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Lectures of Plenary Sessions

Poster Sections

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3RD ANNUAL MEETING OF THE EUROPEAN SOCIETY FOR CLINICAL VIROLOGY

CONTENTS

Acta Microbiologica et Immunologica Hungarica, 46 (1), pp. 1–20 (1999)

CANCER VACCINES WITH EMPHASIS ON A VIRAL ONCOLYSATE MELANOMA VACCINE*

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Biotherapy of malignant diseases has become the fourth treatment modality besides surgery, chemo- and radiotherapy. Whole cell melanoma vaccines with or without BCG and other adjuvants, purified ganglioside and shed antigens, recombinant viruses carrying tumor antigens, dendritic cells pulsed with antigenic peptides etc. are in clinical trials.

Efficacious viral oncolysate vaccines induce the host to mount tumor-specific cytotoxic T-cell response and prevention of relapses is supported by clinical trials. The use of "polyvalent" whole cell vaccines vs. purified or genetically engineered single antigen vaccines is justified as *i.* only very few single tumor antigens are present in all tumors of a given histological type; and *ii.* antigen modulation occurs in tumors rendering them resistant to immune attack generated by vaccine against a single antigen. Thus polyvalent vaccines immunize against several antigens vs. against a selected antigen.

*Part of this paper was presented at the scientific meeting on May 22, 1997 at Semmelweis University Medical School in honor of professors Ilona Szeri and István Nász 70th birthday. Invited paper to commemorate the 50th anniversary of the Department of Microbiology Semmelweis University Medical School.

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Introduction

The occasional observations of the often short-lived spontaneous remissions with inflammatory infiltration in melanomas and renal cell carcinomas since long time have suggested the existence of tumor-specific antigens and the presence of tumor-specific immunity and recognized the pivotal role of the immune system in the control of malignant tumors.

In their native forms naturally occurring tumors seldom generate strong, rejection type tumor-specific immunity. Our perception of the interaction between the immune system and the tumor has gone through significant changes during the last decades. We envisioned the role of the host defense as immune surveillance, continuously seeking and destroying emerging malignant cells and protecting against developing tumors. We know by now that the encounter between the immune system and the weakly antigenic tumor cells does not necessarily result in protective immunity; it might generate immunological tolerance or even "tumor enhancement" as well. The outcome of the encounter depends on the way the antigen is presented to the immune system. Even the presence of strong delayed type hypersensitivity reaction can be associated with failure to control tumor growth due to adverse circumstances, as low level of expression of MHC antigens or immune modulation in the target cells, production of immunosuppressive substances by the tumor, etc. [1, 2].

The anergy or tolerance toward week tumor antigens can be overcome if such antigens are presented in an "unnatural way" to the immune system. The immune response generated by the vaccine depends on the adjuvant used as well. While alum – widely used adjuvant in classical vaccines against microbial infections – promotes the generation of humoral immune response, BCG applied with or scarified over the site of the anti-cancer vaccination generates cellular immunity considered to be of primary importance for efficient anti-tumor immunity. DETOX (detoxified endotoxin of *Salmonella minnesota* and cell wall skeleton of *Mycobacterium phlei*, squalane and emulsifier) is used in Mitchell's Theraccine [3]. The use of interferons (IFN), G-CSF/GM-CSF and IL-2 as adjuvants to vaccine is gaining significance [4, 5]; maybe IL-12 will be used in the future.

SYNOPSIS OF TUMOR IMMUNOLOGY

Tumor antigens

Tumor specific and tumor associated antigens of malignant melanoma are among the most studied ones in human tumor immunology. The first melanoma antigens were recognized by use of patients' sera. The recent wave of new melanoma associated antigens was discovered using tumor-specific $CD8^+$ T-cell clones and peptides eluted from the MHC complex of the tumor cells [6–8].

Tumor specific antigens are seldom present in normal or embryonic tissues and generally are products of genetic mutations (chromosomal translocation, point mutations

etc.) such as the abl-bcr fusion protein in Philadelphia chromosome$^+$ chronic myelogenous leukemias or they are expressed from the genomes of transforming viruses (E6 and E7 in human papillomavirus-transformed cervical cancer, LMP of EBV in AIDS associated non-Hodgkin lymphomas as well as in endemic Burkitt's lymphoma). Tumor-specific antigens can be generated by irregularly spliced transcription of tissue specific antigens such as the tyrosinase-related protein, which is recognized by CTL [9]. Mutated p53 is also antigenically different from wild type p53. Tumor-specific antigens are good candidates to be used as cancer vaccines [10]. *Shared tumor antigens* are specific to the same type of tumor and are not or weakly expressed in normal tissues. The MAGE family of antigens are expressed in the majority of melanomas and only weakly in the testis [11, 12]. These antigens are good candidates for tumor-specific vaccine development.

Tumor-specific differentiation antigens are non-mutated proteins expressed in the tumor and in normal tissue from which the tumor originated. Tyrosinase, MART-1/melan-A and gp100 melanoma antigens are non-mutated proteins expressed in melanocytes as well but still generate T-cell response in patients with melanoma.

Antigen presentation

Cytotoxic lymphocytes identify protein antigens with low efficiency but recognize antigenic peptides, processed by antigen presenting cells (APC) and presented at the groove of the MHC, with high efficiency. Professional APC (dendritic cells, macrophages, etc.) express accessory co-stimulatory molecules such as HLA B7.1 and B7.2 which link to specific receptor CD28 on the lymphocyte surface. Such event happens as an introduction to antigen presentation (1st signal). This is followed by the lower affinity TCR-antigen binding to antigenic peptide in the MHC pocket (2nd signal) and results in "capping" of TCR and concludes finally in stimulation and proliferation of the antigen-specific T-cell clone. Antigens presented by non-professional APC such as tumor cells without co-stimulatory molecules result in apoptotic death of involved lymphocytes and tolerance will develop [13]. However, when CD70 (CD27L) and/or CD40L (CD154, expressed on T cell membrane and essential for dendritic cell activation as well as for T- and B-cell help) are transduced into murine tumor cell lines the transplanted tumor cells generate protective anti-tumor immune response [14].

A frequent finding is that HLA antigens in human tumors are expressed weakly or not at all [15–21]. Sometimes the loss of HLA expression is a consequence of chromosome rearrangements and involves peptide transporter molecules TAP1 and TAP2, transporting peptides from the cytosol to the endoplasmic reticulum [22]. These melanoma cells require IFN induction of TAP1 and -2 as well as transduced HLA-A2 to replace the rearranged allele to be targets to CTL. When such tumor cells act as APC they cause tolerance and themselves are poor targets for cytotoxic T lymphocyte attack as well. Melanoma cells freshly extracted from a metastatic lesion of one of our patient expressed MHC Class I antigens (tested with antibodies specific to its β chain known also as β-2-microglobulin). During immunization with an unmodified whole cell autologous

melanoma vaccine relapse occurred: tumor cells from that new metastatic lesion ceased expressing β-2-microglobulin (Figure 1). These cells were poor targets for and escaped to be killed by cytotoxic T-cells generated by the vaccine.

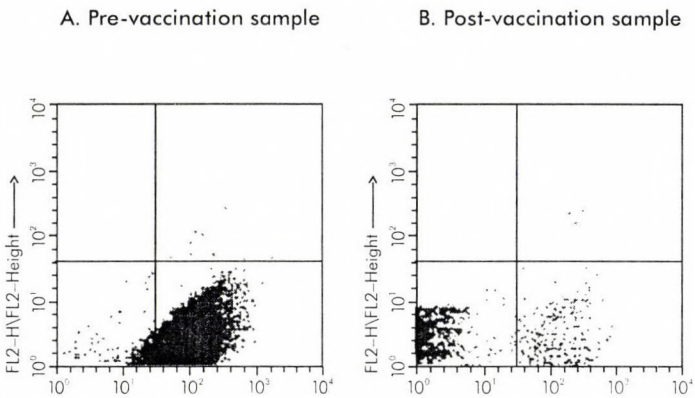

Fig. 1. Flow cytometry analysis of melanoma cells obtained from metastatic lesions from a patient before (A) and after (B) active immunization with an autologous unmodified melanoma vaccine. The cells were stained with FITC labeled human β-2-microglobulin specific mouse monoclonal antibody

Dendritic cells (DC) are the most potent APC. They present antigenic peptides to T-lymphocytes through Class I and Class II HLA molecules (for $CD8^+$ and $CD4^+$ lymphocytes, respectively). The source of DC determines which kind of cytokines they produce and whether Th1 or Th2 type response is generated (regulated by IL-12). Activated DC of systemic origin (spleen, etc.) induce IFNγ and IL-2 production and TH1 type (cell mediated, delayed type) response in T-lymphocytes whereas DC from Peyer's patches (mucosal DC) promote IL-4 and IL-6 production and Th2 (predominantly humoral) immune response [23]. As DC can be cultured, in vitro from peripheral blood and can be pulsed (empty MHC grooves dressed in vitro) by antigenic peptides or tumor cell lysates their significance as components of cancer vaccines is increasingly recognized (*vide infra*).

CANCER VACCINES

Crude whole cell or cell lysate vaccines

Whole cell based vaccines contain large amount of normal cell components, however, they also represent a wide range of tumor-specific antigens. Early passage autologous tumor cell vaccines carry all tumor antigens expressed in the patient's resected

tumor but immune modulation or mutation in a metastasis developing later can render it resistant to immune attack generated by the vaccination. The use of allogeneic vaccines is justified by the existence of common (shared) tumor-specific antigens; to cover as many existing antigens specific to the particular tumor type as possible, however, such vaccines contain a mixture of several different tumor cell lines or lysates (from 2 lines in Mitchell's cell lysate vaccine to 8 cell lines in Cassel's viral oncolysate). Mitchell's allogeneic melanoma cell lysate vaccine induced objective responses (average duration 21 months, one lasted over 6 years) in a therapeutic setting in 25/106 patients with metastatic disease, including choroidal melanoma [24–26]. Patients suffering from stage IV metastatic melanoma with measurable disease responded better to chemotherapy than to Mitchell's vaccine in a therapeutic setting (12.3 months OS, 2CR, 5PR, 19 SD vs. 9.4 months OS, 2CR, 3 PR and 5 SD, respectively) but the quality of life was better and there were no toxic side effects in the vaccinated group. The patients who failed on the Theraccine vaccine responded better to IFN-α treatment than non-vaccinated patients [27]. Expanding clones of tumor-protecting suppressor cells can be eliminated by low dose cyclophosphamide [28]. Morton's polyvalent whole cell allogeneic vaccine is admixed with BCG and the patient is premedicated with cimetidin, indomethacin and/or cyclophosphamide, to block histamine receptor$^+$ suppressor T-cells and prostaglandins [29]. This vaccine induced regressions in 23% of patients with measurable disease and increased the median survival as well as the 5 years survival of patients suffering from stage III disease with 20% and is claimed to have reduced the incidence of brain metastases when used in adjuvant setting.

To increase immunogenicity the tumor cells are xenogenized with dinitrophenol (DNP) in Berd's autologous vaccine [30] or are infected with oncolytic viruses (*vide infra*). Autologous melanoma vaccine xenogenized by DNF induced delayed type hypersensitivity (DTH) reactions in vaccinated patients. The 5 year survival of patients with positive DTH to untreated melanoma cell antigens was 71% compared to 49% of patients with negative DTH [31]. The DNP-modified vaccine induces clonal expansion of CTL with TCR-Vβ14 in existing metastases, capable of lysing melanoma cells [33]. Preliminary results with DNP-modified autologous ovarian carcinoma vaccine are also promising.

Anti-idiotype monoclonal antibodies as cancer vaccines

Anti-idiotype antibodies bearing the internal image of a tumor associated antigen offer unique opportunity as cancer vaccines. Active tumor-specific immunotherapy with the mouse monoclonal antibody MK2-23 bearing the image of high molecular weight melanoma associated antigen promotes longer survival of vaccinated patients [33].

Cellular vaccines genetically engineered to express tumor antigens, lymphokines or transplantation antigens

Preclinical studies with murine transplantable tumors demonstrated that the immunogenicity of weakly antigenic tumor cells can be efficiently increased if tumor cells, after transduction with retroviral vectors encoding cytokine genes, secrete functionally active cytokines. Tumor cells double-transduced with IL-7 and HSV

thymidine kinase (TK) cause augmentation of cytotoxic T-cell production and down-regulation of immunosuppressive TGFβ; TK provides the way of eliminating the injected vaccine cells with gancyclovir treatment [34]. Local production of lymphokines potentiates APC function (IFN, GM-CSF) and activates lymphocytes (IL-2) without the side effects of systemic lymphokine therapy. Human clinical trials are in progress using cytokine gene-transduced autologous kidney cancer or melanoma cells as vaccines [35–37]. IL-4 transduced dermal fibroblasts coinjected with irradiated tumor cells are in a phase I/II clinical trial [38]. Tumor-antigen transfected DC (with or without cotransfected cytokine genes) joined the rank [39, 40]. These settings work well in preclinical trials and generate clinical responses in patients with advanced disease. Several clinical trials are in progress to demonstrate their usefulness in generating long-term tumor-specific protective immunity.

Partially purified tumor antigens

Bystryn's melanoma vaccine consists of antigens shed by 3 human and 1 hamster melanoma cell lines. The disease free interval (DFI) of patients with stage III melanoma who developed high anti-melanoma antibody titer to vaccination with shed melanoma antigens was 52% and OS was 71% vs. 31% and 44% among patients with no antibody response to the vaccine [41]. Several melanoma-specific proteins, identified using sera of vaccinated patients, represent candidate antigens for potential new vaccines [42].

Ganglioside tumor antigens

Gangliosides are continuously shed from tumor cell membranes and are immunosuppressive: binding to IL-2R they interfere with IL-2 binding and inhibit T-cell activation [43]. The role of a ganglioside based vaccine is to raise antibodies capable of removing and neutralizing circulating gangliosides shed by tumor cells. High titer of antibody response to GM2 ganglioside antigens conjugated to keyhole limpet hemocyanin (KLH) correlated with longer disease free and overall survival [44–46]. A multicenter randomized trial is ongoing to compare the effectiveness of a KLH-conjugated GM2 vaccine with high dose IFNα in patients with deep stage II and stage III melanoma.

Tumor-antigen derived T-cell specific peptides

Since technology became available to identify tumor-specific antigenic peptides, presented in the groove of MHC molecules and capable to induce cytotoxic T-cell response, several approaches evolved to use peptides as tumor vaccines. Patients with evidence of metastatic melanoma received MART-1 and gp100 specific synthetic peptides (modified to increase binding to MHC molecule) admixed with incomplete Freund adjuvant. When the vaccine was administered with a single cycle of high dose IL-2 13 (42%) out of 31 patients experienced clinical response after at least 2 vaccine cycles [47].

Dendritic cells (native or engineered to express cytokines), pulsed with tumor cell lysates or peptides or fused with tumor cells

Use of the non-professional antigen presenting tumor cells fused to bone marrow derived dendritic cells offers a unique opportunity for tumor vaccine. Fusion of murine MC38 adenocarcinoma to dendritic cells renders the new entity non-tumorigenic and morphologically dendritic cell-like. Vaccination of mice with the fused cells provided 100% protection against tumor cell challenge by generating cytotoxic T-cells able to lyse tumor cells [48]. In 9 out of 10 animals with established tumors the generated CTL could eliminate metastatic disease as well. These very impressive results are yet to be confirmed in human clinical trials.

Tumor lysates (without any further preparation of tumor-specific antigens) pulsed onto dendritic cells increased cure rate and survival from the parent transplantable tumors [49]. A European study using such approach documented strong clinical response to a therapeutic vaccine consisting of cultured dendritic cells pulsed with melanoma cell lysates or tumor-specific peptides, recognized by T-cells [50]. The vaccine generated delayed type hypersensitivity reaction (DTH) and objective responses were observed in 5 out of 15 patients with advanced melanoma (2CR, 3 PR).

Recombinant viruses encoding specific tumor antigens

The first reports about the use of recombinant viruses encoding tumor-specific antigens for cancer vaccine, which generate DTH and tumor-protection in preclinical studies appeared in the late 80s. Such antigens included retrovirus *env* genes, EBV latent membrane protein, polyoma virus T antigens and melanoma specific g97 protein in vaccinia virus [51–54]. Poliovirus vectors joined the rank as they proved to be able to elicit cytotoxic T cell mediated tumor-specific immune response in mice (55). Canarypox virus vectors encoding mutant or wild type p53 were found to be equally efficient in protecting mice against the challenge with highly tumorigenic fibroblast tumor cell line expressing mutated p53 [56]. However, tumor cells down-regulating p53 expression escaped the immunologic rejection. Immunization with a recombinant vaccinia virus encoding human prostate specific acid phosphatase generates CTL responses in rats: can cross-species immunization cure prostate cancer or prevent relapse [57]? E6 and E7 proteins from human papillomavirus type 16 and 18 in a live recombinant vaccinia virus induced virus specific antibodies in patients with late stage cervical cancer in a Phase I/II trial suggesting a feasible way to fight this virus-induced tumor [58–60]. A vaccinia virus vector encoding the human prostate specific antigen (PSA) generated cytotoxic lymphocyte response in rhesus monkey (94% amino acid homology between the rhesus and human PSA); human clinical trials will certainly follow [61].

Viral vectors themselves generate immune response that can limit their repeated use. When different viral or plasmid vectors are used for priming and boosting with tumor antigens the anti-tumor immune response exceeds the one obtained with a single vector system [62].

DNA vaccines

DNA vaccines utilize plasmid or viral DNA constructs containing a potent eukaryotic promoter and a gene encoding a tumor antigen or costimulatory molecule. Such a construct encoding a tumor antigen may be directly injected to muscle cells rendering them to produce the corresponding antigen protein and generating humoral and cellular anti-tumor immune response [for a review, 63]. Gold particle bound plasmid constructs can be introduced to skin by "gene gun" technology [reviewed in 64]. When Allovectin-7, the HLA-B7 allogeneic class I MHC encoding plasmid, formulated with cationic lipid was injected into metastatic melanoma lesions with CT guidance, CTL response was generated and out of 10 injected patients 6 showed clinical response (1 durable complete response). Bystander effect was also observed, i.e. lesions not injected also diminished in size [65, 66].

Table I

Chemo-immunotherapy of patients with metastatic sarcomas (Oct '76 - Feb '77)

Treatment	No. of patients	Complete remission	Partial remission and stable disease	Progressive disease (death)	% of progressors
Chemotherapy[1]	49	10	3	36 (17)	72
Chemotherapy[1] + BCG	19	3	6	10 (4)	53
Chemotherapy[1]+ BCG + VO	19	4	9	6 (3)	32

[1] vincristine, cyclophosphamide, adriamycin/actinomycin D, DTIC

VO = sarcoma viral oncolysate prepared from allogeneic cell lines with PR8 influenza A virus [70]

Patients with metastatic sarcomas either received standard adriamycin-based chemotherapy or chemotherapy plus BCG scarifications. The third group of patients received in addition autologous (when available) or allogeneic sarcoma viral oncolysates prepared with the PR8 influenza A virus (which was shown to replicate in human sarcoma cells). In the third group of patients, BCG was scarified over the intra- or subcutaneous injection sites of the viral oncolysate vaccine administration. In this third group of patients progression of disease was halted and a remission rate better than in the first and second group of patients was induced [70].

VIRAL ONCOLYSATE VACCINES

First trials

Mice injected with virus-infected transplantable tumor lysates reject a following tumor cell challenge; postoncolytic immunity develops [67, 68]. The first clinical trial with *PR8 influenza A virus* was conducted at MD Anderson Hospital (Houston TX) by

one of us (JGS) and showed its effectiveness in sarcomas and solid tumors [69]. The patients developed antibody- and cell mediated immune response against allogeneic and autologous (when available) tumor cells. In patients with metastatic disease chemo- and immunotherapy were sequentially used, immunotherapy being used when patients rebounced from myelosuppression. The chemo-immunotherapy induced remissions of long duration and prevented late relapses [70–74]. Here we re-produce some of the original tabulated results (Tables I, II and III). It is noticeable that relapses happened in all three groups of patients treated with sarcomas, but the least frequent recurrences occurred in the group of patients receiving chemotherapy with BCG and VO. Patients with stage IV metastatic melanoma receiving chemo-immunotherapy in a therapeutic setting had the longest survival compared to patients receiving chemotherapy with or without non-specific immunostimulation (BCG). These results are among the first documentations of the fact that pulsed chemotherapy combined with immunotherapy can be additive against a targeted tumor.

Table II

Time intervals (in months) for patients with Stage IV melanoma receiving chemo-immunotherapy

Treatment	Length of survival (mo)
Chemotherapy[1]	5.1
Chemotherapy[1] + BCG	6.25
Chemotherapy[1]+ BCG + VO	12.8

[1] DTIC (dacarbazine), vincristine or nitrosourea, actinomycin D
VO=viral oncolysate of allogeneic melanoma vaccine prepared from well-characterized cell lines with PR8 influenza A virus, harvested, disintegrated and UV irradiated [71]

Metastatic malignant melanoma was treated in the early 1970s with nitrosourea and dacarbazin. Administration of BCG in addition to chemotherapy did not significantly prolong survival (mean 5 vs. 6 months). Patients also receiving autologous or allogeneic PR8 influenza A viral oncolysate vaccine (melanoma cells grown in culture and lysed by the virus) experienced significant prolongation of survival: mean 12 months. This often occurred coincidentally with partial remissions, minor responses and stabilizations of disease and only seldom consequentially to complete remissions. In this report treatment schedules for adjuvant therapy and better "no evidence of disease" survival in stages II and III disease are also listed favoring the administration of viral oncolysates over controls. Without prospective randomization of patients, only a favorable trend for better responses and longer survivals for patients receiving viral oncolysate vaccine could be reported [71].

W. Cassel (Emory University Atlanta GA) used the Lederle's strain 73T (which he adapted to mouse ascites tumor) of *Newcastle disease virus* (NDV) to prepare viral oncolysate melanoma vaccines. Patients with stage II metastatic melanoma, rendered clinically disease free by surgical resection, received autologous or allogeneic vaccine in an adjuvant setting to prevent late recurrence of the disease. No adjuvants were used for

his vaccine and the patients received no chemotherapy. Surgical samples from central nervous system (CNS) recurrences showed extensive inflammatory infiltration of the tumor; such infiltration was absent in all brain metastases from non-vaccinated patients. The 10 year follow-up of 83 treated patients showed and unprecedented 61% survival rate compared to 33% survival in historical controls [75, 76]. These excellent results are grossly overlooked and seldom cited.

Table III

Chemo-immunotherapy for Stage III melanoma

Treatment	Patients	NED 2nd year	Relapses with melanoma 1978–80	NED 1981 (5th year)
Chemotherapy+BCG	34	17	7	10 (6m, 4f) 29%
Chemotherapy+BCG+VO	30	13	0*	11 (5m, 6f) 36%

[1] DTIC (dacarbazine), vincristine or nitrosourea, actinomycin D

VO=viral oncolysate of allogeneic melanoma vaccine prepared from well-characterized cell lines with PR8 influenza A virus, harvested, disintegrated and UV irradiated (72)

* 2 deaths with prostate and colon cancer (no melanoma)

In a group of patients with stage III malignant melanoma (massive lymph node metastases surgically removed) chemotherapy with BCG or chemotherapy, BCG and PR8 viral oncolysate autologous or allogeneic melanoma vaccines were given. The viral oncolysate vaccinations were administered in between courses of chemotherapy when blood counts were returning toward normal. In the group of patients receiving chemotherapy and BCG relapses continued up to the 5th year; in the group of patients receiving chemotherapy, BCG and melanoma viral oncolysates relapse rate was reduced and no relapses occurred after the 3rd year [72]. Interpretation: patients destined to relapse due to clinically undetectable but major metastases relapsed equally in both groups; patients with micrometastases were protected from relapse by VO vaccine.

In vitro studies documented increased lymphocyte-mediated cytotoxicity toward autologous (when available) and allogeneic melanoma cells in long-term survivors of the disease [for review see 113].

Current trials

A Phase II clinical trial of an allogeneic melanoma oncolysate made with *vaccinia virus* suggested a longer DFI and overall survival (OS) of vaccinated patients with Stage III melanoma compared to the control group receiving a vaccine containing the vaccinia virus only. A multicenter randomized Phase III trial, however, found no significant difference in DFI and OS between the OV vaccinated group of patients and patients receiving the virus only. The subset of men having 1–5 nodes and aged 44–57 years,

however, enjoyed 21% higher 5 years survival than the control group of same gender and age [77, 78].

The autologous renal cell carcinoma viral oncolysate vaccine prepared with Cassel's strain of NDV was extensively tried and found successful to prevent recurrence in a clinical trial in Germany. As adjuvant subcutaneous low dose IL-2 and IFNα were applied. Over 200 patients were included with locally advanced renal cell carcinoma. An interim evaluation of the patients who received the vaccine for at least 22 months found that the relapse rate was 18%, lower than in the historical control group [79].

Based on extensive preclinical studies a virally modified vaccine made with the non-lytic Ulster strain of NDV was used by Schirrmacher and his group in Germany for active tumor-specific immunotherapy of a range of different tumors in an adjuvant setting. Tumor cells prepared from surgical samples were used directly, without culturing. Cryopreserved cell suspensions were thawed immediately before use, lethally irradiated and infected with NDV-Ulster for 1 h and injected intradermally. Patients immunized with virus-modified, irradiated autologous tumor cells developed delayed type hypersensitivity reaction against non-modified tumor cell antigen. The efficiency of the vaccine depended on the viability of the injected tumor cells. VO vaccine extended survival of patients with mammary carcinoma and with ovarian carcinoma [80, 81]. Stage II melanoma patients received the NDV-Ulster VO vaccines and experienced extended overall survival depending on the quality (number, viability, etc.) of the cells in the applied vaccine. Out of 23 patients vaccinated with the NDV modified autologous colon cancer vaccine 13 developed DTH reaction to unmodified tumor cell antigens; after 18 mo follow-up 61% recurred vs. 87% in patients treated only surgically [82]. Tumor cells were disseminated to the bone marrow of patients with mammary (4), stomach (7), pancreas carcinomas (2) and glioblastomas (2). The bone marrow of 90% of these patients became tumor cell free after active tumor-specific vaccination [79].

At MD Anderson Cancer Center clinical trials elegantly demonstrated the efficacy of PR8 oncolysate vaccines to treat vulvar squamous cell carcinoma and applied intraperitoneally to treat ovarian carcinoma with peritoneal carcinomatosis. Application of the vaccine is followed by IL-2 and TNFβ (lymphotoxin) secretion in the ascites or *in vitro* by the lymphoid cells. $CD4^+$ T cells respond with proliferation to NDVO and tumor cell lysate proving that the vaccine raises $CD3^+$ $CD4^+$ T cell immune response against specific tumor antigens [83–85].

At St. Joseph's Hospital Cancer Institute patients suffering from malignant melanoma with high risk of recurrence are treated with autologous (if available) or allogeneic NDVO in an adjuvant setting. NDVO is found to be an efficient and safe vaccine. No serious side effects are observed and no infectious virus is secreted following application of the vaccine. Since the oncolytic potential of various NDV strains is highly variable, Cassel's 73T strain was used in these clinical trials.

The clinical data of vaccinated patients support a beneficial effect of the vaccine for Stage II and III patients who enjoy extended disease free intervals and overall survival. Relapsed patients likely to have had multiple relapses, higher numbers of positive lymph nodes, thicker and more invasive primary tumors (average 5.5mm vs. 2.3 for patients with no evidence of disease) in their history.

The vaccine gave little benefit to patients with Stage IV disease. Out of 5 patients 2 had metastases in the brain, 1 in bone and liver and 3 in the lung before starting the vaccination.

DISCUSSION

Postoncolytic immunity

The mechanism of the postoncolytic immunity following application of NDVO is not clearly understood. The adjuvant effect of the virus is well demonstrated. Inoculation of PBMC with NDV enhances their cytotoxicity against tumor cells *in vitro*. IFNα and TNF production is induced in infected PBMC [86]. Schirrmacher's group elegantly demonstrated that mouse lymphoma cells infected with the non-lytic Ulster strain generated tumor-specific CTL which effect was inhibited with anti-IFN sera showing that IFN was responsible for the adjuvant effect [87]. NDV-infected or NDV HN transfected mouse fibroblasts (non-professional APC!) pulsed with antigenic peptides generate six times more efficiently CTL than unmodified fibroblasts [88]. In mice NDV induces MHC class I gene expression through the IRF1 and KBF1/NFκB cooperative action, by a mechanism partly different from IFNγ [89]. Some viruses generate virus-specific $CD8^+$ CTL in CD4 depleted mice or in animals homozygous (-/-) in the disruption of class II major histocompatibility complex glycoprotein bypassing the need of $CD4^+$ cells [90]. Does NDV have direct $CD8^+$ CTL activating capabilities? The NDV induced NO synthesis is also activated through the NFκB pathways [91]. NDV is a potent inducer of heat shock proteins (HSP), a family of stress-induced proteins [92]. HSP are recently found to be efficient presenters of tumor-specific antigenic peptides to T-cells and are being in clinical trial as vaccines [reviewed in 93, 94].

The co-presentation of NDV and tumor antigens to naive or committed T cells is also subject of speculation. Is it presented in the MHC pocket as *i.* a virus-tumor antigen fusion peptide or *ii.* two TCRs of the same T-cell with two different specificity recognize the side-by-side presented viral and tumor antigenic peptides on the APC? Our preliminary data suggest that *iii.* one or more virus proteins might act as superantigens linked to TCR and MHC at the same time. Do they serve the role of an accessory molecule, making the cell-cell connection stable and result in immunity instead of tolerance? Or could some NDV gp antigens mimic those of melanoma cells?

While NDV strains may widely differ in their oncolytic activities, Cassel's NDV 73T behaves as an antineoplastic agent; 73T preferentially multiplies and lyses tumor cells while normal cells such as fibroblasts are spared. Normal and fully transformed lymphoid cells bear the same number of receptors for NDV, the virus, however, preferentially causes lytic infection in lymphoma cells compared to lymphoblastoid cell lines (immortalized, not fully transformed) or PBL [95, 96]. Human neuroblastoma and fibrosarcoma xenografts transplanted to athymic nude mice regress completely after intratumoral injection of this virus [97].

Critical comments

What justifies the use of NDVO instead of other types of melanoma vaccines? Cytokine transfected or transduced tumor cells as vaccines are promising. However, at least in mouse experiments the results are controversial, as none of the IL-2, -4, -7, IFNγ or TNF or their combination was found to be superior to irradiated tumor cells admixed with *Corynebacterium parvum* to protect mice against live mouse plasmacytoma cell challenge [98]. It is still uncertain, what are the antigens relevant to develop protective anti-tumor immunity; are the antigens represented in a vaccine present in the particular patient's tumor as well? If the loss of a tumor-specific antigen does not impair the functions of the tumor cell it can be down-regulated rendering the tumor cell resistant toward the immune response generated by a monospecific vaccine. The use of vaccines specific to a single tumor specific antigen seems to be limited to virus induced tumors (E6 or E7 of human papillomavirus in cervical carcinoma or LMP in EBV associated lymphomas of the immunosuppressed patients) and malignancies where the tumor antigen generated by mutation represents a new entity (such as the fusion protein ABL-BCR generated by chromosomal translocation) and is presented in every tumor of that kind [99, 100].

The role of adjuvants cannot be accentuated enough. The HER2 oncoprotein itself was ineffective to protect mice against HER2-transfected fibrosarcoma while animals immunized with the same protein complexed with a hydrophobized polysaccharide nanoparticle developed humoral as well as CD8+ cytotoxic T-cell mediated protective immunity. Combination of vaccines with interferons and other cytokines as adjuvants can efficiently boost the immune response and make the vaccine more effective [4, 101, 102].

In spite of good *in vitro* signs tumor cells escape the immune surveillance and relapses occur. The known reasons outnumber the presently available resolutions.

Melanoma cells often express immunosuppressive biologically active substances. In the presence of IL-10 it is a Th2 type immunity what develops with preferentially antibody production instead of tumor specific cytotoxic immunity. IL-10 decreases the production of IFNα [103]. CD40, CD86 and HLA-DR expression is reduced in IL-10 treated macrophages; such APC fail to activate T-cells [104]. MHC antigens are often underexpressed on human tumor cells, however, interferons γ, and (less efficiently) α and β increase MHC expression [105]. Immune lymphocytes eliminate invading tumor cells by the perforine-granzyme B pathway or by expressing the ligands FasL and TNF inducing programmed death [106]. Coupling of these ligands to their receptors on the Fas/APO-1$^+$ tumor cells induces pre-programmed molecular events leading to DNA fragmentation, cytoplasm membrane blebbing, cell shrinkage and ultimately cell death followed by phagocytosis practiced by the surrounding cells. Melanoma cells produce FasL and counterattack Fas$^+$ lymphocytes and thus protect themselves from being killed [107–111]. A recent report demonstrates, however, that CD4$^+$ and CD8$^+$ lymphocyte populations kill melanoma cells with the perforin-granzyme B mechanism and overcome the $FasL^{MC} \rightarrow FasR^{Ly}$ counterattack [112]. Successful immunotherapeutic modalities have to address these problems. In our next generation NDVO we plan to include IFNα and/or

IL-2 as adjuvants to overcome decreased MHC expression and induce activation and proliferation of cytotoxic T-cells generated by the vaccine.

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STUDIES ON INFLUENZA- AND NEWCASTLE DISEASE VIRUS AT THE UNIVERSITY'S INSTITUTE OF MICROBIOLOGY (BUDAPEST) IN 1948–50*

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In 1946–48 the Institutes of Pathophysiology and Microbiology functioned as one faculty under the directorship of professor Sándor Belák. This marvellous old building at Hőgyes Endre street 7–9 housed previously the laboratories of Endre Hőgyes who produced the Pasteur-Roux type rabies vaccine; and those of Hugó Preisz who described first developmental forms of bacteria that were later referred to as pleuropneumonia-like growth phase (but professor Preisz called these developmental forms "Pettenkofferia").

After professor Belák's death, a separate Institute of Microbiology was formed under the directorship of professor Ferenc Faragó. Adjunct professor dr. László Berta and I, now a newly appointed assistant professor with MD degree, joined professor Faragó in 1948.

It was Dr. Berta who initiated research in virology at the Institute of Microbiology. In the immediate postwar period he completed a one year fellowship in virology in Moscow. Professor Faragó supported our virological studies and provided space and equipment for this purpose. The first report [1] appearing from the Institute described rapidly advancing pneumonias in mice pretreated with colloidal copper and inoculated intranasally thereafter with influenza A virus strains received from C. Andrews (London) and A. Smorodintsev (Moscow). We probably blockaded the very first steps in antiviral immunity in these mice, i.e. the presentation of viral antigens in the MHC of dendritic cells to T lymphocytes. These antigen presenting cells might have been totally incapacitated by the intravenously administered colloidal copper. At that time we could

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not explain the mechanisms by which complete lack of antiviral immunity allowed unopposed viral replication in these mouse lungs.

After the tragic and untimely death of dr. Berta in 1949, professor Faragó appointed me to the rank of adjunct professor and soon Erzsébet Molnár and Ilona Szeri joined me to continue work with viruses. Our unpublished works concerned lymphocytic choriomengitis (LCM) virus isolated from patients (Molnár); mumps virus isolated from saliva in the amniotic cavity of chicken embryos (Sinkovics); serial passages and retitration of influenza A (PR8) and B (Lee) virus strains in the mouse lung (Szeri); the passage, purification and concentration of the Lansing poliomyelitis virus strain (Sinkovics). Published works were the isolation of fowl plague (Newcastle disease) virus (NDV) from human conjunctivitis referred to as "oculoglandular syndrome" (work initiated by dr. M. Radnót professor of ophthalmology) [2] and studies of interference between NDV and influenza viruses in the mouse lung [3, 4]. With the horrible death of professor Faragó in 1950 our work was abruptly interrupted but virus-research was resumed soon thereafter by doctors István Nász and Ilona Szeri and their associates. In the mid-50s projects conceived and/or started at the Institute but brought to completion elsewhere were published. These are reports on the eclipse phenomenon of influenza virus multiplying in the mouse lung [5]; virus neutralizing antibody production in the mediastinal lymph nodes of mice after intranasal instillation of viruses (influenza and NDV) or the lack thereof (LCM) [6, 7]; the interrelationship of mumps, NDV and influenza viruses [8]; phenotypical mixing of NDV and influenza viruses [9] and enhancement of the oncolytic properties of NDV after its adaptation to the brain of newborn mice [10]. Studies were completed on a photodinamically inactivated influenza virus vaccine using the equipment and supplies of the Virology Section of the Institute in 1851–53 [11]. Work initiated at the Institute (and continued at the Department of Virology, The State Institute of Public Health, Budapest, 1954–56), was instrumental in the publication of a textbook of virology both in Hungarian and in German [12, 13]. The lectors (doctors Z. Alföldi, E. Farkas and G. Ivánovics) and foreign reviewers of this volume praised it highly because it dealt with theories on the origin and evolution (including genetic recombinations) of viruses; the then much debated issue of correlation of viruses with filterable forms of bacteria (a topic also discussed elsewhere) [14, 15]; the phenomena of viral interference; and the growth of viruses in tumor cells often resulting in the death of the tumor cell. It united basic science with practical issues and technologies. It revealed the hidden original Russian literature to the West for the first time in the postwar period. Both German (for example A. Marchionini and Th. Nasemann) and Russian (for example professor V. Zhdanov) reviewers gave this volume their highest scores.

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CHROMIUM-RESISTANT SOIL ACTINOMYCETES: THEIR TOLERANCE TO OTHER METALS AND ANTIBIOTICS

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Chromium occurs widely in most soils, but generally in trace amounts. Actinomycetes, one of the important components of the microbial population in soils interact with a variety of metals including chromium. This study was aimed to evaluate the tolerance of soil actinomycetes to Cr^{6+}, other metals and antibiotics. Thirty-two actinomycete isolates were screened for their tolerance to Cr^{6+} on tryptone yeast extract agar medium supplemented with Cr^{6+} at concentrations ranging from 100 to 2000 μg ml^{-1}. Thirteen Cr-tolerant isolates were selected on the basis of their growth at the highest concentration, but their performance was not satisfactory in Cr^{6+} containing liquid salts medium. Resistance of these isolates to other metals and antibiotics was assessed using agar-cup assay and disc diffusion technique, respectively. The sequence of metal toxicity for the actinomycete isolates was in the order $Hg^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+} > Cd^{2+}$, but the Cr^{6+} resistance of the isolates could not be correlated with their antibiotic-resistance profile.

Actinomycetes are widely distributed in a variety of natural and man-made environments and constitute a significant component of the microbial population in most soils. In soil they are primarily concerned with degradation of plant, animal and microbial polymers and can also respond to a range of unnatural compounds introduced to soil. They are also exposed to heavy metals in a variety of ways [13], especially when the agricultural fields are subjected to treatment with sewage sludge and industrial effluents [5, 26]. The ability of actinomycetes to survive and grow in the presence of high metal concentrations may involve adsorption to cell walls and other constituents, extracellular precipitation or complexation, internal compartmentation and transport [12]. Actinomycetes strains isolated from zinc contaminated soil showed an increased tolerance to Zn [15], but isolates from lead mine waste did not show increased lead tolerance [25]. However, it has been reported that actinomycetes are more tolerant than other bacteria to

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cadmium [3]. Abbas and Edwards [1] assessed the tolerance of mesophilic and thermophilic streptomycetes to various metals and the order of toxicity was Hg > Cd > Co > Zn > Ni > Cu > Cr > Mn. *Streptomyces sp.* have been shown to remove uranium, copper and cobalt from solutions, the order of efficiency being Uo_2 > Cu > Co [19, 20]. Similarly streptomycete species that are able to detoxify Hg^{2+} to volatile Hg^{o} by means of mercuric reductase enzyme have also been isolated [23, 24].

Chromium, a transition metal occurs widely in soils. Its concentration ranges from 2–60 mg Kg^{-1} in most soils [10, 27]. Naturally occurring chromium is always present in the trivalent (Cr^{3+}) state, but almost all hexavalent chromium (Cr^{6+}) in the environment is derived from human activities. These include sewage sludge deposition and dispersal of wastes from industries that utilize chromium compounds for ferrochrome production, electroplating, pigment production and leather tanning [16].

Hexavalent chromium (Cr^{6+}) is considered much more toxic and mutagenic for most organisms than trivalent chromium (Cr^{3+}) [16]. Microbial reduction of Cr^{6+} to Cr^{3+}, therefore, could be a useful mechanism in reducing Cr^{6+} toxicity. Bacterial strains isolated from chromium-contaminated sediments and sewage sludge have been shown to tolerate high levels of Cr^{6+} [4, 14, 17, 18]. In addition to chromate reduction, Cr resistance has been correlated with the presence of plasmid DNA in several species of bacteria [6, 7]. Plasmid determined chromate resistance in *Pseudomonas fluorescence* has been reported to be due to reduced uptake of CrO_4^{2-} by the resistant cells [21].

This study was aimed to evaluate the chromium resistance potential of a range of actinomycetes strains isolated from natural soils of West Bengal during the course of screening of antifungal actinomycetes. Attempts were also made to evaluate their tolerance to other metals and antibiotics.

Materials and methods

Bacterial strain and maintenance. A collection of 32 different actinomycetes isolates were obtained from the culture collection of the Microbiology Laboratory, Department of Botany, Calcutta University, Calcutta. The actinomycete strains were isolated from natural soils of West Bengal and were maintained on glucose asparagine agar slants that contained (gL^{-1}) glucose, 10; K_2HPO_4, 0.5; asparagine, 0.5; and agar, 20 (pH 6.8).

Screening for chromate resistance. Qualitative assessment of the chromate resistance of the isolates was made following the method of Luli *et al.* [18]. The isolates were grown on tryptone yeast extract agar medium (TYE) that contained (gL^{-1}) tryptone, 5; yeast extract, 5; NaCl, 5; glucose, 1; and agar, 20 (pH 7.2) and was supplemented with Cr^{6+} at concentrations ranging from 100 to 2000 μg ml^{-1}. Homogenous spore suspensions from the sporulated cultures, prepared in 0.1% (w/v) sterile Tween 80 solution, were streaked in the form of a narrow line on Cr^{6+} incorporated plates and incubated at 30 °C for 3–7 days for visible growth.

Secondary screening. Degree of resistance of the selected isolates was also evaluated in the liquid salts medium that contained (gL^{-1}) K_2HPO_4, 6; K_2HPO_4, 2; $(NH_4)_2SO_4$, 3; $MgSO_4$. $7H_2O$, 0.02; $FeSO_4$ $7H_2O$, 0.01; glucose, 10 and was amended with 0.1% yeast extract (pH 7.4). Filter-sterilized K_2CrO_4 solution was added to the sterilized liquid salts medium to attain a Cr^{6+} concentration ranging from 250 to 2000 μg ml^{-1}. Twenty ml of medium per 100 ml flasks were inoculated with 1 ml of freshly prepared spore suspensions and incubated on a rotary shaker (120 rpm) at 30 °C for 5d. Growth of the

organisms was determined as dry weight of mycelia and the relative growth was expressed as a percentage of those obtained in untreated control cultures at the same time, which was taken as 100%.

Resistance to other metals. Resistance of Cr-tolerant isolates against a number of metals was tested by agar-cup method [22] using TYE medium. Cups (12 mm diam.) were scooped at equidistant places on TYE agar plates seeded with individual isolate and 0.1 ml of metal solutions were added to each cup, allowed to diffuse for 3–4 h at 10 °C and then finally incubated at 30 °C for 24 h. Sensitivity to different metals was scored from the diameter of inhibition zones.

Resistance to antibiotics. To determine the antibiotic sensitivity of the Cr-resistant isolates, antibiotic impregnated discs (6 mm diam., Hi-media) were placed on freshly prepared lawns of each isolate on TYE agar medium. The plates were incubated at 30 °C for 24 h. Based on inhibition zones, the organisms were categorised as resistant, intermediate and sensitive according to antibiotic disc sensitivity testing method as described in DIFCO Manual [8].

Results

Primary screening. Chromium tolerance of 32 actinomycete isolates in solid medium indicates that up to a concentration of 500 μg ml^{-1} of Cr^{6+} all the isolates showed growth almost equivalent to control (Figure 1). At the highest concentration (2000 μg ml^{-1}), 13 isolates (40% of the total number tested) were tolerant to Cr^{6+}. Higher concentrations of chromium also inhibited the sporulation of all the tolerant strains except

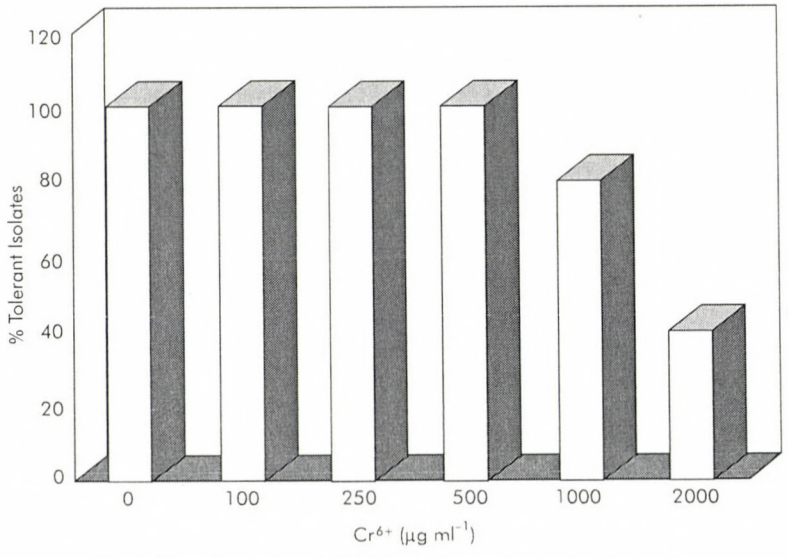

Fig. 1. Chromium tolerance of soil actinomycetes in solid medium

DI-06 and R.A-05; production of diffusible melanoid pigment by the strains DI-05 and DI-25, however, remained unaffected (data not shown).

Secondary screening. Thirteen isolates showing moderate to good growth at 2000 μg ml^{-1} of Cr^{6+} were secondarily screened in liquid salts medium. Only 50% of the selected isolates attained 20–65 g relative growth at 500 μg ml^{-1} of Cr^{6+} (Table I). The performance of the isolates in liquid salts medium with 1000 μg ml^{-1} of Cr^{6+} was very poor, five isolates showed only slight growth, the others were totally inhibited. None of the isolates examined showed detectable growth at 2000 μg ml^{-1}.

Table I

Chromium tolerance of some selected actinomycetes isolates

Isolate	Relative growth, % Cr^{6+} in medium μg ml^{-1}			IC_{50} values of Cr^{6+} for growth, μg/ml
	250	500	1000	
DI-01	21.00	0.00	0.00	177.8
DI-04	56.42	25.93	14.81	281.8
DI-06	61.75	16.67	0.00	295.1
DI-08	13.11	7.55	0.00	169.8
DI-22	68.00	36.96	0.00	371.5
DI-25	71.45	22.20	14.81	338.8
DI-29	78.10	64.81	0.00	588.8
KA-01	21.00	0.00	0.00	177.8
KA-02	64.00	30.56	11.11	316.2
RA-05	73.05	24.07	0.00	346.7
SA-05	49.58	19.61	13.73	251.1
SI-49	35.75	9.52	0.00	199.5
SU-05	38.00	8.33	4.16	208.9

Each value represents average of duplicates

Resistance to other metals. The selected isolates also showed varied degrees of tolerance to 5 different metals tested (Table II). Mercury was by far the most toxic metal, all the strains with the exception of one (RA-05) were totally inhibited at the lowest concentration (5 μg ml^{-1}). A number of strains for example, DI-01, DI-04 and DI-22 showed a wide range of sensitivities to Ni, Cu and Co and most of the strains tested were tolerant to Cd. Strains DI-08 and RA-05 were resistant to Cu^{2+}, Co^{2+} and Cd^{2+}. The chromium-resistant strains KA-02 and SA-05 on the other hand were sensitive to all 5 metals tested. The distribution profile of the sensitivity of all species examined against different metals revealed that the order of toxicity were $Hg^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+} > Cd^{2+}$.

Resistance to antibiotics. Antibiotic sensitivity profile of the actinomycete isolates have indicated that isolate RA-05 was resistant to all the antibiotics except cycloserine (Table III). Resistance of isolate RA-05 to a wide range of antibiotics was also correlated with its multiple metal resistance character. On the other hand, isolates DI-01, DI-22 and DI-25 were highly sensitive to most of antibiotics tested. The rest of the isolates exhibited a varied degree of sensitivity and resistance to different antibiotics.

Table II

Susceptibility of Cr^{6+}-resistant actinomycetes to other metals

Isolate	Diameter of inhibition zone, mm									
	Cu^{2+}		Ni^{2+}		Co^{2+}		Cd^{2+}		Hg^{2+}	
	50	100	50	100	50	100	50	100	5	10*
DI-01	17.5	22.0	20.0	30.5	22.0	31.0	NI	NI	25.0	28.0
DI-04	20.0	26.0	18.0	24.5	NI	20.0	NI	NI	25.0	30.5
D1-06	NI	13.5	NI	NI	NI	NI	16.0	20.0	28.0	32.0
DI-08	NI	NI	18.0	26.0	NI	NI	NI	NI	43.0	49.0
DI-22	NI	16.0	16.0	24.5	26.0	29.0	NI	NI	30.5	35.0
DI-25	NI	17.0	24.0	28.5	NI	17.5	NI	NI	25.0	30.5
DI-29	NI	25.5	22.0	24.5	NI	20.0	18.0	20.0	26.0	31.0
KA-0l	NI	18.0	20.0	24.5	NI	NI	NI	NI	28.5	32.0
KA-02	NI	22.0	22.0	27.5	22.0	29.5	18.0	20.0	26.5	31.0
RA-05	NI	NI	22.0	29.0	NI	NI	NI	NI	NI	23.5
SA-05	NI	21.0	18.0	26.0	NI	22.0	NI	20.0	34.0	40.0
SI-49	NI	16.0	NI	18.0	33.5	44.0	NI	22.0	26.0	31.0
SU-05	N1	19.0	NI	18.0	20.0	24.0	NI	NI	23.5	28.5

*Concentration of metal μg ml^{-1}

NI = No inhibition

Each value represents average of duplicates

Discussion

This study shows that actinomycetes isolated from natural soils were more tolerant to Cr^{6+} when tested in solid medium compared to that in liquid salts medium. Such a differential response may be due to the complex nature of the solid medium, the components of which could bind metal ions. The metal binding ability of the complex organic constituents of the solid medium – if any – is yet to be determined. Angle and

Table III

Antibiotic sensitivity profile of chromium-resistant actinomycetes isolates

Antibiotic Disc (conc, µg)	Diameter of inhibition zone, mm Isolate												
	DI-01	DI-04	DI-06	DI-08	DI-22	DI-25	DI-29	KA-01	KA-02	RA-05	SA-05	SI-49	SU-05
Ampicillin (10)	35 (S)	38 (S)	13 (R)	40 (S)	33 (S)	34 (S)	22.5 (I)	28 (I)	18 (R)	NI (R)	32 (S)	28 (I)	24 (I)
Bacitracin (10)	30 (S)	28 (S)	17.5 (S)	20 (S)	27 (S)	24 (S)	28 (S)	28 (S)	40 (S)	NI (R)	28 (S)	40 (S)	23 (S)
Chloramphenicol (30)	40 (S)	30 (S)	24 (S)	40 (S)	48 (S)	28 (S)	28 (S)	33 (S)	30 (S)	NI (R)	48 (S)	48 (S)	28 (S)
Chlortetracycline (30)	30 (S)	32 (S)	30 (S)	38 (S)	35 (S)	40 (S)	35 (S)	36 (S)	44 (S)	NI (R)	36 (S)	44 (S)	30 (S)
Cycloserine (200)	30 (S)	24 (S)	21 (I)	22 (S)	26 (S)	28 (S)	20 (I)	30 (S)	32 (S)	22 (S)	30 (S)	48 (S)	22 (S)
Erythromycin (15)	35 (S)	30 (S)	06 (S)	40 (S)	36 (S)	38 (S)	26 (S)	30 (S)	16 (S)	NI (R)	34 (S)	13 (R)	24 (S)
Gentamycin (10)	36 (S)	36 (S)	42 (S)	34 (S)	42 (S)	32 (S)	36 (S)	34 (S)	38 (S)	NI (R)	32 (S)	36 (S)	29 (S)
Neomycin (30)	28 (S)	24 (S)	28 (S)	26 (S)	33 (S)	22 (S)	41 (S)	31 (S)	26 (S)	NI (R)	26 (S)	26 (S)	22 (S)
Penicillin G (10)	40 (S)	40 (S)	15 (I)	38 (S)	36 (S)	40 (S)	27 (S)	34 (S)	10 (R)	NI (R)	42 (S)	32 (S)	28 (S)
Rifampicin (5)	28 (S)	22 (R)	29 (S)	22 (R)	22 (S)	25 (S)	NI (R)	38 (S)	18 (R)	NI (R)	18 (R)	43 (S)	30 (S)
Streptomycin (10)	40 (S)	40 (S)	20.5 (S)	36 (S)	37 (S)	20 (S)	28 (S)	37 (S)	NI (R)	NI (R)	38 (S)	28 (S)	33 (S)
Vancomycin (30)	42 (5)	35 (S)	35 (S)	42 (S)	34 (S)	40 (S)	28 (S)	38 (S)	48 (S)	NI (R)	35 (S)	46 (S)	34 (S)

NI = No inhibition
S = Sensitive
I = Intermediate
R = Resistant

Chaney [2], however, concluded that the activity of free metal ions in undefined growth media is affected by processes such as binding to, or chelation and/or complex formation with organic components of the media yielding erroneously high tolerance data.

The mechanism of Cr^{6+} tolerance of the actinomycete isolates is likely to involve surface binding, intracellular transport and accumulation via a specific Cr^{6+} transport system or production of biomolecules like metallothioneins, siderophores and analogous compounds which could complex with Cr^{6+}. The reduction of chromate (CrO_4^{2-}) to Cr^{3+} and its subsequent precipitation could not be ruled out as the possible mechanism. Moreover, Cr resistance has been correlated with the presence of plasmid DNA in several isolates.

Results as shown in Table II are in good conformity with those of Babich and Stotzky [3] who found that actinomycetes as a group are more tolerant to cadmium than other bacteria. It is also interesting to note that the order of heavy metal toxicity to actinomycetes as identified in this study differs significantly from those reported by Duxbury [9] and Abbas and Edwards [1] which were ordered as Hg > Cd > Cu > Ni > Zn and Hg > Cd > Co > Zn > Ni > Cu > Cr > Mn, respectively. The major differences would appear to be the relatively low position of cadmium as a toxic metal for streptomycetes and the corresponding higher toxicity of nickel.

The antibiotic resistance may also reflect the ability of the isolates to produce the antibiotic(s) to which they are resistant. Moreover, the multiple antibiotic resistance of some of these isolates also indicates the possible acquisition of a plasmid – conferred antibiotic resistance factors by the specific isolates. The results of our study show that these resistant actinomycete isolates might be useful in the transformation of toxic Cr^{6+} to less toxic Cr^{3+} or biosorption of chromium both from natural soils and contaminated sediments. Studies on the evaluation of chromium biosorption by resistant actinomycete isolates are in progress.

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CRYPTOSPORIDIOSIS IN IMMUNOCOMPROMISED PATIENTS IN A TURKISH UNIVERSITY HOSPITAL

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The prevalence of Cryptosporidiosis in 18 immunosuppressed diarrheic patients was evaluated by examination of fecal samples by direct staining (Modified Kinyoun and Giemsa), direct and indirect immunofluorescence methods. Forty patients (10 nondiarrheic immunosuppressed, 10 nondiarrheic immunocompetent, and 20 diarrheic immunocompetent) were included in the study as the control group. 11 of 18 samples were positive for cryptosporidial oocysts by at least one of the methods. Oocysts were detected in all (n=7) of the AIDS patients. This high frequency was attributed to a probable nosocomial infection. None of the samples from control subjects were found positive for Cryptosporidium. Our results indicate that Cryptosporidial oocysts should be detected particularly in immunosuppressed patients with diarrhea. Modified Kinyoun staining method is practical and reliable for this purpose. Immunofluorescence staining methods can be applied for confirmation of the results.

Cryptosporidium parvum is a coccidian protozoan, which has recently been recognized to be a human pathogen. The parasite has been detected to be nestled between the microvilli of gastrointestinal and respiratory epithelial cells. Cryptosporidial oocysts can be transmitted from human-to-human and animal-to-human via oral-fecal route by consumption of contaminated water and raw milk. Day-care centers and nosocomial outbreaks highly facilitate person-to-person spread [1–3].

Cryptosporidiosis is more commonly encountered in malnourished children, elderly and immunocompromised patients in underdeveloped countries [4]. While diarrhea is self-limited and lasts not more than 1-2 weeks in immunologically intact hosts, the clinical course is life-threatening in immunocompromised individuals. Profuse and long-lasting diarrhea, dehydration, electrolyte imbalance and extraintestinal invasion is met particularly in patients with AIDS [5, 6].

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The present study was undertaken to determine the prevalence of Cryptosporidiosis in immunocompromised patients with diarrhea and comparatively evaluate the use of various methods in detection of Cryptosporidium oocysts in fecal samples of the corresponding patients.

Materials and methods

Patients and control subjects. The study group consisted of 18 patients who were immunosuppressed due to various disorders and/or immunosuppressive drugs. Fecal samples were sent to Parasitology Laboratory, Department of Microbiology, Hacettepe University in a period of 17 months between July 1995 and January 1997 for detection of Cryptosporidium and other parasites. Forty patients constituted the control subjects of the study; these were chosen among immunosuppressed non-diarrheic patients (n=10), immunocompetent subjects who were examined in the hospital for signs and symptoms other than diarrhea and whose fecal samples were referred to Parasitology Laboratory for routine examination (n=10) and immunocompetent subjects who were admitted for diarrhea (n=20). Characteristics of the patients and the control subjects enrolled in the study are shown in Table I.

Fecal sample. The samples were stored at –30 °C in 10% formalin solution until tested and centrifuged prior to processing. Three samples were collected from each patient.

Detection of cryptosporidial oocysts

Direct staining methods. Fecal smears, stained by Modified Kinyoun Acid-Fast (MAF, Figure 1) and Giemsa method were examined under light microscope and Cryptosporidial oocysts were defined as described previously [7].

Direct fluorescence antibody stain (DIF). The method was performed by a commercially available "Merifluor Cryptosporidium Giardia kit" (Meridian Diagnostics, Inc. Ohio 45244) and the results were evaluated by fluorescence microscopy. Application of the test and interpretation of the results were done according to the instructions of the manufacturer [8, 9].

Indirect fluorescence stain (IIF). Mouse monoclonal antibodies (IgG and IgM culture supernatants) specific to *Cryptosporidium* oocysts were kindly provided from San Francisco General Hospital, Division of Infectious Diseases. The test was employed according to the instructions of "Merifluor Cryptosporidium indirect immunofluorescence kit" (Meridian Diagnostics, Inc. Ohio 45244) and the results were interpreted under fluorescence microscope, as described previously [8, 9].

Routine parasitological examination. Fecal samples of all patients included in the study were examined for the probable existence of egg and/or cyst form of commonly encountered parasites other than Cryptosporidium, by standard methods [10].

Results

Cryptosporidial oocysts could be detected in fecal samples of 11 out of 18 immunosuppressed diarrheic patients with at least one of the methods included in the study. Both direct staining and direct and indirect immunofluorescence methods were positive for 6 of 11 patients. Oocysts were detected by direct staining and direct immunofluorescence tests in 4 patients. The fecal sample of the remaining patient revealed the presence of oocysts by immunofluorescence methods only. All AIDS patients (n=7) were found to carry Cryptosporidial oocysts in faeces by at least one of the methods

Table I

Characteristics of patients enrolled in the study

Characteristic	Value
Patients	
No. of patients (M/F)	18 (10/8)
Age range in years (mean)	24–56 (39.7)
No. with immunosuppression and diarrhea	18
AIDS	7
AML	5
NHL	2
ALL	1
SLE	1
RT	1
Myelofibrosis	1
Control subjects	
No. of patients (M/F)	40
Age range in years (mean)	17–62 (36.6)
No. with immunosuppression and without diarrhea	10
AML	4
RT	3
ALL	1
NHL	1
HL	1
No. without immunosupression and without diarrhea	10
No. without immunosupression and with diarrhea	20

AML: Acute myeloblastic leukemia
ALL: Acute myeloblastic leukemia
NHL: Non-Hodgkin lymphoma
HL: Hodgkin lymphoma
SLE: Systemic lupus erythematosus
RT: Renal transplantation

used in the study. Time for hospitalization and detection of cryptosporidial oocysts in stool samples of AIDS patients were as follows: June 1995 (n=2), January 1996 (n=1), March 1996 (n=1), April 1996 (n=2), and May 1996 (n=1). All of the patients were hospitalized in AIDS Unit. Table II summarizes the clinical features and results of the Cryptosporidial oocyst detection tests of the 11 patients with cryptosporidiosis.

Cryptosporidial oocysts could be detected in none of the samples obtained from the control subjects whereas routine parasitological examination of these samples yielded the presence of Giardial cysts in two.

Discussion

The role of *Cryptosporidium parvum* in life-threatening diarrhea particularly in patients whose immune system has been suppressed due to various disorders or iatrogenic reasons is now well-established [5]. Detection of the definitive agent of diarrhea not only facilitates the direction of appropriate therapeutic approach but also avoids the use of unnecessary treatment modalities. The significance of the entity is more emphasized, depending on the data showing that the mortality in AIDS patients is due to opportunistic infections in about 90% of the cases. Cryptosporidiosis has been defined to cause a seriously debilitating clinical picture characterized by cholera-like profuse diarrhea, dehydration and electrolyte imbalance in patients with AIDS. Since asymptomatic carriage state has been reported in HIV-infected persons, it has been recommended that the fecal samples of such patients should be examined for detection of Cryptosporidial oocysts even in the absence of gastrointestinal symptoms [11–13].

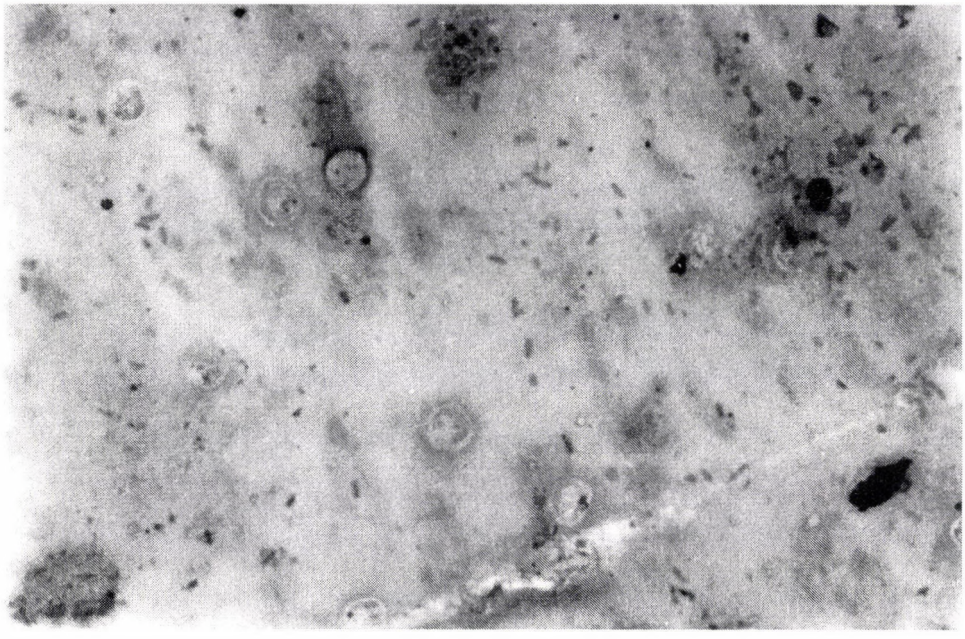

Fig. 1. Cryptosporidium oocysts by MAF (×1000)

Table II

Clinical features and the results of direct staining and immunofluorescence methods of the patients who were found positive for Cryptosporidium

Case No.	Age	Gender	Underlying disorder	Clinical features	Giemsa	MAF	DIF	IIF
1 NÖ	29	F	AIDS (heterosexual transmission)	Watery, profuse diarrhea for 4–5 months, 4–5 times/day	±*	+	±	–
2 FN	35	M	AIDS, PCP (heterosexual transmission)	Watery, profuse, mucus containing diarrhea for one year, 10 times/day	+	+	+	+
3 HÜ	42	M	AML, BMT	Watery, mucus containing diarrhea for 2 days, 2–3 times/day	±	+	+	+
4 GE	24	F	SLE	Watery diarrhea for 10 days, vomitting	+	+	+	–
5 TT	38	F	AIDS (heterosexual transmission)	Watery diarrhea for one week, 4–5 times/day	±	±	+	–
6 GG	55	M	AIDS (heterosexual transmission)	Diarrhea for 3 months; 3 times/day	+	+	+	+
7 SY	34	M	NHL	Diarrhea containing mucus for 3 days, 2–3 times/day, vomiting	±	+	+	+
8 EC	56	M	AIDS (transmission by blood transfusion)	Intermittent diarrhea for one year	±	+	+	+
9 VÖ	30	M	AIDS, CMV retinitis (IV drug user)	Watery diarrhea for one month, 3–5 times/day	±	+	+	–
10 ES	45	F	RT	Intermittent watery diarrhea for 2 years, 6–8 times/day	–	–	+	+
11 ÜN	32	F	AIDS (heterosexual transmission)	Watery diarrhea for 6 months, 5 times/day	+	+	+	+

M: Male
F: Female
AML: Acute myeloblastic leukemia
NHL: Non-Hodgkin lymphoma
SLE: Systemic lupus erythematosus
RT: Renal transplantation
BMT: Bone marrow transplantation
PCP: Pneumocystis carinii pneumonia
CMV: Cytomegalovirus
* If structures which resembled but were not exactly typical for the cryptosporidial oocysts were visualized, the result was denoted as ±

Our results indicated the presence of oocysts in fecal samples of all patients with AIDS included in the study by at least one of the methods used. CDC has reported a rate of 3.6% positivity among 19,182 AIDS patients in a survey carried out in 1986. This has been followed by detection of the parasite in 15–60% of AIDS patients, the incidence being notably higher in underdeveloped and developing countries. Laughon et al. [14] detected the parasite in 15.6% of 388 homosexual subjects with diarrhea. Smith et al. [15], on the other hand reported that 3 out of 20 diarrheic AIDS patients were positive for oocysts. The results of another study carried out in Haiti [16] indicated that 11 out of 29 AIDS patients with chronic diarrhea had Cryptosporidiosis. The incidence varied significantly among different countries, being 3% in Denmark, 11% in England, 12% in Brazil, 21.2% in France, 30% in Zaire and 32% in Zambia [17]. The number of AIDS patients is relatively low in Turkey compared to other countries (630 confirmed cases). Although the number of AIDS patients included in our study is low, our preliminary results indicate that the rate of positivity is considerably high. However, since the time for hospitalization and detection of oocysts is close for most of the AIDS patients and the patients were hospitalized in the same ward, it is almost impossible to rule out the existence of nosocomial spread. This high frequency thus was attributed to both a probable nosocomial infection as well as being a similar finding to the relatively high frequencies previously reported from other developing countries.

There have been reports in literature concerning the prevalence of Cryptosporidiosis in organ transplantation patients. Weisburger et al. [18] detected Cryptosporidium in jejunal biopsy specimen of a diarrheic renal transplantation patient taking high dose immunosuppressive therapy. Our study included one renal transplantation patient whose fecal sample was positive for Cryptosporidial oocysts.

Individuals who have underlying neoplastic disorders and are treated with immunosuppressive agents constitute another population with an increased susceptibility to development of opportunistic infection due to Cryptosporidium. A previous study carried out in Turkish patients with neoplastic diseases and gastroenteritis indicated Cryptosporidium oocysts in 16.9% of the study group [19]. 2 out of 9 patients (one acute myeloblastic leukemia and one Non-Hodgkin lymphoma) with neoplastic disorders were found positive for Cryptosporidium in our study. These results indicate that Cryptosporidiosis should be considered to be present in patients with neoplastic disorders.

Laboratory diagnosis of Cryptosporidiosis can be established by various techniques. Differentiation of oocysts and the yeast cells displays difficulties in some instances, which was also the case in our study, particularly during examination of Giemsa stained smears. Those samples were evaluated as positive-negative to avoid over and underestimations. MAF staining method is superior to Giemsa as far as this problem is concerned. However, false positive results were detected by prolonged application of the MAF stain, decreasing the sensitivity of the method [20].

Direct and indirect immunofluorescence tests which use monoclonal antibodies directed against the oocyst wall are known to be sensitive and rapid. Some of the studies showed that immunofluorescence is superior and more sensitive because of the ability of detection of low number of oocysts [8, 9, 21]. However, there have been reports claiming that the morphologic appearance of the oocyst may sometimes be difficult to recognize and nonspecific fluorescence may cause false-positive interpretation [22]. This and the

other factors stated above might be responsible for the differences between the results of direct staining and immunofluorescence methods in our study.

In conclusion, facilities to detect Cryptosporidial oocysts should be carried out in routine parasitology laboratories, particularly for examination of fecal samples of immunosuppressed diarrheic patients. MAF staining method appears to be practical and reliable for this purpose. Immunofluorescence techniques may also be used for confirmation, if possible, since the excreted oocysts may be low in number in some cases.

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BASIDIOCARP AND MYCELIUM MORPHOLOGY OF *GANODERMA LUCIDUM* KARST. STRAINS ISOLATED IN HUNGARY

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Morphological, anatomical and cultural characteristics of 14 *Ganoderma lucidum* (Fr.) Karst strains isolated in Hungary have been investigated. Macroscopically the basidiocarps of the Hungarian strains are absolutely identical with those of described previously about the *Ganoderma lucidum* species-complex. Microscopic features of the fruitbodies and basidiospores showed some differences from the typical *G. lucidum* species. Pilocystidia, forming a homogenous layer on the surface of the pileus, have smooth heads without protrusions and stalks not ramifying. Cell wall pillar density and width of the basidiospores also differ from that of regarded to be characteristic to *G. lucidum*. Although according to several authors chlamydospore formation is a characteristic feature of *G. lucidum* it has not been observed in mycelial cultures of the Hungarian strains.

Antagonistic reactions between the Hungarian and Far Eastern *G. lucidum* isolates were mostly similar to the interspecific reactions between the two species *G. lucidum* and *G. applanatum* and corresponded only in a few cases to the interactions within one species.

Our results suggest that the Hungarian strains significantly differ from the Far Eastern strains. To determine the taxonomic degree of this divergence genetical examinations should be carried out.

The genus *Ganoderma* had been established by Karsten [1] with a single species called *Ganoderma lucidum*.

Murrill [2] declared first that identification of the species is impossible by morphological investigations only and a complex system of criteria is to be elaborated. This is necessary because of the heterogenity within the species and the similarity between the different species of the genus. The macromorphologically similar species have been contracted by him to the *Ganoderma lucidum* species-complex, while keeping their individual species names.

Since this establishment a lot of investigations has been carried out to determine which properties are necessary and sufficient to distinguish the members of the genus

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Ganoderma. The most characteristic features used to identify *Ganoderma lucidum* species are the morphology of the fruit body [2], the host [2], the geographical area where the fruit body was collected [2], the morphology of pilocystidia [3], structure of the spore wall [4–7] and the properties of mycelial cultures [8–10].

Adaskaveg and Gilbertson [11] tried to synthesize the investigations on the most characteristic features known up to that time. The *Ganoderma lucidum* species-complex has been divided into two groups: to *G. lucidum* living on hardwood and *G. tsugae* living on softwood. As the most distinctive characteristics, the properties of the basidiospore and the parameters of mycelial cultures were investigated, and hybridization probes were carried out. All isolates of the *G. lucidum* group formed terminal or intercalar chlamydospores but none of those of the *G. tsugae* group. They demonstrated that the morphology of the pilocystidia and the density and size of the spore interwall pillars is specific [12]. Later Pegler and Young [4] found that all transient forms of pillars could be detected within the individual specimens. Wang and Hua [13] confirm that *G. tsugae* isolates do not form chlamydospores but they have not found them in all strains of *G. lucidum*, either.

The phenomenon of intraspecific antagonism has been known only for a few decades. Adams and Roth [14] recognized for the first time that a barrier can arise between the dikaryotic hyphae of the same species on agar plates. Antagonism is less intensive between genetically similar strains. According to Brasier the process has a polygenic regulation [15]. Examining this reaction of isolates collected from different sampling sites can help us to determine the taxonomic distance between two strains.

Adaskaveg and Gilbertson [15] carried out experiments with *G. lucidum* and *G. tsugae* strains to investigate intraspecific antagonism. Different stages of antagonism were detected. *G. lucidum* pairs always showed a strong reaction independently from being collected from spots near to or far from each other.

Ganoderma lucidum is common also in Hungary. The aim of our work was to investigate the macromorphological and anatomical features of Hungarian isolates considered to be characteristic for the species and compare them with the data known from literature.

Materials and methods

Strains investigated. Fruitbodies of *Ganoderma lucidum* have been collected from different locations of Hungary. All samples have been collected in deciduous (mainly oak) forests. Collection data are shown in Table I. Mycelial cultures have been isolated by cutting pieces of about 2×2 mm from the inner parts of the fruitbodies with a sterile scalpel and placing them on malate agar plates. The isolates were maintained on malate agar (MEA) at 26 °C. Beside our own isolates three strains from the Culture Collection and Research Centre, Taiwan (CCRC) have been studied. The strains are deposited in the collections of the Plant Anatomy Department of Eötvös Loránd University, Budapest and the Hungarian Natural History Museum.

Light microscopy (LM). Samples for light microscopy were prepared from mycelia of different age grown on malate agar (MEA) and potato-dextrose agar (PDA) or from basidiospores obtained from the basidiocarps. Mycelial samples were taken from the central (old) and marginal (young) parts of two-

week-old and six-week-old cultures. Preparates were mounted in lactophenol (lactic acid : glycerol : phenol : distilled water = 2 : 4 : 2 : 2). Staining with 1% aniline blue was applied occasionally. The samples were examined by Nomarski microscope using immersion objective of ×100 magnification.

Table I

Designation and place of collection of the Ganoderma lucidum strains used in our investigations

Strain	Sampling place
C17	Budakeszi
C64	Budakeszi
C114	Bükk
C115	Pilis, Kevélynyereg
C116	Pilis, Kevélynyereg
C118	Kópháza-Nagycenk
C119	Kópháza-Nagycenk
C120	Budakeszi
C122	Budai-mountains, Normafa
C123	Mátra, Valley of Tó-réti stream
C124	Mátra, Valley of Tó-réti stream
C125	Mátra, Valley of Tó-réti stream
C126	Mátra, Valley of Tó-réti stream
C127	Budai-mountains, Farkasvölgy
C129	Budai-mountains
C130	CCRC 36111
C131	CCRC 36144
C132	CCRC 36021

Scanning electron microscopy (SEM). Mycelial cultures, basidiospores and pilocystidia were examined by Hitachi 2300-N scanning electronmicroscope.

Agar blocks of 2×2 mm size cut from the mycelial cultures were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium-tetroxide. After washing in 0.07 M phosphate buffer (pH=7.2) a stepwise dehydration in ethanol (25%, 50%, 75%, 90%, 96%, absolute) and amyl-acetate was carried out. The samples were critical point dried, followed by coating with carbon and gold layers according to the slightly modified method of Wang and Hua [13]. The spores were dehydrated in xylol before coating.

Spore size was determined based on the SEM photos. Results are given as the averages of ten spores in three repetitions.

Pilocystidia were prepared for SEM investigations by solving the covering laccate layer with acetone from the surface of 5×5 mm pieces cut from the pileus. Treatments of 24, 48 and 72 h were used according to Adaskaveg and Gilbertson [12]. After this the samples were treated as the mycelial samples.

Density of interwall pillars were determined by counting them in a 2×2 μm quadrate of the spore surface. An average of twenty squares was calculated. As no exact density values of interwall pillars

have been published in the literature we tried to compare our data to those of other authors by determining pillar density from their published photos using the same calculating method.

Estimation of optimal growth temperature. The temperature optimal for growth was determined in two culture media (MEA and PDA [13]). Agar plates of 85 mm in diameter (15 ml medium) were inoculated in the centre with small mycelial discs. The diameter of the colonies grown on seven different temperatures (14 °C, 18 °C, 22 °C, 26 °C, 30 °C, 34 ° C and 38 °C) was measured on the seventh day. The experiment was carried out three times in three repetitions. Two values were treated as identical if their difference was not greater than 3 mm, which is about the error of the measurement.

Investigation of intraspecific antagonism. To investigate intraspecific antagonism besides our own *Ganoderma lucidum* strains a *Ganoderma applanatum* (C117) and a *Trametes versicolor* (own isolates) were used. The strains were inoculated in pairs to Petri dishes of 85 mm in diameter containing MEA. Mycelia of the same strains were inoculated together for control. Cultivation was carried out on 26 °C for 8 weeks.

Results

The basidiocarps, which the strains had been isolated from, were completely identical with the descriptions of Adaskaveg and Gilbertson [11]. All fruitbodies were collected from the soil of foliage forests, mainly oakwoods. As these associations are composed of different tree species usually no host could be identified.

The pilocystidia of the basidiocarps investigated are clavate, with shafts narrow at the base, rarely branched. The apical part is spherical, with smooth surface. No other type of cells appear in the surface layer (Fig. 1).

Table II

The size of the basidiospores in Ganoderma lucidum based on data from the literature and own measurements

Spore size	Treatment of spore	Sampling place	Author
9–(11.5)–13 × 6–(7)–8 µm	10% KOH solution	Great-Britain	Pegler and Young [4]
9.5–12 × 6–6.5 µm	drying	East-England	Corner [7]
10–13 × 6.8–8 µm	KOH solution	East-England	Corner [7]
10.6–(11.5)–11.8 × 6.8–(7.4)–7.8 µm	2% KOH	North America	Adaskaveg and Gilbertson [11]
7.0–10.5 × 5.0–7.0 µm	nd	Hungary	Igmándy [19]
9–12 × 6–7 µm	SEM-fixed	North America	Mims and Seabury [20]
7.7–(9.5)–10.8 × 5.5–(6.2)–6.7 µm	drying	Hungary	own results

The average values are in brackets if published.
nd = no data

Corresponding to the characteristic form of the genus, basidiospores are ovate, with truncated apex when dried and brown in colour. The surface of the spores is slightly dimped, uneven (Fig. 2). Spore wall has two layers with inner-wall pillars. The average number of pillars in a 2×2 μm quadrate is 7.8. The size of the spores corresponds to that measured previously [4, 7, 12, 19, 20] (Table II).

The mycelial cultures of the Hungarian strains were homogenous in their growth parameters. The optimal growth temperature was different on the two media investigated (Fig. 3, Table III). The optimal temperature for the majority of the strains on PDA was 22 °C. 26 °C was better by the use of MEA although in the case of two strains growth on 22 °C and 26 °C was equal. At extreme temperatures the rate of growth was quite similar in all strains. These rates were about the half or the third of the maximal values.

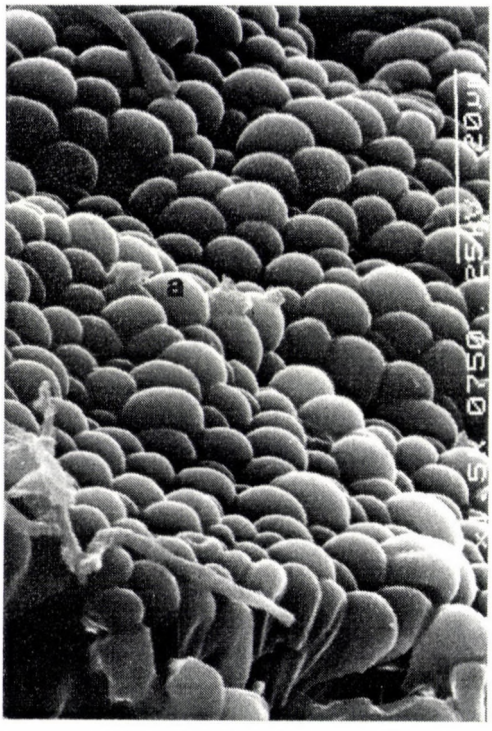

Fig. 1. Pilocystidia form a homogenous surface. No other types of cells occur. The pilocystidal apices are smooth, without any projections (SEM). a = pilocystidal apices

Generative hyphae and skeletal hyphae of different thickness could be observed in the mycelial cultures (Fig. 4). Cuticular cells are characteristic to all strains. They can occur as young, thin-walled or old, thick-walled forms. Incorporation of a yellow pigment

into the walls of the old cuticular cells and the densely branched staghorn hyphae (Fig. 5) is common. Arthrospores could be found in several strains (Fig. 6) but none of the Hungarian isolates produced any chlamydospores.

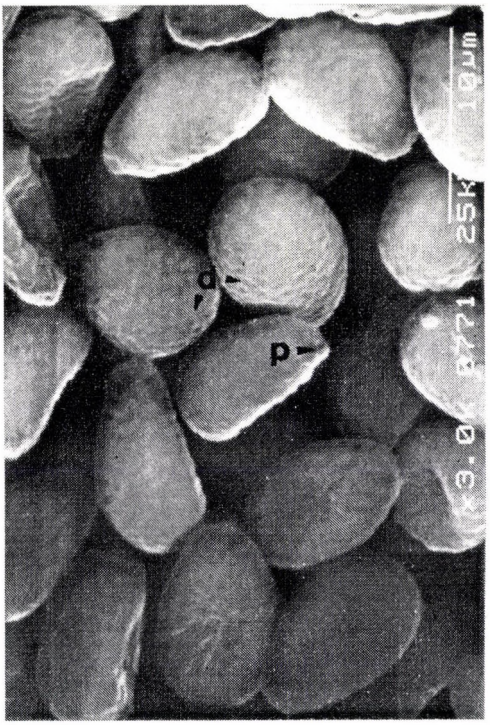

Fig. 2. Basidiospores of *Ganoderma lucidum* (SEM). Depressions indicating the peaks of the interwall pillars are visible. The apical part of the spore is slightly truncated. d = depression indicating the pillar, p = truncated apex

The intraspecific antagonism of 15 Hungarian and 2 Far-Eastern *Ganoderma lucidum* strains has been investigated as well as their interactions with *Trametes versicolor* and *G. applanatum*. The latter two species grew much more intensively than the *G. lucidum* isolates. Within two weeks they completely grew over the mycelium of *G. lucidum*. No special structural alteration in the mycelia of the different species was visible macroscopically. This confirms the observations of Rayner et al. [16] who detected a similar interaction between two strains of different species.

The Far-Eastern strains grew also much faster than the Hungarian ones. The Hungarian isolates inhibited the growth of the Far-Eastern ones unequally. A barrier of mycelium emerging from the medium has been produced but the Far-Eastern strains

covered this in most cases by the end of the experiment. This phenomenon corresponds to an intraspecific reaction (as a barrier arises) and also with an interspecific reaction (the mycelia of Far Eastern strains covered the Hungarian ones).

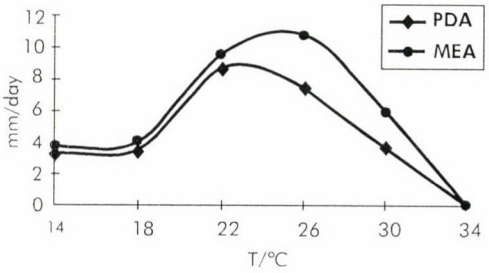

Fig. 3. Growth of Hungarian *Ganoderma lucidum* strains on PDA and MEA at different temperatures. Growth on MEA is more intensive than that on PDA

Table III

Growth of mycelial cultures of Ganoderma lucidum and G. tsugae at different temperatures (comparison of published data with own results)

Species	*Ganoderma lucidum*			*Ganoderma tsugae*	
Author	AG [11]	Wang and Hua [13]	own results	AG [11]	Wang and Hua [13]
Growth on MEA	7.8	9.1	10.6	1–3	1.8
Growth on PDA	nd	7.8	8.6	nd	nd
Optimal growth	30–34	26–30	22–26	20–25	22–26
Temperature range of growth	<10–42	14–38	10–30	10–30	14–30

nd = no data
AG = Adaskaveg and Gilbertson

Antagonistic reaction between the Hungarian isolates had fully developed by the end of the sixth week. During the first week, similarly to the result of Rayner and Todd [17], only normal anastomoses could be observed like within the mycelia of the same

strain. The antagonistic reaction started in the second week. In some cases a macroscopically visible white or brown barrier emerged from the mycelium during the second or third week. This changed its colour or height later. In other cases at first a white, slightly emerging mycelial barrier arose which was decomposed between the fourth and sixth week and a macroscopically empty zone was formed. This zone was bordered by two white mycelium zones, slightly thicker than the normal mycelium. In some cases (e.g. strains C64 and C125) this zone has arisen also between two barriers. Among strains collected in Hungary no correlation between the type of antagonism and the origin of the strains could be demonstrated although isolates from distant places have been investigated. The formation of barrier was more frequent than the formation of empty zone. Thus some strains (e.g. C114, C118, C122) are rather of the latter type.

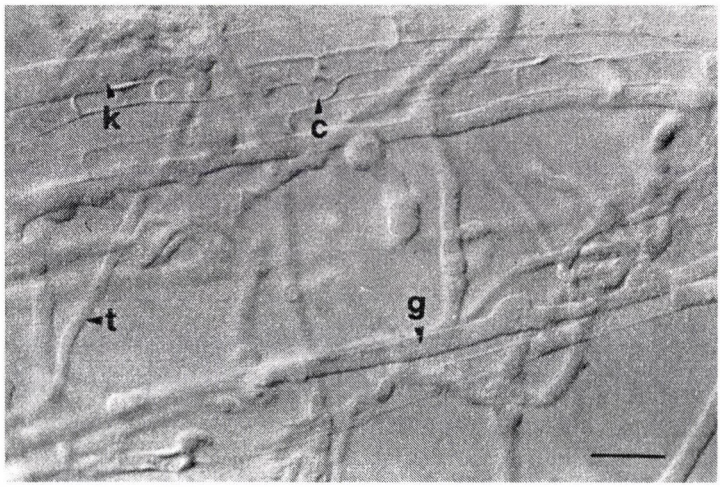

Fig. 4. Different types of hyphae from the mycelial culture of *Ganoderma lucidum*. Generative hyphae with clamps and skeletal hyphae of different thickness are visible. Nomarski IM. g = generative hypha, c = clamp, t = thin skeletal hypha, k = thick skeletal hypha, bar = 10 μm

Combining the isolates within themselves always formed normal anastomoses. The mycelial structures developing during the antagonistic interactions have been investigated also microscopically. The mycelial barrier arising between two strains mainly consisted of pseudoparenchym containing a brown pigment. Both sides of the frontier were absolutely similar to the normal lateral zone of the young mycelium.

In the other type of antagonism, which is characterized by the decomposition of the meeting hyphae, a loose, submers mycelial net is formed in the macroscopically empty zone. At the border of the submers zone and the part covered by hyphae no swollen or densely branching cells could be seen as it had been observed by Adaskaveg and Gilbertson [15] in other *Ganoderma lucidum* samples. Only the hyphal net was denser at

the frontier. The border of the submers and the superficial parts was similar to the mycelium observed at the border of Petri dishes. Submers-type antagonism caused intensive arthrospore-formation in strain C114. (This strain does not form arthrospores alone.) This phenomenon could not be observed in other strains. No chlamydospores in any of the Hungarian strains have been formed but they always could be detected in the Far Eastern strains.

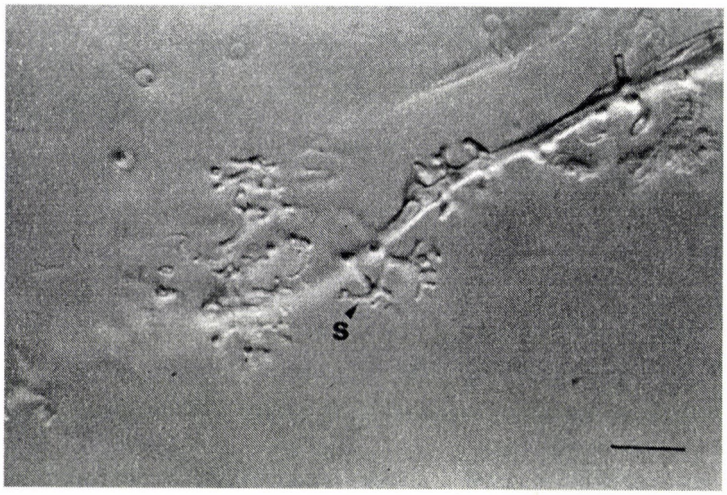

Fig. 5. Densely branched, well-developed staghorn hypha. Yellow pigment is incorporated into the cell walls. Nomarski IM. s = staghorn hypha, bar = 10 μm

Discussion

Our results show significant differences in specific characteristics of *Ganoderma lucidum* previously described by other authors.

The structure of the pilocystidial layer differs from that of observed by Adaskaveg and Gilbertson [12]. They suggested that the simple structure, composed by a single cell-type, (having been found also by us) is characteristic to the group *G. tsugae* although the shape of the cells is similar to that of the *G. lucidum* group. Also pillar density of the spores, considered to be an important property, differs slightly from that of visible in the photos of Adaskaveg and Gilbertson [11], where the number of pillars in a 2×2 μm quadrat is 5.9 in *G. tsugae* and 10.0 in *G. lucidum*. The Hungarian strains show transient values of these two characteristics.

Fig. 6. Arthrospores from the mycelial culture of *Ganoderma lucidum*

The characteristics of mycelial cultures are used for more precise identification. The optimal temperature of growth of the Hungarian isolates was lower than that of any previously published isolate (Table III). The growth of our strains on 26 °C was faster than that of investigated by Adaskaveg and Gilbertson [11] and Wang and Hua [13] and even faster at the temperatures optimal for their strains. Similarly to our results Adaskaveg and Gilbertson [11] observed significant growth also beyond 14 °C while Wang and Hua [13] could demonstrate growth at 14 °C by one out of 12 strain. 34 °C was the optimal temperature for several strains of these authors while none of ours could even grow at this temperature. The temperature range adequate for the Hungarian isolates was significantly more restricted in both media investigated than those of determined by Adaskaveg and Gilbertson [11] and Wang and Hua [13].

A basic difference has been found in chlamydospore formation, considered by Nobles [10] and Adaskaveg and Gilbertson [11] as extremely suitable for differentiation between species. None of the Hungarian isolates formed any chlamydospores. However, arthrospores mentioned also by Wang and Hua [13], had been produced also in some of the Hungarian isolates.

The interaction of *G. lucidum* strains with *Trametes versicolor* and *G. applanatum* was similar to that described by Rayner et al. [16] as interspecific reaction. However intraspecific reactions of our strains differed significantly from those of described previously. Within *G. lucidum* and *G. tsugae* [15] and within *T. versicolor* itself only [18] a reaction resulting in an empty zone had been observed. Whereas the majority of our strains produced a previously unknown reaction called by us "barrier-type" antagonism. Taken also into consideration the microscopic structure two possible mechanism is suggested. In both cases excretion of a substance is supposed to cause the antagonism as quite a long time is needed for its development.

According to the first theory, significant part of the mycelium in the meeting zone, leaving only a loose mycelial net, will be degraded if much excreted substance of great activity is produced. Out of the zone of this effect a normal mycelium develops. If the excreted substance is few or less active a weak reaction takes place. The strains can live quite close to each other but a more differentiated structure separating the two strains arises at the border.

According to the other theory in typical barrier-forming strains a pseudo-parenchymatous barrier arises faster than in submers-type ones. The inhibiting substance, which causes the degradation of the loose, thin-walled mycelium cannot invade into the pseudoparenchymatous tissue formed by the thick-walled cells. Therefore the thin-walled mycelium will be degraded but the barrier already developed cannot degrade. In case of both antagonistic reactions outside the zone of the inhibitor the structure of the mycelium is similar to the normal type. The exact clearing up of these processes should be carried out by the methods used for examining interspecific allelopathic reactions. Confirmation or denial of these theories need further investigations and could be a possible continuation of this work.

Based upon microscopic characteristics, used generally in the literature for interspecific distinction, we may state that the Hungarian *Ganoderma lucidum* isolates form a distinct category at a species or subspecies level. This is especially verified by the absence of chlamydospores in Hungarian isolates, a characteristic to be stated as specific by all authentic authors. In addition the pilocystidal layer, the spore-wall structure and the morphological type of the intraspecific antagonistic reactions are also different from strains known from the literature.

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PARTIAL CHARACTERIZATION OF TWO LYMPHOCYTE-SPECIFIC NATURAL AUTOANTIBODIES ISOLATED FROM NEWBORN MICE

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The early antibody-repertoire is mainly composed of multi- and polyreactive IgM autoantibodies. Some of them (about 10%) also react with cell surface antigens. It is supposed that these latter antibodies promote – particularly under the maturation of the immune system – the expansion of different lymphocyte populations and subpopulations, namely the filling of the secondary lymphoid organs by immunocompetent cells. In order to verify this hypothesis we established hybridoma clones from newborn Balb/c mice. Almost all IgM antibodies produced by these hybridomas (94%) were found to be polyreactive, but only two – the 4/5 and the 8/8 – react with cell surface structures. The 4/5 IgM recognizes a glicerin-phosphathydil-inositol (GPI)-anchored membrane component expressed on the early thymocytes. The 8/8 antibody reacts with resting splenic B lymphocytes as well as B and T cell blasts via an about 40 kD protein. They do not influence the spontaneous or the mitogen induced proliferation of the lymphocytes. Therefore we cannot confirm the hypothesis that increase of the expansion of peripheral lymphocytes would be the main function of these neonatal natural autoantibodies.

B1 cells represent a unique subpopulation of B cells with distinct phenotypic, developmental and functional properties. Uniformly they are IgM^{high}, IgD^{low}, $B220^{low}$, a part of them expresses CD5 (in mouse called Ly-1), a pan T cell marker and they possess autonomous proliferative capacity and a long life span [1–4]. B1 cells appear earlier during the ontogeny than the conventional B (B2) cells, this is why in embryo and newborn the majority of B cells are 1-type. During maturation of the immune system the rapidly dividing B2 cells prevail but B1 cells are also present in the adult organism. They are mainly located in the peritoneal cavity and they are also present at a low frequency in the spleen [5, 6].

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The most important question concerning B1 cells is: what is the function of them within the immune system? It is widely accepted that they mainly produce polyreactive IgM autoantibodies [7] that may be involved in setting up an idiotypic network [8, 9] and could act as a first line of defence against a wide range of microorganisms. A high proportion of the natural autoantibodies reacts with a range of intracellular structures including the cytoskeleton, cell nuclei and mitochondria [10]. Some of them (about 10%) also react with surface antigens of thymocytes, T and B lymphocytes [11–14]. A hypothesis was supposed [15, 16] that the main function of B1 cells via autoantibodies would be the promotion of the expansion of different lymphocyte populations and subpopulations – particularly under the maturation of the immune system –, namely the filling of the secondary lymphoid organs by immunocompetent cells.

In this work a monoclonal antibody panel was created from hybridomas derived from the fusion of the spleen cells of 4-day-old Balb/c mice and Sp2/0 myeloma cells. Antibodies which proved to be autoreactive were studied whether they react with cell surface structures and influence the activation and the spontaneous or mitogen induced proliferation of T or B lymphocytes verifying the previous hypothesis.

Materials and methods

Animals. We used newborn (4-day-old) and young (1–2-month-old) inbred Balb/c, DBA/2, 129 SW, CBA, C3H and C57B1/6 mice derived from LATI (Gödöllő, Hungary). LOU/M/Wsl rats were bred in our animal facility.

Antibodies and reagents. Neonatal autoantibody-producer hybridomas were established from the fusion of the spleen cells of 4-day-old Balb/c mice and Sp2/0 myeloma cells by the method of Köhler and Milstein [18]. Antibodies were purified on 2,4,6-trinitrophenyl (TNP)- or fluorescein isothiocyanate (FITC)-bovine serum albumin (BSA)-coated Sepharose 4B column. FITC, BSA, TNP, lipopolysaccharide (LPS), phytohemagglutinin (PHA), concanavalin A (ConA), nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rat-anti-mouse-B220-FITC, hamster-anti-mouse-CD3-FITC and phycoerythrin-labelled goat-anti-mouse IgM derived from Pharmacia Fine Chemicals (Uppsala, Sweden). Peroxidase-labelled goat-anti-mouse IgM and alkaline phosphatase-conjugated goat-anti-mouse IgM were obtained from Sigma.

Enzyme immunoassay. For screening the monoclonal autoantibodies indirect ELISA was used. Polystyrene ELISA plates were coated with 10 µg/ml TNP-BSA, FITC-BSA, BSA, DNA and actin (50 µl). The antigen-coated plates were washed thoroughly with phosphate buffered saline (PBS) containing 0.1% Tween-20, and were saturated with PBS containing 1% BSA. After washing plates were incubated for 1 hour at 37 °C with 32 distinct monoclonal antibody in 1, 5 and 25 µg/ml concentration in 50 µl. After five washings with PBS-Tween the plates were incubated for 1 hour at 37 °C with peroxidase-labelled goat-anti-mouse IgM in 50 µl. Reacting antibodies were visualized by reaction with ortho-phenylen-diamin (OPD) containing H_2O_2. The resultant colour was determined by MR 700 microplate reader.

Flow cytometry analyses. For FACS analyses 10^6 cells were incubated for 15 minutes with 10 µg/ml neonatal autoantibodies or 10 µg/ml FITC-labelled rat-anti-mouse-B220, or 10 µg/ml hamster-anti-mouse-CD3 in 1% BSA, and 0.1% sodium azide containing PBS. When we used the neonatal autoantibodies we applied a phycoerythrin-labelled goat-anti-mouse IgM as a second antibody. Before measuring cells were washed twice in PBS/BSA. Cell debris and dead cells were excluded by light

scatter parameters and propidium iodide gating. Isotype-matched, irrelevant antibodies were used as negative controls. Analyses were undertaken in a FACScan analyser (Becton Dickinson, Mountain View, CA, USA).

Ca^{2+} measurement. 5×10^6 cells were loaded with 5 μM fluo-3 in 1 ml RPMI 1640 for 40 minutes at 37 °C in waterbath by shaking. Then 10 volume RPMI (without FCS) was added and cells were incubated for 40 minutes at 37 °C. Cells were washed twice and were diluted to 5×10^5 cells/ml and 1 ml samples were measured with flow cytometer in the presence of 5 μg/ml monoclonal autoantibodies. As controls we used anti-Thy-1.2 antibody on thymocytes and goat-anti-mouse IgM $F(ab)'_2$ on B cells.

In vitro cell cultures. Spleen cells and thymocytes prepared from newborn and adult Balb/c mice were cultured in 5% CO_2-containing incubator at 37 °C in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA), L-glutamine (2 mM), Na-piruvate (1 mM), non-essential amino acids (Gibco), 2-mercapto-ethanol (5×10^{-5} M), HEPES buffer (20 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cell concentration was 2×10^5 in 200 μl/wells in a 96-well-microtiter plate (Costar 3596, Costar Data Packaging, Cambridge, MA, USA). Cells were stimulated with 20 μg/ml LPS, 5 μg/ml ConA or 5 μg/ml PHA in the presence or absence of 5, 10 or 20 μg/ml of monoclonal autoantibodies. After 48 hours incubation 0.5 μCi [^{3}H]-thymidin was added to the cultures. Cells were harvested after a further 18 hours incubation. [^{3}H]-thymidin incorporation was measured by a fluid-scintillator.

Western blot analyses. Balb/c and C57Bl/6 spleen cells were disrupted by 3×5 seconds bursts of sonication. Membrane was obtained by centrifuging with 186 000 g for 40 min. Membrane extracts (20 μg) were separated on 12.5% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Blots were incubated with 8/8 monoclonal antibody or rat-anti-mouse-B220 and an alkaline phosphatase-conjugated goat-anti-mouse IgM, as a second antibody. Reacting bands were visualised by reaction with NBT- and BCIP-containing reagent.

Results

With the fusion of the spleen cells of 4-day-old Balb/c mice and Sp2/0 myeloma cells hybridoma clones were established. Thirty-two clones were picked out from 70 and antibodies were purified. Thirty of the 32 antibodies proved to be poly- and autoreactive (tested with TNP-BSA, FITC-BSA, BSA, DNA and actin) (Table I) and only two – the 4/5 and the 8/8 – IgM-s reacted with cell surface structures.

A GPI-anchored protein on the early thymocytes is the antigen of 4/5 IgM. Thymocytes, bone marrow, spleen and lymph node cells were tested with FACS analysis to react with neonatal autoantibodies. It was found that 4/5 IgM binds to thymocyte surface antigen. The binding is weak but dose dependent (data not shown) and is neither strain- nor species-specific (performed with Balb/c, 129 SW, CBA, DBA/2, C3H, C57B1/6 mouse and LOU/M/Wsl rat strains) (Fig. 1).

Mice were injected intraperitoneally with 2.5 mg hydrocortisone. Only $CD4^+$ and $CD8^+$ mature T lymphocytes survive this treatment, the early T cell progenitors die [19]. After two days thymocytes were preparated and their 4/5-binding capacity was measured. 4/5 IgM does not bind to mature T lymphocytes (Fig. 2).

10^6 Balb/c thymocytes were treated with 1 U phosphatydyl-inosilol specific phospholipase C (PI-PLC) at 37 °C for 1 hour. After PI-PLC treatment 4/5 IgM does not bind to GPI-anchored protein-digested thymocytes (Fig. 3).

Table I

MAb	TNP-BSA	FITC-BSA	BSA	DNA	ACTIN
1/1	▨	▨		▨	
1/4	▨				
1/5	▨				
1/11	■	▨			
2/3	■	□	▨	▨	▨
2/5	▨				
2/10	□	□		▨	
2/13	▨	▨		▨	▨
2/15	▨			▨	
2/21	▨		▨		
3/9	□	▨		▨	
3/17	▨	□			
3/25	□	□			
4/5	▨	▨			
4/6	□	□		▨	
4/12	□	▨			
4/18					
4/20					
4/21	□	□	▨	□	□
4/26	▨				▨
6/17	□	▨			
6/26	□				
6/31	□	□	▨	□	□
6/34	□	□	▨	▨	▨
7/1					
7/8	□				
7/14					
7/16	▨	□			
7/20	□	□	▨	□	□
7/21	□	▨			
8/8	□	□		□	□
8/14	▨	▨	▨		

Reactivity pattern of monoclonal IgM antibodies: Binding strength is indicated by the filling pattern of the squares. Filled (black) squares represent positive reaction (OD > 0.1) at a concentration of 1 µg/ml; striped squares represent positive reaction at a concentration of 5 µg/ml; pointed squares represent positive reaction at a concentration of 25 µ/ml; and open squares represent no detectable binding.

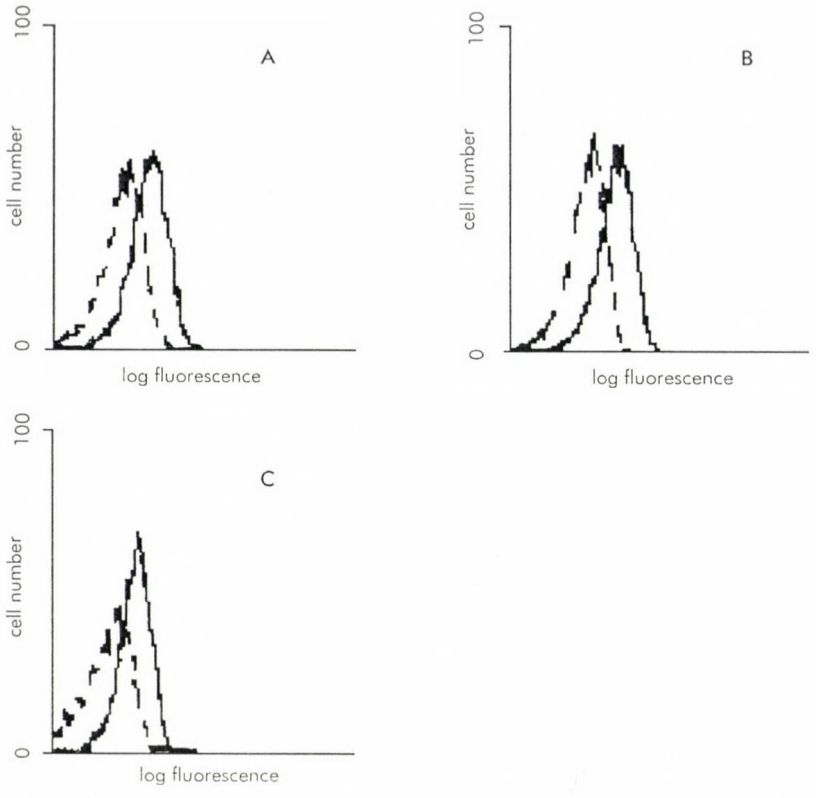

Fig. 1. Immunfluorescence staining of thymocytes derived from Balb/c (a), 129 SW (b) mice and LOU/M/Wsl rat strain (c) with 4/5 mAb

8/8 IgM recognises a 40-kD protein on splenic lymphocytes. 8/8 IgM was found to react with a part of resting splenic B lymphocytes but not with T lymphocytes (Fig. 4a, b). When spleen cells were activated with LPS, ConA or PHA 8/8 recognised the B and T cell blasts (Fig. 4c, d). In these experiments spleen cells derived from C57B1/6 mouse strain were used because its IgM molecules have the b allotype. Neonatal IgM molecules derived from the Balb/c strain are IgM^a. With biotin labelled DS1 antibody – a mouse-anti-mouse IgM^a – neonatal IgM-s were easily distinguishable from the IgM-s on B lymphocytes.

In Western blot analysis blots with the membrane protein extracts of spleen cells derived from Balb/c and C57B1/6 mice were incubated with 8/8 monoclonal antibody, ratanti-mouse-B220 and an alkaline phosphatase-conjugated goat-anti-mouse IgM as a second antibody. 8/8 IgM binds specifically an approximately 40 kD molecular weight protein (Fig. 5), which is supposed to be expressed on the resting splenic B lymphocytes and splenic T and B lymphoblasts.

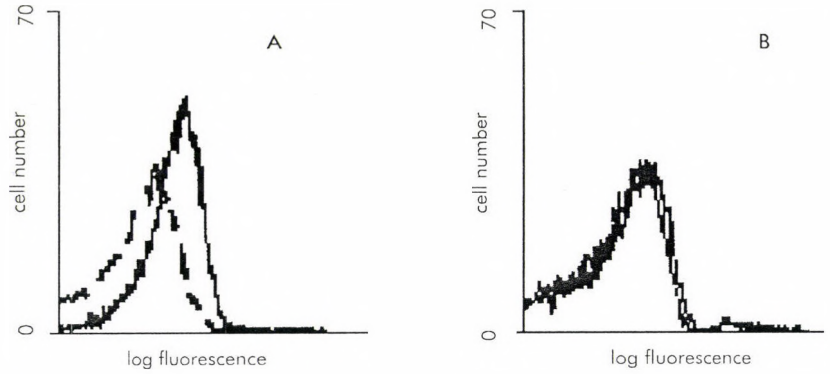

Fig. 2. Immunofluorescence staining of thymocytes derived from Balb/c mice with 4/5 mAb before (a) and after (b) hydrocortisone treatment. Balb/c mice were injected with 2.5 mg/ml hydrocortisone intraperitoneally. After two days thymocytes were preparated. 10^6 cells were incubated with 10 µg 4/5 IgM in 10 µl and after washing cells were incubated with phycoerythrin-labelled goat anti-mouse IgM. Flow cytometry analysis was performed beside untreated control cells. 4/5 IgM does not bind to hydrocortisone-resistant thymocytes

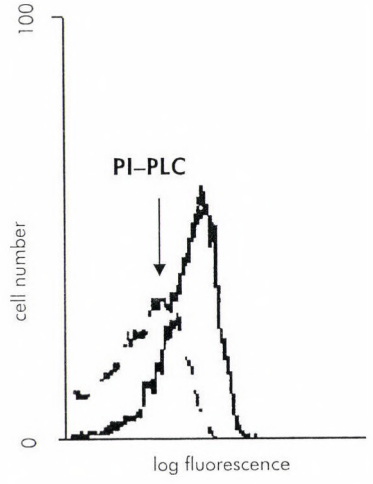

Fig. 3. Immunofluorescence staining of Balb/c thymocytes with 4/5 mAb before and after PI-PLC treatment. 10^6 Balb/c thymocytes were treated with 1 U PI-PLC in 100 µl medium supplemented with BSA (2 mg/ml) and 2-mercapto-ethanol (5×10^{-5} M) at 37 °C for 1 hour. Before measuring cells were washed twice with cold PBS/BSA. After PI-PLC treatment 4/5 IgM does not bind to GPI-anchored protein-digested thymocytes

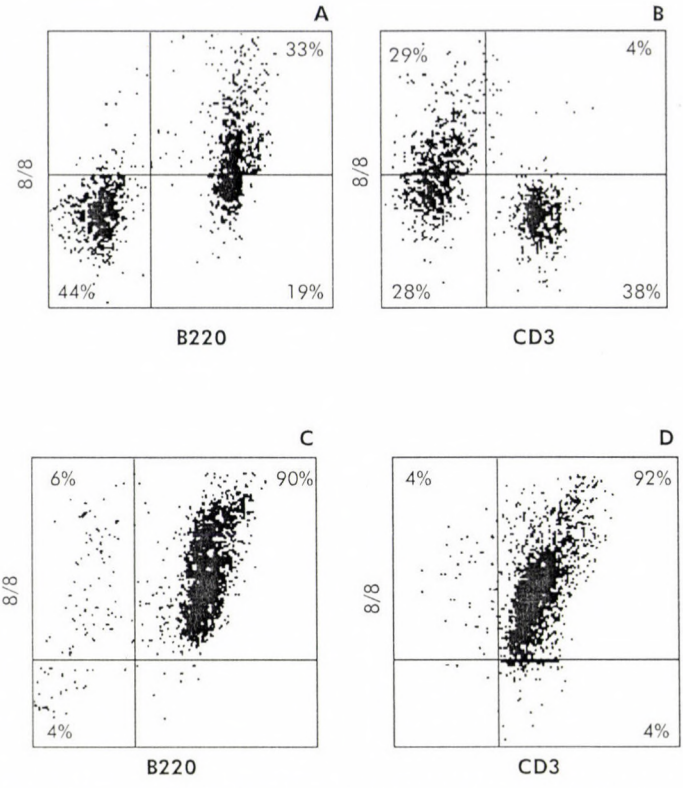

Fig. 4. Immunofluorescence staining of resting and activated spleen cells with 8/8 mAb + anti-IgM^a-phycoerytrine and anti-B220-FITC or anti-CD3-FITC mAb. 8/8 IgM reacts with a part of resting splenic B lymphocytes (a) but not with T lymphocytes (b). After LPS or PHA-activation of the splenic cells 8/8 IgM binds both of the B (c) and T (d) cell blasts

8/8 and 4/5 autoantibodies do not induce Ca^{++} release in adequate cells. Thymocytes and splenic cells were loaded with fluo-3 and in the presence of 4/5 or 8/8 IgMs Ca^{++} release was measured. We used anti-Thy-1.2 antibody on thymocytes and goat-anti-mouse IgM $F(ab)'_2$ on B cells as controls. 8/8 and 4/5 autoantibodies do not induce Ca^{++} release in the adequate cells (data not shown).

Neonatal autoantibodies do not influence the spontaneous or mitogen induced proliferation of lymphocytes. All the 32 monoclonal autoantibodies were tested whether they influence the spontaneous or mitogen (LPS, ConA and PHA) induced proliferation of lymphocytes derived from the thymus or spleen of neonatal (5–10-day-old) and adult mice. The presence of the autoantibodies (5, 10 or 20 μg/ml) in the in vitro cell cultures had no significant and reproducible effect on spontaneous or mitogen-induced proliferation (data not shown).

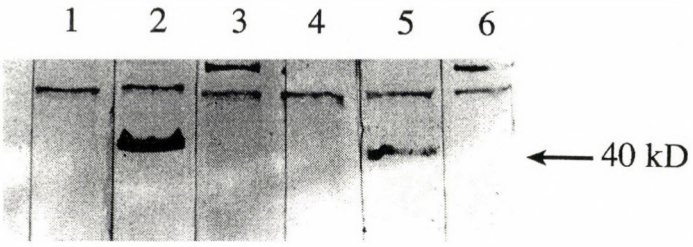

Fig. 5. Membrane protein extracts of spleen cells derived from Balb/c (lanes 1–3) and C57Bl/6 (lanes 4–6) mice were incubated with alkaline phosphatase-conjugated goat-anti-mouse IgM (GAM-IgM-AP) (lanes 1–4), with 8/8 IgM + GAM-IgM-AP (lanes 2–5) and with rat-anti-mouse-B220 + GAM-IgM-AP (lanes 3–6). 8/8 IgM binds specifically an approximately 40 kD molecular weight protein

Discussion

It was found that two antibodies from a monoclonal IgM autoantibody panel derived from hybridomas of the spleen cells of 4-day-old Balb/c mice and Sp2/0 myeloma cells bind to cell surface structures. One is the 4/5 IgM which binds to a GPI-anchored membrane component expressed on the early (cortisone sensitive) thymocytes. This membrane protein can be found neither on bone marrow, spleen and lymph node cells, nor on cortisone resistant thymocytes. Similar autoantibody (NMT-1) was described by J. R. Underwood et al. [11]. They have also found association between the expression of the cell surface autoantigen identified by the NMT-1 antibody and thymocyte maturation. They supposed the autoantigen to be a unique glycosylated form of the Thy-1 molecule. A. Lehuen [13] gave an account of three other monoclonal autoantibodies which recognize a 100 kD protein. The expression of that protein depends on the presence of the Thy-1 antigen. The specificity of the 4/5 antibody established by us also could be related to the Thy-1 molecule. Another antibody, the 8/8 IgM, reacts with resting splenic B lymphocytes as well as B and T cell blasts via an about 40 kD protein. Additional experiments are in progress to identify this protein.

Although some authors have argued that B1 cells may just represent a vestigial arm of the humoral immune system, it is hard to imagine that a population of B cells producing more than 50% of the "natural" serum IgM serves no purpose. This is the reason of enormous variations of hypotheses referring to the possible function of these cells. It was supposed [15–16] that the main function of B1 cells (via autoantibodies) would be the promotion – particularly during maturation of the immune system – of the expansion of different lymphocyte populations and subpopulations, namely the filling of the secondary lymphoid organs by immunocompetent cells. Studying in vitro the spontaneous or mitogen induced proliferation of lymphocytes derived from the thymus or spleen of neonatal (5–10-day-old) and adult mice by our 32 neonatal IgM autoantibodies,

we could not verify this hypothesis. Neither the two lymphocyte-specific (4/5 and 8/8), nor the other polyreactive autoantibodies were able to stimulate normal lymphocytes under the culture conditions used.

Of course, we cannot exclude the possibility that these antibodies reacting with self determinants do not play a major role in self-tolerance in vivo, either by binding to self-antigens and thus precluding the activation of other self-reactive T and B cells or through interaction within the idiotype network [14, 17]. Therefore, it should be of interest to investigate whether the lymphocyte-specific natural autoantibodies might be able to promote some autoimmune diseases such as spontaneous anaemia of NZB mice or systemic lupus erythematosus (SLE) of (NZB × NZW)F1 animals. This possibility is currently under investigation in our laboratory.

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MISKOLC, AUGUST 24–26, 1998

ABSTRACTS OF PAPERS AND POSTERS

Acta Microbiologica et Immunologica Hungarica, 46 (1), pp. 65–160 (1999)

AGRICULTURAL AND FOOD MICROBIOLOGY

M. JUHÁSZ-ROMÁN

Physiological examination of probiotic lactobacillus starters

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Lactobacillus species constitute a significant proportion of probiotic cultures used in developed countries in microbial adjunct nutrition. Fermented milks – containing *Lactobacillus acidophilus*, which has a good colonisation effect in the intestine – are made using this species alone or in conjunction with other Lactic acid bacteria or *Bifidobacteria.*

These reform yoghurts and milks are well known in the Western diets as aids to the maintenance of good health for several years. The "Acidophilus milk" may by useful in counteracting the effect of antibiotics on the normal intestinal flora as a therapeutic product.

However the over-sour fermented milks are not too popular in Hungary, therefore a special kefir – supplemented with intestinal *Lactobacilli* – seems to be better to recover the balance of the gut.

Antibiotic resistance, biochemical characteristics of selected strains of *Lactobacillus acidophilus* and *Lactobacillus helveticus* were examined in details. These thermophilic *Lactobacilli* had a less growth rate at the room temperature than mesophilic culture of kefir species. After the investigation of temperature-optimum, which was very important for biotechnology, the next parameters were obtained in the fermented milks: acidity, organoleptic characters, cfu of Lactic acid bacteria and kefir-yeasts.

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The acidity and cfu values were higher at 30 °C after 15 hours than at 25 °C. On the other hand, the production of aromatic compounds was more favourable at the lower temperature.

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Morphological and biochemical characterisation of *Bifidobacterium* species and their role in the human health

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Nowadays, consumers look more and more for foods with high nutritional value, safe in term of toxicity, but also beneficial for health. It is in these contexts that the term "functional food" has evolved. Physiologically functional foods developed in Japan during the past ten years.

The functional food is a food, which effects the functions of the body in a target way so as to have positive effects because it contains health-enhancing ingredients. The functional foods can be classified as probiotics and prebiotics. Probiotics are alive microbial food or feed supplement, which beneficially affects the host organism. They adhere to tissue, contribute to intestinal microbial balance and play a positive role in the maintenance of the health as well as hinder the unfavourable processes leading to diseases. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activating one or a limited number of bacteria in the colon, and thus improves host health.

In order for a food ingredient to be classified as a prebiotic, it must neither be hydrolysed nor absorbed in the upper part of the gastro-intestinal tract and be selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated. A new concept for the future is the combination of both probiotics and prebiotics. This is named as synbiotics. Synbiotics may be defined as: a mixture of pro- and prebiotics which beneficially affects the host by improving the survival and implantation of alive microbial dietary supplement and selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria and thus improving host welfare.

The main aim of the work is to create novel functional foods. To fulfil this task first *Bifidobacterium* strains were purchased characterised and their cultivation technique optimised. Moreover, a model system was elaborated for the evaluation of natural and biosynthetic oligosaccharides as potential prebiotics. In this system *Bifidobacteria* were used as test organisms.

Five type strains: *Bifidobacterium longum* ATCC 15707, *B. breve* ATCC 15700, *B infantis* ATCC 15697, *B. adolescentis* ATCC 15703, *B. bifidum* NCTC 10471 have been investigated. The cultivation was carried out anaerobically at 37 °C. An attempt was made to cultivate *Bifidobacteria* species in all media used in the clinical practice and the food

industry. The best growth was achieved in TPY (Trypticase-Phytone-Yeast Extract), RCM (Reinforced Clostridial Medium), anaerobe base and Beef Holman media. Colonies of the *Bifidobacteria* are smooth, convex with entire edges, cream to white, glistening with soft consistency. The cellular morphology of the tested strains was observed by scanning electronmicroscopy. The individual strains showed various, characteristic cellular morphologies. All investigated strains of Bifidobacterium are Gram-positive, nonspore-forming, nonmotile and catalase and indol negative.

An agar diffusion system was developed for the evaluation of the oligosaccharides.

The first task was to find a basic medium where the Bifidobacterium strains do not grow without carbohydrate. The most proper medium for this purpose was Peptone Yeast Extract nutrient agar. The system was tested by both glucose and oligosaccharides.

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Studies to improve standardized techniques in quantitative mycological control of feeds

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The Czapek-Dox medium (CD) adopted as standard prescription in the Hungarian Standard MSz G977 and versions in the technique of inoculation and steps of sample-preparation were compared. ISO 7954 Yeast-Glucose-Chloramphenicol and malt-extract-agar, MAEA media vs. CD, different ways of inoculation, milling action, shaking time were tested.

The main goal of the study was to validate a more rapid and sensitive medium with technical advantages over the CD being compatible with the EU-practice. In addition, it was attempted to find a combination in technical parameters reducing variance and resulting in maximum CFUs.

Comparing the media, Yeast-Glucose-Chloramphenicol according to ISO 7954 proved to be more rapid than CD.

Chloramphenicol at a level of 0.01% could prevent bacterial growth safer than the combination of penicillin and streptomycin specified in the former standard or oxytetracyclin.

The comparison of the Koch and Drigalski technique indicated that inoculation upon the surface can render results equivalent to the plating and this may enable the automation of the inoculation step.

As steps of sample-preparation, wet mortar grinding and milling and shaking with the diluent for different times were compared.

The highest number of CFU could be attained following milling action and shaking of 10 min. with the diluent.

Data suggested that the warming of the sample during milling did not affect significantly the titer obtained.

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Selenium enriched yeast

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It was cleared undoubtedly in the middle of the eighties that selenium (Se) in organic (complex) bond can be utilized in human and animal organisms better than inorganic selenium (e.g. Na-selenite). In addition to better utilization, selenium of this kind has more favourable feature from respect of toxicology. Selenium in organic bond can be produced on two ways: in form of Se-contained amino acids (Se-Met, Se-Cys), or accumulating in yeast (Se-yeast). Under suitable circumstances the baker's yeast (*Saccharomyces cerevisiae*) is able to take up selenium and form organic bonds (min. 90%) at concentrations several times higher (1000–3000 µg/g) than the normal level (0.05 µg/g).

Chemical analysis of Se-enriched yeast revealed a further interesting information: The protein content increased by 15–30% as compared to the normal yeast. This increase can be of importance in industrial production of yeast for foodstuff, because it makes possible to utilize the carbon course more economically. In the course of accumulation we tried to optimize the circumstances to get high rate of organic selenium incorporation. For approximate control of incorporation selenium containing cells were disrupted in ultrasonic wave equipment. We also used another method, the so-called differential extraction for approximate control of incorporation and cellular distribution of selenium.

Our feeding experiments have also proved the favourable features of Se-yeast. In a field study the effect of Se-yeast treatment for weaned piglet has been tested. The trial lasted 5 months and the concentration of application was Se 0.5 mg/kg foodstuff. The final average body weight of the treated piglets was higher by 1–2% and the feed utilisation was higher by 8–11% than that of the control. In the slaughterhouse the 82 per cent of the treated animals had first class quality according to the European Union Standard. In the case of control animals this rate was 55 per cent only.

The potential field of application of selenium enriched yeast is quite wide: supplement of food products; supplement of feed products and production of premixes; in paramedicinal products; in cosmetics.

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M. PÁLMAI

Competitive growth of *Listeria monocytogenes* in the presence of *Lactobacillus casei* in broth and skim milk

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The growth of *Listeria monocytogenes* and *Lactobacillus casei* (not bacteriocin producer) was examined in artificial broth and in skim milk by traditional selective plate counting method. The initial cell-count of *Listeria* was 10^3 cfu/ml in all cases. In the mixed cultures different initial cell-counts of *Lb. casei* were present i.e. 10^3, 10^4 and 10^6 cfu/ml. Growth temperature of the mixed cultures was 13 °C.

In broth and in skim milk the formation of the growth curves showed similar properties. The growth of *L. monocytogenes* was not inhibited at all when *Lactobacillus* was present in equal proportion. Moreover, *L. monocytogenes* grew more rapidly than *Lb. casei*. In the presence of tenfold and thousandfold amount of *Lb. casei* the maximum population density (MPD) of *L. monocytogenes* was suppressed to approx. 10^8 and 10^7 cfu/ml (respectively) in milk and to 10^8, 10^5 cfu/ml (resp.) in broth. Thousandfold amount of *Lb. casei* decreased the growth rate of *L. monocytogenes* by 20% comparing to the control *L. monocytogenes* (grown in single culture).

Lb. casei kept the maximum population density of *L. monocytogenes* relatively low (10^5 cfu/ml) only when it was present in cell numbers thousand times greater and there was 0.7% lactic acid in the broth.

Both of the mixed culture experiments showed that *L. monocytogenes* is competitive enough to reach high levels of MPD under the examined conditions and is not inhibited when its initial cfu/ml is close to that of *Lactobacillus*.

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Novel combinations of nisin with other biopreservatives to improve the safety of mildly preserved food

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The aim of this research is to develop novel combinations of nisin with other biopreservatives that can improve the safety of mildly preserved food. Nisin is produced by *Lactococcus lactis* and to it is date the only bacteriocin that has been accepted as a preservative in food. In the experiments combination of nisin with essential oils (carvacrol, thymol, carvone) were used towards *Bacillus cereus* and *Listeria monocytogenes* at three different temperatures. As a food product chicory endive was used. In case of *Bacillus cereus* strains nisin works better at lower temperature. Essential oils alone have no effect on the viable count. There is synergism between nisin and

essential oils, from the three oils carvone is the least effective one. *Listeria monocytogenes* is able to grow on the product. Nisin in combination with carvacrol inhibits the growth of *Listeria monocytogenes* at 8 °C, under controlled atmosphere storage.

J. KROMMER, G. ZSARNÓCZAY, G. SZABÓ

The use of *Lactobacillus sakei* and *Leuconostoc gelidum* strains and their bacteriocins as starter culture to control the growth of *Listeria monocytogenes* in meat products

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The aim of this research was first to analyze the effect of *Lactobacillus sakei* and *Leuconostoc gelidum* strains and their bacteriocins as well as *Lactobacillus jensenii* (a non-bacteriocin producing starter culture already used in the Hungarian meat industry) alone or in combination with lactate towards *Listeria monocytogenes* in meat model system, secondly to determine the effect of these strains on the microbial, physical and organoleptic properties of dry fermented sausages.

Lactobacillus jensenii and *Leuconostoc gelidum* strains alone have no inhibiting effect on *Listeria monocytogenes*, but combined with lactate and bacteriocin have an inhibitory effect. In contradiction to this *Lactobacillus sakei* strain alone inhibits the growth of *Listeria* although to a smaller extent than the bacteriocin produced by this train.

Dry fermented sausage product was inoculated with the strains and their bacteriocins. During the incubation and ripening weight loss, pH, water and salt content and water activity were also determined. There was no significant difference between the chemical composition and the microbiological status of the samples. The bacteriocin producing strains have no deteriorative effect on the organoleptic properties of the dried sausages.

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Effect of irradiation on shelf life of aerobic- and vacuum-packed chicken meat

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Food irradiation in large scale is used in many countries in the last decade. Today it is a well-known fact that irradiation is a very effective treatment against pathogen and spoilage causing bacteria.

Our aim was to determine the irradiation dose for extension of the shelf life of chicken meat. The chicken wing was packed in PE foil (aerobic) and in PE-PA-PE foil

(vacuum). The samples were irradiated by Co-60 with 1, 2, 3 and 4 kGy and stored at 2–3 °C temperature. Colony counts of aerobic mesophilic microbes, *Enterobacteriaceae*- and *Pseudomonas* were determined using Spiral Plater technique.

In case of aerobic packaging the count of mesophilic aerobic microorganisms decreased by 1 log cycle per kGy in dose range 1–3 kGy, in vacuum packed samples treated with 1 kGy the reducing was 2, with 2 kGy 3 and with 4 kGy 4 log cycles. The count of *Pseudomonas* of unirradiated vacuum-packed samples was 10^6 g^{-1} at beginning. This value was reduced immediately after irradiation with 1 kGy by 5 log cycles and the number of surviving fraction increased 1 magnitude order during 23 days of storage time. Colony formation was not detected with 2 kGy treated samples. Count of *Enterobacteriaceae* was reduced by 3.5 log cycles after treating with 1 kGy, the surviving fraction reached to 10^5 g^{-1}, with 2 kGy to 104 CFU g^{-1} till the end of the storage time. The shelf life of aerobic packed samples increased 4–8 times, in case of vacuum-packed samples 4–13 times as a function of irradiation dose.

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Effect of Arbuscular endomycorrhizal fungi on the heavy metal tolerance of clover and barley

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The effect of Arbuscular endomycorrhizal fungi (AMF) on the metal uptake was studied in a pot experiment, when various metal rates, such as 0-, 30-, 90- and 270 mg kg^{-1} Cd, Zn and Ni (as sulphates) were given separately in three replicates. Using the above-mentioned metal rates, calcareous chernozem soil of Nagyhörcsök (TI) was contaminated in 1991. Soil samples were originating from this long-term field experiment 6 years later, and clover (*Trifolium pratense* L.) was grown in the pots. The other soil (TZ) (brown forest soil of Gödöllő) was contaminated only prior to the cultivation of the barley (*Hordeum vulgare* L.). After three months, the biomass production, the macro- and microelement contents and the mycorrhizal colonization of the AMF infected hosts were examined, comparing to the AM-free controls.

A high infection frequency (F %) of AM fungi was found at all the metal rates given, using the chernozem soil of Nagyhörcsök (T_1). Number of arbuscules (A %), however, has increased as a function of increasing doses of Ni and Zn. In case of the Cd on the other hand the arbuscularity index, the effectivity of AM symbiosis has collapsed beyond the 90 mg kg^{-1} dose. Although the metal content of the AM infected clover was found to be low, the biomass production has increased as compared to the mycorrhiza-free plants. In case of the other soil (T_2) as a result of a direct, short-term effect of the same metal-rates, the opposite tendency was realized: lower biomass and higher concentration of metals with AMF infection. This fact was attributed to the different soil physical chemical characteristics, which were affected by the direct or a long-term contamination of metals. Tolerant AM fungi have been selected during the 6 years of

metal enrichment at the calcareous soil (T_1), which reduced the harmful metal effects on the host.

Effectivity of microsymbiotic systems therefore should be a main parameter in estimation of deterious effects of heavy metals.

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N. HARKAI, E. TÓTH

Detection of enthomopathogenic *Bacillus* species in the habitat of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae) and in the developmental stages of the fly

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Myiasis is a disease of warm-blooded animals caused by different fly larvae. *Wohlfahrtia magnifica* (Diptera: Sarcophagidae) causes the main problem in whole Eurasia, so in Hungary, too. Larvicide chemicals used so far cannot prevent the reinfection of the wound and the frequent treatment of the animals is expensive and time consuming. To solve this problem methods used in biological control for example applying entomopathogenic *Bacillus* species against *W. magnifica* can be useful.

The aim of our investigation was to isolate and identify *Bacillus* strains (taking additional care of entomopathogenic species) from the developmental stages and from the habitat of *W. magnifica*. We tested the composition of *Bacillus* species in the samples originating from different maggots, pupae and soil from the habitat of the G sheep.

160 representative strains were selected from 400 *Bacillus* isolates of heat treated samples of the pupae of *W. magnifica* and of the soil. 25 *Bacillus* strains were isolated from larvae. We tested the basic phenotypic characteristics of these bacteria, then from the strains which were selected from the soil and pupae 50 representative ones were chosen to test them with Biolog Metabolic Fingerprint System (Biolog Inc., Hayward, CA) together with the 25 bacterial strains of the larvae. Then some confirmative diagnostic tests were made.

B. subtilis appeared in all samples. Differences in the species composition of the samples originating from the soil and pupa could not been proven: *B. brevis*, *B. sphaericus*, *B. licheniformis*, *B. pumilus* and *B. subtilis* occurred. Microorganisms belonging to the *B. thuringiensis/cereus* group could be isolated only from the sample of the maggots of the third stage. These two species cannot be differentiated with phenotypical tests, so the probes of pathogenicity have to be evaluated later.

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Bacterial communities in the salivary glands of the third stage larvae of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae)

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Wound myiasis of warm-blooded vertebrates caused by *Wohlfahrtia magnifica* is termed as wohlfahrtiosis. Most of flystrike diseases in Hungary is induced by this parasite. The results of previous immunological studies made evident that antigen prepared from the salivary glands of *W. magnifica* larvae are much more specific and reactive with sera of infested sheep than cuticular, intestinal or whole larval antigen samples.

The aim of our work was to identify bacterial species from the salivary glands of third stage *W. magnifica* maggots. Moreover according to the observations so far the wounds containing fly larvae do not suppurate. In the second part of our studies therefore we tested whether bacterial strains isolated from the salivary glands have special role in preventing the multiplication of pyogenic bacteria, first of all Staphylococci.

Composite samples aseptically prepared from salivary glands of *W. magnifica* larvae were made. These samples were diluted and plated onto different agar media. Developed colonies were isolated on a random manner then representative strains were selected for further investigations such as detailed phenotypical assays and Biolog test (Biolog Inc., California).

The dominance of Gram negative strains (95.3% of the representative strains) was characteristic. The main part (70.2%) of these bacteria was identified as *Providencia stuartii* (*Enterobacteriaceae*). The other part of the microorganisms (characteristic bipolarly stained bacterial strains) belonged to the genus *Acinetobacter*: *A. johnsonii* and *A. junii* or *Acinetobacter genospecies* 11 or 15. The ratio of other genera (*Micrococcus*, *Kocuria* etc.) was relatively low.

The results of antimicrobial assays against pyogenic Staphylococci (authentic *S. aureus* and *Staphylococcus* sp. originated from skin surface of sheep) showed that *Providencia stuartii* prevent the multiplication of these microorganisms.

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Algological investigation in the Soroksár-arm of the river Danube near Budapest

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The algological investigation of Ráckeve (Soroksár)-Danube started in the 20s, but the regular phytoplankton analysis started some twenty years ago. The reed-periphyton has been investigated in the 30s before this study.

Reed-periphyton and phytoplankton samples were taken in November 1996, January, April and July 1997 from the Ráckeve (Soroksár)-Danube near Dunaharaszti (39 river km), to study the seasonal and vertical distribution of periphytic algae both on old and green reed-stems, concerning the taxonomic composition, abundance, biomass and chlorophyll-a content, among others. In addition, the abundance, chlorophyll-a content and taxonomic composition of phytoplankton was calculated.

Altogether 289 species and varieties were identified, among them 171 were diatoms. Diatoms dominated both the periphyton and phytoplankton during the year. The abundance, biomass and chlorophyll-a concentration of the periphyton showed the highest values in April connected to optimal conditions for algal reproduction and were the lowest in July, when a serious flood washed out many algae from the surface of the stems. Considering the season the abundance of periphyton was high in January, in spite of low temperature and the thick ice-covering, due to the rich nutrient supply and big transparency of the water.

On the green reed-stems the abundance, biomass and chlorophyll-content were less in all depths than on the same depths of the old reed, caused by the shorter time for algal growth on green-reed.

Concerning to vertical distribution in November, January and April, these values were the lowest just below the water surface and above the sediment, had a maximum somewhere on the upper part of the stem and decreased more or less evenly toward the bottom. This tendency was the opposite in July along the reed-stem, as the flood had slighter effect on the lower parts of the reed.

According to the chlorophyll-a concentration of phytoplankton, the trophic state of the side-arm was mesotrophic in November and July, meso-eutrophic in January and eutrophic in April.

MINISYMPOSIUM: MYCOLOGY AT MOLECULAR LEVEL

A. MARÁZ

Structure and organization of fungal genomes at molecular level

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Fungi, the most simple eukaryotic organisms have small but complex genomes. In the period of classical genetics, beside *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, there were only a few yeast species that were known at genomic level. Filamentous fungi were similar, considering the narrow range of species having mapped genome (i.e. *Neurospora crassa*, *Aspergillus niger*), what was even more narrow if one takes into account the genome structure and diversity of mitochondrial genome.

Early genetic molecular techniques were developed for prokaryotes, mainly for *E. coli* that were applied quickly for yeasts and filamentous fungi. This resulted in an explosion of our knowledge concerning the genome structure and complexity of fungi both vertically and horizontally. At the same time fungi, as model organisms, have got more into the focus in comparison with higher eukaryotes.

Present lecture will show the importance of molecular techniques, which revolutionized genome research studies in fungi. Also, it will present some examples showing large-scale biodiversity of fungi at genome structure and organization level.

Chromosome sequencing, physical mapping and functional analysis of single genes in the case of *Saccharomyces cerevisiae* are in close connection of the Yeast Genome Project.

Autonomy of extrachromosomal genome and its relationship with the chromosomal one illustrate that fungi have developed as the consequence of a long evolutionary process.

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A PCR-based method for the detection of isolates belonging to the *MAT*$^{+}$ mating type of *Gibberella fujikuroi*

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Fusarium anamorphs of the ascomycetous fungus, *Gibberella fujikuroi* are common inhabitants of cereals and other cultivated plants as weak pathogens or saprophytes. Some of these fungi produce various mycotoxins, such as fusarin C,

moniliformin and fumonisins which may pose serious health hazards to humans and animals.

Seven different mating groups, termed A–G have been identified within *G. fujikuroi* and a correlation has been established between certain mating populations and their host plant affinity. The mating behaviour within each mating group was found to be heterothallic and controlled by a single locus with two alleles (Leslie, 1991).

The mating type (MAT) genes of most ascomycetes are structurally complex idiomorphs containing large regions of unique DNA sequences (Turgeon et al., 1995). Although there is only a weak similarity among the MAT genes known from different genera, the DNA binding proteins encoded by these genes contain a conserved motif called HMG (high mobility group) box.

Two degenerated oligonucleotide primers (NcHMG1 and NcHMG2) were synthetized based on the HMG sequence of *Neurospora crassa* and *Podospora anserina* (Arie et al., 1996) and used to amplify by PCR the potential MAT sequence of *G. fujikuroi* A-00999 (mating population A^+). A 242 bp fragment, named *fusmat1* has thus been obtained, cloned and subjected to sequence comparisons. Data base search revealed 57 and 65% similarity between *fusmat1* and the conserved regions of the *mt a-1* and the FPRI genes, that control the a and the mat mating type in *N. crassa* and *P. anserina*, respectively (Staben and Anofsky, 1990; Debuchy and Coppin, 1992). These similarity values suggested that *fusmat1* is a fragment of one of the two opposite mating type genes in *G. fujikuroi* mating population A.

The same primers were used to amplify the conserved part of the MAT gene in seven pairs of the authentic mating type testers (A–G) of the *G. fujikuroi* aggregate. The 242 bp PCR amplificatum corresponding to the *fusmat1* segment has always been detected in only one member of the tester pairs. This method is thus suitable for the rapid detection of mat^+ individuals in large mass of *Fusarium* (section *Liseola*) samples and may be useful in identification of strains exerting atypical mating behaviour. The identification of additional mating populations could also be facilitated by this PCR based procedure.

T. DEÁK

Molecular taxonomy of fungi

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In the last two decades the application of molecular techniques has revolutionized the classification of microorganisms. Studies on DNA and/or DNA/RNA relatedness have become the basis of the differentiation of species and genera. Molecular fingerprinting methods such as restriction fragment length polymorphisms, random amplified polymorphic DNA analysis, pulsed field gel electrophoresis of chromosome DNA and others allow intraspecific differentiation and typing. The most far reaching, however, has been the introduction of ribosomal RNA sequencing that has made for the first time

possible the assessment of phylogenetic relationship among microorganisms. Extensive investigations of small and large ribosomal subunit RNAs or their genes has radically changed our conception of kingdoms of living organisms.

Compared to bacteria, the use of molecular techniques for the study of fungi has been less extensive yet, nevertheless data obtained so far have also made a large impact on the classification of filamentous fungi and unicellular yeasts. Examples will be given to show how the various molecular techniques made changes in the taxonomy of this group of microorganisms.

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Determination of strain relatedness of opportunistic pathogenic *Candida* spp. by molecular genotyping

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Antibacterial antibiotic treatments suppress bacteria of the intestinal tract for mucosa resulting in potential growth of the opportunistic pathogenic *Candida* strains which can be found in the environment and in some foods. Immunocompromised patients have also increased risk of candidiasis.

It is very important to find the sources of *Candida* strains becoming infectious and causing disease and to recognize their virulence factors. It is a frequent question whether clinical isolates are from nosocomial infection. The recently developed and promising molecular typing methods offer a new and efficient way for molecular epidemiological studies and for finding the potential and real reservoirs of *Candida* infections.

Clinical and food isolates, culture collection strains of *Candida* glabrata and *C. krusei* were involved in our molecular epidemiological studies in which intraspecies relatedness of them was determined by the following typing methods: RFLP analysis of rDNA gene sequences (ribotyping); RAPD-PCR analysis; Electrophoretic kariotyping; Chromosomal Fragment Length Polymorphism.

Clinical isolates an collection strains of *Candida glabrata* formed two, completely homogenous groups which showed similarity at 35% level according to the results of ribotyping. RAPD-PCR analysis followed by cluster analysis resulted in the same grouping, while similarity of the two groups was much less than by ribotyping. Chromosomes of the strains separated into 6–11 bands during PFGE, but only two karyotypes, representing the previous two groups, were recognizable. These results indicate that there was no greater similarity between the clinical isolates than among these and other strains on the basis of molecular genotyping, making less probable their epidemiological relation.

Clinical isolates and type strain of *C. krusei* showed a small degree of chromosome length polymorphism by electrophoretic separation of intact chromosomes, but

differences of strains disappeared when the chromosomes were fragmented with restriction enzymes. Ribotyping and RAPD-PCR analysis showed homogenous clusters of strains, no differences were found at genome sequence level. Before concluding that the clinical isolates were resulted by nosocomial infection we have to find suitable primers for PCR amplification of sequence(s) of type strain and clinical isolates.

L. HORNOK

Molecular aspects of fungal pathogenicity to plants

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The most advanced phytopathogenic fungi penetrate through the plant cuticle by means of special structures, i.e. appressorium and penetration peg. Several appressorium-specific genes have been cloned; they were found to control either attachment behaviour or the melanin biosynthesis. Some of these genes are triggered by topographical signals, while others are induced in the presence of specific wax components of their host plant. Extracellular hydrolytic enzymes may also be required for penetration; cutinases seem to be the most important but not essential factors of pathogenicity.

Genes, encoding enzymes able to detoxify antimicrobial plant compounds are determinants of fungal aggressiveness. Preformed plant defence compounds, such as cyanide (in sorghum) or avenacin (in oat) are converted to nontoxic compounds by fungal hydrolytic enzymes. Induced defence compounds (phytoalexins) are decomposed by cytochrome P-450 demethylases; one of the phytoalexin degrading genes (*pda*) coding for such a demethylase is located on a dispensable chromosome of *Nectria haematococca*, and if this chromosome is lost, the resultant isolate becomes nonpathogenic. Random chromosome loss (or gain) may thus contribute to the variability of phytopathogenic fungi.

Genes, encoding host specific fungal toxins are virulence determinants. A large body of molecular information is available on HC-toxin (produced by *Cochliobolus carbonum*) and T-toxin (synthesized by *C. heterostrophus*).

Fungal avirulence genes trigger the hypersensitive defence reaction (HR) in plants. The HR inducing fungal compounds are collectively named as elicitors. The most specific elicitors are small cystein-rich molecules, fragments of essential fungal hydrophobins. Genes encoding for AVR (avirulence) compounds in *Cladosporium fulvum* were found to be extremely variable, and this is the major cause of the lack of recognition of the AVR elicitor by the susceptible host plants.

Novel resistance breeding strategies based on the molecular background of fungal pathogenicity have been elaborated in the recent years.

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Cloning the gene of a novel type ser/thr protein phosphatase from *Neurospora crassa*

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Protein phosphorylation is a universal regulatory mechanism in eukaryotic cells. The phosphorylated state of a protein is affected by the conflicting activities of protein kinases and phosphatases. Nearly all Ser/Thr specific dephosphorylation reactions can be attributed to four classes of protein phosphatases (PP's): PP1, PP2A, PP2B, PP2C, which are differentiated on the basis of their inhibitor sensitivity and metal ion dependence. In addition to these classical enzymes there are several novel type phosphatases, which do not fit into the above categories.

We have isolated a gene encoding for a novel-type phosphatase from *Neurospora crassa* using a *Drosophila* PP1 cDNA clone as a heterologous probe. The deduced amino acid sequence of the isolated gene showed high degree of homology to *Saccharomyces cerevisiae* PPZ1 and PPZ2 and *Schizosaccharomyces pombe* PPZ (novel-type phosphatases which have been suggested to play a role in osmotic stability and the PKC mediated signal transduction pathway), and was therefore designated PZL-1 (PPZ-like). The corresponding yeast proteins are considerably larger (77.5 and 78.5 kD) than the *N. crassa* PZL-1 (predicted size: 58 kD). On the basis of comparison to a corresponding cDNA clone, two introns were identified close to the 3' end of the gene and one in the untranslated leader sequence. *pzl-1* was mapped by RFLP analysis to the left arm of chromosome I, close to the *cyt-21* locus. Transcript of the gene was detected with RT-PCR during conidial germination and the early phase of hyphal growth. Our aim is to inactivate the *pzl-1* gene by gene disruption or RIP (repeat induced point mutation) to learn if the gene is essential for the fungus.

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Isolation and metal induced expression of a metallothionein gene from *Trichoderma hamatum*

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Metals play a double-faced role in biological systems: they serve as essential cofactors in basic biochemical reactions, while high concentrations of the same metals may be toxic to cells. Microorganisms have developed metal sensing proteins able to

respond to the stress exerted by increases in the external metal concentrations. The signal received by these sensing proteins activates the expression of metal detoxifying genes. The products of these genes are directly or indirectly involved in the transportation and/or sequestration of the metal ions.

In fungi, a class of small cysteine rich, metal binding proteins named metallothioneins (MTs) is of primary importance in responding to cellular metal stress. MTs are encoded by a multigene family and their synthesis is controlled by various stimuli including heavy metals and different stresses. The signal transduction mechanisms and the transcriptional induction of the metallothionein genes are largely unknown.

Our strategy of cloning the copper MT gene from *T. hamatum* was based on known MT-sequences, that allowed the synthesis of two unique 21-mer oligonucleotide primers. Genomic DNA and enriched, copper-induced metallothionein mRNAs were used as templates in direct and reverse transcribed PCR reactions for amplification of MT-specific DNA fragments. The amplified DNA fragments were cloned, sequenced, and compared to published MT genes. Northern analyses were used to determine whether these clones could detect specific metal induced expression in response to different metals (Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^{+}, Pb^{2+}, Cr^{3+}, Mn^{2+}). One of the PCR products, designated *hamMT* detected a rapid expression induced by copper, silver, zinc and manganese. In contrast, the MT genes known from *Neurospora crassa* and *Saccharomyces cerevisiae* are primarily induced by copper. Southern hybridization of the *hamMT*-probe to restriction enzyme digested genomic DNA samples revealed that the *hamMT* sequence exists in a single copy in the genome of *T. hamatum*. Organization and characteristic sequence motifs of *hamMT* indicate that it belongs to the MT-I class methallothionein genes which contain a non-conserved intermediate region flanked by cystein-rich regions at both ends.

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Caffeine and multidrug resistance

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Caffeine is one of the most frequently consumed drug. Several aspects of effect of caffeine on living organisms are studied. The latest results showed that caffeine could not cause cancer alone but it could increase effectively the carcinogen effect of several compounds. In addition, caffeine is a well-known toxin of cells with different effects on cells. The most common changes in phenotype following addition of caffeine is the prolonged cell cycle and appearance of elongated cells.

To understand these effects we isolated caffeine-resistant mutants that could tolerate 4–5 times higher concentration of caffeine than the wild-type cells. One hundred mutants isolated were brought into four complementation groups. One representative was chosen from each group for further study. Analysis of these mutants revealed several dissimilarity to wild-type cells such as prolonged cell cycle, elongated cells, UV sensitivity, γ-resistance, pH-sensitive growth, damaged meiosis, starvation sensitivity and

other drug resistance. These results suggest a very complex system exist in the cells that is able to reduce the toxic effect of caffeine. To our mind there should exist only one common biochemical pathway in which a mutation can reduce the toxic effect of caffeine. We also found that *S. pombe* cells could adapt oneself to higher concentration of caffeine. This adaptation is reversible. It seems to be reasonable that there is a relation between the mechanism of adaptation and the genes that could cause caffeine-resistance by mutation. To understand this phenomenon more clearly we have cloned three of the four *caf* genes and sequenced them. Molecular study shows that the gene product of *caf2* (*crm1*) is responsible for formation of compact chromosome structure. Deletion of this gene is lethal for cells. The *caf1* (*hba1*) gene product is located in the nucleus and seems to be a new type of protein. The *caf4* (*trr1*) gene encodes thioredoxin reductase enzyme. The cloning of the fourth *caf* gene is in progress now. Cells containing the disrupt alleles of *caf1* or *caf4* genes get caffeine sensitive as wild-type cells and the generation times are prolonged extremely depending on the pH of medium. The *caf* genes interact with several different genes. For example the mutation of the *caf2* (*crm1*) gene causes caffeine-resistance through the *pap1* gene and the p25 protein. In addition there is interference between the *caf1* and *caf2* genes. A mutation in the *caf4* (*trr1*) gene can cause inhibition of the effect of the wild-type p53 human protein that inhibits the cell growth in *S. pombe*. The *caf3-89* dominant mutation increases the morphological changes in phenotype caused by *sep1* and *stey* while the *caf3-89sep13* double mutation is lethal. The *caf3* gene even interacts with some auxotroph gene.

Mutations in *crm1* (*caf1*, *bar1*) can even cause resistance to brefeldin A (an antifungal antibiotic that inhibits the Golgi complex), staurosporin (inhibits the protein kinase C) and leptomicin B (an antifungal antibiotic that inhibits cell cycle arrest). The wild-type allele of *hba1* (*caf1*) can cause brefeldin A resistance on high copy plasmid.

S. BIRÓ

The application of recombinant DNA techniques for the production of medically important β-lactam antibiotics in filamentous fungi

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The medically important β-lactam antibiotics are produced by a wide variety of prokaryotic and eukaryotic microorganisms. Their biosynthesis is one of the most thoroughly studied antibiotic pathways. The availability of recombinant DNA techniques provides new ways complementary to the conventional random mutagenesis and rational screening programs in yield improvement and the production of useful novel β-lactam compounds.

Published examples and potential benefits, as well as unexpected difficulties will be discussed using the biosynthesis of β-lactam antibiotics in filamentous fungi as an example, since most of the genes involved in their biosynthesis have been cloned and sequenced.

I. PFEIFFER, J. KUCSERA

Characterisation of DNA plasmas in *Phaffia rhodozyma*

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Phaffia rhodozyma is a red-pigmented basidiomycetous yeast that can be cultivated only at low temperature [1, 2]. It has become a well-studied organism because of its carotenoid (astaxanthin, (3-carotene) content [3]. A series of articles has been published concerning its astaxanthin production and strain improvement. Nevertheless, little is known about the genetics of this species. The existence of linear DNA plasmids in a *Phaffia rhodozyma* strain was proved earlier [4].

Our study demonstrates the localisation of these plasmids in the crude mitochondrial fraction. These plasmids were protected against DNAse when mitochondria were treated with DNAse in the presence of an osmotic stabiliser. After *Bal*31 treatment the plasmids were degraded suggesting their linear structure. Though yeast linear DNA plasmids are generally insensitive to ethidium bromide, *P. rhodozyma* strains could be efficiently cured by ethidium bromide treatment.

DNA hybridization study revealed strong sequence homology among some plasmids belonging to the same and different strains. No homology was found between the *P. rhodozyma* and other linear DNA plasmids. Some of the fragments of the plasmids were sequenced and data were compared by databases. Significant homology was not detected to any of the sequences.

The only known function of yeast linear DNA plasmids is that they confer killer character to the host cells (*Kluyveromyces lactis*, *Pichia acaciae*). No such activity could be detected in any of the *P. rhodozyma* strains. Further studies are in progress to establish the correct structure and explain the function of these DNA elements in *P. rhodozyma.*

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VIROLOGY

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Cloning the entire genome of herpesvirus saimiri strain C-484 in *E. coli*

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Group C strains of Herpesvirus saimiri (HVS) can induce malignant lymphomas in rabbits and immortalize human peripheral blood T lymphocytes. We now successfully cloned the entire 112 kb unique region (L-DNA) and one unit H-DNA of the viral genome of strain 484-77 DNA in pBeloBac (Shizuya et al. Proc Natl Acad Sci U.S.A. **89**, 8794–8797). Five clones were confirmed to contain full-length 484-77 HVS L-DNA. DNA of the five clones containing full-length L-DNA were found to be infectious. Analysis of immortalizing potential of cloned HVS DNA is in progress. Two genes encoding the Saimiri Transforming Protein (STP) and the Tyrosine Interacting Protein (TIP) are required for both oncogenesis and immortalization. Expression vectors containing these genes gave repeatedly negative results in immortalization assays suggesting that other genes are required. Mutagenesis of the HVS clone in *E. coli* is expected to be very rapid which will allow identification of additional genes required for immortalization and oncogenesis. This system can be also used as an expression vector via a unique restriction site already available in our pBeloBac-HVS clones. Chimeric KSHV-HVS viruses can be also readily constructed for the study of genes shared by the two viruses.

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LBD, the minimal domain of herpesvirus saimiri tip viral protein, constitutively activates P56lck

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Herpesvirus saimiri (HVS) is a primate virus and a model for leukemogenesis. HVS is found in the squirrel monkey of South America and induces rapid and malignant lymphomas and leukemias in other species of monkey. HVS also has the ability to immortalize and transform human T cells into an IL-2 independent phenotype in vitro. Tip (Tyrosine kinase Interacting Protein) is one of the viral genes known to play a role in HVS transformation of T cells. We have previously shown Tip to interact and upregulate the kinase activity of P56lck in a multi-protein complex. P56lck is a major tyrosine kinase in T cells essential for signalling from the T cell receptor. We now show that only a

subfragment (55 residues) for Tip termed LBD (Lck Binding Domain) is needed to increase the kinase activity of a Gst-Lck fusion protein in vitro, and also increase in vivo p56lck activity when expressed transiently. The LBD fragment of Tip contains two subdomains: an 8 amino acid domain with homology to scr-family kinases and 10 amino acid region which is a putative SH3 binding domain. By in vitro kinase assays, we show that both of these domains are necessary for activation of p56lck. Therefore, these experiments suggest that interaction of Tip with p56lpck alone, without a requirement of other proteins in this system, is sufficient for constitutive activation of tyrosine kinase activity of p56lck. These data provide a further understanding of the signalling disregulation which occurs in DNA tumor virus transformed cells.

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Monitoring HHV-8 antibodies after renal transplantation

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The role of human herpesvirus-8 (HHV-8) in the pathogenesis of Kaposi's sarcoma (KS) is widely accepted and some routes of the possible mechanisms have been described recently. The seroepidemiology of HHV-8 is still debated, but it's clear that the prevalence of antibodies to latent nuclear antigens (LANA) fits to the epidemiology of KS and it has been also described that seroconversion of antibodies to HHV-8 LANA precedes the development of KS in AIDS patients. In post-transplant KS-patients it has been observed that HHV-8 seroconversion occurs prior to transplantation but in one case the transmission of HHV-8 with the transplanted organ has been observed. Formerly we showed that renal transplantation and previous transfusions of the patients do not increase the risk for HHV-8 transmission.

In this study we provide additional data that HHV-8 reactivation occurs in transplant recipients parallel to the immunosuppressive therapy. We screened serum samples of 139 renal transplant recipients with indirect immunofluorescence assay. We found 5 patients to be seropositive for anti-LANA antibodies. With subsequent serum samples of the patients we recorded elevation of titres of IgG antibodies to LANA. Two of the five patients developed post-transplant KS.

HHV-8 antibody monitoring seems to be informative to predict KS development in transplant recipients, too. Possible effects of drugs, HLA and other factors on post-transplant KS development will be discussed.

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Pathogenetic role of HHV-8: sequence polymorphism and seroepidemiology

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Kaposi's sarcoma (KS) has sporadically been present in the Eastern/Central European region for more than a century. KS associated human herpesvirus or HHV-8 has been identified in all forms of KS. A variety of viral homologues of cellular genes that may play a role in tumorigenesis have been discovered in HHV-8, but its etiological role in the pathogenesis of KS has not yet been established.

We analyzed the presence and DNA sequence variability of ORF26 and the K1 ORF regions of HHV-8 in different clinicopathological subtypes of cutaneous and visceral forms of KS lesions and lymphocytes from 27 AIDS-associated, iatrogenic and classical KS cases. Moreover, the seroprevalence of HHV-8 antibodies in KS risk groups – including 521 dialysed patients waiting for renal transplantation – as well as in the normal population have been determined.

HHV-8 specific DNA regions were detected by PCR followed by Southern blot. Amplified ORF26 and K1 ORF were cloned and sequence variability was analyzed by SSCP followed direct sequencing. The presence and prevalence of HHV-8 antibodies in sera of 1.379 individuals including 600 healthy subjects was determined on HHV-8 positive BCBL-1 cells by immunofluorescence, and also with Western blot of non-stimulated and PMA stimulated BCBL-1 cell lysates and concentrated HHV-8 virions.

KS has been developed in <10% of AIDS patients in our region, but HHV-8 DNA was identified in >95% of the KS/AIDS cases. On the other hand KS is diagnosed in >22% of all malignancies observed after organ transplantation, with a ~100% HHV-8 positivity. ORF26 sequence analysis revealed differences only in two nucleic acids at positions 1033 (C/T) and 1160 (T/C), showing a high degree homology to database sequence of HHV-8 (KSU 18551). In the K1 ORF sequences, however more variability (>6%) was detected in the 5' segment (115–463 bp), especially in the regions of 125–132 and 293–304 bps.

Antibodies to HHV-8 were detected in 22.2% of HIV-1 infected non-KS patients, in 1% of their HIV negative non-KS contacts and other STD patients, and in >80% of HIV-1 negative KS patients. Among the normal population 2.1% exhibited presence of HHV-8 antibodies with titres of 1:100–1:6000.

We identified and confirmed the presence of HHV-8 DNA in our patients with AIDS associated, iatrogenic and classical KS and also in the normal population. Seroepidemiological data obtained make it likely at the present that only a very small number of distinct infectious exogenous isolates have been transmitted horizontally. The differences in the pathogenesis in population groups in our region, however, may be attributable to HHV-8 strain variability besides various additional factors.

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Simultaneous visualization of the resident chromosome site of EBV genome and EBV transcripts in interphase nuclei by FISH

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Visualization of integrated copies of EBV DNA on chromosomes and specific viral RNAs within nuclei of cells latently infected with EBV was achieved by FISH (fluorescent in situ hybridization) with BamHI W and K fragment of the EBV genome.

In two subclones of the Burkitt lymphoma cell line NAMALWA differentional chromosomal localization (chromosome X and 1, respectively) of EBV genome was shown. In EBV-transformed lymphoblastoid cell line IB4 containing integrated but no episomal viral DNA the integration site was found on chromosome 4. In most experiments, transcripts synthesized from an integrated EBV genome in NAMALWAs and IB4 were investigated. Several hundred kb of specific transcripts is sharply restricted to a small region of the nucleus, frequently in a curvilinear "track". Results of simultaneous detection of specific chromosome domains and nuclear RNAs indicate that RNA "tracks" extend from the site of transcription into the nuclear periphery and that RNA transport may be coupled to transcription.

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Possible role of placental macrophages in transplacental transmission of human cytomegalovirus

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Transmission of human cytomegalovirus (HCMV) from mother to child is the most common cause of congenital viral infection, but the detailed mechanisms of transmission through the placenta are still unknown at the cellular level. The placental syncytiotrophoblast (ST) layer serves as the first line of defense of the fetus against viruses. HCMV can enter ST cells but virus replication is restricted at the level of immediate-early (IE) and early (E) gene products in these cellular targets. This study was designed to reveal the role of placental macrophages (Hofbauer cells) in transplacental transmission of HCMV. The ST cells were infected at a multiplicity of infection of 1.0

PFU per cell. At various times after infection the ST cells were cocultured with Hofbauer cells. Then the cocultivated macrophages were monitored for HCMV infection with immunofluorescence using monoclonal antibodies to IE and late (L) viral proteins. Release of infectious virus was assessed by PFU assay in fibroblasts. Results showed that coculture of infected ST cells with Hofbauer cells caused transmission of HCMV to the macrophages. Low level of virus release (10^2 PFU/ml) was observed from cocultivated

macrophages. Cytokine activities capable of upregulating HCMV replication could be detected in the coculture system of ST and Hofbauer cells. These data suggest that interaction of HCMV-infected ST cells and placental macrophages may play an important role in the transmission of HCMV from mother to the fetus.

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Cytomegalovirus specific CTL response in healthy individuals

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We have analyzed memory CTL responses of 32 healthy blood donors. Peripheral mononuclear cells (PBMC) were isolated and restimulated with recombinant canarypox viruses expressing cytomegalovirus (CMV) proteins pp65, pp150, IE-1 exon 4 and gB or with autologous fibroblasts infected with: CMV. Cytolytic activity of the restimulated PBMC was tested on EBV transformed autologous and heterologous lymphoblast as well as on fibroblast target cells. CMV-infected autologous fibroblasts effectively restimulated pp65 and IE-1 exon 4 specific CTL and were good targets for pp65 and IE-1 exon 4 specific cytolysis. We investigated the MHC class I restriction of IE-1 exon 4, pp65 and pp150 specific CTL by using target cells of several individuals characterized for HLA haplotype and determined new HLA alleles restricting IE-1 exon 4, pp150, and pp65 specific lysis. Precursor frequency of IE-1 exon 4 and pp 65 specific CTL was analyzed by limiting dilution assay and was found to be comparable in the two donors tested. We concluded that not only pp65 specific but IE specific CTL may also play an important role in CTL mediated immunity after natural infection.

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Role of INTERLEUKIN 8 and transforming growth factor β1 in enhancement of human cytomegalovirus replication by human T-cell leukemia-lymphoma virus type I in dually infected macrophages

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Human cytomegalovirus (HCMV) infection of macrophages was shown to be nonlytic and exclusively cell associated. Our previous studies have demonstrated that coinfection of macrophages with HCMV and human T-cell leukemia-lymphoma virus type I (HTLV-I) significantly enhances HCMV replication, resulting in release of

infectious HCMV from dually infected cells. The *tax* gene product of HTLV-I alone was capable of upregulating HCMV production. In the present study, we tested whether activation of HCMV by HTLV-I in macrophages could be mediated by the induction of cytokines. Supernatants of virus-infected and control cultures were assayed for interleukin 8 (IL-8) and transforming growth factor β1 (TGF-β1) with the use of ELISA kits. Anti-IL-8, anti-TGF-β1 neutralizing antibodies and chemically modified anti-Tax antibodies were used as a means of blocking cytokine and Tax activities. Although HCMV alone was able to induce IL-8 and TGF-β1 release, coinfection of macrophages with HTLV-I resulted in a rapid production of massive amounts of both cytokines. Treatment with anti-IL-8 and anti-TGF-β1 antibody resulted in a 20-fold and 50-fold decrease in HCMV production, respectively. When anti-IL-8 and anti-TGF-β1 treatments were combined, an about 800-fold inhibition of HCMV release by macrophages was observed. Release of infectious HCMV could be totally suppressed by anti-Tax antibody treatment. Similar results were obtained when macrophages were transfected with a tax expression vector and then infected with HCMV. These data show that a substantial part of the stimulatory effect of HTLV-I Tax on HCMV replication is mediated by IL-8 and TGF-β1.

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HHV-7 and HHV-8 infection in pityriasis rosea

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Pityriasis rosea (PR) is a distinctive, acute, inflammatory, papulo-squamous skin disorder occurring in young adulthood, once in life time, but may show recurrences in < 4 per cent of the cases. The clinical features, the course of the disease with a primary large region followed by secondary dissemination, the seasonal occurrence, the spontaneous resolution suggest a viral origin, but previous studies failed to determine a putative causative agent.

To study the possible role of human β-herpesvirus HHV-7 and γ-herpesvirus HHV-8 in the pathogenesis of PR we performed serological and PCR analysis of the sera, peripheral blood mononuclear cells (PBMC) as well as of the skin lesions.

IgG and IgM antibodies to HHV-7, and LANA antibodies to HHV-8 were determined by indirect immunofluorescence on infected Sup-T1 and BCBL-1 cells, respectively. For the detection of HHV-7 DNA a nested PCR was used amplifying 183/143 bp of HHV-7 specific region homologous to U42(KA3L) of HHV-6 gene. Presence of HHV-8 DNA was detected by PCR of 233 bp portion of ORF26.

HHV-7 specific antibodies, namely IgG (> 1:640) and IgM (> 1:640) were detected in 60 per cent of the patients (all female). In the PBMCs of the same patients HHV-7 DNA was detected, while in the skin lesions the presence of HHV-7 could not be verified. In two PR patients beside the HHV-7 infection the presence of HHV-8 DNA in the PBMCs were also detected, however the related skin lesions were unaffected. Sera of these patients contained HHV-8 specific antibodies (max. titre: 1:6000). On the contrary,

our patient with erythema annulare centrifugum (Darier) was negative for the two herpesviruses infection. In the control group matched in age and gender, neither HHV-7 IgM nor antibodies to HHV-8 could be detected in the sera, or viral DNA in the PBMCs. However HHV-7 IgG could be found in low titre in some control cases. Our results suggest that Pityriasis rosea could be the consequence of i) reactivation of the latent, persistent HHV-7 infection occurred in childhood, or ii) acute HHV-7 infection in the young adulthood. Based upon our observation according to which the relevant skin lesions were unaffected by the virus(es), other factors – like immunosuppression or cytokine signals – should also be considered in the pathogenesis of PR.

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Genetical differences between an echovirus type 11 strains causing hemorrhagic syndrome of newborn babies in Hungary and the echovirus 11 (Gregory) prototype strain

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An echovirus 11 epidemic occurred in 1989 in Hungary, which caused hemorrhagic hepatitis of lethal outcome in 13 newborn babies. The molecular comparison of the prototype Echovirus 11 (Gregory) strain with the pathogenic virus isolates was the aim of this study.

Six different regions of the isolates were sequenced in the 5'-untranslated region, the 1A/1B (VP4/VP2) and the 1C/1D (VP3/VP1) capsid protein region, the 1D, the 3D and the 3'-end (untranslated region). After PCR reactions the sequencing was carried out by Pharmacia and ABI Prism automatic sequencer with the help of fluorescent labelled primers.

5'-untranslated regions of 12 isolates were sequenced. The twelve different isolates were homologous in 99% but according to the fastA analyses the similarity between the Gregory strain and the isolates was only 90%. Interestingly, the strains of the swine vesicular disease viruses (SVDV) were the most similar to the Hungarian strains (94.7%). The Gregory prototype strain contains a 12 nucleotides long poly-U stretch (might be involved in the initiation of the translation) absent in any other sequenced enterovirus and in the Hungarian isolates. The translation starts at the last AUG codon (nt. 752–754), and the favourable Kozak context (AXXAUGG) was also identified in the field isolates.

The 1A (internal VP4 polypeptide) region is highly conserved among three of our sequenced isolates, but the similarity among them, the Gregory and SVDV strains were equally 75%.

The 1C/1D (VP3/VPl capsid protein) and the 1D are also very conserved in the isolates originated from different counties of Hungary (98% at nucleotide and aminoacid

level). The same data for prototype strain were 78 and 94% respectively, but only 64% similarity was observed in the case of the SVD viruses at nucleotide level, which is explicable, because the field isolates proved to be Echovirus 11 serologically. Originally we had difficulties in the serological detection, what is explicable by four aminoacid changes in the VP1 region. There is no any aminoacid change in the enterovirus group specific anti-VP1 monoclonal antibody (5-D8/1) epitope region (VPALTAVETG); the strains could be detected as an Echovirus 11 strain by the commercially available DAKO IIF test.

The sequence data of the 3D region of one strain and of the 3'-end of eight strains show bigger similarity to the SVD viruses than to the Gregory strain (86 and 77%) again, but the field isolates were homologous in 98%.

Phylogenetical studies were carried out of every sequenced region and the Hungarian echovirus 11 isolates, the SDV, Coxsackie A9, Echovirus 9, 6, 12, Coxsackie B 1, 3, 4, 5 viruses were clustered in the same group.

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Detection and molecular identification of different avian leukosis virus (ALV) subgroups in vaccines

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Avian leukosis viruses (ALVs) are members of the family Retroviridae, and belong to the genus of avian type C retroviruses. They are highly pathogenic and induce a wide spectrum of disease in infected animals. ALVs isolated from chickens fall into five subgroups A, B, C, D and E on the basis of serological properties of the viral envelope gp85 glycoprotein. Viruses of subgroups A, B, C and D occur as pathogenic exogenous viruses, whereas the subgroup E is known to be endogenous.

Our aim was to detect the ALV contamination of our vaccines produced in embryonated eggs and cell cultures derived from chickens. Because ALVs have RNA genome we have used a reverse transcriptase PCR amplification assay (RT-PCR). The following viral strains were used as controls in our experiments: RAV-1 (ALV-A), RAV-2 (ALV-B) and RAV-O (ALV-E). The RNA had been isolated from control viral strains and vaccines, then cDNAs were produced by moloney murine leukemia virus reverse transcriptase. Subsequently, a 466 bp region of gp85 *env* gene was amplified. The envelope glycoprotein (*env* gp85) is the main antigen determinant and responsible for subgroup classification. Based on sequence differences in the gp85 *env* gene that was amplified by primers for its conserved sequences, subsequent digestions with Bsa I, Ava II, Eco 47 III restriction enzymes provide distinctive electrophoresis patterns suitable for differentiation between subgroups.

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Infectious agents and development of atherosclerosis

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Heart disease, including atherosclerosis (AT), is the leading cause of death all over the world. Recent studies indicate that development of AT begins during childhood. One of the theories of the development of AT suggests that the artery's inner layer, the endothelium, is injured by an insult, which leads to the development of early AT plaques. By the contribution of risk factors early lesions progress into "complicated" plaques. The second theory suggests that a plaque develops from a single cell and is thus a non-cancerous tumor growing inside the arterial wall.

A common viral and/or bacterial infection(s) may be the first step in the complex process of the development of AT, a concept consistent with both theories. Cytomegalovirus (CMV), and *Chlamydia pneumoniae* (*C. pneumoniae*), an obligate intracellular bacterium, have been strongly associated with AT. Further candidates are herpes simplex virus, coxsackie B virus, *Helicobacter pylori* and *Mycoplasma pneumoniae*.

CMV particles, antigens and DNA sequences have been described in AT plaques of biopsy and autopsy material. The involvement or reactivated CMV in restenosis of coronary arteries, an accelerated form of AT, following angioplastic surgery has been suggested. CMV infection usually occurs in childhood, paralleling the pattern of appearance of early AT lesions; by the young adult age 50–100% of individuals are CMV seropositive. Animal studies have shown that the Marek disease herpesvirus induces AT in chickens and rat CMV induces early AT lesions in immunosuppressed rats. Our results have shown that intranasal inoculation of BALB/c mice with murine CMV resulted in the replication of the virus in the endothelial cells of aorta and initiated the development of early AT plaques. The mechanism(s) is complex. CMV is known to induce expression of several types of molecules that are involved in the initiation and maturation of AT plaques, e.g. growth factors, heat shock proteins, cytokines and adhesion molecules.

C. pneumoniae requires an intracellular habitat for growth and causes acute and chronic respiratory tract infections with possible bacterial spread to aorta and heart by macrophages. Population antibody prevalence studies have shown that more than 50% of young adults world-wide have antibody. Recently *C. pneumoniae* has been associated with AT based on both seroepidemiology and data demonstrating the presence of the organism in AT plaques. Evidence of the persistence of *C. pneumoniae* in atheromatous plaques has been obtained by electron microscopic studies, immunohistochemical staining and PCR testing. *C. pneumoniae* activates growth factors and changes lipoprotein metabolism of infected cells.

The etiologic significance of infectious agents in the development of AT can be ascertained by further studies in experimental animal models. Large, prospective eradication trials with antimicrobial therapies, currently being designed for *C. pneumoniae*, will also help to finally clarify what role these microbes play in AT diseases.

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PCR based typing of human papillomaviruses from gynecologic samples processed for Hybrid Capture™ test

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The Hybrid Capture™ human papillomavirus test (Digene) is used in some laboratories in Hungary. The test is based on nucleic acid hybridization. For the routine diagnostics, two mixes of nucleic acid probes are used to detect low risk (5 types) and high risk (9 types) oncogenic human papillomaviruses (HPV). Some clinical samples scored positive with both probes, indicating multiple papilloma viral infection of the cervix uteri. However we cannot exclude cross-reaction with other rare types.

To address this question, we developed a method that neutralizes and precipitates the clinical sample already denatured for the Hybrid Capture™ test. The precipitate is dissolved and subjected to a one step PCR amplification with the consensus HPV primer pair MY09-MY11, which is able it detect broad spectrum of anogenital HPV infections. Multiple infections are demonstrated by polyacrylamide gelelectrophoresis, where the mobility of nucleic acids is influenced also by the base composition under non-denaturing conditions. Typing is based on restriction fragment polymorphism. The results of the Hybrid Capture™ test and the PCR typing will be compared.

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Multicentre study for detection of human papillomaviruses with hybride capture assay in Hungary

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The human papillomaviruses (HPV) are regarded as the etiological agent of cervical carcinoma. A multicentre study was organized to determine the prevalence of HPV in the fertile female population in Hungary.

Simultaneously with the clinical sample collection, a questionnaire interview was performed to acquire data on the life style, sexual practice, etc. Between 1 April and 1

July 1997, 1200 women were examined colposcopically and cervix samples were collected for cytology and the detection of HPV DNA (Digene Hybrid Capture™ System).

17.4% of the samples were HPV-infected. 3.9% of the patients had acquired low-risk, and 10.2% high-risk HPV types. 3.4% of the women were simultaneously infected with both low-risk and high-risk HPV types. The correlation of the results of colposcopy and the HPV hybrid capture assay was 7.5%, whereas that of the results of cytology and the HPV hybrid capture assay was 73%. Statistically significant differences in HPV prevalence were detected as functions of age ($p<0.001$), age at first sexual intercourse ($p<0.01$), the number of sexual partners ($p<0.01$), a changing in sexual partner in the last 3 months ($p<0.001$), marital status ($p<0.001$), parity ($p<0.001$) or smoking ($p<0.001$). No statistically significant differences in HPV prevalence were detected with regard to ethnic group, education, socioeconomic status, dietary habits, working with chemical or radioactive agents, use of oral contraceptives, age at first birth, the previous performance of induced abortion, or the occurrence of spontaneous abortion.

Parallel performance of cytology and the HPV hybrid capture assay increases the certainty of the carcinoma prevention diagnostics and serves as a quality control of the cytological diagnosis.

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Functional significance of sequence variation in the E2 gene and the long control region of human papillomavirus type 16

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The long control region (LCR) and the E2 protein of human papillomaviruses (HPV) are the most important viral factors regulating the transcription of the viral oncogenes E6 and E7. Sequence variation within these genomic regions may have an impact on the oncogenic potential of the virus. We studied sequence variations in the LCR and in the E2 gene of human papillomavirus type 16 (HPV 16) isolates originating from cervical cancer patients from East Hungary. In 30 samples, sequencing and/or single-strand conformation polymorphism (SSCP) analysis revealed variants belonging to the European (E) variant lineage of HPV 16. These variants differed only slightly in the E2 and LCR sequences from the reference European clone. Three samples represented variants belonging to the Asian-American (AA) group. These differed at several positions in the LCR and E2 regions from the published reference sequence. Compared to the reference clone, the LCR clones of the European isolates showed very similar transcriptional activities, while that of an Asian-American isolate had ~1.8-fold increased activity. Most of the increased activity of the Asian-American isolate could be ascribed to nucleotide changes found at the 3'-end of the LCR (nt 7660–7890). The transcriptional transactivation potential of the HPV 16 E2 isolates differed only slightly from each other, and the differences seemed to be independent of the taxonomical position of the isolates.

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Genotyping of human cytomegalovirus strains isolated from prenatal infections

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Human cytomegalovirus (CMV) is the most common cause of viral congenital infection. Most of the infected infants are asymptomatic at birth, but in 10% of cases develop cytomegalic inclusion disease with a 20–30% mortality rate.

At present there is not enough clinical and laboratory evidence to predict the fetal outcome after CMV infection, therefore the question of whether an infected fetus is going to be damaged remains unanswered.

Certain regions of the envelope glycoprotein B (gB) gene are variable between different virus strains. Based on the sequenal variation of the gB gene, CMV strains can be classified into 4 gB genotypes. Initiated by the assumption that the gB genotype of CMV may have an influence on the outcome of prenatal infection we carried out genotyping on strains isolated from congenital infections.

Sixty pregnant women were included in our study. Thirty pregnancies were normal and in the other half the fetuses or newborn babies were highly suspected to have viral infection.

For detection of CMV in the amniotic fluids and a few cases in urine samples shell vial method and polymerase chain reaction (PCR) were used.

Of the amniotic fluid samples from the patients suspected to have viral infection 4 showed CMV by shell vial method and 7 by PCR. Amniotic fluid was not available from two patients of this group, but after birth CMV was detected in the urine specimens by both methods. There was no detectable CMV with shell vial method in the amniotic fluid specimens of the individuals in the normal group, however a weak CMV specific sign was observed by PCR in two cases. The gB genotypes of the CMV strains were determined by restriction fragment length polymorphism (RFLP) analysis of the isolates were PCR amplified CMV DNA. The genotypes of the related to the clinical findings.

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Apoptotic death of Vero cells following rubella virus infection

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The clinical course of natural childhood rubella is usually mild and frequently asymptomatic, whereas establishment of rubella virus infection of the fetus may cause profound damage in the ontogeny of fetal organs. We investigated the molecular events implicated in the cytopathogenicity of rubella virus in an effort to gain some insight into the teratogenic effect of the virus. To determine whether rubella virus-induced cell death is apoptotic, we examined the effect of rubella virus on Vero cells, which efficiently support viral replication and exhibit extensive cytopathic effect following infection. Our results demonstrate that apoptotic cell death occurs in 60–70% of cells infected with the To-336 strain of rubella virus. UV-irradiation of the virions abolished the capability of this virus to elicit the apoptotic response, indicating that productive infection is necessary to trigger cell death. p53 and p21 protein levels were highly elevated in rubella virus-infected cells. On the other hand, p53 gene expression was unaffected by rubella virus infection, while transcription of p21 gene significantly increased. These results suggest that post-transcriptional regulatory mechanisms accounted for the increased levels of p53 protein but the increased level of p21 protein is caused by transcriptional activity of p21 gene. Rubella virus infection slowed down cell proliferation and inhibited cell cycle progression, consistent with the induction of p53 and p21. These data implicate a p53-dependent apoptotic pathway in the cytopathogenicity of rubella virus, thereby suggesting a mechanism by which rubella virus exerts its teratogenic effects.

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Isolation of the first domestic hantavirus strain from a rodent

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Korean hemorrhagic fever was discovered in 1951 (Lee et al. 1989). The clinical appearance of the disease is hemorrhagic fever with renal syndrome (HFRS) in the Old World. Epidemic form of the disease occurred in Hungary as early as in the 1950s (Keleti et al. 1955), serologic diagnosis of HFRS started in the late 1980s (G. Faludi 1992). The causative agent of the hemorrhagic fever with renal syndrome is hantavirus. It belongs to

the Bunyaviridae family. Domestic occurrence of some bunyaviruses was already proven serologically in Hungary (Koller et al. 1969; Molnár et al. 1976). In contrast to the other related viruses (genera of the Bunyaviridae family), hantaviruses are transmitted without arthropod vectors and are assigned to several serogroups. At least two different types of hantaviruses (Hantaan /HTN/ and Puumala /PUU/) proved to be the causative agent of Hungarian human HFRS cases by serological methods (Takenaka et al. 1985). The possibility that the Seoul /SEO/ serotype may be present in our country has arisen, too (Faludi and Ferenczi, 1995). The presence of different types and variants of hantaviruses has been demonstrated in Yugoslavia, Slovakia and most recently in Austria (Avsic-Zupanc et al. 1991; Gligic et al. 1992; Taller et al. 1993; Bowen et al. 1997). These results suggest that we may expect emergence of further different hantavirus serotypes (e.g. Dobrava, Tula) with a high probability. The discovery of the hantavirus pulmonary syndrome in the USA (CDC, MMWR 1993) gave a new impulse to the research of these infections.

Study project: The essence of our collaboration was to demonstrate specific antibodies against viruses in rodents living at natural foci considered as infected on the basis of epidemiological data. We intended to isolate viruses, determine their identity and identify their host species.

Methods: Rodents were caught by trapping. Tissue-, blood- and organ-samples taken from these animals were investigated by serological, virological and molecular biological methods. For the serological tests we applied the following reagents: Hantadia (particle agglutination reaction specific to Hantaan virus), ELISA and indirect immunofluorescent tests specific to PUU and HTN viruses. The smears of spleens were investigated by indirect immunofluorescent assay using human Hantaan-specific antisera. Virus isolation attempts from the organs were done on Vero and Vero E6 cell lines by cocultivation, while the detection of viral genome has been accomplished by RT-PCR.

Results: Rodents trapped for the investigations were members of the following species: *Clethrionomys glareolus*, *Microtus arvalis*, *Apodemus sylvaticus*, *A. flavicollis*, and *A. agrarius*. Smears of spleens from several *Clethrionomys glareolus* and *A. flavicollis* were found to be positive using indirect immunofluorescent assay. In at least one case the isolation of the virus was successful, but identification is yet to be done. We succeeded in the detection of viral RNA. The "nested PCR" products from a *Cl. glareolus* tissue yielded PUU virus sequences. Further studies are needed for characterization of enzootic hantavirus types possible in natural foci.

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The effect of chloroquine on the early events of adenovirus entry process

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The chloroquine disrupts the transvesicular pH gradient in the endosomal system due to its alkaline nature and puts up resistance to the acidifying action of H^+-ATPase.

Adenovirus entry is a receptor internalization based process and the virus passes through the endosomal membrane directly on way to the nucleus, which process' detailed nature is unknown. In our experiments we followed the luciferase gene expression of a recombinant adenovirus (human adenovirus type 5) in the presence of chloroquine. This reporter gene made possible to detect the more subtle effects on the internalization of the virus as it can be detected in the traditional assay systems. In the presence of 0.1 mM chloroquine we could not detect the published inhibitory effects on the virus entry but 0.5 mM chloroquine was effective to prevent the virus internalization. Nevertheless both concentration was toxic to the cells. Moreover, changing the time of post-infection addition of chloroquine to the assay, different effects could be observed, under certain circumstances even the enhancement of the luciferase activity could be detected.

A. JÓZSA, J. FODOR, Z. KIRÁLY

Role of reactive oxygen species and antioxidants in plant host-parasite interactions with different symptom expressions

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Generation of reactive oxygen species (ROS) in higher plants, like in other living organisms, is a natural process. The most frequently occurring ROS are the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). Antioxidant prevention against ROS in plant cell is provided by enzymes and non-enzymatic molecules. However, transient and rapid generation of ROS sometimes exceed the antioxidant capacity of cells in infected plants. In this case ROS could affect many cellular processes and damage important macromolecules.

The aim of our work was to investigate and compare the role of ROS in different plant-virus interactions:

Chenopodium quinoa - tobacco necrosis virus (TNV) with local necrosis,

Ch. quinoa - tobacco mosaic virus (TMV) with local chlorosis, and then necrosis,

Ch. quinoa - zucchini yellow mosaic virus (ZYMV) with local chlorosis,

Ch. quinoa - sowbane mosaic virus (SoMV) with systemic chlorosis.

Generation of $O_2^{\bullet -}$ was measured by p-nitrotetrazolium blue dye. For the detection of H_2O_2agar plates containing KI-starch were applied. Large amount of both $O_2^{\bullet -}$ and $H_2O_2$was detected in the interactions with necrotic symptoms (*Ch. Quinoa*-TNV or TMV). Activity of antioxidant enzymes: ascorbate peroxidase, dehidroascorbate reductase, glutathione S-transferase, glutathione-reductase, catalase and the amount of ascorbate were measured spectrophotometrically. Interactions producing chlorotic symptoms showed decreased antioxidant enzyme activities. However, in the case of necrotic symptoms significant increases in enzyme activities were measured. In summary, increased antioxidant activities and large amount of ROS were detected only in the interactions which showed necrotic symptoms.

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Juvenile laryngeal papillomatosis: virological aspects

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Juvenile laryngeal papillomatosis (JLP) is the most common benign tumour of the larynx in childhood showing an ability of recurrence. Non-oncogenic human papillomavirus types 6 and 11 are the specific etiological factors of the disease. In the present study we demonstrate the two cases of recent years from the Department of Otolaryngology, University Medical school of Debrecen. The at present 6-year old male and 6-year-old female underwent microlaryngoscopic papilloma removals 5 times each after periods of hoarseness, stridor and dysphonia. As a medication they receive leukocyte interferon (IFN-α).

The aspects of our study were as follows: 1. *maternofoetal transmission.* In the parental heteroanamnesis we examined the signs of genital parental HPV infections. The conditions of delivery were important as vaginal delivery is much more frequent in the group of JLP patients in comparison with normal population. The mother of the female at the age of 3 was examined for the presence of HPV DNA by polymerase chain reaction (PCR). 2. *Sites of papilloma predilections* in the larynx. 3. *Histopathological signs* of HPV infection and a possible malignant degeneration in the tissues removed from the patients were researched, too. 4. The formalin-fixed and paraffin-embedded tissues were reviewed for HPV DNA by PCR applying type-specific primers. In our former study tissues from the first operations reviewed for HPV 6, 11, 16 and 18 DNA contained HIV 11 DNA, so materials from further removals were reviewed exclusively for this type.

Although parental heteroanamnesis was negative and maternal cervix proved to be negative for HPV we cannot exclude the possibility of a subclinical HPV infection being present at the time of delivery. Papillomas showed predilection sites in the normal squamociliary junctions of the larynx. Histopathological examination showed koilocytosis and parakeratosis characteristic for HPV infections. We did not find any signs of malignant alteration, which is a rarity in laryngeal papillomas. The 70 bp amplimer deriving from the L1 region of the genom of HPV 11 was present in each tissue. On the basis of localisation and the results of histopathological and molecular studies we consider our patients as typical cases of juvenile laryngeal papillomatosis.

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Examination of B-cell development in bursa of fabricius using PCR and northern blot methods

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We examined the gene expression of the chB1 lectin from bursa of Fabricius of embryonic and several-day-old chickens using RT-PCR and Northern blot methods. ChB 1 lectin is a cell-surface marker, which is expressed only in bursal B-cells at the stage of B-cell maturation in which the gene conversion process generates the antibody repertoire. Recent studies suggest that the expression of this gene is highly dependent on the bursal microenvironment, which influences the maturation of the B-cells. In the present state of our study we examine the physiologic status of the chB1 expression in normal B-cell development in order to analyze the influence of the in ovo applied infectious bursitis (IBD) vaccines to B-cell development through the change of expression of chB1 gene.

J. NEMES, J. SZABÓ, Z. BECK, A. BÁCSI, I. ANDIRKÓ, J. KISS, F. D. TÓTH

Interactions between human immunodeficiency virus (HIV) and human t-cell leukemia-lymphoma virus (HTLV) in monocyte-derived macrophages

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Coinfection with HIV type 1 (HIV-1) and HTLV type I (HTLV-I) is associated with a high frequency of tropical spastic paraparesis (TSP) or an increased risk of development of AIDS. Since in vitro dual infection of CD4+ T-lymphocytes with HIV-I and HTLV-I was reported to result in a moderate enhancement of replication of both viruses, we investigated the interactions between HIV-1 and HTLV-I in cultures of monocyte-derived macrophages. The lymphocytotropic IIIB and macrophagetropic Ada-M strains of HIV-1 were used. The HTLV-I was prepared from the supernatants of the virus-producing MT-2 cell line. Since matured macrophages resist to infection with lymphocytotropic HIV, 5-day-old macrophages were used throughout our studies. The type of interactions between HIV-1 and HTLV-I depended on the tropism of the HIV-1 isolate used for coinfection. The HTLV-I could stimulate the replication of the lymphocytotropic HIV strain but not that of the macrophagetropic Ada-M strain. In contrast, both IIIB and Ada-M HIV isolates were capable of enhancing HTLV-I release from dually infected macrophages. To study the mechanism of the stimulatory effects, we transfected macrophages with plasmids pcTAX or pTAT and then infected them with HIV-1 or HTLV-I, respectively. It was shown that reciprocal stimulatory interactions were mediated by the trans-activating proteins of these retroviruses. Our data strongly suggest that (i) significant interactions between HIV-1 and HTLV-I may occur in

macrophages, and (ii) the in vivo consequences of such coinfections are likely determined by the tropism of HIV-1 strain interacting with HTLV-I in these cellular targets.

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The use of NASBA/NucliSens for the monitoring of antiretroviral treatment in HIV infected children

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NASBA was selected for follow-up of virus-RNA level in AIDS patients in Hungary. As Hungary belongs to low prevalence countries with HIV, most patients – including children – are involved in monitoring of the antiretroviral treatment. In this study we demonstrate the quick change in RNA copy number at the beginning of the treatment, and at change of drug during therapy in children living with HIV infection. CD4 cell count and virus phenotype were determined in correlation with RNA level measurements.

Plasma and lymphocytes were separated from whole blood of children representing symptom-free HIV infected, pre AIDS and AIDS stages of the disease. Measurements of RNA copy number were performed (3–10 measurements from the same patient) according to the manufacturer's instructions (Akzo Nobel). CD4 cell count was determined using standard flow cytometry (Becton Dickinson) simultaneously. Virus phenotype investigations were completed by cocultivation of peripheral lymphocytes with MT2 cells.

The RNA level decreased shortly (1–2 weeks) following the start of the therapy, or change of the drug applied. It is a quick process compared to the changes in CD4 cell counts. Virus isolation was successful in all cases, when high viral copy number was measured. In most of the cases involved in this investigation SI (syncytium inducing) HIV-1 strains have been found.

We concluded that NASBA (recently NucliSens, a more sensitive generation of the method) is a suitable help in monitoring the health status of HIV infected patients.

É. BARABÁS, R. GONZÁLEZ, A. HORVÁTH, K. NAGY

Alteration of intracellular cytokine synthesis in CD4+ and CD8+ cell subsets during progression of HIV disease

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Aims: Intracellular cytokines IFNγ (type 1) and IL-4 (type 2) at the single cell level were measured by flow cytometry in he several stages of HN infection and role of

alteration in proportion of cytokine producing cells in progression of disease was investigated.

Study groups: 1. Control subjects (n=5). Asymptomatic HN infected individuals: 2. with CD4 > 500 cell/μl (n=5), 3. with CD4 < 200 cell/μl (n=5).

Methods: Flow cytometric three-color analysis (Fastimmune Cytokine System, BD) a novel method in Hungary was used. Peripheral blood mononuclear cells stimulated for 4 hours with PMA/Ionomycin and brefeldin-A was used to interrupt intracellular cytokine transport. Cell surface was stained with CD4/CD8 PerCP for 15 min. After cell membrane permeabilization intracellular staining applied for 30 min with IFNγ/FITC and IL-4/PE. Cells were fixed in 1% paraformaldehyde. Intracellular cytokine production was analyzed by gating on CD4+, CD8+ cell subsets respectively.

Statistical analysis: Student's *t*-test.

Results: 1. After 4-hour stimulation of PBMCs less than 3% of CD4+ and CD8+ cells were IL-4 positive (type 2) or double positive (type 0) in all groups. 2. In CD4 subsets, 20% of the activated cells in the control and 17.5% and 12% of the cells, respectively in HIV infected groups showed IFNγ production (NS). 3. In CD8 subsets, however, 53% of the control cells showed IFNγ synthesis compared to that of 32% ($p=0.01$) and 10.5% ($p<0.001$) positivity in HN infected groups. Intracellular IFNγ production by cells from patients with CD4 count < 200/μl was significantly less ($p=0.005$) than in cells from those with CD4 count > 500/μl.

Conclusions: 1. Decreased IFNγ production of CD8+ cells during the course of HIV infection developed as a consequence of the effect of the virus induced progressive dysfunction in CD8+ population, which may have a significant role in immunodeficiency. 2. Our data demonstrate a deficiency in type 1 cytokine production. A type 2 phenotype switch, however, could not be established after PMA/Ionomycin stimulation in our system.

B. KEMÉNY, K. NAGY, A. HORVÁTH

Frequency of 432 mutation of HIV co-receptor CCRS in HIV infected and healthy population

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When infects the target cell, HN binds to two receptors on the cell surface. One of them is the CD4 receptor; the second one is determined by the type of the virus; the T cell tropic, syncitia inducing (SI) HIV uses the CXC chemokine receptor 4 (CXCR4), while the macrophage tropic, NSI variant of the virus prefers the CC chemokine receptor 5 (CCR5).

In the European population a mutant allele of CCR5 can be found, with a deletion of a 32 bp region. The reading frame is erroneous and the protein translated from this gene cannot function properly as a receptor, therefore it is not able to bind HIV. Persons who are homo- or heterozygotes for this deletion have a partial resistance against the HIV infection, and in those who are infected, the progression of the disease is slower. We

investigated the frequency of the allele 432 in the Hungarian population in the following study groups: i) healthy (HIV negative, with normal risk of infection) population ii) HIV infected people, with standard progression of the disease, iii) HIV positive persons with slow progression of the infection iv) HN negative sexual contacts of the infected patients. In our study PCR was used for the detection of the polymorphism of the CCRS gene in DNAs from the PBMCs of 157 individuals.

Our results:

	wt/wt	wt/Δ32	Δ32/Δ32	Σ	Allele frequency
Norm. population	59	16	2	77	0.1299
HIV+, progressor	47	10	0	57	0.0877
HIV+, nonprogr.	3	2	0	5	0.2000
HIV–,contactus	15	3	0	18	0.0833

We demonstrated that the 432 deletion is less frequent among HIV infected persons than in the normal population. Here we also provided evidence that Δ32 mutation is significantly higher in HIV infected long-term non-progressors. However in sexual contacts of HIV infected individuals – those who remained uninfected albeit that they belong to high risk groups – the higher frequency of Δ32 deletion could not be verified. The mutation of the CCRS gene is indicative that by blocking the coreceptor the entry of HIV into the cell could be inhibited, which may open up new approaches in the therapeutical intervention of HIV/AIDS.

BACTERIOLOGY

P. BIACS, Á. KARDOS

Environmental effects of genetically modified microorganisms

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The genetically modified organisms (GMO) have generated a powerful resistance among the food consumers, though the scientific public opinion and the economic life have been looking on a wide-ranging application of them with great expectations. In case of the plants of general production (corn, soybean, tobacco) these plants have taken over from other organisms the surplus task (e.g. BT corn) by the help of which they can protect themselves against parasites (insects) and pathogens (viruses).

The gene-technical law having been accepted recently in our country remedies not only the legal sort-comings but ensures – due to the composition of the approval committee to be created – the intervention of the interested social group (environment protectors, consumer protectors). In international practice, mainly by the immunological testing of new protein components and the detection of the new DNA fragment (PCR-technics) the possibility has been made available for the demonstration of genetical modifications. Actually, the work of the transformation of qualitative testing to quantitative determination has started. About 40 European laboratories have got invitation to give account within the scope of circular-scale laboratory tests of their through grounding and applied technics they are using for the GMO demonstration built in the new food basic materials. The common research center of the European Union (JRC) have evaluated already 30 of them and data of more than 20 instructions have been judged to be acceptable. The about 2000 measurement data are providing possibilities for the public opinion to be able to form a judgement of the preparedness of demonstrating supposed or declared genetic modifications.

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Soil microbial biomass-C as a possible bioindicator near two Hungarian power plants

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Indication value of microbial biomass-C was studied in case of two presumably contaminated soils by power plants at Visonta and Százhalombatta region in Hungary. The seasonal changes of four soil microbiological parameters, suggested by novel studies, microbial biomass-C, biomass specific respiration rate, relative proportion of biomass-C to organic-C, and the formation of biomass-C during a 30 days incubation with lucerne meal were studied. Soils were sampled from five locations at seven times from spring 1995 to spring 1996. Although the dust deposition rate was not high in the above regions, some accumulation of inorganic pollutants could be observed, however this was below the legal environmental limit. The low level of pollution did not cause significant changes in microbial biomass-C. The results of our investigations showed strong differences in microbial biomass-C during the season which can be probably related to the nutrient state of the soil rather than the soil moisture or temperature. Biomass specific respiration of soils significantly differed from site to site but there is no evidence whether it can be attributed to the soil contamination. The relative proportion of biomass-C to organic-C varied significantly during the season but it can be related to the different clay content of the soils. Carbon incorporation rates into microbial biomass were also changed during the year in spite of the equal incubation conditions and they were not altered by the soil contamination.

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G. SZABÓ, M. VARGHA, K. MÁRIALIGETI

Microbiological examination of the filtering capacity of the Danubian gravel-bed

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Examination of Danube bankwall-filtered wells, which play an important role in providing drinking water for Budapest, has been the aim of many long-term studies at the Department of Microbiology. In this study, a laboratory model was built in order to investigate the filtering capacity of the gravel-bed under laboratory conditions. The model contains sediment sample from the catchment area of the bank-filtered wells by Kisoroszi, and it is being supplied continuously by Danube water.

Main chemical and hygienic parameters substantial in drinking water quality assessment were followed. Results showed that the concentration of nitrite and ammonia and the bacterial germ counts significantly decreased, as it had been expected considering the usual microbial processes in the sediment. Identification of bacteria isolated from inlet and outlet water can give information on the microbial changes during filtration. On the basis of morphological, physiological and biochemical investigations, most of them proved to be characteristic water and sediment organisms which support us simultaneously with data on the possible biotransformation capability of the sediment column.

Atrazine was used to examine the special xenobiotic degrading capacity of the system. This herbicide is constantly present in Danube water, due to its wide agricultural usage. A single atrazine pulse load appeared in the outlet in an unexpectedly short time. This result suggests that water movement throughout the diameter of the column is by no means uniform. Supposedly, high-speed "channels" intercalate with stagnating cores. In few days, the total amount of pulse loaded micropollutant passed through the sediment sample, thus measurable biodegradation was not detected in such a short time. However, continuous atrazine supply may initiate biodegradation. Utilization of atrazine as a carbon and energy source by characteristic strains isolated from outlet water has also been investigated. Incubating these strains in media containing atrazine in different concentrations, various levels of growth inhibition were detected in different strains. However, some identified strains (*Tsukamurella paurometabolum*, *Janthinobacterium lividum*, *Pseudomonas* sp.) seem to be able to utilize atrazine as a sole carbon source.

A. SZABÓ

Significance of morphological and morphogenetical studies in Ciliata (protozoa)

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In taxonomy and ecology identification of species or other taxa is indispensable. In the classic period of Protozoological research – until the end of the 30s – description of taxa (species, genera, families, etc.) was based mainly on morphological characters. Wide-scale usage and perfecting of staining methods made possible a more accurate description and identification of species.

The aim of the earlier staining methods (e.g. silvering, staining of Klein, Párducz, Gelei) was to make the important characters of Protozoa (nucleus, contractile vacuole), necessary for identification visible.

Due to new methods – which frequently are the modifications of old ones – the accuracy of identification is remarkably increased.

The dry silvering method of Klein (1926) was modified by Foissner (1976, 1981). Corliss proposed a modification of Chatton-Lwoff's wet silvering method. Foissner et al. (1984) have modified the silver-carbonate method of Fernandez-Gaizano.

The protargol impregnation method described by Drageso and Tuffrau was improved by Wilbert (1975) and later by Foissner (1982).

These methods (modifications) made possible the identification of morphological characters of species, and furthermore the study of the details of morpho- and stomatogenesis (reproduction of forms and ultrastructure) and reproduction of their individuals.

By these methods taxonomical relationship of several species have been clarified. Different species, described earlier only by morphological characters, were reduced into one (e.g. Euplotes sp.), new taxonomical relationships were described and it was possible to re-describe some species as well (e.g. Foissner, Hemberger, Curds, Petz, Weibo).

Recently the morphogenetical description of species has great significance in addition to the morphological characters.

This is demonstrated here by the morpho- and stomatogenesis of a Ciliata (Protozoa, Hypotrichida) species, *Onychodromus grandis* Stein 1859. Morphogenesis of this species is similar to that of other species belonging to the genus (*O. acuminatus*, *O. quadricornutus*, *O. indica*). Similarities are found to the morphogenesis of *Laurentiella acuminata* and *Stylonychia vorax*, but with remarkable differences in morphological characters and in the development of marginal cirrus "anlagen".

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Investigations on gill bacterial communities of eel nematode infected European eel

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The causes of mass eel kill in Lake-Balaton are still unknown. Among the possibly multifactorial causes, beside eel nematode, bacterial infections play certainly a role. The focal point of invasion of obligate or opportunistic bacterial pathogens is supposed to be the gill, since the nematode infection deeply influences the respiration of eels. The comparative characterization of gill microbiota in eels differing in the severity of eel nematode (*Anguillicola crassus*) infection could provide us with important informations.

During the summer of 1997 eels were collected three times. Eels were dissected to excise gills, which were then dropped into sterile phosphate buffer, and washed carefully. One g of gill tissue was homogenized in phosphate buffer, diluted and plated onto blood, chocolate, glutamate, yeast supplemented nutrient, and RCA agars. Plates were incubated at room temperature under aerobic and anaerobic conditions. From a total of 8 eels with different level of eel nematode infection 250 aerobic or facultatively anaerobic and 50 obligately anaerobic bacteria were isolated. These were grouped using ARDRA, and group representatives were identified by BIOLOG test and/or by partial 16S rDNA sequence analysis. 16S rDNA based identifications were also made from total DNA isolations.

In case of eels characterized as fully healthy based on parasitological investigations gill aerobic germ counts were higher and the morphotypes of colonies proved to be more numerous. On contrary to this, the gill bacterial communities of healthy fish were characterized with the dominance of a single bacterium, *Aeromonas veronii*. Other species occur only sporadically. In parasitologically ill fish, in gills *Pseudomonas* species, namely *P. putida* and *P. stutzeri* dominate, while aeromonads significantly decrease in numbers. Moreover a series of other "aquatic or soil" microbes invade the gills, like *Dietzia maris*, *Shewanella putrefaciens*, *Acinetobacter* spp., *Plesiomonas shigelloides*, *Comamonas* sp. Besides, the anaerobic germ counts significantly increase.

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The role of soil microbiota during colonization of different soil types by *Trichoderma* fungi

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The effect of physico-chemical parameters of different soil types and soil microbiota on the colonization of soil by *Trichoderma* was studied. Mathematical model of soil colonization by *Trichoderma* fungi was built-up and it was stated that the sensitivity of trichodermas to the soil microbes (especially to bacteria) may play an important role during the colonization process. This factor might cause the significantly different colonization of different soil types comprising presumably different microbiota. Chernozem soils were not colonized, whereas sandy soils were always grown over by the applied strains. In brown forest, salt affected and meadow soils medium colonization values were obtained. Significant correlations with colonization value were found in the case of humus content (r=–0.75), K_2O content (r=–0.65) and P_2O_5 content (r=–0.58). These factors may not directly affect the colonization of soil. They rather have an impact on the activity of microbial communities, which may be higher in soils with better nutrient maintenance. The more active microbiota allowed less colonization by trichodermas. It was supported by the result of the treatment of soil with a fungicide metalaxyl selective against oomycota or a broad-spectrum antibacterial streptomycin. They caused markedly enhanced soil colonization by *T. atroviride* and dropping of correlation coefficients.

H. M. ROFAAT, K. MÁRIALIGETI

Comparative studies on the actinomycete communities of cattail and papyrus rhisosphere

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Wetlands, and among them especially floating mats play an utmost importance in the fossilisation pathway of plant residues. They are effective sinks of atmospheric CO_2, since in the strictly anaerobic environment the characteristic local N and S cycles together with low pH, etc. strongly inhibit mineralization of organic debris. It is by no means a wonder that most of microbial activities concentrate into the rhisospere of macrophyton, where aeration occurs.

Samples were taken from the root region of cattail (*Typha augustifolia*) growing in a floating mat of Soroksár Danube arm, and of papyrus (*Cyperus papyrus*) growing in a floating mat on the river Nile near Alexandria. Root washing fluids were used for plating and isolation on different actinomycete isolation agars. The bacterial counts in case of *Typha augustifolia* were in order of 10^4/g, where in case of papyrus they were higher by

one order of magnitude. Actinomycete counts proved to be 10^2–10^3/g in both samples. The germ counts report of a low aerobic bacterial activity. In cattail samples polysporic and monospore actinomycetes were isolated in equal numbers (with a *Streptomyces* sp., and *Micromonospora coerula* in dominant position), whereas papyrus rhisospheres were characterized by the dominance of *Streptomyces* sp.

The ecological tolerance levels of the strains isolated tested under laboratory conditions enable us to suppose their vegetative growth in the root environment. The characteristic difference between the two rhisosphere environments is most possibly a function of root physico-chemical properties, first of all aeration levels.

A. MICSINAI, A. BORSODI

The bacterial communities participating in the biodegradation of reed rhizomes

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The degradation of reed rhizomes in their natural environment has been studied. The rhizomes harvested in early spring 1997 were kept underwater until collected in July and October 1997. Dilutions of scrapings taken from the surface of the degrading rhizome pieces were spread and cultivated on cellulose containing media in order to gain insight into the biofilm community structure. With 102 surviving representative strains physiological, biochemical and ecological tolerance test were carned out and sole-carbon-utilisation patterns were recorded with the Biolog metabolic fingerprint method.

The species identified in the summer sample included: *Aeromonas hydrophila, A. media, Pseudomonas pseudoalcaligenes* and *Shewanella putrefaciens.* The species composition of the autumn sample was the following: *Alcaligenes latus, Al. eutrophus, Hydrogenophaga palleronii, Micrococcus luteus, Ancylobacter aquaticus, Xanthobacter flavus, Corynebacterium* sp., *Bacillus circulans* and *A. hydrophila.*

The characteristic differences in the species composition of the two samples can be explained by a seasonal change in the bacterial community structure, and by the different media used for isolation and cultivation.

The dominant species of the summer sample were facultatively anaerobic, fermentative bacteria whose presence on the surface of the degrading rhizomes might be connected to the higher productivity of warm waters during the summer period. The autumn sample contained relatively more facultatively chemolithotrophic bacteria along with chemoorganotrophic organisms. These organisms are potentially able to establish a local nitrogen cycle within the biofilm on the reed surface. These biofilm bacteria are active participants of the community level metabolism, which eventually results in the degradation of the cellulose containing materials.

S. SZABÓ, CS. FENYVESVÖLGYI, S. BALÁZSY

Impacts of algal species on duckweed *Lemna gibba* during competition

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Axenic *Lemna gibba* L. (duckweed) cultures grown on autoclaved communal wastewater were inoculated by four green algal, one diatom and five cyanobacterial species. These species were obtained from a local minipond containing wastewater covered by duckweeds. In a parallel experiment, *Lemna* cultures were grown on algal treated solution in such a way that there was no direct contact between the fronds and algal cells. After ten days of incubation, the total chlorophyll content and wet weight of the duckweed cultures were measured. The bioproductions of *Lemna*-alga co-cultures were compared with the bioproductions of *Lemna* cultures grown on algal treated algal free solution. The influence of water and ether soluble extracts of algae on *Lemna* cultures was also measured.

When the duckweed cover was incomplete, with the exception of the diatom species, the bioproduction of *Lemna gibba* cultures decreased significantly on solutions which had been treated with the examined algal species. The result of the experiments showed that algae inhibit the growth of duckweeds by their alkalization activity and by the removal nutrients from the solution. *Sphaerellopsis* sp., *Chlamydomonas ehrenbergii* and *Oscillatoria redekei* had inhibitive contact effect on *Lemna* cultures.

These algae formed biofouling on the fronds and the roots of duckweeds. The examined algal species did not excrete duckweed inhibiting allelopathic substances into the medium. The water and ether soluble extracts of algae did not show inhibitive impact on *Lemna* cultures. The water soluble extracts of *Protococcus viridis*, *Oscillatoria redecei*, *O. pseudogeminata*, *Lyngbya limnetica* and *Nostoc* sp. significantly stimulated the bioproduction of duckweeds.

[1]A. GRALLERT, [1,2]M. SIPICZKI

Investigation on *sep9* gene of *Schizosaccharomyces pombe*

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The *sep9* gene of *Schizosaccharomyces pombe* was identified in genetic screen for genes important in the control of cytokinesis. It encodes a 406 amino acids long protein which shows homology with the *SPT8* transcription factor of *Saccharomyces cerevisiae* and has a highly conserved domain found in a number of other eukaryotic proteins, among them the β subunit of heterotrimeric G proteins. The *sep9* gene is not essential.

The *sep9-307* mutation causes a pleiotrop phenotype. It induces a defect in sexual differentiation, which causes sterility. The mutant cells are defective both in conjugation and in meiosis, but the cells are able to produce both mating pheromones. The mutant is

also defective in the cytokinesis, which results in the formation of short mycelia. The structure of the cell wall and the septa is normal and the *sep* phenotype does not result by changes in the interphase actin or tubulin cytoskeleton. The number of cells containing duplicated SPBs is much higher than in the wild type cells. At the restrictive temperature a lot of abnormal septa were observed and the mutant cells are defective in chromosome separation, they form a normal mitotic spindle and the chromosomes are condensed but mitosis does not occur.

[1,2]E. ZILAHI, [2]Á. GRALLERT, [1,2]M. SIPICZKI

Investigations of *sep15* gene of *Schizosaccharomyces pombe*

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S-phase, mitosis and cytokinesis are the landmarks of eukaryotic cell cycle. *Schizosaccharomyces pombe* is an eukaryotic haploid organism. Fission yeast cells grow by elongation at their ends and divide by binary fission after forming a centrally placed septum. We have isolated and identified a number of cytokinesis mutants called *sep* mutants. *Sep* mutants are able to form septa, yet they are unable to complete cytokinesis. Since the daughter cells do not separate, the mutants form short mycelia. We were particularly interested in *sep15* mutant, which is sterile in addition to displaying the *sep* phenotype.

Using classical genetic methods it was established that the inheritance of *sep* phenotype and sterility is a recessive feature encoded by the same gene.

We have tried to find out any functional interaction creating double mutants between the *sep15* and other *cdc* (cell division cycle) mutants.

Using immunofluorescent techniques it was found that the distribution of actin and tubulin cytoskeleton is normal but the separation of chromosomes is aberrant at restrictive temperature.

In addition to the classical genetic methods we have cloned the *sep15* gene. The phenotype of the *sep15* mutant cells made us to develop a direct selection system for cloning. We have used conventional genomic DNS library called pSP1 for the selection. As a result of the cloning we have found a 5.5 kb fragment which carried the $sep15^{+}$ information. After the physical mapping of this fragment we started the subcloning of it using pUR18N shuttle vector to define the gene providing the above-mentioned functions.

Determining the DNA sequence of *sep15* gene, additional molecular investigations are required to reveal what process the gene is involved in. The complete characterization of *sep15* gene may give further understandings of the regulation of cytokinesis.

L. MAJOROS, C. MISZTI, B. SZABÓ

Identification and determination of antimycotic sensitivity of *Candida* species isolated from clinical specimens

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The authors isolated 510 *Candida* yeasts from 22.512 clinical specimens during a 10 months period in the University Hospital of Debrecen. The identification of species was performed by culture on CHROM-agar, biochemical reactions (bioMérieux, ATB system) and germ tube test. The determination of antimycotic sensitivity of isolates was carried out in the clinically necessary cases and upon request of the physician of the patient.

The 510 isolates could be included into 14 *Candida* species: 62% to *C. albicans*, 10% to *C. glabrata*, 5% to *C. tropicalis*, 3% to *C. krusei* and 20% to other *Candida* species.

The sensitivity to flucytosin, amphotericin B, nystatin, miconazol, econazol and ketoconazol was tested by determination of MIC values. Ninety-nine percent of *C. albicans* strains was sensitive to amphotericin B, whereas the ketoconazol proved to be the less effective drug. Hundred percent of *C. glabrata* strains were sensitive to the 6 drugs tested, while the *C. tropicalis* isolates showed complete sensitivity only to flucytosin.

The frequency of isolation of different *Candida* species in immunosuppressed persons varied from 30 to 50 percent. Analysis of epidemiologic aspects of our data by determination of karyotype of *Candida* strains by pulse-field electrophoresis is going on in our laboratory.

[1]J. ZALA, [2]A. ZALATNAI, [3]G. SÁNDOR

Coccidioidomycosis in Hungary

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In the year 1997 a coccidioidomycosis was diagnosed in Hungary. There were some earlier publications from the 50s about coccidioidomycosis cases in this country, but the origin could not been clarified. Thus the recently observed case could be regarded as the first imported one in this country. The 61 years of age white male US citizen patient came from the desert area of Arizona, USA, where he lived between 1993–96. Subsequently he has been accepted as a teaches in Hungary. During two weeks a painless bump have been developed on his chest wall in the spring of 1997. A tumor-like lesion in the anterior thoracic wall on the right side of the sternum was observed regarded as a tumor of indeterminate origin and this subcutaneous mass has been surgically removed.

However, histologically no neoplastic proliferation was seen, but there were a lot of PAS positive randomly distributed spherules in the specimen. It proved to be *Coccidioides immitis* infection, which was supported serologically with counterimmunoeletrophoresis as well.

[1]G. GICZEY, [2]J. KUKOLYA, [1,2]L. HORNOK

Characterization of extracellular lytic enzymes of the biocontrol fungus *Coniothyrium minitans*

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Coniothyrium minitans is an effective mycoparasite of sclerotium forming plant pathogenic fungi, therefore it can be used in plant protection as a biological alternative to chemical fungicides. Despite of its potential as a powerful biocontrol agent, the mechanisms responsible for the hyperparasitic activity of this fungus are rather unknown.

During the parasitic process, the hyperparasite penetrates through the external pigmented layer of sclerotia, a process followed by inter- and intracellular growth of the fungus. The penetration involves both physical pressure and enzymatic degradation of the sclerotial wall. Fungal cell wall degrading hydrolytic enzymes, such as chitinases, β-1,3-glucanases and proteases have been suggested to play a major role in the biological activity of biocontrol fungi.

The aim of the present study was to characterize the chitinolytic and glucanolytic enzyme systems of *C. minitans*. We determined the time course of the extracellular N-acetyl-β-D-glucoseaminidase, endochitinase and β-1,3-glucanase activities of the fungus grown in a synthetic medium supplemented with various carbon sources, such as glucose, colloidal chitin and sclerotial wall preparation of *Sclerotinia sclerotiorum*. Chitinase and β-1,3-glucanase isoenzymes produced by the fungus on these carbon sources were detected in gels after SDS-polyacrylamide gel electrophoresis. Four N-acetyl-β-D-glucoseaminidases, one endochitinase and three β-1,3-glucanases were observed in the samples. The induction of these enzymes depends on the carbon source used. Assay for N-acetyl-β-D-glucoseaminidase activity after isoelectric focusing of the proteins produced in the presence of chitin as a sole carbon source revealed two isoenzymes with approximate pI points 4.0 and 4.5, respectively. Chromatographic purification and further characterization of these hydrolytic enzymes are in progress.

GY. TURÓCZI, J. FODOR, T. ÉRSEK

Detection of free radicals in the parasitic relationship of fungi

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The importance of free radicals produced in animal and plant tissues as a consequence of stress has been known for a long time. Reactive oxygen species are for example formed during the infection of plants by pathogenic fungi.

We hardly (or not at all) know anything about the occurrence of free radicals in the kingdom of fungi. For this reason we decided to detect hydrogen peroxide in the antagonist-parasitic relationship of different fungi. H_2O_2 is a reactive oxygen species in plants, and since it gets out of cells, it can be easier detected than other, in the cell decomposing free radicals and reactive oxygen species.

Microscopic fungi in the genus *Trichoderma* are well-known hyperparasites of many plant pathogenic fungi. In their antagonism either mycoparasitism, antibiosis and saprotrophic competition are important. So we have tried to detect H_2O_2-production in the in vitro interaction of *Trichoderma* strains from our strain collection and of plant pathogens like *Bipolaris sorokiniana*, *Fusarium oxysporum*, *F. solani*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*, respectively. As reagent we employed the solution of potassium-iodide/starch solidified with agar. (Molecular iodine formed in the reaction of H_2O_2 and KI gives intensive blue color with the starch.)

We have developed a new method for the application of potassium-iodide/starch reagent: the concentration of the reagent and other circumstances have been optimized. H_2O_2 production could be detected in each pairing of *Trichoderma* strains and *S. sclerotiorum*. The age of colonies and pairings strongly influenced the color reaction, but it was independent of the media used. The color reaction was uncertain in other pairings. Axenic cultures and non-antagonist (i.e. not a parasite-host) pairings never produced color reaction. This supports our theory that the detected H_2O_2 was released from the parasited host cells during mycoparasitism.

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Microbial inoculations: success and failure among various environmental conditions

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Beneficial microbes, such as associative and symbiotic N_2-fixers (Azospirillum, Rhizobium), PGPR Pseudomonads and arbuscular endomycorrhizal fungi (AMF) should make a great influence concerning the plant growth, development and health, i.e. the plant

fitness. That is why it is much more highlighted nowadays to develop a common legislation policy in Europe both for the fundamental and for the applied aspects of inocula production (COST Action 8.30: "Microbial inocula in the agriculture and the environment").

Regarding the unsuccessfulness of microbial inoculations, numerous side effects do exist, such as the changeable environmental conditions, site specific soil properties, abundance and effectivity of indigenous microbes, soil-born plant pathogens, xenobiotics or other environmental pollutants, overfertilized soil conditions, low competitiveness of inocula components... etc.

The possible advantage of using microbial inocula components is to be presented in the lecture; the role of the beneficial microbes, and also their synergistic effect on the plant growth and development as a function of various environmental circumstances; the necessity of using numerous in vitro screening procedures for selecting the most appropriate homologous isolates as potential inocula components to the various stressed environmental conditions (Biró et al. 1995).

Using a new fluorescens induction in vivo technique (OJIP test) (Strasser et al. 1997) it was possible to evaluate the physiological stage of the hosts and the effectivity of microbial inoculation already at the early stages of experiment.

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I. LENTI

Mushrooms infected by parasitic fungi in the "Bátorliget" reserves

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When studying mushrooms of the Bátorliget Nature Reserves it was found that the large mushrooms were heavily infected by microscopic fungi. As several publications have appeared about this topic (Arnold 1969–89, Helfer 1991), I decided to examine the relationships among mushrooms and their microscopic fungi.

In Hungary this field has not been thoroughly studied promising interesting new results.

Intrahymenial parasitic fungi interweave the texture of the large mushrooms and appear on their surface in a variety of colours and forms. Biotrophic parasitic fungi infect live mushrooms and when the mushrooms die, so do the parasites. Purely saprophytic fungi only colonize the body of the mushroom when the mushroom has died.

I have found *Sepedonium chrysospermum* (Bull.) Fr. microscopic parasitic fungi on 11 *Boletus*, 5 *Xerocomus*; and one on species of *Gyroporus*, *Gyrodon*, *Leccinum* and *Tylopilus* species.

Cladobotryum tulasnei (G. Arnold) W. Helfer comb. now. is a parasitic microscopic fungus that "specializes" exclusively to *Lactarius* species. Four of their eleven species identified so far in the area of the Bátorliget Nature Reserves are regarded as a host to *Cladobotryum*. No data to the occurrence of *Cladosporium tulasnei* in Hungary has been found in domestic literature.

Spinellus fusiger (Link: Fr.) van Tiegh. is a parasitic of certain *Lactarius* species; *Syzygites megalocarpus* (Ehrenb.: Fr.) is parasitic for *Boletus* and *Xerocomus* genera, but some "common" microscopic fungi have been isolated such as *Cladosporium herbarum* (Pers.: Fr.) Link, *Botrytis cinerea* Pers. and *Penicillium expansum* Link: S.F. Gray from the same group of microscopic fungi.

The forms of microparasitic fungi imperfecti have not yet been identified it can be supposed, however, that they can be found on the infected, rotting remains of mushrooms in the Bátorliget Nature Reserves.

I started the isolation of interhymenial parasitic species from certain genera of the *Piptoporus*, *Polyporus*, *Armillaria*, *Ganoderma*, *Scleroderma* and *Cortinarius* mushrooms.

A. PENYIGE, N. DEÁK, A. KÁLMÁNCZHELYI, GY. BARABÁS

Signal transduction and differentiation in *Streptomyces griseus*

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The initiation of sporulation of bacteria is a complex cellular event. This developmental decision requires the integration of both external and internal stimuli, including nutritional, cell density, metabolic and cell cycle signals. In *Streptomyces* the sporulation also requires communication between cells, mediated by chemical signals, like A-factor (AF), in *S. griseus*. An important question is, how does a cell monitor these signals and convert that information into developmental decision, especially interesting is to study the role of the cellular membrane in signal transduction.

The identification of several GTP-binding proteins (GBPs) (showing homology to eukaryotic G proteins) in the plasma membrane of *S. griseus* 2682 raises the possibility that *S. griseus* possess GBP mediated signal transduction systems, like eukaryotes. This suggestion was supported by the findings that in vivo activation of GBPs by GTPγS or AlF_4^- restored sporulation in a nonsporulating (spo^-) but AF responsive mutant of *S. griseus* 2682, imitating the effect of AF. Even the age dependence of their effect was similar to that of AF. Mastoparan, a cationic amphiphilic oligopeptide – known to activate membrane bound GBPs – also effectively initiated the morphological differentiation in our mutant. In addition, AF stimulated a high affinity GTPase activity present in the cellular membrane of our mutant, in an age-dependent manner. We have also found that AF and mastoparan stimulated binding of [α^{32}P]GTP to a 37 kDa GBP of *S. griseus* 2682. At higher concentration (10^{-7}–10^{-6} M), however, AF stimulated GTP-binding affinity of all detectable GBPs. The effector systems regulated by GBP are very diverse in different organisms, they include enzymes or ion channels (for K^+, Ca^{2+} or Na^+). Studying the

effect of AF and GTPγS on the electrophysiological state of the cellular membrane we have found that both agents induced, an immediate depolarization of the membrane potential.

These findings suggest that AF and GBP-activating agents could modify ion transport processes and ion distributions, across the cellular membrane and membrane potential depolarization is always an extremely important signal in the control of the opening of other voltage gated ion channels. Using ionophores and specific ion channel blockers, we were able to demonstrate that by modulating intracellular K^+ and Ca^{2+} levels it was possible to initiate aerial mycelium and spore production in the spo$^-$ mutant. Very likely intracellular [Ca^{2+}] plays a major role in the sporulation process since initiation of sporulation in *S. griseus* 2682 displayed sensitivity to calmodulin-antagonist and low extracellular [Ca^{2+}]. Indeed, using an in vitro assay system we have found calmodulin-analog activity in *S. griseus* 2682 and we have isolated two calmodulin-binding proteins (M_r=54 and 52 kDa). The major 54 kDa protein proved to be an N-terminally modified protein.

These observation suggest that activation of GBPs could initiate morphological differentiation in *S. griseus* 2682 and the intracellular mediators generated by the specific signal transduction pathways might be changes in intracellular ion levels. It is possible that alteration of the transmembrane potential by AF may provide an initial signal to trigger sporulation in *S. griseus* 2682.

ZS. EGYED, CS. ROMSICS

Measurement of the natural pollutant degrading activity and characterization of the biodegradative bacterial communities of hydrocarbon contaminated soils

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One of today's biggest problems is environmental pollution. Among several pollutants that get into the environment, different hydrocarbon compounds are of utmost importance. Monitoring the fate of these substances in the environment (degradation, accumulation, etc.) is a very important task.

During our work we parallelly used different methods to measure the biodegradative capacity and the species diversity of different types of soils contaminated by diesel oil (determination of total count and the hydrocarbon degrading one, cumulative CO_2 production, ARDRA). Enumeration of microorganisms was carried out by a miniaturized MPN method, using 96-well microtiter plates, while biological CO_2 production was detected in "Biometer" flasks. The method of van Elsas and Smalla has been adapted for performing ARDRA.

To isolate microorganisms that take part in the biodegradation of aliphatic and aromatic hydrocarbons present in the polluted environment special mineral media containing oil or naphthelene as sole carbon and energy source were used. The derived isolates were characterized by molecular biological methods, among others the plasmids

playing possibly a role in hydrocarbon metabolism were investigated. Identification at species level of bacterial strains was based on the analysis of their 165 rDNA sequences.

According to our results, severe hydrocarbon contamination seriously changes the microbial diversity of the examined soils: the diversity decreases. Meanwhile the number of those microbes increases significantly that tolerate the effect of the contaminant, survive its effects and presumably utilize hydrocarbons. This increase in germ counts and the presence of hydrocarbon (diesel oil) decomposition is well shown in a rise of CO_2 production.

H. E. A. F. BAYOUMI HAMUDA, M. KECSKÉS, B. OLDAL, A. KHILF

Establishment of *Rhizobium leguminosarum* bv. *viciae* and *Vicia faba* symbiosis at different pH levels

Environmental Microbiological Research Group of the Hungarian Academy of Sciences, Budapest and University of Agricultural Sciences, Gödöllő, Hungary

In vitro differentiation among the strains of *Rhizobium leguminosarum* bv. *viciae* was conducted in response to the effect of pH values on their multiplication in yeast extract mannitol broth medium using phosphate buffer. The growth of the strains was evaluated in term of optical density (λ=550 nm) after 48 h incubation in rotary shaker (150 rpm) at 28 °C and compared with the control at pH 6.8. The investigation showed that *Rhizobium* strain Lóbab Z was the most tolerant strain to pH variations, while HB-3841*str*$^+$ was the most sensitive one.

The ability of the strains of *R. leguminosarum* bv. *viciae* to fix the nitrogen with horse bean plants (*Vicia faba*) was tested in agroecosystem symbiotic model of mesocosm experiments for 8 weeks under greenhouse conditions at different soil (brown forest, Gödöllő) pH levels (5.3, 6.6 and 8.3).

The results indicated that the inoculation of horse bean plants with *Rhizobium* strains at pH 6.6 resulted in the optimalization of the symbiosis and nitrogen fixation on the basis of plant biomass and nodulation potential.

The Hungarian strains (Lóbab Z and Bükköny 75/4) preferred the acidic pH more than alkaline one to fix the atmospheric nitrogen and forming nodulation with the tested plants. The Libyan (HB-3841*str*$^+$) and English (E 1012) strains could establish the symbiotic performance more in alkaline soil than in the acidic one.

Statistical analysis tests were carried out to evaluate the tested model of agroecosystem symbiotic using the ANOVA and linear regression tests.

J. MAKK, É. ÁCS, G. KOVÁCS

Investigation of the Danube gravel biofilm diatom-associated bacterial communities

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The epilithon microbial communities which develop on gravels in riverbeds significantly contribute to changes in the chemical-biological quality of water that penetrates through the sediment. The metabolic activity of attached populations has deep influence on the water quality of drinking-water supply based on bank wall filtered wells. Biofilm consists of bacteria, algae (where light level permits), protozoa and fungi etc. Among algae diatoms are found to be the dominant group of the epilithon communities of River Danube. As producer organisms they play an important role in the cycling of elements and energy flow in aquatic ecosystems. On the other hand the surfaces of diatoms serve as habitats for bacteria.

Gravel samples were collected from the northern water gaining area of bank wall filtered wells of Budapest at the northeastern shoreline of Szentendre Island, in the main arm of River Danube. On the island there are rows of bank wall filtered wells, which supply with drinking water the capital of Hungary. The collected gravel washing fluid samples were plated on three different algal media in order to isolate diatom-associated bacterial strains. Isolates were purified and representative strains were selected on the basis of cultural-morphological features, certain biochemical investigations, and ARDRA analysis. Selected group representative strains were identified by BIOLOG phenotypic fingerprint method and 16S rDNA sequencing.

Our results have shown that dominant members of Gram negative communities are *Pseudomonas*, *Cytophaga*, *Aeromonas*, *Acinetobacter*, *Flavobacterium*, *Psychrobacter*, *Rhizobium*, whereas of Gram positive ones *Corynebacterium*, *Streptomyces* and *Rhodococcus-Nocardia* group.

Laboratory diatom cultures and original gravel samples were examined by scanning electron microscopy. We have found adhered appendaged bacteria adhered to surfaces of diatoms (*Caulobacter* spp., *Hyphomicrobium* spp.), which may play a crucial role in development and functions of biofilms.

E. TÓTH, G. KOVÁCS, A. EKKER

Changes in the composition of the bacterial communities of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae) during the developmental stages

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The obligate parasitic fly, *Wohlfahrtia magnifica*, Schiner (Diptera: Sarcophagidae) is the main species causing traumatic wound myiasis in many countries of

Eurasia. In our previous studies the bacteriological background of wohlfahrtiosis had been examined in detail, but for the better understanding of the ethiology of this disease and behaviour of this fly the changes in the composition of bacterial communities during the development of *W. magnifica* was investigated.

In the summer of 1996 larvae of different stage of *Wohlfahrtia magnifica* were taken out from wounds of 5 sheep and from them the following samples were made: composite samples separately from L1, L2, L3 (first, second and third stage larvae); L3 larvae were induced to pupate aseptically – from them we used the whole pupa as sample; L3 larvae were pupated under non-sterile condition, from them (after triplicate washing) aseptically prepared internal liquid material and imago before hatch-out were used. From these samples bacterial communities were isolated and identified with the following methods: classical phenotypical tests; Biolog Metabolic Fingerprint System (Biolog Inc., CA); chemo- and genotaxonomical investigations.

Both Gram positive and Gram negative strains appeared in all samples. Among Gram negative bacteria there were characteristic differences during the development of the fly (up to the pupa stage) the ratio of Gram negative aerobic rods (*Pseudomonas* sp., *Acinetobacter* sp. etc.) decreased, later their number increased again. The members of the family Enterobacteriaceae (genera: *Proteus*, *Serratia*, *Enterobacter*) are present in all samples though in different ratios.

Among Gram positive microorganisms a big quantity of *Bacillus* species, some *Corynebacterium* sp., *Arthrobacter* sp. and *Staphylococcus* sp. are present almost in all samples. In the beginning of the pupation *Enterococcus faecalis*, later some *Rhodococcus*-like microorganisms seem to be dominant. By the end of the development the ratio of Gram positive bacteria was relatively low.

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Investigations on antimicrobial effect of essential oils by automated conductimetry

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In the frame of the INCO-COPERNICUS "PlantChem" EU research cooperation project, the effects of essential oils of several species (thyme, peppermint, oregano, cassia, dill weed and dill seed) as well as linalool and hexanal have been studied on cultures of a number of food-borne bacteria and one yeast (*Saccharomyces cerevisiae*). After inoculation, the inhibitory or inactivating effects were monitored by conductimetry in Don Whitley impedimetric broth during incubation at 30 °C in a RABIT-type instrument. The conductimetric data were compared with changes of viable cell counts estimated after 48h of incubation. The results obtained showed that the kinetical method based on automated conductimetry provides a tool for characterization of the antimicrobial activity and the area value under the conductimetric curves described most effectively the overall antimicrobial effects. The yeast seemed to be more sensitive for the effects of most

essential oils than bacteria. However, essential oils with aldehydic main components (e.g. cassia oil) proved to be quite effective also against Gram negative bacteria (e.g. *Salmonella typhimurium*, *Pseudomonas fragi*), whereas essential oils with phenolic main components (e.g. thymian) were promisingly effective against Gram positives such as *Bacillus cereus* and *Staphylococcus aureus*).

H. E. A. F. BAYOUMI HAMUDA, M. KECSKÉS, I. JEVCSÁK, H. ABDORIHIM

Selection of Al^{3+}-tolerant *Rhizobium leguminosarum* bv. *viciae* strains and their symbioses with *Vicia faba*

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Selection of aluminium tolerant strains of *Rhizobium leguminosarum* bv. *viciae* was carried out in vitro. The multiplication of the strains in broth defined basal medium was dependent on the Al^{3+} ions. The growth of the strains was evaluated in term of optical density (λ=550 nm) after 48h incubation in rotary shaker (150 rpm) at 28 °C.

The investigation showed that *Rhizobium* HB-3841*str*$^+$ and E1012 strains were not able to grow at 25 µM $KAl(SO_4)_2$, but they grew at 25 µM Al_2O_3. In addition, HB-3841*str*$^+$ and Lóbab Z strains were the most tolerant strains at 50 µM $Al(OH)_3$ and the other strains were inhibited as the concentrations were increased. The results indicated that the multiplication of Rhizobium strains (except E1012) was not affected by 100 µM $Al_2(SO_4)_3$, while the growth of the strains (except Bükköny 75/4) was affected by 50 µM $AlCl_3$.

The increasing inhibitory order of investigated Al compounds was found as follows: $Al_2O_3 < Al_2(SO_4)_3 < KAl(SO_4)_2 < AlCl_3 < Al(OH)_3$.

The symbiotic effectiveness of *R. leguminosarum* bv. *viciae* strains was tested for their symbiotic performance with *Vicia faba* plants, carried out in pot experiment at different soil (brown forest soil of Gödöllő with pH 5.3) Al^{3+} levels (50–400 µM/kg soil) in the form of $Al_2(SO_4)_3$.

It turned out that the higher in Al^{3+} concentrations were used and the lower were the values of symbiotic parameters. The maximum symbiotic features were at 50 µM/kg soil. The two Hungarian strains showed more symbiotic performance to the Libyan horse bean plants, while the Libyan and English strains were less effective to this plant.

Statistical analysis tests (ANOVA and linear regression) were conducted to identify the efficiency of the studied model.

A. SZABÓ, M. ÁRNYASI

A study of biologically decomposable plastics

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The large-scale usage of synthetic materials resulted in an increase of synthetic waste. Environmental protection has to more and more face with the problem of depositing, processing and recycling of these waste materials.

Conventional plastic products manufactured in Hungary (PE, PVC, PP) are not accessible (not decomposable) for microorganisms living in the soil and water, thus they can remain unchanged and pollute the environment "till the end of time".

Recently starch and cellulose aggregates are given to plastics, by which these materials become microbiologically decomposable. The microbes break down and decompose the plastics by their enzymes and the final products are released into the environment in the forms of recyclable and non-toxic materials (humus, water, CO_2, elements).

Microbiological degradation of plastic foils and products of different starch content (CABOT, ECOSTAR, MATER BI) has been studied.

In the course of our studies applying a preliminary EU directive, the burying method, the culture-receiver experiment and the modified activated sludge (SCAS) method were used in a complex way. Experiments were set in 3–5 repeatings in chernozem and sandy soils. According to our data biodegradation of the CABOT and ECOSTAR foils was not efficient enough (0.2–1%), thus their decomposition can last for a long time (5–10 years). Biodegradation (weight-loss) of MATER BI foils has reached 80–100% in chernozem, and 53% in sandy soils. By the end of the study period the foils broke down into small pieces or completely disappeared. Laboratory experiments provided similar results to that of the field ones, where a 50–80% decomposition of Mater BI foils was found. Using the activated sludge method (SCAS) biodegradation has reached 55% within only 3.5 rnonths!

It is worth to mention that Mater BI foils have been used as a nutrient by several groups of organisms (worms, mite) and by all the edaphon components. In this way all of these organisms took part in the breaking down and decomposition process.

Field studies (in soil and sewage water) have proved the high biodegradability of Mater BI products without the production of toxic materials, hence the number of bacteria (*Cellfalcicula* sp., *Cellvibrio* sp.) and fungi (*Penicillium*, *Aspergillus*, *Chaetomium* sp.) together with Protozoons increased in the environment studied.

In our opinion Biologically Degradable Plastics from an environmental point of view need peculiar further attention. Their introduction and usage would mean a new alternative in waste management.

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Effect of *Pseudomonas fluorescens* bacteria and *Trichoderma viride* soil fungi to wheat of *cv. Tavasz'* qualitative attribute

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We studied the influence of *Pseudomonas fluorescens* bacteria and *Trichoderma viride* soil fungi to wheat of *cv. Tavasz'* developments, after the seeds were treated in plots growing in the garden.

We observed the weight of wheat seeds, then the thousand seeds category; the weight of the chaff; the weight of the straw and the yield of moisture. Milling %; wet gluten; dryness gluten was also measured.

We have found that the quality of the wheat improved substantially as a result of microorganisms' treatment compared to the control group.

T probe was used for mathematical analysis. Treatment with *Pseudomonas fluorescens* and with *Trichoderma viride* significantly improved the qualitative values of wheat of *Tavasz*. The humidity of the crop was consequently lower in both treated seeds.

[1]GY. GUNICS, [2]S. FARKAS, [3]J. MOLNÁR

Synergistic effect of antiplasmid compounds with antibiotics

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The antibacterial and F'-lac plasmid eliminating effects of phenothiazines and structurally related compounds were studied.

Synergism between the antibiotics (Ampicilline, Penicilline-G, Chloramphenicol, Doxycycline, Gentamicine and Streptomycine) and antiplasmid compounds (Promethazine, Chlomipramine, Verapamil) were investigated on several laboratory strains and clinical isolates by Checkerboard method. The minimal inhibitory concentrations (MIC) of various antibiotics and antiplasmid compounds on the *Escherichia coli* K 12 LE 40, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* strains were measured with microdilution method. Synergistic effect between the Ampicilline-Promethazine and Erythromycine-Promethiazine were found on *Escherichia coli*. Additive effect was found in the Ampicilline-Promethiazine and Doxycyline-Promethazine combinations on *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* strains.

The MIC of Ampicilline increased, in contrast MIC of Erythromycine decreased in the presence of different concentrations of Verapamil.

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Mechanism of PMS-resistance of a *Staphylococcus aureus* isolate from Hungary

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Plasmid pEP2104 originates from a 1977 clinical isolate of *Staphylococcus aureus* from Hungary. The plasmid mediates inducible resistance to PMS-antibiotics: partial macrolide [the 14-membered macrolides erythromycin (EM) and oleandomycin, the 16-membered macrolide mycinamicin (MCM)] and type B streptogramin antibiotics such as mikamycin-B (MKM-B).

The sequence of 31 amino acid residues obtained by N-terminal analysis of the 63kDa protein (MsrSA) present in the membrane of 8325(pEP2104) cells, whose PMS-resistance was induced by a concentration of 1.3 5 mg EM/L [EM-induced 8325(pEP2104)], was identical to the corresponding sequence of the membrane-associated MsrA protein known to promote efflux of [^{14}C]EM [J. I. Ross et al., Mol. Microbiol., 4, 1207 (1990)].

No inactivation of EM in the EM-induced 8325(pEP2104) or its constitutive PNIS resistant derivative 8325(pMC38) [obtained in the presence of 1 mg MCM/L] was observed. Moreover, poly(A)-directed polylysine synthesis by a cell-free system containing ribosomes from EM-induced 8325(pEP2 I 04) cells and S 100 from *Escherichia coli* was inhibited by not only EM but spiramycin and MKM-B [M. Matsuoka et al., Biol. Pharm. Bull., 16, 1288 (1993)]. In addition, ribosomes from both EM-induced 8325(pEP2104) and 8325(pMC38) strains showed about the same affinity to EM as those from the host strain, NCTC8325.

These results suggest that, like the MsrA protein, active drug-efflux due to the MsrSA protein may be responsible for PMS-resistance. How can the 8325(pMC38) strain discriminate PMS-antibiotics from most of 16-membered macrolides and lincosamides? A possible explanation is discussed in terms of the pKa-value related to the physicochemical nature of the antibiotics.

Recently, we determined the nucleotide sequence of the PMS-resistance gene region of both pEP2104 and pMC38. An open reading frame right upstream of the PMS resistance determinant of pEP2104 was completely absent from the corresponding area of pMC38. This suggests that a leader peptide upstream of the MsrSA-open-reading frame determines the inducible expression of PMS-resistance.

[1]G. KISKÓ, [2]J. REICHARDT, [2]A. NÉMETH, [3]J. FARKAS

Effect of bacteriocins on the growth of *Listeria monocytogenes* at various environmental conditions

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The effect of bacteriocins produced by *Lactobacillus sake* and *Leuconostoc gelidum* has been investigated in broth, at various environmental conditions (various temperatures, a_w and pH values and nitrite concentrations) on the test organism *Listeria monocytogenes*. The growth of the test organism could be successfully inhibited with bacteriocins at a given combination of the above-mentioned experimental conditions. By moving off from the optimum temperature, this inhibitory effect was markedly increased and a pH drop further enhanced the sensitivity of *Listeria monocytogenes* against temperature decrease. At low temperature and pH the favourable combined effect of NaCl and $NaNO_2$ could also be observed.

M. P. ARNÓTH, E. PUSKÁS

Pasteurella isolations in our laboratory from 01. 01. 1997 to 15. 05. 1998

Public Health Institute, Miskolc, Hungary

During the period from 01. 01. 1997 to 15. 05. 1998 we isolated 24 *Pasteurella* strains from 19 patients in our institute. There were 15 *P. multocida*, 2 *P. aerogenes* (from the same patient), 1 *P. haemolytica* and 6 non-identified *Pasteurella* species. Most of the strains were isolated from wounds and one was isolated from peritonsillar abscess. We do not know whether the patients from whom *Pasteurella* was isolated had a history of contact with animals.

All of the strains were susceptible to penicillin, ampicillin and cephalosporins. A few strains were resistant to sumetrolim and gentamicin.

There are only few data about human infections caused by *P. haemolytica*. We isolated *P. haemolytica* from a young man whose hand had been hurt by a swine bone. It was a T biotype of *P. haemolytica* and was intermediately susceptible to penicillin. The man had got purulent arthritis on his III finger. A surgical intervention and Zinacef therapy was effective.

J. KISS, I. HERCZEG, I. BÁNHEGYI

A short overview of the microbiological quality control tests

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The quality requirements of drug products and some articles used in medical practice (syringes, needles, etc.) are strictly regulated by the pharmacopoeias. Part of the quality requirements is related to the microbiological purity of the products.

The pharmacopoeial microbiological quality tests can be divided into two basic categories: sterility tests for the products having a sterility requirement and microbiological purity tests for the products having a defined limit for the total microbe count and in which the occurrence of certain microbes is prohibited.

Our laboratory performs a great number of the above tests (that are described slightly differently in several national or international pharmacopoeias) according to the methods of generally accepted pharmacopoeias (Ph Hg. VII, USP 23, BP 93, Ph Eur 3, etc.).

In addition to pharmaceuticals we perform microbiological tests of other substances, medicated products, cosmetics, animal feed, etc. according to the relevant quality requirements of the product. Control testing of clean areas of different classes and microbiological stability testing of pharmaceuticals is also included in our test lists.

In the presentation a brief summary is given of the microbiological quality control tests and of our experience obtained by the use of the tests.

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Consequence of lipopolysaccharide pretreatment on the hypersensitive necrosis causing capability of phytopathogenic bacteria, comparative analysis of lipopolysaccharide of some xanthomonas strains

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Lipopolysaccharides (LPS) represent a crucial component of the Gram negative bacterial outer membrane for the early steps of bilateral plant-microbe recognition processes in pathogenesis or in resistant reactions of the plant. Compared to the well-established role of bacterial LPS in other organisms (animals and men) it is surprising that we know so little about their effect on plants at the cellular, biochemical and genetic level.

In our planta experiments we pretreated Xanthi tobacco leaves with LPS extracted from *Xanthomonas campestris* pvs. *vignicola* 56a (Xcv56a). It turned out that the

normally appearing hypersensitive response (HR) was prevented if 125–250 pg/ml of LPS extracts were applied prior to Xcv56a (10^7–10^8 cfu/ml) bacterial inoculation.

HR was also absent by similar concentrations of *Pseudomonas syringae* pv. *syringae* syringae 61 if LPS of Xcv56a was applied 3–6 hours prior to challenge inoculation of the same leaf area. The non-appearance of HR was greatly influenced by the LPS concentration, the physiological state of the leaf/plant and the incubation temperature throughout the experiment. When we shortly disturbed the protein synthesis of a leaf by heat shock (50 °C, 13 sec), the HR always appeared in spite of the LPS pretreatment.

Based upon these data we concluded that due to the LPS pretreatment specific mechanisms were activated in plant cells associated with protein synthesis and probably reflecting a pathogen recognition/protection process (elements of the earlier discovered locally induced resistance).

LPS constituents of 2 strains of *X. c.* pv. *vignicola* and one strain of *X. c.* pv. manihotis were characterised by: gel electrophoresis, KDO-(2-keto-3-deoxi-octolosonic acid) test, hexoseamine-, phosphate-, fatty acid-, neutral sugar- and uronic acid analysis.

D. SZAKÁL, GY. SCHNEIDER, T. PÁL

The aetiological diagnosis of bacillary dysentery with a colony immunoblot technique

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With the aid of a monoclonal antibody specific to an invasion plasmid coded protein, IpaC, of shigellae the detection of these pathogens from contaminated stool samples was attempted. Tryptic Soy agar plates inoculated with the samples were covered with nitrocellulose membranes. The antigen was detected in the prints of colonies growing under the membrane during an overnight incubation period. Shigella colonies were clearly marked by an intensive red colour. The sensitivity of the method equals that of the traditional techniques. Its advantage is that it can be performed directly from the primoculture plates giving results within 18–24 hours after the receipt of the sample. Since bacteria in colonies left of the plate stay alive, it also allows their further typing, as well as the determination of their antibiotic sensitivity.

J. SZENTANDRÁSSY, K. GERDEI, K. KATONA

Pasteurella multocida meningitis (case report)

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We isolated *Pasteurella multocida* strain from the liquor of a 5-month-old infant. The isolation was performed using the Bio-Merieux API 20 NE. In addition to the disk

diffusional examinations, the MIC values of Penicillin, Ampicillin, Ceftriaxon and Ceftazidim were determined.

During further examinations we could cultivate the *Pasteurella multocida* also from salina of the family's dog and its resistance was the same as that of the strain isolated from the liquor.

Z. TIGYI, B. PÁLMAFY, T. PÁL

A possible mechanism of the blocking effect of Congo Red on *Shigella sonnei* type 7 colicin

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Previously we have shown that Congo Red blocks the toxicity of certain bacteriocins. Although this blocking effect seems to be independent of the mode of action of the particular colicins, it can be mostly observed at those acting on the same receptor, i.e. btuB, the B12 receptor. In this study we have demonstrated that pre-incubating the sensitive cells with Congo Red, the killing effect of *Shigella sonnei* type 7 colicin decreases in a dose-dependent manner. However, this was true only in the presence of free dye, the blocking effect can be removed by washing the cells after preincubation with Congo Red. This treatment did not decrease the colicin binding capacity of the cells. Our results suggest that Congo Red may act on the colicin itself and not on the receptor of the sensitive cell.

A. KISS, H. MILCH, ZS. RUZSICS

Genomic fingerprinting of *Klebsiella* spp., *Serratia marcescens* and *Escherichia coli* isolates using arbitrarily primed PCR (AP-PCR)

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For fingerprinting epidemic and sporadic isolates of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens* and *Escherichia coli* an arbitrarily primed PCR (AP-PCR) method was used. AP-PCR [1] belongs to the multiple arbitrary amplicon profiling (MAAP) methods widely used for fingerprinting microbial genomes [2].

Bacterial DNA was extracted by the use of Wizard Genomic DNA Purification Kit (Promega). Template amplification was performed in an AP-PCR protocol with the use of ERIC-2 primer [2] designed from the central inverted repeat of the enterobacterial repetitive intergenic consensus (ERIC) sequence located in extragenic regions of various members of *Enterobacteriaceae* family.

On the base of the electrophoretic patterns of the amplicons the isolates were classified into genomial types. In the case of species represented with sufficiently great number of isolates the discrimination indices (based on the Simpson's index of diversity) were calculated [3] for both the AP-PCR typing and the phage typing.

Our results confirm that AP-PCR is a powerful method allowing further subtyping of many species of *Enterobacteriaceae* family, and especially it is very useful for fingerprinting isolates not typible by other methods (e.g. phage typing).

The protocol presented seems to be very useful for high-discriminatory fingerprinting further species of *Enterobacteriaceae* family, too.

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Spontaneous mutation causes non-motile phenotype in *Escherichia coli* O157:H7

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Spontaneous Rifampicin-resistant mutants were isolated from prototype strains 7785 and EDL933 of *E. coli* O157:H7. Rif^r mutants showed pleiotropic changes and a new phenotype was detected. Almost all (45/48) of these independent mutants showed reduced motility or no motility at all demonstrated in semisolid medium. 7785-5, a Rif^r mutant had reduced motility and less FliC flagellar protein of 60.5 kDa was present in the exoprotein fraction demonstrated by SDS-polyacrylamide gelelectrophoresis (PAGE). Mutant strains 7785-15 and 933-3 derivatives of 7785 and EDL933, respectively lost their motility and no FliC production could be detected by either SDS-PAGE or immunoblot analysis. 7785-5 had four times lower anti-H7 titre than parent 7785 and 7785-15 had no detectable H7 antigen examined by tube agglutination. Exoprotein fractions of additional wild type strains of serogroup O157 were analysed. Strain C57 of serotype O157:H7 produced FliC, but strain C81 of serotype O157:NM did not produce detectable amount of FliC flagellar protein. Phage sensitivity of the mutants changed as well: 7785-15 and 933-3 turned to be sensitive to an *E. coli* and a *S. sonnei* phage, while 7785-5 became only sensitive to an *E. coli* phage. These phages lysed none of the *E. coli* parent strains. 7785-5 and 7785-15 grews lower than 7785. Our results give evidences that Rif^r mutation effects a wide variety of phenotypes including production of H7 flagella. This phenotype may provide selective advantages for *E. coli* 0157 and could explain the increasing frequency of predominantly atypical O157:NM strains among *E. coli* 0157 isolates.

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Examination of the postantibiotic effect of Meropenem on outer membrane proteins of Gram negative bacteria by capillary electrophoresis

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Postantibiotic effect (PAE) is the persistent inhibition of bacterial growth after a short exposure to an antimicrobial agent. Postantibiotic effect of β-lactams is often substantial for Gram positive bacteria, but minimal for Gram negatives. The exceptions are penems and carbapenems, which might induce a significant postantibiotic effect in Gram negative bacteria as well. The clinical importance of PAE is that it has impact on the dosage regimens. Drugs with longer PAE might be administered with longer dose intervals without loss of efficacy and with reduced toxicity and cost.

During the PAE several bacterial functions change: the microbial adherence, the ultrastructure of the cells, the synthesis of proteins and nucleic acids, and production of some enzymes and virulence factors. The aim of our work was to determine the changes in outer membrane protein profiles during the PAE of meropenem. Examinations were performed both by conventional SDS-PAGE, and by a new, modern and exceptionally fast method, capillary electrophoresis. Meropenem at suprainhibitory concentrations (8x MIC) was able to induce PAE in the Gram negative strains examined. The time interval of PAE was between 1.2 and 2.3 hours.

During the PAE we could demonstrate significant changes in the outer membrane protein profiles by "dynamic sieving" capillary electrophoresis. Profiles were recorded always within ten minutes. The patterns obtained were reproducible, characteristic for the bacterial strains and comparable to gel electrophoretic results. Therefore we presume that capillary electrophoresis provides a new, accurate, reproducible and fast technique in the examination of bacterial proteins.

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GVVPQ-like fimbriae on enteroaggregative *Escherichia coli*

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We have shown by electronmicroscopy that several enteroaggregative *Escherichia coli* strains identified by aggregative adherence to HEp-2 cells and EAggEC-PCR express GVVPQ-like fimbriae. Earlier we detected this fimbria type on *Salmonella enteritidis* strains and *E. coli* isolates from protracted infantile diarrhoea. The same fimbria species was also described as "curli" on *E. coli* strains causing bovine mastitis. In this way

enteroaggregative *E. coli* is the fourth nosological unit producing this type of thin aggregative surface appendages.

Unlike *S. enteritidis* and *E. coli* from protracted infantile diarrhoea, the enteroaggregative *E. coli* strains express GVVPQ-like fimbriae only at an incubation temperature below 37 °C with an optimum of ~30 °C. In this respect they show an analogy with bovine mastitis strains. Accordingly, bacterial colonies sticking to the surface of culture plates and intensively binding the hydrophobic dye congo red appear on 1% Tryptone medium (T-plates) only when incubated at this lower temperature. Fimbriate bacteria exhibit a thirty times higher capacity to adhere to HEp-2 cells than their non-fimbriate counterparts. The presence of these appendages also increases the ability of bacteria to interact with collagen types I and IV.

ELISA reactivity of the fimbria positive strains with specific immune serum to purified GVVPQ-fimbriae of *S. enteritidis* has also been shown.

Taking together we conclude that enteroaggregative *E. coli* strains may produce a fimbria species related to GVVPQ fimbriae by morphology, antigenicity and other biological characters. Purification and N-terminal amino acid sequence determination of these surface structures is, however, needed to prove that they are not only related but also identical to GVVPQ-fimbriae. Experiments to elucidate this question and to assess the possible pathogenetic role of these appendages are in progress.

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Comparison of pathogenicity of human and equine *Rhodococcus equi* strains

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Three human *R. equi* strains (1 from AIDS patient and 2 from immunocompromised persons) and 9 equine strains (2 from foal septicaemia and 7 from foal pneumonia) were isolated from lung biopsies and blood. The studies on HeLa cell adherence, hydrophobicity and beta-lactam resistance were performed parallel with the determination of the protective effect of two cytokines (tumour necrosis factor [TNF] alpha and interferon [INF] gamma) against *R. equi* infection.

Data show that the equine isolates are characterized by less adherence capacity, hydrophobicity and beta-lactam resistance as compared to the strains of human origin. On the contrary, no significant differences could be observed between the effect of anti- INF gamma and anti-TNF alpha on bacterial colony forming units in *R. equi* infected C57 Black mice.

This work was partially supported by grant OTKA T 017702.

Z. PÉTERFI, I. MAZÁK, B. KOCSIS, S. VÖRÖS

Immunochemical and serological analysis of *Proteus morganii* strains

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Proteus morganii strains are Gram negative. Their outer membrane contains endotoxin, which is responsible for the O-specific serologic feature of the strains. Many years ago Prof. Károly Rauss started to investigate the antigenic structure of different *Proteus morganii* strains and divided them in serogroups.

The goal of our work was to extract endotoxic lipopolysaccharides (LPS) from characteristic strains, to analyse their sugar content, to produce immune sera in rabbits, to compare the LPS-s extracted from different strains by modern and sensitive serologic methods, to find serologic cross-reactions with other members of Gram-negative *Enterobacteriaceae.*

Methods: We identified the *Proteus morganii* strains isolated from different samples by biochemical assays, cultivated in fermentor, extracted the LPS by phenol-water method described by Westphal. The purified intact LPS-s were investigated by polyacrylamide gel-electrophoresis (PAGE). The LPS-s were hydrolysed by hydrochloric acid and their sugar content was analyzed by thin-layer and gas-chromatography (TLC and GC). Hyperimmune sera were produced against bacterial strains and their LPS-s in rabbits and they were investigated and grouped by serologic methods: W be and indirect haemagglutination, ELISA.

Results: The modern serologic methods verified the serogrouping and serologic cross-reactions of *Proteus morganii* strains determined earlier by tube agglutination and the cross-reactions are caused by common sugar components. There are many serotypes, so we could not finish our work. We want to continue these investigations.

[1]ZS. CSUKÁS, [2]K. TÖRŐ, [3]I. JANKOVICS, [1]F. ROZGONYI

Microbiological examinations in cases of Sudden Infant Death Syndrome

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The potential role of microbiological agents was investigated in 10 cases of Sudden Infant Death Syndrome (SIDS) in Budapest between November 1996 and December 1997.

Autopsy, histological examinations and microbiological tests were performed on samples of blood, cerebrospinal fluid, pharyngeal and bronchial samples from infants under six month, died suddenly, without previous diseases.

The multifactorial pathomechanism of SIDS was suggested by detection of Parainfluenza 2 type virus antigen, isolation of toxin producing *Staphylococcus aureus*-, *Enterobacteriaceae*- and *Candida albicans* strains in large number of more samples of the same infant.

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Investigation of *Klebsiella pneumoniae* virulence in mouse models

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Klebsiella pneumoniae is a common inhabitant of the human and animal intestine. It may also occur as a saprophyte of soil, water and vegetation. As an opportunistic pathogen it is incriminated for causing respiratory and urinary tract infections, wound infection, septicaemia and newborn meningitis. Evidence has accumulated that *K. pneumoniae* may also be the causative agent of bacterial endophthalmitis. The aim of the present study was to develop an animal model for studying the pathomechanism of *Klebsiella* endophthalmitis. As polysaccharide capsule is a well-documented factor of virulence a wild type isolate and its capsule deficient mutant were compared in several mouse assays. Expression of the capsular material was detected by topo-optical reaction of the oriented carbohydrate molecules under polarization microscope. As endogenous bacterial endophthalmitis develops most probably by haematogenous dissemination from respiratory or urinary infections these two routes and direct intravenous injection of bacteria were applied in the models.

Respiratory infection was performed by intranasal instillation of 3×10^7 bacterial cells. No death occurred and no symptoms developed during the two weeks of observation.

In the intravenous model there was a marked difference in the LD_{50} values for the favour of the wild type strain. In the survivors abscesses developed in the kidney and liver but lower doses were needed to evoke these pathologic signs in the case of the wild type isolate. No endophthalmitis occurred in any of the animals.

Urinary tract infection was produced by intravesical injection of 10^5 bacterial cells into the bladder of suckling mice. This dose of both the wild type strain and the capsule negative mutant caused the death of some animals, and progression of the infection was verified by positive cultures from the kidney. However, endophthalmitis developed only in a portion of mice infected with the original capsule positive isolate. The infection of the eye was not the part of an ongoing bacteriaemia but rather the sequel of a transient presence of bacteria in the bloodstream. This idea is supported by negative cultures of blood samples taken parallelly with organ sampling.

The above results indicate that the intravesical mouse infection model may serve as a useful assay system to study the pathomechanism of *Klebsiella* infections. Further, these

preliminary data suggest a pathogenetic role for the capsule in the development of endophthalmitis.

MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

A. GÁCSER, I. PFEIFFER, J. KUCSERA

Molecular and phenotypic characterization of *Cryptococcus hungaricus* isolates

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Cryptococcus hungaricus was described by Zsolt (1957) as *Dioszegia hungarica*. This species has reddish colonies due to carotenoid pigments of unknown constitution. It is unable to grow above 25 °C and does not ferment glucose. Its perfect state is not yet known. Intraspecific variabilities were examined in six strains from CBS culture collection.

Strains were characterized by a combination of morphological and physiological properties; RFLP patterns of the total DNA after double digestion with *Hae*III and *Bg*III enzymes; and by RFLP patterns of isolated mitochondrial DNA (mtDNA).

The results showed that the strains but CBS 6324 and CBS 6576 were very inhomogeneous. mtDNA was characterized in the type strain CBS 4214. Its molecular weight proved to be 27.47 kb by estimation of molecular weights of the fragments generated by different restriction enzymes.

Detailed physical and functional map is under construction.

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ZS. PALÁGYI, CS. VÁGVÖLGYI, Á. NAGY, L. FERENCZY

Carbon source utilization patterns of wild-type *Phaffia rhodozyma* isolates

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Phaffia rhodozyma is an important yeast species because of its growing biotechnological importance. As this fungus could be a natural source of astaxanthine (3,3'-dihydroxy-β,β-carotene-4,4'-dione), several investigations concerning its genetics and taxonomy were carried out in the recent years [1–3]. In the present work the

applicability of carbon source utilization patterns for the characterization of *Phaffia* isolates were studied to obtain further data of theoretical and practical importance.

The abilities of 16 *P. rhodozyma* strains of diverse origin to utilize 98 individual compounds as sole carbon sources have been tested. As the investigated strains revealed substantial differences in this respect, the coded results of the experiments were unified into a data matrix and subjected to a numerical analysis to construct a dendrogram. The value of this approach to provide complementary data for the characterization of *P. rhodozyma* isolates has been demonstrated.

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Investigation of the taxonomic position of *Rhizomucor tauricus*

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The genus *Rhizomucor* belongs to the Zygomycetes and involves the species *R. tauricus*, *R. miehei* and *R. pusillus* [1]. These fungi are of value in both theoretical and applied microbiology. They are found among the etiological agents of human and animal mucormycosis, while some other strains are good producers of different extracellular enzymes (e.g. proteases).

As *Rhizomucor tauricus* is represented by a single isolate, its status as a separate species was sometimes questioned [1]. The purpose of this study was to characterize this *R. tauricus* isolate and to determine its true taxonomic position.

One *R. tauricus*, 1 homotallic and 1 heterotallic *R. pusillus*, 1 *R. miehei* and 1–1 strain of 3 other species (*Absidia glauca*, *Rhizopus stolonifer* and *Mucor piriformis*) were studied. They were subjected to carbon source utilization tests, tests isoenzyme and RAPD (random amplified polymorphic DNA) analysis. Results were evaluated by methods used in numerical taxonomy.

Though both the morphological traits and the ability to grow on single carbon sources of *R. tauricus* were highly different from other *Rhizomucor* strains, there was some similarity in the growth on certain compounds of differentiating value (sucrose, phenylalanine and β-alanine; 2) between *R. tauricus* and *R. pusillus*. The results obtained from other experimental approaches suggest even closer connection; e.g. the six enzyme systems tested revealed no differences among *R. tauricus* and the heterotallic *R. pusillus*

isolate. On the basis of these results, the strain described originally as *R. tauricus* is a mutant heterothallic *R. pusillus* strain and does not represent a separate species.

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Analysis of the mitochondrial DNA of the postharvest pathogen *Mucor piriformis*

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Members of the genus Mucor are considered as plant pathogenes of minor importance. However, *Mucor piriformis* Fischer can cause serious postharvest losses in various agricultural products. High water content fruits and vegetables undergoing prolonged storage at low temperature are especially threatened by this type of decay.

The mitochondrial (mt) DNA of *M. piriformis* wild-type strain (NRRT, 26211) was purified from isolated mitochondria. To establish a circular restriction map, it was digested with several enzymes as single and double digests. The size of the mtDNA (33.6 kbp) was found to be substantially lower than that of *M. racemosus* (63.4 kbp; Schramke and Orlowski 1993), a closely related *Mucor* species. Heterologous hybridizations with cloned *Aspergillus nidulans* mitochondrial genes (SrRNA, *nad2*) was used to locate coding regions.

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Transmission of mitochondria between vegetative incompatible *Aspergillus japonicus* strains

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A. japonicus is a frequently occurring cosmopolitan species of black *Aspergilli* (section *Nigri*). The isolates belonging to this species exhibit wide range of genetic diversity and heterokaryon incompatibility due to the absence of sexual reproduction. The mitochondrial genoms of these strains display highly variable RFLP patterns [1]. The 80

collection strains and field isolates investigated could be classified into eight different rntDNA RFLP groups (one of the eight types represented by some *A. aculeatus* strains, which are closely related with *A. japonicus*).

Mitochondrial oligomycin resistant mutant (oliR) was isolated from the most frequent mtDNA type of strains. Applying the resistance marker and the variable RFLP patterns transmissions of mitochondria were carried out between oliR donor and sensitive recipient strains by using protoplast fusion technique. These transfer experiments resulted in oliR progeny in each case of intraspecific combinations, but it failed when *A. aculeatus* was the recipient partner. Following the successful mitochondrial transfers applying different recipient strains three different resistant populations of progeny could be isolated with various frequencies: 1. strains harbour the nuclei of recipient strains together with the unchanged RFLPs of donor mtDNA, 2. progeny carry the nuclei of recipient partners and harbour either donor mtDNA or various recombinant mtDNAs, 3. protoplast fusion products with low frequencies those proved to be mtDNA recombinants displaying various RFLP patterns. For interpretation of recombination processes physical and functional maps of mtDNAs of parental and most frequent recombinant types of strains were developed. The data have been compared with those obtained previously concerning *A. niger* species aggregate [2] and the possible reasons of variable RFLPs and frequencies of recombinants will be discussed.

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Breeding of *Trichoderma* strains by protoplast fusion

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Several isolates of the *Trichoderma* genus have promising properties to be used as biofungicides. These species also take part in the degradation of plant debris. Most of the *Trichoderma* strains are mesotherm, consequently they cannot provide protection against plant pathogenic fungi or decompose post harvest plant residues during late autumn, winter or early spring, when the temperature is low. Another problem is the high level of benomyl in the agricultural soil treated with chemical agents, which inhibits the growth of *Trichoderma* strains. To resolve this problem a mesotherm biofungicide *T. harzianum* strain T95 was breeded for fungicide resistance. This strain can grow even at 20 µg/ml benomyl concentration.

The aim of our work was to combine some favourible properties of *Trichoderma* strains by protoplast fusion techniques. In these experiments the benomyl resistant *T. harzianum* strain T95 was crossed with cold tolerant strains belonging to the *T. harzianum* or *T. viride* aggregate. The benomyl resistant progeny of the intra or interspecific fusions are similar to the cold tolerant strains in their phenotype and temperature sensitivity. Germination of conidia on selective medium C suggests non-heterocariotic hifae. The restriction enzyme digested mitochondrial DNA patterns of the fusion products were the same as that of the cold tolerant parental strains, recombination was not observed.

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Gain-of-function mutations in the activation domain of the yeast transcription regulator Pdr3p

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Mutations in the yeast PDR3 transcriptional regulation locus are responsible for overexpression of three ABC transporter genes PDRS, SNQ2 and YOR1 associated with the appearance of multiple drug resistance. Hydroxylamine mutagenesis was used to introduce mutations into the plasmid-born PDR3 gene and seven independent clones with mutations in w the carboxy-terminal part of this gene were isolated and characterized. All the pdr3 mutants were resistant to cycloheximide, sulfomethuron methyl, fluconazole, chloramphenicol, mucidin and oligomycin. The pdr3 mutant alleles conferred multidrug resistance also in the presence of chromosomal wild-type PDR3 gene. No synthetic-lethal interactions were observed among different pdr1 and pdr3 mutant alleles. In transactivation experiments the mutated forms of Pdr3p induced an increased expression of the PDR3-IacZ fusion gene. DNA sequence analysis of isolated clones revealed six single amino-acid substitutions in the C-terminal protein segment (amino acids 725–957) involving the activation domain of the Pdr3p. This region may play an important role in protein-protein interactions during activation of transcription by Pdr3p.

A. BOZSIK, M. SIPICZKI

Genetic analysis in a dimorphic fission yeast

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The fission yeast *Schizosaccharomyces japonicus* var. *japonicus* shows a dimorphic cycle regulated by extracellular and intracellular signals. It alternates between the unicellular yeast phase and the filamentous mycelial phase. Under specific growth conditions the yeast cells can conjugate and form zygotes that undergo meiosis and sporulation. Since the second meiotic division is followed by mitotic divisions of the

meiotic nuclei, most asci contain eight spores. Octad analysis of asci produced by yeast cells carrying different auxotrophic markers revealed a 4:4 marker segregation, suggesting that the yeast phase is haploid. Diploid yeast strains could be obtained only by forced fission of protoplasts with complementary auxotrophic markers. These did not need conjugation for sporulation but converted directly into asci (azygotic asci) on the sporulation medium. The spores isolated from their asci showed diploid segregation.

Polyploid hybrids were not detected. Numerous mutants auxotrophic for uracil or leucine were isolated and subjected to complementation and segregation analysis. Three ura^- and four leu^- complementation groups were identified. One mutant of each group is now used for selecting yeast vectors suitable for transformation and cloning in this species.

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Genetic and cytological investigation of *Schizosaccharomyces pombe* mutants defective in cytokinesis and sexual differentiation

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The *Schizosaccharomyces pombe* unicellular eukariota is an excellent model organism for cell cycle studies. Processes explored by its studies are evolutionary conserved. The rod-shaped cells grow by elongation at their ends and divide by a medical septum. Many mutants have been identified which are defective in septum formation. We isolated and started to investigate a novel category of mutants which can form septum but do not dissolve it therefore do not undergo cytokinesis. This leads to formation of short mycelias. We named these mutants to *sep* mutants. The *sep* mutants can divide into several groups, in this poster we present the results of the genetic and cytological investigation of *sep6-sep16* group. These mutants are also defective in sexual differentiation.

Based on fluorescent septum staining and EM studies of the mutants we found that the cytokinesis defect is not the result of change in the structure of septum. Changing of the cytoskeleton can also cause cytokinesis defect, so we made cytological studies with immunofluorescent techniques to specify the localization of actin and microtubules. According to these studies the interphase cytoskeleton is normal. Therefore the cytokinesis defect is presumably a cell cycle regulation defect, and it is supported by mitotic defects (chromosome separation defect, SPB – centriolum equivalent – duplication defect) found in the mutants.

Based on the defect in sexual differentiation, mitosis and cytokinesis defects we might suppose that *sep6-sep16* genes are elements of a general regulation system which play a role in the regulation of very different processes.

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The role of the *cdc25/pyp3* genes in the timing of mitotic onset in *Schizosaccharomyces pombe*

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In proliferating cells, the size homeostasis is maintained by size control mechanism(s). The underlying principle of these controls is that there are some events in the cell cycle, which might be executed only after the cell has reached a critical mass. The fission yeast *Schizosaccharomyces pombe* is a classical example of mitotic size control: the onset of mitosis has a size requirement. For nearly twenty years it was thought that this control operates at the end of G2 phase, near the G2/M boundary.

Using time-lapse microphotography we have recently studied length growth in wild-type fission yeast cells and concluded that mitotic size control acts much earlier than it was previously believed, somewhere in the middle of G2 phase (Sveiczer, Novak and Mitchison. J Cell Sci 109, 2947–2957, 1996). Also analysing length growth in numerous cell cycle mutants we have argued that the 'sizer' period in the first part of G2 depends on the activity of the wee 1 tyrosine kinase. On the other hand, the second part of G2 is a 'timer' which may correspond to the preMPF → MPF conversion.

If our assumption is true, then the timer period depends on the activity of the cdc25 and pyp3 phosphotyrosine phosphatases. Therefore, using the same method we have analysed the position of mitotic size control in the *cdc2-3w cdc25Δ* double mutant. We have found that in this genetic background this control acts in the second part (probably at the end) of G2, in contrast to wild-type cells. To explain this discrepancy we suppose that the known hyperactivity of the cdc2-3w protein means that even its preMPF form has a considerable kinase activity. In the absence of the cdc25 phosphatase, this active preMPF is the only form of the kinase which can accumulate proportional to cell mass, and after reaching a critical level in activity it can drive the cell into mitosis. As there is no preMPF → MPF conversion, the timer at the end of G2 is missing.

We have found similar results with the *cdc2-3w cdc25Δ pyp34Δ* triple mutant where even pyp3, the backup phosphatase of cdc25, is missing. The diploid wild-type strain was analysed as a control, which has a cell length similar to these mutants. According to our results, the mechanism of mitotic size control in these diploid cells is similar to the haploid wild-type cells. So, the difference described above in the mutants is definitely a consequence of their different genotype. As a consequence, the preMPF → MPF conversion in the timer period at the end of G2 phase is really a rate limiting step, and the duration of this period depends on the activity of the cdc25 and pyp3 phosphatases.

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Characterization and izoenzyme analysis of some *Candida albicans* morphological mutants

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Candida albicans ATCC 64385 was used to receive colony morphology mutants after UV treatment. Four genetically stable lines were further investigated and biochemical characteristics compared with the parental strain.

The structures and ultrastructures of these different types of colonies were determined not only in terms of the proportions of blastospores, hyphae and pseudohyphae, but also with regard to the mode of budding of blastospores and the positions of these particular cell types within the colonies. Hyphae with an atypical appearance and branching characters were observed both in regular-wrinkled and in irregular-wrinkled mutant colonies. Smooth colonies of the parental strain and the mutants exhibited the same hyphal network within the agar, suggesting that microenvironmental factors in the agar overcame the effects of these mutations.

The assimilation and fermentation tests with 19 carbon sources showed, that difference could be observed only at the rate of citrate assimilation of mutants compared with parental strain.

The minimal inhibitory concentrations of nystatin were the same for the mutants and parental strains, but mutants growth activity was detectable higher at subinhibitory concentrations of this drug. However the results with amphotericin-B and miconazol were opposite to this.

Isoenzyme pattern of the morphological mutants were also investigated. Among the five enzyme activities (acid phosphatase, catalase, β-glucosidase, glutamate dehydrogenase and malate dehydrogenase) tested, only glutamate dehydrogenase pattern of mutants were different from those observed for the parental strain.

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Partial comparative investigation of virulence factors of two *Candida albicans* strains

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The opportunistic pathogenic yeast *Candida albicans* is an obligate associate of human beings and other warm-blooded animals. It is frequently encountered as a harmless commensal of the digestive system and vaginal tract. However the delicate balance between the host and this otherwise normal commensal fungus may turn into a parasitic relationship resulting in the development of infection, called candidiasis. The fungus is not a mere passive participant in the infectious process; a hypothetical set of virulence

factors for *C. albicans* has been proposed and supported by various studies. Numerous antifungal agents have been developed, one of them is fluconazole, a triazole derivative. Because of its effectiveness and of the lack of side effects, fluconazole is used extensively. But simultaneously with its usage a problem has appeared: in case of *Candida albicans* this molecule has a resistance-inducing effect. This is why we investigated the changes accompanying the phenomenon of resistance in case of this yeast.

Our aim is a partial morphological and physiological comparative study of a fluconazole sensitive and a fluconazole resistant *Candida albicans* strain (with the smallest genetic differences possible), first of all concentrating on virulence factors.

For that purpose we have generated a fluconazole resistant *Candida albicans* strain from a fluconazole sensitive (OKI) one. We compared fluconazole resistance, growing features and certain virulence factors (adhesion ability, and germinal tube production) of the two strains and we found considerable differences. The hydrolytic enzymes aspartic protease and α-glucosidase are also considered to be virulence factors. In case of aspartic protease enzyme we have found differences both between enzymatic activities and the speed of enzyme synthesis of the two strains. Inducing α-glucosidase enzymes on different carbohydrate sources, we found that they were induced differently in the resistant and the sensitive strains. The highest α-glucosidase activities were measured on sucrose as carbohydrate source in case of both strains. The enzyme kinetic parameters, molecular weight, pH optimum and ability of induction and repression of α-glucosidase enzymes from the two *Candida albicans* strains are similar. On the basis of our experiments we do not support the assumption that α-glucosidase enzyme of this yeast is a virulence factor. α-,β-,γ-cyclodextrines were bad inducers of α-glucosidase enzyme (In the presence of cyclodextrines we have detected filamental growth of this yeast [Mycoses **40**, 451–453 1997]).

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Methods for detecting and enumerating yeast from foods

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The harmful role of some yeasts in the spoilage of food has long been recognized. Hence, it is necessary to detect and enumerate them both in raw materials and finished food products. The dilution spread plating in the most generally used traditional method in the food industry to follow the contamination with yeasts. The selectivity of this procedure is achieved by using different diluents and media.

First, we measured the growth of five yeast species of industrial significance (*Saccharomyces cerevisiae*, *Kloeckera apiculata*, *Kluyveromyces marxianus*, *Zygosaccharomyces bailii* and *Debaryomyces hansenii*) using 5 different diluents (distilled water; 0.85% NaCl; 0.1M Na-phosphate buffer pH=7; 0.1% peptone and 0.1% peptone in 0.85o% NaCl) and a general medium (glucose-yeast extract agar) with dilution

spread plating technique. The results were statistically evaluated by StatGraphics v.5.1 computer program.

There was no single diluent found effective for the detection and enumeration of all yeast species, and the diluent resulting in the highest number of cells differed with each yeast species. This may be attributed to the difference in physiology of the investigated yeast species.

In the next series of experiments we determined the counts of three strains of the xerotolerant food spoilage yeast, *Zygosaccharomyces rouxii* in three low a_w food (honey: a_w=0.92; concentrated milk: a_w=0.91 and orange syrup: a_w=~0.92) on three different media (TY10G agar : tryptone-yeast extract-30% glucose agar; DG18 agar; dichlorane-18% glycerol agar; MY50G agar; malt extract-yeast extract-50% glucose agar) and using three kinds of diluents (0.1% pepton; 40% glucose and 30% glycerol) and here, too, dilution spread plating was applied.

The most effective diluent-medium combination was 30% glycerol and TY10G agar. 30% glycerol proved to be the most effective of all diluents. Although the difference between the media was small, the TY10G agar combined with the 30% glycerol give the largest counts. In foods where bacterial contamination is high the 40% glucose diluent with MY50G agar can also be recommended. The least effective combination for detection and enumeration of *Zygosaccharomyces rouxii* was the 0.1% pepton - MY50G agar, which resulted only in small amounts of yeast cells in each food tested.

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S^{35}-sulphate uptake of selenate-resistant *Schizosaccharomyces pombe* mutants

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Selenate resistance of *Schizosaccharomyces pombe* mutants is accompanied by their inability for sulphate utilization. Selenate-resistant mutants were proved to belong to the same complementation group according to the complementation analysis performed (Bánszky and Maráz, 1997).

Selenate-resistant mutants of *Aspergillus niger* and *A. nidulans* belong to two complementation groups (sB^- and sC^-) which can be clearly differentiated by their resistance to chromate. Chromate-resistant mutants have a defect in sulphate-permease, while the sensitive ones in the sulphate-reductase enzyme complex (Buxton et al. 1989). We could not apply this distinction, however, in the case of *Schiz. pombe* selenate-resistant mutants, because wild type strains were highly tolerant for chromate.

Activity of sulphate permease was checked by measuring the S^{35}-sulphate uptake of sulphate starved cells in the case of selenate-resistant (mutant) and sensitive (wild type) strains. Sulphate uptake of the wild type showed a saturation kinetics while selenate-resistant mutants took up only a limited amount of S^{35}-sulphate. These results confirm that all the selenate-resistant mutants studied have got mutation in the same gene, i.e. they

belong to the same complementation group. Considerably decreased sulphate uptake of selenate-resistant mutants clearly indicates that mutation of the sulphate-permease gene is responsible for this phenotype.

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Intracellular distribution of zinc, iron and selenium in *Saccharomyces cerevisiae* cells enriched in these microelements

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In human organism there are many essential micro- and macroelements. Zinc, iron and selenium are in a distinguished position. They have role in many functions of the human organism, e.g. zinc in growth, tasting, synthesis of blood cells, blood coagulation, lipid and protein metabolism, cerebral activity; the iron in the synthesis of blood cells, cerebral activity, while selenium in growth, activation of the immune system and in the regeneration of liver.

For satisfactory function of the organism it is necessary to maintain suitable microelement level.

Yeast cells enriched in microelements are frequently used as paramedicinal drugs. Yeasts are able to take up certain microelements and to incorporate into organic molecules which are more favourable to both human and animal organisms.

In the course of our experiments we determined the effects of the ionic forms of zinc, iron and selenium on the growth of *Saccharomyces cerevisiae* when these ions were applied in higher than physiological concentration. We determined specific growth rate and generation time from the exponential phase of growth.

Fe(III)-ammonium-citrate did not have any influence on the multiplication of yeast cells, while Na_2SeO_4 was slightly inhibitory in 5–20 mmol concentration. $ZnSO_4$ was toxic for the cells in 1 mmol concentration, it completely blocked growth, but its inhibitory effect decreased by lowering the concentration. When microelements were used in slightly inhibitory concentration, the microelement content of yeast cells were as follows: Zn 7100 μg/ml, Se 2440 μg/ml and Fe 1700 μg/ml.

Cytoplasmic and vacuolar membranes of yeast cells were successively permeabilized using DEAE-dextrane and localization and the ratio of free and organically bound microelements were determined. More than half of the intracellular microelement content was organically bound in all cases.

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Catabolite repression in *Penicillium chrysogenum*

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β-Galactosidase, a glycoside-hydrolase, is present in animals, plants and microorganisms. The β-galactosidase in bacteria, yeast and filamentous fungi is intracellular and can be induced by lactose.

Glucose and other easily metabolised sugars inhibit the synthesis of β-galactosidase. This phenomenon is known as catabolite repression. Catabolite repression is best studied in *E. coli*. Until recently, only one mechanism of carbon catabolite repression, a mechanism dependent on cAMP was understood in molecular detail. The effect of glucose is mediated by the level of cAMP. This mechanism of catabolite repression involves adenylate cyclase and a cAMP receptor protein (CRP).

Two cAMP-independent catabolite repression mechanisms are currently under study. One such mechanism is found in *E. coli*. Here, a catabolite repressor/activator protein has a key role. The other mechanism, found in *B. subtilis*, involves a catabolite-activated, ATP-dependent protein kinase, a phosphocarrier protein (Hpr) and a transcription factor (CcpA).

The mechanism of glucose repression in yeast and other fungi is entirely different from that of bacteria. Glucose repression is not mediated directly by cAMP and its role if any differs completely. In the mechanism of catabolite repression zinc-finger type proteins have a key role.

We studied the mechanism of catabolite repression in an industrial strain of *Penicillium chrysogenum*. Using different carbon sources the enzyme activity and the intracellular levels of different nucleotides were monitored. From the data we try to conclude the possible mechanism of carbon catabolite repression.

E. SÁNDOR, E. FEKETE, L. KARAFFA, I. PÓCSI

Effect of methionine on the glutathione metabolism and cephalosporin-c production of *Acremonium chrysogenum*

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Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is one of the most widely distributed low molecular weight thiols in nature, which takes part in numerous biochemical processes. The chemical structure of GSH is homologous to that of the β-lactam-forming tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) and, therefore, GSH is a potent inhibitor of the β-lactam biosynthetic enzymes ACV synthase

and isopenicillin-N synthase. On the other hand, GSH may provide the biosynthetic enzymes with a suitable reducing milieu and, at least in part, may fuel the biosynthetic machinery as a sulphur source.

The intracellular GSH concentration is influenced by many factors including the quality and the quantity of the carbon, nitrogen and sulphur sources in the culture media. In the industrial scale production of cephalosporin-C (CPC), which is one of the most important currently used β-lactam antibiotics, the filamentous fungus *Acremoniurn chrysogenum* is grown on culture media supplemented with methionine. According to our investigations, the microorganism metabolised the sulphur-containing amino acid in the exponential and stationer phases of growth which resulted in a very significant increase in the intracellular GSH concentration while the biomass production also exceeded those found in control cultures. Concomitantly, the formation of *A. chrysogenum* cells with characteristic yeast-like morphology was observed. In the idiophase, a significant CPC production and a decreasing intracellular GSH concentration were recorded. These findings may refer to a likely GSH-~CPC sulphur transfer while a simultaneous and progressive decrease in the GSH repression of the biosynthetic enzymes also seems to be probable. It is worth noting that the redox status of the cells characterized by the GSH/GSSG ratios remained essentially unchanged during the idiophase.

Obviously, these experimental data may serve as a valuable and interesting contribution to the concepts which consider methionine as an important inducer of the ACV, isopenicillin-N and deacetoxicephalosporin synthase genes. It is possible that methionine also plays an important regulatory role at the level of the enzymes which manifests itself through the changes in the intracellular GSH concentrations.

L. KARAFFA, K. VÁCZY, E. SÁNDOR, I. PÓCSI

Regulation of the alternative respiration by hydrogen-peroxide in *Acremonium chrysogenum*

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Most fungi and higher plants possess a cyanide-resistant, non-phosphorylating alternative mitochondrial respiratory pathway. The existence of this path has earlier been demonstrated in the filamentous fungus *Acremonium chrysogenum* as well.

In many cases the inhibition of the cytochrome path coincides with increasing alternative respiration activity. The mechanism that triggers this effect may involve hydrogen-peroxide (H_2O_2), since the increase in the reduction level of the cytochrome path following its inhibition results in a significant intramitochondrial H_2O_2 production. In this study the effects of H_2O_2 added into the fermentation broth on the alternative path and on the metabolism of *Acremonium chrysogenum* were investigated.

First, we established that the spontaneous decomposition of H_2O_2 was negligible, while in the presence of cells the elimination of H_2O_2 was extreme fast (200 mM H_2O_2 diminished in less than 30 minutes). Cells exhibited a significant tolerance against H_2O_2,

which was largely, in our opinion, due to the constitutive and high intracellular catalase activity.

Specific growth rate of cells exposed to increasing H_2O_2 concentrations has declined, reaching a negative value at 200 mM. This phenomenon was accompanied with emerging specific glucose consumption rates, which indicates that the viability of cells was not affected.

Cyanide-resistant alternative respiration of cultures increased steadily, reaching a maximum in the third hour following the addition of H_2O_2. Since this increase in alternative respiration could be suppressed by cycloheximide, we suggest that the enhancing effect of H_2O_2 was due to de novo protein synthesis.

To sum it up, it was established that *Acremonium chrysogenum* cells have an efficient protective system against H_2O_2, in which the enzyme catalase plays a crucial role. The presence of H_2O_2, however, also coincides with the de novo synthesis of alternative oxidase. The over-reduction of the cytochrome path results in increased intramitochondrial H_2O_2 production in plants. It is very likely that the physiological significance of the alternative route is the take-over of electrons in excess, playing therefore a preventive role in the protection against oxidative stress. Nevertheless, the question whether H_2O_2 has it effect directly or through other molecules, remains to be elucidated.

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Nitrate assimilation and oxidative stress responses in *Penicillium chrysogenum*

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Both the nitrate assimilation and the glutathione (GSH) dependent elimination of reactive oxygen species require high quantities of NADPH, which is provided primarily by the pentose phosphate pathway (PPP). Therefore we investigated how these three major metabolic processes are co-ordinated in the filamentous fungus *P. chrysogenum*.

Physiological studies revealed that both nitrate reductase (NR) and nitrite reductase (NiR) were induced by the substrates nitrate and nitrite and were repressed by ammonia, glutamine and glutamate. When *P. chrysogenum* mycelia were challenged with oxidative stress caused by high concentrations of either hydrogen peroxide (450 mM), *tert*-butylhydroperoxide (1 mM), menadione (500 μM), diamide (1.3 mM) or phenoxyacetic acid (40 mM) the intracellular peroxide concentrations of the cells increased significantly. Under these conditions the microorganism could survive but ceased to grow on culture media containing nitrate or nitrite as a sole nitrogen source and NR and NiR activities could not be detected in the cell-free extracts any longer.

In terms of GSH metabolism and PPP we found that in the presence of either nitrate or nitrite the specific glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) activities increased significantly when glucose was used as a sole carbon source. Most likely, these changes contributed profoundly to keeping the

intracellular peroxide levels low and, hence, to avoiding the onset of any GSH/GSSG redox imbalances. The specific activity of several other GSH metabolic enzymes including GSH peroxidase, GSH S-transferase, γ-glutamyltranspeptidase and the GSH producing activity remained unchanged under these circumstances. When glucose was replaced with lactose the GR and G6PD activities did not increase in the presence of nitrate or nitrite but in this case the intracellular GSH concentration was twice higher than that observed with glucose. It is known that the de novo GSH synthesis is regulated by glucose repression, which is diminished with lactose.

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Increase of glucoamylase production of *Thermomyces lanuginosus* by the induction of 2-deoxy-d-glucose resistant mutants

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During the last decades there has been an increasing demand for those polysaccharide degrading enzymes, which retain their activities at higher temperatures. Therefore to meet the current demand and to improve the economically feasible processes thermophilic microorganisms are applied as a source of thermostable enzymes.

The aim of the present study was the induction of 2-deoxy-D-glucose resistant (DGR) mutants of a thermophilic fungus, *Thermomyces lanuginosus.* Among these strains one can isolate catabolite derepressed mutants which show higher glucoamylase production than the parental strain.

The minimal inhibitory concentration (MIC) of 2-deoxy-D-glucose was determined in case of wild-type strains. On the basis of the results DG^R mutants were isolated on asparagine-glucose medium containing 1.25% of 2-deoxy-D-glucose.

Mutagenic treatment by UV irradiation or by ethyl-methanesulfonate (EMS) and methyl-nitro-nitrosoguanidine (MNNG) were ineffective. Germination of conidia did not enhance lethality off MNNG. To support the transport processes and metabolic activities, mutagenic treatment was carried out at the optimal growth temperature of the fungus. In this way the desired result was achieved. The effective mutagenic treatment can be summarised as follows: Conidia are germinated for 4-6 hours, then the mutagenic treatment is carried out in presence of 250 μg/mL MNNG at 50 °C for 30 minutes.

As the result of the mutagenic treatment, 11 genetically stable DGR mutants originated from various *Thermomyces lanuginosus* were obtained. All of them showed higher glucoamylase production than the original ones. The best mutant performed about 3–5 times higher glucoamylase activity after 96 hour of fermentation than the parental strain. This activity was stable maintained until the end of fermentation. To support high glucose concentration during fermentation fed-batch technique was applied and various carbohydrates (glucose and/or starch) were added to the culture broth. In spite of the

increasing glucose concentration the effect of carbon catabolite repression could not be observed.

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Investigation of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus*

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Nowadays, the microbial enzymes are applied more and more widely in food industry. A thermophilic fungus, *Thermomyces lanuginosus* that synthesises various extracellular enzymes has been chosen for our present studies. Among them amylolytic enzymes and cellulase free xylanases can be found depending on the applied inductors. The methods based on starch hydrolysis (starch-iodine complex) and on amount of released glucose were used for enzyme activity assays during fermentation.

One of 18 *Thermomyces lanuginosus* strains that performed outstanding amylolytic activities according to the results of screening experiment was selected for further studies. To maximize the enzyme production optimization of media composition was carried out. As a result of the optimization experiments a batch fermentation technology was developed. It can be applied in both shaken culture cultivation and in laboratory fermentor as well. The pH optima of amylolytic and glucoamylase activity were pH=5.0 and pH=4.6, respectively. The temperature optimum was 70 °C for both enzyme activities at the relevant pH optima. This is very promising because the temperature optimum is about 10 °C higher than that of the industrially applied glucoamylase enzymes.

The extracellular proteins produced by *Thermomyces lanuginosus* with ATCC 34626 have been precipitated by organic solvents and the protein components were separated by ion-exchange chromatography. Certain protein fractions can be identified as α-amylase or glucoamylase type enzyme, α-amylase and glucoamylase gave bands corresponding in size to 47–55 kDa and to 70–78 kDa of relative molecular mass on SDS-PAGE, respectively.

After fermentation the extracellular protein components of the culture filtrate was precipitated and dissolved in buffer. This solution was used as crude enzyme preparation to carry out the hydrolysis of starch. The hydrolysis profile was very similar to that obtained by the industrial enzyme originated from *Aspergillus niger*.

On the basis of the recent results the investigated *Thermomyces lanuginosus* strain can be candidate for production of the multi-enzyme preparation that contains both α-amylase and glucoamylase. Taking into consideration that the optimal temperature and the pH values of the amylolytic enzymes produced by the fungus are very similar, operating conditions can be determined where both enzymes work perfectly and their synergistic effects can make their way.

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Production of L(+)-lactic acid by *Rhizopus oryzae*

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L(+)-lactic acid production has received much attention recently because lactic acid can be a starting material of polylactic acid (PLA), a new biodegradable plastic. Though industrial production of lactic acid still relies mainly on *Lactobacillus*, research on lactic acid production by *Rhizopus* has continued primarily because of the ease of product purification and the ability of the fungus to utilize both complex carbohydrates arid pentose sugars. Fifteen *Rhizopus* and *Rhizomucor* strains (*Rh. oligosporus*, *Rh. oryzae*, *Rh. stolonifer*, *Rhizomucor pusillus*) have been screened for lactic acid production in shake flasks on a medium containing 10% corn meal and 3% calcium carbonate. Three *Rhizopus oryzae* strains (NRRL 395, ATCC 6204 and ATCC 34121) produced 5.0–5.2% lactic acid in 6 days at 30 °C. Fumaric acid (0.6–1.2%), tartaric acid (0.01–0.02%), ethanol (0.25–0.80 µl/ml) and methanol (0.03–0.07 µl/ml) were also produced. In shake flasks on a medium containing 20% corn meal, 6% calcium carbonate and 0.05% Termamyl 240 L, Novo-Nordisk A/S (heat stable bacterial α-amylase to help starch liquefaction during sterilization), *Rhizopus oryzae* NRRL 395 produced 8.6% lactic acid in 9 days at 30 °C.

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Production of cellulase and xylanase by solid substrate fermentation (SSF)

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Lignocellulolytic fungi were tested for growth and enzyme production on solid substrates such as extracted sweet sorghum pulp, wheat straw, corn stalk, corn seed hull (corn fiber), brewery spent grains and pine kraft pulp. Crude SSF enzymes (cellulases and/or xylanases) may be used directly in agrobiotechnological applications such as ensiling, feed additives and retting, and in the paper industry for biobleaching and in the biofuel industry for hydrolysis of cellulose. In situ SSF enzyme production may be incorporated into such biotechnologies with great saving in process cost. Solid substrate fermentations were performed on lignocellulose substrates supplemented with nutrients at 67–80% moisture content and 26 °C for 3–12 days. *Trichoderma reesei* Rut C30 produced on corn fiber, brewery spent grains and wheat straw/wheat bran 9:1 mixture 30.3, 46.5

and 18.1 Filter Paper Unit (FPU)/g dry weight (DW) cellulase activity and 444, 2200 and 5220 IU/g DW xylanase activity in 12, 10 and 10 days, respectively. FPA and xylanase activities were assayed for *Trichoderma reesei* Rut C30 at pH 4.8 and 50 °C. Fast growing wild type isolates (*Gliocladium* sp. TUB F-498 and *Trichoderma hamatum* TUB F-105) secreted 5–7 FPU/g DW cellulase and 800–3000 IU/g DW xylanase activities on wheat straw/wheat bran 9:1 mixture, corn stalk, corn fiber or extracted sweet sorghum pulp in 3–5 days. The Filter Paper Activity/Beta-Glucosidase Activity (FPABG) ratio was 2.3–3.5 in *Trichoderma reesei* Rut C30 cultures indicating a suboptimal production of beta-glucosidase. The same ratio was in the range 0.15–0.36 in *Gliocladium* sp. TUB F-498 and *Trichoderma hamatum* TUB F-105 cultures. Crude SSF cellulase-xylanase enzyme mixture of fungal origin was successfully used for ensiling green forages such as alfalfa and bromegrass. Also, fermentable reducing sugars have been produced from corn fibre and brewery spent grains for bioalcohol production by SSF lignocellulolytic enzymes.

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Solid substrate fermentation by *Trichoderma* spp. at 10 °C

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Recycling crop residues to soil and employing biological agents for plant protection and growth promotion are key elements in sustainable agriculture. Some *Trichoderma* strains may grow at low temperatures such as 5–10 °C and show antagonistic properties towards plant pathogenic fungi. These *Trichoderma* strains may be valuable tools for the biodegradation of post-harvest residues and bioprotection of plants in the fall and winter season.

Five cold-tolerant *Trichoderma* isolates (*T. harzianum* T66, *T. viride* T114, *T. aureoviride* T122, *T. viride* T228 and *T. harzianum* T334) were tested in solid substrate fermentation (SSF) for production of lignocellulose degrading enzymes (cellulase, endoglucanase, beta-glucosidase and xylanase). SSF experiments were carried out on wheat straw and corn stalk supplemented with inorganic nutrients at 75% humidity and 10 °C for 36 days. Enzyme activities were determined from the culture extracts at pH 4.8 and 10 °C.

The peaks in enzyme activities were detected from the 28–30 day SSF cultures. The following enzyme activities were measured by internationally recognized methods: cellulase activity (expressed as Filter Paper Unit), $3.9–6.9\times10^{-2}$ FPU/g dry weight (DW) substrate; endoglucanase activity, 89–183 EGU/g DW; beta-glucosidase activity, $4.1–9.6\times10^{-1}$ International Unit (IU)/g DW; xylanase activity, 72–208 IU/g DW. The pH values were in the range of 6.0–6.6 during fermentation.

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Study of practicability of turbidimetry for determination of growth optimum of *Lactococcus lactis*, *Listeria monocytogenes* and *Bacillus cereus*

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Turbidimetry is generally applied as a quick method for determination of kinetics of microbial growth. In addition to determination of growth optimum, we studied how the conventional living cell count and turbidimetric measures are in accordance with each other.

Growth of *Lactococcus lactis* BFE 920, *Listeria monocytogenes* SLCC 2540 and *Bacillus cereus* CCM 2010 was followed with Bioscreen C turbidimetric analyser (Labsystem Oy, Finland) in ST-I bouillon at different pH value and temperature combinations. These value combinations were the followings:

pH: 4.5; 5; 5.5; 6; 6.5; 7; 7.5; 8 and

temperature: 37 °C; 30 °C; 25 °C; 20 °C; 15 °C; 10 °C.

Analyser supplied Detection Time (DT) was applied for the estimation of microbial growth. In the case of *Listeria monocytogenes* and *Bacillus cereus* the values were the lowest at pH 6.5–8, at 37 °C while the *Lactococcus lactis* strain gave the lowest results at pH 6–8 at 37 °C.

After 168 hours the turbidimetric measures at 10 °C – independently of pH value – did not show any growth of *Bacillus cereus* strains.

In all the cases of the three examined strains the connections between the logarithm of initial Colony Forming Unit (CFU) and Detection Time (DT) were studied by linear regression. The correlation coefficients at pH 7.5, 30 °C were the followings:

$r_{L.\ lactis} = 0.9928$, $r_{L.\ monocytogenes} = 0.9979$, $r_{B.\ Cereus} = 0.9902$

Our results suggest that turbidimetric measures make possible the quick determination of growth optimum of single cell cultures and the evaluation of initial living cell count of the examined microorganism in unknown samples after taking calibration curve.

J. HORVÁTH, M. KRISTON, I. BÁNHEGYI

Antibiotic activity assays with different international reference standards

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Microbiological activity assays are required by the Pharmacopoeias for the determination of active ingredient content of antibiotic drug substances and finished products.

Two types of activity assay are used, the agar diffusion and the turbidimetric method. In both cases the activity of samples is determined by comparative methods against the declared activity of reference standard substances. The reference substances are issued by internationally recognized pharmacopoeias (USP, Ph. Eur.) or international regulatory bodies and they are generally called "international reference standards".

In the present work the activity assay results of Vancomycin, Gentamycin, Nystatin and Thiostrepton antibiotics obtained with different "international reference standards" are compared. Significantly different activity results were obtained for the same sample, depending on the reference standard used. The activity differences in some cases were outside the accuracy and confidence limits required by the pharmacopoeias.

The results call attention to the fact that there is no internationally accepted unified system for the activity assays of antibiotics, and the activity assay results can only be evaluated against the specific reference standard used.

The lack of unified international reference standards may cause evaluation problems both for the manufacturers and the pharmaceutical control authorities.

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Investigation of heavy metals and microorganisms transported by *Ambrosia elatior* pollen

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One of the most spectacular changes which causes pollution is the qualitative and quantitative modification of plant cover allowing metals to aggressively accumulate in soil. Generally the autochthonous flora is progressively replaced by tolerant plants, often characterized by an increased attitude to concentrate these metals. Some plants could even be used to build up barrages against the extension of heavy metals or to extract them from polluted soils. Plants can be highly polluted even on industrial wastelands, which are considered to be rehabilitated. Furthermore, saprophytes responsible for decomposition later disseminate metals initially stocked by plants, at a high concentration.

In our current work we have studied the accumulation and transport of heavy metals in *Ambrosia elatior*, which is widespread in eastern Hungary and causes serious

allergic problems. We are also studying the relationship between uptaken heavy metals, microorganisms colonizing plants and on the pollen of *Ambrosia elatior*. We found that heavy metals in soil were transported through the plant to its pollen, where it was concentrated to above average level. From the surface of the pollen we isolated bacteria from *Bacteriodaceae*, *Enterobacteriaceae*, *Micrococcaceae* and *Neisseriaceae* genera and bacteria from *Bacillus* species. The fungi genera were mainly *Alternaria* sp. and *Cladosporium* sp. could isolate the *Acremonium* sp., *Aspergillus* sp., *Fusarium* sp., *Humicola* sp., *Penicillium* sp. and *Phytophtora* sp. of fungi from the surface of the pollen. The degree of the soil pollution had not decreased the diversity and its amount of fungi species found on the surface of pollen. In the case of bacteria from plants grown on industrial wastelands the Gram negative microorganisms were dominant. We have concluded that about every 500–1000th pollen transported bacteria, and every 1000–2000th transported fungi. Another aspect of this work directly concerns human environment, and the coincidence between the presence of plants of a given fungal microflora and allergic or respiratory diseases.

PÁL ÁLDÁSY MEMORIAL SESSION

G. SZIGETI

In memoriam dr. Pál Áldásy

Central Veterinary Institute, Budapest, Hungary

This is a very brief reminiscence of an outstanding researcher and diagnostician of Hungarian Veterinary medicine on the occasion of his early death ten years ago.

Pál Áldásy was born in Szombathely in 1928, where he attended elementary and grammar schools, then continued his studies at the University of Veterinary Sciences of Budapest. He graduated in 1951 with a qualification "summa cum laude". First, he worked at the department of Pathology of the Central Veterinary Institute, and in 1952, he became post-graduate student of professor Manninger at the department of Epidemiology of the University. In 1956, by defending the thesis entitled "Determination of the types of pathogenic *E. coli* bacteria", he acquired the degree of Ph.D. In 1955, he became the first director of the Animal Health Institute of Miskolc established at that time. He served as the director of the Institute for 32 years until his retirement in 1987.

Results of his investigations and his wide experiences were published in prestigious national and foreign professional journals, always providing his reader with very useful new ideas.

The main areas of his scientific work were as follows:

– investigation of diseases of viral or bacterial origin in young calves and possible methods of protection,

– pathological and causality studies of infectious and non-infectious abortion in cattle,

– diagnosis and prevention of metabolic disorders of cattle,

– appearance and significance of mycoses and mycotoxicoses in domestic animals,

– epidemiology of the rabbit myxomatosis, establishment of an attenuated vaccine,

– diagnosis of certain contagious diseases of sheep, including scrapie.

The results of Áldásy's scientific research work are of much value even today: his registered vaccines against myxomatosis and adenoviruses causing lung and intestine inflammation of calves are still in use.

For his professional and scientific activities, Áldásy was awarded several prizes, including Excellent Worker Price, Labour Medal for Merit, Honour of Labour, Hutÿra Commemorative Medal, Loránd Eötvös Prize and Excellent Inventor Prize of Hungarian Academy of Sciences.

He played significant role in different scientific committees, in scientific qualification boards, was active in the post-graduate training of veterinarians and in the education of veterinary students. Áldásy's scientific importance, however, is much greater than his actual activity: he had colleagues, disciples who, together with significant representatives of related institutions, faithfully follow his ideas and inwardness thus actively and creatively serving the animal health in Hungary. Áldásy was an intuitive, open-minded epidemiologist interested in both theoretical and practical matters with a sense to perceive internal rules and interconnection of events who had excellent rhetoric skills for education.

This session of the Hungarian Society for Microbiological Association is a commemoration of the creative, scientific expert who founded a school in the field of veterinary diagnostics and proved that outstanding results can be achieved even in the provinces.

I. LONTAI

Human rabies cases in Hungary since 1950

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At the beginning of the 1950s, the control system for domestic rabies was restored again, after a period of disorder, as a consequence of the war, thus, there were only 2 home-originated (1–1 in 1951 and 1967) and 1 imported case (in 1978) between 1951 and 1984.

Since 1985 – already in the silvatic rabies era – there were 5 human rabies cases registered. There was 1 fox originated case in county Somogy and 4 cat originated cases from the northeastern part of Hungary. None of them had the correct postexposure prophylaxis.

In two cases (one in 1985, in Debrecen and an other in 1991 in Miskolc) the cat brain material was tested and proved to be negative both in immunofluorescence and in mouse inoculation test. The human autopsy brain material in both cases was negative in

immunofluorescence test, but the mouse inoculation test results verified the diagnosis of rabies.

As the tests done in Debrecen and Miskolc gave negative results, the examinations were repeated in the State Veterinary Institute's laboratory. On the basis of the repeated and negative test results, the presence of a rabies virus serotype, other than the European Serotype 1 was supposed, but the characterization of the agent isolated from the human autopsy brain material in 1991 proved the presence of the European serotype 1 rabies virus (P. Surean, Pasteur Institute, Paris).

By the recommendation of WHD, the expositions caused by an animal, the brain material of which results in a negative immunofluorescence test, is not considered as rabies infection-suspect event, thus the specific prophylaxis is not prescribed.

On the other hand, on the basis of thousands of immunofluorescent test results, it is proved that about in 1% of cat, and in 0.4–0.5% of dog, cattle and fox immunofluorescence test negative results, the mouse inoculation tests give rabies positive result – thus the decision based only on the negative results of the immunofluorescent test – may cause human rabies because of the lack of the appropriate postexposure prophylaxis.

GY. SZŰCS, M. ÚJ

Caliciviruses in and around us

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The caliciviruses (CVs) are a family of viruses of which the molecular biology, replication scheme and genome organization are just beginning to be understood. They take their name from the characteristic cup-shaped depressions on the 'classic' CV virions. Recently, they have been separated into four genera on the basis of sequence relatedness and genomic organization [4]: 1. Norwalk-like viruses (SRSV, small round-structured viruses, e.g. Norwalk, Southampton, DSV, Snow Mountain, Hawaii, MX, Lordsdale viruses), 2. Sapporo-like viruses (Sapporo, Manchester, London, Houston, Parkville viruses), 3. Vesiviruses (SMSV, San Miguel sea lion viruses, VESV, vesicular exanthema of swine viruses, FCV, feline CVs, and primate CVs), 4. Lagoviruses (RHDV, rabbit hemorrhagic disease viruses and the EBHSV, European brown hare syndrome viruses). Hepatitis E virus has been removed from the CVs, and placed in a 'site uncertain' designation [4]. Members of the Caliciviridae are non-enveloped viruses. The virions consist of a single polypeptide and possess a positive-sense polyadenylated RNA genome of approximately 7.5 kb [1].

Progress on the molecular characterization of the human CVs has been slow because of the lack of cell culture systems. FCV, SMSV and other CVs of animal origin can be readily propagated in tissue culture cells, however, the application of molecular techniques, e.g. PCR, recombinant antigen-based ELISA, etc., has led recently to recognition of the genetic diversity and changed assessment of the epidemiologic importance of CVs. Moreover, the discovery of movement of SMSV from ocean

reservoirs to terrestrial hosts led to the conclusion that fish and perhaps other ocean products provide a vehicle for transmission [2]. CVs are now established as the causative agents of a number of veterinary and human diseases. Human cases are associated mainly with food-borne and water-borne outbreaks of gastroenteritis in people of all ages and on all continents.

In Hungary, the observation of calicivirus-like particles in animal fecal samples, limited unpublished investigations on RHDV and EBHSV [5], and results of serosurveys for Norwalk [3] and MX viruses in human sera collected from different geographic areas demonstrated the circulation of these viral pathogens in the country. A lack of an official reporting system for viral gastroenteritis in humans precludes an estimate of how many cases of non-bacterial diarrhea may be associated with CV infection in the country.

Many of the above-mentioned factors, including hosts, zoonotic potential, transport, disease spectrum and requirements for new and better diagnostic tools and epidemiologic assessments for pathogenic CVs will be discussed.

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Prevalence and pattern of feline coronavirus subpopulations in asymptomatic shedders

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102 cats were randomly sampled by rectal swabbing to estimate the occurrence of feline coronaviruses in the area of Debrecen. The samples were subjected to a nested RT-PCR specific for the conservative nucleocapsid region of the virus genome then the amplified products were investigated by SSCP and nucleotide sequencing. The obtained sequence informations were compared to those that have been deposited in the GenBank so far.

30% of the sampled population proved to carry and shed feline coronavirus by the RT-PCR investigations.

Our results will serve as a starting database for a longitudinal study where the positive cats are monitored monthly to record their state concerning virus shedding and the phylogenetic analysis of the shed viruses – in accordance with the clinical data. It has already been shown that feline enteric coronaviruses (FECV) can establish persistent infection in cats during natural infection. FECV is not an important cause of morbidity in

cats, however, it is believed that FECV presents a considerable health risk to cats because it is the parent of feline infectious peritonitis virus (FIPV), the causative agent of a consistently fatal disease. FIP viruses arise as a result of certain mutation/recombination events during the intestinal replicative phase of FECV infection from the persisting subpopulations of the FECV quasi-species. Having acquired the ability to replicate in macrophages, FIPV becomes an intracellular pathogen of the monocyte/macrophage system and, consequently, develops the different clinical forms of FIP.

The mutation/recombination event(s) and the site(s) of it (these) have not yet been determined. Therefore, it is reasonable to monitor asymptomatic feline coronavirus shedders because they are the potential victims of fatal FIP, thus, the source of FIP inducing coronavirus(es). Comparing the parental "enteric" coronavirus with the FIPV should reveal the difference(s) between them.

T. PÁL

The role of epithelial cell Invasion in the pathogenesis of enteric bacterial infections

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Various microbial strategies have been evolved during the evolution to secure the survival of bacteria in the intestinal tract. One of the most effective colonisation mechanisms exhibited by several pathogens is the infection of the cells of the host. The most important invasive enteric pathogens are salmonellae, yersiniae, shigellae, and the enteroinvasive *Escherichia coli* strains. The target cells of these bacteria could be professional phagocytes, M cells and/or epithelial cells of the intestinal tract. These microbes differ from each other in their interaction with their respective target cells, particularly as far as the mechanism of invasion, the microbial factors involved, their genetic background, the receptors on the target cells, and the intracellular fate of the bacteria are concerned. In order to understand the pathogenesis and the clinical picture and to develop effective therapeutic and preventive measures and diagnostic tools it is inevitable to understand the modes of invasion shown by these pathogens. The molecular basis of the invasive capacity of some of the important enteric pathogens will be discussed.

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Salmonella typhimurium DT104 in animals and man in Hungary

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In light of reports about internationally emerging significance of multiresistant *Salmonella* in animals and men studies were conducted to estimate the significance of multiresistant *Salmonella typhimurium* phage type DT104 in Hungarian animal population. A collection of 173 strains have so far been tested (isolations from late 1997 to early 1998) and 15% of them proved to be multiresistant (resistance against ampicillin, chloramphenicol, streptomycin and tetracyclin). Phage typing of these strains revealed 15 strains belonging to phage type DT104. *S. typhimurium* DT104 was isolated from turkey (5/11), pork (4/27), geese (1/87), duck (2/20), from other animals (1/9) and from animal environment (2/6), while isolations from chicken were – so far – negative (0/13). Resistance to quinolones was frequently detected, but no strains was found with fluoroquinolone resistance. Plasmid profile analysis detected the serovar specific large plasmid (with some small plasmids in some strains) but no plasmid profile characteristic to *S. typhimurium* DT104 could be found. Strains proven to be DT104 have fallen into the "phage/bio types" of 2c/3, 2/3 or 35/3 of the Felix-Callow phage typing system used in Hungary. The presence of such strains was low (2.5%) in 1985, but by 1995 it increased to 54% of the total (animal and human) *S. typhimurium* isolates phage typed in Hungary. Since then it seemed to stay around that level. It can be concluded that turkey seems to be at present the main animal reservoir for *S. typhimurium* DT104 for men in Hungary. Although this is the first report about this phage type in Hungary our data indicate the presence of this DT for more than 10 years.

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Diagnosis of *Contagious agalactia* of sheep and goats in Hungary

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There is no incidence of *Contagious agalactia* of sheep and goats in Hungary. Introduction of an epidemic happened 50 years ago, but the infected stock had been stamped out.

At the beginning of August 1997 a massive outbreak of the disease occurred on an Eastern Hungarian farm among 200 mother goats and 500 ewes. Approximately 150

animals got sick in a month. Among the sick animals there was keratoconjunctivitis leading to opacity and perforation of the cornea, purulent keratitis and iridocyclitis and blindness. At the worst forms there was polyarthritis in the carpal joints resulting in lameness, abasia, lairing and interstitial mastitis leading to atrophy of mammal glands.

The clinical signs related to Mycoplasma caused disease. The differential diagnosis requires consideration of bacterial and chlamydial keratoconjunctivitis, polyarthritis, mastitis, CAE. These diseases have been excluded.

Mycoplasma were detected from the synoviae of sick goats and sheep by PCR technique using general Mycoplasma primer pairs.

In order to culture Mycoplasma we tested 14 synoviae and milk samples and conjunctiva swabs. In all but one sample we isolated Mycoplasma. We identified them as Mycoplasma agalactiae by their biochemical characteristics and growth inhibitory test made by positive blood serum.

Biological Research Institute of Szeged sequenced a 270 bp PCR product originated from a pure Mycoplasma broth culture. The sequence of the amplified DNA fragment showed 100% homology with corresponding part of a gene coding for 16S rRNA of *M. agalactiae.*

Based upon our examinations we excluded diseases caused by *M. mycoides* ssp. *mycoides* LC, *M. capricolum* ssp. capricolum and *M. putrefaciens*, and we diagnosed contagious agalactia.

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New type of immune stimulant to increase antibody production generated by viral and bacterial vaccines

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It has been recently discovered that certain compounds significantly stimulate the function of the immune system. Using additives for feed acidification, garlic extract and microbial cell materials, a liquid product was developed and applied in a concentration of 1 ml/litre in drinking water before and during immunization.

The application of the experimental product started 2–3 days before vaccinations and was provided for 17–20 days. According to data available so far the product increased in several poultry models, (on the basis of geometrical mean titer values) the antibody production generated by parenterally applied inactivated goose parvovirus, Newcastle and Gumboro disease virus, egg drops syndrome (EDS) virus vaccines, as well as vaccines prepared from killed *Salmonella enteritidis*, *Pasteurella multocida* and *Leptospira pomona* bacteria by 40–300%.

On the basis of the data obtained it could be concluded that the humoral immune response of treated animals increased in comparison to the controls. Furthermore, in the treated groups, all of the animals seroconverted.

After optimizing the efficacy and formula of the product, currently designated "IM 326", the authors intend to register it with a name that would reflect their respect for Dr. Pál Áldásy.

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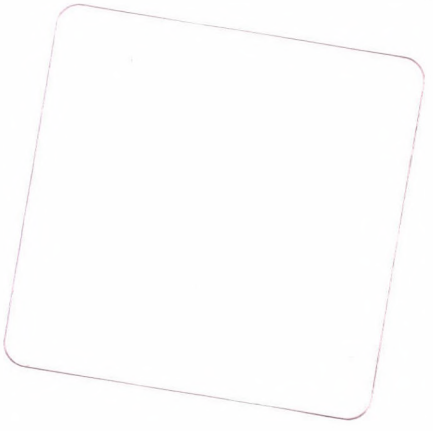

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CONTENTS

Lectures of Plenary Sessions

Poster Sections

Lectures of Plenary Sessions

Acta Microbiologica et Immunologica Hungarica, 46 (2–3), p. 161 (1999)

PREFACE

Welcome to the First Hungarian Conference of Mycology at the Hungarian Academy of Sciences. We are happy to see you at this meeting which is the first occasion in Hungary gathering people working in all fields of mycology. More than 100 people registered for the meeting. Twenty-five lectures and about 120 posters are presented. We are pleased meeting here our keynote speakers, Professor Franz Oberwinkler, President of the International Mycological Society and Professor John F. Peberdy, Former President of the British Mycological Society. We are glad to greet our Masters in mycology here and it is a pleasure to see so many young mycologists from all over the country.

The aim of this Conference was to give a board overview of the present state of mycology and Hungarian mycological research. In the last two decades mycology evolved in such an extensive way and our knowledge about fungi changed so radically that its significance must be emphasized in an increased degree. Revaluating the traditional judgement and position within biology, mycology should be defined, separated from botany, as an independent branch of science. The subject is diverse, covering most areas of fungal cell biology, genetics, evolution, taxonomy, ecology and applied fields like medical, agricultural and industrial mycology. To fulfil this task we decided to have a general introduction to the main current topics by invited speakers as well as to give the possibility to everybody to present his/her results in the poster section. The proceeding contains the abstracts of oral presentations in the sequence of the conference program. Poster abstracts are divided into three main thematical groups containing the items in alphabetical order.

The conference could not have been taken place without the support of the Hungarian Academy of Sciences, the Eötvös Loránd University, the Hungarian Microbiological Society and the Hungarian Mycological Society. The financial assistance we received from our sponsors listed above is also much appreciated.

Thank you all, participants, organizers and supporters, for making this meeting possible. May our increased knowledge of fungi be the benefit of your efforts!

The Organizing Committee

Acta Microbiologica et Immunologica Hungarica, 46 (2–3), p. 163 (1999)

RECENT RESULTS OF FUNGAL TAXONOMY

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Fungi in the traditional sense are heterogeneous eukaryotes in several phylogenetic lineages. Oophyta will be addressed briefly for comparison. *Basidiomycota* will be taken as the main group of true fungi to be discussed in detail. *Cryptomycocolax* is a key-taxon to understand connections of *Basidiomycota* and *Ascomycota*. Also the transfer of *Mixia* into *Basidiomycetes* allows new insights in the origin of *Basidiomycota*. Other basal basidiomycetous taxa are the *Agaricostilbales* and *Atractiellales*. The evolution in the *Uredinales* is closely bound to their hosts, mainly to conifers and angiosperms. Coevolutionary trends in some groups of rusts are used as representative examples. Several plant and insect parasites belong into the *Uredinales* relationship. Smuts are now considered to fall into two major groups, the *Microbotryum*-relationship and the true smuts. In the *Microbotryanae* also mycoparasites are included which share colacosomes as unique cell organelles. The *Ustilaginomycetes* have been rigorously reinterpreted recently. The new system of smuts will be explained referring to the main groupings and some new orders. Within the *Hymenomycetes* we treat some heterobasidiomycetous taxa, e.g. the *Tremellales*, *Dacrymycetales*, *Auriculariales*, and *Tulasnellales*, and the former *Homobasidiomycetes*. The latter are still the most difficult ones to be grouped in monophyletic taxa. How to connect them in a meaningful evolutionary sequences is a future challenge. The *Hymenochaetales*, *Russulales*, *Boletales*, and *Thelephorales* can be circumscribed adequately. But still better delimitations are needed. To demonstrate the problems, also the traditional *Aphyllophorales*, *Agaricales*, and *Gasteromycetes* will be considered. Final remarks concern the distribution of plant parasites and symbionts within the *Basidiomycetes* and their coevolution with the hosts.

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EXTRACELLULAR PROTEINS IN FUNGI: A CYTOLOGICAL AND MOLECULAR PERSPECTIVE

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Protein secretion is a vital process in fungi. For many, the secretion of hydrolytic enzymes provides a crucial step in their nutrition in nature. However, in recent years the list of different types of secreted proteins that have been discovered has extended significantly. These have been shown to have a diversity of functions including toxic molecule transport and control of desiccation. The majority of secreted proteins are glycosylated and our understanding of this aspect of fungal biochemistry has also extended in recent years. This review addresses the process of protein secretion from the cytological, biochemical and genetical standpoints. Advances in technology in many areas of scientific approach have enabled a better and understanding of this important process in fungi.

Most, if not all filamentous fungi actively secrete proteins during vegetative growth. These molecules are diverse in their function, they include enzymes which play a key role in nutrition and proteins associated with the cell wall, where they may have some structural or recognition role or a protective function as in the case of the recently described hydrophobins, or in the plasma membrane.

The secretion of enzymes is related to the invasive growth that occurs in both saprotrophic and biotrophic filamentous fungi. The former play a key role in the breakdown of plant wastes especially cellulose and lignocellose and many fungi are known to produce the enzymes which hydrolyze these polysaccharides. Both facultative and obligate biotrophs are found associated with plant hosts, and the former also with insects, crustaceans and nematodes. Not surprisingly the plant biotrophs also produce cellulases as well as pectinases, whereas the insect and nematode pathogens produce proteases, lipases and chitinases. In laboratory cultures secreted enzymes are normally detected in the culture medium, however, whether this is true in the natural environment is unknown.

Several enzymes secreted by fungi have realized commercial potential and have application in a variety of industrial processes. Strain development in the fungi used for these purposes has resulted in the production of hyper-secreting strains, which produce yields reportedly as high as 40 gl^{-1} of extracellular protein [1]. This characteristic has highlighted a further application in the production of recombinant proteins [2]. To date

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only two such products have been commercialized, bovine chymosin [3] and lactoferrin [4], however, the yields produced in industrial fermentation are of the order of 1.5 to 2 g, which represents at best only 10% of the high production of a native protein.

The roles and importance of other types of secreted proteins described above have come to a more detailed understanding in recent times and will, along with a discussion on the processing of proteins for secretion, form the basis of the review that follows

Cell biology of protein secretion

The first definitive studies on the ultrastructure of the fungal hypha, which aided our understanding of the secretory pathway in filamentous fungi, were published some two decades ago [5]. With the exception of Golgi structures, organelles involved in the post-translational processing of proteins, prior to secretion, present in mammalian cells were also demonstrated in hypha although their spatial distribution was quite different and related to the growth associated polarized organisation which these cells exhibit. The presence of Golgi bodies in filamentous fungi is a matter of debate. Most studies based on transmission electron microscopy have not shown the presence of stacks of Golgi cisternae, but rather single cisternae that have been regarded as Golgi equivalents. This divergence from the typical eukaryotic organisation is further exemplified in studies with the inhibitor Brefeldin A, which was found to cause gross distortion of the endomembrane system at concentrations which inhibit hyphal growth [6, 7]. In contrast ultrastructural examinations on appresoria of fungal pathogens show the presence of this organelle [8]. It is possible that this difference relates to the differentiation of the hyphal tip for the infection step.

The Spitzenkorper is a structure first identified at the tip of the fungal hypha by light microscopy and subsequently shown in transmission electron microscopy to be a complex of apical vesicles [5]. The location of vesicles at hyphal tips suggested a highly polarized release of proteins at growing hyphal apices. Clearly these techniques reveal nothing of the dynamics of such a structure, however, recent studies in which video-microscopy was used showed the Spitzenkorper to be a "pleomorphic complex constantly changing size and shape" [9]. A co-ordinated role for this vesicle complex in the polarized processes of hyphal tip growth and protein secretion is a reasonable hypothesis that explains the invasive growth characteristic of filamentous fungi. Evidence to confirm this view was made available in studies, which involved the immunostaining of hyphal tips of *Aspergillus niger* that were secreting glucoamylase [10].

The mechanism of vesicle translocation to the hyphal apex still requires definitive investigation. A reasonable organelle for involvement is the cytoskeleton. Studies involving the use of inhibitors such as methyl benzimidazole-2-yl carbamate suggest a role for both microtubules [11–13] and actin [13], but genetic studies to confirm this are still required. Such data does, however, come from an alternative approach with a video-microscopic study on a kinesin-deficient strain of *Neurospora crassa*, which exhibits extensive distortion to hyphal morphology and concomitant failure to assemble a Spitzenkorper [14]. Assuming that kinesin in *Neurospora* has the same property of

organelle translocation along microtubules as in mammalian cells, then this data supports the hypothesis of cytoskeleton mediated vesicle translocation.

The role of the secretory pathway organelles has been extensively described in *Saccharomyces cerevisiae* through studies of conditional mutants defective in secretion and post-translational modification [15]. Studies now being pursued in *Aspergillus niger* and *Trichoderma viride,* which will enable a similar confirmation of the functional pathway in filamentous fungi. These investigations will undoubtedly be important in the future with the need to manipulate protein secretion and enhance the process.

Role of the cell wall in protein secretion

The hyphal cell wall is clearly a porous structure allowing the free flow of small molecules into and out of the cell, however, even the smallest secreted proteins e.g. hydrophobins, are too large to move out of the cell on a simple diffusion gradient. The accumulation of vesicles at the hyphal tips suggests that protein molecules, which have been processed during their passage from the ER to the tip vesicles, will be discharged at the apex as the vesicles fuse to grow the plasma membrane. The apical tip is also the site of nascent wall synthesis, but in this region it is assumed that the different polymers of the wall, i.e. chitin and β-glucan are independent layers. In the sub-apical region the polymers become cross-linked by covalent and non-covalent bonding to create a rigid wall. The hyphal tips are therefore very plastic and it can be supposed that proteins released on the plasma membrane surface are carried in the flow of cell wall material eventually reaching the outer surface of the wall, where they either remain or are released into the surrounding medium [16].

This model is supported by a variety of studies, which focus on the hyphal wall. Treatment of fungal hyphae with enzyme cocktails used in the isolation of protoplasts confirms the view that proteins are trapped in the more rigid regions of the wall [17]. Studies on the secretion of enzymes by protoplasts demonstrate clearly that without a wall more than 80% of a secreted protein is found in the external medium [17]. Mutants which affect hyphal morphology and also show a modified wall composition as part of their phenotype may either secrete less or more than the wild type [18] and some cellulose hyper-secreting mutants of *T. reesei* have been shown to have a modified cell wall [19].

Secreted proteins in the plasma membrane and cell wall of fungi

Since the earliest reports on cell wall composition it has been known that the hyphal wall of fungi contains proteins, however, the chemical format of these molecules and their functional significance were not known. More recent studies have benefited from the technological developments in the past decade and have shown that many of the proteins are glycosylated molecules. Some of these are enzymes destined for secretion, but become entrapped, others are part of the cell wall itself and generally are found at the

hyphal surface. In the last decade a new type of proteins which have unique properties, called hydrophobins, have been discovered.

The demonstration of the entrapment of enzymes has been described above, however, it is not unlikely that some enzymes are integral parts of the wall and are retained there by covalent or other types of linkages. Such enzymes could be significant in the invasive growth of fungi. Because enzymes can be found free, for example in culture filtrates of growing fungi, it is assumed that this is the norm for the breakdown of extracellular insoluble substrates. Recent studies with *Aspergillus fumigatus* would tend to suggest otherwise. This pathogen although commonly associated with infections of the lung can be very invasive into deeper body tissues in immunocompromised patients. As the fungus is known to produce a number of proteinases it was reasonably assumed that these were important in tissue invasion. Mutants blocked in the production of these enzymes were, however, found to be still invasive. Analysis of these strains revealed the presence of aspartic and serine proteases in the wall at hyphal tips that could provide a localized mechanism for tissue invasion [20].

Glycoproteins at the cell surface are thought to have a role in recognition or as elicitors. Such proteins are now being discovered in fungi, which have some form of association with plants. One such example is the pathogen of tobacco, *Phytopthora parasitica* var. *nicotianae* in which a 34 kDa glycoprotein has been found and purified [21]. This molecule was shown to be an elicitor when tobacco plants exposed to the molecule through their roots showed an up-regulation of lipoxygenase activity and the accumulation of a hydroxyproline-rich glycoprotein. The timing of expression of this glycoprotein was not determined, however, in *Colletotrichum lindethianum a* proline rich protein, with two putative glycosylation sites, has been found which is expressed in the fungus only when in the host plant [22]. It is suggested that the molecule forms cross-linking components in the cell wall.

Cell wall proteins with recognition functions have been described in ectomycorrhizal fungi in studies on the symbiosis of *Psilothus tinctorius* and *Eucalyptus* spp. [23]. Dramatic changes in the cell wall proteins of the fungus were seen at the early stages of the interaction, which implied significant spatial and temporal phenomena. Analysis of the protein profile revealed an increase in a 32kDa acidic polypeptide and a decrease in a 95kDa glycoprotein.

The discovery of the unique group of proteins, the hydrophobins is one of the major advances in fungal biochemistry in this decade. Since their discovery in 1991 [24] there have been reports for their presence in many fungi [25] which implies they are probably ubiquitous in terrestrial species. These molecules are generally small peptides comprised of ca 75–90 amino acids, always including 8 cysteine residues, which contribute to the hydrophobic properties. When released onto the surface of aerial hyphae, fruit bodies and other structures exposed to the atmosphere, the proteins self-assemble to form rodlet structures. It is also possible that hydrophobins have a role in adherence of hyphae to surfaces as they grow over them [26] and are involved in plant fungus interactions both symbiotic [23] and pathogenic [27]. When fungi are grown in submerged liquid culture the hydrophobins are released into the growth medium. Molecular analysis of hydrophobin genes has shown that many fungi produce several of these proteins that may be expressed at different stages of development and have different functions. In

Schizophyllum commune at least four hydrophobins have been identified, SC1, SC3, SC4 and SC6, and their expression shown to be developmentally regulated. SC3 is, so far, unique in that it is the only hydrophobin expressed in the monokaryon of *S. commune*. Synthesis of the protein is suppressed in dikaryons with compatible B mating genes. Expression of SC1, SC4 and SC6 is associated with the dikaryon and fruit body. In these basidiomycete reproductive structures, hydrophobins are important as surface coverings for the fruit body and lining the air cavities in these structures [28]. Families of hydrophobins have also been reported in *Agaricus bisporus* [29–31], *Trichoderma reesei* [32–34] and *Coprinus cinereus* [35].

The importance of glycoproteins in plasma membranes has arisen from quite divergent work on fungal pathogens of animals and plants. The pathogen of mammals *Pneumocystis carinii* [36] recently designated as a fungus, and the entomopathogen *Entomophaga maimaiga* [37] have both been shown to have glycoproteins, of yet unknown significance in their plasma membranes. In both *Aspergillus fumigatus* and plant pathogens, the plasma membrane is the location for glycoproteins that are homologous to ABC transporters of mammalian cells that are responsible for the efflux of drugs from cells [38]. In fungi transcription of the genes encoding these proteins is enhanced in the presence of fungicides suggesting a role in multi-fungicide resistance [37].

Extracellular secreted proteins

Probably the best-known examples of proteins secreted from fungal hyphae are hydrolytic enzymes, those which attack plant polysaccharides such as cellulose, hemicellulose, starch and pectin being the most prevalent. However, enzymes that degrade a range of other biological macromolecules and other organic compounds are also found. These enzymes have a significant function in the nutrition of fungi.

Genes encoding many of these enzymes have been cloned and extensively characterized. Two examples of these, which emphasize the advances and developments of recent years, will be described here. These enzymes also represent two good examples of the commercialization of fungal enzymes.

Trichoderma reesei is well known as a highly cellulolytic fungus [40]. The hydrolysis of this polysaccharide is very complex involving three classes of enzymes, cellobiohydrolase (CBH), endoglucanase (EGL) and β-glucosidase. Genes encoding all three enzymes have been isolated and extensively characterized and as a consequence small families of *cbh* and *egl* genes have been discovered. When grown on cellulose, the expression levels of these enzymes in *T. reesei* are characteristically unequal with the CBH1 protein amounting to 60% of the total activity produced and secreted. Glucoamylase is an important enzyme produced by *Aspergillus niger* [41]. In culture the enzyme is produced and secreted when the fungus is grown on starch, maltodextrin or maltose. As far as is known, there is only one form of the protein, which is encoded by the gene *glaA*. The two proteins show similarities in both structure and in regulation of their synthesis. Each has three domains, a binding domain, a catalytic domain and a bridging domain. How universal this structure is for secreted enzymes remains unknown.

Protein processing for secretion

Proteins destined for secretion are synthesized on ribosomes located on the cytosolic surface of the endoplasmic reticulum. The first twenty or so amino acid residues of the translated protein form the signal peptide sequence which has the key role of ensuring translocation of the nascent protein into the lumen of the ER. This is achieved by the docking of the signal peptide with the signal recognition protein, which traverses the ER membrane and mediates the transfer of the nascent protein through the membrane into the lumen of the ER. The complexity of the recognition protein is apparent from the discovery of several genes being involved in *S. cerevisiae*, but recently a gene encoding a protein component of the recognition particle has been isolated from *A. niger* [42].

Glycosylation and protein folding

To ensure the secreted proteins to be properly functional when they achieve their destination, several key changes to the molecules are necessary before they are released across the plasma membrane. These changes are known collectively as post-translational modification, but it is becoming increasingly apparent that two of the processes, N-linked glycosylation and protein folding, are probably closely interactive and co-ordinately regulated.

N-linked glycosylation of nascent proteins probably begins very soon after the putative glycosylation sites appear as the polypeptide grows into the ER lumen. The process is best conceived in two stages, the addition of the core glycan to the protein and the maturation of the glycan. The core glycan is comprised of seven sugar residues, two N-acetylglucosamine (GlcNAc), which provide the peptide link between the oligosaccharide and the polypeptide at an asparagine residue, and five mannose (man).

This core molecule is synthesized on the cytosolic ER surface on a transmembrane carrier dolichol phosphate (dol-PP). Synthesis of the core commences with the addition of the two GlcNAc residues to dol-PP catalyzed by the enzyme UDP N-acetylglucosamine:phosphodolichol N-acetylglucosaminyl-1-phosphotransferase (GPT). This enzyme can be found in a microsomal fraction from *Aspergillus niger* [43] and is sensitive to the antibiotic, tunicamycin. A gene encoding the enzyme has recently been cloned and is currently under investigation (T. Sørenson and J. F. Peberdy, unpublished data). Addition of the five man residues from a GDP-man carrier, catalyzed by dolichol phosphate mannose:$Man_5GlcNAc_2$ PPDol mannosyl transferase, completes the core glycan with two branches, one with a single man and the other with four man. The molecule is then translocated into the ER lumen where its maturation takes place by the addition of four additional man residues, by making two branches of two residues from single residue branch, and three glucose residues to the original three man branch. The mature glycan is now transferred to the nascent protein to generate the asparagine/N-linked structure, a reaction controlled by oligosaccharide transferase.

Simultaneously the nascent polypeptide interacts with chaperone proteins and enzymes that carry out the folding process. Whilst these have been well described in yeast

and mammalian cells, reports in filamentous fungi are more recent and arise from the isolation of encoding genes, e.g. for BiP and TIG. BiP is a chaperone, which is soluble in the ER lumen; it is constitutively expressed but expression is enhanced by heat shock and exposure of the cells to tunicamycin [44, 45]. Up-regulation by tunicamycin is interesting in view of the target for the antibiotic. The implication is that impaired glycosylation enhances the need for increased BiP levels in the lumen suggesting a link between the addition of the glycan and protein folding. The role of TIG is less well defined, but again the encoding gene is upregulated when glycosylation is inhibited [46].

Alongside these chaperones several enzymes are also involved in the folding process. The one identified so far in filamentous fungi is protein disulphide isomerase (PDI), the gene for which has been cloned [47]. The oxidative environment of the ER lumen is crucial to the functionality of this enzyme.

Further modification to the glycan chains may take place, the timing of which relative to the folding process is not clear. Additional man residues and galactose residues may be added, or indeed removed by mannosidases, resulting in a high degree of heterogeneity to the glycan chains on a particular protein. However, before the protein is released for secretion it is given a "quality control check" in a process involving further enzymes and chaperones. First the two terminal glucose residues are removed a by glucosidases I and II, the protein is then caught with the remaining glucose binding to a membrane associated chaperone calnexin. Interestingly yeast seems to lack this chaperone, but it is present in the mammalian ER together with calreticulin, a lumen-located chaperone. Attempts to clone a gene encoding this protein from *A. niger* proved to be unsuccessful (J. Lambert and J. F. Peberdy, unpublished data). Whilst bound to calnexin the protein is subjected to the repair of misfolding and then released by the action of glucosidase II cleaving the terminal glucose from the glycan chain. If in the first place all three glucose residues are removed, or the repair of misfolding is incomplete, another enzyme UDP-glucose:glycoprotein glycosyl transferase (GGT) can add a glucose molecule to the glycan chain to enable binding to the chaperone. Evidence for these functions in filamentous fungi is available from the cloning of genes encoding calnexin and GGT (J. Lambert and J. F. Peberdy, unpublished data) and the detection of GGT activity in microsomal fractions (G. Wallis, F. W. Hemming and J. F. Peberdy, unpublished data). The existence of this quality control system is interesting. It is unknown to what degree misfolded proteins are produced in the secretory pathway, the presence of such a complex means of control would imply that it might be a common occurrence.

The above description indicates the importance of N-linked glycosylation in the processing of proteins prior to secretion. However, in both mammalian and fungal cells a second type, O-linked glycosylation also occurs. In this case the process and glycan structures are much less complex. Five membered mannose chains are synthesized, again with a dol-PP GlcNAc anchor but involving the enzyme dol-PP man synthase, which are bonded to threonine-serine residues in the protein. The importance of O-linked glycosylation for secretion is unknown, but its occurrence in specific domains of secreted proteins e.g. the bridge domain of *T. reesei* CBHI and glucoamylase of *A. niger* may be significant.

Concluding remarks

The discussion pursued in this review makes it abundantly clear that the secretion of proteins is a vital process in fungi. It is not surprising therefore that our understanding of the process of protein secretion in filamentous fungi has expanded significantly in the past decade. This reflects the technological developments in all areas relevant to this research from microscopy to molecular biology. The realization that proteins secreted by fungi have a diversity of roles, much wider than enzymes emphasizes even more the importance of the process and the need for continued study. Up to five years ago our interpretation of the secretion pathway in filamentous fungi drew heavily on the extensive genetic studies with *S. cerevisiae*. Whilst there are similarities, it is apparent there are differences not least in the diversity and functional significance of the proteins themselves. Extensive gaps still remain in our knowledge which is clearly very fundamental to either our further exploitation of fungi or for developing new strategies for controlling them in pathogenic situations. It is vital therefore that research on this complex area of fungal biology continues to be pursued.

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ORIGINS, PHYLOGENIES AND RELATIONSHIPS IN THE FUNGAL KINGDOM

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The "true" fungi have been referred to as the Kingdom *Fungi,* the Kingdom *Eumyceteae,* or the *Eumycota* [1]. The fungi are eukaryotic organisms, characterized by: (i) a diversity of microbodies; (ii) cell walls that have a great similarity of architecture; (iii) hyphae that have a major chitin component, extended apically, and divide by centripetal invagination of the plasma membrane; (iv) lomasomes: sponge-like intumescences seen on the inside of the cell wall; (v) complete absence of the Golgi organelle in the terrestrial assemblages (zygomycetes, ascomycetes, and basidiomycetes) and some of the aquatic taxa; and (vi) nuclei in which most, if not all, gene products involved in mitosis probably have higher eukaryotic paramologues but which, in other ways, are exceptional [2]. Fungi are reproducing sexually or asexually, the diploid phase generally short-lived. Fungi parasitize a wide range of plants, animals, and other fungi [3].

The number of known species of fungi is about 70,000, but species of the fungal world are conservatively estimated to be 1.5 million [4]. The fungi are of great consequence agronomically, bioindustrially, medically, and biologically. In spite of their importance, their taxonomic inventory is poor. In addition, very little is known about the phylogeny and evolution of fungi and about the correlations between these and other organisms [1].

Sexual and asexual reproductive structures have provided important phenotypic characters to measure relatedness and evolutionary affinities among fungi. If they lose these structures, accurate taxonomic assignment is quite difficult.

To date, phylogenetic speculations and taxonomies for the fungi have been based mainly on analyses of morphological data sets. In the 1980s, development of molecular biological techniques (DNA:DNA hybridization, DNA fingerprinting, nucleic acid sequencing, isozyme analysis), proliferation of high performance computers, and improvement of molecular evolutionary analysis programs have extended studies of relatedness, phylogeny, and evolution of organisms, including fungi, at the molecular level [5–8]. Nucleic acid characters, as genotypic characters, are ubiquitous and are not dependent on the expression of reproductive structures. Nuclear DNA base composition and nuclear DNA relatedness, as nucleic acids characters resolve only to the genetic sibling species level [5].

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Ribosomal RNA sequence comparisons, as a nucleic acid character, offer a means for estimating more distant relationships [9]. In recent years, molecular phylogenetic analysis of the fungi shifted to the small (18S) and large (23S to 28S) subunit rRNAs. Phylogenetic analysis among distantly related taxa, using 18S rRNA gene sequences, has contributed to well-resolved and statistically supported conclusions. In the early 1990s, such an approach has steered fungal taxonomy towards fungal molecular systematics [10–17]. Thus, studies on fungal phylogeny and evolution have entered a new era.

Among earlier phylogenetic speculations concerning the fungi and related organisms that have been made during the past ten years, Cavalier-Smith [1] provided a framework for a taxonomic system and phylogeny for the fungal kingdom. His system included only the *Chytridiomycetes, Zygomycetes, Ascomycetes,* and *Basidiomycetes* in the Kingdom *Fungi.* These four fungal groups are characterized by chitinous cell wall and the alpha-aminoadipic acid (AAA) lysine biosynthetic pathway. The *Oomycetes, Hyphochytrids, Labyrinthulida, Thraustochytrids,* and slime molds, which are cellulosic and have the diaminopimelic acid (DAP) lysine biosynthetic pathway, are excluded from the *Fungi.* The former four major groups have been accommodated in the *Pseudofungi* and the latter in the slime moulds in the kingdom *Protozoa* [18]. He concluded that all major eufungal taxa, e.g., the *Endomycota, Ascomycota,* and *Basidiomycota,* evolved from the *Entomophthorales* from a chytridiomycete ancestor by loss of cilia (flagella). Molecular phylogenetic analysis [19, 20] confirmed that the chytrids were true fungi and the true fungi, the ascomycetes, the basidiomycetes, the zygomycetes, and the chytridiomycetes, formed a monophyletic group, distinguished from slime molds and the oomycetes. On the basis of 18S rRNA sequence analysis, Hendriks et al. [21] indicated that the red algae and the higher fungi did not possess a common ancestor. Wainright et al. [22] suggested that animals and fungi share a unique evolutionary history and that their last common ancestor was a flagellated protist similar to extant choanoflagellates. According to the molecular evidence, the fungi may have originated from protozoan ancestors before the Kingdoms *Animalia* and *Plantae* split [23].

Berbee and Taylor [11] calculated that the three main fungal phyla diverged from the *Chytridiomycota* approximately 550 Myr ago, that the *Ascomycota/Basidiomycota* split occurred at about 400 Myr ago after plants invaded the land, and that many ascomycetous yeasts and moulds (e.g. *Eurotiales)* evolved after the origin of angiosperms in the last 200 Myr. These results are broadly supported by fossil evidence, the main difference being that remains of *Ascomycota* go back to the Silurian (c. 440 Myr ago) while the oldest definite *Basidiomycota* occurred at about 380 Myr ago [24].

Data of the latest molecular phylogenetic analysis, using 18S rDNA sequence divergence, shows that a monophyletic kingdom for the *Fungi* contains the *Chytridiomycetes* and the *Zygomycetes* as lower fungi, and the *Ascomycetes* and *Basidiomycetes* as higher fungi. They suggest great phylogenetic divergence among the chytrids and the entomophthoralean fungi of the lower fungi and loss of flagella within several lineages of the lower fungi. On the other hand, the molecular and morphological data set clearly indicate existence of two monophyletic Divisions, *Ascomycota* and *Basidiomycota.* The former comprises the *Archiascomycetes,* as a new concept, the *Hemiascomycetes* (ascomycetous yeast), and the *Euascomycetes,* whereas the latter contains the *Ustilaginales* smut (or smut fungi), the simple septate *Basidiomycetes,*

including most of the basidiomycetous yeasts, and the *Hymenomycetes.* Analyses of more taxa within these lineages and more sequence data are required to elucidate evolutionary relationships among the fungi, from the lower to higher fungi, in the light of the extensive fungal species diversity that has been revealed to date [13].

The other organisms previously regarded as "fungi" belong to Kingdom *Protista,* which for phylogenetic reasons should be divided into four separate kingdoms: *Archeozoa, Euglenozoa, Protozoa,* and *Chromista* [18].

In Kingdom *Euglenozoa* we find the *Acrasida* – a group of cellular slime moulds. Kingdom *Protozoa* contains two independent lineages of *fungus-like organisms.* One is phylum *Mycetozoa* encompassing class *Myxogastrea* (the true slime molds) and the small classes *Dictyostelea* (cellular slime moulds differ from *Acrasida)* and *Protostelea.* The other is the enigmatic phylum *Plasmodiophoromycota* which probably links with the *Ciliophora* in the alveolates. In Kingdom *Chromista* we also find two independent lineages. One leads to division *Labyrinthomorpha* comprising slime mould-like organisms. The other is the division *Pseudofungi* with the two classes *Oomycetes* and *Hyphochytridiomycetes.* Its closest relatives are probably yellow-green algae in *Xanthophyta* (25).

Out of the proposed classifications of higher categories including fungi and fungus-like organisms we cite the following [26]:

PROTOZOA
- Acrasiomycota
- Dictyosteliomycota
- Myxomycota
  - Myxomycetes
  - Protosteliomycetes
- Plasmodiophoromycota

CHROMISTA
- Hyphochytriomycota
- Labyrinthulomycota
- Oomycota

FUNGI
- Ascomycota
- Basidiomycota
  - Basidiomycetes
  - Teliomycetes
  - Ustomycetes
- Chytridiomycota
  - Zygomycota
  - Trychomycetes
  - Zygomycetes

The *Deuteromycota* (Mitosporic fungi) is not accepted as formal taxonomic category in this proposed classification; they are not a monophyletic unit, but are fungi which have either lost a sexual phase or which are anamorphs of other phyla (mainly *Ascomycota,* some *Basidiomycota).* Of the three kingdoms *(Chromista, Fungi, Protozoa)* only *Fungi* consist exclusively of fungi; the *Chromista* and *Protozoa* mainly comprise non-fungal phyla. Some authors unite the *Chromista* and *Protozoa* into a single highly polyphyletic Kingdom *Protoctista* (syn. *Protista),* but that conclusion is not supported by molecular, biochemical, and other evidence. Cavalier-Smith [18] and Corliss [27] both retain *Chromista* and *Protozoa* as separate kingdoms.

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MOLECULAR TAXONOMY OF YEASTS

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In the last two decades the application of molecular techniques has had a major impact on the classification of yeasts. The nuclear DNA relatedness has become the basis of species delineation. Molecular fingerprinting methods such as analysis of restriction fragment length polymorphisms, random amplified polymorphic DNA, PCR-amplified sequences and fragments, pulsed field gel electrophoresis of chromosome DNA and others allow intraspecies differentiation and typing. The most far reaching method has been the sequencing of various parts of ribosomal DNA that has made for the first time possible to assess the phylogenetic relationships among yeasts at different taxonomic levels. Based on the molecular data obtained so far several changes have been introduced in the classification of yeasts, however, substantial restructuring of current taxonomic schemes with the consequence of numerous nomenclatural changes must await further studies.

Introduction

Molecular techniques are increasingly used in the taxonomic study of yeasts. Historically, the DNA base composition (G+C mol %) was first used for species definition, later, however, DNA relatedness (DNA/DNA homology, nuclear DNA hybridization) has become the standard basis to delineate species. Comparisons of ribosomal RNA and its gene (rDNA) have made the largest impact on yeast taxonomy, as the variable and conservative sequences of rRNA genes allow to assess both close and distant relationships at various taxonomic levels. Moreover, these studies have also led to establish phylogenetic and evolutionary relationships among yeasts and their connections with other fungi [1]. Various other molecular methods, such as analysis of restriction fragment length polymorphisms (RFLP), pulsed field gel electrophoresis of chromosomal DNA (PFGE), random amplified polymorphic DNA (RAPD), as well as PCR-based techniques have been used extensively for typing species, because their sensitivity reveals intraspecific relations. Some of these and other molecular techniques (e.g. species-specific DNA-probes and sequencing of specific rDNA regions) can be used for yeast identification [2].

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In this review the impact of the use of molecular techniques on yeast taxonomy will be discussed at decreasing levels of taxons. First, the place of yeasts in the kingdom of fungi and among the fungal divisions will be outlined, followed by the overview of higher taxa of yeasts (orders and families). Examples will be given for the recent classification of genera and species, and for the typing of strains at intraspecies level.

Place of yeasts among fungi

Based on the evidence from 5S and 18S rDNA sequences it is now a consensus in the taxonomy of fungi that the true fungi comprise four monophyletic groups: chytridiomycetes, zygomycetes, ascomycetes and basidiomycetes. Yeasts belong to the latter two groups (the so-called higher fungi), and in contrast to the majority of these organisms, which are hyphal and have chitinous cell walls, the yeasts are traditionally defined as unicellular organisms with mannan and glucan in the cell wall. In addition, yeasts are usually considered as fermentative organisms that vegetatively reproduce themselves by budding. This definition of yeasts is clearly imprecise, and the name "yeasts" can be used only as a practical term. The borderline between filamentous and unicellular, budding forms is not sharp as many fungi, both ascomycetes and basidiomycetes, may be dimorphic and have either hyphal or yeast-like growth phases. Traditionally, those fungi are considered yeasts that exist primarily in unicellular state, although a number of them can also produce hyphae. The number of described species in the successive taxonomic treatises on yeasts has steadily increased as more fungi with budding states are accepted as yeasts, which currently have 100 genera and over 700 species [3].

Phylogenetic analysis of rDNA sequences have revealed three major evolutionary lineages in both ascomycetes and basidiomycetes, and yeasts or yeast-like organisms are found in each of these groups [4, 5].

The *Ascomycota* is composed of three classes: the *Archiascomycetes*, the *Hemiascomycetes* and the *Euascomycetes*. The *Archiascomycetes* (basal or early ascomycetes) diverged prior the separation of the other two major ascomycetous lineages [6]. They include diverse taxa among them the fission yeasts. One of the most striking result of molecular studies has been the demonstration of the deep phylogenetic distance between the fission yeast (genus *Schizosaccharomyces*) and the budding yeasts (*Saccharomyces* and similar genera).

The ascomycetous true yeasts have traditionally been grouped in *Hemiascomycetes*, that also included the taphrinas. The latters having transferred to *Archiascomycetes*, the *Hemiascomycetes* now becomes limited to the single order, *Endomycetales*. However, the identity of the type species of the genus *Endomyces* is doubtful, and recently the ascomycetous yeasts are classified in the order *Saccharomycetales* with the type genus *Saccharomyces*. Several yeast-like forms occur in cultures of filamentous ascomycetes fungi (Euascomycetes). These, among them the so-called black yeasts (e.g. *Aureobasidium*) are, however, not considered as yeasts. The main

distinctive character between hemiascomycete yeasts and euascomycete fungi is the formation of fruiting bodies in the teleomorphic states of the latters, although their anamorphs may be confused with yeasts.

Molecular analysis of basidiomycetous fungi has resulted in conflicting results with the traditional classification based mainly on basidial morphology. It has been demonstrated that higher taxa characterized with the formation of phragmobasidia or holobasidia or teliospores do not represent natural phylogenetic groups. Evidence from the 18S rDNA sequences for the three main classes of basidiomycetes is rather corroborated with ultrastructural and chemotaxonomic features, such as septal pore, spindle pole body or cellular carbohydrate composition [5]. Accordingly, *Urediniomycetes*, the rusts, are characterized with simple septal pore and fucose or rhamnose in cell walls, *Ustilaginomycetes*, the smuts, may also possess simple pores but no pentoses occur in the cellular carbohydrates, whereas the third group, *Hymenomycetes* are characterized with complex septa and xylose in the cell wall. Basidiomycetous yeasts are distributed in all three phylogenetic lineages and can be integrated with filamentous forms. However, the earlier criteria used for the classification of basidiomycetous yeasts, such as the formation of teliospores or ballistoconidia do not correspond with the phylogenetic grouping. Yeasts with teliospores and/or ballistoconidia can be found in all three classes, however, in *Hymenomycetes* they are confined only to the subgroup of tremellaceous fungi.

In the traditional classification of fungi, species lacking sexual reproduction were assembled in the form-taxon *Deuteromycota*, and anamorphic yeast forms were placed in *Blastomycetes*. The current trend is to combine anamorphs with teleomorphs (mitosporic forms with meiosporic ones) into one classification [7]. This meets great difficulties with filamentous fungi possessing several different anamorphs corresponding to a given teleomorph, and vice versa. With yeasts, the conditions are more favourable, and by the concerted use of morphological, chemical and molecular data, a unified phylogenetic classification can be attained.

Higher taxa of yeasts

In the framework of fungal classes outlined above, yeasts can be placed in several orders and families. Limited sequence data make, at present, their circumscription uncertain and the classification at these levels is far from complete [8].

Members of the *Archiascomycetes* appear to be in rather distant phylogenetic relationships, hence they are differentiated at ordinal level. One of the four orders is *Schizosaccharomycetales*, which can be contrasted with the single order *Saccharomycetales* (synonym *Endomycetales*) comprising all ascomycetous yeasts and yeast-like organisms (*Hemiascomycetes*). The inclusion among these the hyphal yeasts, families of *Endomycetaceae, Dipodascaceae, Saccharomycopsidaceae, Ascoideaceae, Cephaloascaceae* and *Eremotheciaiceae* is well supported by molecular data [9]. An interesting case is the last family, the members of which are characterized by long, needle-shape spores, in common with the species of *Metschnikowia*. Hence they were formerly included in one family, *Metschnikowiaceae*. Contrary to the apparent morphological

similarity, they form distinct phylogenetic lineages representing separate families. A similar case may be the family *Saccharomycodaceae,* in which the bipolar budding is a shared morphological character of the genera included. Nevertheless, they fall on different branches of the 18S rDNA phylogenetic tree. The *Lipomycetaceae* form such a deep phylogenetic branch among the ascomycete yeasts that may justify their elevation to the rank of an order. The largest family of true yeasts is the *Saccharomycetaceae,* and its subdivision into monophyletic genera will need further molecular studies. Together with the ascospore-forming families can be classified the family *Candidaceae* including anamorphic forms of ascomycetous yeasts.

The basidiomycete yeasts with simple septal pore are placed in the order *Sporidiales* of the *Urediniomycetes* [10, 11]. Formerly, it was related to *Ustilaginales*, but no phylogenetic relatedness has been demonstrated between the two groups. The related anamorphs are included in the family *Sporobolomycetaceae* that is no longer confined only to ballistoconidial species. Yeasts with dolipore septa are divided according to the type of basidia: *Tremellales* with septate basidia whereas *Filobasidiales* with aseptate basidia. *Cryptococcaceae* is the family that corresponds to anamorphs in both orders.

Delineation of genera and species

Traditionally, yeast genera were mainly differentiated by morphological criteria, particularly the type of sexual spores, whereas species were delineated using physiological features such as the fermentation and assimilation of substrates. Information provided by molecular studies has exerted the largest impact at these taxonomic levels [1]. It is now generally accepted that strains showing 70% or greater nuclear DNA homology are conspecific. The degree of nDNA relatedness usually corresponds to the ability of interfertility, on the other hand, species with high DNA relatedness may show differences in phenotypic characters. This has led to reducing many species and even genera to synonymy, e.g. the nitrate assimilating species of the former genus *Hansenula* has been placed among the nitrate-negative species of the genus *Pichia.* The DNA/DNA homology studies have also been very useful in establishing teleomorph - anamorph connections, e.g. between species of *Hanseniaspora* and *Kloeckera* as well as *Dekkera* and *Brettanomyces.*

The taxonomy of the genus *Saccharomyces* has undergone significant changes with the use of molecular analysis [12, 13]. The once 41 species has been reduced to 7 and recently increased to 16 due to re-examination by molecular methods. The species currently accepted in the genus can be divided into two groups: *Saccharomyces* sensu stricto, and *Saccharomyces* sensu lato, in addition, there is an outlying species, *S. kluyveri.* The *Saccharomyces* sensu stricto complex includes four species (*S. cerevisiae, S. bayanus, S. pastorianus,* and *S. paradoxus*), which have been established by DNA relatedness and are indistinguishable from each other by the conventional morphological and physiological tests. Sensitive molecular methods (karyotyping, mitochondrial DNA restriction analysis, RAPD, PCR-ribotyping) differentiate most of the *Saccharomyces*

species, with the exception of the very closely related species pair, *S. bayanus* and *S. pastorianus.*

Phylogenetic analysis based on the rRNA genes and their intergenic spacer sequences (ITS_1, ITS_2) showed that the genus *Saccharomyces* is heterogeneous and its species are interdispersed on the phylogenetic tree with members of related ascomycetous genera such as *Torulaspora, Zygosaccharomyces* and *Kluyveromyces* [14]. E.g. *S. kluyveri* forms a group, which includes two *Kluyveromyces* and two *Zygosaccharomyces* species. Clearly, the current phenotypic delineation of species and genera differs markedly from the phylogenetically related groups and awaits taxonomic restructuring.

Identification and typing

Molecular techniques offer the opportunity for rapid and reliable identification of yeasts. One way is to develop oligonucleotide probes that hybridize with species-specific sequences [15]. These so-called signature sequences have so far been determined for relatively few species, and a larger database is required for successful application of the method. More promising is the comparison of 18S rDNA sequences of unknown isolates with those obtained from type strains. The latter can be retrieved from computerized databases that contain an increasing number of complete sequences. Instead of complete sequences, analysis of restriction fragments of PCR-amplified rDNA (ribotyping) is also an effective way of identification, this too, however, requires a prior database of known species.

Different molecular approaches mentioned before are sensitive enough to detect intraspecific differences and provide means to type strains that are applied in industrial production, attributed to specific ecological niches or involved in clinical cases. Polymorphisms is common for nuclear and mitochondrial DNA sequences and fragments, and can be analysed with various methods that are relatively easy to perform, accurate, reproducible and rapid. With yeasts, karyotyping of chromosomal DNA and PCR-based methods using appropriate primers have been effectively applied for biotyping and characterizing intraspecies diversity of strains [16, 17].

Conclusions

Molecular studies have had a great impact on the taxonomy of yeasts and over the last decade their classification has started to undergo major alterations. One of the most significant contributions has come from nDNA reassociations which have brought the precise delineation of several species, re-establishing a number of them while reducing others to synonyms. The other most significant contributions have been provided by sequencing of various rDNA regions which allowed to assess phylogenetic relationships at both lower and higher taxonomic levels. The data accumulated show already that restructuring to a large extent of current taxonomic schemes will be necessary. As a

consequence, however, numerous nomenclatural changes are inevitable, and this is a major obstacle in constructing a phylogenetically sound classification that also provides a reliable base for the identification.

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QUESTIONS OF CLASSIFICATION OF BASIDIAL FUNGI

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Surveying mycological works dealing with great number of species about basidial fungi published in the last hundred years (Kalchbrenner [1], Istvánffi [2], Hollós [3], Moesz [4, 5], Bohus et al. [6], Bánhegyi et al. [7], Ubrizsy [8], Bánhegyi et al. [9], Babos [10, 11], Rimóczi–Vetter [12], Rimóczi [13]) the great variety and changes of applied systems is conspicuous. In all works the doubt is expressed whether the currently applied systematic lists and nomenclatural solutions are the best, and whether they have chosen the most appropriate systematic theories.

Hungarian authors have never created an own system though it is conspicuous that their knowledge about foreign mycologist taxonomist's works of the given period is thorough. Critical usage of their elements and critical review in the own works is rare (Ubrizsy–Vörös [14], Ubrizsy [15]).

Aims

This paper aims to show systematic principles and nomenclatural changes applied in the foreign, principally European mycological works of the last decade. This paper is not intended to be complete. This is true even with considering only narrower circles of basidial fungi: *Hymenomycetes* and *Gasteromycetes*. This paper is intended to make recommendations only for works dealing with Hungarian macrofungi, and therefore will not address systematic and nomenclatural questions concerning *Uredinales* and *Ustilaginales*.

Discussion

More than half of macrofungal life of Hungary is unknown. The work must focus on identification of species, and on their role played in ecosystem. After all, answering the question "what is this" is the base of all morphological, physiological and cultivation-physiological works, and this basic question requires a systematic and nomenclatural

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approach. This approach is often formulated with difficulty in the level of species (Rimóczi [16]).

- Bresinsky [17] presents five phyla of fungi: *Acrasiomycota*, *Myxomycota*, *Plasmodiophoromycota*, *Oomycota*, *Eumycota*.
- Courtecuisse [18] includes fungi into four phyla: *Gymnomycota*, which includes slime molds. Phylum *Mastigomycota* includes oosporal fungi with flagellate spore and known generative reproduction, while phylum *Amastigomycota* includes all fungi the spores of which are not flagellate, and the generative reproduction of which is known (subphyla *Zygomycotina, Ascomycotina* and *Basidiomycotina*). The author classifies all other fungi with yet unknown generative reproduction into phylum *Deuteromycota*.
- Webster [19] separates only two phyla: *Myxomycota* and *Eumycota*.
- Ubrizsy and Vörös [14] divides fungi also into two phyla: *Myxophyta* and *Mycota*, but they include the greater part of slime molds into the latter as a subphylum (*Myxomycotina*). This opinion is still in use, because e.g. Folk–Glits [20] write: "Fungi belong to the phylum *Mycota*, and are divided into two subphyla: the one is subphylum *Myxomycota*, where class *Myxomycetes* belongs to; the other is subphylum *Eumycotina*, where classes *Phycomycetes, Ascomycetes, Basidiomycetes* and *Deuteromycetes* belong to".

It follows from the before mentioned that one taxon within basidial fungi (e.g. *Hymenomycetes*) can belong to more kinds of systematic categories. For instance by Kreisel [21] *Basidiomycetes* is a class, which includes three subclasses: subclass *Phragmobasidiomycetidae*, in which two orders by each other are *Auriculariales* and *Uredinales*. Subclass *Hymenomycetidae* includes eight orders, where *Poriales* includes the major part (16 families) of fungi with not lamellate fruiting body, e.g. *Peniohoraceae* and *Meruliaceae*, but *Thelephoraceae* and *Ganodermataceae*, too (i.e. extremely different morphological and life-mode types). Thereupon in order *Polyporales* only two families: *Polyporaceae* and *Schizophyllaceae* remain. Order *Cantharellales* includes 13 families (even families *Fistulinaceae* and *Bondarzewiaceae*), where *Hydnaceae* includes *Cantharellaceae*, too. Orders *Boletales, Russulales* and *Agaricales* are separate, in the latter families *Lepiotaceae* and *Agaricaceae* separates, similarly to the work of Bon [22] 21 years later.

At Kreisel [21] the third subclass is *Gasteromycetidae* with 11 orders, where *Lycoperdales* and *Geastrales* are separate. So Kreisel does not use the name *Holobasidiomycetidae* beside *Phragmobasidiomycetidae* neither later (Kreisel [23]).

Also by Bresinsky [17] *Basidiomycetes* is a class, in which beside subclass *Heterobasidiomycetidae* there is *Holobasidiomycetidae*, and he does not indicate *Gasteromycetidae*.

Within *Heterobasidiomycetidae Ustilaginales* and *Tilletiales* are superorders, where superorder *Exobasidiales* is in close relation with the latter. Bresinsky amalgamates *Uredinales* into superorder *Tremellanae*, because production of sprout cells is not present here, which in spite was more or less characteristic to the former superorders. At the same time he takes also *Dacrymycetales* here as a superorder, because though the long sterigmae – on the tip of which basidiospores develop – arise from undivided basidia, and basidiospores are able to bud.

Subclass *Homobasidiomycetidae* by Bresinsky, where dolipore on the transversal wall of hyphae is more complex in comparison with the former, includes *Lycoperdanae* and *Phallales* as superorders, i.e. all the puff-balls, beside superorders *Porianae* (syn. *Aphyllophoranae*) and *Agaricanae*.

Internal classification of puff-balls shows more similarity with the system of Kreisel [21]: genera *Geastrum* and *Lycoperdon* belong to separate orders, but Bresinsky considers the morphological and structural transition of the gastroid fruiting body, appearing in many different ways, towards the pileate (cap shaped)–stipitate (stalked) fruiting body, as one way development of basidiocarpium realizing in more different ways from the gymnocarp to the hemiangiocarp (*Agaricus – Endoptychum*) or angiocarp (*Entoloma – Richoniella*).

There is no exagerrated importance of separation by the morphology of the hymenophore in the orders of *Agaricanae* (e.g. lamellate families of *Boletales*). Since among puff-balls with gastroid fruiting body there are both lamellate gleba (*Galeropsis, Montagnea*), which can be originated from *Agaricales*, and porous (*Chamonixia*), which is linking to *Boletales*.

These puff-ball species, showing transitional (common) characteristics are included by Moser [24] into the families of *Boletales* and *Agaricales*. Jülich [25] also describes them within the same orders as monotypic genera, but as genera of separate families. So by him out of *Russulaceae Russulales* includes *Elasmomycetaceae,* too with four gastroid genera by Pegler–Young [26].

Although the system of Bresinsky is in complete harmony with newest results of cytology and physiology – and presumably it may stand closest to the phylogenetic reality –, it does not appear in any determinant newer mycological work.

Breitenbach and Kränzlin [27] take over certain elements from Jülich [25], but basically they follow Moser [24] and Singer [28].

Therefore here *Basidiomycetes* is a subphylum, and classes are *Heterobasidiomycetes* (syn. *Phragmobasidiomycetes*), *Holobasidiomycetes* and *Gasteromycetes*. The expression "*Hymenomycetes*" is not used, because they do not attribute such a taxonomical importance to basidium layes covering the surface as a continuous membrane, since e.g. on the surface of the lamella of a *Pluteus* species (*Agaricales*) basidia form a membrane (i.e. hymenium) just as on the lamella of a *Montagnea* (agaricoid *Gasteromycetes*), or on the wall of the gleba-chamber of a *Langermannia* (epigeic *Gasteromycetes*).

In contrast Arnolds et al. [29] mark *Hymenomycetes* and *Gasteromycetes* as classes. After Hawksworth et al. [30] they also consider *Basidiomycotina* as a subphylum.

The two subclasses, *Phragmobasidiomycetidae* and *Holobasidiomycetidae* are separated within the class *Hymenomycetes*. By Arnolds and his colleagues [29] admit the exaggerating character of these contractions with listing five groups within *Aphyllophorales* clearly on morphological basis, without mentioning taxonomic category: "Cantharelloide fungi", "Corticoide fungi", "Clavarioide fungi", "Hydnoide fungi" and "Poroide fungi".

Krieglsteiner [31] in his general work, declares clearly that he intentionally chose alphabetical list not only for lucidity, but he would like to avoid taxonomic classification

and taking a stand on "natural system" of basidial fungi. With this expression in quotation marks he fully reveals his opinion about this question.

Though Krieglsteiner mentions that his work is in accordance with systematic works of Moser [24], Jülich [25] and Kreisel et al. [23] in many aspects, he says "one should take this as his identifying with these systems, or as an opinion against other systems by no means".

Clemençon in his latest work elaborates the anatomy of *Hymenomycetes* through 996 pages (Clemençon [32]). His systematic opinion is very careful and reserved. According to him *Hymenomycetes* is a class with two orders: *Agaricales* and *Aphyllophorales*. He sharply separates *Gasteromycetes* from *Hymenomycetes* for two reasons: either because the inner mycelium, or because active spore scattering is absent in puff-balls, which he considers characteristic to *Hymenomycetes*.

On the topic of the formation of the system of basidial fungi producing hypogeic fruiting body, from the scientific achievement of Vittadini [33], about changing of their system and their present diversity the best summary is given by Montecchi and Lazzari [34].

Hungarian macrofungus taxonomic books either completely take the system of Moser [24] and Jülich [25] over Babosné [11] or in addition to these they they use the works of more other taxonomists to greater or lesser extent mainly for interior analysis of certain families (Rimóczi–Vetter [12], Rimóczi [35]).

In the textbook written for the agrarian higher education (Rimóczi [36]) I described the world of fungi following the system of Webster [19], though in the system of Bresinsky [17] characteristics being qualified as really phylogenetical (e.g. separation of *Oomycota* on the highest level) rather prevail. I have chosen the former for didactic reasons and with regard to aspects of practical use of the textbook.

Appearance of taxonomic and nomenclatural changes are continuous and regular in the level of families and genera of macrofungi, too. One part of these – more significant in Hungarian practice – have been summarized (Rimóczi [16]). A more recent summary of changes should be done in the immediate future.

Summary

The author presents the present state of the system of basidial fungi mainly through the taxonomic judgement of *Hymenomycetes* and puff-balls. The diversity of systematic approaches derives from the fact that certain authors assert data concerning phylogenetic characteristics of fungi and practical didactic relations of their works in the most different rate.

Authors dealing with the largest number of species definitely avoid systematic statements concerning higher taxa. Hungarian mycologists, dealing with macrofungi, can also do this, moreover the unexplored state of the Hungarian fungal world directly requires placing priority on species-level investigations.

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FUNGAL SYMBIOSES

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Demonstrating different types of biotrophy fungi are outstanding within eukaryotes. All types of symbioses sensu deBary [1] (parasitism, mutualism, amensalism, commensalism, neutralism, antagonism) and all possible transient forms of these are represented by them. Furtherly the word "symbiosis" will be used, as commonly, in the strict concept as mutualistic symbiosis.

It has been long debated on whether biotrophic or saprophytic forms could be the ancestors of terrestrial fungi. Evolutionary evidences show that both types existed already within the first fungi conquesting land in the same period as plants and animals. Finds of the first vascular plants (e.g. the ancient fern Rhynia) of the early Devonian period certify that VA-type mycorrhizal symbiosis had already been evolved up to that time [2] and spreading of plants provided further expansion for symbiotic endophytes. Production of large amounts of terrestrial organic debris (e.g. cellulose, lignin, keratin) must have been a great evolutionary challenge for saprophytic fungi at the same time. Therefore fungal life had to evolve parallelly with plant and animal evolution on land. It is coevolution of symbiotic fungi and their hosts which has created the now existing fungal symbioses. Understanding the diversity, structure and function of modern symbiotic relationships is only possible on the basis of their evolutionary history [3].

Symbioses of fungi are highly diverse. Coevolution with different groups of animals resulted in such strange relationships like the symbiosis between fungus-growing ants and their fungi [4], some truffles and truffle-flies or groups of hypogeous basidiomycetes (e.g. *Rhizopogon*) with some mammals (e.g. mice) [5] dispersing their spores by mycophagy. Thus, fungus-plant symbioses based on the mutualistic metabolic cooperation between the photobiont and mycobiont are of greater ecological importance than fungus-animal symbioses.

One of the main types of plant-fungus symbioses is lichenes, which are terrestrial thallophytes composed by fungi with algae. The about 14,000 lichen types are formed by 21 genera of green and blue algae with a wide variety of obligatory biotrophic fungi (mainly ascomycetes) [6]. Symbiotic partnership between algae and fungi are known also from marine environments involving some red algae and ascomycetes. A series of more or less strict to loose symbiotic associations existing between algae and fungi in the upper

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intertidal zone (the so-called "littoral lichenes") are more resistant io desiccation than algae alone.

The most widespread and diverse plant-fungus symbioses are mycorrhizae. At about 240,000 species, 90% of terrestrial plants (livermosses, ferns, gymnosperms and angiosperms) take part in mycorrhizal associations with more than 6,000 fungal species [7]. Enhancing fitness of both partners, mycorrhizal state can be regarded as the normal physiological condition of plants and fungi in natural environments.

Different morphological and functional forms of mycorrhizae with distinct fungus-host specificity have been evolved. The most widespread is the vesicular-arbuscular type (VAM) formed by about 130 species of the *Gomales* (*Zygomycota*) with about 80% of the plant species [8]. Ectomycorrhizal relationships involving mainly basidiomycetes are characteristic to gymnosperms and angiosperm trees in moderate climates [9]. Ericoid, arbutoid and orchid mycorrhizae are more specific than the former ones restricted to a few families of plants and a smaller group of fungal partners [10].

Symbiotic fungal associations are widespread and frequent on Earth. Tundra-vegetation dominated by lichens occupies about 8% of the continental area. Ubiquitous VAM-associations are of special importance in grasslands and tropical woods. Coniferous and deciduous forests of moderate climates are highly dominated by ectomycorrhizal associations. In the heatherlands of alpine and subarctic regions ericoid mycorrhizae play an important role in element and energy transfer [11]. The function of the common but up-to now weekly known so-called "dark septate endophytes" must also be significant in the northern temperate zone [12].

As the result of more than 400 million year's evolution fungal symbioses play a basic role in maintaining the balance of natural ecosystems in the biosphere [13].

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THE ROLE OF FUNGI IN THE CARBON- AND NITROGEN CYCLES

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The green plants fix a great amount of carbon (CO_2) and accumulate as different organic molecules mainly as lignocelluloses. Fungi belong not only to an independent group of Eukaryots but they have – in most of the cases – a specific biochemical, physiological ability to decompose organic molecules, to produce substrates of respiration and to mineralize these for the biological cycles of ecosystem.

Different groups of lower and higher fungi are able to degrade cellulose. Such genera of lower fungi are: *Trichoderma*, *Penicillium*, *Fusarium*, *Aspergillus*, etc.; the most powerful fungi are the so-called xylophageous or wood decaying fungi [1–3]. The classical mycological and biochemical investigations demonstrated the production of a synergistic, cellulase decomposing enzyme system. The lignin degrading ability is the property of different fungus taxa. Lignin degradation studies of white-rot fungi were performed extensively but basic questions remained problematic. The observations have led to the conclusion that the lignin degrading system is associated with the secondary metabolism of the fungi [4, 5]. This system consists of ligninase (lignin peroxidases), manganese peroxidase, phenoloxydase and peroxide producing enzymes. The lignin decomposition is regulated by various nutritional (N supply, Mn level) and ecological (pH, temperature) factors.

A lot of fungi have the required genetic information of the degrading enzyme system. After an adaptation time the following processes are going on: (1) uptake and utilization of soluble, free sugars, (2) increase of biomass, formation of new hypha- and mycelial system, starting of cellulose- and hemicellulose decay, (3) increase of secretion of organic acids, (4) increase of quantity and activities of intra- and mainly of extracellular oxidases, (5) cellulose and lignin decay are leading later to the complete decomposition (to mineralization) of lignocellulose substrates.

The ecological N-cycle is composed of different processes (nitrogen fixation, nitrification, nitrate-reduction, etc.). The fungi belong to the organisms of relative high N content (15–40% dry weight crude protein) but their common substrates contain low N level. There are three possibilities (6): a specific biological adaptation, a dynamic

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reutilization of nitrogen of older cells into the young mycelium or the utilization of N from different sources including the posibility of N fixation. The role of fungi in the N cycle is undoubtful, they can be important components of the soil-plant-animal chain

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THE RESEARCH OF LICHENIZED FUNGI IN HUNGARY

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A brief historical account on the Hungarian lichenology is given in three stages. The early stage involves the oldest collections and the lichenological activity of F. Hazslinszky. In the mid-stage a separate, independent lichen collection was established in the Natural History Museum, and a rapid development of floristical and taxonomic research took place. Recent lichenological research in Hungary follows several fields: taxonomy, biogeography, bioindication and ecophysiology.

The early stage of Hungarian lichenology

The first signs of the interest on lichens in Hungary are realized in the oldest herbarium specimens (*Cetraria islandica,* collected by H. J. N. Crantz in 1762, and *Cladonia rangiferina, Peltigera leucophlebia,* collected by J. J. Winterl in 1766 in the Alps) kept in the Hungarian Natural History Museum (Budapest). These collections were established in the era of the Swedish doctor, Erik Acharius (1757–1819), who is called "the father of lichenology", because he gave the basic methods for study and classification of lichens (Acharius [1]).

The academician, teacher and polymath Frigyes Hazslinszky [2] published the first detailed lichen flora of the Carpathian Basin based on the results of his own microscopic studies and field experience accummulated in ca 50 years. He also studied and reviewed the herbarium specimens of Lumnitzer, Rochel, Kitaibel, Sadler, Bothar, Jermy, Kalchbrenner, Borbás, and Lojka. Chemical characters and spot reactions were regarded unreliable by him, thus he followed the Körber's system as well as his own concepts. Together with the most valuable, earlier botanical collections (Crantz, Kitaibel, Sadler, Lumnitzer, Albach, Haynald, Lojka) Hazslinszky's lichens were also placed to the museum.

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The mid-stage of Hungarian lichenology

The progress of Hungarian lichenology cannot be separated from the history and development of the Hungarian Natural History Museum, Budapest (Rajczy and Buczkó [3]).

A separated, independent "Collectio Lichenum" within the Natural History Museum was founded only in 1909, several decades after Hazslinszky's work. The lichenological literature was relatively well concentrated here and microscopes indispensable for the scientific study were also available. Therefore the curators (Gy. Timkó, V. Gyelnik, Ö. Szatala) of the lichen herbarium were the first professional lichenologists in Hungary (for more details see Verseghy [4]). They could devote most of their time to the scientific investigation of lichens both in the field and in the herbarium. Their comparative, mainly morphological studies resulted in the description of several new species. Gyelnik (1906–1945) himself described ca 1,300 new taxa (among them 264 new species). From his descriptions it is clear that he applied the usual chemical spot reactions (K, I and C) regarding them of great importance in taxonomy. Szatala (1889–1958) published 583 new names (among them 73 new species). He prepared the second lichen flora of the Carpathian Basin (unfortunately only three parts were published because of his death).

The diversity of lichens is so high that recently ca 13,500 species is known world-wide and the number of possibly existing species is estimated as much higher (ca 20,000) (Hawksworth et al. [5]: 247). The main purpose of the first lichenologists was the description of these variable organisms. The Hungarian lichenologists followed the accummulated taxonomic and systematic knowledge of the world, corresponding and exchanging lichen specimens with the greatest specialists of their age (e.g. A. Zahlbruckner, E. A. Vainio, H. Magnusson).

Klára Verseghy (1930–) was the first curator of the lichen herbarium who did also, but more than floristics (Bakony, Balaton-felvidék, Hortobágy NP, Kiskunság NP, Őrség, Vendvidék, Villányi-hegység, Zempléni-hegység) and taxonomy (*Caloplaca*, *Gasparrinia*, *Ochrolechia*, *Squamaria*) before she wrote the synthesis of her 35 years in lichenology, The handbook of the lichen flora of Hungary [6], containing 715 species from Hungary. She participated in the research project of IBP. She measured the production of *Cladonia* species of Bugac, Csévharaszt (Kiskunság) in the 1970s. Together with Edit Láng she founded the Hungarian lichen ecophysiological research. Later on Verseghy led air pollution investigations in Budapest. Her students carried out lichen mapping and heavy metal measurements on transplanted lichens (Verseghy and Farkas [7], Farkas et al. [8]).

Ferenc Fóriss (1892–1977) became famous for his enormous collecting activity, almost 40,000 specimens over 25 years, 15,000 records of ca 1,400 taxa only from the Bükk Mts (unpublished). László Gallé's (1908–1980) greatest and also internationally important work is the lichen coenological system of Hungary (Gallé [9]), containing 47 associations. Anna Kiszely-Vámosi (1935–) made the lichen flora of Mátra and Bükk Mts better known. Péter Solymosi (1945–) made lichen floristical research in the Gerecse, Vértes and Buda Mts, along the Danube, and he also used lichens as bioindicators of air

pollution. Tamás Kiss (1953–) studying this problem in Szombathely and its surrounding area established a life strategy system for lichens in West Hungary.

Recent topics of lichenological research

The recent lichenological research is even more specialized than that of the 1970s and early 1980s, follows several research lines and has been carried out in various institutions: Institute of Ecology and Botany, Hungarian Academy of Sciences (Vácrátót), Department of Botany, Hungarian Natural History Museum (Budapest), Department of Plant Taxonomy and Ecology, Eötvös Loránd University (Budapest), Department of Botany and Plant Physiology, University of Agricultural Sciences (Gödöllő), Eszterházy Teachers' Training College (Eger), Aggtelek National Park.

Lichens as indicators of air pollution were applied in various investigations (mapping, SEM-EDXRA, photoregistration of permanent quadrats) at the Budapest agglomeration area and at the Pilis and Visegrád Mts (Farkas [10]). It was proved that lichens are sensitive orgarnisms to the quality of environmental conditions, especially to air pollution level and the corresponding meso- and microclimatic changes. The occurrence and distribution of *Lecanora conizaeoides* and several other toxitolerant and rare species were shown on ca 200 maps. For bioindication purposes the study of the gonidial layer was suggested for *Hypogymnia physodes* samples by SEM-EDXRA. The permanent quadrat investigations presented considerable differences between the percentage cover of lichen thalli (*H. physodes*) on moderately polluted and control sites.

Zoltán Tuba and Zsolt Csintalan applied lichen samples for the bioindication of air pollution from industrial sources and the traffic near agricultural areas.

Lichen mapping studies were continued later on Hungarian and European level (also as a part of the International Lichen Mapping Project). Farkas and Lőkös [11] prepared the European distribution maps of the terricolous *Cladonia magyarica*, a Pannonian endemism and a rare loess inhabiting lichen species, *Solorinella asteriscus*. Half of the species involved in the ILM Project were extinct from the Hungarian flora, other data documented, that distribution patterns of lichens in Hungary is insufficiently known. Also these results inspired the preparing of a preliminary list of rare and endangered lichens of Hungary (Lőkös and Tóth [12]). Surveys on the flora and fauna of the Hungarian National Parks (e.g. Kiskunság, Bükk, Aggtelek) also included lichen inventories carried out by Lőkös since 1985. From these and several additional, smaller areas approx. 50 lichen species new for Hungary were detected in the last decade. On the basis of the old and new distribution patterns of the species, the amount and trend of the air pollution can be considered. A computerized database system for the present ca 30,000 records and a mapping application for the distribution analyses were organized, elaborated and managed in the Hungarian Natural History Museum.

The maintenance of the tropical biodiversity is essential for the future of the biosphere. Tropical lichenological research is a little contribution to this problem. The extended field work of Tamás Pócs, Attila Borhidi and partly by Edit Farkas resulted in collections of tropical (mainly Tanzanian and Cuban), mainly leaf-inhabiting lichens. New

species of epiphyllous lichens were described from these collections by Edit Farkas and Antonín Vezda. The bibliography and checklist of foliicolous lichens were compiled (Farkas [13, 14]) and regularly updated (Farkas and Sipman [15, 16]). The first international IAB and IAL *Symposium on foliicolous cryptogams* (Eger, Hungary, 1995) was initiated and organised by Edit Farkas (Farkas and Pócs [17]). It served the better understanding of the natural ecosystems in the tropical rainforests.

In 1994 a complex biodiversity study started on corticolous and foliicolous representatives of both Hungarian and tropical *Bacidia s. str.* and related crustose genera (Farkas et al. [18]). HPTLC is introduced for chemical analysis of *Bacidia* species.

The research line of Klára Verseghy and Edit Láng studying various aspects (drought-tolerance, light-shade conditions, etc.) of autecology and ecophysiology of lowland *Cladonia* species is continued by Katalin Mázsa (Verseghy et al. [19]) and Zoltán Tuba and their colleagues. Recently a new field of science, synphysiology was outlined by Tuba et al. [20] using also lichens in the experimental basis for the arguments.

An increasing worldwide interest in lichenology is reflected in the number of participants of the symposia of the International Association for Lichenology: IAL Symposium of Tropical Lichenology, London (1989) – ca 60 participants, IAL 2, Båstad (1992) – ca 230 participants, IAL 3, Salzburg (1996) – ca 350 participants.

Fortunately more and more students become interested in the study of lichens also in Hungary, since there is a lot more to learn about lichens.

Lichen substances are analyzed routinely in several institutions of the world, even new substances are described every year. In Hungary HPTLC is introduced recently for chemical analysis of only a few lichen substances (Farkas et al. [18]).

There are successful lichen culture experiments in some institutes in the United States and Europe. No similar experiments exist in Hungary.

The first seminar on molecular studies of lichens was held in Graz between 11–15 August, 1998. In Hungary no molecular research of lichens has been started.

Lichenology in Hungary is still in a descriptive phase. As in the past the lichenologists take great efforts in following the results of the world's lichenologists and only in rare minutes are at least at the international level. A continuous activity is necessary and a hope for increasing possibilities of the forthcoming lichenologist generations.

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FROM EDIBLE TO USEFUL MUSHROOMS – AN ATTEMPT FOR THE NEW ECONOMICAL ASSESSMENT OF LARGE FUNGI

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According to the stand of the modern applied mycological research the most commonly used term "edible mushroom" does not express all significant aspects large fungi can be used for. Additionally to bioconversion for food and animal feed production there are at least three other fields where large fungi may also get economical relevance: for establishing of ectomycorrhiza, for medical application and for soil decontamination including environmental engineering. This new situation justifies the introduction of a new, all-embracing designation for large fungi. The term "useful mushroom" will be suggested. The various options of the use of mushrooms will be introduced and briefly discussed in this paper.

Introduction

Edible mushroom cultivation has greatly increased worldwide in the last 15 years. Not only has the production volume risen dramatically, but the focus of edible mushroom cultivation has altered as far as range is concerned. Also noteworthy is the geographical shift in production from the West (Europe, North America) to the East (East Asia). At the same time the significance of the white mushroom *(Agaricus bisporus)* has declined appreciably.

The value of worldwide edible mushroom sales was some $ 9.8 billion in 1994 [6]. However, we can still expect the consumption and production of edible mushrooms to grow further in the future. Extensive, as yet untapped consumer potential exists in the eastern central European countries, the Baltic states, Russia, as well as in the Arab nations, Africa and Latin America. The cultivation and consumption of mushrooms is set to rise continually in these regions. The reasons for the expansion in production will differ, however, from country to country. But they can be summarized as follows

– Worldwide general change in eating habits
– Consumers' desire for higher-grade foods as a consequence of higher standards of living

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- In some parts of the world, the necessity to ensure an adequate supply of food for the population
- The variety of available options for edible mushroom cultivation and the exploitation of cultivation methods matched to practically all conditions
- Efforts to obtain foreign currency by exporting mushrooms and/or mushroom products

We could thus be content and simply await future developments. On the other hand, the outlook for mushroom growers in the industrialized nations is gloomy. The prospects for creating wealth are restricted by the problems of general economic conditions in the European Union (increasing costs, stagnating prices, more stringent regulations and many other factors), predatory competition in the EC and competitive pressure from eastern central Europe and Asia.

It is therefore an appropriate time to take stock of mushroom cultivation and the opportunities it offers and to seek ways in which the industry can succeed by way of crisis management. The practices of mushroom cultivation and, of course, research, have to be redefined. We must carefully think about the feasible prospects of the sector and provide researchers with fresh impetus.

In this paper we would like to engender and to continue a clarifying discussion involving as many interested parties as possible.

Definition of "useful mushroom"

The first thing to do is to demarcate the principal area of interest and describe the object of discussion: the mushroom or mushrooms.

Throughout the world mushroom cultivation involves only large mushrooms; that is to say, those we commonly call "mushrooms", also referred to as macromycetes and macrofungi. The framework in which mushroom growers operate is determined merely by the size of the mushrooms, not by their type or any other criteria.

The large mushrooms cultivated by mushroom growers worldwide are defined as follows:

The mushroom is a macrofungus with a distinctive fruiting body and large enough to be seen with the naked eye and to be picked by hand [7].

Chang [5] extends this definition as follows: Thus, mushrooms need not be Basidiomycetes, nor aerial, nor fleshy, nor edible. Mushrooms can be *Ascomycetes*, growing underground, having a non-fleshy texture and need not be edible.

According to Chang, therefore, the macrofungus category does not constitute a systematic entity. Rather, the definition crosses boundaries, while complying with certain morphological characteristics. Moreover, it focuses on the objective of economic utilization. On the basis of his definition, Chang [5] forms four categories of macrofungus:

- Those, which are fleshy and good to eat: edible mushrooms (e.g. *Agaricus bisporus*)

– Those, which can be used in medicine: medicinal mushrooms (e.g. *Ganoderma lucidum*)
– Those which are poisonous (e.g. *Amanita phalloides*)
– Those whose attributes are as yet entirely or largely undefined

Chang's definition of large mushrooms is acceptable and can therefore be used as the basis for further observations. His categorization into four groups is, however, not very useful. It completely ignores some important aspects and instead relates specifically to the situation in East Asia and the demands made on large mushrooms in this region.

To do justice to the outstanding economic role they play in the industrialized nations, we have to take a more differentiated look at large mushrooms and describe them more comprehensively. To this end, however, we must detach ourselves as much as possible from existing notions and also turn our attention to new unconventional fields of application. Without doubt, the way for mushroom cultivators in the industrialized nations to overcome the present crisis lies in the answer to the following question: For what purposes other than consumption could large mushrooms be produced and utilized?

The exploitation of additional applications justifies the introduction of a new, all-embracing designation. My suggestion is based on a familiar and generally recognized technical term in the fields of botany and zoology. Plants and animals of which man makes various use in agriculture, forestry and other sectors of the economy are called "productive" or "useful". In line with this designation, it would be purposeful to introduce a definition of useful mushroom(s):

Useful mushrooms are thus all large mushrooms used by man wholly or in part (mycelium, fruiting body or both); that is to say, all cultivated mushrooms and wild mushrooms of economic use.

The term most commonly used until now, edible mushroom, is restrictive and incomplete, even if many large mushrooms are produced primarily for human consumption. Eating mushrooms is, however, only one of several significant potential uses. Depending on the species, they can also be used in medicine, environmental engineering and ecosystem protection. "Useful mushroom" thus opens up new dimensions. We must investigate these before we can give answers to compelling questions of the future.

Worldwide potential applications of large mushrooms

At present there are four relevant options for using large mushrooms:

1. *Bioconversion for food and animal feed production*

The most significant characteristic of mushrooms is their ability to convert the enormous quantities of cellulose and lignin that make up the bulk of the vegetable biomass. This attribute is used worldwide to produce a mushroom biomass with a high physiological food value from agricultural and woodland waste that is often unsuitable for further use. Concerning the food value the most important substances in mushroom

biomass are amino acids (also essential amino acids), carbohydrates, vitamins (thiamin, riboflavin, niacin, folic acid, pantothenic acid, calciferol) as well as minerals (potassium, phosphor, iron).

It is worth to take a close look at this process. Plant growing produces food by deriving organic material from inorganic resources. In livestock farming, high-grade biomass is converted from one form into another with an input:output ratio of at least 3:1, even with modern production methods. In addition, both activities give rise to large quantities of waste. Mushroom cultivation is exceptional, however, in that it produces high quality food by degrading organic materials. Mushroom cultivators use and recycle the waste and residual materials generated by crop and livestock farmers. They contribute a great deal to waste avoidance and disposal.

At present mushrooms are still produced mostly for human consumption in both the western and the eastern hemispheres. It is therefore unnecessary to describe this method of use in greater detail here.

The use of large mushrooms to produce animal feed, on the other hand, has to be explained briefly. The ability of some large mushrooms (white rot fungi) to degrade lignin in lignocellulosic material can directly and substantially enhance the value of the plant as a source of dietary energy. The partial breakdown of the lignin component by the mushrooms allows the intestinal flora to make better use of cellulose. Ruminants in particular can benefit from this advantage [9, 19, 33, 34, 35].

2. *Establishing of mycorrhiza*

The symbiotic relationship between mushrooms and higher plant forms is referred to worldwide as *mycorrhiza* (mushroom root) after Frank [12]. Its most common form, which occurs on trees in the forests of central Europe, is ectotrophic mycorrhiza. From a morphological viewpoint, it is regarded as a unified organ [12].

Ectotrophic mycorrhiza result primarily from basidiomycetes and some ascomycetes. Among the ectotrophic mycorrhizal fungi are well-known forest mushrooms and some highly valued edible mushrooms, such as truffles, *Boletus edulis* and chanterelles. Most of the capable mycorrhizal fungi, however, are of no value from the layman's viewpoint and largely unknown: *Paxillus involutus, Scleroderma vulgaris* (common earth ball), *Pisolithus tinctorius* and others.

The physiological benefits of the symbiosis between tree and mushroom are very important for the tree. It allows the tree to transpire if the ground is dry and promotes more efficient photosynthesis. The hyphae of the mushroom colonize the soil much faster than the tree's roots. This can increase the nutrient and water-absorbing surface area as much as 1,000-fold and also enlarge the exploited soil volume. The absorption of soil nutrients, including those that are present in a form that plants cannot exploit, is intensified. The mycorrhizal fungi produce enzymes and organic acids, which increase the solubility of the minerals in the soil. In addition, the mushrooms keep company with nitrogen-fixing anaerobic bacteria. The supply of nitrogen to the tree is ensured by this "hypersymbiosis" and the absorption of mineral nitrogen [10, 15, 20, 23, 27, 30, 31].

The mycorrhizal fungus gives the tree very substantial protection by diverse mechanisms [4, 22, 26, 29, 36]. It also has a positive effect on the roots' environment, the mycorrhizosphere, which is distinct from the rhizosphere. The mycorrhizosphere has been

found to contain more water-resistant soil aggregates, which increase the soil's ability to retain water and improve its aeration [2].

We are now aware of the scientific and technical preconditions for the specific application of mycorrhizal fungi in forestry. We anticipate the worldwide onset of commercial, large-scale production of a mycorrhizal fungal inoculum. According to Becker [1], this inoculum could give rise to a considerable market in Germany. Assuming 10 million hectares of woodland in Germany, an average rotational period of 100 years and 50% artificial regeneration, and given an average of 5,000 plants per hectare, the annual demand is 250 million plants. If each of these plants is worth DM 1 on average and the additional cost of producing mycodendrons from them is 10%, this use of large mushrooms could generate annual turnover of some DM 25 million in Germany alone [1].

3. *Medicinal applications*

Some of the early botanists (Hieronymus Bock, Adamus Lonicerus, Peter Melius) who wrote books on herbs which were well-known in Europe in the 16th and 17th centuries recommended large mushrooms for medical applications. Some of these, such as the paste produced from the spores of the *Lycoperdon* genus of fungi, are still mentioned today in homeopathic literature. Measured against the significance of medicinal mushrooms in East Asia, however, their use in the West has been very modest.

In East Asia some large mushrooms have been valued for centuries as effective remedies. During the Ming Dynasty (1368–1644), Whu Shui was already praising shiitake (*Lentinula edodes*) as an elixir of life that cured colds, stimulated the circulation and built up stamina. Various preparations derived from the jew's-ear (*Auricularia polytricha)* have been recommended to treat weakness after childbirth, blocked blood vessels and numbness.

The tonic qualities of mushrooms are still highly regarded in East Asia [14]. In China, Japan and Korea, for example, the current annual sales of drugs made from *Lentinus edodes, Schizophyllum commune, Coriolus versicolor* and others are estimated at $ 3.6 billion [6].

The objective of traditional chinese medicine (TCM) is to support and promote positive factors in the patient and to strengthen the body's immune system in order to prevent disease. Besides, other activities, research into and testing of new antitumor drugs follow this basic principle. The aim is to find biological response modifiers (BRM), substances that promote the positive factors and eliminate the negative factors from the human body. These include interferon, interleukin-2 and lentinan. In addition to surgery, chemotherapy and radiotherapy, BRM have become the fourth principal form of conventional cancer treatment [32].

Several experts believe that the medical fraternity could regard large mushrooms much more favorably in Germany in the future [11, 21, 24, 25, 28]. The natural remedy industry has already started to take an interest in large mushrooms, and customers react very positively to the notion that many large mushrooms could be used as natural remedies.

The further development of this application could therefore spawn a market for medical mushrooms in Germany in the foreseeable future.

4. *Soil decontamination, environmental engineering*

Bumpus and Aust [3] and other authors stated that *Phanerochaeta chrysosporium*, a white rot fungus, is able to break down a large number of structurally different organic substances. These include many pollutants that are difficult to degrade. They concluded that this singular biodegrading capability was linked to the mushroom's lignin-degrading system and was unspecific and extracellular in nature. In laboratory experiments, Bumpus and Aust [3] succeeded in breaking down several aromatic compounds (containing a benzene skeleton), such as vanillic acid, and even lindane and DDT.

As far as quantity is concerned, the principal naturally occurring substance that consists mainly of benzene rings is lignin. It is a component of nearly all parts of plants; wood, for instance, contains an average of 30% lignin. As stated above, the enzymes of white rot fungi enable them to decompose and mineralize lignin in particular. The enzymes are highly non-specific and effective with practically all aromatic compounds [16].

Experiments concerning the practical use of this ability of white rot fungi – to decompose hazardous aromatic compounds in the soil and air – have focused primarily on the easily cultivated oyster mushroom (*Pleurotus ostreatus*). First of all, an active mushroom substrate that remains stable for a long time was produced from cereal straw.

After colonization by the oyster mushroom mycelium, the straw is mixed with contaminated soil according to a certain volume content. The ecophysiological parameters are subsequently controlled so that the mushroom mycelium remains active for as long as possible. The hazardous substances in the soil are then decomposed together with the straw substrate. Contaminated air passed through a layer of this type of substrate can also be substantially cleaned if the rate of flow and layer thickness are matched to one another [16–18].

Although very limited research has been conducted so far into this potential application, which is highly topical from an environmental policy viewpoint, large-scale commercial projects have already been implemented in Germany. Noteworthy in this context is an installation near Hamburg, where several thousand tons of tar-contaminated soil have been cleaned. Here again, the mycelium of the oyster mushroom that was used had been cultivated on cereal straw. Several hundred tons of oyster mushroom substrate was used. The average contamination of more than 180 mg PCAH (polycyclic aromatic hydrocarbons) per kilogram dry soil was reduced to below 40 mg within 16 months.

The consequences for research and practice

Both researchers and practitioners have to respond to the new outlook on mushroom cultivation as described above. At the same time, all the energies and capacities of the large mushroom research sector must be brought together. Many species of mushroom have two or even three potential applications (food production and naturopathy or food production, environmental engineering and animal feed production). Moreover, many similarities and overlaps exist as regards the laboratory and research work into the various uses of large mushrooms. Many years of experience have shown

that close cooperation between those working in research into useful mushrooms, notwithstanding their diverse objectives, is very stimulating and beneficial to all parties.

Scientists must also learn to fill in the considerable gaps that exist in the general knowledge of large mushrooms and mycology in general; the public, politicians, public authorities and association officials all have to be educated. It is a telling observation that most people do not have any notion of what a "medicinal mushroom" is. Only very few spontaneously make the rather obvious – association with medicinal plants. Most people think that mushrooms are either edible or poisonous and otherwise uninteresting.

Mycologists themselves are, regrettably, partly to blame for this lack of understanding. Thus far they have been unable to distinguish themselves from botanists and establish mycology as an independent field. Despite the abundance of pertinent facts, they have even failed to assert the overdue distinction between plants and mushrooms and have not constituted a discipline dedicated exclusively to mushrooms. This diffuse approach has caused people to classify edible mushrooms as vegetables and to teach production methods as a subject for vegetable growers, if at all. Misleading concepts such as "plant protection in mushroom cultivation" have been coined in this context. This lack of regard for mycology probably explains the general unwillingness of foresters to accept that symbiosis can exist between the fragile mushroom and the mighty tree

The scientific reputation and evaluation of large mushroom research is still so slight in Germany as well as in Hungary that not a single chair or principal scientific post exists in the universities and specialist colleges of further education. At the same time, several professorships for Tibetan Studies have been created at German universities. A dramatic change has to be accomplished on a broad front as soon as possible.

The situation outlined above foreshadows far-reaching consequences for practical mushroom cultivation. Growers will have to be far more flexible, prepared to cultivate several mushroom species and able to adjust the focus of their activities according to changing situations. Process engineers are required to standardize cultivation methods as far as possible and to elaborate unified methods for different species. This will make it easier for cultivators to switch production from one species to another or to grow several species at the same time, depending on demand.

The scope embraced by the term "mushroom cultivation" must be broadened in the future. Mushroom growers will no longer be producing only edible mushrooms. Since the potential applications of large mushrooms are not restricted to their fruiting bodies, but also generate demand for large quantities of substrate colonized by mycelia, for the purposes of soil decontamination or the establishment of mycorrhiza, for example, some enterprises could exist alongside fruiting body growers and produce "only" mycelia.

Well-established structures will inevitably change and the industry will have to set itself new objectives and goals. As well as exploiting its opportunities in the food sector, it will have to develop and utilize the various options available to large mushrooms in various other branches of economic activity.

In addition, the mushroom industry must step up its commitment to research with more financial investment or by subsidizing the work of research institutes. The utilization of useful mushrooms in the new fields mentioned above has so far been hampered or held back principally by a dearth of scientific groundwork. If a lack of support causes this situation to persist, the mushroom industry will be doing itself a disservice.

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PRODUCTION OF BIOACTIVE COMPOUNDS BY DIFFERENT FUNGAL SPECIES

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Production of cholesterol-lowering drugs

Nowadays in Hungary and all over the world there is an increasing demand on cholesterol-lowering drugs. Highest risk factor of atherosclerosis and especially coronary occlusion is the high cholesterol level of the plasma. In the recent two decades the 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC.1.1.1.34) as the rate limiting key enzyme of the cholesterol biosynthesis in the organism was extensively examined. Mevinolin and other related compounds (compactin, pravastatin, simvastatin) are the competitive inhibitors of the HMG-CoA reductase enzyme. Our investigations were focussed on finding microorganisms, which would produce HMG-CoA reductase enzyme inhibitors. During screening, covering about 20,000 fungus strains two microorganisms were selected, one of them an *Aspergillus* species was able to biosynthesize mevinolin and another strain belongs to the *Penicillium* genus was able to produce compactin. In the course of our experiments new, economic microbial processes were developed for both cholesterol-lowering agents.

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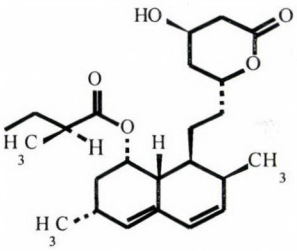
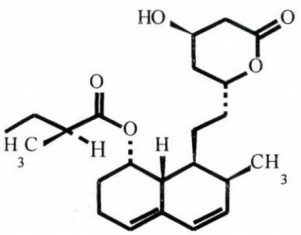
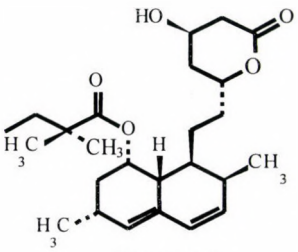


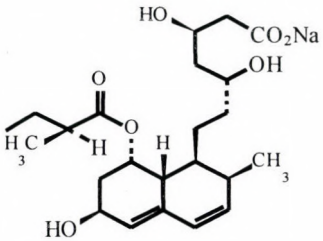
Lovastatin
(MK-803,Mevinolin, MEVACOR)



Mevastatin
(ML-236B, CS-500, Compactin)



Simvastatin
(MK-733, Synvinolin, ZOCOR)



Pravastatin
(CS-514, SQ-31000, Eptastatin)

Production of ergot alkaloids

Ergot is the sclerotial form of parasitic fungi of the genus *Claviceps* growing on wild grasses and cereals. The ergot alkaloids are the secondary metabolites of these fungi.

Claviceps purpurea was responsible for the deaths of thousands of people in Middle Ages, in France and Germany which were the rye-bread eating region of Europe but in the 20th century it has become the source of potent medicines *per se*, and many of their semisynthetic derivates.

The life cycle of *Claviceps* in nature is complex. It consists of an asexual and a sexual phase.

The first consists of a filamentous mycelium that reproduces through conidia. The second starts with formation of a sclerotium. The sclerotium can be considered as the early stage of sexual differentiation of the fungi. In the spring the ergot, while surviving in the soil, has become activated by frost and it germinates with stalked stromata. In the round head of stromata the perithecias can be found in which the asci and the ascospores are visible. The thread-like ascospores infect the flower of rye or host plant.

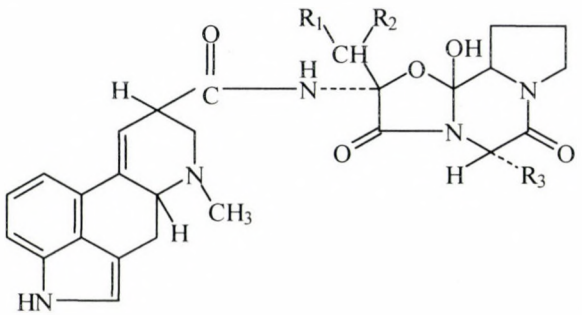
In nature, ergot alkaloids are formed only in sclerotia which contain about 30% lipid and 0.01–0.10% alkaloids. Ergometrine is soluble in water but the so-called peptide alkaloids are soluble only in lipids.

The research on the production of alkaloids in saprophytic conditions took into account and tried to obtain cells *in vitro* with characteristics similar to those of sclerotia.

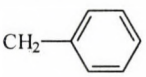
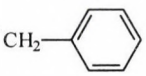
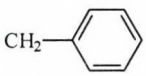
Under saprophytic conditions three phases of growth could be distinguished: colony growth on solid media which could be started from conidia, ascospore or sterile fragment of sclerotium growth of vegetative phase mycelium in liquid media. The last

phase: the ergot alkaloid synthesis in higher amounts is obviously after about 14 days of cultivation in the production media and is accompanied by the typical differentiation of filamentous mycelium into sclerotial tissue like cells with high lipid content. Differentiation toward sclerotia-like cells can be achieved in different ways: using high concentrations of different substrates, after nutrient exhaustion, in presence of osmotic stabilizers or after $NaNO_3$ addition. Besides specific morphological and ultrastructural differentiation, the essential biochemical features are crucial for alkaloid synthesis.

The majority of high-yielding strains were obtained by classical methods of mutation and selection performed either by UV and γ-irradiation or by a wide variety of chemical mutagens. Very successful programs were carried out in successive steps by using various mutagens. The protoplast fusion technique has begun to find useful applications either in producing improved mutant strains by intraspecies crosses or in formation of a novel spectrum of products by interspecies hybrids.



Despite the fact that biosynthesis of ergot alkaloids is a genetically regulated process, genetic studies of the *Claviceps* fungi have received limited attention compared to genetic studies of other industrially important microorganisms. This is probably due to the complex life cycle of *Claviceps* species and their genetic structure, which makes manipulation difficult.

Name	R_1	R_2	R_3	Amino acid sequence of the tripeptide
Ergotamine	H	H		L-Proline-L-Alanine-L-Phenylalanine
Ergosine	H	H	$CH_2CH(CH_3)_2$	L-Proline-L-Alanine-L-Leucine
Ergocristine	CH_3	CH_3		L-Proline-L-Valine-L-Phenylalanine
α-Ergokryptine	CH_3	CH_3	$CH_2CH(CH_3)_2$	L-Proline-L-Valine-L-Leucine
β-Ergokryptine	CH_3	CH_3	$CH(CH_3)CH_2CH_3$	L-Proline-L-Valine-L-Isoleucine
Ergocornine	CH_3	CH_3	$CH(CH_3)_2$	L-Proline-L-Valine-L-Valine
Ergostine	H	CH_3		L-Proline-l-α-aminobutyric acid-L-Phenylalanine

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HYGIENIC MYCOLOGY IN HUNGARY

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In Hungary the medical mycological research concerning the systemic (so-called deep) mycoses started in the National Institute of Hygiene. The Mycology Department has been founded in 1955 in this Institute with nation-wide authority as a routine diagnostic laboratory for clinical specimens from systemic mycotic infections. Three years later its activity was broadened – on ground of the experiences – to cover the total field of hygiene, thus we had to establish the "mycologia hygienica" (hygienic mycology), however, this has not been accepted as an independent discipline. Here a short, selected overview is given of our experiences.

In the following the trends of the activity is demonstrated along the subdivisions of hygiene (epidemiology, environmental-hygiene, professional and occupational-, nutritional and food hygiene). In respect of hygiene, however, the fungi play a Janus-faced (more exactly Dr. Jekyll–Mr. Hyde) role, as they can behave not only harmful but also beneficial.

Epidemiology

Fungi may induce fungal lesions and diseases. Among them are allergies caused by the more than 100 spp. allergenic fungi. Both alimentary and respiratory allergies are frequent in the Hungarian population, especially in children (the bronchial asthma will be discussed later in environment hygiene). Monitoring of the patients with qualitative and quantitative IgE test (RAST and PRIST techniques) were done. *Alternaria* spp. and *Cladosporium* spp. proved to be the most frequent allergenic molds. Out of the previous the *A. alternata* species, however, showed allergen relationship to an other black mold *Phoma betae.*

The problematic of toxicoses (mycetismus; as well as the mold mycotoxicoses) caused by the more than 300 spp. of toxinogenic fungi belong to Alimentation and Food Hygiene (see below).

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The most important human fungal harms are the tissue invasions caused by the more than 200 spp. of invasive fungi (dermato-mycological problems will be touched also in an other review). Clinical specimens from suspected "deep" mycosis or colonization (with opportunistic pathogens) were studied not only with classic laboratory diagnostic and identification techniques (direct microscopy of smears and tissue sections stained with various methods, as well as cultivation on general, selecting and differentiating media), but also with modern serologic-immunologic methods (Radial Immunodiffusion, Counter Immunoelectrophoresis, latex-agglutination, PRIST, RAST, ELISA), while new methods were also developed and introduced (India Ink Immuno Reaction and Enzyme Linked Immuno Sorbent Stain).

In the spectrum of the pathogenic- and colonizing agents (opportunistic pathogens) the prevalence of yeasts and yeast-like fungi was observed and *Candida albicans* proved to be the most frequent although *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were also found in some instances.

Molds and dimorphic fungi, however, were much rare but some interesting cases were also found: *Emericella nidulans* (*Aspergillus nidulellus* sexual reproduction form) in a postoperative lung infection (first report in the literature on a cleistothecial aspergillome), *Acremonium strictum* meningitis, *Coccidioides immitis* arthritis, *Histoplasma capsulatum* generalized infections.

Dermatophytes. This group and the fungal skin diseases were not dealt with in the basic routine studies of the Department. However, some restricted data were collected (e.g. screening on foot dermatophytoses and dermal yeast infections). Last but not least we succeeded to induce budding of *Trichophyton mentagrophytes* in mouse peritoneum after cystein-cultivation, thus simulating the morphogenesis of true dimorphs (and other soil inhabitant geophilic Gymnoascaceae pathogens).

The source of infection is one of the most important factor in epidemiology. In addition to diseased and/or carrier humans and/or animals soil is a real source of infection as it serves as a biotope for many (geophilic) fungal pathogens, contradicting the classic theses of epidemiology (based on bacteriology and virology) which negate the role of soil as being a source of pathogens. Beyond the dimorphic fungi and obligate and potential pathogenic molds, dermatophytes are also inhabitants of soil and it succeeded to isolate from Hungarian – and Vietnam – soils all the worldwide known species of geophilic dermatophytes, moreover many in the teleomorphic (sexually reproductive) state. While the most frequent pathogenic yeast *Candida albicans* was found to survive outside the host only for a restricted period, it cannot be regarded as a real soil inhabitant and at worst may persist in soil within pathologic materials.

Host susceptibility and resistance have also a major role in fungal diseases. Natural (hereditary) susceptibility: only the observed definite nephrotropism and hepatotropism in experimental mice candidiosis is mentioned here. However, regarding human pregnancy, in contrast to the earlier epidemiologic and gynecologic belief the highest incidence of vaginal colonization gets not to the time of parthus but to the second trimester of pregnancy. Nosocomial (acquired) susceptibility is well illustrated e.g. by the significance of oral thrush in newborns and infants. Puerperal mothers' antibacterial, antibiotic and/or iron (Fe) treatment significantly increased the frequency of *C. albicans* mouth colonization and thrush in their newborn.

A new form of "kidney-therapy", the Continuous Ambulant Peritoneal Dialysis (CAPD), gives also an opportunity for nosocomial fungal infections: peritonitis with various mold and yeast ethiology (only in 1989 15 *Aspergillus niger* cases were diagnosed). Sometimes prior of use the fresh dialyzing fluids were found to be contaminated by fungi (because of visible and invisible damages of the sack).

Growth of the pathogen. In the "antibiotic era" an increased incidence of systemic mycoses were observed in the treated patients, thus a direct growth promoting effect of the antibiotics on pathogenic fungi was supposed, moreover observed in (inadequate) experiments. With adequate studies no *in vitro* growth stimulation could be confirmed. While *in vivo*, in experimental candidosis in mice, some antibiotics (chloramphenicol, erythromycin and rovamycin) proved to be aggravating, also with an induction of earlier hematogeneous dissemination. Some others, however, showed a pseudo-curative (pseudo-therapeutic) effect (anti-tuberculotics: streptomycin, viomycin, and INH). Explanation of this protection lays in the induction of intraperitoneal coalescence at the site of administration (thus fixing the pathogen and help to the defense). Other tuberculostatics: pyrazin amide, cycloserin, and p-amino salicylic acid proved to be indifferent.

Referring to antifungal substances both efficiency and mode of action were studied. Out of the preservatives used in foodstuffs and pharmaceutics sorbic acid could only temporally inhibit some of the studied yeasts (*C. alb.*, *C. claussenii*, *C. kefyr*, *C. tropicalis* and *C. utilis*), because of the aerobic degradation (and even assimilation) of it by these species. While the sometimes weak antifungal effect of p-oxybensoic acid methyl- and propyl esters (Nipagin and Nipasol) was due to their protein binding. From disinfectants a comparison was made of 14 commercial products in anti-*C. alb.* efficiency (killing 99% of the cells within 2 min.). The weak, if any, effect of the hexachlorophen containing compositions, Ritosept and Solutio hexachloropheni, originated from their alkali or ethanol content, respectively. A cleansing combination type the "lysoform" (liquor formaldehydi saponatus; containing also ethanol, and potassium-soap) leaving an assimilable residue on the disinfected object makes it with time invadeable for fungi. An easy termination of the effect of the cetyl pyridinium bromide containing preparations (Nitrogenol and Sterogenol) was found in their inactivation by zinc ions. By the combination of low (0.2 M, non-fungicidal and less corrosive) concentration of hydrogen peroxide with low (slightly fungicidic and less toxic) concentration of formaldehyde an efficient disinfectant was achieved. It is regrettable that the best antifungal preservation and disinfectant compound pentachlorophenol had to be disclosed because of its high mammalian toxicity. However, it was earlier used with the best efficiency against molding of painted or paper covered wall surfaces and floor carpets. For the last aim only Eulan Asept P was also a convenient impregnation compound. While PCP was tried to be introduced into disinfecting wash-detergent combinations, its sensitivity to the type of the detergent compound and other additives (viz. emulsifiers) could be observed.

With antifungal chemotherapeutics and polyene antibiotics the efficiency (i.e. the susceptibility against these medicines of the isolated pathogens) are regularly checked because of the natural and induced resistance of the fungal strains. Because of the constitutive and acquired resistance of the pathogenic fungi towards the antifungal drugs (azole derivatives and polyenic antibiotics) the study of the mechanism of action of the later is not irrelevant. Regarding the antifungal antibiotics – the polyenes – in contrast to

the widespread belief on their common mechanism of action: i.e. complexing with ergosterol and inducing transverse physical channels in the plasma membrane of the fungal cells leading to loss of K^+ by passive efflux etc., a more complicated mechanism of action was rendered probable based on the studies of ion movements, amino acid transport and comparison with real membrane damaging agents.

Beneficial roles of fungi in medicine are played in their direct and indirect (recombinant DNA techniques) biotechnological utilization as producers of antibiotics, vitamins, and hormones. In this field for supporting the checking of the first two phases (physical fusion of membranes, and plasmas) in the protoplast fusion technique an India-Ink Immune Reaction and an Enzyme Linked Immuno Sorbent Stain was developed.

Environmental hygiene

Fungal contamination of soil, surface waters, air, inside surface of flat border walls (panel dwelling estates), stored raw materials, intermediary and end products, objects, etc., was extensively studied. In air-hygiene the fungal pollution over 500 particles/m^3 is considered to be a physical overload of the lungs in addition to the danger of invasive, allergenic and toxic effects. Outdoor air pollution was monitored not only in Budapest, but also in agricultural areas, inside and outside villages. Beside molds yeasts were also registered. Among indoor studies the air of hospital boards, nursery, microbial laboratory and many metropolitan flats in merely panel housing estates, were repeatedly sampled. Within the species spectrum considerably high participation (ca. 50%) of the black molds could be observed, however they are more allergenic.

In water hygiene the fungal pollution of fresh water (small streams, rivers and the lake Balaton) were repeatedly investigated for the presence of molds and yeast species. Twenty-seven human pathogen species (while no dermatophytes) were found. It seems that 68 species of 20 genera were hitherto not found in Hungary, while 59 species of 20 genera were not mentioned from surface waters by the most comprehensive US project.

In some cases, however, sewage plants were also studied at recreation areas. In effluents – besides the considerable decrease in population density – *C. famata* and *Sacch. cerev.* were found to be the most frequent. *C. albicans*, however, failed to appear although excrement was abundant in the sewage. Other pathogenic yeasts were also abundant in the influents.

Beneficial role of fungi in respect of environmental hygiene is realized in the decomposition (metabolization, humification) of environment-polluting plastics, sewage, styrene, phenol and formaldehyde by various fungus species. However, the dangerous level of nitrate and/or ammonia could be also eliminated by fungi. Fungal detoxification is an other route of elimination of various toxic or poisonous substances (e.g. pesticides) resulting in atoxic metabolites (e.g. glutaminic acid conjugates). For heavy metals fungal absorption – more exactly accumulation (biofiltration) – comes also into consideration in environmental protection. By adaptation considerably increased tolerance could be achieved with various molds and yeast species, however in the literature extreme value was published with *Trichosporon cerebriforme* (28% $CuSO_4$ in 1.0 H_2SO_4).

Professional and occupational hygiene

Toxic, allergic, invasive injuries through air pollution and/or contact with fungi, may be acquired during occupation. We introduced to distinguish Primary fungal Industries, Factories and occupations (fungi and/or its products are participants or aim of the technology; positive role) such as Biotechnology; food industry or staff of mycologic laboratories; and Secondary fungal ones (fungi are undesirable invaders of the technology; negative role): e.g. non-bio-industries and employment: paper-mills, cellulose factories, tanneries, archeology-museology etc. However, in bio-industry and agriculture the fungal contamination of raw-materials, intermediary and finished products is also frequent. Thus it is very difficult (and expensive) to prevent air-pollution in primary type factories, while very easy in secondary types. The fungal damages and diseases acquired in the primary fungal factories and occupations fall into the group of the specific professional damages and diseases, while those acquired in secondary fungal factories and occupations belong to the non-specific professional damages and diseases. The highest Fungal Air Pollution in numerous primary fungal industries and occupations was found with "blue cheese" mold inoculum cultivation at conidium harvesting (46×10^6 cfu/m^3 air). An interesting fungal air-pollution in secondary fungal industries and occupations was observed in a localization tower (with inside plastic covered walls and boric acid solution molded by *Asp. fumigatus*) in a reactor block of an Energy Enterprise (electric power works) in Trans-Danubium, where the workers were monitored for fungal allergy.

As it was suspected "soaked skin" especially in food-industry proved to be a predisposing factor for dermal yeast infections of the workers' hand, while for "athlete's foot" perspiration was responsible.

Nutritional and food hygiene

Damages of agricultural raw and preprocessed materials are manifested in deterioration of foodstuffs caused by molds.

Out of the food preservatives sorbic acid (see in Epidemiology, antifungal preservatives) and pimaricin (polyenic antifungal antibiotic) were studied. The last one was found to induce giant cell production in *C. albicans*.

Beside the well-known deteriorations it seems worthy to mention an uncommon type of fungal foodstuff degradation. In a vinegar factory a fungal invasion by *Monascus ruber* and *Moniliella acetabutans* was capable of reducing the acetic acid production by assimilating it.

Beneficial effects are not only restricted to the well-known fermented drinks (alcoholic and non-alcoholic beverages) of western, African and far-east types, but fermented foods and foodstuffs are also worldwide involved (cf: Far East: tempeh, shoyu, etc.). Out of soft alcoholic beverages tea-fungus (kombouchа) and Japan crystal both are symbiotic consortium of yeasts and acetic acid bacterium. The latter Japan fungus (white alga) was found to be white, amorphous, hard gel grains of ca. 4–6 mm, grown on sucrose solution supplemented with 1–2 raisins, and was "consumed" as an universal medicine. It

is, however, a symbiosis of *Sacch. cerev.* race *prostoserdovi* or *Sacch. cerev.* race *uvarum*, and *Gluconobacter oxydans.*

[The scientific stuff (long staying visitors also) since 1955, in chronological order: Anna Csillag (head: 1955–57), E. K. Novák (head: 1957–92), Györgyi Vörös-Felkai, Cs. Dobolyi, J. Galgóczy, T. Deák, Anna Pólay, L. Sztankov, L. Kustán, Judit Zala (head: 1992–), Nguyen Ngoc Thuy, Zsuzsa Horváth, J. Bitskey, Eman Hussien Ashour, T. Nagy; while many other colleagues in clinics and hygienic services also helped us.]

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CURRENT TRENDS IN MEDICAL MYCOLOGY

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Public health importance of fungal infections lies in the fact that they occur frequently throughout the world and are very heterogeneous in etiological and nosological point of view. Their relative therapy-resistance and high relapse rate are also remarkable. For this reason, the medical mycology needs an urgent change of attitudes to clinical and diagnostic aspects.

The investigation of predisposing factors, the route of infection, as well as the distribution, ecology and environment of obligatory and facultative pathogenic fungi is necessary for the adequate therapy of fungal infections. The introduction of new laboratory techniques, the standardization and quality control of classic methods also improve the efficacy of prevention and treatment. This lecture overviews some of the actual problems.

One of the most important trends in the dermato-mycology is the natural spectrum change of anthropophilic dermatophytes. *Epidermophyton floccosum* became very rare while *Microsporum (M.) audouinii* has disappeared. Their space have been filled in by the anthropophilic *Trichophyton (T.) rubrum* and the zoophilic *M. canis*, respectively. The main source of etiological agent of favus *T. schoenleinii* has already disappeared in Central Europe together with the horroristic clinical picture.

Sporothrix schenckii shows the widest geographic distribution among the dimorphic fungi. At the beginning of the century it occurred in numerous clinical samples but in next decades became rare. From the fifties it could be isolated only in a few cases. However, the soil and plants are its reservoirs even in Hungary. On the other side, eventual imported cases of coccidioidomycosis and histoplasmosis must be taken into consideration.

Yeast infections show the greatest change in the last thirty years. The increasing number of predisposing factors has led to a dramatic increase in these life-threatening mycoses. In the field of mucosal infections, however, only a shift of proportions can be noticed primarily due to the increasing of drug-induced resistant strains and species.

Little is known about the real pathogenic role of moulds in cutaneous and subcutaneous infections. This is especially true in onychomycosis. The other unexplored field of mould infections is mycotic keratitis where the extremely small clinical sample causes diagnostic problems.

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The standard and the new, mainly molecular methods improve the diagnostic tool of human medicine. The most important thing is, however, the good co-operation of clinicians and laboratory professionals to recognize the necessity of mycological investigation and the correct interpretation of laboratory results to the interest of the patient.

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CHANGING EPIDEMIOLOGY OF SYSTEMIC FUNGAL INFECTIONS AND THE POSSIBILITIES OF LABORATORY DIAGNOSTICS

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Advances in medical and surgical therapy over the past two decades have changed the nature of patient care during hospitalization. Recently developed technologies and therapies, involving bone marrow or solid-organ transplants and chemotherapeutic agents, have become common at many medical centers, resulting in the emergence of many immunocompromised individuals. In intensive care units (ICU) the use of invasive monitoring devices, parenteral nutrition, broad-spectrum antimicrobial agents, and assisted ventilation has helped in the treatment of patients suffering from previously devastating or fatal diseases and has provided opportunities for life to premature neonates previously thought to be non-viable [1]. However, these successes have resulted in the proliferation of a severely ill, immunocompromised, long-lasting hospitalized patient population. The AIDS epidemic has also added patients at risk to this growing population of immunocompromised individuals [2]. The immunocompromised patient is highly susceptible to nosocomial infections caused by organisms such as fungi that were previously considered to be of low virulence or "non-pathogenic" [3]. Besides the well-known endemic fungal pathogens (*Histoplasma capsulatum*, *Coccidioides immitis* and *Blastomyces dermatitidis*), opportunistic *Candida* species have been implicated most frequently in nosocomial fungal infections.

In the mid-1980s, many institutions, including cancer research, university and community hospitals, reported that fungi were becoming common pathogens in nosocomial infections [3, 4]. In addition, during the period 1980 to 1990, hospitals in the US providing data to the Center for Disease Control and Prevention (CDC) National Nosocomial Infections Surveillance (NNIS) system reported a steady increase in the rate of nosocomial fungal infections, from 2.0 to 3.8 per 1000 discharges [5]. The high rate of fungal infections was not limited to oncology wards and high-risk nurseries, but also occurred in cardiac surgery and burn and trauma wards. The data suggest that, although nosocomial fungal infections may be more common in large university-affiliated hospitals, the increase in incidence of these infections may be evident in all types of hospitals.

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The majority of nosocomial fungal infections are reported to be caused by *Candida* spp. Even today, *C. albicans* is by far the most common *Candida* species causing infections in humans, though the prevalence of other *Candida* spp, such as *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae* and *Torulopsis* (*Candida*) *glabrata*, has increased during recent years. Many institutions have reported newly recognized pathogenic fungi, previously thought to be non-pathogenic, including *Malasezzia* spp., *Fusarium* spp., *Trichosporon* spp., *Mucor* spp. and *Acremonium* spp. [6–8]. *Aspergillus* spp. may also be involved in infections of hematology and oncology patients [4].

C. albicans and the *Candida non-albicans* species may cause a similar spectrum of diseases, ranging from thrush to invasive diseases such as arthritis, osteomyelitis, endocarditis, endophthalmitis, meningitis or fungemia [1, 3].

Candidemia is the most extensively studied nosocomial invasive fungal infection. The presentation of fungemia may vary, with pyelonephritis, peritonitis, hepatosplenic abscesses, pneumonitis, myositis, macronodular skin lesions, osteomyelitis, endophthalmitis, meningitis and/or multiorgan involvement. Most serious nosocomial *Candida* infections are believed to be endogenous, acquired through prior colonization of the mouth, gastrointestinal tract, vagina or skin, which act as source [9]. The organs involved in disseminated candidiosis may vary with the route of infection. If a previously colonized gastrointestinal tract is the source of infection through a breakdown in mucosal or epithelial tissue, liver and splenic abscesses will probably result [10]. In contrast, if a colonized central venous catheter is the source, endocarditis or renal involvement is more likely [1]. Patients with candidemia and/or disseminated infection usually develop fever and leukocytosis, unless they are also on immunosuppressive medication. It has been proven that some infections caused by non-albicans *Candida* species such as *C. lusitaniae* or *C. parapsilosis* or by *Aspergillus* spp may be exogenous.

The differentiation between the exogenous and the endogenous acquisition of *Candida* infections is important for determining appropriate control measures to prevent the nosocomial transmission of *Candida* spp. Strain-typing studies are needed to avoid implicating an environmental source solely on the basis of the presence of a single species of *Candida*. Interstrain differentiation should be carried out. Strain-typing may also be important in less serious local infections, such as recurrent vulvovaginitis, to distinguish between recurrent or persistent infection. Various typing methods have been developed for this purpose [11, 12].

Prevention methods aimed at reducing identified risk factors for nosocomial fungal infection are being increasingly advocated. The usefulness of the prophylactic use of fluconazole among selected leukemia or bone marrow transplant patients is supported by recent studies. For the treatment of invasive *Candida* and *Aspergillus* infections, amphotericin B, its liposome-encapsulated form, flucytosine and fluconazole are more widely used. Amphotericin B is the most active and the most toxic antimycotic agent. However, about 80% of the *C. lusitaniae* strains display resistance to this agent. Flucytosine should be used in combination to prevent the early development of resistance. Fluconazole has a similar activity to that of amphotericin B in invasive fungal infections, with very favourable pharmacokinetics and low toxicity, but *C. glabrata* and *C. krusei* are resistant to fluconazole.

Laboratory methods available for diagnostics of fungal infections

A definitive laboratory diagnosis of invasive fungal infections can be made by the histopathologic demonstration of fungus invaded tissue, or by the isolation of *Candida* or other fungi from normally sterile body sites. Isolated *Candida* spp. should be identified at a species level, as some of the non-albicans *Candida* spp. proved to be resistant to antimycotic agents used in the empirical therapy of invasive fungal infection. Direct microscopic examination of the specimen (liquor) may help to identify encapsulated *Cryptococcus neoformans.*

For the primary culturing of fungi, Sabouraud dextrose agar is used most frequently. To ensure maximum recovery of fastidious fungi, brain heart infusion (BHI) agar supplemented with 5% sheep blood and media containing antibacterial agents can also be used with any specimen that might contain normal bacterial flora.

The identification of *C. albicans* can be based on germ tube formation in human serum, and the formation of chlamydospores on the rice agar. *C. albicans* screen plate (BioMerieux, Sanofi Diagnostics Pasteur) provide a rapid identification of *C. albicans* isolates directly in clinical material. Non-albicans species can be identified by the classical assimilation/fermentation method, which requires up to 28 days before the identification can be made [13]. For clinical purposes, more rapid tests are needed, which permit species determination on the same day or within 48–72 hours. Specific exoenzymes of the most frequent human pathogenic *Candida* species can be detected by using CHROMagar (Beckton Dickinson) for the direct culturing of specimens or for the subculturing of non-albicans isolates. The chromogenic substrates incorporated in the agar plate provide well-distinguishable coloured colonies of *C. albicans* (green), *C. tropicalis* (metallic blue), *T. glabrata* (purple) and *C. krusei* (pink). All other species which appear with white colonies can be identified by commercially available identification systems such as the ATB 32C, API 20 CAUX (BioMerieux) strip and the AUXACOLOR, and FUNGSCREEN (Sanofi Diagnostics Pasteur).

To diagnose invasive *Candida* infections, blood cultures may be used. Several blood culture systems are available for recovery of fungi. The lysis-centrifugation method provides a sensitive method, but several fully automated blood culture systems are also currently available (BacT/Alert, Organon Technica; Bactec, Becton Dickinson; Vital, BioMerieux). Positivity may be recorded after a few hours of incubation. Despite the fact that blood cultures remain the basic tool for the diagnosis of fungemia, this method may fail to detect as many as 50% of disseminated candidiasis cases. The detection of massive colonization with the same *Candida* species at different body sites (feces, urine, and oral cavity) may help in the prediction of the dissemination of the *Candida* infection. Even when blood cultures are positive, identification can be attempted several days after isolation. Serological diagnosis of fungemia is problematic, since it is neither sensitive nor specific. Direct antigen detection from serum samples may also be used for diagnosis of systemic fungal infections. Latex agglutination for *Candida*, *Aspergillus* and *Cryptococcus* (Sanofi Diangnostics Pasteur). However, the sensitivity of these tests is also low. ELISA method for detection of *Aspergillus* antigen from serum samples and from bronchoalveolar lavage was developed recently and have a sensitivity of 1 ng/ml (Platelis Aspergillus, Sanofi Diagnostics Pasteur). A PCR-based method can likewise be used to detect pathogenic fungi in human blood [14].

Over the past decade, routine susceptibility testing has played a minimal role in the management of nosocomial *Candida* infections. Many difficulties have arisen as concerning the establishment of a reliable and reproducible susceptibility testing method for *Candida* spp. Factors such as inoculum size, medium type, and temperature and time of incubation influence the results of such testing, and standardized methods have only recently been developed [15]. A comparison of the relative susceptibilities of *Candida* spp to antifungal agents, performed in a blinded fashion, revealed great intra- and interlaboratory variations in the results [16]. However, recent studies demonstrated a very good correlation between susceptibility tests involving Etest strips. The National Committee for Clinical Laboratory Standards (NCCLS) [17] provide new guidelines for the susceptibility testing of antifungal drugs for *Candida* spp.

The increasing number of severe infections caused by different yeasts and other fungi is a great challenge not only for the clinician, but also for the clinical microbiology laboratory. The provision of rapid and reliable methods for these pathogens that are difficult to culture and identify is very important, as at present the clinician's decision must be based on a synthesis of both laboratory and clinical data for the successful management of serious fungal infections.

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RECENT PROGRESS IN MYCOTOXIN RESEARCH

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Mycotoxins are a diverse group of fungal secondary metabolites, which are harmful to animals and humans. The definition does not include toxic compounds produced by the fruit bodies of basidiomycete and ascomycete species (the toxicoses caused by them are called mycetisms), and fungal phytotoxins harmful to plants, although many mycotoxins may also have phytotoxic properties [1]. The first known example of mycotoxicosis was ergotism caused by the consumption of food, particularly rye bread made from flour contaminated with ergot (sclerotia of *Claviceps purpurea*) in the middle ages. Outbreaks continued mainly in Europe, the last being reported in Russia in 1928 [2].

Mycotoxins may have a range of pharmacological effects, partly due to their chemical diversity. These effects include hepatotoxicity and hepatocarcinogenicity (aflatoxins, sterigmatocystin), teratogenicity (aflatoxins, ochratoxins, rubratoxin B), neurotoxicity (citreoviridin, patulin, cyclopiazonic acid), nephrotoxicity (ochratoxins, citrinin, viomellein, xanthomegnin) and tremorgenic effects (penitrem A, fumitremorgens) (for more details, see ref. [3]). Most of the mycotoxin-producing species are filamentous ascomycetes, basidiomycetes or deuteromycetes [4]. *Aspergillus*, *Fusarium* and *Penicillium* are usually considered as the most important mycotoxin-producing genera. Postharvest spoilage caused by these and other fungi is the most important source of mycotoxins in foods and feeds. However, many fungi produce toxins while interacting with living plants as pathogens or as endophytes (preharvest spoilage). Examples of these are the production of the tremorgenic alkaloid lolitrem by the endophyte *Acremonium lolii* in perennial rye grass, and the production of phomopsin A by the pathogenic fungus *Phomopsis leptostromiformis* in *Lupinus* sp., causing lupinosis of grazing animals [1]. *Aspergillus flavus* and *Fusarium* species may be involved both in pre- and postharvest spoilage of agricultural products [1, 5].

Excellent reviews have been published recently on the chemistry, molecular biology and mode of action of mycotoxins [1, 6–10]. The scope of this review is to give a general overview of recent progress in mycotoxin research with special attention to the genetics of production of some selected mycotoxins (fumonisins, trichothecenes,

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aflatoxins and ochratoxins). Finally, some new developments of lowering mycotoxin contamination in agricultural products will be discussed briefly.

Fumonisins

Fumonisin B1 was discovered in 1988 in *Fusarium moniliforme* (teleomorph: *Gibberella fujikuroi*) isolates [11]. Several other maize pathogens of the so-called "*Gibberella fujikuroi* species complex" (*F. proliferatum, F. napiforme, F. nygamai*) have also been reported to produce this or related mycotoxins (fumonisins Ax-Cx). *F. moniliforme* causes stalk and ear rot of corn worldwide, but may also be present in corn without symptoms due to its endophytic life style. Fumonisins are most frequently encountered from stored corn and other cereals, are the casual agents of leucoencephalomalacia, a fatal brain disease of horses, pulmonary oedema in pigs and are also suspected to be responsible for high incidences of oesophageal cancer in South Africa and China [12]. Fumonisins induce a variety of responses in animals including neuro-, renal and hepatotoxicoses as well as apoptosis and cell death.

Fumonisins are amino polyalcohols and structurally related to sphinganine, an intermediate in the biosynthesis of the sphingosine backbone of more complex sphingolipids such as ceramides, gangliosides and sphingomyelo-cerebrosides. Sphingolipids are essential components of the eukaryotic membranes and are also involved in the regulation of cell growth and differentiation. These mycotoxins inhibit sphingosin-N-acetyl transferase leading to altered sphingolipid synthesis and to the accumulation of toxic sphingoid bases. Fumonisins are structurally related to some *Alternaria* phytotoxins (AAL toxins) and to sphingofungins produced by *Aspergillus fumigatus*, which are potent inhibitors of serine palmitoyltransferase [13]. Three genes responsible for fumonisin biosynthesis have been identified by genetic analysis so far; *fum1* appears to be involved in the regulation of fumonisin production, while *fum2* and *fum3* encode two hydroxylases. These genes are linked and appear to form a fumonisin biosynthetic gene cluster on chromosome 1 of *G. fujikuroi* [14].

Trichothecenes

Trichothecenes are sesquiterpenoid mycotoxins produced by several fungal genera including *Fusarium, Trichothecium, Acremonium, Gliocladium, Myrothecium, Trichoderma* and *Stachybotrys*. Trichothecene producing *Fusarium* species have been associated with outbreaks of alimentary toxic aleukia which occurred in Russia at the beginning of the 20th century caused by *F. sporotrichioides* and *F. poae*, with a similar disease called red mold disease or akakabi-byo caused by *F. graminearum* (=*F. roseum*) and with bean hull toxicoses caused by *F. solani* in Japan [5, 8]. Recently a possible association has been suggested between infant deaths due to pulmonary haemosiderosis (bleeding of the lung) in the US, and the presence of a trichothecene-producing fungus, *Stachybotrys atra* in water-damaged buildings where these babies lived (New Scientist, 9

August, 1997, p. 10). Animals fed with contaminated cereals also display several deficiencies including anaemia, immunosuppression, vomiting and feed refusal [5].

More than 30 structurally related tetracyclic trichothecenes are known, among which T-2 toxin, diacetoxyscirpenol, deoxynivalenol and nivalenol are the economically most important mycotoxins. The main producers of these toxins are in the genus *Fusarium* (e.g. *F. acuminatum, F. culmorum, F. graminearum, F. poae, F. solani* and *F. sporotrichioides*). Trichothecenes have dermatotoxic, cytotoxic and phytotoxic properties, and have also been suggested to play a role in plant pathogenesis as virulence factors [8].

Trichothecenes are synthesized from trichodiene through a complex series of steps to structurally related compounds such as deoxynivalenol, T-2 toxin or diacetoxyscirpenol. Trichothecene biosynthesis genes were found to comprise a 25 kbp gene cluster including at least 10 genes in *Fusarium sporotrichioides* [15]. The gene products identified so far include two cytochrome P-450 monooxygenases, a trichodiene synthase, three acyltransferases, a transport protein and a transcription factor encoded by the *Tri6* gene, which contains sequence motifs similar to C_2H_2 type zinc fingers found in many eukaryotic DNA binding proteins [16]. The acetyltransferase which appears to be responsible for the protection of the producing organism from the harmful effects of the toxin has also been identified [17]. Similar clustering of the trichothecene biosynthesis genes was found in *Myrothecium roridum*, which produces macrocyclic trichothecenes [15].

Aflatoxins

Aflatoxins (especially aflatoxin B1) are the most thoroughly studied mycotoxins. In the early sixties, toxicity of animal feeds containing contaminated peanut meal led to the deaths of more than 100,000 turkeys by acute liver necrosis (turkey X disease). Scientists quickly identified the toxin-producing fungus as *Aspergillus flavus*, and the toxic agents as a group of structurally related bisfuranocoumarins that were named as aflatoxins B1, B2, G1, G2, etc. Since then, only two other related species, *A. parasiticus* and *A. nomius*, have been proven to produce aflatoxins in ecologically important quantities, although recent reports indicate that these toxins can also be produced by *A. ruber* [18], *A. ochraceoroseus* and an *Emericella* species (J. Frisvad, unpublished observations). These mycotoxins most frequently contaminate peanut, corn and cereals, but also occur in meat, milk (aflatoxin M1 = milk toxin) and eggs of animals that consumed contaminated feeds. Aflatoxin B1 exhibits hepatocarcinogenic and hepatotoxic properties and is referred to as the most potent naturally occurring carcinogen [19]. Epidemiological studies in China provided strong evidence that aflatoxins increase the risk of human liver cancer and also interact synergistically with hepatitis B virus [8]. Aflatoxin B1 is metabolically activated by the liver phase I detoxification pathway, and the resulting aflatoxin B1-8,9-epoxide binds to the DNA. The molecular hot spot of this compound was identified on the p53 tumor suppressor gene, site-specific mutations of which induce hepatocarcinoma [20]. The epoxide derivative reacts with guanine to form several DNA adducts the principal of which is 8,9-dihydro-8-(N'-guanyl)-9-

hydroxyaflatoxin B1. This adduct promotes depurination leading to G → T transversions [21].

Aflatoxins are produced by a complex pathway involving over 16 steps after the synthesis of the first stable intermediate, norsolorinic acid. In contrast to most polyketide synthases, the starter unit for aflatoxin biosynthesis is hexanoate, which is produced by a fatty acid synthase [22]. In recent years considerable research has been carried out on the genetics and molecular biology of aflatoxin biosynthesis (see [22] for a review). Seventeen genes responsible for the biosynthesis of aflatoxins and sterigmatocystins (structurally related intermediates of the aflatoxin biosynthetic pathway, which are the final products of similar pathways in *A. nidulans*, *A. versicolor*, *A. amstelodami*, *A. ruber*, *A. chevalieri*, *Bipolaris* and *Chaetomium* sp.) have been characterized in the past decade. Most of these genes were found to be closely linked forming a gene cluster. In *A. nidulans*, the sterigmatocystin gene cluster is about 60 kbp long and comprises 25 genes, the transcription of which is regulated by a $Zn(II)_2Cys_6$ DNA binding protein encoded by the *aflR* gene [23]. The functions of the gene products identified so far include the fatty acid synthase and polyketide synthase mentioned earlier, five monooxygenases, several reductases, dehydrogenases, a methyltransferase, and an esterase. Similarly organized aflatoxin gene clusters have also been identified in *A. flavus* and *A. parasiticus* [24]. The *A. flavus* and *A. parasiticus* aflatoxin gene clusters are about 75 kbp long, and consist of at least 17 different genes.

Ochratoxins

Ochratoxins are cyclic pentaketids, dihydroisocoumarin derivatives linked to an L-phenylalanine moiety. Ochratoxin A (OA) was first discovered in 1965 in an *Aspergillus ochraceus* isolate [25]. During subsequent years, 8 species of *Aspergillus* (*A. ochraceus*, *A. alliaceus*, *A. ostianus*, *A. sclerotiorum*, *A. sulphureus*, *A. melleus*, *A. petrakii* and *A. glaucus*), and several *Penicillium* species were shown to produce this mycotoxin. This mycotoxin has also been found recently in some black *Aspergillus* species including *A. foetidus*, *A. niger* and *A. carbonarius* [26, 27], in *A. albertensis*, *A. wentii* and *A. auricomus* [28], and in *A. fumigatus* and *A. versicolor* [29]. OA contaminates different plant products, including stored cereal grains, coffee beans, peanuts, beer and spices. It is receiving worldwide attention because of its nephrotoxic properties. Ochratoxin contamination of plant products is a serious health hazard throughout the world; the tolerable daily intake of this mycotoxin is 5-16 ng kg^{-1} body weight. Contamination of green coffee beans is especially important, since OA is not completely degraded during roasting [30]. This mycotoxin has also been detected in animal and human blood and human milk [31]. Ochratoxins have been implicated in the aetiology of urinary tract tumors, are involved in a disease of swine called porcine nephropathy, and are often cited as a possible casual agent of Balkan endemic nephropathy [32], although recently nephrotoxic glycoproteins produced by *Penicillium aurantiogriseum* and *P. commune* have also been suggested to be responsible for this toxicosis [33]. OA has also been reported to exhibit carcinogenic, teratogenic and immunosuppressive properties in

animals. At the biochemical level, OA inhibits the enzyme involved in the synthesis of the Phe-tRNA complex, thus inhibiting the incorporation of phenylalanine into peptides. OA was suggested to be metabolically activated by the liver cytochrome P-450 enzyme system, resulting in the appearance of 4-hydroxyochratoxin A [34, 35]. Both toxicological and mutagenic effects of OA have been suggested to be linked to biotransformation processes [36].

Research is in progress in our laboratory to clarify the genetic background of OA biosynthesis. Mutants of *A. albertensis*, which do not produce ochratoxins were isolated [37]. These mutants are planned to be used in the future to clone genes taking part in the OA biosynthesis by making use of the differential display technique which has already been used successfully to clone and characterize genes responsible for secondary metabolite production [38]. The effects of a variety of compounds known to affect the aflatoxin biosynthesis in *A. flavus* or ochratoxin biosynthesis in *A. ochraceus* were tested in order to find a compound, which could successfully be used to lower or eliminate ochratoxin production in *A. albertensis*. A conclusion, that ochratoxin biosynthesis is differently regulated in *A. ochraceus* and *A. albertensis* could be drawn from these experiments, since most of the compounds (e.g. caffein) which were found to inhibit OA biosynthesis in *A. ochraceus* had only limited or no effect on OA production in *A. albertensis* (Téren,J., Rigó,K. and Varga,J., unpublished observations).

Other mycotoxins

The ergot alkaloids are produced mainly by *Claviceps purpurea* and some other *Claviceps* species (e.g. *C. paspali, C. fusiformis*), although other species, e.g. *Aspergillus fumigatus*, *A. clavatus*, *Penicillium* species and plants have also been described as producers of these mycotoxins. Since *Claviceps* strains parasitize not only cereals but different kinds of grasses as well, ergot poisoning of grazing animals due to the infestation of different growing grasses is still an economical problem. The common structural characteristic of the more than 100 ergot alkaloids is the tetracyclic ergoline system. This structure is formed from 4-(γ,γ-dimethylallyl)tryptophan (DMAT), the synthesis of which is catalyzed by the prenyl synthase, DMAT synthase. The gene encoding for this enzyme has recently been isolated from *Claviceps fusiformis* [38, 39] and *C. purpurea* [40]. There are certain indications that this gene is part of an alkaloid biosynthesis gene cluster, similarly to the aflatoxin and trichothecene pathway genes [40].

Patulin is an unsaturated lacton and is produced by a number *of Aspergillus, Penicillium* and *Byssochlamys* (*Paecilomyces*) species. Representatives of other fungal genera, such as *Mucor, Mortierella, Alternaria, Chrysosporium, Fusarium, Trichoderma* were also found to produce this mycotoxin [41]. Patulin is toxic to many biological systems. Economically the most important producer of patulin is *Penicillium expansum*, the causative agent of soft rot of apples and other fruits. Patulin is receiving worldwide attention because of its occurrence in unfermented apple juice. The polyketide synthase, 6-methylsalicylic acid synthase responsible for the biosynthesis of patulin has recently been cloned and characterized [42, 43]. Surprisingly, the amino acid sequence of this

enzyme resembles fatty acid synthases from vertebrates more closely than to its own fatty acid synthase.

Several other mycotoxins have been described, which can be considered as health hazards in foods and feeds. Examples include cyclopiazonic acid [44], anthraquinones such as luteoskyrin produced by *Penicillium* species [45], or the hepatotoxic sporidesmins, the causative agents of facial eczema in sheep produced by *Pithomyces chartarum* [1].

Apart from their harmful properties, mycotoxins also have beneficial effects on mankind. Several derivatives of ergot alkaloids are used for medical purposes throughout the world. Other examples of mycotoxins which are of interest to the pharmaceutical industry are the amebicidal fumagillin and the immunosuppressive and antiviral gliotoxin, both produced mainly by *A. fumigatus*. Destruxins, the cyclic depsipeptides produced by *Metarhizium anisoplae* and other fungi pathogenic to insects might be useful as potential control agents of insect pests [46]. In addition, several mycotoxins (e.g. nominine or petromurins) purified from the sclerotia of different *Aspergillus* species (*A. flavus*, *A. nomius*, *A. ochraceus*, *A. tubingensis*) exhibit strong antiinsectan activity that compares favourably with commercial insecticides [47].

Elimination of mycotoxins from foods

Mycotoxin producing fungi may contaminate agricultural products in the field (preharvest spoilage), during storage (postharvest spoilage) or during processing. For lowering preharvest contamination, treatment of field crops with fungicides is the traditional technique. However, more environmental friendly alternatives have been sought for recently. Moss [1] suggested the elimination of the seed-borne toxigenic endophyte *Acremonium lolii* by heat treatment, and its replacement with a nontoxigenic strain in the case of lolitrem contamination of rye grass. Lolitrem is a tremorgenic mycotoxin, which induces rye staggers in grazing animals. The replacement is possible, since the insect antifeedant activity of this endophyte is not dependent on lolitrem. Aflatoxin production of *A. flavus* on a host plant can be inhibited by the plant growth regulator methyl jasmonate or by the use of *A. niger* as a competitor [1]. Cotty [48] suggested the application of nontoxigenic natural isolates of *A. flavus* to outcompete toxigenic strains in cotton fields. However, recent results indicate that recombination takes place in *A. flavus* populations, making this approach doubtful [49]. Another possibility is plant breeding in order to improve the resistance of host plants to fungal infection. Such attempts are promising e.g. in the case of *Fusarium* infection of wheat and corn [50]. Aflatoxin resistant corn, peanut and cotton varieties have also been developed [51]. Investigations to inhibit either growth or aflatoxin formation in crops led to the identification of antifungal peptide genes which are being used in the genetic engineering of cotton and corn to prevent infection by *A. flavus* [52, 53]. Similar experiments are in progress to identify the protein(s) responsible for the resistance of wheat cultivars to *Fusarium* and *Aspergillus* infection in cooperation between the Cereal Research Institute (Szeged) and our laboratories.

For postharvest control, storage conditions should be improved to minimize the mycotoxin content of foods and feeds. Mycotoxin production is dependent on a number of factors, e.g. water activity of the stored product, temperature, gas composition, the presence of chemical preservatives and microbial interactions. An integrated approach for controlling several of these factors could give much more effective control of deterioration without requiring extreme control of any one factor [54]. One possible way of decontamination is the reduction of the population of natural mold contaminants by gamma irradiation, or by the application of antifungal agents (e.g. sorbic acid, acetic acid, propionic acid, natamycin) [55]. The degradation of the mycotoxin itself is another possibility; e.g. ammonia treatment eliminates aflatoxins from stored grains, while H_2O_2 degrades ochratoxins in coffee beans. Ozone was found to be effective in degrading several mycotoxins in contaminated grains [56]. Aflastatin A, a compound produced by a *Streptomyces* sp. completely inhibits aflatoxin production of *A. parasiticus* at low concentrations without affecting its growth rate [57]. Microorganisms could also be used to lower the mycotoxin levels of agricultural products [7]. Hwang and Draughton [58] described a bacterial species, *Acinetobacter calcoaceticus*, which is able to degrade OA to the non-toxic ochratoxin α. Large numbers of *Aspergillus* strains were screened in our laboratory for their ability to degrade OA in liquid and solid cultures. Some *A. fumigatus* and *A. niger* strains were identified which eliminated OA (and also ochratoxin α) from the media within some days. We plan to test the ability of *A. niger* strains to eliminate OA from products such as cereals and green coffee beans. Such an approach has already been used successfully to lower OA levels in milk by using *Lactobacillus* and *Bifidobacterium* species [59]. The levels of other mycotoxins, e.g. aflatoxins, zearalenon and trichothecenes could also be minimized in food products by using various microbes (for details, see ref. [7]).

Potentially toxigenic fungal species are also frequently encountered in the food and fermentation industry. An extreme example is the application of possibly toxigenic strains of *A. flavus* and *A. candidus* for the production of Sierra rice (Arroz fermentado) in Ecuador. Recently, cyclopiazonic acid has been found in the fermentation broth of *A. oryzae*, and a lovastatin overproducing *A. terreus* strain has been proved to produce sulochrin in industrial fermentations [60]. Another example of mycotoxin production by fungi used extensively in the fermentation industry is ochratoxin production by black Aspergilli [26, 27]. *A. niger*, *A. awamori* and *A. foetidus* strains are frequently used in the food industry for the production of different enzymes and organic acids like amylases, pectinases, or citric acid, while *A. carbonarius* is utilized in the production of gluconic acid and urate oxidase. Strains of all of these species were found to produce OA [26, 27]. Due to its low toxigenicity, *A. niger* is one of the few fungal species which has received the GRAS (generally regarded as safe) status from the U.S. Food and Drug Administration. Although black Aspergilli produce only low levels of ochratoxins, which cannot be regarded as a serious health hazard, more attention should be paid to mycotoxin production of fungal strains used in the fermentation industry.

Mould-fermented meat and cheese products pose another problem. The mycotoxins identified in such products involve ochratoxins and cyclopiazonic acid. Some attempts were made to decrease the chance of mycotoxin contamination of meat products. In Germany and in France, non-toxigenic *P. nalgiovense* strains were introduced as starter

cultures for salami ripening under the names of "Edelschimmel Kulmbach", and "Blanche", respectively [61]. In the cheese industry, all *P. camembertii* isolates used in cheese-making were found to produce cyclopiazonic acid. *P. camembertii* mutants unable to produce this mycotoxin have recently been isolated [62]. Another species used in the manufacture of mould-ripened cheeses, *P. roqueforti*, was also found to produce mycotoxins like roquefortine, mycophenolic acid and PR toxin. The long-term toxicity of these metabolites is still unproven.

It is also possible to prevent toxic effects once the toxin is ingested. In the case of OA, the phenylalanine analogue aspartame effectively prevented OA induced toxic effects in rats [63]. Rats could also be protected against aflatoxin B1 hepatocarcinogenesis by supplementing their diet with antioxidants such as ethoxyquin [64]. Glutation-S-transferase contributes to this protection by conjugating glutathion to aflatoxin B1-epoxide. The resultant conjugates are actively extruded from the cell by ATP-dependent export pumps such as multidrug resistance proteins [65].

In summary, although mycotoxicology is a relatively young science, promising results have been achieved in the study of the biology and biosynthesis of a number of mycotoxins involving aflatoxins, trichothecenes, and ergot alkaloids. Understanding the biosynthesis and effects of these compounds could facilitate safeguarding human and animal health. To achieve this goal, further research is needed to clarify the biosynthetic pathways of other economically important mycotoxins such as ochratoxins, zearalenon and fumonisins; to ascertain the role of these mycotoxins in plant and animal pathogenesis; and to develop environmentally safe ways to minimize mycotoxin contamination of agricultural products.

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FUNGI IN FORESTRY – SIGNIFICANCE AND RESEARCH IN HUNGARY

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The importance of the fungi in forest ecosystems and in forestry as well as a short review of the research of fungi with forestry significance in Hungary are discussed in the paper.

The multiple roles of fungi in forest ecosystems is well known. As heterotrophic organisms their nutrition is pathogenic, symbiotic or saprobic. The mode of nutrition determines their forestry significance. As pathogens they can cause diseases, sometimes death of the forest trees, or discoloration and heart rot in their trunk. As symbiotic partners on roots of forest trees (mycorrhiza) they further the nutrition and defense capacity of trees against the root pathogens. The saprobic activity of fungi decomposing the dead wood and leaf litter is very important in humification process. Fungi are the most important organisms in nature, which have cell wall decomposing capacity. They can cause serious damages by staining and destroying of felled trunks or stored and utilized wood products. The fruit-bodies of many fungi growing in forest are comestible representing not negligible sources of human alimentation.

In the followings the role of fungi as pathogens of forest trees and wood decayers are summarized. A large-scale of fungal systematic groups contains species with above-mentioned significance, so this review permits a fairly comprehensive summary of fungal world.

The fungi bearing importance in forestry are shown in actual systematic arrangement [8] (Table I). The review of knowledge and research of these fungi in Hungary was made on the basis of special literature on Hungarian forestry. For reasons of dimensional limit it is possible to cite only a reduced number of selected papers referring mostly to the recent research works. For the same reason the works about the mycorrhiza fungi and non-pathogen pileated fungi which are the subject of other presentations are not cited in this paper.

The research of fungi significant for forestry in Hungary is closely related to the history of Hungarian Academy of Forestry founded in 1808 in Selmeczbánya, the precursor of the University of Sopron. The diseases of forest trees were taught at the

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Table I

System of principal orders of forestry significant fungi from Ainsworth et Bisby's DICTIONARY OF THE FUNGI Eighth Edition (1995)

Kingdom	Phylum	Order
Protozoa	Myxomycota	
Chromista	Oomycota	Pythiales
Fungi	Zygomycota	Entomophthotales
		Mucorales
Ascomycota	Diaporthales	Diatrypales
		Dothideales
		Eurotiales
		Erysiphales
		Hypocreales
		Leotiales
		Microascales
		Ophiostomatales
		Pezizales
		Rhytismatales
		Saccharomycetales
		Taphrinales
		Xylariales
	Basidiomycota	Uredinales
		Auriculariales
		Tremellales
		Agaricales
		Boletales
		Fistulinales
		Ganodermatales
		Hymenochaetales
		Poriales
		Schizophyllales
		Stereales
		Thelephorales
	Mitosporic fungi	

beginning of this academy even if their fungal origin was not known in all cases. The first papers about the fungi pathogenic for forest trees appeared in "Erdészeti Lapok" and "Természettudományi Közlöny", periodicals founded in 1862 and in 1860, respectively, then in "Erdészeti Kísérletek", periodical of the Forestry Research Station founded in 1898. At the turn of the century and in the first decades of the 20th century many articles about a large number of fungi pathogenic for forests were published in the journals mentioned above. The appearance of two epidemic tree diseases in the first half of the

20th century had an echo in Hungarian special literature of forestry: the powdery mildew of oak caused by *Microsphaera alphitoides* Grig et Maubl. and the vascular wilt disease of elm caused by *Ophiostoma ulmi* (Buism.) Nannf. Also the fungi causing heart root of oak were studied in detail at this period of time.

New forest pathological problems appeared and were studies in the second part of our century. The principal objectives were mostly connected to starting of intensive afforestations using hybrid poplars, Scotch and Austrian pine. The forest pathology and wood protection knowledge were synthesized in book form [5, 7].

Recently research themes in forestry mycology are current problems of forest protection as oak decline, death of trees by root diseases, shoot blight of pines etc. The heart rot inducing and wood destroying poroid fungi in Hungary have been systematically investigated [9].

Concerning the mycorrhiza fungi, their importance was known already at the end of the past century. The inoculation of soil with mycorrhiza fungi was a widely applied sylvicultural operation in the 1950s. These fungi are in the focus of current forest mycological researches, too. The importance and composition by species of pileated fungi in forests, as well as the different aspects of their practical utilization (cultivation of comestible fungi, decomposition of different substrata etc.) have been studied.

Systematic treatment

The slime fungi *(Myxomycota)* included in kingdom *Protozoa* have a fairly frequent occurrence in the forests on decaying wood and other organic substrata. Species playing a significant role in diseases of forest trees or in wood protection are not known.

Among the *Oomycota* classified now in the kingdom *Chromista* some *Phytium* and *Phytophthora* species are known as important root pathogens of different forest trees. In Hungary they were observed in course of mycological investigations related to damping-off of conifer and beech seedlings, to the ink disease of chestnut and to oak decline.

Fungi belonging to *Zygomycota* can be found mostly as contaminators of seeds during the storage or germination *(Mucorales).* Some species of insect-pathogen *Entomophthorales* were observed on dead insects in sinking stage of gradations.

Ascomycota is the richest in species among the fungal phyla. Genera significant in forestry and some important species are treated shortly in the followings.

To the order *Diaporthales* belong several leaf-, shoot- and bark disease causing species. Some are weak or mild pathogens having a role in decline processes of different tree species, others are aggressive pathogens. *Apiognomonia* and *Gnomonia* species cause leaf and shoot necrosis of trees mostly of anthracnose type [20]. *Valsa* species living in the bark of forest trees are weak pathogens, contributing to death of branches of weakening trees. They were studied especially in the case of poplars [12]. *Diaporthe* species are also weak pathogens in the bark of different trees. Bark necrosis caused by them was observed on young oaks (*D. leiphaemia* /Fr./Sacc.) and robinia (*D. oncostoma* /Duby/Fuck). *Cryptodiaporthe populea* (Sacc.)Butin is the agent of bark chancre of poplars. Its biology and the conditions of disease were studied in detail by forest pathologists in Hungary [3, 12]. *Cryphonectria parasitica* (Murrill)Barr, a very aggressive pathogen, causes bark cancer of chestnut. Found first time in 1969, now it is present in all the chestnut stands and plantations causing great ravages. The natural hypovirulent strains

of the fungus are found in Hungary, their application in biological suppression of the disease is tested [16].

The order *Diatrypales* includes in the bark living saprobic or weak pathogen fungi. Some species are very common on dead branches in forests. They can cause saprobic wood stain of fresh felled trunks of broad-leaved trees, followed by an incipient decay.

Dothideales contain many species occurring on forest trees. *Cucurbitaria* and *Botryosphaeria* species are weak pathogens living in bark. Some *Botryosphaeria* species were found during the mycological investigations related to oak decline, the pathogeny of *B. stevensii* Shoemaker on young oaks was demonstrated [23–25]. According to recent observations this species is implicated in stock cancer syndrome of turkey oak. *Scirrhia pini* Funk et Parker which recently has appeared in Hungary is a dangerous needle pathogen of pines, especially of Austrian pine [19]. The *Mycosphaerella* species are leaf and needle pathogens on different trees. *Venturia* species are important leaf and shoot pathogens on poplars and willows [12].

Endomycetales, the yeast fungi, occur in the forests primarily in slime-flux of the trees of different origin.

The species of *Eurotiales* are known mostly in their anamorph state *Penicillium* and *Aspergillus.* They contaminate the seeds during the storage affecting their germination capacity and cause a superficial discoloration, mould of wood products stored in wet condition.

Among the *Erysiphales* the powdery mildew of oaks *(Microsphaera alphitoides* Griff. et Maubl.) is very common, dangerous especially in nurseries and young plantations of pedunculate oak. It was found in Hungary for the first time in 1908, its perithecia were detected in 1922 only. The powdery mildews of other forest trees *(Uncinula* spp. on maples, poplars and willows, *Phyllactinia guttata* (Wallr. ex Fr.)Lev. on several tree species etc.) are less important, their symptoms appearing mostly at the end of the growing season.

Some *Nectria* species belonging to the order *Hypocreales* are bark pathogens causing cancer of various trees (*N. ditissima* Tul. on beech, *N. galligena* Bres. on poplars, ash and other trees). Other *Nectria* species are contributing factors to decline diseases as weak pathogens (*N. coccinea* /Pers./Fr. on beech, *N. cinnabarina* /Tode/Fr. on several woody plants). The *Nectria* species important for the forestry were studied in Hungary already in the first years of the 20th century. *N. radicicola* Gerlach et Nilson, a root pathogen of different young trees in nurseries and natural renewals, was recently found in Hungary.

The order *Leotiales* contains several pathogens. *Drepanopeziza* species cause leaf spots of poplars and willows. They were investigated especially on poplars in connection with breeding programs [4]. *Lachnellula willkommii* (Hartig)Dennis causes cancer disease of larch known in Hungary already in the late 19th century. *Cenangium ferruginosum* Fr. is a pathogen of pines causing shoot and twig death. It was identified in relation with the deaths of Austrian pine during the earlier epidemics as well as at the present time [17]. *Botryotinia fuckeliana* (de Bary)Whetz. in its anamorphe state *(Botrytis cinerea* Pers.) is a pathogen causing the death of the young shoots of conifers. The disease was studied and described for the first time in Hungary in 1900. *Ciboria batschiana* (Zopf)Buchw. damages oak acorns causing their mummification.

From the order *Microascales* the *Ceratocystis* species has importance. *C. fagacearum* (Bretz)Hunt causing vascular wilt of oaks in North America does not occur in Europe. Some other species are endophyte or decline pathogens in various trees, as *C. erinaceus* described recently in Hungary [1, 2].

Ophiostoma ulmi (Buism.)Nannf. *(Ophiostomatales)* is the agent of elm vascular wilt. Its epidemic started in Europe at the late 1910s. The disease was found in Hungary in the 1930s. The more aggressive strain of the fungus was reintroduced in Europe from America in the 1960s, causing the present epidemic. It is considered as a distinct species now, named *O. novo-ulmi* Brasier. Other *Ophiostoma* species especially *O. piceae* (Münch)H. Sydow et Sydow are known as contributing factors in decline diseases of oak and beech [1, 23]. Some species cause the blue stain of conifer wood after felling or death of the trees [5].

The order *Pezizales* contains mostly saprobic fungi growing on soil or decaying wood. *Rhizina undulata* is a pathogen Fr. which can attack the roots of conifers under special conditions [18].

Rhytisma acerinum (Pers.)Fr. *(Rhytismatales)* is common in Hungary causing tar spot disease of maples. *Lophodermium seditiosum* Minter, Staley et Millar is one of the most important needle pathogens of pines in nurseries. Its biology as well as the methods of defence against it were studied in detail [13].

Taphrina species *(Taphrinales)* induces hypertrophic symptoms as leaf blister witch's broom or fruit deformation of some broad-leaved forest trees.

Xylariales contains mostly saprobic species common on dead wood. Some of them can affect the wood quality of felled trunks causing saprobic wood stain. *Ustulina deusta* (Hoffm.)Petrak causes discoloration of wood in living trees. *Rosellinia quercina* Hartig is a root pathogen of young oaks.

Phylum *Basidiomycota* contains a great number of fungi important in the forestry: pathogens, mycorrhiza partners, decomposers etc.

In Hungary about 100 rust fungus species *(Uredinales)* have been registered on various forest trees and shrubs. Only a few of them have practical importance for forestry. *Melampsora* species causing leaf rust of poplars were studied in the 1960s and 1970s in relation with breeding of these trees. The investigation of different biotypes of these rust fungi has been started recently in our country [22]. *Cronartium ribicola* J.C.Fisher, the agent of blister rust of white pine causes serious damages in young plantations of this tree species.

The orders *Auriculariales, Dacrymycetales, Tremellales* contain saprobic species occurring on dead, decaying wood, they play some role in decomposition processes in the forests.

Agaricales and *Boletales* include the most part of fungi important as litter decomposers, mycorrhiza partners or edible fungi. The significance of these fungi in the forestry ecosystems as well as their composition by species has been researched by several mycologists.

Armillaria species *(Agaricales)* have importance as root pathogens of forest trees. They represent an important factor in decline processes of forest trees [23]. The identification of species and their hosts in Hungary is going on [21]. *Pholiota* species can attack living trees through the stem wounds, for example *Ph. destruens* (Bond.)Quel., a

frequent wound pathogen and wood stain agent in living poplars. It causes a rapid white decay of wood after felling [12].

To the *Boletales* belong the most dangerous decayers of wood in buildings: *Serpula lacrimans* (Wulf. In Jack.ex Fr.)Schroeter and *Coniophora puteana* (Schum.ex Fr.)P.Karst. They were studied by many specialists primarily regarding defence possibilities [5].

The further orders contain the most important heart rotters of living trees and decayers of wood products. They can cause major losses of wood production. These species have been studied recently in Hungary [9]. Some frequent species are as follows:

Fistulina hepatica (Schaeff.)Fr. *(Fistulinales)* causes butt rot of aged oaks. It is a brown rotter (It decomposes holocellulose, but not lignin).

Ganoderma adspersum (Schulz.)Donk *(Ganodermatales)* is a white butt rotter of many tree species (It decomposes holocellulose as well as lignin).

Inonotus nidus-pici Pilat *(Hymenochaetales)* is a white heart rotter, very frequent on turkey oak *(Quercus cerris L.)*, causing severe losses.

Frequent species from the order *Poriales: Polyporus squamosus* (Huds.)ex Fr. (white rot), *Laetiporus sulphureus* (Bull.ex Fr.)Murrill (brown rot) occur on more tree species. They are wound pathogens causing heart rot of their hosts. *Heterobasidion annosum* (Fr.)Bref. can be considered as one of the most dangerous pathogens in planted pine stands. It attacks the roots thus causing death of the trees. The biology of this species, the possibilities of protection against it and recently its population structure have been objects of detailed studies [14, 15]. *Pleurotus ostreatus* (Jack.)Quel., wound pathogen, causes stain in living trees and white decay after felling. This fungus was studied concerning its utilization for decaying of the stumps before reforestation of the terrain [10]. It is one of the most favourite cultivated fungus. Its breeding and gene bank completing are under study.

Schizophyllum commune (Fr.)Fr. *(Schizophyllales)* occurs frequently on branches of declining or dead trees.

Stereum species *(Stereales)* are mostly saprobic decayers frequent on dead wood in the forests. *Phlebiopsis gigantea* (Fr.)Jülich is utilized in biological prevention of infection with *Heterobasidion annosum* in *Pinus* stands [14].

Mostly saprobic fungi belong to the order *Thelephorales. Thelephora terrestris* Fr. is known to overgrow the conifer seedlings but it can be a mycorrhiza partner at the same time.

The group of Mitosporic fungi is not accepted as a systematic category in the actual fungal system. Only some of the important species are mentioned here:

Sphaeropsis sapinea (Fr.)Dyko et Sutton is the agent of the disease known as sphaeropsis blight of pines. It is very frequent and causes serious damages in Austrian pine stands. Its biology under Hungarian conditions has been cleared recently [11]. The leaf disease causing *Coelomycetes* have been studied concerning the occurrence of species and their hosts [20]. Some *Fusarium* species cause damping-off of conifer seedlings [6]. Bark cancers or vascular discoloration of *Fusarium* origin have been observed on poplars and robinia, respectively on oak. Conidial fungi include some antagonist species *(Trichoderma, Ampelomyces)* employable against the pathogen fungi of forest trees. *Rhizoctonia solani* Kiihn. is a frequent root pathogen on the seedlings.

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CURRENT RESEARCH ON PHYTOPATHOGENIC FUNGI: AN OVERVIEW

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The majority of plant pathogens comprising about 60% of the total are belonging to a group of eukaryotic microorganisms, commonly known as FUNGI although this name obviously covers a number of organisms that are not fungi in a strict sense [1]. Yet, all these plant pathogens, having different systematic positions within the livings, are of significance as far as agricultural crop production is concerned.

Mycologists in the past were primarily interested in identifying fungal diseases, describing the fungi, studying their life cycle in relation to environment and looking for effective control measures. A big step forward was when investigations expanded toward the physiology of plant diseases and disease resistance. Meanwhile fungal genetics has developed providing a better understanding of plant-fungus relations. Recently, with the introduction of molecular techniques, new approaches of research, such as molecular taxonomy and molecular genetics have been established and the molecular methods were applied in other related fields of studies.

For the illustration of changes in research interest worldwide, the main research topics and all the contributions (poster presentations) accepted at and published by the 7th International Congress of Plant Pathology held in Edinburgh, Scotland between 9-16 August 1998 have been scanned. Based on these, some of the most promising research trends with a few examples will be accounted here and then a short overview of what has recently been done by Hungarian mycologists on the subject will be given.

Research tendencies in the World

Detection and identification. No doubt, modern diagnostic technology started in 1976 with the first application of ELISA to plant virus detection. Subsequently, assays have been targeted to the detection of plant pathogenic fungi. ELISA kits are now commercially available and ELISA and other related antibody-based technologies are widely used. As an example, Jennings et al. [2] have raised Mabs (monoclonal antibodies) to either the *Fusarium* genus or individual species, such as *F. avenaceum, F. culmorum* and *F. poae*. Further, the use of protein and enzyme profiles have also been established as

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tools of differentiating between fungal species/strains. Belisario et al. [3] found *Monilinia fructigena, M. fructicola* and *M. laxa*, the brown rot pathogen of fruits distinguishable based on protein patterns.

The practical use of nucleic acid-based detection technologies in plant pathology was limited until the introduction of the polymerase chain reaction (PCR) assay in the late 1980s. Afterwards, however, PCR or reverse transcription (RT) PCR have been developed for a wide range of plant pathogens including phytopathogenic fungi, and the combination of PCR and DNA hybridization allowed the detection of multiple pathogens in a single test. The only limiting factor of widespread application of these techniques is their expensiveness. One of the most promising uses of DNA technologies in the plant pathological practise is the detection of latent (symptomless) infection by fungal pathogens in plants. For example, Whisson et al. [4] were able to produce taxon-specific DNA probes derived from DNA extracted from both *Phomopsis viticola* conidia and infected grapevine tissues. A PCR-based assay then allowed the detection of the fungus from symptomless plants.

Apart from molecular methods, there are improved microscopical techniques, like image analyses or comparative electron microscopy (SEM and TEM) of spores, suitable for discriminating between uncertain species [5, 6].

Genetic and molecular aspects of pathogenicity. Within the last few years, the application of molecular genetic methods has enabled the identification of a variety of genes involved in pathogenesis, such as determinants of early infection stages, colonization and of fungal products killing plant cells. One of the most exciting questions is what are the signals influencing spore germination and host penetration, in other words, what are the factors responsible for mutual recognition and subsequent events leading to disease. With the rice blast fungus, *Magnaporthe grisea*, for example, thigmotrophic sensing appeared to involve a fungal cell-surface protein called Mpg-1 that belongs to the known hydrophobins [7]. Another point of interest relates to the presence of specific proteins of fungal origin, called elecitins, the recognition of which by the host plant is a prerequisite of events leading to either compatibility or incompatibility. By means of molecular methods it was possible to clone elicitin genes from the fungus *Phytophthora sojae* [8].

Necrotrophic fungi require a rapid death of plant cells to get into contact with host nutrients. Therefore, a wide range of secondary metabolites, like phytotoxins and lytic enzymes produced by them prior to, during and after penetration are considered to play a role in pathogenesis. In fact, by isolating and cloning the genes governing the production of pectinases and lipases, Ten Have et al. [9] elucidated their significance.

Since P J G M de Wit and his co-workers [10] were successful in cloning first an avirulence gene from the fungus *Cladosporium fulvum*, additional avirulence genes have been identified in different laboratories, e.g. in Australia [11] and in the UK [12].

Genetic diversity of fungal populations. The mechanisms of population genetics over space and time appear to be rather complex and multifactorial. However, there are increasing experimental evidences that genetic structures of phytopathogenic fungi are basically influenced (determined) by mating systems, immigration (gene flow) and selection [13] as well as by somatic heterokaryosis [14].

From the practical point of view, population changes as variation in virulence, aggressiveness or fungicide-sensitivity are being of particular interest. Accordingly, the number of investigations using either conventional methods (host differentials, mating analysis), molecular techniques (DNA polymorphism) or both have markedly been increased. Recently, Pipe et al. [15] applied, as a new tool, microsatellite markers with *Phytophthora infestans* for the analysis of diversity in population structure.

Response to environment. Ecology of phytopathogenic fungi came to the focus recently in many mycological laboratories. Of particular interest are investigations looking at the survival and growth of fungi in soils of various qualities, as well as the various biotic interactions including those between pathogenic and non-pathogenic species [16]. To study the complexity of such soil systems and to elucidate the processes taking place in the rhisosphere, molecular approaches, e.g. the use of GUS-transformants of *Trichoderma harzianum* have been applied [17]. Since many of the non-plant pathogenic soil microorganisms are considered as potential biocontrol agents, techniques of isolation and characterization and mass-production of the candidates have been the subject of studies worldwide. In addition, as a most promising tool, the so-called molecular breeding of some of the biological antagonists were initiated [18].

Research progress in Hungary

Much of our studies on phytopathogenic fungi, being either basic or applied, have been carried out in research institutes (MTA-NKI, MBK, MTA-MgKI) and agricultural universities (GATE, PATE, DATE, KÉE, SE), respectively. Based on recent publications, a selection of research topics will be shown here to provide with a short and informative overview.

Etiology. Of particular interest are studies related to woody plant diseases (fruit and forest trees, grapevine) where newly appearing pathogens or pathogen complexes are causing severe damage [19, 20], or investigations of the pathogenic mycofloras of natural vs. agricultural ecosystems [21].

Detection/identification. Besides classical methods, molecular techniques in the detection and identification of phytopathogenic fungi have been introduced. For example, the comparison of pectic enzyme zymograms of *Cytospora* species [22], and the esterase isozyme patterns of different *Fusarium* species [23] permitted to distinguish between different taxons, or DNA polymorphism and RAPD analysis verified the taxonomic position of genera and species of the so-called "Helminthosporium" form complex [24].

Population structure and genetics. Much of recent studies have been focussing on inter- and intraspecific variations of some important phytopathogenic fungi and both classical methods and new molecular techniques have been applied. For example, the relative dominance of *Fusarium* species in winter wheat and maize [25, 26], changes in mating type, virulence pattern and fungicide sensitivity of *Phytophthora infestans* [27], evolutionary processes in the virulence character of field populations of *Blumeria graminis*, *Puccinia graminis* and *Plasmopara halstedii* [28–30] have been the subject of investigations in order to combat with these pathogens. Furthermore, PCR-RAPD

techniques allowed to distinguish between *Fusarium* species belonging to the teleomorph *Gibberella fujikuroi* [31], and cloning a repetitive element from *Fusarium poae* made it possible to understand, at least in part, the genetic background of fungus variability [32].

Biological antagonists. The potential of using antagonistic fungi as biocontrol agents in agriculture has prompted Hungarian mycologists to set up and expand research in different laboratories. A wide range of investigations, from the isolation, identification and systematics of such fungi through molecular genetics to the patented bioproduct have been made e.g. with *Trichoderma* spp. [33], *Coniothyrium minitans* and *Ampelomyces* spp [34]. In addition, VCG groups within the Hungarian populations of *Cryphonectria parasitica* have been determined enhancing to characterize population structures and to detect hypovirulence, a phenomenon known to play an important role in the biocontrol process against this pathogen [35]. And another, special aspect of studies on antagonistic fungi resulted in identifying species as potential mycoherbicides [36].

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BIOLOGICAL CONTROL OF AGRICULTURAL PESTS BY FILAMENTOUS FUNGI

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Agricultural use of chemical pesticides has polluted the environment and resulted in resistance among the target organisms. The chemical strategies of pest control are dangerous to both the nontarget organisms in natural habitats and human health. Biological control is an attractive less dangerous possibility for controlling plant pathogens.

Some methods of biological control are becoming now commercially available against plant parasitic fungi, nematods and insects. Among filamentous fungi many candidates with biocontrol potential can be found. Fungal biocontrol agents are less effective and reliable than the synthetic pesticides therefore their use in the agricultural practice requires genetic improvement.

Mycofungicides

There are many fungal genera, which contain species, which effectively antagonize and/or parasitize plant parasitic fungi both in the rhizosphere and phyllosphere [1].

The most effective species can be found in *Trichoderma*, *Gliocladium*, *Fusarium*, *Talaromyces*, *Ampelomyces* and *Coniothyrium* genera.

Mycoparasitic *Trichoderma* and *Gliocladium* strains could be used to control a wide range of plant-pathogenic fungi e.g. *Pythium*, *Rhizoctonia*, *Fusarium* and *Botrytis*.

The ecological, physiological and molecular aspects of biocontrol by these fungi were recently reviewed [2–5, 77–78, 80].

Alternative mechanisms may be responsible for the control effects of these species. Some strains of them are able to produce antifungal antibiotics [6–7], which may have synergistic effect with extracellular fungal-cell-wall degrading extracellular enzymes [8].

The production of cell-wall hydrolyzing enzymes seems to be the most important factor of antagonism. Both *Trichoderma* and *Gliocladium* mycoparasitic strains in some cases constitutively secrete high amounts of β-1,3-glucanases [9], chitinases [10–11] and proteases [12]. The other important phenomenon of mycoparasitic strains is the capability for the production of appressoria which penetrate the cell wall of the target fungus and

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grow inside the target cells. The production of appressoria is an inductive process which require the presence of specific lectins in the cell wall of the target fungus [13]. There are reports that the cell-wall-degrading enzymes act synergistically in the cell-wall hydrolyzing process. Several different enzymes, chitinases, β-1,3-glucanases, β-1,6-glucanases and proteases have been purified from the inductive ferment brothes of *Trichoderma* [14–17] and *Gliocladium* [8] mycoparasitic strains. From the purified enzymes chitinases and β-1,3-glucanases showed the most intensive spore-germination inhibitory effect.

The purified degradative enzymes acted synergistically not only with each the other, but also with antibiotics and chemical pesticides [18–19]. Many *Trichoderma* and *Gliocladium* biocontrol strains could be originally resistant against a wide variety of chemical fungicides frequently used for controlling phytopathogenic fungi.

Combining fungicide resistant biocontrol strains with chemical fungicides could results in decreased fungicide level in soils. This is the base of integrated pest management which cold be very effective, but less dangerous to the ecosystem and human health than the chemical control alone [3].

In some cases the biocontrol strains are also sensitive to the fungicide to be applied (e.g. to the frequently used fungicide benomyl); in this case resistant strains could be isolated by induced mutagenesis [20].

An effective pest control may require 10^5–10^6 propagula to be present in one gram soil in the case of *Trichoderma* and *Gliocladium* [2]. These high amounts of conidia to be present are not desirable either of ecological and economical or public health of aspects.

To solve this problem the biocontrol strains of *Trichoderma* and *Gliocladium* should breed for more effective.

Strains, which do not require a long inductive process for production of degradative enzymes, could be more effective biocontrol agents. Generally the biocontrol strains produce β-1,3-glucanases constitutively, some elements of chitinase systems require induction and the most effective proteases are also produced inductively [12, 21].

Only a limited number of publications deal with mycoparasitic strains obtained by mutagenesis. Some of these investigations report on random isolate tests following only mutagenetic treatments, others publish on obtaining fungicide resistant mutants which could be used in integrated pest management and only some of them deal with true targeted mutagenetic improvement of the enzyme secretion abilities of the strains [22–23].

The mutagenetic methods of breeding are worth to bigger attention as these strains could get registration more easily for on field use from environmental protection agencies than the strains, which are produced by protoplast fusion, transformation or via gene cloning.

Protoplast fusion is a quick and easy method for combining the advantageous properties of distinct promising strains. It was successfully applied for the breeding of *T. harzianum* biocontrol strains [24–25]. The parasexual cycle of *Trichoderma* has some interesting phenomenons, which were first published in *T. reesei* [26]. The parameiotic behaviour was later also published for *T. harzianum* and *T. viride* [27]. Protoplast could be easily produced from the biocontrol *Trichoderma* strains and their induced fusion resulted in genetic recombinants with elevated biocontrol abilities in many instances [28]. Many publications deal with the transformation methodology of *Trichoderma* and

Gliocladium strains. Some of the most frequently applied methods use fungicide resistance, others auxotrophic-complementing or acetamid-catabolitic genes for transformation [29, 67–68, 75–76]. Transformation for benomyl resistance interestingly never resulted in high transformation frequencies even if homologous system was used. The use of hygromycin B selection systems resulted in the highest transformation frequencies. These frequencies were even more elevated if spermin was present in the transformation mixture [29]. Many *Gliocladium* and *Trichoderma* strains are originally resistant to hygromycin B. In these cases the acetamidase or auxotrophic complementation methods could be used.

Many publications are dealing with the cloning of structural genes of enzymes important in the mycoparasitic process. Most frequently chitinase genes have been cloned [30–35].

In some cases protease [36] and a β-1,3-glucanase [37] genes were also successfully cloned from mycoparasitic strains.

The cloned genes in some cases with new constitutive promoters were transformed into *Trichoderma* strains. More efficient biocontrol strains could be found among the transformants [38].

There are many ecological factors which could disturb the effectiveness of biocontrol strains in soils: e.g. other *Trichoderma* strains [39], bacteria [40], low temperature [41] and ionic composition. A more intensive investigation of these fields is very important for the effective practical use of the wild type and the breeded strains.

In on field investigation the monitoring of the biocontrol strains in soils is also very important.

For these purposes resistance markers, RFLP analysis, DNA fingerprinting, PCR and RAPD techniques could be used efficiently [42–44].

Table I

Commercial biocontrol products for use against plant-pathogenic fungi

Biocontrol fungus	Product Name
Ampelomyces quisqualis	AQ10
Coniothyrium minitans	Contans
Fusarium oxysporum	Biofox C, Fusaclean
Gliocladium virens	SoilGard (Formerly GlioGard)
Phlebia gigantea	Rotstop, P.g. Suspension
Pythium oligandrum	Polygandron
Trichoderma harzianum and other *Trichoderma* species	Bio-Fungus, Binab T, RootShield, T-22G, T-22 Planter Box, Bio-Trek, Promote, Supresivit, Trichodex, Trichopel, Trichoject, Trichodowels, Trichoseal, Trichoderma 2000

Besides *Trichoderma* and *Gliocladium* species apathogenic *Fusarium* strains against phytopathogenic *Fusaria* [45], *Coniothyrium* strains against *Sclerotinia* [46] and *Ampelomyces* strains against powdery mildews [47] are used successfully for biocontrol, but few publications are dealing with their biochemical and genetic properties.

Many biocontrol filamentous fungi were patented and their registered on field use were permitted by the environmental agencies. Some important commercially available preparations are listed in Table I.

Myconematocides and mycoinsecticides

There are three types of nematophagous fungi. The first is able to produce trapping hyphae e.g. *Arthrobotrys* species [48], the conidia of second type are germinating on the surface of the nematode e.g. *Verticillium* [49] species, and in the third case the conidia are germinating in the intestinal system of the nematod e.g. *Harposporium* [50] species.

The trapping method of biocontrol is not reliable, as many environmental factors may disturb the differentiation of the trapping apparates [51].

An effective and widespread nematode biocontrol could be accomplished by the use of distinct strains of *Verticillium lecanii*, *V. chlamydosporum* and *Paecilomyces lilacinus* [52–53].

The most important factor of pathogenesis is the extracellular protease system both in *Arthrobotrys*, *Verticillium* and *Paecilomyces* species [54–56]. *Verticillium*, *Arthrobotrys* and *Paecilomyces* species besides their nematocidical effect, might also be effective against fungi and insects.

Many filamentous fungal genera contain insect parasitizing species. From them the species belonging to the *Beauveria*, *Metarhizium*, *Verticillium* and *Paecilomyces* are the most promising [79]. The effectiveness of the strains is related to the secretion of extracellular chitinases and proteinases; these enzyme systems are well characterized in the most strains [57].

The nematocide and insecticide strains were breeded by mutagenesis, protoplast fusion [58] and transformation [59–60]. In some cases structural genes of the degredative enzymes have been cloned [61–62].

There are possibilities for integrated control with chemical insecticides and fungicides by applying fungicide resistant strains.

The modern molecular methods, RFLP, PCR and RAPD for typing, identification and on field monitoring of strains are widely and successfully used [63–64].

Commercially *Beauveria bassiana*, *Verticillium lecanii*, *Metarhizium anisopliae* and *Paecilomyces lilacinus* are available. There are several products that contain *B. bassiana*, including Naturalis® and Mycotrol®.

Hungarian research activities

In Hungary research activities related to fungal biological control are mainly limited to the *Trichoderma* genus. In his pioneering works Vajna has determined the distribution of *Trichoderma* species in Hungary and described some antagonistic isolates [39, 65].

Manczinger in Department of Microbiology, Attila József University, Szeged, investigated the catabolic abilities of many Hungarian isolates and detected species within the species aggregates with the use of assimilation spectra [66], also he explored the transient nature of the somatic diploid state in *Trichoderma* following protoplast fusion [26]. Recently in the microbial ecological working group of this department new biopesticid strains were produced by mutagenic treatment with excellent antagonistic properties [23] and for some *T. harzianum* and *T. viride* strains genetic transformation systems were established [29, 67–68]. Naár in the Department of Plant Science, Esterházy Teachers Training College, Eger investigates the ecological behavior of *Trichoderma* strains in distinct soil types, the effect of heavy metals to biocontrol *Trichoderma* strains and the possibilities for integrated pest management with *Trichoderma* strains [69–72]. Turóczi and others in the Department of Plant Pathology, Plant Protection Institute, Hungarian Academy of Sciences, Budapest and in the Agricultural Biotechnology Center, Gödöllő, respectively, in collaboration investigate the molecular characterization possibilities of *Trichoderma* strains [73–74] and recently they successfully cloned and transformed back a chitinase gene in *T. hamatum* [75].

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SIGNAL TRANSDUCTION IN FUNGI – THE ROLE OF PROTEIN PHOSPHORYLATION

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Living cells are able to respond to the surrounding environment. As a first step in this process, membrane receptors react with an extracellular ligand. There are three main families of cell-surface receptors: (1) Ion-channel-linked receptors, (2) G-protein-linked receptors, and (3) Enzyme-linked receptors that either act directly as enzymes or are associated with enzymes. These enzymes are often *protein kinases* that phosphorylate specific proteins in the target cell. Through cascades of phosphorylations elaborate sets of proteins relay signals from the receptor to the nucleus regulating gene expression. There are two groups of protein kinases: tyrosine- and serine-threonine-specific protein kinases and there are *protein phosphatases* with specificity for the appropriate side chain to match each type of kinase. They can terminate an activation event reversing the phosphorylation caused by a protein kinase.

Receptors, effectors, second messengers, kinases, phosphatases and transcriptional regulators form *signal transduction pathways*, relaying a specific signal to the gene expression machinery or other target sites thus change the cell's behavior. One can recognize significant similarities in signal transduction pathways of lower and higher eukaryotes. E.g. all eukaryotes transduce external signals via a conserved module composed of three protein kinases, the *mitogen activated protein kinase (MAPK) cascade.* Another "classical" pathway includes G-proteins, adenylate cyclase, cAMP and protein kinase A. In this lecture we summarize some of the important fungal signal transduction pathways and briefly describe our own work in the field of *Neurospora crassa* protein phosphatases.

In eukaryotic cells the MAPK cascade lies in the heart of many signalling pathways. Three protein kinases that are highly conserved in all eukaryotes make up this module: MAPK (also known as extracellular signal-regulated kinase [ERK]), MAPK kinase, (MAPKK, also known as mitogen-activated, ERK-activating kinase [MEK]) and MAPK kinase kinase (MAPKKK, also known as MEK kinase [MEKK]). The budding yeast *Saccharomyces cerevisiae* has at least six identified MAPK pathways that regulate response to high or low osmolarity, pheromones, perturbation of cell wall integrity, sporulation and pseudohyphal growth. In *Schizosaccharomyces pombe* the pathways responding to pheromones and osmotic stress were characterized [1, 2].

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Adaptation to high osmolarity is mediated by the HOG (high-osmolarity glycerol) pathway in *S. cerevisiae* and the Sty1 (suppressor of tyrosine phosphatase) pathway in *S. pombe.* The PKC (protein kinase C) pathway is activated by low osmolarity in budding yeast. In both cases the MAPK cascade is activated, which regulates the expression of specific target genes via transcription factors. The direct target of the PKC MAPK cascade is a transcriptional activator (Rlm1) that may regulate the expression of genes for cell wall biosynthesis.

Salt tolerance and maintenance of cell integrity are regulated by associated pathways in yeasts. Two genes, *PPZ1* and *PPZ2*, encoding "novel type" Ser/Thr phosphatases were described, whose overexpression suppressed *Δmpk1* (MAPK) and *Δpkc1* mutations. Deletion of both genes resulted in a temperature dependent cell lysis defect, similar to that observed for MAPK cascade mutants. It was assumed that PKC pathway and *PPZ1/2* may have a common target responsible for the maintenance of cell integrity. *PPZ1* is also an important determinant of salt tolerance and caffeine sensitivity [3]. Functional homologues of Ppz1 with striking similarities were also cloned from *S. pombe* and *N. crassa.* All three proteins have a protein phosphatase 1-like C-terminal catalytic domain and an N-terminal domain, in which the first fifty amino acid residues exhibit certain degree of similarity.

The pheromone response pathway of *S. cerevisiae* mediates cell-cell interaction during mating. This is one of the best-understood signalling pathways in eukaryotes. Studies with this yeast pathway provided a framework for understanding the effect of growth factors on cell cycle progression in mammalian cells. The most important characteristics of this pathway are the following: (1) Peptide pheromones interact with serpentine receptors and initiate the response. (2) Receptors interact with heterotrimeric G protein that transmits the signal to downstream effectors. (3) MAPKs mediate the activation of a transcription factor (Ste12). (4) One of the MAPKs phosphorylates a cyclin dependent kinase inhibitor (CKI), leading to cell cycle arrest. (5) A component of the pathway (Ste5) acts as a scaffold that brings together cascade components and prevents cross talk. (6) Many of the target genes are necessary for cell and nuclear fusion and for cell cycle arrest [1, 2].

There are relatively few data on signal transduction systems operating in filamentous fungi. Germination of conidia is an important developmental event in fungal life. Extra- and intracellular cues trigger the major transitions that occur during this process. A protein likely to mediate the response to the signals activating conidial germination is the cAMP-dependent protein kinase A (PKA) [4].

Most steps in signal transduction pathways involve protein phosphorylation as a signal relaying mechanism. A signal, which was generated by extra- or intracellular changes has to be terminated after some time. Protein phosphatases are responsible for this termination event: dephosphorylate the target proteins reversing the action of protein kinases. Eukaryotic protein phosphatases are classified as Ser/Thr, Tyr and dual specificity phosphatases. Ser/Thr phosphatases are divided into four subclasses (PP1, PP2A, PP2B, PP2C) on the basis of their substrate specificity, metal ion dependence and inhibitor sensitivity. On the basis of their sequence-homology, PP1, PP2A and PP2B belong to the PPP family, whereas PP2C and the related mitochondrial pyruvate dehydrogenase phosphatase are members of the PPM family [5]. Among fungi, protein

Ser/Thr phosphatases are well known in the yeasts, considerable information is available in *N. crassa* and *A. nidulans*, but little is known about other fungal species. Most of the "classical" type fungal protein phosphatases play an indispensable role in the regulation of cell cycle, growth, and morphogenesis.

More recently, many other phosphatases have been discovered that do not fit into the classical categories, although they show considerable homology to some of them. These are the "novel type" protein phosphatases. They are grouped according to the sequence similarity they show to PP1 and PP2A. PP5 seems to be a third, not very closely related subfamily [6]. Three genes encoding classical type (PP1, PP2A, PP2B) and two encoding novel type phosphatases (PPT/PP5 and PPZ-like) were cloned from *N. crassa* to date.

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CHROMOSOMES, KARYOTYPE ANALYSIS, CHROMOSOME REARRANGEMENTS IN FUNGI

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In this review the organization of fungal chromosomes and the methods used for karyotype analysis are briefly summarized. The role of chromosome rearrangement, supernumerary chromosomes and repeated DNA sequences in the genetic change of fungi is evaluated.

Fungal chromosomes

The genome organization of fungi is typical of eukaryotic systems. The chromosomes are composed of DNA, as well as histone and non-histone proteins. The size of the haploid nuclear genome of fungi ranges between 15–60 Mb (megabase pairs), the number of chromosomes varies from three to a few dozens depending on species. The chromosomes are organized into nucleosomes and contain a localized centromere-kinetochore complex (which is the nucleating site for the spindle microtubules), as well as telomeres (that maintain the integrity of the chromosomes and help in directing chromosome pairings). A typical spindle mechanism is the driving force of mitosis divided into a normal prophase, metaphase and a non-synchronous anaphase-telophase taking together 6 min. under optimal conditions. The meiotic process – like in other eukaryotes – includes DNA replication (pre-meiotic S phase), as well as one reductional (meiosis I) and one equational (meiosis II) division. In the majority of fungi the meiotic S phase precedes karyogamy.

Karyotype analysis

Chromosome numbers and sizes (the karyotype) can be determined by light and electron microscopy as well as by pulsed-field gel electrophoresis. The latter technique – used for the first time in 1984 to separate yeast chromosome-sized DNAs [1] – raised a

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revolution in fungal genetics. Since then, numerous electrophoretic karyotypes for a wide range of fungi have been published. Hungarian workers provided the first comparative karyotype analysis for selected species of several economically important genera, like *Fusarium, Mucor, Phaffia* and *Trichoderma* [2–5]. Cloned DNA fragments may be assigned to electrophoretically separated chromosomes by Southern hybridization, therefore molecular karyotyping allows direct gene mapping and identification of linkage relationships. Chromosome-specific libraries may also be prepared after separation of chromosome-sized DNAs. Chromosome-specific probes may be used to recognize homologous chromosomes within polymorphic chromosome patterns. Karyotype analysis may provide additional information for the appropriate taxonomic affiliation of controversial species.

Chromosome polymorphisms

Genomic rearrangements play an important role in the evolution of fungi [6]. Such rearrangements may result in chromosome polymorphisms found to be accompanied by phenotype differences including morphology [7], antibiotic production [8] or host range [9].

Karyotype analysis revealed high levels of variation in chromosome sizes and/or numbers within most species of fungi. According to a persuasive hypothesis proposed by Kistler and Miao [10] the extent of intraspecific karyotype polymorphism is inversely correlated to the frequency of meiosis. Asexual fungi have more extensive polymorphisms than sexual ones, which frequently undergo meiosis, an efficient process selecting against detectable chromosome aberrations. Both meiotic and mitotic processes may lead to chromosome polymorphisms and genomic stress can also account for karyotype variability. Repeated DNA sequences may also generate chromosome polymorphisms (see below).

For asexual plant pathogenic fungi, variation in chromosome sizes and numbers is an efficient non-Mendelian mechanism of genetic change resulting in the emergence of virulent races or pathotypes.

Supernumerary chromosomes

The occurrence of 'extra' chromosomes is very common in fungi. Various terms (B chromosomes, dispensable chromosomes, mini-chromosomes and supernumerary chromosomes) have been used for naming these structures. According to a recent review of this topic [11] the term 'supernumerary chromosomes' was suggested as a preferred name. A chromosome could be qualified as a supernumerary one, if it is not present in one or other viable individual of a given species. In sexual fungi the supernumerary chromosomes are inherited by non-Mendelian transmission, their absence is non-lethal. In spite of this seemingly dispensable nature of these elements there are examples for the long-term mitotic stability of supernumerary chromosomes [12, 13].

The movement of supernumerary chromosomes in pulse-field gels, their structural stability during mitosis and their positive hybridization to telomeric DNA probes indicate strongly that these elements contain centromeres and telomeres. Information on DNA composition of these chromosomes is limited to a few species. A mixture of repeated as well as low- and single-copy DNA sequences have been identified in supernumerary chromosomes of *Colletotrichum gloeosporioides* and *Nectria haematococca* [14, 15]. In our experiments mini-chromosomes in *Fusarium sporotrichioides* (some of them were undoubtedly supernumerary ones) were found to constitute a mosaic composed of repeated DNAs dispersed throughout the genome and unique sequences. These chromosomes were most probably formed by major genomic rearrangements and were preserved during the evolution of the species. The mosaic structure was identified in all genetically isolated strains of the fungus indicating that the mini-chromosomes – although lacking any coding region – could be useful components of the genome [12].

The biological function of supernumerary chromosomes is cryptic except for two notable cases. The 1.6 Mb dispensable chromosome of *N. haematococca,* a facultative plant pathogen carries a gene *(PDA1-1)* encoding the enzyme, pisatin demethylase, which catalyses the detoxification of pisatin, an antifungal phytoalexin of pea. Strains that retained this supernumerary chromosome show a high level of pisatin detoxification activity and are highly virulent on *Pisum sativum* [16]. A supernumerary chromosome of *Cochliobolus carbonum* race 1 isolates carries genes responsible for the synthesis of HC-toxin, a host specific cyclic tetrapeptide which causes disease symptoms on maize [17]; strains lacking this chromosome (and the genetic determinants of HC-toxin production) are avirulent on maize.

Repeated DNA sequences

The fungal genome harbors interspersed repetitive elements, that may be mobile or stably integrated elements. The mobile elements are classified as (i) transposons, which transpose through DNA copies and have an open reading frame encoding transposase activity, (ii) retrotransposons, which duplicate via RNA intermediates and have retrovirus-like structures and (iii) retroposons, which also use RNA intermediates during replication but lack the structural features of retroviruses. The fungal retroposons are further sub-grouped into either as short interspersed nuclear elements (SINEs) or long interspersed nuclear elements (LINEs). The first fungal transposon, Fotl discovered in the plant pathogen *Fusarium oxysporum* was found to share structural similarities with the class of Tcl-like elements [18]. Of the retrotransposons, Ty4 present in *Saccharomyces cerevisiae* was the first element subjected to complete molecular analysis [19]. *CfT-1* isolated from *Cladosporium fulvum* was a pioneer example of the LTR-type (long terminal repeat) retrotransposons in filamentous fungi [20]. A short interspersed nuclear element (Mg-SINE) was isolated from the rice blast fungus, *Magnaporthe grisea* [21], whereas LINE-like elements were for the first time detected in *Neurospora crassa* [22]. Numerous other examples of the mobile repetitive sequences have subsequently been reported and some of these sequences proved to be functional transposable elements.

Of the stably integrated (non-rDNA) repeated DNA sequences in fungi one class is characterized as telomere-associated elements, present on all or almost all chromosomes. Such telomeric sequences, like Ca7 [23] or the Rel-2 element [24] of *Candida albicans* are considered essential for proper chromosome maintenance. The second class of the stably integrated non-rDNA repeats is represented by the *CARE-1* element [25] and the RPS sequences cloned from *C. albicans* [26]. These elements are not present on all resolvable chromosomes. *CARE-1* was identified as a non-telomeric, probably non-essential sequence with a copy number ranging between two and twelve per haploid genome, depending on strain, whereas the RPS 1 element, represented by some 80 copies was found to be present on all but one chromosome of *C. albicans.*

In our laboratory a moderately repetitive DNA element has been cloned from *Fusarium poae a* strictly asexual fungus, which is a secondary invader of small-grain cereals. The element, named ZIT1 selectively hybridized to the polymorphic, 1.0–3.7 Mb chromosomal region of the fungus, but no hybridization signal was observed on any of the large chromosomes. ZIT1 shares only a moderate level of similarity to *gag* genes of fungal retrotransposons, which was mainly due to a small cysteine-reach motif, known as zinc-finger DNA binding domain. No significant homology was found with any other published nucleotide sequence. The novelty of this element is that its distribution is restricted to the polymorphic chromosome region of *F. poae.* ZIT1 could be a remnant of a formerly active foreign DNA that had been eliminated from the large chromosomes by a defensive mechanism suitable to prevent the disruption of genes encoding vital functions [27].

Repetitive DNA sequences are regarded to be important determinants of genetic variability in clonal eukaryotic organisms, like most hyphomycete fungi in which recombination through sexual or parasexual cycles is rare or, in fact absent. Transposable elements have been shown to cause chromosome rearrangement and various aberrations like deletions, inversions and translocations. Repeated DNA sequences, even if they are unable to transpose may generate DNA rearrangements through recombination among their homologous sequences scattered throughout the genome.

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*Due to spatial limitations of this review the coverage of literature is only partial. Non-intentional omissions could not be avoided.

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EXTRACHROMOSOMAL GENETIC ELEMENTS IN FUNGI

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All fungi like eukaryotes possess mitochondria, which are the sites of the oxydative phosphorylation. As eukaryote evolution depends on oxygenic atmosphere, these organisms are primarily aerobic. Except a small group of strict anaerobes (those which lost the capacity of oxydative pathways living in special milieu in association with rumen of grass-eating animals) all fungi can utilize various compounds as carbon sources via oxidative phosphorylation pathways resulting in high energy yield. Certain groups of fungi – i.e. most of the yeasts – under anaerobe conditions, are able to supply themselves with lower levels of fermentation energy, too exhibiting a slow growing capacity utilizing the same amount of carbon source. The mutation of mitochondrial genome or mitochondrial functions encoded by nuclear genes of these fungi might result in a so-called *petite* phenotype producing small colonies on solid media due to their slow growing capacity. These mutants can utilize only fermentable carbon sources. Filamentous fungi have only limited possibilities to produce such phenotypes. Except *Zygomycetes* (where the shortage of oxygen induces dimorphic transitions) filamentous fungi can grow and develop their vegetative and sexual reproductive structures only in aerobe milieu. However among *Neurospora* species there are several mitochondrial mutations resulting in morphological phenotypes. These are due to the lower energy level provided by the reduced capacity of cytochrome-oxidase enzymes. These mutants (e.g. *poky*, *stopper*) can be considered as *petite* analogues. The complete loss of mitochondrial functions – such as *rho zero* character in yeast – cannot be survived by filamentous fungi. *Podospora anserina* and some of its close relatives exhibit a so-called *senescence* phenotype, which means that the growing hyphae in the youngest part of the colonies stop growing and start to die within a short period of time. This phenomenon – discussed below – is also connected to reduced function of mitochondria.

The first part of this paper gives a short overview of the genetic organization of mitochondria of fungi, based on the most recent data of three filamentous fungi: *Aspergillus nidulans*, *Neurospora crassa* and *Podospora anserina*. Their data are compared to those of the well-characterized *Saccharomyces cerevisiae*. In the second part we summarize what we know about other extrachrosomal elements, such as DNA plasmids of

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various origins and structures, and dsRNAs or virus like particles (VLP). Also discussed are their roles and/or putative functions in the life of the fungi.

Mitochondrial DNA

The fungal mitochondrial genomes vary in size between 17.6 and 172 kb represented by *Schizosaccharomyces pombe* and *Agaricus bitorquis*, respectively [1, 2]. This range puts the fungi in the middle in respect of sizes of mtDNAs between animals and plants. Animals (including humans) exhibit the smallest size of mtDNAs between 14–36 kb (but most of them range in size between 16–18 kb). These very compact genomes proved to be intronless, and size differences can be due to the variability of their replicating (*ori*) sequences. Plants possess the largest mtDNAs (from 218 to 2400 kb). Despite their large sizes they have no additional functioning genes and their intron content is abundant resulting in very complex RNA editing [1, 3].

MtDNA organisation in fungi has been reviewed by several authors [1–11]. Despite some organisational differences among various groups of fungi several generalisations can be made. The configuration of mtDNAs is considered to be double stranded circular, but recently there have been some doubts about this general statement. Based on various molecular techniques verifying the structure of mtDNAs they are now considered to have linear variations especially during their replication [12].

The most common mitochondrial functions encoded by mitochondrial sequences are as follows: 1) A part of the translation apparatus with minimum set of 22 tRNAs and ribosomal RNAs: *rns* and *rnl*, small and large subunits of rRNAs, respectively; one protein of the small ribosomal subunit (*var1p*); a 9S RNA component of mitochondrial RNase in *S. cerevisiae*; 2) membrane associated proteins responsible for catalyzing oxidative phosphorylation: seven subunits of NADH dehydrogenase complex (*ndh*) (except *S. cerevisiae* and most of the yeasts); apocytochrome b (*cob*) (cytochrome b, c1 complex); three subunits of cytochrome c oxidase (*cox*) and ATP synthase subunits 6, 8 and 9 (*atp*) [1, 4, 6, 8, 11]. Additionally URFs are present with unknown functions.

As only a limited number of mitochondrial proteins (about 8–10% of them) are encoded by mitochondrial genes it follows that all the others related to mitochondrial functions and structural elements have to be encoded by the nucleus. Distribution of functioning genes of ATP-ase subunits is a good example for co-operation between mitochondria and nuclei. *Podospora anserina* has no mitochondrially encoded *atp9* gene. Both in *S. cerevisiae* and *S. pombe* the *atp9* subunits are active in mitochondria, but there are no such functioning nuclear genes of these yeasts [1, 11]. *A. nidulans* possess *atp9* genes both in nuclei and mitochondria. It seems that the mitochondrial *atp9* gene proceeds transcription, but translational product is not produced especially during the vegetative growth similar to *N. crassa* [4, 6]. Some of the nuclear encoded proteins can enter mitochondria, across the specific double membrane boundaries. Studies on these processes e.g. the role and functions of mitochondrial heat shock proteins, chaperons have become one of the most exciting fields of research in modern cell biology [13].

Mitochondrial introns make mosaic structure of the functioning genes, they have self-splicing activity and usually contain open reading frames (ORF). Two main groups of introns were distinguished on the basis of self-splicing excision processes. These group I and group II introns are characterized by specific secondary structures which strongly determine their splicing processes. The introns generally carry ORFs with endonuclease, or reverse transcriptase activity in the cases of group I and group II introns, respectively. There ORFs are responsible for mRNA maturing processes (maturase activity) and the mobility of whole introns or only their ORFs [14]. Of course a great part of the splicing of mitochondrial primary transcripts *in vivo* is dependent on nuclear encoded maturases. While group I introns encode endonucleases, *rnl* intron's ORF of *N. crassa* encode a ribosomal protein (S5).

Except *Zygomycetes*, most of the fungi, possess mtDNAs with exceptionally high AT-rich sequences when compared with the nuclear ones (this provides an easy separation of them by density-gradient ultra centrifugation) [15]. Among the ascomycetous yeasts *S. cerevisiae* possesses relatively large mtDNA (75 kb) but only about one-fifth of the total mt genome represents exon sequences. The size of mtDNAs can be influenced by the number of introns in specific genes in addition to the intergenic spacer regions [9–11]. In the long non-coding spacers (intergenic sequences) of *S. cerevisiae* the AT distribution ranges 85% but even coding regions can contain about 75% AT sequences. However, the total GC content is only about 18%; large numbers of short GC clusters could be identified in intergenic spacers and in some functioning regions of it, such as *ori/rep* sequences (their numbers are at least 8, scattered around the whole genom). GC clusters can be classified into different groups, can form short duplications and direct repeats and have function in intramolecular recombinations and excision of *petite* mtDNA.

The mtDNAs of filamentous *Ascomycetes* are also AT-rich as compared with coding regions. The intergenic regions are highly variable, their sequences are species specific. In the mt genomes of well-studied *N. crassa* and *P. anserina* dispersed repeated elements have been detected. These short sequences with high GC content are present in several dozen copies: e.g. *Pst*I palindromes in *Neurospora* [1, 6], MUSEs elements in *P. anserina* [20]. These special motifs are thought to be involved in intramolecular processes of mitochondrial sequences.

Comparing mitochondrial functions and gene content with the sizes of mtDNAs in several species some generalizations can be made.

Aspergillus nidulans has mtDNA with the size of 31.5 kb, nearly the full sequence is known. Its tRNAs are located in two big clusters except seven of the total 28 tRNA genes. All seven *ndh* subunits are present, beside cob and three *cox* subunits (*cox1*, *cox2*, *cox3*), all the three *atp* subunits (*atp6*, *atp8*, *atp9*) are also present in mtDNA. A nuclear gene encodes the *atp9* function, too. *A. nidulans* possess five group I introns, their locations are as follows: one-one is in cob and *rnl* regions and three are situated in *cox1* subunit, together they form a mosaic gene structure. Mitochondrial drug resistant mutants are known such as oligomycin-, chloramphenicol-, mucidin resistance and cold-sensitivity [4]. MtDNA polymorphisms have not been reported even between vegetative incompatible *A. nidulans* isolates. Only its closely related species (*A. quadrilineatus* and *A. echinulatus*) exhibited slight intronal variations [16]. Black *Aspergilli* (sectio *Nigri*) exhibit a wide range of mtDNA polymorphism [17–19]. Beside the existing great intra-

and interspecific variations of RFLPs the sizes of the mtDNAs also differ greatly due to the actual intron content.

Neurospora crassa laboratory strain (Oak Ridge 74-OR-23-1A) bears 62 kb mtDNA. Its 27 tRNAs are clustered similarly to *A. nidulans*. The gene content in other respects is also similar to it, but *N. crassa* possess five URFs (*A. nidulans* has one URF: unidentified reading frame) and 9 introns, too. The *cox1* region is intronless, *cob* and *ndh5* genes have two introns, one can be detected in each of the *ndh1*, *ndh4*, *ndh4l* and *atp6* subunits and every one of this intron bears a single ORF. The intron situated in *ndh3* subunit has two ORFs due to frame shift transcription. All introns of laboratory strains are proved to be group I types encoding endonucleases as maturase functions. Similarly to *A. nidulans* small numbers of promoter sequences (five) have been identified, this resulted in a limited number of long pre-mRNAs. This finding is contrary to those of the yeasts where more promoters can be recognised. No drug resistant mutants could be isolated, but some morphological mutants could be produced. The phenotypes of *poky* or *stopper* mutant strains are due to the reduced level of oxydative phosphorylation. Ethidium-bromide, acriflavine, nitroso-guanidine derivatives can induce such mutant phenotypes, deleting specific parts of mitochondrially encoded enzyme genes resulting in various levels of deficiency in cytochrome *aa3* and b, but cytochrome c is usually not effected by these treatments. All three types of morphological mutants can be identified. Two of them cannot be suppressed by nuclear gene functions, in these cases the energy requirements are generated by the cyanide insensitive alternative respiration system. Among wild isolates of *Neurospora* strains considerable mtDNA polymorphisms have been observed [6, 7]. In a wild-type strain a group II type intron with reverse transcriptase activity has been detected in *cox1* gene [21].

Podospora anserina: mtDNAs vary in size between 80–102 kb. Well-studied "s" and "A" heterokaryon incompatible strains bear 94.192 and 100.314 bp, respectively. Nearly all of the *mt* genomes are sequenced. The laboratory strains possess 33 group I type and 3 group II type introns. About 60 kb from the total 95 kb mtDNA are covered by intron sequences. In particular the *cox1* gene is interrupted with at least 16 introns, therefore this mosaic gene itself represents 22.9 kb size. *P. anserina* mitochondrial DNA codes for 25 tRNA genes, but has no *atp9* subunit [8]. So-called mitochondrial ultra-short invasive elements (MUSE) have been detected (GGCGCAAGCTC), thought to be the sites of intramolecular recombinations [20]. Among the wild isolates the mtDNA polymorphism are exceptionally rich due to their variable intron contents [22].

Senescence phenomenon (progressive loss of growth potential ends in death) has been extensively studied in *P. anserina* where this is a normal event in wild type strains [23–26]. Senescence is correlated with the appearance of circular multimeric plasmid like elements in mitochondria. Senescence DNAs (senDNA) are amplificates of specific mobile intron sequences. When the introns are excised they are called plasmid-like DNA. There are several types of senDNAs, α, β are the most common ones. The α-senDNA derives from the first intron of cox1 gene by the precise excision of it, therefore it always has the same monomeric size around 2.5 kb. This intron can be grouped with class II introns, which have ORFs coding reverse transcriptase-like proteins. The size of putative α-protein is about 96–100 kD. Various natural senescence cultures may exhibit different α-introns, but all of them proved to be reverse transcriptase homologues. Following

excision the amplified α-introns can rearrange the mitochondrial genome. As only about 30 kb of the total mtDNA remains in original structure, this rearrangement could result in reduced activity of cytochrome a3 and b proteins. Excised β-senDNAs are larger than the α-introns, they differ in length but share a common sequence of 1.1 kb which is located between the 3' end of *cox1* and *nadh4l* genes. β-senDNAs can be classified as group I introns. Their common region has three ORFs X, Y and Z, the last encodes for a glycine-rich ssDNA-binding protein. The reason of senescence is the specific structure of the mtDNA itself, but the occurrence of this phenomenon depends on many other factors. There is a special nuclear control, e.g. in *grisea* and *vivax* double morphological mutants the senescence phenotype has never been observed, and could not occur in liquid phase. The amplified sequences of mobile introns result in a decreased energy level, by the rearrangement of functioning *mt* genome that induces a complex ageing process. This process can be suspended by ethidium-bromide treatment, probably by the selective inhibition of the excised intronal DNAs. Some natural occurring "rejuvenation" events have also been observed, e.g. in presence of a linear mitochondrial plasmid (pAL2-1) the senescence cultures turn into juvenile ones.

In *Neurospora* besides the *poky* and *stopper* mutant phenotypes the real senescence phenomenon can be studied in wild type plasmid bearing strains [27–29]. One-third of natural isolates of *N. intermedia* from the Hawaiian island of Kauai exhibit senescence caused by the so-called *kalilo* plasmid (or *kal*DNA) its size is about 9 kb. This is a linear protein primed replicating plasmid that has no homologous region with *mt-* or *nuc*DNAs. It has two ORFs encoding for DNA and RNA polymerases, respectively. This element could exist either in a free form (AR-*kal*DNA) or integrated into various sites of the *mt* genome (IS-*kal*DNA), most frequently into the LrRNA gene. In contrast to *Podospora* the amplified free plasmids integrate into the intact *mt*DNAs. The senescence can be observed only in the presence of this plasmid, but the rearrangement of mtDNAs itself caused by the integration does not result in death. Some nuclear controls are also involved in this process. Another linear mitochondrial plasmid is the so-called *maranchar* derived from an Indian isolate of *N. crassa*. Although *mar*DNA and *kal*DNA do not show homology to each other, they have the same basic structure and use the same mechanism for replication. Both plasmids have terminal inverted repeats (TIR) which play a role in the integration. In contrast to *kalilo* and *maranhar* of *Neurospora*, linear plasmids of other filamentous fungi are not associated with senescence.

In *Aspergillus amstelodami* the so-called *ragged* (*rgd*) mutant exhibits a senescence-like phenotype. Various regions of mtDNAs are excised and amplified and form head-tail repeated circular concatamers. In the presence of these molecules the level of oxydative phosphorylation decreases. In contrast to *Podospora* the *A. amstelodami rgd* strains contain intact mitochondria, too [30].

Fungal plasmids

Senescence, as it is demonstrated above, involves the presence of plasmids or plasmid-like elements as liberated mobile introns. These plasmids belong to the group of

mitochondrial plasmids because of their location. They are called defective plasmids or defective sequences owing to causing progressive loss of growth. Most of the fungal plasmids are neutral they do not result in any phenotypical changes in the host strains. In contrast to the mostly circular dsDNA plasmids of bacteria the fungal ones are quite different resulting in various phenotypic consequences. They differ in their basic structures, types of nucleic acids, their origin and location, and finally in their functions (they could be cryptic or responsible for certain phenotypes) [27, 28, 31, 32].

Plasmids of mitochondrial origin occur most frequently among filamentous fungi. These plasmids can be classified into the following groups:

1) mtDNA origin defective sequences: as sen-plasmids in *Podospora*, kalilo-maranhar-plasmids in *Neurospora*.

2) so-called true mitochondrial plasmids having no homology to *mt-* or *nuc*DNA. This group involves circular and linear plasmids.

Well-known circular plasmids were described from various natural isolates of *Neurospora*. Among them *Mauricewille* and *Varkud* were studied in detail, they originated from *N. crassa* and *N. intermedia* with nearly the same size of 3.6 and 3.7 kb in monomeric conditions, respectively. They exhibit a high level homology, having nearly common ORFs encoding a reverse transcriptase, which is a typical class II intron characteristic. These plasmids replicate thorough mRNA intermediates, they also bear class I type intron-like sequences, and *Pst*I palindromes which are characteristic in *mt*DNA, too. Various origin of *N. tetrasperma* strains possess other homologous group of circular plasmids e.g. *surinam*, *hanelei*, while *fiji* plasmid from *N. intermedia* also belongs to this group with their sizes of around 5 kb. *LeBelle* plasmid also derived from a *N. intermedia* strain from another geographic location representing another homologous group. This is only one circular *mt* plasmid, which shares homologous regions with the *mt*DNA. The fourth homologous group of *Neurospora* circular elements the so-called VS-plasmids (*varkud satellit*) have relatively small sizes varying between 1.8 and 2.6 kb. VS-RNAs are transcripts of VS-plasmid exhibiting a self-splicing activity *in vitro*. The presence of these plasmids cannot be correlated directly with any phenotypes, but as mobile retroelements are able to induce rearrangement of genomes resulting in genetic instability during the vegetative growth. Some other practically important fungi as *Cochliobolus heterostrophus*, *Trichoderma viridae*, *Ophiostoma novo-ulmi* also possess circular mitochondrial plasmids. Except the last one no phenotypic character appears to be present in these elements.

Linear mitochondrial plasmids of fungi have a common structure called: protein primed replicating elements. They have terminal protein (TP) at the 5' end of the genome and terminal inverted repeats (TIR) with different sizes. The best studied of this type are *kalilo* and *maranhar* resulting in senescence, but many others are cryptic. *Claviceps purpurea*, *Ascobolus immersus*, *Ascosphera apis*, various *Fusarium* strains and basidiomycetous *Lentinula edodes*, *Agaricus bitorquis*, *Pleurotus ostreatus*, *Rhizoctonia solani* contain linear protein primed plasmids without real phenotypic consequences. Their sizes vary between 1.1 and 10 kb. In presence of plasmids some *Fusarium* strains do not produce trichotecin type mycotoxins probably due to the decreased energy level caused by plasmids resulting in inability of epoxidation step that would be required during mycotoxin synthesis. The pAL2-1 plasmid of *P. anserina*, which results in rejuvenation in

senescence cultures, also belongs to this class of plasmids [32]. True slime mould *Physarum polycephalum* has mitochondrial linear plasmids with an essential function. These plasmids direct fusion of mitochondria during sexual mating and also direct the mtDNA recombination between parental strains which is a unique characteristic among the lower eukaryotes [33].

Linear protein primed replicating plasmids can also be localized out of mitochondria either in cytoplasm or in nuclei. The best example for cytoplasmatically localized linear plasmids are *Kluyveromyces lactis pGKL1* (8.9 kb) pGKL2 (13.4 kb) which are responsible for killer phenomenon in this species. Details are presented in an other paper [34].

The well-studied 2 μ plasmid of yeast represents a completely different type of such extrachromosomal elements, they are circular and located in the nucleus. Until now it is the best eukaryotic vector for transformation, its ARS elements are frequently used in shuttle constructions. Its completely sequenced gene's functions are known, but their role in host cells has not yet been elucidated.

dsRNAs and VLPs in fungi

Most of the fungal viruses are dsRNAs containing VLPs. The majority of them have no influence on the fitness of the fungal growth (in contrast to the first discovered fungal virus causing so-called "*la France disease*" symptoms in *Agaricus bisporus*). They are so-called endogenous, non-pathogenic viruses, their presence do not result in any advantage or disadvantage for host cells. They can be transmitted under experimental conditions, in nature they spread via cytoplasm fusion and are inherited maternally in general. Taxonomically the dsRNA fungal viruses can be grouped into three families. The best-studied members of *Totiviridae* is the so-called killer virus (or RNA killer plasmids) of *Saccharomyces cerevisiae* [35]. It consists of two linear dsRNAs, one of which is responsible for toxin production and carries a gene for immunity (MdsRNA). The other RNA (L-dsRNA) is a 4579 bp molecule with two long ORFs. ORF1 encodes for a 76 kD viral particle major coat protein. ORF2 occupies the 3' part of the L-A positive strand and overlaps ORF1 in –1 frame by 130 bases, and apparently encodes for an RNA-dependent RNA-polymerase, similar to that of (+)ssRNA viruses. Similar RNA bearing killer phenomena were described in connection with some other ascomycetous and basidiomycetous fungi e.g. *Ustilago maydis*, and *Cryptococcus* species. The members of the *Partitiviridae* family became well known as viruses of the *Penicillium chrysogenum*-group. Some other ascomycetous fungi also possess cryptic dsRNA viruses with segmented genomes e.g. *Gaeumannomyces graminis* and many *Aspergillus* and *Penicillium* species. The functions of these viruses are not known but there are some hypotheses that they might influence the gene expression of host as effector molecules responsible for silencing and co-suppression [36]. *Cryphonectria parasitica*'s hypovirus is the most studied member of the *Hypoviridae* family where the viruses are not always encapsidated. This fungus is a causative agent of chestnut blight and has killed millions of chestnut trees in America. Natural virus transmission is inhibited by vegetative

incompatibility, therefore its possible application as a biocontrol agent has not yet been successful. The molecular background of hypovirulence has been extensively studied. Virus free *C. parasitica* transformed by cDNA of hypovirus can induce phenotypic changes characteristic to hypovirulence strains. Hypovirulence is attributed as a loss of aggressive extracellular enzyme activities of the pathogen fungus [37].

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IMPACT OF YEAST GENETICS AND MOLECULAR BIOLOGY ON TRADITIONAL AND NEW BIOTECHNOLOGY

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Developments in yeast genetics, biochemistry, physiology and process engineering provided bases of rapid development in modern biotechnology. Elaboration of the recombinant DNA technique is far the most important milestone in this field. Other molecular genetic techniques, as molecular genotyping of yeast strains proved also very beneficial in yeast fermentation technologies. *Saccharomyces cerevisiae* is the most exploited eukaryotic microorganism in biotechnology but non-*Saccharomyces* species are becoming more and more important in the production of perfectly translated heterologous proteins.

Importance of yeasts in traditional biotechnology is enormous. In food and beverage fermentation processes a wide range of yeast species are playing a role, but *Saccharomyces cerevisiae* is undoubtedly the most important species among them. New biotechnology is aiming to improve these technologies, but besides this, a completely new area of yeast utilization has been emerged, especially in the pharmaceutical and medical areas. Without decreasing the importance of *S. cerevisiae*, numerous other yeast species, e.g. *Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Schizosaccharomyces pombe* and *Yarrowia lipolytica* have gained increasing potentialities in the modern fermentation biotechnology.

Developments in yeast genetics, biochemistry, physiology and process engineering provided bases of rapid development in modern biotechnology, but elaboration of the recombinant DNA technique is far the most important milestone in this field. Other molecular genetic techniques, as molecular genotyping of yeast strains proved also very beneficial in yeast fermentation technologies, because dynamics of both the natural and inoculated yeast biota could be followed by these versatil DNA-based techniques.

Impact of yeast genetics on alcoholic beverage technologies

Roots of classical yeast genetics go back to the early work of Lindegreen in the 1930s, who studied thallism, sporulation and inheritance of wine yeast strains belonging to *S. cerevisiae*. Consequent mutation and hybridization of heterothallic *S. cerevisae* strains resulted in the discovery of life cycle and mating type system, as well as

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construction of the genetic map. Elaboration of induced mutation and controlled hybridization of yeast strains opened up new possibilities for the genetic analysis of technologically important properties and for the production of improved industrial strains, but a big drawback was the widely different genetic properties of laboratory and industrial yeast strains. Genetic analysis and mapping of industrial strains were generally hindered because of homothallism, poor sporulation and/or low spore viability of brewing and wine yeast strains [1, 2]. In spite of this there are a few examples of the application of sexual hybridization in the study of genetic control of important technological properties, e.g. sugar utilization, flocculation and flavour production in brewing yeast strains [3], or in the improvement of ethanol producing *S. cerevisiae* strains [4]. Rare mating and application of karyogamy deficient (*kar*$^-$) mutants also proved useful in strain improvement [5].

Hybridization by protoplast fusion is circumventing most of the difficulties arising from polyploidy, homothallism and non-mating of the majority of industrial strains. Novel genetic properties can be effectively transferred to industrial yeasts from strains belonging to the same or closely related species (e.g. *S. cerevisiae* and *S. bayanus*). New wine yeast strains were constructed by fusion of genetically different wine yeast strains, when natural genetic markers and mitochondrial mutations were used in the selection of hybrids [6]. An other example is the construction of dextrin fermenting brewing yeast strain when the glucoamylase *(STA)* gene was transferred from an auxotrophic mutant of *S. cerevisiae* var. *diastaticus* into a lager yeast strain [7]. Karyotype analysis of hybrids and mitotic segregants obtained proved to be very useful in the selection of those descendants which harboured the most chromosomes of the original brewing yeast strain. A major drawback of the method is, however, that undesired properties are generally also transferred, so a labourious, time-consuming postselection work is needed. Cytoplasmic genetic determinants, as killer dsRNA molecules can also be easily introduced into industrial strains by induced fusion of protoplasts without transferring chromosomal genes [7].

Application of molecular typing methods in identification, classification and population studies of biotechnologically important yeasts

Yeast genome consists of chromosomes and mitochondrial DNA, sometime plasmids and viral nucleic acids are also found in the cells. Number and organization of chromosomal sets are typical for distinct species, while polymorphism of homologues chromosomes reflects difference or identity of strains belonging to the same species. Electrophoretic karyotyping is the most promising method in karyotype analysis, especially in industrial strains belonging to the Saccharomyces genus. Reproducable chromosomal fingerprints are obtained when intact chromosomes are separated under identical conditions of electrophoresis. Combination of electrophoretic karyotyping and Southern hybridization of special genes, Tamai and cowerkers [8] showed that the bottom fermenting brewing yeast, *S pastorianus* has two types of chromosomes, *S. cerevisiae*-type and *S. bayanus*-type, and that these chromosomes co-exist independently without undergoing reciprocal rearrangements. *S. pastorianus* is thus a natural hybrid of *S. cerevisiae* and *S. bayanus* as was thought earlier by Vaughan Martini and Kurtzman [9].

Population kinetics of yeasts responsible for alcoholic fermentation of must depend on specific factors such as climate conditions, soil, wine grape varieties and also viticultural and enological practices used.

Electrophoretic karyotyping in combination with mitochondrial restriction fragment length polymorphism (RFLP) and PCR amplification of Ty elements were used by Versavaud and cowerkers [10] to study the variability, phylogenetic affinities and biogeographical distribution of wild *S. cerevisiae* enological yeast strains in a natural wine producing district, Charentes (France). They found only limited correlation between geographical location and genetic affinity, but one strain seemed to be distributed over the entire area surveyed.

In collaboration with a Slovenian research group we made comparative analysis of indigenous yeast biota on vine grapes and studied the yeast population kinetics during spontaneous and induced fermentation of "refosk" musts in selected cellars in Costal and Carst regions of Slovenia during two subsequent seasons. Among more than 500 isolates tested, extremely high inter- and intraspecific heterogeneity was observed on the basis of chromosome length polymorphism (CLP) of yeasts isolated from vineyards during different fermentation stages and also in starter culture induced fermentation. Only limited correlation was found between indigenous yeast flora in vineyards and wine fermentation, because all yeasts isolated from grapes were non-*Saccharomyces* yeasts. They belonged to the genera of *Rhodotorula* and *Cryptococcus.* In spite of this, *Saccharomyces cerevisiae* strains clearly dominated in all middle fermentation phases tested. Sequential substitution by *Saccharomyces cerevisiae* strains was observed along fermentation on the basis of electrophoretic karyotyping and RAPD-PCR analysis of these yeasts.

Killer yeasts

Killer yeasts are closely associated with the winemaking process. A number of wild killer yeasts from various genera have been isolated from grape must so far, some of these strains have caused stuck or slow fermentation. Occurrence of killer activity has been observed also among strains, which are employed in the commercial fermentation of wine (see the review by Shimizu [11]). Production of Tokaj wine is typical to the Hungarian wine-producing district, Tokajhegyalja. This technology involves extraction of overripe botrytized grapes either by dry wine or fermenting must. This is followed by a secondary fermentation with the help of wine yeast strains that survived the elevating ethanol concentration. Fermentation is finished by a long maturation, when the typical flavour and aroma compounds develop.

Killer, sensitive and neutral phenotype of the fermenting yeast populations was checked and killer strains were isolated from different stages of wine fermentation. Killer yeasts belonged to two phenotypes: one of them showed killing activity only at pH 4, while the other was active at pH 5, too.

Genetic determinants for the toxin production were found to be dsRNA plasmids in every killer strain that differed in the size of M dsRNA molecules. Killer toxins of activity at pH 4 were associated with different M dsRNA molecules, but killer strains from the same fermentation always harboured M dsRNA of the same size.

Successive changes of the fermenting killer yeast populations were also monitored by molecular analysis of their nuclear genotypes. Electrophoretic karyotyping showed high degree of heterogeneity when yeast strains from different fermentation were compared, while more similarity was found when strains from the same fermentation were analysed. Both similarity and dissimilarity in the PFGE karyograms of the isolates were

reflected in the cluster analysis of the RAPD-PCR profiles but more detailed distinction of strains that belonged to the same PFGE group was achieved by the latter method.

We used mitochondrial drug resistant mitochondrial mutants for studying the role of killer toxin produced in the competition of killer, sensitive and neutral wine yeast strains. Mitochondrial point mutations causing resistance against diuron were induced by manganese treatment of the killer wine yeast strains that were used in different ratio together with sensitive or neutral wine yeast strains in model fermentation. As the control, killer plasmid cured, diuron resistant strains were applied making possible to study the role of other competitive factors than the killer toxin.

Yeasts as cloning hosts in biotechnology

Yeasts are far the most frequently used cloning hosts in both the basic research and biotechnology among the eukaryotic microorganisms. The leading role of *S. cerevisiae* is indisputable, but numerous other species are becoming more and more important. The main reason of this is that post-translational modification of mammalian proteins by *S. cerevisiae* is not always perfect, especially O-linked glycosylation of maturing proteins is different, which endows antigenic property of mammalian proteins produced. *Pichia pastoris, Hansenula polymorpha* and *Schwanniomyces occidentalis*, however, glycosylate proteins similarly as higher eukaryotes [12]. There are some other advantages of the application of the formerly mentioned species; e.g. utilization of cheap carbon sources during propagation, lack of catabolite repression or excretion of heterologous proteins after having been synthesized. Undoubtedly, however, the well developed vector system, a big set of well-characterized mutants and mapped genes and the fully sequenced genome make *S. cerevisiae* the most important eukaryotic microorganism.

Brewing yeasts

Brewing yeasts can be considered as fully domesticated strains. Producing strains that are applied in controlled technological processes of beer production are the results of successive selection activity of the brewers. In spite of this, they are still not the ideal strains that could fulfil the demands of the technologists. Genetic modification by classical methods have contributed to the improvement of strains only in limited range, mainly due to the loss of meiosis hindering efficient application of mutagenesis and hybridization. Molecular biology opened up new possibilities for both the genetic analysis and strain improvement, what have been utilized in many areas. Promising genetically engineered brewing yeasts have been produced in several laboratories, which were tested also in semipilote or pilot scale beer fermentation's. Table I contains some examples of genetically engineered brewing yeast strains.

Wine yeasts

Fermentation of wine is attributed to the activity of many yeast species. *Kloeckera apiculata, Hanseniaspora uvarum, Candida stellata, Torulaspora* and *Pichia* spp. start fermentation and predominate in the early phase, while *Saccharomyces cerevisiae* becoming dominant soon, when the elevating ethanol content reaches 5%. Selected wine yeast strains have been used as starter cultures, which overgrow the natural yeast biota

Table I

Practical application of recombinant DNA technique in the improvement of industrial yeasts

Type of industrial yeast	New properties	Gene cloning
Brewing yeast	Fermentation of maltodextrins	Cloning of glucoamylase genes from *Schwanniomyces* spp. and *S. cerevisiae* var. *diastaticus*
	Degradation of ß-glucan derived from malted barley in wort – improvement of filtration	Cloning of ß-glucanase genes from bacteria, fungi and barley
	Hydrolysis of wort proteins – beer haze prevention	Genetic engineering for supersecretion of protease
	Reduction of diacetyl level in beer – decrease of maturation time	Cloning of ALD, ALS and AR genes from *Enterobacter* sp. and *Klebsiella* sp.
	Reduction of H_2S formation by yeast	Cloning of cystathionine syntase gene
	Increase of SO_2 production acting as antioxidant and anticontaminant	Site directed mutagenesis of sulphite reductase gene (MET10)
	Increase of flocculation level at the end of fermentation	Cloning of FLO1 gene
	Derepression for glucose	Manipulation of MAL genes
	Improvement of wine filtration and fruity aroma	Cloning of pectinases and glucanases from fungi
Wine yeasts		
	Malolactic fermentation	Cloning of lactate dehydrogenase from *Lactobacillus* sp.
	Malo-alcoholic fermentation	Cloning of malate permease and malic enzyme from *Schizosaccharomyces* sp.
	Increase of glycerol level	Cloning of DHAP reductase and glycerol phosphatase
	Construction of double killer toxin producing yeast	Transformation of cDNA of K1 preprotoxin gene into K2 killer strain
	Improvement of flocculation of a champagne yeast	Cloning of FLO5 gene from *S. cerevisiae*
	Utilization of raffinose in molasses	Cloning of melibiase gene from brewing yeast
Baker's yeast	Increase of dough-raising ability	Cloning of maltose permease and maltase genes with strong promoters
	Avoidance of glucose repression of maltose utilization genes	Cloning of manipulated MAL gene from yeast
	Derepression of maltase and invertase synthesis	Cloning of multiple copies of MAL and SUC regulatory genes

Information mainly from Walker [15]

and complete fermentation of must within a short time. Commercial starters are the results of not only selection but strain improvement, too. Improvement, what is accepted by the wine producers without any restrictions, can be achieved by mutagenesis and several varieties of hybridization, including protoplast fusion. Major achievements of such classical breeding experiments are new hybrids and killer wine yeast strains [13]. Recombinant DNA technology is in the phase of to revolutionize wine yeast strain improvement; the results are, however, mainly in experimental stage. Some examples of genetically engineered wine yeast strains are shown in Table I. Main directions of strain improvement are cloning heterologous enzyme genes of bacterial, yeast and fungal origin, and use the site-directed mutagenesis to modify metabolic genes having a role in flavour and aroma production.

Baker's yeasts

Baker's yeast manufacture is using selected *S. cerevisiae* strains, which have an accelerated growth in molasses containing sucrose as a carbon source. High dough leavening activity, cryotolerance and good storage stability are the most important characteristics what are expected from the yeast during bread making. Strain improvement by rDNA technique is aiming to increase biomass yield, to reduce fermentation time and to increase leavening activity. Genetically modified baker's yeast strain, constructed and patented by the Gist Brocades, contains derepressed promoters of maltase genes, and as a consequence, its leavening activity has been enhanced significantly. The British Government in 1991 for use in baking has approved this strain. There are other achievements of gene cloning in baker's yeast improvement, some of them is shown in Table I.

Application of gene cloning in the production of enzymes and therapeutic proteins by yeasts

Yeasts are generally considered as very poor sources of industrially useful compounds and enzymes, because they excrete just a few enzymes and overproduction of metabolites is very rare. This is true if someone concentrates only to the *Saccharomyces* species, but this picture is more promising if the wide natural biodiversity of non-*Saccharomyces* species are analyzed more carefully from biotechnological aspect (see the reviews by Wolf [14] and Walker [15]). Several non-*Saccharomyces* species seem also to be near the ideal host in cloning of heterologous genes of eukaryotes, as mentioned previously. More research is needed to utilize the gene pools of these species.

Starch hydrolyzing activity is very significant in some yeast species. Cloning the glucoamylase genes of *Schwanniomyces* and *S. cerevisiae* var. *diastaticus* in industrial ethanol producing strains of *S. cerevisiae,* bioethanol producing strains from industrial starch containing waste and raw material were constructed. The renewable energy sources, cellulose and hemicellulose can be directly converted into ethanol by *S. cerevisiae*, containing cloned cellulase and xylanase genes of various yeasts and fungi.

One of the major achievements of modern food biotechnology was the production of recombinant chymosin by *Kluyveromyces lactis.* Not only this product could substitute the calf rennin, but it was acceptable by certain religious people, who are not aloud to eat cheese processed by the calf rennin.

Recombinant vaccine against Hepatitis B was the first approved therapeutic yeast protein produced by Merck in 1986. Since that time several other antiviral, antibacterial and antimalaria proteins have been produced by *S. cerevisiae* and other yeast species. Recombinant human blood proteins, hormones, growth factors and antibodies are now available for medical purposes, or a yeast expression and production system has been successfully developed. Significance of yeasts as cloning hosts for the production of recombinant pharmaceuticals is increasing.

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GENETICS, PHYSIOLOGY AND CYTOLOGY OF YEAST-MYCELIAL DIMORPHISM IN FISSION YEASTS

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The order *Schizosaccharomycetales* contains a dimorphic and two yeast species. *Sch. japonicus* can form both yeast cells and mycelium, depending on the substrate and the culturing conditions. *Sch. pombe* is a strictly unicellular organism, but it can be forced to form mycelial cell chains by inactivating members of the *sep* gene family. The mutations in most of the *sep* genes confer pleitropic phenotypes indicating functional involvement in MAP-kinase-mediated signalling pathways. Two of them were found to encode transcription factor homologues of other eukaryotes.

The fission yeasts are unicellular ascomycetes that are becoming increasingly popular as model organisms for molecular genetical and cytological studies. They comprise an order (*Schizosaccharomycetales*) with a single family (*Schizosaccharomycetaceae*) which contains three species: the four-spored *Schizosaccharomyces pombe* and the eight-spored *Sch. octosporus* and *Sch. japonicus*. The four-spored species has two varieties such as *Sch. pombe* var. *pombe* and *Sch. pombe* var. *malidevorans*. *Sch. japonicus* is also subdivided: *Sch. japonicus* var. *japonicus*, *Sch. japonicus* var. *versatilis* and *Sch. japonicus* var. *longobardus* (for a review see [1]). Sequence comparison of rRNAs, tRNAs and phylogenetically conserved proteins revealed that the lineage leading to the present-day fission yeasts must have separated from the lineage of the *Saccharomyces*-type budding yeasts some 1000 million years ago, soon after the *Ascomycota* separated from the lineage leading to the *Metazoa* [1]. The phylogenetic gap that separates the two groups of yeasts now is almost as wide as the gaps that separate them from the metazoans. The common ancestor of the fission yeasts is supposed to have been a filamentous fungus that colonized solid substrates but could also fragment into arthroconidia. During a later adaptation to fluid environment, the fragmented (single-cell) form has gradually become the dominating growth morphology

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[2]. *Sch. japonicus* is still dimorphic, because it has retained the ability to form invasive true mycelia on solid substrates [3]. The other two species propagate as yeasts both in liquid and on solid substrates, although *Sch. pombe* can be forced to form mycelium by mutations in certain genes [4–6]. The yeast cells of *Sch. pombe* and *Sch. japonicus* have elongated, cylindrical form and grow by polar extension (for a review see [7]). Cell division begins with the formation of a trilaminar septum halfway between the poles (septation) and concludes with the degradation of the central layer of the mature septum (cell separation or cytokinesis) [8]. The polar growth and the dimorphic transitions make the fission yeasts an excellent model for the investigation of cell polarity, one of the basic phenomenon of all living organisms.

The environmentally controlled dimorphic cycle of *Sch. japonicus*

Both varieties of *Sch. japonicus* can alternate between yeast and mycelial morphology. On solid media, the yeast phase is unstable and can switch to mycelial growth [3]. The transition is induced by starvation of nitrogen, and the growth of the hyphae is directed by the gradients of the nitrogen-containing nutrients in the substrate. The mycelial phase is not stable either; the mycelium can fragment into arthroconidia or return to the yeast form of growth in response to environmental changes [3]. The yeast-to-mycelium transition involves a changeover to unipolar growth associated with asymmetric division, the development of large polarly located vacuoles, the reorganisation of the actin and microtubular cytoskeleton and the repression of cell separation after septation [9]. In the yeast phase, the cells usually grow at both poles, whereas the mycelial cells extend only unipolarly, either at the old end (the end that has already grown as the end of the mother cell) or at the new end (the end formed from the septum at the division of the mother cell) [9]. Several lines of evidence suggest that the morphological transitions are cAMP-regulated. For example, the supplementation of the growth medium with caffeine or with cAMP inhibits the yeast-to-mycelium transition and promotes the mycelium-to-yeast conversion [9].

Polar growth of *Sch. pombe* cells

No physiological conditions are known that could be induce mycelial growth in *Sch. pombe*. Its polarly extending cylindrical yeast cells divide by forming medial septa and then separate to form two physically independent yeast cells (for a review see [7]). In exponentially growing cultures, cell extension is usually initiated from the old end. The new end starts growing much later, at the point in G2 called NETO (*n*ew *e*nd *t*ake *o*ff) [10]. However, growth can also start from the new end or from both ends simultaneously as shown by time-lapse photomicrography [11, 12]. Interestingly, caffeine, that elevates the intracellular cAMP level by inhibiting the degradation of cAMP, makes cell growth more unipolar because it extends the pre-NETO part of cell extension [13]. However, it cannot cause a complete transition to unipolar growth and cannot block cell separation

either. Thus, the caffeine-treatment drastically changes the growth pattern but cannot change the yeast morphology to mycelial.

Mycelial mutants in *Sch. pombe*

Mycelial morphology can be provoked in *Sch. pombe* by mutations in genes which directly or indirectly participate in cell separation (cytokinesis). The first mycelial mutant identified defined a novel gene, *sep1*$^+$ [4]. The *sep*$^-$ cells formed septa (could divide) but did not undergo cell separation (could not cleave the septum) and thus developed cell chains morphologically similar to mycelium. Unlike the transient mycelial phase of *Sch. japonicus*, the *sep1*$^-$ mycelium was stable and could not revert to yeast morphology. The description of *sep1*$^+$ was followed by the identification of eleven more *sep* genes [5, 6]. In spite of the mycelial growth, the mutant cells retained the bipolar extension pattern, which is also an important difference from the *Sch. japonicus* mycelium. Since the cell poles were usually covered by unsplit septal material, cell extension could take place only from subapical positions (at both cell ends) and in lateral directions. Thus, the mycelium formed was usually highly branched. This bipolar branching pattern and the lack of vacuolation in the hyphal cells indicate that the *Sch. pombe* mycelium is not an equivalent of the mycelial phase of *Sch. japonicus*.

sep1$^+$ encodes a homologue of HNF3 transcription regulator and interacts with M-phase genes

sep1$^+$ has been cloned and found to encode a homologue of the HNF-3/forkhead family of tissue-specific and developmental gene regulators common in higher eukaryotes [14]. The aminoacid sequence of the conserved DNA-binding region of the Sep1 protein showed 56% homology with the corresponding region of the human HNF-3 protein.

sep1$^-$ synthetically interacts with early mitotic and cell division mutations, such as *wee1*$^-$, *cdc2-1w*, *cdc25-22* and *cdc4-8* [4, 15, 16], which suggests that the Sep1 protein is not involved in cell separation *per se*, but in some early event(s) of division initiation and therefore its inactivation causes a delay of the whole process of division and shifts its last event, the separation of daughter cells, out of the cell cycle. The inactivation of *wee1*$^+$ in *sep1*$^-$ background had a peculiar effect: the cells frequently skipped cell division and entered a new cell cycle without septation. The wee1$^-$ sep1$^-$ double mutants formed dikaryotic cells at high frequency [16] Thus, *sep1*$^+$ seems to be a positive, but indirect regulator of cell separation.

Contribution of the analysis of cytoskeleton in mycelial mutants to the better understanding of the determination of cell polarity

The altered cell morphology of the intrafilamental *sep*⁻ cells provided a possibility to address the role of the microtubular cytoskeleton in the determination of cell polarity. Based on the correlation between the defects of the cytoplasmic microtubules and the deformations of the cell shape, it has been hypothesized that the interphase microtubules play a central role in the determination of the growing poles [17]. The analysis of the re-organisation of the microtubules after cell division in the branched *sep*⁻ cells, however, revealed that they are directed by the cell shape "inherited" from the mother cell. This dependence on cell shape was particularly obvious in the multipolarly growing syncytial cells of the *sep1*⁻ *spl1*⁻ *cdc4-8* triple mutant [15]. Furthermore, cell extension cannot be initiated at regions where the cell wall is covered by septal material [16], suggesting that there must exist some communication between the cytoskeleton and the cell wall before establishing a growing site. Thus, the microtubular array is only one member in a multi-component mechanism responsible for the positioning of the growing cell poles [15, 16].

sep2⁺ is involved in cell separation and division site positioning

The rest of the *sep* gene family also seems to have complex functions. The *sep2-SA2* mutant cells frequently form twin septa separated by anucleate minicells [5]. The longer the cells are, the more minicells can be formed. Presumably *sep2*⁺ is required not only for cell separation but also for the correct placement of the division plane. The dependence of the number of septa (division planes) on the lengths of the cell further suggests that there must be a polarly generated intracellular signal which forms a gradient decreasing towards the cell middle. Where it falls below a critical value, a septum is formed. *sep2-SA2* might be impaired in the production of this signal [5]. The supposition of the existence of such a polar signal is in conflict with the model proposed by Chang and Nurse [17]. They suggest that the division plane is positioned only by the position of the premitotic nucleus. The polar-oriented signalling mechanism which we suppose to exist in *Sch. pombe* might be the equivalent of that believed to determine the position of the cleavage furrow in mammalian cells [18]

Mutations in *sep6*⁻ to *sep16*⁻ confer complex phenotypes reminiscent of defects in signal transduction pathways

The mutants *sep6*⁻ to *sep16*⁻ showed not only aberrant cell separation but also impaired sexual activity [6]. None of the mutants could conjugate normally and three of them were also defective in meiosis-sporulation. The examination of the production of sexual pheromones by halo-test revealed that all of them could produce M-factor, but *sep8*⁻, *sep11*⁻ and *sep15*⁻ were defective in P-factor production. The mutations in *sep8*⁺,

sep11$^+$ and *sep16*$^+$ suppressed the *pat1-114*-driven haploid meiosis, the forced entry into meiosis provoked by the inactivation of Pat1, a negative regulator of meiosis initiation [19].

In addition to the defects in cell separation and sexual differentiation, the *sep6*$^-$ to *sep16*$^-$ mutants are all super-sensitive to the presence of higher concentrations of chloride in the medium and to short heat shocks [6]. These pleiotropic effects suggest that this group of *sep* genes might act in or interact with a multiple overlapping network of regulatory modules. Mutations in genes of MAPK-mediated signalling pathways frequently show similarly complex phenotypes. For example, in the budding yeast *Saccharomyces cerevisiae* signalling pathways have been described which participate in the regulation of pseudohyphal growth, cytokinesis, hyperosmotic stress response and cell shape (for a review see [20]). Little is known about homologous signal transduction pathways in fission yeasts. One of the few examples is the recently identified Spm1/Pmh1-Mkh1 MAP-kinase cascade, whose defects enhance stress sensitivity, cause occasional cytogenesis defects and confer reduced fertility, but do not elicit mycelial growth associated with complete sterility [21, 22]. The products of the *sep6*$^+$ to *sep16*$^+$ genes might be involved in this or other signal transduction pathways, which have not been identified yet. In the eukaryotic cells the mitogen-activated protein kinases and their upstream regulators couple upstream signals to the modulation of co-ordination of various cellular processes and to the activation of the genes involved in these processes. Consistent with this view, two of the five cloned members or the *sep6*$^+$ to *sep16*$^+$ group show high degree of sequence homology with transcription regulators. *sep9*$^+$ is a homologue of the *Saccharomyces cerevisiae SPT8* [unpublished result], a protein that encodes a putative TATA-binding protein [23]. Inactivation of *SPT8*, also causes defects in growth, mating and sporulation. Cloning and sequencing of the rest of the *sep* genes is in progress.

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INDUSTRIAL MICROBIOLOGISTS IN THE AREA OF MICROFUNGI

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The microbial fermentation industry employs microorganisms to produce different desired valuable products. A number of commercial products – including antibiotics, organic acids, steroids, vitamins, enzymes – are made by fungi. Many of these products can be produced both microbially and by chemical synthesis. The choice of which process to employ generally depends on economics. (The decision is dictated by variable costs.)

Below a short but not complete list of the most important primary and secondary metabolites which are produced by fungi.

Product	Microorganism
Penicillins	*Penicillium chrysogenum*, *P. notatum* Greek: *χρυσοζ... γενοζ*
Cephalosporin	*Acremonium chrysogenum*
Fumagillin	*Aspergillus fumigatus*
Griseofulvin	*Penicillium griseofulvum*, *P. urticae*, *P. nigricans*
Lovastatin	*Aspergillus terreus*, *A. oryzae*, *A. niger*, *A. nidulans*, *A. fumigatus*, *A. flavus*, *A. obscurus*, *Syncephalastrum nigricans*, *Mucor hiemalis*, *Pleurotus sapidus*, *P. saca*, *P. ostreatus*, *Coniothyrium fuckelii*
Cyclosporin	*Trychoderma polysporum*, *Tolypocladium inflatum*, *T. geodes*, *T. tundrense*, *T. terricola*, *T. cylindrosporum*, *Fusarium solani*, *F. javanicum*, *Neocosmospora vasinfecta*, *Beauveria nivea*, *B. bassania*, *Cylindrocarpum lucidum*, *Sesquicilliopsis rosariensis*
Riboflavin	*Eremothecium ashbyi*, *Ashbya gossypii*
Ergot alkaloids	*Claviceps purpurea*
Amino acids: (Tryptophan)	*Hansenula anomala*, *Candida humicola*, *Saccharomyces cerevisiae*, *Claviceps purpurea*
Giberellins	*Giberella fujikuroi*, *Sphaceloma manthoticola*, *Neurospora crassa*
Citric acid, Gluconic acid	*Aspergillus niger*
Ethanol	*Saccharomyces cerevisiae*

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Important activities of industrial microbiologists include the search for microorganism to produce substances of commercial importance, or creating specific strains of microorganisms that yield sufficient quantities of the desired product to permit commercial production economically, design the optimal production process and in the batch reactor to optimize the physical and chemical environmental conditions in order to achieve maximal product yields.

Production process technology includes defining the medium – containing the least expensive components – that will produce the highest yield of the desired product, recognizing that very often the presence or absence of even trace amounts of a component will vastly alter the yield of the desired product.

Microorganisms can carry out a number of chemical modifications more efficiently than synthetic chemical methods. For example: a number of fungi can introduce oxygen specifically at the position 11 in a single reaction into the steroids resulting in the desired products

Hydrocortison from Cortexolone	*Curvularia lunata, Cunninghamella blakesleana*
11α-hydroxyprogesterone from progesterone	*Rhizopus nigricans, R. arrhizus*

Different essential intermediates are produced for organic chemical synthesis by stereospecific reduction. For example: *Saccharomyces uvarum, S. bayanus, Zygosaccharomyces rouxii, Rhodotorula rubra, Geotrichum candidum, Aureobasidium pullulans, Schizosaccharomyces pombe, Hansenula anomala* and a number of other strains.

Various microbial enzymes are produced for commercial or industrial applications

Amylases	*Aspergillus oryzae*
Glucamylase	*Aspergillus niger*
Glucose oxidase	*Aspergillus niger*
Cellulase	*Trichoderma reesii*
Invertase	*Saccharomyces cerevisiae*
Lactase	*Kluyveromyces fragilis*
Lipase	*Saccharomycopsis lipolytica, Syncephalastrum racemosum, Pythium ultimum, Candida rugosa, Rhizopus niveus, Penicillium cyclopium, Aspergillus niger, Ustilago maydis, Geotrichum candidum, Fusarium oxysporum*
Pectinases and proteases	*Aspergillus foetidus*
Naringinase	*Penicillium decumbens, Aspergillus niger*
Microbial rennin (chymosin)	*Mucor pusillus, M. miehei, Rhizomucor* spp.

Most of them are used by food industry. Amylase is applied for bread baking and for breakfast foods. Pectinases are useful for clarification of wine. Fungal glucose oxidase is used for removing glucose from eggs prior to drying. Removing the glucose stabilizes and prevents deterioration of the dried egg product. Glucose oxidase is also used to

remove oxygen from different other products preventing any oxidative colour and flavor changes. Naringinase (α-L-rhamnosidase) can be used to remove bitterness from grapefruit juice. Rennin is used for curdling milk in the production of cheese.

The applied microbiology is economically feasible only if full advantages of the biological capacity of microorganisms to convert any kind of substrates into desired products are taken. A good knowledge of microbial physiology (metabolism and its control) with its molecular biological background is therefore of primary importance. It seems that we have to know more about this well regulated metabolic network. Unfortunately there are many important problems in metabolic regulation which have not been solved. The promise of recombinant DNA technology to permit the incorporation of foreign genetic information into microorganisms support the belief that microorganisms will become increasingly important in the production of many goods by microbial fermentation.

Poster Sections

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POSTER SECTION 1

TAXONOMY, EVOLUTION AND ECOLOGY

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Lichenological research in Transylvania in the last decades of the century

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Biological research declined in Romania in the years 1970–1990 as having been considered unprofitable from an economic point of view. Lichenology was, of course, in the same situation. As a consequence, just one lichenologist is now active in Transylvania (and also in Romania). Her share of interest is rather wide, in close connection with the projects performed by the Institute of Biological Research of Cluj.

The main research fields are: studies of lichen flora and vegetation in Romania (Apuseni-, Retezar-, Cozia-, Calimani Mts.); taxonomical, ecological and corological studies of the *Cyanophilinae* lichen-group (*Lobariaceae*, *Peltigeracea*, *Nephromataceae* etc.); bioindication and biomonitoring of heavy metals and radioactive substances using lichens in some heavily polluted industrial areas of Transylvania.

A short review of these studies has been presented here.

R. BÁN, F. VIRÁNYI

Distribution of fungal pathogens on reed (*Phragmites australis* (Cav.) Trin ex Steud) in relation to host characteristics and environmental factors

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Although common reed (*Phragmites australis* (Cav.) Trin ex Steud) is one of the best studied plant species in natural ecosystems, the distribution and damage caused by reed parasites have been poorly investigated.

The aims of our investigations were to study the distribution of fungal pathogens in reed stands in relation to quantitative characteristics of the host plants (stem diameter, height and density) and environmental factors (temperature, precipitation).

Investigations were carried out from June to October in 1996 and 1997 in seven different reed stands at Lake Balaton and Lake Velencei. Diseases were assessed monthly

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by using a 1–5 scale five-grade prepared for the main reed pathogens, and infection indices were then calculated.

The occurrence of *Puccinia phragmitis* (Schum.) Koernicke showed positive correlation with reed stem diameter, while that of *Deightoniella roumequerei* (Cav.) O.Const. with shoot density. *Puccinia magnusiana* Koernicke and *Polythrinciopsis phragmitis* Walker caused severe infections in the warm seasons.

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New and rare hypogeous fungi of Carpathian Basin

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After the death of the world famous Hungarian truffle researcher, László Szemere there was a long break in Hungarian truffle research. His book (L. Szemere: Földalatti gombavilág, 1970) contains the latest summary of the hypogeous fungi in Carpathian Basin. From the early 90s our group have been trying to continue the mycological work of L. Hollós and L. Szemere. Use of truffle hunting dogs in the last 3 years leaded to some surprising results. A general overview of hypogeous mycota supplemented with new species is presented here.

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Tolerance of composting thermophilic fungal isolates to toxic heavy metal ions

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The presence and the role of thermophilic fungi in composting process is very special and significant. The different communal, industrial and agricultural wastes, as raw materials of composting technologies, may contain noteworthy amounts of toxic heavy metal ions. Therefore their inhibitory effect on composting mycota is to be studied.

19 samples of the perfect composts made from communal waste, communal sewage, industrial sewage and raw leather were cultured to reveal the thermophilic fungal populations. 166 isolates were obtained with the use of streptomycin-containing potato-dextrose agar. *Thermomyces lanuginosus*, *Malbranchea cinnamomea* and *Myceliophtora thermophila* proved to be the most common species, and 7 further species could be differentiated.

Cadmium, arsenic and lead ions inhibited the fungal populations and changed their diversity. On the effect of 50 ppm Cd^{2+} the number of thermophilic fungal propagules, in

in vitro experiments, decreased with 45–62%, the same effect of 30 ppm As^{3+} was of 50–55%, while that of 100 ppm lead was of 43–54%.

The sensitivity of the tested species to the toxic heavy metal ions markedly differed: the growth of *Thermomyces lanuginosus* was affected by even 9 ppm As^{3+}, 24 ppm Pb^{2+}, 18 ppm Cd^{2+}, and totally inhibited by 100 ppm As^{3+}, 200 ppm Pb^{2+}, and Cd^{2+}; while the growth of *Malbranchea cinnamomea* was affected by 20 ppm As^{3+}, 50 ppm Pb^{2+}, 60 ppm Cd^{2+}, and totally inhibited only by 200 ppm As^{3+}, 500 ppm Pb^{2+} and 300 ppm Cd^{2+}.

Further and comprehensive investigations of the effect of heavy metal ions on thermophilic fungi are necessary.

E. FARKAS

Complex biodiversity studies on the tropical material of the Hungarian lichenological collections

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Tropical E-African and Cuban collections of Tamás Pócs, Attila Borhidi and the present author initiated the study of foliicolous and corticolous lichens. Twelve species (all leaf-inhabiting) new for science and two new combinations (1 foliicolous, 1 corticolous) were published since 1987. The bibliography and checklist of foliicolous lichenized fungi were compiled and regularly updated also in the last decade.

An international IAB & IAL Symposium on Foliicolous Cryptogams was held in 29 August – 2 September 1995, Eger in Hungary. The goal of the meeting was to assemble cryptogamist specialists – mainly bryologists and lichenologists – of the phyllosphere organisms in natural ecosystems of tropical and subtropical forests.

Bacidia s.l. species of recent tropical collections of André Aptroot, Harrie Sipman, Tamás Pócs and myself form the basis of a complex biodiversity study – concerning morphology, chemistry, phytogeography and ecology.

E. FARKAS[1], L. LŐKÖS[2], K. MÁZSA[1]

Introducing HPTLC analysis for screening of lichen substances in Hungary

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In the 1970s the Culberson-laboratory worked out a standard method of thin-layer chromatography (TLC) for studying the lichen chemistry. A detailed description of the more sensitive high performnce thin layer chromatography (HPTLC) method and application for lichen substances was published by Arup and his coworkers in 1993. This

method was introduced to the Hungarian lichenological research without any former considerable background.

From 1997 we concentrated on the identification of atranorin, zeorin and norstictic acid, most important for the analysis of *Bacidia* species. *Umbilicaria* species were also investigated in a smaller study. Therefore gyrophoric acid was also necessary to identify.

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Lichens as indicators of air pollution in Hungary

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Lichens are known as sensitive organisms to the environmental quality, especially to air pollution for more than 150 years. Lichen mapping around cities and other pollution sources is based on the presence and absence and the distribution of the sensitive and toxitolerant species. The classic zone maps (patten of distribution) consist of a central lichen desert, one or more struggle zones around that and at last a normal zone. In Hungary the first lichen map like that was prepared by Felföldy in 1942 in Debrecen. It was followed by the maps of Szeged by Gallé in 1979 and Budapest by Farkas in 1982.

Recently the maps of Komárom and Miskolc are under preparation.

Chemical analysis of accummulated heavy metals in transplanted lichen samples was carried out in Budapest by Lőkös in 1983. A remarkable amount of lead content was detected by atomic absorption method from the samples transplanted close to the roads. Lichens of the Budapest Agglomeration and the Pilis and Visegrádi Mts were investigated in a more detailed study: lichen mapping, SEM-EDXRA and permanent quadrat analysis at differently polluted sites.

L. FODOR[1], I. RIMÓCZI[2]

The Fungi are a new component in the Hungarian Biodiversity Monitoring System

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The monitoring of biodiversity is carried out by regularly repeated and standardized measurement of selected characteristics of chosen organisms or communities on a long-term basis. HBMP follows the hierarchy of biological organisations: landscape, community, and population level are sampled. Due to the very high number of species and habitats, it is impossible and unnecessary to monitor everything, and everywhere. In the design of the HBMS the following key areas were given: the monitoring of endangered

and protected natural values, the observation of elements with a diagnostic value in assessing the general state of the biota and communities, the study of the direct and indirect effects of human-induced changes, and changes of the environment. This year some new projects are being elaborated, regarding new objects, including Fungi. In the future the monitoring sites, methods, the parameters to be recorded for the information system must be chosen. This shows some problems: how can the locality and the plot area on the ground netted by micelia be standardised, the fruiting bodies should be found in these sites at every recording time, and how should the monitoring frequency be determined in the changeable weather conditions.

J. GÖNCZÖL, Á. RÉVAY

Studies on the aquatic Hyphomycetes of the Morgó stream, Hungary

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Conidial populations of aquatic hyphomycetes at six sampling sites along the main channel of the Morgó stream system in the Börzsöny Mts., NE Hungary were sampled by membrane filtration. In total 69 fungal species, mainly Ingoldian fungi, were detected by water filtration. The structure of the hyphomycete communities gradually changed along the stream. Species numbers and conidial concentrations of aquatic hyphomycete species increased simultaneously downstream up to Site IV, and then generally decreased. Fungal species richness and diversity were always the highest at Sites III and IV. At this stream section the neutral or slightly alkaline pH together with increased richness and diversity in riparian vegetation and moderate current velocity probably offers optimal conditions for growth and sporulation of more fungal species than in any other part of the stream.

The longitudinal distribution of individual fungal species differed greatly. 38% of all species were found at all sites and more than 10% were restricted to specific sections of the stream. Only three species, *Alatospora acuminata*, *Flagellospora curvula* and *Heliscus lugdunensis*, occurred frequently at all the six sites. The longitudinal distribution patterns of the most important species are presented.

E. JAKUCS, L. MAGYAR

New Ectomycorrhizae from Hungary

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Ectomycorrhizae play a key role in element and energy transport of forest ecosystems in moderate climates. It has been proved that distribution of the fruitbodies shows no correlation with that of mycorrhizae in the rhizosphere, therefore direct investigations of the ectomycorrhizae from soil and root samples are essential to study

diversity of mycorrhizal relationships in natural environments. Using the widely accepted morphological and anatomical methods of AGERER about 300 ectomycorrhizae have been comprehensely described up to now. The majority of these species has been isolated from needle and beech forests of Northern America and Western or Northern Europe but litte is known about the typical ectomycorrhizae of semiarid continental woods of Middle Europe.

The short original descriptions and documentations of the following new ectomycorrhizae collected in Hungary are presented:

"Fagirhiza vermiculiformis" + Fagus sylvatica L.
Genea verrucosa Vitt. + Quercus sp.
Hebeloma ammophilum Bohus + Fumana procumbens (Dun.) Gr. Godr
Inocybe heimii Bon + Fumana procumbens (Dun.) Gr. Godr
"Quercirhiza fibulocystidiata" (Tomentella subtestacea) + Quercus ssp.
Rhizopogon vulgaris var. intermedius Svrcek + Pinus nigra Arn.
Scleroderma bovista Fr. + Populus alba L.

E. JAKUCS, É. MAJOROS

Ectomycorrhizae of Populus alba l. on the Hungarian plain

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Although using artificially mycorrhizated seedlings has produced outstanding results in forestation in Western Europe, America and the tropic regions these techniques are almost unknown in Hungary. As Hungarian long-term forestation program aims that 25% of the country's territory be covered with forests within 20 years an intensive planting schedule of 15–20 000 ha/year is to be realized. In this program weakly fertile and dry-soil areas of the Hungarian Plain are mainly concerned. As according to experiences ectomycorrhizal seedlings have the greatest benefit under bad environmental conditions introducing these methods would be economic.

A project for investigating ectomycorrhizal relationships in naturally grown forests of the Great Plain and evolving methods for using mycorrhizated seedlings in forestation has started a year ago in cooperation of the Departments of Plant Anatomy and Plant Physiology of Eötvös Loránd Univesity (Budapest) and the KEFAG Rt. (Center for Production of Forestry Propagation Materials, Kecskemét) with the support of the Ministry of Agriculture. As one of the potential plants suitable for the forestation program is *Populus alba* L., a native plant of the region, the natural ectomycorrhizal partners of this species have been investigated. The following ectomycorrhizae detected as being characteristic to *Populus alba* in the Kiskunság region have been presented:

"Quercirhiza fibulocystidiata" (Tomentella subtestacea)
+ Populus alba L.
Tomentella pilosa (Burt) Bourdot et Galzin + Populus alba L.

black-brown ectomycorrhizae of the family Thelephoraceae + Populus alba L.
Tuber rapaedorum + Populus alba L.
Xerocomus armeniacus (Quél.) Quél + Populus alba L.
Scleroderma bovista Fr. + Populus alba L.
Lactarius controversus Pers. + Populus alba L.
ectomycorrhizae of the group Russula foetens + Populus alba L.

I. KIRÁLY, Z. LUKÁCS

New data on the hypogeous flora of Hungary

Department of Plant Physiology, Eötvös Loránd University, Budapest, Hungary

In Hungary, there are relatively few recent data on the occurrence of subterranean mushrooms. This study reports two species new to the Hungarian flora and new habitats of infrequently reported species. Among the three ascomycota presented here, *Geopora schakii* P. Hennings – as that has been postulated by Szemere, – do occur in Hungary, but no one was able to substantiate this conjecture so far. It grows under cold climate, in beech forest. *Elaphomyces virgatosporus* Holl., an apparently endemic, very rare species, was found predominantly with *Carpinus betulus*. *E. granulatus* Fr. known exclusively from the north-western region of Hungary, occurring under *Pinus silvestris* and *Picea abies*, on acidic soils. The basidiomycota *Gautieria morchelliformis* Vitt. was reported only by Hollós long ago; we found it on a warm, south-facing slope, on calcareous soil, under oak trees; *G. mexicana* (Fischer) Zeller et Dodge known also from a relatively warm habitat, under *Picea abies*. *Octavianina asterosperma* (Vitt.) O. Kuntze seems to be an acidophilic fungus. *Phallogaster saccatus* Morg. specimens were excavated in a mountain area, near to *Carpinus* trees.

I. KIRÁLY, Z. LUKÁCS

Comparative description of *Tuber mesentericum* Vitt.

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The morphological features of *T. mesentericum* Vitt. from three habitats in comparison to data available in the technical literature are presented. Features different from that of *T. aestivum* Vitt. were: 1. shape and size of the carpophore; 2. smaller size of peridial warts and the lack of fine cross-streaks; 3. basal folding; 4. the dark venae give a darker gleba colour; 5. smell and taste of fruit bodies. The bright black colour of fruit bodies associated with *Corylus avellana*, *Quercus* sp. and *Carpinus betulus* persists even after drying. The spore ornamentation revealed by scanning electronmicroscopy features further subdivisions into fragments of *T. mesentericum* spore reticulum, just as that can be seen on light microscope photos of *T. aestivum* spores. The speciation of *T. aestivum* to

different species and varieties probably still continues today and, in our opinion, *T. mesentericum* represents an advanced stage of speciation. As *T. mesentericum* is an unfavourable accompagnion of *T. aestivum*, causing eventually disagreeable odour of the crop, there is a need to discriminate these two species on the basis of characteristic morphological traits.

Zs. Lázár

Contributions to the study of mycoflora in the pine-forests of Gyergyói mountains (Transylvania)

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The forests of the Gyergyói mountains are formed mainly by different spruce coenoses. Beside the natural coenosis there can be found also spruce, pine and – in more restricted territories – larch plantations. In the investigated territory three pine plantations can be distinguished, commonly characterized by a south- or south-west exposure, high slope and thin soil stratum. Between 1996–1998 I collected 64 mushroom species altogether that can be divided into the following overlapping groups: 31 species in Bothvára, 27 in Magasbükk and 26 in Csere. Among these species there are generally occurring ones, mushrooms living in coniferous forests and species that occur only with Pinus genera. To this latter group belong the saprotrophic *Auriscalpium vulgare*, *Strobilurus tenacellus* and mycorrhizal *Gomphidius rutilus*, *Lactarius deliciosus, Suillus bovinus, S. granulatus* and *S. luteus* species. These planted pine forests contain fewer mushroom species than the surrounding spruce forests but some rarely observed species, like *Catathelasma imperiale* and *Strobilurus tenacellus,* could be found, too.

Zs. Lázár

Saprotrophic fungi of pine-cones in the Gyergyói mountains (Transylvania)

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In the coniferous wood ecosystem saprotrophic superior fungi breaking down pine-cones (*Ascomycota, Basidiomycota*) have a strait ecological niche. Though only a few species, representing taxonomically very different genera are able to break down this very resistant vegetal material, they are the significant representatives of the early-spring mushrooms. In the investigated territory where *Hieracio rotundatii – Piceetum, Chrysanthemo rotundifolium – Piceetum* coenosis and planted pine forests (*Pinetum cultum*) can be found, *Rutstroemia bulgaroides*, *Strobilurus esculentus* habiting the spruce and *Auriscalpium vulgare*, *Strobilurus tenacellus* abiting the pine have been proved to be specific saprotrophic species, breaking down cones. All these species represent new data

for the Gyergyói mountains and the occurrence of *Rutstroemia bulgaroides* is a new observation for East Transylvania, too. Some of these cone-saprotrophic fungi have already been described in Romania: 11 species have been observed living on cones (Bontea, 1986) but 2 acicolous species have also been occurred on cones (*Ramaria abietina* and *Mycena rosella*).

L. KISS

Molecular systematics and ecology of *Ampelomyces* spp., hyperparasites and biocontrol agents of powdery mildew fungi

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Pycnidial fungi belonging to the genus *Ampelomyces* are common intracellular hyperparasites and also the most studied biocontrol agents of powdery mildew fungi. Currently, they are generally considered as belonging to a single species, *A. quisqualis*. However, RFLP analysis of the nuclear rDNA ITS region in a world-wide collection of *Ampelomyces* isolates showed the existence of seven RFLP groups [1]. Furthermore, divergence values among ITS sequences suggested that isolates belonging to different RFLP groups may represent different taxa which are possibly not even congeneric [2]. We have also shown that graminicolous powdery mildews are natural hosts of *Ampelomyces* [3], but their natural occurrence in these fungi is rare compared with their incidence on powdery mildewed dicotyledons [4]. Our studies suggest that these host-parasite-hyperparasite interactions are much more complex than previously believed.

1. Kiss,L.: Mycol Res **101,** 1073 (1997). 2. Kiss,L., Nakasone,K.K.: Curr Genet **33,** 362 (1998). 3. Kiss,L.: Can J Bot **75,** 680 (1997). 4. Kiss,L.: New Phytol **140,** 709 (1998).

L. KISS

New records of powdery mildew fungi in Hungary

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Powdery mildew fungi are obligate parasites of many plants worldwide and, thus, ubiquitous and damaging pathogens of many crops. In the past five years, three new records of powdery mildew infection were reported from Hungary. Firstly, a close examination of a powdery mildewed begonia *(Begonia tuberhybrida)* has shown that it was infected by *Erysiphe cichoracearum*, not by the anamorph of *Microsphaera begoniae*, the common powdery mildew fungus of *Begonia* spp. Even the teleomorph of this heterothallic fungus was found [1]. Secondly, the occurrence of an *Erysiphe*-like anamorph on tomato *(Lycopersicon esculentum)* was also reported for the first time from

Hungary [2]. This pathogen has spread rapidly worldwide since the early eighties causing serious losses in greenhouses, but its clear identification still requires further studies. Finally, an *Oidium* sp. belonging to the genus *Erysiphe* sect. *Galeopsidis* was found on dollar-plant *(Crassula ovata)*. This accidental observation [3] represents the first report of powdery mildew on *C. ovata*.

1. Kiss,L.: Acta Phytopathol Entomol Hung **29,** 57 (1994). 2. Kiss,L.: Plant Dis **80,** 224 (1996). 3. Kiss,L.: Plant Dis **83,** in press (1999).

L. LŐKÖS[1], E. TÓTH[2], E. FARKAS[3]

The study of *Bacidia* species (Lichenized fungi) in Hungary

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Twenty-three *Bacidia s.l.* species are known from Hungary. A complex biodiversity study based on a considerable amount of recent collections and historical collections of Hungarian herbaria (BP, DE, EGR, JPU, SZE, SZO, VBI) covers morphological, chemical, phytogeographical and ecological investigations.

The recent generic concept is explained through Hungarian representatives of *Bacidia s. str.*, *Bacidina* and *Mycobilimbia*. The main morphological and anatomical differences are illustrated and distribution maps containing several new localities are given for the most important species (*Bacidia fraxinea*, *B. rosella*, *B. rubella*, *B. subincompta*, *Bacidina phacodes*, *Mycobilimbia sabuletorum*).

Fourteen permanent quadrats were studied in the Aggtelek National Park in 1995–1998 to reveal ecological and coenological differences between *Bacidia fraxinea* and *B. rubella*. Substrate and habitat features are analysed to detect preferences by various lichen associations dominated by these species.

D. MAGYAR[1], S. TÓTH[2], Á. SZÉCSI[3], ZS. HORVÁTH[1], F. VIRÁNYI[2]

Examination of microscopic fungi in the forest "Budakeszi"

[1]Országos Közegészségügyi Központ, Országos Környezetegészségügyi Intézete, [2]University of Agricultural Science, Gödöllő and [3]Department of Plant Pathology, Plant Protection Intstitute, Hungarian Academy of Sciences, Hungary

The mycoflora in the forest "Budakeszi" was examined in the years 1995–98 according to five aspects as follows: environmental protection; allergenic and pulmonary pathogens; plant protection and fungal species spectrum. The forest has been selected for the examination because it plays an important role in the ventillation of the Hungarian capital, so the place can be denominated as the lung of Budapest. There are several hospitals and pulmonary sanatoria in the forest as well. 89 strains (79 species) were

collected with the traditional collection method (stereomicroscopic examination and cultivation) and 78 by means of different air sampling methods as Koch-type sedimentation, air sampling devices (VPPS-1000-Lansoni, RCS-Biotest).

J. MÁTÉ

Polypori in the Nyírségense district

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Nyírségense district is found in the eastern part of Hungary, in Szabolcs-Szatmár-Bereg county. Sand-dunes of county bears in its history and development some important stages of the Hungarian Plain's evolution, geomorphological features and successional transformation. Since the vascular flora and fauna of the Nyírség is almost entirely known, we know very little of the fungi culture and beard-mosses of this area.

Our research work of more than 3 years was directed towards the registering of 'oak–silver linden forest' and 'oak–ash–elm park-forests'. 125 species were identified of 55 genera of the vascular tilth families belonging to the order of polypori (*Aphyllophorales* s.l.). In a specially big species number are the representatives of several genera of *Polyporaceae* s. str. family found in this area 33 spreading fungi species were identified in the Nyírség up to present. A relevant part (23 species) of them are of the *Corticiaceae* s.l. family.

We concluded that the park-forest of the Nyírség are rich in tinder fungi species. 15 of them are obligate parasites which prove the health of the tree-stand in district.

K. MÁZSA[1], T. KALAPOS[2], R. MÉSZÁROS[2]

Ecophysiological characteristics and microhabitat preference of soil-living lichens

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Soil-living lichens contribute significantly to the biomass and species diversity of the seminatural sand grassland-juniper scrub vegetation mosaic characteristic in lowland Hungary. This landscape provides a small-scale habitat heterogeneity with dry open grassland patches and the more humid juniper canopy shade, between those lichen species appear to differentiate. This research aimed at exploring the lichens' microhabitat preference and searching for its ecophysiological background, mostly in water relations and photosynthesis. Photosynthetic response to light and water content were determined in laboratory, while daily and seasonal courses of photosynthesis were followed in the field. In their natural habitat, the photosynthetic activity of lichens is limited by light intensity and thallus water content mostly. Species differing in the degree of drought adaptation

showed different compensation points and saturation values of the photosynthetic response. Lichen species living in different microhabitats did not differ significantly in their photosynthetic light response, as each studied species behaved as shade plants reaching light saturation at light intensities 1/3–1/6 of full sunlight.

Z. NAÁR[1], M. NEMES[2], M. KECSKÉS[2]

Competitive saprophytic ability of *Trichoderma* fungi in various soil types

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The competitive saprophytic ability of a fungus cannot be characterized by an absolute value because it depends on the interaction with its environment. We carried out soil inoculation experiments (supported by OTKA IF025924) with conidial suspensions of *Trichoderma* fungi. It was observed that the type of soil is a predominant factor during its colonization by *Trichoderma*. The various bacterial communities of different soil types had different inhibiting effect on *Trichoderma* fungi. This effect may considerably decrease their success in the competition for nutrients with oomycetous fungi. The special ecophysiological features of *Trichoderma* fungi have a great role only in cases if they are not suppressed by the activity of microbiota or antagonistic microbial groups. These microbiological parameters depend on the physicochemical properties of soils which, in turn, can influence the competitive saprophytic ability of *Trichoderma* fungi indirectly, too. On the basis of predictable competitive success of introduced Trichoderma, we classified the 23 investigated soil types into 3 groups: receptive, slightly receptive and suppressive.

M. NEMES[1], Z. NAÁR[2], M. KECSKÉS[2]

Occurrence and abundance of *Trichoderma* species in the rhizosphere of some arable plants

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Trichoderma fungi are wide-spread saprophytic soil microorganisms. They play an important role in natural biocontrol of soil-borne plant pathogens and are potential agents for biological plant protection. Strains isolated from the rhizosphere of a particular plant species may be more active against the root infecting pathogens. Therefore we used soil samples from the rhizosphere of potato, lupin and vetch, and from the soil far from roots, for isolation *Trichoderma* fungi. Amongst 7 species (*T. koningii*, *T. harzianum*, *T.*

stictipilis, *T. virens*, *T. pubescens*, *T. spirale*, *T. hamatum*) *T. pubescens* and *T. koningii* proved to be the most frequent. Higher number of isolates was detected in the rhizosphere than in the non-rhizosphere soil. Biodiversity (Shannon-index) of samples varied between 0.254–1.067. There was no clear trend in difference of the diversity of rhizosphere and non-rhizosphere soil.

F. PÁL-FÁM

Macrofungi occurring in calcifugous beechwoods in Mecsek Mts.

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Investigations were made during 1995-1998 concerning the macrofungi vegetation of various types of forests in Mecsek Mts. Up to now 233 species were registered in this area. From these results the paper presents the 61 species occurring in three calcifugous beechwoods (*Deschampsio-Fagetum mecsekense* Soó 1960). The characteristic species of the early summer (*Boletus calopus Fr., Amanita rubescens (Pers.:Fr.)Gray, Cantharellus cibarius Fr., Dermocybe phoenicea (Bull.:Mre.)Mos.)* and the autumn aspect (*Albatrellus cristatus (Pers.:Fr.)Kotl. et Pouz., Amanita gemmata (Fr.)Bertillon, Amanita muscaria (L.)Pers., Cantharellus tubaeformis Fr., Cortinarius orellanus Fr., Lactarius chrysorrheus Fr., Ramaria botrytis (Fr.)Ricken, Ramaria formosa (Fr.)Quél., Ramaria aurea (Schaeff.:Fr.)Quél., Russula emetica Fr., Russula fellea Fr., Sarcodon scabrosus (Fr.)Karst.)* are given. The associations are characterized by the most abundant and dominant species too (*Russula nigricans (Bull.)Fr., Cantharellus tubaeformis Fr., Hydnum repandum L.:Fr., Hypholoma fasciculare (Huds.:Fr.)Kumm.*). The 23 species included in the "Red Data List of Hungarian Macrofungi" (Rimóczi, 1998) are indicated.

F. PÁL-FÁM

Macrofungi species recommended to be protected in Belső-Cserehát

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Floristical and coenological investigations have been made during 1995–1998 in 8 habitats in the territory called "Nyéstai-erdő" in Belső-Cserehát. The area is mostly characterized by deciduous and coniferous forests originated from the forestry utilization and transformation. Until now the 210 macrofungi species occurring in these habitats are the only data from this territory (Pál-Fám, 1999, in press). From this species-list 62 are included in the "Red Data List of Hungarian Macrofungi" (Rimóczi, 1998). Only *Hygrophorus pustulatus (Fr.)Fr.* belongs to the "threatened by extinction" category. Six species (for ex. *Flammulaster limulatus (Weinm.:Fr.)Watl., Flammulaster muricatus*

(Fr.)Watl., Lactarius deliciosus Fr. and Tricholoma virgatum (Fr.:Fr.)Kumm.) are in the "highly endangered", 45 (for ex. *Amanita ceciliae (Bk. et Br.)Bas., Chalciporus piperatus (Bull.:Fr.)Bat., Clavariadelphus pistillaris (Fr.)Donk, Cortinarius bulliardi (Fr.)Fr., Hygrocybe coccinea (Schaeff.:Fr.)Kumm., Lepiota aspera (Pers.:Fr.)Quél., Otidea onotica (Pers.)Fuckel, Pseudocraterellus sinuosus (Fr.)Corner:Heinemann*) in the "endangered" and 10 (for ex. *Clitocybe geotropa (Bull.:Fr.)Quél.*) in the "potentially endangered" categories.

F. PÁL-FÁM, ZS. LÁZÁR, I. RIMÓCZI

Contributions to the mycoflora of some East-Transylvanian peat-bogs

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In the Eastern Carpathians oligotrophic peat-bogs have formed in several places. These can be characterized by *Sphagno-Betuletum pubescentis* and/or *Pino-Sphagnetum magelanicii* encircled by *Sphagno-Piceetum* associations. During 1998 we organized several expeditions to three different places: Mohos, Lucs and Nyíres. We have collected altogether 62 macrofungi species, many new for the territory. The *Pino-Sphagnetum magelanicii* habitats were characterized by the occurrence of *Galerina paludosa*, while *Dermocybe sphagnogena* occurred only in the *Sphagno-Betuletum pubescentis.* The mycorrhizal species of both habitats are: *Russula paludosa, R. emetica, R. decolorans, Lactarius vietus, L. helvus, L. bresadolianus, L. sphagneti, L. glyciosmus, Laccaria bicolor.* The *Sphagno-Piceetum* coenosis can be characterized by *Amanita fulva, A. porphyrea, Lactarius turpis, L. picinus, L. lignyotus, Mitrula paludosa, Xeromphalina campanella, Leccinum oxydabile* and *L. vulpinum.* In the Nyíres we have found partly the species described also for the other habitats, besides we have found *Russula anatina, Hygrocybe clorophana, H. ortoniana* and *Lactarius uvidus* as characteristic species.

T. PÁTKAI

SEM-EDXRA analysis of mycorrhizal *Ophrys sphegodes* Mill. (Orchidaceae) roots

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Nutritional studies on orchidacean mycorrhizae concentrate mostly on organic compounds, data on the mineral nutrient-content of mycorrhizal roots are scarce; applying SEM-EDXRA techniques to these problems seemed and proved to be a promising approach. Slices of excised roots were surface-sterilized, liophylised, then sprayed with type Polaron 803 conductive spray. The SEM-EDXRA analysis was performed by using an ISI SEM equipped with a KEVEX energy dispersive X-ray analyser. According to the

SEM-EDXRA analysis, mineral constituents in the de facto heterokaryon and heteroplasmon, endomycorrhiza-harbouring root cells proved to be different qualitatively as well as quantitatively even from the neighbouring, non-infected cells of the secondary cortex. The overall mineral nutrients content was higher by about 40% in the mycorrhiza-harbouring tissue regions than either in the central vascular bundle or in non-infected regions of the secondary cortex. In infected areas, K/Ca ratios were considerably lower and these regions seem to be "leaky" in both directions. This situation alone can be responsible for the altogether higher overall mineral nutrient transport rate of the whole plant.

G. PÉTER, J. LEHOCZKI-TORNAI, D. DLAUCHY

Three new yeast species from Hungary

National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary

The number of newly described yeast species is steadily increasing. Up to now more than 800 species were described. During the investigation of different decaying wood materials in Hungary, several yeast strains were isolated, including strains of three hitherto unknown species. One of them was already described as *Candida novakii,* while the remaining two are under description. They were provisionally designated as "*C. methanolocapsulata*" and "*Sympodiozyma* sp.".

The two strains of *C. novakii* were isolated from decaying wood of *Quercus* sp.. They are nitrate assimilating, weak fermenters with septate hyphae. Due to the several physiological and morphological differences *C. novakii* can easily be separated from other known *Candida* species even on phenotypic base. "*C. methanolocapsulata*" (one strain) was isolated from sawdust. It is a methanol assimilating species, which forms slimly capsular material. It ferments glucose, assimilate nitrate, does not form pseudohyphae or septate hyphae. Its high (more than 50%) G+C content is rather rare among yeasts with ascomycetous affinity. This species can also be separated from other *Candida* species on the basis of phenotypic characters. The only strain of "*Sympodiozyma* sp." was isolated from decaying wood of *Pinus nigra.* It shows clear basidiomycetous affinity and it is characterized by bud formation on sympodial manner. Although its taxonomic position is not clear yet, may be it is only distantly related to the genus *Sympodiomycopsis* and a new genus "*Sympodiozyma*" might be established.

I. RIMÓCZI

Some pages from the planned "Red Data Book of Hungarian Macrofungi"

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For the international opinion the existence and contents of Red Books always qualifies the situation and the professional level of a particular branch of science and its place in the environment protection in a country. Up to now Red Books containing macrofungi species were published in Europe in six countries. Moreover, in two countries they are forthcoming. The condition of the compilation of a Red Book is the legally valid Red List. In our country the Red List has already been made, so it can be expected that in this year the list of protected plant and animal species will be supplemented by the list of protected macrofungi. The Red Book will contain 470 taxons, on the basis of the IUCN recommendations and the ECCF (European Council Conservation of Fungi) experiences. It must characterize the listed taxa by the criteria given below: 1. Official Hungarian name, 2. Former or widespread local name, 3. Scientific name, synonyms and taxonomic classification, 4. Life-form type, 5. Distribution, characterization of its area, 6. Occurrences in the vegetation-map, 7. Ecological, cenological and fenological data, 8. Its state in nature conservation, the causes and the measure of its endangerment, 9. Its conservation in Europe, 10. References, 11. Remarks. In this poster I present some examples.

I. RIMÓCZI

First data about the macrofungi vegetation of "Fertő-Hanság" National Park and its environs

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I examined the appearance of macrofungi in the autumn aspect in woodstepp and bog associations in the National Park (MTB 8368) and its environs. I noted 57 species belonging to 27 genera in the area. The number of mycorrhizal species (with *Populus, Quercus, Betula* and *Alnus*) was relatively high. For the 8 *Agaricus* species gathered the occurrence of *A. pseudocretaceus Bon* in a *Fraxino pannonicae-Ulmetum* represents the first data for Hungary. Up to present the occurrence of *A. impudicus (Rea)Pilát* in *Alnetum glutinosae* associations has not been observed. In this association I gathered numerous fruitbodies of *Macrotyphula fistulosa (Holmsk.:Fr.)Petersen* too, for the first time in Hungary. The soil with high organic substance content was favourable to the appearance in mass of *Langermannia gigantea (Batsch:Pers.)Rostkow.* in the neighbourhood of Osli village. The macrofungi vegetation of the National Park can be characterized by high species diversity.

I. RIMÓCZI

Macrofungi in the territory of "Duna-Ipoly" National Park

Department of Botany, University of Horticulture and Food Industry, Budapest, Hungary

Four year-investigations have been made in the environs of Dejtár and Ipolyszög(MTB 7981) referring to the occurrence of macrofungi in woodstepp associations. Until now no researches in this topic in the area were made. Our investigations were performed in spring, summer and autumn aspects in sand-grassland turfs of Ipoly valley and poplar plantations set upon these, and in remains of soft- and hardwood galery forests supplied ordinarily with high level of soil water. We noted the species composition, the measure and "mode" of the appearance and the characteristics of the substrate. On the basis of our data it is possible to characterize macrofungal communities by life mode-type and the compilation of list of species recommended to be protected. Up to now we recorded 131 data of 81 macrofungi species belonging to 43 genera and 18 families. So the species diversity is remarkable. *Sowerbyella unicolor (Gill.)Nannf., Laccaria fraterna (Cke. et Massee)Pegl., Leucopaxillus rhodoleucus (Romell)Kuehn.* and *Hebeloma leucosarx Orton* can be pointed out as rare and characteristic species.

I. RIMÓCZI[1], I. LENTI[2], J. MÁTÉ[2]

New species of Hungarian macrofungi in the "Bátorligeti-ősláp"

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Four year-systematic mycological gatherings have been made in the territory of the "Bátorligeti-ősláp". As a result of our work we can characterize the occurence of 517 macrofungi species collected in *Fraxino pannonicae-Ulmetum* and *Quercetum robori tilietosum* associations by the measure and the "mode" of appearance and the characteristics of the substrate. Herbaria has been made, too for the collected species. In this respect it can be started that the "Bátorligeti-ősláp" has a remarkable value from mycological point of view, too. Many species are recommended to be protected, they must be taken to the "Red Data List". We present some new species for the macrofungi vegetation of Hungary: *Tulasnella violacea (Olsen in Brief.)Juel., Calocybe ionides (Bull.:Fr.)Donk var. obscurissima Pears., Cytidia salicina (Fr.)Burt, Cortinarius alnetorum (Vel.)Mos., Hebeloma collariatum Bruchet* and *Psathyrella populina (Britz.)v. Wav.*

I. RIMÓCZI[1], I. LENTI[2]

Taxonomic and coenosystemic characterisation of macrofungi attacked by mycophage microfungi

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During the investigations of "Bátorligeti-ősláp" we had the possibility to study these carposoma damaging microfungi. We review in this paper the results of a 4-year investigation. We have found 23 species of parasitic microfungi, belonging to 15 genera, being present on 45 macrofungi species of 20 genera. Infected carposomas can be found in spring, summer and autumn periods, though most of them can be observed at the end of summer and in autumn on the mushrooms belonging to *Boletaceae* (20 species) and *Russulaceae* (7 species). On the tinders that seem resistant, with hard flesh we could observe mycophage fungi on 5 species of 4 genera, analogous with the species of *Ramaria*, *Auricularia* or *Peziza*. Most of the infected mushrooms were found in the coenosis of *Quercetum roboris*, but they were also detected in the *Fraxino pannonicae-Ulmetum*.

I. SCHWARCZINGER[1], W. L. BRUCKART[2], L. VAJNA[1]

Mycobiota of *Salsola kali* ssp. *ruthenica* and some wild *Centaurea* species in Hungary

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As part of a joint project between Hungary and the United States (US), field surveys have been conducted to find fungal pathogens on *Salsola kali* ssp. *ruthenica* and on some wild *Centaurea* species (*C. solstitialis, C. pannonica. C. jaceae* and *C. micranthos*). The objectives of this project were to collect fungal pathogens and select those species, that might be useful for biological weed control against some introduced noxious weeds, including *S. iberica*, *S. paulsenii, S. vermiculata* and *C. solstitialis* in the US.

Field surveys for diseased *Salsola* and *Centaurea* species were made in Hungary from 1994 to 1999. Specimens were collected from Szarkás, Bugac and Szigetszentmiklós, Hungary.

During our survey we found 31 species of fungi on the examined weed species which are demonstrated here.

I. SILLER[1], ZS. MAGLÓCZKY[2]

Mycological investigations in the "Kékes North" forest reserve

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Knowledge of the decaying processes is necessary to understand the ecological mechanisms of natural forests. Since macroscopic fungi play an important role in the decomposition processes of temperate forests, investigation of their occurrence and abundance may shed light on the biogeochemical cycles characteristic of the forest ecosystem.

In the present study the occurrence of macroscopic fungi was investigated in the Kékes North forest reserve situated in the Mátra Mountain, Hungary. The core area of the reserve is 69 ha. The age of the trees, forming a nature-close, unmanaged montane beech forest (Aconito-Fagetum), consisted of patches of different developmental phases, is 1–195 years. Four sampling areas were established in the core area, one from each developmental phases (renewing, optimal, aging and collapsing phases) and one permanent plot was pointed out in the managed buffer zone. The investigation began in April 1998. The sample plots were visited in every two-three weeks, and 1–2 fruit-bodies of the found species were collected for identification as well as preparation. Considering that the reserve is strictly protected, productivity measurements were not carried out, however, the abundances of the species were estimated by observation.

Preliminary results show that the species composition is different in the managed and unmanaged sample areas as well as during the distinct developmental phases. The species richness of the core area, consisting of species typical for montane beech regions, is very high, while the buffer zone is dominated mainly by ordinary, frequent species. Most species are present during the collapsing phase, which is absent in managed forests, and is characterized by a large amount of dead wood. In areas under collapsing phase some rare species were also found.

Our results suggest that species composition strongly depends on the forest structure, even if the climatic and soil conditions as well as the forest vegetation types are the same. Forest reserves with their highly diversified structure seem to be suitable for both the preservation and the scientific investigation of the ecological role of fungi in forest ecosystems.

The present study was supported by the KKA.

M. SZÁNTÓ[1], M. STEENACKERS[2]

Preliminary data from *Melampsora* species in Hungary

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In the Hungarian poplar stands *Melampsora* species cause rusts on leaves. One of the most important species of the genus is *Melampsora larici-populina,* but there is another species *Melampsora allii-populina,* which should be also very harmful. In the nurseries or in mother stands a high infection causes early leaf fall, resulting in an early stop of growth. Several physiological races of *Melampsora larici-populina* have been identified in Europe, so in that case a high resistance to rust species is one of the main aims of poplar breeding and selecting. The occurrence of these species in Hungary is very important because plants become sensitive to other parasites of stems and branches such as *Dothichiza populea.* The preliminary results of our work show that these two *Melampsora* rust species is present in Hungary and the occurrence of several physiological races seems to be credible.

GY. SZEDLAY[1], CS. ROMSICS[2], I. BOLDIZSÁR[1], E. JAKUCS[1]

Specific characteristics of Hungarian *Ganoderma lucidum* (Fr.)Karst. strains

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Ganoderma lucidum has been used in Far-Eastern folk medicine for hundreds of years. The phylogenetic analysis based on DNA-sequences of the ITS region and cultural characteristics of isolates collected in different countries show that isolates referred as *Ganoderma lucidum* are sometimes not conspecific [1]. The aim of our work was to investigate the taxonomic status of Hungarian *G. lucidum* isolates and specific morphological characteristics accepted for identification.

Hungarian *Ganoderma lucidum* strains are genetically and morphologically homogenous. They form a separate group within the *Ganoderma lucidum* species complex according to the result of parsimony analysis of the ITS nucleotide sequence. Three groups with intragroup nucleotide sequence variation lower than 2.5% and intergroup variation higher than 6% can be separated. Two groups are homogenous based upon cultural characteristics, but group I, where the Hungarian isolates belong to, is heterogenous.

This work was supported by OTKA grant No. F020919.

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T. TAKÁCS, I. VÖRÖS

Influence of Cd, Zn and Ni on the diversity of arbuscular mycorrhizal fungi

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Mycorrhizas are the most widespread associations between fungi and higher plants in both natural and agricultural ecosystems. Arbuscular endomycorrhizal fungi (AMF) are associated with about 80% of the plant species of the world. The fungi may affect the water regime, macro- and microelement uptake of host plants. Diversity of indigenous AMF was studied in heavy metal loaded soil samples taken from a long-term field experiment on a calcareous chernozem soil in Nagyhörcsök. Soil samples were taken in 1998 from plots contaminated with soluble Cd, Zn, and Ni salts in 1991, at rates of 0, 30, 90, 270 mg metal kg soil^{-1}, and AMF-spores were isolated by wet sieving and decanting. Five Glomus and a Sclerocystis species were identified. The highest diversity of AMF was found in the control soil, but the number of spores did not differ significantly. The number of AMF-species declined at increasing metal rates, following the sequence of Zn < Ni < Cd. The two species adapted mostly to the heavy metal stress were *Sclerocystis sinuosa* (absent only at 90 mg Ni kg soil^{-1}) and a straw-yellow *Glomus* sp. in loose bunch (absent only at 270 mg Cd kg soil^{-1}). The indigenous AMF species, being in contact with high metal levels for seven years, went through an adaptation, and possibly metal tolerant AMF species were selected.

K. TURI[1], CS. DOBOLYI[2], K. SZOMBAT[3], E. SZALAI MÁTRAY[1]

Distribution of *Ascosphaera apis* populations in different regions of Hungary

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The spore-cyst fungal species *Ascosphaera apis* (Maassen ex Claussen) L. S. Olive and Spiltoir is an economical important pathogen of honey bee (*Apis mellifera* L.). It has been spread all over the world, causing the chalk-brood disease of honey bee larvae. Being a heterothallic species, isolation and identification of its axenic cultures can be carried out only with the use of special methods and media.

Revealing the distribution of the fungus in Hungary seemed to be an important ecological and epidemiological task.

26 samples from 19 areas of apiary in Hungary were collected and cultured for revealing the presence of the pathogenic fungus *A. apis*. 106 isolates were obtained from the necrotic larvae of honey bee with the use of a modified potato-dextrose agar. 41

isolates proved to be heterothallic *a*, 44 *b*, whereas 21 of them could produce the typical spore-cysts as fruiting bodies without outer mating relation.

A moderate diversity was found on the base of the physiological-biochemical characteristics of 14 representative isolates. Each tested strain could assimilate diagnostic mono- and disaccharides. Difference among them was experienced in their assimilation spectra of organic acids, requirement of vitamins and the optimal temperatures of growth.

The groups of our isolates provide an available stock for detailed taxonomical and epidemiological investigations.

G. VASAS

New mushroom taxa (Basidiomycetes, Agaricales) from Hungary

Botanical Department of the Hungarian Natural History Museum, Budapest, Hungary

Four new species and one new variety have been described by the author in the last ten years.

Macrolepiota citrinascens Vasas [4]

Tricholoma eosinobasis Babos, Bohus & Vasas [1]

Rhodocybe mundula var. *rubescens* Locsmándi & Vasas [2]

Agaricus annulospecialis Bohus, Locsmándi & Vasas [3]

Tricholosporum subgoniospermum Bohus &Vasas [3]

Mushroom preparations (holotypes) are stored at the Botanical Department of the Hungarian Natural History Museum.

The author gives a detailed discussion and presentation of the described species.

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POSTER SECTION 2

CELL BIOLOGY, BIOCHEMISTRY AND GENETICS

Zs. Antal[1], L. Manczinger[2], L. Kredics[2], L. Ferenczy[1,2]

Mitochondrial plasmid in a *Trichoderma harzianum* strain

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Mitochondria were isolated, and nucleic acids were purified by lysing the mitochondria of a mycoparasitic *Trichoderma harzianum* strain. MtDNAs were found to be 32 kb in size. In the undigested mtDNA preparation a simple ladder-like banding pattern was observed. This pattern was produced by single molecules 2.6 kb in size that were supercoiled to various degrees and formed concatamers in the large molecular weight fraction. The pattern was resistant both against RNase and S1 nuclease treatment indicating the double stranded DNA nature of this plasmid. There were no sequence homologies between the plasmid and the mitochondrial DNA.

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I. Boldizsár, D. Dara, Gy. Szedlay

Examination of polysaccharides in wood-rotting fungi

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High-viscosity polysaccharides of plant-extracts have been used for years in the therapy of gastric ulcer, inflammations of the respiratory organs and injuries. Extracts of polysaccharides of several plants are used in the therapy of these diseases, e.g. the extracts of *Linum usitatissimum* (L.), *Malva silvestris* (L.) and *Althaea oficinalis* (L.) [1]. Among fungi *Flammulina velutipes* has been used in the therapy of the mentioned diseases up to now. The quality and quantity of sugars, uronic acids, proteins and the high viscosity of the polysaccharides takes a prominent part in the medical effect of extracts. Our aims were to analyse the extracts of Hungarian wood-rotting fungi and search for potential medical products. The quantity of sugars [2], proteins [3] and uronic-acids [4] was determined in polysaccharide-extracts and the viscosity of these polysaccharides was investigated [5]. It is established that 4 species (*Auricularia mesenterica, Auricularia auricula-judae, Armillariella mellea, Pleurotus ostreatus*) from the examined 14 wood-rotting fungi have good influence in therapy of the mentioned diseases.

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I. BOLDIZSÁR, GY. SZEDLAY

Relationship between the molecular weight of polysaccharides and colour of *Coriolus versicolor*

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Polysaccharides of *Coriolus versicolor (Fr.) Quel.* are used extensively in therapy based on their strong immunostimulant and antitumor activity [1]. These effects depend on the molecular weight of the polysaccharides. The highest molecular weight fraction (Mw > 200 kD) has the greatest activity [2].

The fruitbodies of *Coriolus versicolor* have different colour. We established that the colour of the fruitbody depends on lighting. Fruitbodies growing in darkness are light-coloured. Those ones growing in light are dark. The molecular weight of polysaccharides of dark- and light-coloured fruitbodies are different. Our data indicated that the polysaccharide extract of darker fungi contained lower molecular weight polysaccharides (Mw > 200 kD) than the extract of light-coloured fungi. We suppose that light has a prohibitory effect on the synthesis of high molecular weight polysaccharides. This conception was supported by *in vitro* performed experiments. Our result suggests that light coloured fruitbodies are more valuable from pharmaceutical point of view than fruitbodies pigmented darker.

This work was supported by a grant of OTKA (no. F020919).

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I. BOLDIZSÁR[1], D. DARA[1], GY. SZEDLAY[1], I. MOLNÁR-PERL[2]

Antioxidant effect and chemical composition of proteoglycans from mycelium cultures of *Ganoderma lucidum*

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Ganoderma lucidum (Fr.) Karst. is used in the Far Eastern medicine because of its immunostimulant and anticarcinogenic proteoglycans [1]. The antioxidant effect and chemical composition of water-soluble proteoglycans of 13 mycelium cultures isolated from different places was examined. The sugar, sugar-alcohol and acid-composition of proteoglycans was analysed after hydrolysis with trifluoroacetic acid in GC-MS system [2]. The protein-quantity was mesured according to Folin-Lowry [3]. The antioxidant effect was examined in Fe/ADP/Ascorbate system on the basis of the induced lipidperoxidation [4]. It is established that the sugar, sugar-alcohol, acid and protein-composition of proteoglycans of mycelium-cultures originating from different places differ from each other. The total sugar content of proteoglycans varied between 50 and 80%, the total protein content between 14 and 25%. The antioxidant effect increased with the increase of the total protein content and it decreased with the increase of the total sugar content.

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I. BOLDIZSÁR, D. DARA

The antioxidant substances of *Coriolus versicolor*

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The fungus *Coriolus versicolor (Fr)* Quel. produces medically active proteoglycans. These products are used because of their immunostimulant and antitumor effect [1]. It is established that a part of water-soluble proteoglycans of *Coriolus versicolor* decreases the level of lipidperoxidation in ADP/Fe/ascorbate system of mitochondria [2]. The pH of the extracting water influences the chemical composition of isolated proteoglycans in the extract made from the fruiting body. The proteoglycans – especially below molecular weight 50-kD – extracted by neutral distilled water have low antioxidant effect. It is proved that proteoglycans extracted by alkaline solvents have the most intensive antioxidant effect. In this case, those proteoglycans have the highest antioxidant effect the molecular weight of which is under 200 kD. After the quality

analysis of extracted proteoglycans we have found connection between the level of the antioxidant activity and the quality of proteoglycans. Our data show that antioxidant activity increases in correlation with increase of protein/saccharide ratio.

This work was supported by OTKA grant F020919.

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J. C. CARRILLO, G. SZAKÁCS

Screening for fungi producing cellobiose dehydrogenase enzyme

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Cellobiose dehydrogenase (CBDH) is probably a link between cellulose and lignin biodegradation. CBDH has been shown to reduce quinones and phenoxyradicals released during lignin degradation concomitantly oxidizing cellobiose and other cellodextrins released during cellulose degradation to their corresponding lactones.

Twenty-nine filamentous fungi have been screened for extracellular CBDH production in shake flasks on two media. The fungi belonged to the genera (number of isolates in brackets) *Aspergillus* (10), *Ceratocystis* (1), *Chaetomium* (4), *Cladosporium* (1), *Gliocladium* (2), *Myrothecium* (1), *Penicillium* (6), *Phanerochaete* (2) and *Trichoderma* (2). The two *Phanerochaete chrysosporium* strains produced the highest enzyme activities, but *Chaetomium globosum, Myrothecium verrucaria* and *Aspergillus terreus* strains also showed significant CBDH secretion. Enzyme production was equally good on Solka Floc SW 200 (pure amorphous cellulose) and on lignocellulose substrate (equivalent mixture of ground wheat straw and corn stalk). *Trichoderma* and *Gliocladium* strains have been shown to produce very low amount or nil of CBDH on both carbon sources.

K. CZAKÓ-VÉR, G. BÖJTI, ZS. KOÓSZ, ZS. FEKETE, T. MAGYAR, M. PESTI

Characterization of *Schizosaccharomyces pombe* chromium(VI)-sensitive and resistant mutants

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The *Schizosaccharomyces pombe* strains lys1-131h$^+$ (CRW-6) and leu1-32h$^-$ (CRW-9) were used to obtain 84 chromium(VI)-sensitive (CRS) and resistant (CRR) mutants by induced mutagenesis. The chromium sensitivities were determined by minimal inhibitory concentrations of $K_2Cr_2O_7$ on complete nutrient agar and by the live-cell numbers in chromium-containing complete liquid medium.

The mutants were tested for their auxotrophic markers, temperature-sensitivities, back mutation frequencies, ploidities and mating types. The mutants exhibited cross-sensitivity and resistance of various types to Ni^{2+}, Se^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+}. Generation times, cell volumes and DNA contents were determined. On the basis of the results the two wild-type strains and two mutants, a sensitive CRS-6.51, and a resistant CRR-6.66, were selected for further crossing experiments to examine the genetic background of chromium(VI) resistance.

T. EMRI, É. LEITER, I. PÓCSI, A. SZENTIRMAI

Effect of phenoxyacetic acid on the glutathione metabolism of a high β-lactam producer *Penicillium chrysogenum* strain

Department of Microbiology and Biotechnology, Kossuth Lajos University, Debrecen, Hungary

Although penicillins G and V are among the first known antibiotics we still have surprisingly few data on the metabolism of their side-chain precursors, phenylacetic acid and phenoxyacetic acid (POA). Studying the glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) metabolism of an industrial strain of *Penicillium chrysogenum* we obtained the following results: (1) In the presence of 0.5% POA the intracellular concentration of GSH, which is an effective inhibitor of penicillin synthesizing enzymes, decreased profoundly. (2) POA induced both glutathione S-transferase and γ-glutamyl-transpeptidase, which are the first two enzymes in the GSH-dependent detoxification of xenobiotics. (3) 4-Hydroxy-POA, a naturally occurring intermediate of the oxidative degradation of POA, had no effect on the GSH metabolism of and, in contrast to POA, was not toxic to *P. chrysogenum*. We assume that, similarly to the detoxification of aromatic hydrocarbons, a reactive intermediate generated by the oxidation of the aromatic ring of POA induced the GSH-dependent detoxification pathway and, consequently, decreased the intracellular GSH level. This process was crucial to relieve the GSH inhibition of the penicillin synthesizing enzymes.

T. EMRI, I. PÓCSI, A. SZENTIRMAI

The protective role of glutathione against oxidative stress in a high β-lactam producer *Penicillium chrysogenum* strain

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Of the enzymes and intermediates of the penicillin biosynthesis, isopenicillin N synthase and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine are sensitive to oxidation and, therefore, the effective protection against oxidative stress is likely an important condition for a satisfactory antibiotic production. *Penicillium chrysogenum* was resistant to the oxidative stress caused by high concentrations of either H_2O_2 (350–700 mM) or *tert*-butyl

hydroperoxide (0.5–2.0 mM), which could be explained with high levels of catalase and glutathione (GSH) peroxidase (GPx) activities. Interestingly, menadione (MQ), which is a superoxide generating agent, caused sensible cell injuries even at 500 μM concentration. While a significant increase in the GSH synthesis and in the GSH reductase activity was observed after exposing mycelia to either peroxides or MQ, the specific catalase and glucose-6-phosphate dehydrogenase activities remained unchanged. The specific GPx and γ-glutamyltranspeptidase activities increased significantly only when the intracellular peroxide levels were high, whereas the GSH S-transferase and superoxide dismutase activities were induced only in the presence of MQ.

N. FARKAS[1], J. BELÁGYI[2], M. PESTI[1]

Effect of chromium on plasma membrane of sensitive and resistant mutans of *Schizosaccharomyces pombe strains*

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Lysine and leucine auxotrophic, heterothallic (h^+,h^-) strains of *Schizosaccharomyces pombe* were used to obtain chromium(VI)-sensitive and resistant mutans. The effect of Cr(VI) anions on the plasma membrane was studied *in vivo* by applying electron paramagnetic resonance (EPR) spectroscopy. 5-doxyl stearic acid (5-SASL) and 3-doxyl butyric acid (HO-185) spin probes were used to label the membrane. The order parameter S from the EPR spectra was calculated at different temperatures (0–25 °C) to characterize the internal dynamics of the membranes. Mutants in control experiments exhibited altered structural transitions both in 5-SASL- and HO-185-membrane in comparison with their parental strain suggesting alterations in membrane composition of these mutans. Addition of 225 μM $K_2Cr_2O_7$ significantly decreased the phase transition temperatures of the 5-SASL-membrane of wild-type strain, CRW-6 and sensitive mutant, CRS-6.51, but slightly increased phase transition temperature was obtained for resistant mutant, CRR-6.66 as revealed by the HO-185 label.

CS. FEKETE, K. POSTA, L. HORNOK

Isolation and characterisation of metallothionein genes from *Trichoderma hamatum*

Agricultural Biotechnology Center, UAS, Gödöllő, Hungary

Metallothioneins (MT) are low-molecular-weight, cysteine-rich proteins with a high metal binding capacity. These proteins are important in responding to cellular metal stress. Our strategy of cloning the copper MT genes from the biocontrol fungus, *T. hamatum* was based on known MT-sequences, that allowed the synthesis of degenerate oligonucleotide primers. The amplified DNA fragments were cloned, sequenced, and compared to published MT genes. The amplicons, whose steady-state levels of expression changed 5–10-fold in response to different metals were used to screen cDNA and genomic libraries of *T. hamatum*. Sequence comparisons of the positive clones revealed three distinct MT genes designated as *ThMT-1*, *ThMT-2* and *ThMT-3*. Based on the characteristic features of MT genes we found that genes *ThMT-1* and *ThMT-2* belong to the MT-I class, whereas *ThMT-3* is a member of the MT-II class of the metallothionein genes.

K. FEKETE-FORGÁCS[1], L. GYÜRE[2], B. LENKEY[1]

Characterisation of an induced fluconazole-resistant *Candida albicans* strain

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We investigated a fluconazole-sensitive ($MIC_{fluconazole}$ 5 µg/ml) clinical isolate (FS) and a fluconazole-resistant ($MIC_{fluconazole}$ > 80 µg/ml) laboratory mutant *Candida albicans* strain (FR) developed from the sensitive one. We studied putative virulence factors of the two strains as well as their growth. The fluconazole-resistant strain proved to be superior in all the virulence factors tested than the original strain: 1. the resistant strain considerably surpassed the FS strain in germinating capacity. 2. the FR strain showed ~3 times higher adhesion to buccal epithelial cells compared with the FS strain. 3. the aspartic proteinase activity of the FR strain was more than ten times higher, the phospholipase activity of this strain was twice higher than these values of the sensitive strain. The higher virulence of the fluconazole-resistant strain was also supported by mouse model. These results suggest that the development of fluconazole resistance can accompany with serious morphological and physiological changes: several putative virulence factors, moreover the *in vivo* virulence can change and become significantly superior.

A. GÁCSER, I. PFEIFFER, J. KUCSERA

Molecular and phenotypic characterization of *Cryptococcus hungaricus* isolates

Department of Microbiology, Attila József University, Szeged, Hungary

Cryptococcus hungaricus was described by Zsolt as *Dioszegia hungarica* (Zsolt, 1957). This species has reddish colonies due to carotenoid pigments of unknown chemical structure. The fungus is unable to grow above 25 °C and does not ferment glucose. Its perfect state is not yet know. Intraspecific variabilities were examined in six strains from the CBS culture collection. Strains were characterized by a combination of morphological and physiological properties as well as RFLP patterns of isolated mithochondrial DNA (mtDNA). Polymorphism was also analysed by amplifying and double digesting of the ITS region of nuclear ribosomal DNA. Carbohydrate-assimilation patterns were examined, and five types of C-sources were found to be useful for differentiation among isolates. Results showed that all strains but CBS 6324 and CBS 6576 were very inhomogeneous. This with the results of the RFLP pattern of the isolated mtDNA and the ITS region of the nuclear ribosomal DNA. Mithochondrial DNA of the type strain CBS 4214 was characterized in detail. Its molecular weight proved to be 27.47 kb by estimation of molecular weight of the fragments generated by different restriction enzymes. The physical and functional map of mtDNA of this strain is fully constructed.

Zsolt,J.: A new yeast *Dioszegia hungarica* nov.gen.et sp. Botanikai Közl 47, 63–66 (1957).

G. GICZEY, J. KUKOLYA, L. HORNOK

Detection of chitinase and glucanase isoenzymes from culture supernatants of the biocontrol fungus, *Coniothyrium minitans*

Agricultural Biotechnology Center, UAS, Gödöllő, Hungary

Coniothyrium minitans is an efficient mycoparasite of sclerotium forming plant pathogenic fungi. Despite of its potential as a powerful biocontrol agent, the mechanisms responsible for its hyperparasitic activity are rather unknown. During the parasitic process, the hyperparasite penetrates through the external pigmented layer of sclerotia. Penetration involves enzymatic degradation of the sclerotial wall that contains chitin and β-1,3-glucan as major constituents. The aim of the present study was to characterize the mycolytic enzyme system of *C. minitans*. We determined the time course of the extracellular exo- and endochitinase as well as β-1,3-glucanase activities of the fungus grown on various carbon sources, such as glucose, colloidal chitin and sclerotial wall

preparation of *Sclerotinia sclerotiorum*. Chitinase and β-1,3-glucanase isoenzymes produced by the fungus on these carbon sources were identified in gels after SDS-polyacrylamide gel electrophoresis.

Zs. Hamari[1], B. Tóth[2], Á. Juhász[2], L. Ferenczy[1,2], F. Kevei[2]

Interpretation of recombination events of mtDNAs after transmission of mitochondria among vegetative incompatible *Aspergillus japonicus* strains

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[2]*Department of Microbiology, Attila József University, Szeged, Hungary*

The mitochondrial genomes of the strains of *A. japonicus* belonging to the imperfect black *Aspergilli* display highly variable RFLP patterns. The 80 collection strains and field isolates investigated could be classified into eight different mtDNA RFLP groups (one of the eight types represented by some *A. aculeatus* strains which are closely related to *A. japonicus*). Applying a mitochondrial oligomycin resistant mutant (oliR) strain transmissions of mitochondria were carried out between oliR strain as standard donor and sensitive recipient strains with different mtDNA RFLP patterns by using protoplast fusion technique. These transfer experiments resulted in oliR progeny with recombinant and/or unchanged donor mtDNA in each case of intraspecific combinations, but it failed when *A. aculeatus* was the recipient partner. For interpretation of recombination processes physical and functional maps of mtDNAs of a recombinant and its parental strains were constructed. The mtDNA of recombinant strain consists of basically the donor mtDNA with additional recipient sequences. On the basis of sequence analysis the sequences derived from recipient mtDNA proved to be introns. It is suggested that the recombination events are due to the mobility of certain group I introns. Both intron acquisition and loss play a role in development of the recombinant character.

This work was financially supported by OTKA grant T025849.

L. Karaffa, K. Váczy, E. Sándor, I. Pócsi, A. Szentirmai

Connection between the cyanide-resistant alternative respiration and the intracellular peroxide levels in *Acremonium chrysogenum*

Department of Microbiology and Biotechnology, Kossuth Lajos University, Debrecen, Hungary

The aim of this work was to establish whether the intracellular hydrogen-peroxide levels enhanced by other means than the inhibition of the cytochrome path coincides with increased cyanide-resistant respiration. For this purpose, effects of exogenously added

H_2O_2 and salicylic acid (known to be the inhibitor of the enzyme catalase) were investigated.

When mycelia were exposed to increasing H_2O_2 or salicylic acid concentrations, increasing glucose consumption rate and increased alternative respiration activities were accompanied with decreasing specific growth rates and declining biomass productions. This could well be explained by the enhanced alternative respiration rates. Stimulation could be inhibited by cycloheximide, suggesting a de novo protein synthesis in this process.

Intracellular H_2O_2 levels also increased in a slow, concentration-dependent manner, achieving a maximum level in 4 hours in both cases.

It was concluded that increased intracellular H_2O_2 level is an important factor in the stimulation of the alternative respiration.

L. KREDICS, ZS. ANTAL, L. MANCZINGER

Influence of water activity and temperature on growth and enzyme activities of *Trichoderma harzianum*

Department of Microbiology, Attila József University, Szeged, Hungary

Influence of water activity (a_w) and temperature on linear mycelial growth and on activity of β-glucosidase, cellobiohydrolase, β-xylosidase, exochitinase, and chymotrypsin enzymes of *Trichoderma harzianum* was studied. Nearly linear correlation was found between water activity and linear growth rate at 25 °C and at 10 °C with higher growth rates at higher temperature and water activity values. Enzyme activities were significantly affected by water activity and temperature. All enzyme activities were lower at lower temperatures. In case of some enzymes a second activity peak was found at lower water activity possibly indicating the presence of xerotolerant isoenzymes. Significant relative enzyme activity was measured for most of the enzymes even at a_w=0.900 which is below the water activity limit of mycelial growth. This fact can make possible the use of mutants with improved xerotolerance for biocontrol purposes in lower water activity soils.

M. LÁDAY[1], F. BAGI[2], Á. SZÉCSI[1]

Isozyme evidence for two populations of *Fusarium graminearum*

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Thirty isolates of *F. graminearum* representing two populations (Group 1, Group 2) of this fungus were characterized by isozyme electrophoretic patterns. Cellulose-acetate electrophoresis (CAE) was used to investigate the isozyme variation among isolates.

Twenty-six enzyme systems were screened, and four of them (*alkaline phosphatase* E.C. 3.1.3.1, *NADP-dependent glutamate dehydrogenase* E.C. 1.4.1.4., *peptidase D* E.C. 3.4.13, *phosphoglucomutase* E.C. 5.4.2.2.) proved to be suitable for distinguishing between members of Group 1 and Group 2. The best results were obtained from patterns of *peptidase D* and *phosphoglucomutase* which are excellent diagnostic tools for the identification of two populations, as well. This is the first report offering an isozyme based genetic evidence for the difference and independence of the two groups of *F. graminearum*.

Due to short run time, minimal need of equipment, small sample volume and staining requirements CAE generally provides a rapid and accurate method for isozyme analysis of *Fusarium* species. CAE is also adequate for diagnostic and taxonomic purposes.

É. LEITER, T. EMRI, I. PÓCSI

Effect of the Fe(III) transport on the antibiotic production of a high β-lactam producer *Penicillium chrysogenum* strain

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Isopenicillin N synthase (IPNS), which is a key enzyme of the penicillin biosynthesis, requires high concentrations of Fe(II) cofactor [1]. All the experimental conditions which increase the intracellular Fe concentration may therefore result in an improved penicillin yield. Experiments were performed to see how changes in the extracellular Fe(III) concentration influenced the β-lactam production of an industrial strain of *Penicillium chrysogenum*. We found that the antibiotic yields increased markedly with increasing $FeCl_3$ concentrations starting from 15 μM in the culture media. On the other hand, $FeCl_3$ concentrations higher than 150 μM caused no further stimulation of the penicillin formation. Interestingly, the siderophore, mainly coprogen, production of the fungus was very low at any Fe(III) concentration tested under β-lactam producing conditions. Consequently, both the uptake of Fe(III) and the intracellular Fe(II) concentration seemed to be highly suboptimal during penicillin fermentations.

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Z. NAGY[1], A. SZENTIRMAI[1], A. J. CLUTTERBUCK[2], S. BIRÓ[1]

Isolation and mapping β-galactosidase negative mutants of *Aspergillus nidulans* and *Penicillium chrysogenum*

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We initiated the study of the carbon catabolite repression in filamentous fungi. In earlier expriments the intracellular production of β-galactosidase, using different carbon sources, and the intracellular levels of different nucleotids were monitored.

In order to study the genetic background of β-galactosidase production, and to clone its cognate gene, we isolated β-galactosidase negative mutants of *Penicillium chrysogenum* and *Aspergillus nidulans.*

Characterization of the mutants and mapping of the mutation(s) in *Aspergillus nidulans* by mutant complementation and cleistothecium analysis is on the way.

E. K. NOVÁK

Microscopic visualization of physical phases of fungal protoplast fusion

Department of Mycology, "Béla Johan" National Center for Epidemiology, Budapest, Hungary

With protoplast fusion experiments often used in biotechnology when no regeneration is observed it would be advantageous to know which stage of the process is defective. Thus stages before the successful melting together of the genetic stocks (the criterion for regeneration) are interesting to study. Therefore the following techniques were applied: (i) for plasmalemma fusion: postfusion India Ink Immune Reaction or enzyme Linked Immuno Sorbent Stain with species specific immune-serum (to one partner), (ii) for fusion of cytoplasms: Safranine prestaining (one partner only); for intermixing of cytoplasmic "organelles" production of intracytoplasmic inclusion bodies (blue formazan particles) by preincubation (one partner) with triphenyl tetrazolium violet. Results are demonstrated by the biologically incompatible intergeneric fusion of *Candida albicans* with *Saccharomyces cerevisiae.*

ZS. PALÁGYI, Á. NAGY, L. FERENCZY, CS.VÁGVÖLGYI

Genome typing suggests considerable natural ploidy differences in *Phaffia rhodozyma*

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Phaffia rhodozyma is a yeast species that produces astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) in considerable quantity. This carotenoid could be used as a feed ingredient for salmonid fish produced in aquaculture. The first investigations by pulsed field gel electrophoresis suggested the presence of chromosomal length polymorphism (CLP) in this species; surprisingly, the type strain of *P. rhodozyma* revealed more definite differences both in the genome size and in the number of chromosomal DNA in comparison with other *Phaffia* strains [1]. The aim of the present study was to investigate further *Phaffia* strains to obtain a clearer picture of the extent of CLP. The electrophoretic karyotypes of 7 *Phaffia* strains not analysed earlier revealed a very high level of CLP. Seven, nine, ten, eleven or fifteen distinct DNA bands were resolved. The total genome sizes of the *P. rhodozyma* strains studied varied between 15.38 and 32.28 Mb. The most probable explanation of the up to twofold difference in genome size between these strains is the difference in their ploidy level. These new investigations suggest that the genome size of the *P. rhodozyma* type strain is in the range observed for other *Phaffia* strains.

This study was supported by OTKA grants F021242 and D29113.

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T. PAPP, M. VASTAG, CS. VÁGVÖLGYI

Genetic variability of the postharvest pathogen *Gilbertella persicaria*

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Random amplified polymorphic DNA (RAPD) and isoenzyme polymorphisms among 16 isolates of *Gilbertella persicaria* were examined. Seven different 10-bp primers were used to determine the extent of intraspecific genetic polymorphism. Thirteen composite amplification types were identified. Numerical analysis revealed three clusters, which correlated with the mating types and places of origin of the isolates. Among the enzyme systems tested, catalase, α-esterase, glucose-6-phosphate dehydrogenase, malate dehydrogenase and superoxide dismutase were used for analyses. The variability in the isoenzyme patterns was very low. In contrast with RAPD analysis, no correlation was found between the isoenzyme markers and the mating type of the *Gilbertella* isolates. Correlation between these molecular markers and the growth and pathogenicity characteristics of these isolates were also tested.

This study was supported in part by OTKA grant F/4 017677 and Soros Foundation grant 230/1/676.

M. PESTI

Metal ion resistance in yeast

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All cells are presented with the challenge of living with metal ions. Cobalt, copper, iron, molybdenum, nickel, and zinc ions are essential for biological function, while ions of cadmium, lead, chromium, and mercury are not essentials. Cells need to maintain a balance between levels of metal ions that are nutritious and those that are toxic. They have to maintain their homeostasis in variable environments.

Most of these ions are transported into the cell by constitutively expressed ion uptake systems of broad specificity. Therefore, in case of a heavy metal stress, uptake of the toxic ions cannot be reduced by a simple down-regulation of the transport activity. Other than mutations limiting the ion range of the uptake system, only two main mechanisms of resistance to these ions are possible and were developed by evolutions: (i) intracellular complexation of the toxic metal ions by phytochelatins and metallothioneins, and (ii) reduced accumulation based on an active efflux of the ions.

Other specific mechanisms for resistance to Cu^{2+}, Cd^{2+}, Ni^{+}, Hg^{2+}, and $(CrO_4)^{2-}$, might involves extracellular chelation, chemical reduction, increased oxidative stress responses, and exclusion.

M. PÉTER, Z. PÉTER

A new simple *in vitro* cytotoxicity assay

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The authors have recommended a simple *in vitro* cytotoxicity test in order to complete *in vivo* methods or use it as an alternative technique for the prediction of acute toxicity. The substance studied is treated in bidistilled water with the suspension of living cells of a *Candida albicans* strain, and then the changes in the number of surviving cells is followed up daily. The result is expressed as a percentage of the initial number of living cells in comparison with the controls. The number of surviving cells is inversely proportional to the toxicity of the substance studied. The method is simple and cheap.

I. PÓCSI, T. PUSZTAHELYI

An opinion on the physiological classification of fungal *N*-acetyl-β-D-hexosaminidases (HexNAc'ases), *N*-acetyl-β-D-glucosaminidases (GlcNAc'ases) and chitobiases

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Our physiological and enzymological experiments indicated that the HexNAc'ase of *Penicillium chrysogenum* was likely a vacuolar/cytoplasmic hydrolase which was released into the culture medium during autolysis without any modification. The formation of the enzyme was not induced by either chitin or N-acetyl-D-glucosamine (GlcNAc) but was subject to carbon source repression. The selective inhibition of *P. chrysogenum* HexNAc'ase *in vivo* did not result in any morphological or physiological change in any growth phase. Substrate specificity investigations revealed that the hydrolase splits effectively N,N'-diacetylchitobiose but was not active against higher chitooligomers and, surprisingly, did not liberate GlcNAc units from β-D-galactosidase-treated asialofetuin either. The overall picture on the physiology and enzymology of *P. chrysogenum* HexNAc'ase shows some resemblance to analogous enzymes found in pathogens and in both growing and autolysing hyphae of saprophytes. These hydrolases should therefore be divided into subgroups only circumspectly, especially if this classification is based on the possible physiological functions of the enzymes.

J. REZESSY-SZABÓ, Q. D. NGUYEN, E. BUJNA, Á. HOSCHKE

Production of α-galactosidase by *Thermomyces lanuginosus*

Department of Brewery and Distillery, University of Horticulture and Food Industry, Budapest, Hungary

Filamentous fungi produce numerous enzymes, which are capable to degrade and transform various materials. Sixteen strains of the thermophilic fungus *Thermomyces lanuginosus* were screened for the production of α-galactosidase. On the basis of enzyme activity 3 strains were selected for further studies. The optimum parameters for the activity assay were determined. The temperature optimum was measured in the range of 55–58 C. The pH optimum was observed at pH=4.0. The effects of the different carbon sources on α-galactosidase activity were studied. Three variable, two-level factorial design was applied in order to determine the optimum composition of the medium for the maximum production of α-galactosidase. The investigated variables were raffinose, L-asparagine and $MgSO_4$. Only raffinose exhibited significant effect on the enzyme activity in the tested range. The produced enzyme was precipitated by ammonium sulphate and dissolved in minimum volume of buffer McIlvaine. The maximum specific activity was reached at 60% saturation.

K. RIGÓ[1], J. TÉREN[2], J. VARGA[1]

Degradation of ochratoxin A by *Aspergillus* strains

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Ochratoxin A (OA) is a mycotoxin discovered in 1965 as a secondary metabolite of *Aspergillus ochraceus* strains. In the subsequent years several other *Aspergillus* and *Penicillium* species were described as producers of this toxin. OA was proved to exhibit nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties. OA contamination of green coffee beans and other plant products such as barley, wheat and bread is a serious health hazard throughout the world. Elimination of OA from agricultural products including poultry feed, cereals and green coffee beans can be accomplished by chemical methods (e.g. H_2O_2 treatment), or by microbes (e.g. *Acinetobacter* sp.). During our studies, several *Aspergillus* species were examined for their ability to degrade OA. Some *A. fumigatus* and *A. niger* strains could eliminate OA effectively from the culture media. *A. niger* is one of the few fungal species which has received the GRAS (generally regarded as safe) status due to its low toxigenicity. An atoxigenic *A. niger* strain could degrade OA both in liquid and solid media within 4 days and the degradation product, ochratoxin α, was also eliminated in the next two days. Further studies are in progress to examine the ability of this strain to degrade OA in agricultural products.

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L. SÁMI, T. EMRI, A. SZENTIRMAI, I. PÓCSI

Protection against oxidative stress in *Penicillium chrysogenum* cells grown on nitrate or nitrite

Department of Microbiology and Biotechnology, Kossuth Lajos University, Debrecen, Hungary

Adding NO_3^- or NO_2^- to *P. chrysogenum* cultures influenced the NADPH production and the glutathione (GSH) metabolism of the cells in addition to the induction of the NO_3^- assimilatory pathway. The concomitant induction of glucose-6-phosphate dehydrogenase, a key enzyme of the pentose phosphate pathway, and GSH reductase, which reduces GSSG to GSH, by using either NO_3^-or NO_2^- clearly demonstrated the efforts made by the cells to maintain simultaneously both the NADPH and GSH concentrations at physiological levels. As a result, no elevation in the intracellular peroxide concentrations and no redox imbalances were observed during the assimilation of these nitrogen sources. On the other hand, the specific activity of several other GSH metabolic enzymes including GSH peroxidase, GSH S-transferase, γ-glutamyltranspeptidase and the GSH producing activity was not effected by NO_3^- and NO_2^-. When *P. chrysogenum* mycelia were challenged with oxidative stress caused by

high concentrations of H_2O_2, *tert*-butyl hydroperoxide, menadione, diamide or phenoxy-acetic acid the intracellular peroxide concentrations increased significantly, and the nitrate reductase and nitrite reductase activities were eliminated.

E. SÁNDOR[1], A. JUHÁSZ[1], L. KARAFFA[1], P. C. GOPAL[2], I. PÓCSI[1], S. BÍRÓ[1], C. R. THOMAS[2], A. SZENTIRMAI[1]

Application of the fluorescent dye "Acridine Orange" as a research tool for the investigation of fungal physiology

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In this study we tried to find out whether there is a correlation between the physiological activity of *Acremonium chrysogenum* and *Aspergillus nidulans* cultures and their staining with the fluorescent dye Acridine Orange (AO).

In the exponential phase, mycelia exhibited filamentous morpholo-gy and a green colour, whereas the stationary phase could be characterized with cells stained red. Stationary phase cells supplied with glucose reinitiated growth, which coincided with enhanced respiration rate and a more green-like AO staining. During soybean supply, no growth, but significant cephalosporin C production in *A. chrysogenum* could be observed, while cells exhibited an overhelmingly green colour upon AO staining.

In chemostat cultures, specific growth rate was inversely proportio-nal to the ratio of areas stained with red and green, respectively.

E. SÁNDOR, E. FEKETE, L. KARAFFA, A. SZENTIRMAI, I. PÓCSI

Effect of sulphur-containing amino acids on the glutathione metabolism and cephalosporin-C production of *Acremonium chrysogenum*

Department of Microbiology and Biotechnology, Kossuth Lajos University, Debrecen, Hungary

Methionine (Met) added in optimal, 7.0 g l^{-1} concentration to *A. chrysogenum* cultures increased all the biomass production, the intracellular glutathione (GSH) concentration and the cephalosporin-C (CPC) yield. When cysteine (Cys) was applied in an equimolar concentration no increase in the GSH level and CPC production was observed but stimulated the biomass production. On the other hand, cystine, the oxidized form of Cys, hindered profoundly even the mycelial growth. Therefore, we concluded that the transient elevation of the intracellular GSH concentration observed only in the presence of Met in the trophophase was a prerequisite of the satisfactory CPC production. Moreover, sulphur was most likely transferred *via* the Met→GSH→CPC pathway in the idiophase. Similarly to other organisms and unlike to Met, Cys was thought to inhibit the

de novo synthesis of GSH resulting in a reduced intracellular GSH level. With cystine, the formation of high concentrations of mixed GSH-Cys disulphides and, consequently, the onset of redox-imbalances were likely responsible for the inhibition of biomass production.

Á. SVEICZER[1], B. NOVÁK[1], A. CSIKÁSZ-NAGY[1], B. GYŐRFFY[1], M. MITCHISON[2], J. J. TYSON[3]

Quantized cell cycles in fission yeast

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The fission yeast, *Schizosaccharomyces pombe* is a classical test organism for studying cell cycle. A very simple method often used is taking time-lapse films of growing cultures in a thermostated photomicroscopic field. We have recently analysed many cell cycle mutants by this method. The most interesting case was the *wee1-50 cdc25Δ* double mutant where the main inhibitor and activator of mitotic onset are both absent. We have found that the cycle time is quantized in this population and the cycle time of a cell is not determined by its birth length. Those cells having long cycles abort mitosis from an early stage and return back to G2 phase. We have developed a mathematical model in which *mik1*, the back-up enzyme of *wee1* is activated during early mitosis, therefore the mitosis promoting factor (MPF) becomes inactivated, causing an improper exit from mitosis instead of separating the sister chromatids.

G. SZAKÁCS

Trichoderma biodiversity in TUB culture collection

Department of Agricultural Chemical Technology, Technical University of Budapest, Budapest, Hungary

Trichoderma species are ubiquitous in the environment, especially in soils. They have been used in many human activities, including commercial applications in production of enzymes (e.g., cellulase, xylanase) and biological control of plant disease. The type of climate, soil, plant, fungal pathogen, etc. may influence the usefulness of *Trichoderma* as biological control agent. Culture collection at the Technical University of Budapest (TUB) comprises 2100 microorganisms with emphasis on lignocellulose degrading fungi and actinomycetes. For isolation and screening programs, 285 soils are stored at +5 °C. They were collected in 52 countries. There are approximately 480 *Trichoderma* strains in TUB collection. They have been isolated worldwide representing 36 countries. *Trichoderma* spp. isolated from soils at high elevations in the Himalayas region and Rocky Mountains in Colorado grow at low temperatures such as 5–10 °C.

These strains do not grow at 30 °C. On the contrary, some *Trichoderma* spp. were selected from tropical soils and decaying plant materials at 37–40 °C. *Trichoderma* spp. tolerating limited oxygen supply were isolated from river sediments and muds.

J. TORNAI-LEHOCZKI, D. DLAUCHY, G. PETER

Differentiation of brewing yeast strains

National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary

Brewing yeast strains are important factors in producing consistent and characteristic products in the brewing industry. Recently more and more companies started to use more than one strain of brewers' yeast, particularly if ale and lager beer are being brewed or if brewing agreements with other companies require the use of alternate strains of yeast. In such cases microbiological purity of the strains both from each other as well as from nonbrewing "wild" yeast strains is necessary to maintain consistency in fermentation performance and in overall product quality.

Modern molecular biology techniques, Random Amplified Polymorphism DNA analysis (RAPD-analysis) and chromosomal DNA karyotyping using Pulsed – Field Gel Electrophoresis and ribosomal DNA – Restriction Fragment Length Polymorphism (rDNA-RFLP) were used. The active fructose transport was tested to reveal differences between *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* species.

The above-mentioned methods were used for several ale and lager brewing yeast strains and for *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* type strains.

The results show that for separation of ale and lager yeast strains the chromosomal DNA karyotyping d RAPD analysis proved to be appropriate methods however these strains belong to the same species on basis of their entire rDNA-RFLP patterns.

B. TÓTH[1], ZS. BEER[1], ZS. HAMARI[2], F. KEVEI[1]

Detailed physical maps of mitochondrial DNAs of *Aspergillus niger* strains and their intraspecific recombinants

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Intra- and interspecific mitochondrial DNA (mtDNA) polymorphisms are frequently observed among imperfect black *Aspergillus* isolates. Successful mitochondrial transfers were carried out between incompatible strains possessing different mtDNA patterns by selecting for the oligomycin resistance carried by the mtDNA of the donor parent. Each intraspecific transfer resulted in a single type of recombinant RFLP profile, while interspecific transfers resulted in the appearance of a number of different

recombined mtDNAs. Physical and functional maps of some strains of the *A. niger* species and their recombinants were constructed. These maps of the recombinant mtDNAs are similar to those of the mitochondrial genomes of the donor strains, with the exception of the region containing the *cox1* gene, which displayed size differences in the examined strains. Sequence analysis of this region and the *atp6* gene, which carries the oligomycin resistance, let us localize the sites where recombination took place in these mtDNAs.

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Production of amylase and amyloglucosidase with *Rhizopus oryzae* strains by solid substrate fermentation on brewing spent grains

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Annually, 651,000 dry tons of brewing spent grains (BSG) are produced in the US alone. The by-product contains 23% hemicellulose, 22% lignin, 18% protein, 15% cellulose, 12% starch and 10% other constituents. BSG can be converted to value-added products, such as animal feed additive and/or crude (*in situ*) enzyme by microbial fermentation. Solid substrate fermentation (SSF), a relatively simple and economical method is especially suitable for this purpose.

Eleven *Rhizopus oryzae* strains have been screened in SSF on BSG wetted with salt solution. The SSF was performed at 25 °C and 67% moisture content for 1–3 days. The best amylolytic enzyme producers (*Rh. oryzae* NRRL 1981, NRRL 3562, NRRL 6201 and NRRL 6400 strains) secreted 127–162 International Unit (IU)/g dry matter (DM) α-amylase and 47–65 IU/g DM amyloglucosidase activity. The amount (activity) of simultaneously produced lignocellulose degrading enzymes (cellulase, endoglucanase, xylanase) was negligible.

Z. VARECZA, T. EMRI, T. PUSZTAHELYI, I. PÓCSI

Allosamidin inhibits the fragmentation and autolysis of hyphae in ageing cultures of *Penicillium chrysogenum*

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The pseudotrisaccharide allosamidin, which is a well-known, specific and potent inhibitor of chitinases, retarded significantly the fragmentation and autolysis of hyphae in ageing carbon-depleted cultures of an industrial β-lactam-producing strain of *P. chrysogenum* [1]. Moreover, allosamidin also hindered, even after the addition of an extra

dose of glucose, the germination of the surviving hyphal fragments, which were round-ended and usually consisted of two cells as described before [1]. In spite of this, no difference in the glucose utilisation rates was found in allosamidin-treated and control cultures. Under antibiotic producing conditions, allosamidin did not have any effect on the penicillin yields.

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M. VASTAG, T. PAPP, K. ÁCS, CS. VÁGVÖLGYI

Intraspecific variability of thermophilic *Rhizomucor* species as assessed by randomly amplified polymorphic DNA

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The genus *Rhizomucor* comprises three species: *R. pusillus*, *R. miehei* and *R. tauricus*. In spite of their importance, little is known about the extent of genetic variability in these species. Twenty-three *Rhizomucor* isolates (9 *R. miehei*, 13 *R. pusillus* and 1 *R. tauricus*) from various sources were studied. Random amplified polymorphic DNA (RAPD) analyses were performed with seven different 10-bp primers. These data were supplemented with mating studies and were used in numerical analyses. The genetic variability was found to be different among *R. pusillus* and *R. miehei* isolates; the latter displayed less intraspecific polymorphism. The *R. tauricus* isolate (the only known isolate of this species) showed no substantial difference from the heterothallic *R. pusillus* isolates: a similar situation was observed earlier when isoenzyme and ITS-RFLP markers were investigated [1]. The RAPD amplification allowed the creation of composite amplification types to differentiate *Rhizomucor* isolates at the strain level.

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CS. VÁGVÖLGYI, M. VASTAG, T. PAPP, ZS. KASZA

Isoenzyme and random amplified polymorphic DNA (RAPD) analysis of the homothallic *Mucor genevensis*

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Mucor genevensis is of special interest as it exhibits both dimorphism and homothallism. The aims of the present study were to investigate the intraspecific variability of this species and to obtain data concerning its taxonomic relationships. Ten *M. genevensis* strains, each strain of the recently described new homothallic species (*M. meguroense* and *M. hachijyoensis*) [1] and strains of *M. hiemalis* and *M. piriformis* were investigated. Five different enzyme systems (CAT, GDH, LDH, MDH and SOD) and six 10-bp random primers were used in isoenzyme and RAPD analyses, respectively. Data from these studies were used for numerical analyses. Substantial intraspecific variability was detected in *M. genevensis* by using any of the methods. Although strains of *M. meguroense* and *M. hachijyoensis* revealed characteristic differences, they grouped closer to the homothallic *M. genevensis* strains than to the heterothallic *M. piriformis* and *M. hiemalis* isolates.

This study was supported in part by OTKA grant F/4 017677 and Soros Foundation grant 230/1/676.

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POSTER SECTION 3

APPLIED MYCOLOGY

K. BÓKA[1], J. JAKAB[2], I. KIRÁLY[2]

Application of fungal elicitors in *Rubia tinctorum* tissue culture

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The use of fungal mycelium wall extracts is a well-known method to elicite secondary metabolite production in plant cell suspension cultures. Here we show results on the elicitation of alizarin production in *R. tinctorum* cell culture by various fungal mycelium wall fractions of *Bothrytis cinerea, Pythium debaryanum, Verticillium dahliae.* The amount of different oligosaccharins was determined photometrically following column chromatography. Elicitors contained mostly 67–1,5 kD oligosaccharins. The pH raised from 4.76 to 6.27 in a 96 hours exposition period while the alizarin content both in the cell fraction and the supernatant was significantly higher. The elicitation process was accompanied by typical stress symptoms of the plant cells, including alkalinization of the medium and changes of defence related enzyme activities. During the 10–16 hours period of elicitor treatment a moderate raise in peroxidase activity level, peaking at 14 hours was detected, while superoxide dismutase level was constant.

L. FEHÉR, J. CSATLÓS, L. TANÁCS

Residue testing of winter-wheat species formerly treated by fungicide

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The degree of contamination by mycosis in wheat fields has increased because of the lack of expertise and capital due to the privatisation process of agro-economy in the last nine years.

Besides the pathogenic effect of smut and rust fungi fusarium contamination is the primarily most significant phenomenon in mycoses. These factors make effective plant protection necessary. Using pesticides the dosage measure of the applied fungicides is important, as well as the selection of the right phenophase.

Without pesticides 24.4% of the world's wheat-crops would be ruined by different weed-fungi and animal pests (Hoffman, 1984). The accurate extent of the sowing area of

pesticide-treated wheat cannot be estimated at present, but in 1983–84 the treated wheatfield reached the size of 1.2M ha-s (Gimesi and Hunyadi, 1987).

The applied fungicides are mainly triazole-type compounds having a well-known broad effect-spectrum against mycoses and remain effective for long. In addition they improve the efficacy of photosynthesis via their favourable physiological side-effects causing increase of crops (Petróczi et al., 1996).

Our objectives were to detect the residue in flour and bran grist of different winter wheat species improved by GKI (GK-Ötthalom, GK-Góbé , GK-Délibáb and GK-Olt) on the external surface of the husk and to determine the distribution of fungi below the husk and on the external surface of husk on genus level.

Altogether 432 basic chromatographic analyses done from the extract of 18 components studying of four winter wheat species and 12 fungicide-treatments.

In treatments 4–9 and in (V.4.) I-J phenophase (besides the applied Glean (chlorosulphuron) herbicide) out of the six applied fungicides only the triazole residue could be detected successfully. In treatments 10–15 and in (V.17) L-M phenophase again some triazole residue was detected.

It can also be concluded that the triazole type compounds got into the flour-fraction in greater proportions than into the bran fraction.

It is interesting to note that it was possible to detect residue in nine cases (I-J) in the phenophase further in time from the data of harvesting whereas the same kind of detection was possible only in six cases in the L-M phenophase.

This latter result is also interesting because the translocation of the applied fungicides into the grain crop was much more moderate in the phenophases closer in time to the full development of the generative part than in the earlier (I-J) phenophases despite the fact that the fungicides were spread on the ears of wheat.

When studying the microflora an essential difference between the fungi on the external surface of the grain and below the pericarp was shown.

E. FODOR, I. BOLDIZSÁR, B. DÁNOS, GY. SZEDLAY, M. LÁSZLÓ, I. GYURJÁN

Elicitation of anthraquinones in *Rubia* root culture by fungal treatment

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Rubia tinctorum L. like other species of the Rubiaceae is well known due to its pharmaceutical (*e.g.* phytoncide, spasmolytic, litholytic) effects [1, 2] as a consequence of abundant production of anthraquinones [3]. Previous results showed that anthraquinone synthesis could be stimulated by stress conditions activating defense mechanisms of the plant. The purpose of our studies was to investigate whether the formation of these anthraquinones can be affected by fungal polysaccharides. An autoclaved polysaccharide solution of *Coriolus versicolor* (*Fr.*) Quel. prepared by hot water extraction and ethanol precipitation [4] was added as elicitor to the *Rubia* root cell culture [5]. The amount of

secreted anthraquinone in the cell culture medium was quantified by spectrophotometry. The addition of the fungal polysaccharides to the *Rubia* cell culture induced a significant (more than 50-fold) increase of the anthraquinone content in correlation with the concentration of polysaccharides. Analysing the concentration as a function of time we have found that the largest quantity of anthraquinones could be measured on the 4th day of the incubation. We fractionated the fungal polysaccharide solution by gel filtration chromatography and showed that only the fractions containing polysaccharides with higher molecular weights (MW > 60 kDa) have considerable eliciting activity. Our findings show that fungal polysaccharides have potent stimulatory effect on the anthraquinone synthesis of *Rubia tinctorum* and are potent candidates for biotechnological and pharmaceutical use.

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K. GARAS[1], M. VASTAG[1], F. SOMOGYVÁRI[2], CS. VÁGVÖLGYI[1]

Applicability of the ATB Fungus system for rapid antifungal susceptibility testing of *Rhizomucor* isolates

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Rhizomucor species may be agents of rapidly progressive and frequently fatal opportunistic mycotic diseases [1]. The ATB Fungus system (BioMerieux) was used for the *in vitro* susceptibility testing of different yeast pathogens [2]. The aims of the present study were to evaluate this system as a rapid *in vitro* antimycotic test for *Rhizomucor* strains and to gain data about the variation of the *in vitro* susceptibility against different antifungal agents within this genus. The effect of 6 antifungal agents (amphotericin B, econazole, flucytosin, ketoconazole, miconazole and nystatin) against 15 *Rhizomucor* strains representing all the currently accepted species of the genus (*R. miehei*, *R. pusillus* and *R. tauricus*) was studied. The results demonstrate the applicability of this micromethod for the simple antifungal susceptibility testing of these opportunistic fungal pathogens.

This study was supported in part by PFP grant 0304/1998.

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J. GEML[1], I. RIMÓCZI[2]

Attempts at cultivating wild strains of various *Agaricus* species

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The most important cultivated mushroom of the world is *Agaricus bisporus*. Other *Agaricus* have been examined also by mushroom breeders, although growing them is not in practice yet. The importance of using wild varieties of *A. bisporus* and other *Agaricus* species in breeding new commercial strains has been realized by several researchers. These wild types can be used to improve the commercial strains' growing characteristics, resistance to pests etc.

In our laboratory we have collected wild varieties of several species and made some initial observations on culturing them. In this paper we introduce the main *Agaricus* species of Hungary, including their descriptions and habitats under artificial conditions.

K. HALMY

Results of internal treatment of onychomycosis

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In the past five years patients with onychomycosis in an open survey were treated with terbinafine, itraconazole and fluconazole. 94 patients received terbinafine continuously. Fingernail and toenail infections were treated for 6 and 12 weeks, respectively. At week 36 clinical and also mycological cure was achieved in 85.7% of the fingernails, whereas the toenails were cured in 88% clinically and in 91.5% mycologically. Side effects were noted in 2.1% of the cases. Itraconazole was given to 47 patients. In fingernail and toenail infections two and three intermittent treatments were applied, respectively. At week 36 clinical and mycological cure of fingernails was observed in 90% and 95%, respectively, while in the case of toenails the therapeutic results were 92% and 81.5%. Side effects were found in 4.2% of the patients. A 150 mg weekly dose of fluconazole was given to 27 patients with onychomycosis for 6 months. At week 36 fingernails were clinically cured in 100%, whereas mycological cure was achieved in 95%. As regards toenails, in 6 out of 7 patients both mycological and clinical cure was achieved. Side effects were noted in 3.7% of the patients. The applied antimycotics had favourable effects in the treatment of onychomycosis. Terbinafine is recommended to use in dermatophytic, whereas itraconazole and fluconazole in yeast infections.

K. HALMY, M. HALMY

Investigations and treatments in vaginal mycosis

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30% of the diseases transfected through sexual ways are candida vaginitis. The chronic recurrent vaginal mycosis occurred in 5% of cases. Decrease of cellular immunity could also be detected in these patients. According to our five-year-survey, 7–14% of the mycological diseases (1400 cases/year) were vaginal mycoses and a quarter of them were chronic. Clinical, mycological and immunological examinations were carried out at 30 patients with vaginal mycoses (12 chronic, 10 acute, 8 control). Predisposition and clinical symptoms of both the chronic and acute form occurred in the similar proportion. The pathogen proved to be *Candida albicans* in all cases. Results obtained from the cellular immunity examinations were not different in the acute and chronic groups. The serum levels of the candida antibody IgG were elevated in both forms compared to the control. IgA levels increased only in the chronic cases. The amounts of candida antibodies IgG and IgA were higher in the vaginal fluor of chronic patients. *C. albicans* blastospores and germ tubes were found to be more adherent in both groups of patients in contrast to control. *Candida* could be cultured only from the faeces of chronic patients. Both itraconazole (Orungal) and fluconazole (Diflucan) therapy had favourable effects.

A. HEGEDŰS[1], HOSAM BAYOUMI H.E.A.F.[2], M. KECSKÉS[3]

Dressing of Gerbera root with *Trichoderma* spp. to study its effect on plants production

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We studied the effect of *Trichoderma viride* on the growth of nine *Gerbera* cv. (Macho, Zigone, Snowball, Rebecca, Fame, Robijn, Parade, Pascal, and Fanny). The Gerbera plants were taken from tissue culture and were grown – under greenhouse conditions – in sterilised peat pots before the treatments of plant roots with *Trichoderma viride* solution. Roots were immersed into the spore suspension of fungus for ten minutes. Plants were then watered with nutrient solution every day. The young plants were grown for eight weeks then they were planted into sterilized peat soil in 10-litre plastic containers. We measured chlorophyll and carbohydrate content of the leaves, and estimated yield and flower quality of the plants.

Significant differences were observed among the growth of treated and control plants.

I. KIRÁLY[1], G. KÁLMÁN[2], Z. LUKÁCS[3]

Growing gourmet- and medicinal mushrooms

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Mushroom growers in Hungary produce considerable amounts of only two species: *Agaricus bisporus* and *Pleurotus ostreatus*. Recently market for some new species (*Lentinula edodes, Pleurotus citrinopileatus, Pleurotus djamor*) is developing. All around the world, attention paid to the production of gourmet- and medicinal mushrooms is increasing. Here we show results on growing new mushroom species under laboratory conditions. *Ganoderma lucidum*, the well-known medicinal mushroom, is a slow-growing species with very complex requirements towards the environment. Classic carpophore form is generated only by a three step fruit-body induction strategy. The favoured shape and size of *Flammulina velutipes* may be reached by elevated CO_2 level, supplied in a special shape growing vessel. Such a way the cap remains less glutinous, and a mild stem develops. *Hericium erinaceum* tends to form conidiospores, early in the colonisation period; inoculation by liquid inoculant effectively prevented this inconvenience. *Agrocybe aegerita* is a prolific carpophore-producing gourmet mushroom, requiring a relatively long resting period between flushes and a complex substrate rich in nitrogen.

D. KRISTÁLY, C. RADULESCU

Genital candidiasis – a risk factor for postcesarian complications

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Objectives: Vulvovaginal candidiasis is very common during pregnancy. It occurs in 35–55% of women during the third trimester. The yeasts, like other organisms present in vagina or cervix, are risk factors causing postcesarean complications.

Methods: A study was performed in a cohort of 189 pregnant women in the period of 1995–1998, selected for Cesarean intervention. Samples from vagina and cervix were taken for laboratory diagnosis of bacterial, mycotic and *Trichomonas vaginalis* infections.

Results: The incidence of one or more agents was 87.3%. The prevalence of yeasts was 20.6%, that of *Trichomonas vaginalis* 9.69%, *Ureaplasma urealyticum* 62.4%, *Mycoplasma hominis* 10.9%, *Chlamydia trachomatis* 9.1% and *Gardnerella vaginalis* 21.8%. Other infections (streptococci, staphylococci, Gram-negative bacilli and anaerobs) were identified in 49% of the positive samples. Biochemical identification of yeasts with API strips was performed. *Candida albicans* was found in 61.7%, *C. glabrata* in 20.5%, *C. parapsilosis* in 11.7%, *C. guilliermondii*I and *C. krusei* in 1% of the samples.

Conclusion: For efficient specific prophylactic drug therapy of maternal and neonatal postcesarian complications the microbiological control of the genital flora is necessary.

E. K. NOVÁK

Sick buildings – flat wall molding – bronchial asthma

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Although the water demand of fungi is lower than that of bacteria (water activity higher than 0.8) yet the ability of fungi to colonize in-door flat walls depends on the humidity of it (other circumstances – nutrients, temperature, pH, aeration – are convenient for fungi). In addition to accidental pipe bursts and rain insulation defects in panel buildings excess moisture appears by vapor condensation on the surface and/or in the capillaries of the internal front of the external walls, which in turn is the consequence of architectural faults in the temperature insulation leading to temperature bridges through the wall. The water activity $a_w = 0.6<$ in the "substrate" causes fungal growth resulting in extreme in-door air pollution by fungal propagules (up to 41,000 c.f.u./m^3) endangering with bronchial asthma of the tenants, especially children. Into the fungal colonies, often fungal lawns, however, i.a. mites as well as nematodes could settle. The situations are visualized by macro- and micro-photos.

M. OPREA

Early drying grapevine in Romania induced by lignicolous fungi

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Ecological and biological decline of grapevine in Romanian vineyards massively appeared during the last 15 years. Examination of diseased materials (branches, multiannual shoots, rootstocks, roots) revealed 10 lignicolous fungal species involved in early drying of grapevine, namely: *Eutypa lata* (57.8%), *Phomopsis viticola* (24.8%), *Cytospora vitis* (8.4%), *Phoma uvicola* (4.8%), *Diplodia viticola* (4.8%), *Stereum hirsutum* (3.8%), *Verticillium dahliae* (3.6%), *Spaeropsis malorum* (2.4%), *Roesleria hypogea* (1.2%), *Pestalozzia vitis* (1.2%). Decline appeared mainly in wine cultivars, where attack frequency was 5–95% and less in table grape cvars. Plants affected had poor vegetation, delayed budding, great shoots proliferation in spring were noticed only at vegetation outset, without determining normal evolution in autumn; branches exhibited fanleaf symptoms; plants apparently healthy wilted in full-summer, yield being diminished up to disappearance.

Developmental biological parameters of these lignicolous fungi have been framed within the same values under laboratory conditions, their ecological requirements being close, this explaining their evolution in autumn – winter – spring period (October – April) under the pedoclimate conditions prevailing in this country.

I. RIMÓCZI

Biological research on bringing the giant puffball (*Langermannia gigantea* [BATSCH: PERS.] ROSTK.) in cultivation

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Being an edible, tasty mushroom with pharmacological importance, the *Langermannia gigantea* is urged to bring into cultivation. Its requirements for soil types, the fenology and the environmental factors of fructification, and the cenological rules of its occurrence have been examined for ten years in more than 81 habitats.

More than 2 000 data, gained from soil components of 58 habitats, give a guide for preparing suitable substrate for the cultivation of *Langermannia gigantea*, with the requested climatic conditions for fructification. The *Langermannia gigantea* is exclusively saprophyte, even its facultative mycorrhiza connection is unlikely. Its spontaneous appearance in cultivated vineyards and orchards supports the possibility of its cultivation.

T. E. SESAN[1], N. CSÉP[2], E. PROCOPOVICI[3], S. RARANCIUC[4], M. GURAN[4]

Biocontrol of white rot (*Sclerotinia sclerotiorum*) with antagonistic fungi (*Trichoderma* spp., *Coniothyrium minitans*)

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Investigations have been performed in multiannual field trials (1991–1998) with *Trichoderma harzianum*, *T. viride* and *Coniothyrium minitans* and similar bioproducts based on these fungi (Trichodex 25 WP – *T. harzianum*, isolate T-39, Maktheshim Agan-Israel; Trichosemin 25 PTS, *Trichoderma*-pellets, *T. viride* – isolate Td 50, RIPP Bucharest-Romania) to protect sunflower, soybean and beans against white rot (*Sclerotinia sclerotiorum*).

All biological seed or soil treatments (*Trichoderma* spp., *Coniothyrium minitans*) showed good efficacy in protecting sunflower, soybean and beans from white rot (*S. sclerotiorum*), however lower than the specific standard chemicals (Sumisclex 50 WP,

Metoben 70 PP, Tiramet 60 PTS). Seed amount harvested in biological treatment trials were higher than seed yield obtained from untreated controls.

Although efficacy of biological products was lower than that of chemicals, the former ones are advantageous by protecting agroecosystems of sunflower, soybean and beans and environment, as well.

Á. SUHAJDA[1], B. JANZSÓ[1], J. HEGÓCZKI[2], G. VERECZKEY[2], Á. BATA[3], A. MARÁZ[4]

Micro-element enriched yeast

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To provide widely available micro-element sources for humans and animals it is a possibility to improve the mineral substance content of food and feed products or to prepare micro-element containing paramedicinal products. For this purpose there are different attempts, one of them is micro-element enrichment in yeast cells. In appropriate circumstances yeasts (e.g. baker's yeast *Saccharomyces cerevisiae*) are capable of accumulating large amounts of micro-elements and incorporating them into organic or complex compounds. It has been found that introduction of water soluble salt of a micro-element as a component of the nutrient medium for yeasts produced by conventional batch processing, results in a substantial amount of micro-element absorbed by the yeast.

Micro-element enrichment in yeasts can be performed by two essentially different methods, either adding the water soluble salt of micro-element during the growth phase or to resting, but still respirating cells after cultivation.

In the first method salts that can be added either in small quantities periodically or continuously in the exponential growth phase, in some cases can have a strong inhibitory effect for the final yield of the yeast. Another aspect is the rate of uptake, because in the yeast cell proportion of organic and inorganic form of micro-element in question considerably depends on the rapidity of building in. The conditions of uptake must be exactly chosen by varying cultivation parameters, such as media components, pH and aeration.

In the case of certain micro-elements the second method can be used that have several advantages in comparison with the method mentioned above. Biomass production and micro-element uptake occur separately, therefore there is no inhibitory effect; conditions for the maintenance of yeast cells have less influence for micro-element uptake and the amount of micro-element to be added can be calculated more exactly, thus no significant level of micro-element remains in the medium after yeast cells have been harvested.

G. SZAKÁCS[1], R. P. TENGERDY[2], K. URBÁNSZKI[1], É. SUGÁR[1], P. O. SAKARIASSEN[3], A. KERTENICS[1]

Production of cellulase, xylanase and laminarinase with *Trichoderma* and *Gliocladium* strains by solid substrate fermentation

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Lignocellulolytic enzymes may be produced economically and efficiently by solid substrate fermentation (SSF). The advantage of SSF is its operational simplicity and economy in a water restricted environment and possibility for use of the product with little or no downstream processing. Crude SSF enzymes may be used directly in agrobiotechnological applications such as ensiling, feed additives, retting, soil additives, and in biotechnological industries such as paper industry and biofuel industry.

SSF laboratory experiments were carried out with *Trichoderma reesei* Rut C30, *Trichoderma hamatum* TUB F-105 and *Gliocladium sp.* TUB F-498 strains on corn fiber, brewery spent grains, wheat straw and Norvegian seaweed (*Laminaria digitata*) substrates. Depending on the strain and substrate, the filter paper degrading cellulase activity was 3–54 FPU/g dry weight (DW). Simultaneously, 320–5100 International Unit (IU)/g DW xylanase activity was also produced. The production cost of the crude (*in situ*) enzyme was about ten times less than the cost of commercial enzymes produced by submerged fermentation.

G. SZAKÁCS[1], K. URBÁNSZKI[1], R. P. TENGERDY[2], J. C. CARRILLO[1]

Screening for metabolites produced by filamentous fungi: lactic acid, lovastatin and fumagillin

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L(+)-lactic acid production has received much attention recently because lactic acid can be a starting material of polylactic acid, a new biodegradable plastic. Sixteen *Rhizopus* strains (*Rh. oligosporus, Rh. oryzae, Rh. stolonifer*) have been screened for lactic acid production in shake flasks on a medium containing 10% corn meal and 3% calcium carbonate. Three *Rhizopus oryzae* strains (NRRL 395, ATCC 6204 and ATCC 34121) produced 4.7–5.2% lactic acid in 6 days at 30 °C.

Lovastatin (also known as mevinolin, Mevacor®) lowers the concentration of cholesterol in the plasma of humans and animals. Of 68 strains of *Aspergillus terreus*, three produced lovastatin with equivalent or better yield than strain ATCC 20542 originally described for lovastatin production. In shake-flasks with optimized medium

containing 4% lactose, 400 µg lovastatin/ml was produced by *Aspergillus terreus* strain TUB F-514 in 7 days at 30 °C.

Sixty-five wild-type *Aspergillus fumigatus* strains have been tested for fumagillin production in shake flasks. The best two isolates produced 450 µg fumagillin/ml on optimized medium.

G. VERECZKEY[1], J. HEGÓCZKI[1], P. SÁTORHELYI[2]

Investigation of bioactive compounds in *Lentinus edodes* (Shiitake) mushroom

[1]*Central Food Research Institute, Budapest, and* [2]*University of Horticulture and Food Industry, Budapest, Hungary*

The edible mushroom Shiitake (*Lentinus edodes*) is one of the most widespread varieties in the world. This can be accounted for its intensive volatile and various bioactive (antiviral, antitumor and hypocholesterolemic) compounds. Japanese researchers proved these effects from the aqueous extracts of the fruiting body, many experiments were done by animals and volunteers.

This mushroom was produced in submerged culture (not by the traditional technology) at the Bioengineering Department of the Central Food Research Institute. We used shaker flasks and fermenter (10 dm^3 medium) to the experiments. In this way a considerable reduction could be achieved in the time required for mushroom production as compared to the traditional technologies.

Following the production of Lentinus edodes mycelium, the biomass was analysed with GC-MS technique at the University of Horticulture and Food Industry. We investigated the presence of eritadenine and some of cyclic sulphur containing compounds in the samples. On the basis of the GC-MS measurements it was proved that eritadenine was produced in the mycelium, too (not only in the fruiting body). Furthermore we found lenthionine (the main aroma component) and other cyclic sulphur containing compounds in the biomass, too.

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J. ZALA[1], ZS. GÁLFFY[2], K. KISS[1], T. NAGY[1], B. FRIGY[1], J. JÁKICS[2], A. TELEKES[3]

Problems in the antimycotic susceptibility testing of the fungal isolates; the influence of the therapy and other factors

[1]Department of Mycology, "Béla Johan" National Center for Epidemiology, [2]Clinic of Organ-Transplantation and Surgery, Semmelweis University of Medicine, Budapest, and [3]National Institute of Oncology, Budapest, Hungary

Standardisation has been recently one of the main goals in the medical mycology. However, the antifungal susceptibility testing seems to be the most problematic field. Despite of a lot of research and effort the adequate solution has not born yet. The reason among other things may be that there are a lot of factors influencing the result.

In our study different sensitivity testing methods (E-test, Fungitest, macrodilution method etc.) were compared and the role of some possible *in vitro* modifier factors was investigated using several antifungal drugs (e.g. amphotericin B, fluconazole, miconazole, ketoconazole, nystatine).

According to our results amphotericin B had always clear and well determined MIC, while the results with fluconazole were very confusing. The base of these problems was confirmed by kinetic experiments carried out with several Candida species. The results proved the fungicid effect of amphotericin B and the fungistatic effect of fluconazole.

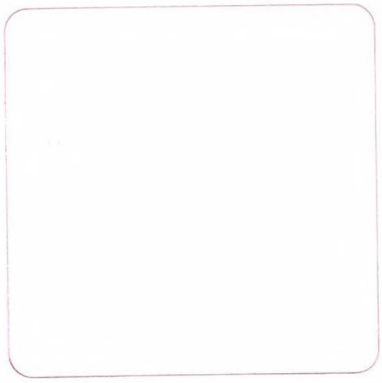
J. ZALA, ZS. HORVÁTH, T. NAGY, E. K. NOVÁK

Occupational hygienic investigations in a Hungarian power plant

Mycology Department, "Béla Johan" National Center for Epidemiology, Budapest, Hungary

In a power plant of South-Hungary in the localization towers on the plastic coated walls of the air-trap and boric acid solution containing chambers, as well as in the boric acid solution heavy fungal invasion was observed in consequence of the indoor high humidity (RH = 63–80%). As a consequence of the relative high temperature (31–34 °C) *Aspergillus fumigatus* proved to be the main member of the fungus population. It survived well the relative short yearly meeting with radioactivity, however, some sign of suffering from it was seen. The situation lead to an extreme fungal air pollution (80000 < c.f.u./m^3), endangering the workers with lung mycosis or at least bronchial hyper-sensitization during the yearly performed keep up. Upon our advices (substitution of plastic coated walls with stainless steel ones, decreasing the humidity, filtering of the boric acid solution

and uv. irradiation of the surface layer of the last one) under 5 years the air pollution could be decreased to "normal" value (< 200 c.f.u./m^3). The immunologic state of the employee was monitored by seasonal serum CIE, PRIST, and RAST tests.



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ABSTRACTS

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I. H. A. ABD EL-RAHIM[1], U. ABD EL-HAKIM[1], M. HUSSEIN[2]

An epizootic of Rift Valley fever in Egypt in 1997

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An epizootic of Rift Valley fever occurred in Egypt from April to August 1997. Infected cattle and sheep showed high fever, icterus, bloody diarrhoea and abortion. Aborted sheep foetuses and sera from the affected herds were collected in the Aswan and Assiut provinces, Upper Egypt for virological and serological examination. A cytopathic effect (CPE) was detected in vero cell cultures 48 hours after inoculation with the foetal liver and spleen suspensions. The same suspensions caused paralysis and mortalities 2–3 days post-intracerebral injection in mice. The isolated virus was identified using an agar gel precipitation test (AGPT) and a direct fluorescent antibody technique (FAT). Serological examination revealed that all tested sheep (57) and cattle (93) were serologically positive using a complement fixation test (CFT), serum neutralisation test (SNT) and indirect fluorescent antibody technique (IFAT); while only 48 out of 57 sheep sera and 69 out of 93 cattle sera were positive using an agar gel precipitation test (AGPT). Titration of the serum samples indicated that SNT is more sensitive than CFT. The present study concluded that the haphazard importation of RVF-infected animals from Sudan and failure of the locally applied RVF vaccination program are the main causes of reoccurrence of RVF epizootics from time to time in Egypt. Continued RVF outbreaks among domesticated ruminants, i.e. in 1977, 1978, 1993 as well as in 1997, indicate that the virus had became enzootic in Egypt. To control the disease in Egypt we suggest: 1) Prevention of introduction of RVF-infected ruminants, especially camels, from northern Sudan into southern Egypt (Aswan province), 2) Avoid importation of ruminants from RVF enzootic-African countries, 3) All ruminant animals (camels, cattle, buffaloes, sheep and goats) should be annually vaccinated with an effective RVF vaccine, 4) Trials for controlling the insect vectors especially in summer season, 5) Application of an intensive TV-education programmes to inform the farmers and animals owners about the vaccination programmes and its importance.

C. AITKEN[1], W. BARRETT-MUIR[1], C. MILLAR[2], J. THOMAS[3], D. JEFFRIES[1], M. YAQOOB[2], J. BREUER[1]

The use of molecular assays in the diagnosis and monitoring of CMV disease following renal transplantation

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Method. We have compared 2 commercial molecular assays (Murex Hybrid Capture CMV DNA assay; HCA, version 2, and the Roche Amplicor assay, plasma PCR)

with a standard shell vial assay in terms of CMV disease detection and prediction in a group of renal transplant patients, and assessed the role of viral load measurements in their management.

Results. The sensitivity of the Hybrid Capture and Amplicor assays in terms of disease detection was 100% compared to 71% for the shell vial assay. Both the HCA and PCR detected all cases of disease 11 and 12.5 days before the onset of symptoms, respectively. Significantly higher viral loads were detected in those with symptoms (7.9×10^5 copies/ml) compared to those without symptoms (7.9×10^4 copies/ml; $p<0.0001$). There was also a trend to higher viral loads in those with a primary infection (7.8×10^5 copies/ml). Successful treatment with ganciclovir was associated with >90% reduction in viral load.

Conclusion. Both of these new assays are sensitive and easy to use. Comparison of accurate quantitation is also useful in monitoring response to antiviral therapy.

M. ALTINDIS[1], A. SIMSEK[2], S. YAVRU[3]

The hepatitis B and C frequency in staff working in veterinarians faculty of Selcuk University in Konya, Turkey

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The aim of this work is to start an immunization program to the academic and management workers of Veterinary Faculty, Selcuk University by the results of hepatitis B virus and hepatitis C virus markers.

This study is made on 107 person (17 female 15.8%, 90 male 84.2%). They filled a questionnaire form and HBsAg, anti-HBs, anti-HBcIgM and anti-HCV by EIA was detected in their blood serum.

Six people (5.6%) HbsAg (+), 16 people (14.9%) anti-HBs (+), 4 person (4.7%) anti-HbcIgM (+) and one person (0.9%) anti-HCV (+) revealed as a result.

Program of immunization and control was started.

M. ALTINDIS, H. KOC, E. ATAS, M. BAYKAN, I. REISLI, A. TASTEKIN, I. ERKUL

Seroprevalence of mumps antibodies in children at 1–15 years of ages in Konya, Turkey

Departments of Pediatrics and Microbiology, Faculty of Medicine, Selcuk University, Konya, Turkey

In order to determine the incidence of mumps and susceptibility to it, 896 children from Konya city center and near villages were included in the study.

Mumps antibody with Bioclinica Mumps IgG (Biobak Laboratory) by EIA was detected by their blood serum.

No significant difference in seropositivity was detected between girls and boys. Seropositivity was found to be 25% in 1–2 age group while it was 89.3% in 13–15 age group. There was a statistically significant increase ($p<0.001$) in the rate of seropositivity with advancing age through the group of 10–12 age. In 7–9 and 10–12 age groups, children living in the city center and suburbs were found to be infected by disease more than peers residing in villages. Attending nursery or day care center did not add additional risk for the mumps infection. However, infection rate was found to increase with the number of siblings. Results of the present study suggested that mumps vaccination, with its very low side-effects and high preventive value, should be included in the routine immunization schedule. The most appropriate time for initial application seems to be 12–15 months of age.

H. AL-ZAHRANI[1], P. J. VALLELY[1], P. E. KLAPPER[2], R. F. T. MCMAHON[1]

Development of an *in situ* PCR assay for the detection of hepatitis C virus genome in biopsied liver

[1]*School of Medicine, University of Manchester and* [2]*Clinical Virology CMHT, Manchester, United Kingdom*

Hepatitis C virus is a major cause of chronic hepatitis with liver-related death occurring in 20–25% of patients who develop cirrhosis. Interferon α is currently used to treat chronic hepatitis C infection. However the efficacy of such treatment is variable and as little is known about the localization of the virus in the liver, monitoring is difficult.

The aim of this study was to develop an assay for localization of HCV RNA in liver and to adapt this for use in liver biopsies. The assay was developed using formalin fixed paraffin embedded liver taken from 17 hepatitis C positive patients. A nested, solution polymerase chain reaction assay using primers directed against the 5' end of the HCV genome was adapted to *in situ* use. Five micron tissue sections were dewaxed, rehydrated and pretreated with pepsin for 1 hour at 37 °C. Amplification was performed using a Perkin Elmer GeneAmp *in situ* PCR System 1000. Two amplification stages of 20 cycles each were carried out, the first using rTth enzyme and the second using Taq polymerase. Amplified product was hybridized to a 67 bp biotin labeled probe and

detected via streptavidin/alkaline phosphatase and dye precipitation with nitro-blue tetrazolium (N bromo-chloro-indolyl phosphate (BCIP)). Ten of the seventeen blocks gave a positive reaction for HCV genome. Positive sites included hepatocytes, inflammatory cells, bile duct and Kupffer cells.

The assay was then further validated using liver biopsies taken from well-characterized HCV antibody positive and negative patients.

This assay appears to provide a powerful procedure for detection of HCV genome in liver biopsies, and a potentially invaluable tool for monitoring responses to antiviral therapy.

B. AVIDOR, Y. MESHULACH, G. EFRAT, A. FATTAL, M. WEINBERG, A. J. JAFFA, M. GILADI

Detection of CMV DNA by PCR in amniotic fluids of asymptomatic women with recurrent CMV infection

Laboratory for Molecular Biology, Tel-Aviv Sourasky Medical Center, Tel Aviv, Israel

Background. PCR is a sensitive method for detection of CMV DNA in amniotic fluid. It has been evaluated primarily in acute CMV infections.

Objective. To assess the use of PCR in detecting intrauterine CMV infection asymptomatic pregnant women.

Design. 89 amniotic fluids samples were collected from 88 asymptomatic women. Maternal sera were tested for anti-CMV IgG and IgM antibodies. Amniotic fluids were cultured for viral growth and tested by a seminested PCR for the presence of CMV DNA.

Results. CMV DNA was detected in 2 amniotic fluid samples (2.3%). Both from women with positive IgG, negative IgM, and negative amniotic fluid cultures, suggesting recurrent CMV infection. One pregnancy was terminated due to the presence of intracranial calcifications by ultrasound, and the other woman delivered a healthy baby.

Conclusions. Recurrent CMV infection in asymptomatic pregnant women may result in viral shedding in the amniotic fluid. PCR can detect CMV DNA in amniotic fluid in the presence of negative cultures.

A. AZZI, K. ZAKRZEWSKA, S. CESARO, R. FANCI, A. BOSI

Monitoring of BKV viral load in urine of bone marrow transplantation patients and haemorrhagic cystitis

Department of Public Health, Microbiology Section, University of Firenze, BMT Unit, University of Padova, BMT Unit, Careggi Hospital of Firenze, Italy

Human polyomavirus BK (BKV) urinary shedding has been shown in more than 90% of bone marrow transplantation (BMT) patients with late haemorrhagic cystitis (HC)

by the use of a very sensitive PCR. Moreover, virus concentration in urine from HC patients is higher than in urine from asymptomatic BMT recipients. We have performed a longitudinal study on few BMT patients for monitoring BK viruria before, during and after the HC episode. At this purpose urine specimens have been obtained at different times after BMT from 7 patients, who developed HC. In these specimens the amount of viral DNA was determined by a quantitative competitive PCR using a target sequence mimetic. The target sequence and the internal standard, constructed in our laboratory by site directed mutagenesis, were amplified by the same primers and differed from one another only in a restriction site in the internal standard, absent in the target sequence. The results suggest that the onset of HC is coincident with a burst of viral load, which persists at high levels during the episode and then slowly decreases after the clinical recovery. A sudden rise of viruria level could be prognostic of HC in BMT patients and could allow a timely preventive or therapeutic approach.

A. BÁCSI[1], J. ARANYOSI[2], P. EBBESEN[3], F. D. TÓTH[1]

Placental macrophage contact potentiates the complete replicative cycle of human cytomegalovirus in syncytiotrophoblast cells

[1]Institute of Microbiology and [2]Department of Obstetrics and Gynaecology, University Medical School, Debrecen, Hungary, and [3]Department of Virus and Cancer, The Danish Cancer Society, Aarhus, Denmark

Maternofetal transmission of human cytomegalovirus (HCMV) is the most common cause of congenital viral infection. How HCMV crosses the syncytiotrophoblast (ST), the barrier between maternal blood and fetal tissue in the villous placenta, is unknown. Although syncytiotrophoblasts can be infected by human cytomegalovirus (HCMV), ST cells do not support the complete viral reproductive cycle or HCMV replication may occur in less than 3% of ST cells. On the basis of these observations we investigated whether placental macrophages might enhance activation of HCMV carried in ST cells and infected ST cells would be capable of transmitting virus to neighbouring macrophages. For this purpose, we studied HCMV replication in ST cells grown alone or cocultured with uninfected placental macrophages. Our results demonstrated that HCMV gene expression in ST cells was markedly upregulated by coculture with macrophages, resulting in release of substantial amounts of infectious virus from primarily infected cells. After having become permissive for viral replication, ST cells delivered HCMV to the cocultured macrophages as evidenced by detection of virus-specific antigens in these cells. The stimulatory effect of coculture on HCMV gene expression in ST cells was mediated by marked interleukin 8 and transforming growth factor β1 release from macrophages, an effect caused by contact between the different placental cells. Our findings indicate an interactive role for the ST layer and placental macrophages in the dissemination of HCMV among placental tissue.

D. BAETENS, L. VAN RENTERGHEM

Coupled particle light scattering (COPALIS™I): new technology for serodiagnosis of Epstein-Barr virus infection

Laboratory for Bacteriology and Virology, Gent, Belgium

Objective. Comparison of the COPALIS™ technique with other tests for detection of IgM and IgG (VCA, EBNA and EA) antibodies against EBV. Evaluation of the analytical performance.

Materials and methods.

– Copalis™: EBV-M Antibody assay, MuItipIex™ EBV Antibody assay (DiaSorin, Italy)

– ELISA's: EBV IgM and IgG (Dade-Behring, Germany), EBNA and EA (DiaSorin, Italy)

– IgM-IFA: immunofluorescence assay for detection of EBV IgM antibodies (Gull)

– IgG avidity determination: 8 M urea procedure

281 selected patient sera were tested and divided into the following subpopulations: acute primary infections (33), non-primary infections (57), seronegatives (20), other infections and rheumatoid factor (Rf) positive samples (59), 22 follow-up panels after primary infection or reactivation (74), IgM positive samples routinely found by ELISA in a hospital population (47).

Results.

– Acute primary infections (IgM-IFA pos, heterophile Ab pos, lymphocytosis with atypical lymphocytes): on Copalis™ primary infections were confirmed by IgM positivity and EBNA negativity, except for 3 equivocal IgM results and 2 low positive EBNA's

– Negatives: 2 samples were VCA positive (1 IgG-IFA positive) and 2 were equivocal on Copalis™. On reanalysis the IgG-IFA positive sample was equivocal in ELISA.

– Interferences: 4 of 12 CMV IgM positive samples were EBV-M positive on Copalis™ (7 in ELISA): 1 sample was IgM-IFA positive with a IgG avidity index of 100%. In the Rf positive population (21 samples) we found 9 Copalis™ positive IgM samples (0 in ELISA) of which 6 were positive in IgM-IFA. In all 9 sera the IgG avidity index is high.

– Non-primary infections (IgM neg, IgG pos): On a total of 57 sera, 5 were IgM positive on Copalis™ of which 3 were negative in IgM-IFA. Eight samples were IgM equivocal. All samples were VCA positive and 8 were EBNA negative on Copalis™.

– Comparison to IgM-IFA (Gull) and IgG ELISA (Dade-Behring).

	IgM	IgG
Sensitivity	97.1%	99.4%
Specificity	61.5%	74.1%

– Analytical performance: IgM within-run CV was 2.83%, and between-run CV was 3.96%. The average between-run CV for IgG (VCA, EBNA, EA) was 11%.

Conclusions. The Copalis^{TM}I proved to be an easily maintainable, reproducible and pleasant technology for routine purposes. The availability of 3 quantitative IgG results in 1 run is the main advantage of the technique. The minimal frequency for calibration and control must be further evaluated. IgM has a good sensitivity, however specificity needs further investigation.

J. E. BANATVALA

Eradication of congenitally acquired rubella worldwide: is this feasible?

Guy's, King's and St Thomas' School of Medicine, London, United Kingdom

Industrialized countries, which have implemented rubella vaccination programmes, have shown a marked reduction, almost to the point of elimination, of Congenital Rubella Syndrome (CRS). Data from developing countries show that the proportion of rubella susceptible women (15–20%) is similar to the proportion in industrialized countries in the pro-vaccination era. Studies conducted in parts of the developing world show that that incidence of CRS per 1,000 live births was usually considerably higher (range 0.6–2.2 per 1,000 live births) than in the UK during the early years of rubella vaccination (0.14 per 1,000 live births during epidemics, 0.08 at other times). Thus, it is likely that LRS is an important cause of blindness and deafness in developing countries and created a considerable burden on scarce health resources.

An ideal opportunity to implement rubella vaccination programmes in developing countries now exists because, globally, measles vaccination uptake rates average about 80% or more and some regions have adopted measles elimination goals. A single dose of combined measles and rubella vaccine should be given to children aged 1–2; however, it is also important to ensure that women of child-bearing age are also targeted in vaccination programmes. Any vaccination programme must be accompanied by sound surveillance data and, for rubella, this applies to postnatally as well as congenitally acquired infection. Rapid tests suitable for use in the field are required to distinguish postnatally acquired rubella from measles, dengue and perhaps human parvovirus B19 infection. Although a diagnosis of CRS may be established on clinical grounds (which must be supported by such laboratory investigations as the detection of rubella specific IgM), such investigations are unlikely to be of value after about 6–9 months of age. A diagnosis can then be established by the detection of low avidity IgG antibodies or by PCR on lens aspirates from surgically removed cataracts. Such investigations would be restricted to specialized reference laboratories with expertise in rubella diagnosis.

Although it should be feasible to mount rubella vaccination programmes in developing countries, emphasis on polio eradication, competition from new vaccines, e.g. pneumococcal and rotavirus, and financial constraints imposed by recession (particularly

in the Far East and Latin America), may restrict development of rubella vaccination programmes.

W. BARRETT-MUIR[1], C. MILLAR[2], M. YAQOOB[2], J. BREUER[1], C. AITKEN[1]

A comparison of CMV load measurements in the plasma and leukocyte fractions of a group of renal transplant recipients

[1]*Department of Virology and* [2]*Nephrology, Royal Hospitals Trust, London, United Kingdom*

Objective. To measure the CMV load in the plasma and leukocyte fractions in a group of renal transplant patients, and to assess the effect of ganciclovir (GCV) on viral load.

Method. EDTA anticoagulated blood samples were collected on a weekly basis for 12 weeks following renal transplantation in 52 adult patients. The viral load was measured in plasma using the Roche quantitative PCR assay and in the leukocyte fraction using the Murex Hybrid Capture Assay (Version 2.0).

Results. Plasma DNAemia was detected in 192/491 (39%) samples and leukocyte DNAemia in 227/467 (46%) samples. Thirty-seven patients had detectable DNAemia in both fractions. This was measured first in the plasma fraction in 7 patients and in the leukocyte in 11 patients. In the remaining 19 patients the detection of CMV DNA occurred in leukocytes and plasma at the same time. The median peak viral load in plasma was 4.0 $\log_{10}$ compared with 5.1 $\log_{10}$ in the leukocyte fraction. Following the introduction of GCV the viral load fell in both compartments, but became undetectable in the plasma fraction first.

Conclusions. Both plasma and leukocyte fractions may be useful for CMV load measurements in renal transplant recipients with the peak viral load coinciding in plasma and leukocytes in most patients. Successful antiviral therapy is mirrored by a decline in CMV load.

R. BECK[1], H. SCHÄFER[2], I. WESTDÖRP[1], H. EINSELE[2], L. KANZ[2], G. JAHN[1]

Detection of EBV-DNA in plasma of patients with lymphoproliferative disease after allogeneic BMT/PBSCT

[1]*Department of Medical Virology and* [2]*Department of Internal Medicine II, University of Tübingen, Germany*

Epstein-Barr virus (EBV) is a ubiquitous herpes virus that persistently infects humans. In immunocompromised patients reactivation of virus can result in EBV-associated lymphoproliferative disease (LPD). In previous studies elevated levels of EBV-

DNA were demonstrated in peripheral blood mononuclear cells of patients with EBV-LPD. Using a nested PCR assay we investigated the presence of EBV-DNA in plasma from 6 patients with histopathologically proven LPD after allogeneic BMT/PBSCT. All patients presented with fever and lymphadenopathy on a medium of day 84 (range day 44 to day 160) after transplantation. For 5 patients plasma samples obtained during the clinical course of LPD were available. Cell-free EBV-DNA was detected in all 5 patients. Furthermore, in 4 patients EBV-DNA was detected in plasma on a medium of 25 days (range 7 to 50 days) prior to the diagnosis of EBV-LPD. Although further analysis concerning the specificity of our assay is necessary, we conclude that detection of cell-free EBV-DNA in plasma might be useful as a sensitive marker for the detection of EBV-LPD in patients after allogeneic BMT/PBSCT.

S. S. BIEL, H. G. BAE, H. R. GELDERBLOM

External quality assessment schemes: preparing diagnostic electron microscopy for the future

Robert Koch Institut, Berlin, Germany

Unlike other diagnostic techniques in infectious diseases, electron microscopy (EM) by its undirected "open view" is a catch-all-method, detecting also double infections and even agents not previously considered to be involved.

To save the rapidity and the "open view" of EM also for the future, we have started a regular External Quality Assessment Program (EQA) on EM Viral Diagnosis (EQA-EMV) in 1994, comprising two EQA runs per year with both, ready-made specimens and particle suspensions to be prepared by the participants.

Presently with EQA-EMV 7, there are some 80 participants from 20 countries worldwide registered, mainly from Germany, United Kingdom and other European countries. The ratio of correct results is increasing for both ready-made specimens (EQA 6: 77%) and particle suspensions (EQA 6: 71%).

The raising number of participants and participating countries as well as the ongoing improvement of virus detection rates show the future need for this programme.

S. S. BIEL, A. NITSCHE, H. G. BAE, W. EBELL, T. HELD, W. SIEGERT, H. R. GELDERBLOM

Quantification of polyomavirus in urine of bone marrow transplant recipients

Robert Koch Institut, Berlin, Germany

Hemorrhagic cystitis (HC) is a complication occurring after bone marrow transplantation (BMT). In the literature HC is often described as linked to the excretion of human polyomavirus BK and/or JC.

To elucidate the association of HC and polyomavirus excretion we present a PCR assay using the Taqman® technique for the detection and quantification of polyoma viral DNA from crude urine using general primers for both BKV and JCV. It is shown that after freezing and dilution of the urine specimens only little inhibitory effects are evident. The PCR assay can detect up to 10 copies per assay, i.e. 2000 copies per millilitre.

To differentiate between polyomavirus BK and JC, a seminested PCR assay has been developed using BK/JC specific primers only for the second round, enabling the direct PCR differentiation by different amplicon sizes.

A. BIOLCHINI, S. CURRELI, M. ZICCHEDDU, C. SERRA, A. DOLEI

CXCR4 and CCR5 HIV co-receptors in adherent cells

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HIV-1 strains are T- or M-tropic, based on macrophage or lymphocyte tropism and on CCR5 or CXCR4 coreceptor usage. We showed that in T cells the CC-chemokines increase binding and replication of T-tropic HIV strains, as they increase CXCR4 expression (Dolei et al.: AIDS **12**, 183 1998). Co-receptor expression mid modulation by proinflammatory cytokines were studied by RT-PCR and Western blotting, in various cell types, either as such, or after infection by T tropic HIV-1MSaT or M-tropic HIV-BaL strains. Cells from solid tissues are all CCR3(+); some of them express either CXCR4 or CCR5 or both, and in different ratios. Upon cytokine treatments, CXCR4 and/or CCR5 modulation is observed, particularly with TNFalpha, IFNgamma and IL-6. Dichotomous findings were observed in epithelial cells exposed to IFNbeta and IFNgamma, where CXCR4 expression is reduced while that of CCR5 is stimulated, dose-dependently. Also TNFalpha reduces CXCR4 expression in these cells, in terms of both RNA transcripts and protein accumulation. These findings indicate different responses to inflammatory stimuli in calls from various tissues that may provide selective advantages for binding of HIV strains with different tropism.

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An improved DOT ELISA to detect viral antigens

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The sensitivity of dot immunobinding reaction significantly depends on the substrate used. This paper reports on the examination of a lot of substrate mixtures to detect ortho- and paramyxovirus antigens. The serial dilution of viral antigen was dotted onto the nitrocellulose paper and allowed to react with a conjugate of specific antibodies to these antigens with horseradish peroxidase. Positive reactions were easily visualized as clearly stained spots on the membrane after enzyme degradation of the substrate. The

results obtained showed that the sensitivity of tile dot immunobinding reaction significantly depended on generation of large substrate complexes of limited diffusion ability. The highest sensitivity is observed using benzidine and diamine-phenol combinations due to the reaction of the coupled oxidation (NADI). Substrate mixtures which could be involved in reactions of oxidative combination were found to increase the sensitivity of dot ELISA 4–8 times compared to either of the compounds without addition of the other ingredients.

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Inflammatory responses in respiratory, and syncytial virus (RSV) infection in relation to clinical severity

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Aim. To study whether an immune pathological mechanism may play a role in the development of RSV bronchiolitis we investigated the relationship between clinical severity and inflammatory parameters measured in plasma and cells present in the respiratory tract, in infants younger than six months of age.

Clinical data and plasma samples were obtained from 111 children with respiratory tract infection suspect for RSV. 95/111 patients were RSV positive. 55 of the RSV positive children were defined as having a severe disease and 41 as having a mild disease. Inflammatory cytokines in plasma samples were found only in low concentrations. No differences were found between the RSV positive and negative children in this respect. In RSV positive children the general pro-inflammatory cytokines IL-6 and IL-8 were found more often and at higher levels in more severely affected patients. No significant differences were found for IL2, IL4, IL5, IL10, TNF-α and IFN-γ. The cellular infiltrates in nasopharyngeal washings consisted mainly of polymorphonuclear granulocytes and monocytes. Cells related to allergic reactions (eosinophils, IgE positive cells, basophilic cells) were found sporadically.

Conclusions. Children with a more severe clinical infection showed signs of more general inflammation as indicated by higher levels of general pro-inflammatory cytokines. Indications for a skewing in cytokine response or an eosinophilic infiltrate like that found in murine models, using formalin inactivated RSV vaccination, were not found in severe naturally occurring RSV bronchiolitis.

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Fecal hepatitis A virus shedding as detected by PCR

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In The Netherlands, hepatitis A has changed from an endemic childhood disease to an epidemic import disease. Most of the new hepatitis A virus (HAV) infections are possibly caused by children of immigrants who return from holidays in the country of origin (often Morocco or Turkey). Many school related outbreaks in early fall and into the winter may be caused by this import. The development of a RT-PCR has increased the sensitivity of detection of HAV infection. In addition, it enables to establish the duration of fecal excretion after the development of clinical symptoms. In Amsterdam, fecal samples were collected during 1997 through February 1998 from persons with serologically confirmed (IgM HAV) hepatitis A. Single stool samples were obtained from 19 persons, whereas sequential daily stool samples (day 6 to day 27 after clinical symptoms) were collected from 8 persons. Also single samples from two families were included (6 persons in total). Using two nested primer sets located in the VP3-VP1 and VP1-VP2A regions, we set up the conditions to amplify HAV RNA, which was isolated by an adapted Chomzynski method. Successful amplification was achieved using random hexamer primers and the MMLV RT enzyme, followed by two PCR reactions (nested setting, 30 cycles). By spiking of a part of the stool sample with cultured virus and processing it in parallel with non-spiked sample we controlled PCR inhibition. Stool samples from most patients (n=28) yielded positive PCR reactions in the VP1-VP2A region, probably because this region is genetically highly conserved. From the total of 33 patients, 21 had both primer regions PCR positive, 1 with only the VP3-VP1 region and 7 with only the VP1-VP2A region positive. Four patients were negative for both regions (no PCR inhibition); possibly the primer sets used did not recognize all circulating viruses. In one case 4 siblings could be studied; 3 were PCR positive for both regions at 21 days, whereas the fourth sibling was negative at day 27. The maximum duration of fecal HAV shedding, as established with this RT-PCR, was 27 days. The viral load was probably variable among the patients, because some patients were PCR positive at all time points tested, whereas in others the PCR was positive only on alternate days. We thus conclude that hepatitis A virus sequences can still be present in fecal excretions at 27 days after clinical symptoms. Sequencing of the obtained PCR products is in progress to try to see epidemiological relationships.

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Examination of synovial tissue cells for capacity to support B19 entry and persistence

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Parvovirus B19 infection is associated with a growing spectrum of clinical manifestations. In addition to the classical fifth disease (erythema infectiosum), transient aplastic crises, fetopathic complications, and transient arthopathies are well-established consequences of some B19 infections. There is additionally some evidence that B19 may play a role in induction of rheumatoid-like chronic arthritis.

Of importance to this last point is previous work from this lab (Söderlund et al.: Lancet 349, 1063 1997) showing evidence for long-term persistence of B19 viral DNA in synovia. To elucidate this phenomenon, we have examined individual cell types within human synovia for their levels of globoside, the putative B19 receptor. We report here the development of a new microscale globoside (Gb4) measurement assay and the levels of Gb4 in synovial tissue subtypes as determined with this assay. We have preliminary data on detection of B19 capsid binding to cell membranes as an adjunct to Gb4 measurement. In addition, we have examined the capacity of isolates of synovial cell subtypes to support either viral replication or nonreplicative DNA persistence in cell culture.

We have also characterized the full coding sequence of B19 DNA from several synovial samples where it was persisting, and summarize these results in comparison with available B19 sequence data from other sources.

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Inflammation and advanced atherosclerotic lesions after MCMV infection, increased histopathology after consecutive infection with MCMV and *Chlamydia pneumoniae*

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Murine cytomegalovirus (MCMV) is involved in two of the mechanisms that lead to the development of atherosclerosis: immune injury at the site of virus infection in the mouse aorta, and an increased percentage of serum LDL-cholesterol (Berencsi et al.: Cell Adhesion Commun 5, 39 1998). Here we show that MCMV-induced inflammatory foci in the mouse aortic wall decreases in number and size by 2–4 months after infection, however, more advanced subendothelial necrotic lesions develop, indicating progression

of early histopathological changes. We also show that inoculation of mice first with MCMV, then with *C. pneumoniae*, a timely order suggested for human infections with these common pathogens, induces more inflammatory foci in the aorta, for a longer period of time; and in a higher number of mice, than inoculation with MCMV or *C. pneumoniae* alone. Both MCMV and *C. pneumoniae* mRNA, as detected by *in situ* hybridization, as well as viral/bacterial proteins, as seen by IF test, are expressed in the aortic wall. These results support the etiologic role of dual infections with CMV and *C. pneumoniae* in the development of atherosclerosis.

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Parvovirus B19 DNA, IgM and IgG antibody investigations in febrile Danish children

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Background. Parvovirus B19 is a 5.4 kb single stranded DNA virus that was first described in 1975. Since 1984 it has been known as the etiologic factor of "5th disease" with erythema infectiosum among various other clinical manifestations or it may be subclinical.

Patients and methods. Serum from 228 children, aged 3 days to 14 years, admitted to the pediatric Department due to febrile illness in the period August 1996 until November 1997, were investigated for Parvovirus B19 DNA by PCR and by a Parvovirus B19 IgM and IgG EIA for antibodies.

Results. None of the serum samples 0/228 (0%) had Parvovirus B19 IgM antibodies or Parvovirus B19 DNA due to acute infection, but 56/228 (24.6%) of the children had Parvovirus IgG antibodies indicating maternal antibodies or a previous Parvovirus B19 infection. The majority of the children admitted to the pediatric department due to fever were under 6 years (male:female = 146:82).

Age range (months)	0–3	>3–12	>12–24	>24–36	>36–48	>48–60	>60–72	>72
No. investigated	40	51	76	32	12	2	5	10
IgG antibodies (n)	17	12	14	4	0	1	1	7
% of each group	43%	24%	18%	13%	0%	50%	20%	70%

Discussion. Parvovirus B19 IgG were detected in 24.6% of the investigated children. Most of them were less than 36 months indicating that nurseries and kindergartens are reservoirs for this droplet infection, but the high percentage with detectable Parvovirus B19 IgG antibodies in their first period of life is probably also due

to maternally transferred Parvovirus B19 IgG. The reason for not finding any acute cases could be that Parvovirus B19 infections is epidemic, with approximately 3 years in between outbreaks.

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Astrovirus as a cause of gastroenteritis: our experience

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Introduction and objectives. It is a relatively recent acknowledgement of medical importance of astrovirus as a cause of viral diarrhea in humans. The development of immunoenzimatic techniques for detection of this viral antigen made the diagnosis of gastrointestinal infections by astrovirus easier. It is our objective to present our experience in detection of astrovirus as a cause of diarrhea.

Material and methods. A total of 231 children between 8 days and 9 years of age were studied over a period of 22 months (May 1997 to March 1999) to determine the etiological role of virus in acute diarrhea. The stool samples were tested for the presence of Rotavirus/Adenovirus/Astrovirus by use of respective diagnostic kits from Dako®, which use an immunoenzimatic method to detect viral antigen in stool samples.

Results. From the 231 samples studied, 121 (52.4%) were negative for any of the studied viruses and 110 (47.6%) were positive. The positive samples were: 91 (39.3%) for rotavirus, 2 (0.9%) for adenovirus and 17 (7.4%) for astrovirus. In 5 cases there was coinfection by rotavirus and astrovirus. Infections by astrovirus peaked in August and September. About half cases affected children between 2 and 5 months of age and the other half between 1 and 5 years old.

Conclusions. As stated by other studies, in our survey astrovirus is an important cause of gastroenteritis, being the second more frequent cause of viral gastroenteritis.

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Virological diagnosis of acute pneumonia in the immunocompromised host. What viruses should be sought?

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Acute pneumonia is a frequent, possibly life-threatening complication in immunocompromised (IC) adult patients. Viruses, including cytomegalovirus (CMV), herpes simplex virus (HSV), respiratory syncitia! virus (RSV), parainfluenza viruses (PIV), adenoviruses, and influenza viruses are implicated in a significant number of cases.

Accurate diagnosis of viral infections in these patients is mandated because therapeutical solutions may exist. The objective of this work was to evaluate the respective roles of these viruses in acute pneumonia in a population of consecutive IC adult patients referred to our canter, in order to clarify whether or not their systematic detection in bronchoalveolar lavages (BALs) is needed. Direct markers of CMV, HSV, RSV, PIV, influenza virus and adenovirus infections were sought in 85 BALs from 75 consecutive IC adult patients with acute pneumonia (including 13 allogeneic and 2 autologous bone marrow recipients, 17 HIV-infected patients and solid organ transplant recipients). RSV, PIV, influenza and adenovirus antigens were sought by direct immunofluorescence in BAL cells using specific monoclonal antibodies. BALs were inoculated: (i) on MRC-5 cell line for rapid shell vial assay to detect CMV and HSV replication, and (ii) on Hep-2 cell line to detect adenovirus replication by rapid shell vial assay and conventional culture. Adenovirus DNA was detected by PCR followed by hybridization. Markers of active viral infection were detected in 26 BALs (31%). CMV was present in 18 cases (69%), HSV in 6 cases (23%), and influenza virus in 2 cases (8%). Markers of adenoviruses, PIV and RSV infection were not detected.

In conclusion, the most frequently detected viruses in acute pneumonia of adult IC patients are CMV and HSV and, occasionally, influenza viruses, whereas RSV, adenoviruses and PIV appear to be rarely implicated. In this context first line systematic screening of BAL for RSV, adenoviruses and PIV may not be needed out of seasonal outbreaks.

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Serological diagnosis of NPC by recombinant Epstein-Barr virus replication activator fusion protein specific ELISA

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Nasopharyngeal carcinoma (NPC) patients sustain high levels of a broad spectrum of Epstein-Barr virus (EBV) specific serum antibodies, particularly viral capsid antigen (VCA) and early antigen (EA) which are traditionally determined by immunofluorescence (IF). This outstanding feature has been usually applied in diagnosis of NPC. A new recombinant glutathione transferase (GST)-replication activator (ZEBRA) fusion protein was synthesized and assessed in ELISA for serum IgG response in NPC patients and normal healthy donors. In 74 confirmed NPC patients, 72 (97.3%) had IgG anti-ZEBRA antibodies and 2 (2.7%) had indeterminate results. In contrast, only 1/81 (1.2%) serum sample from healthy donors was indeterminate for IgG antibodies to ZEBRA protein. The three suspected positive serum were further analysed for IgA antibodies to EBNA-1 fusion protein by ELISA. One serum collected from NPC patient was positive for IgA antibodies to EBNA-1, while the other two including one healthy donor sera were negative for IgA antibodies to EBNA-1. The sensitivity for diagnosis of NPC thus is

increased to 98.6%. We conclude that the high sensitivity and specificity of this ELISA assay for IgG antibodies to ZEBRA protein can be used as first screening assay for the detection of NPC. With the combination of second assays specific for IgA to EBNA-1 protein, the sensitivity of serological diagnosis of NPC can be substantially enhanced. Therefore, a combination of the two immunoassays can permit an early diagnosis of the cancer. In addition, the advantage of ELISA is to avoid the assay artifacts relating to subjectivity of the traditional IF methods.

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Human Herpesvirus 8 in women with abnormal PAP smears

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Objectives. Examine the presence of HHV-8 in cervical scrapes and its association with cervical lesions.

Methods. Cervical scrapes were collected from women with abnormal Pap smears for HHV-8 DNA detection by nested PCR using primers KS1, KS2 (outer) and NS1, NS2 (inner) targeting the ORF26. Positive samples were confirmed by another PCR amplifying a non-overlapping region at ORF25.

Results. 8.5% (37/434) of patients were positive for HHV-8 DNA, and 7 (18.9%) of them had a biopsy-proven high-grade cervical lesion (3 patients with invasive squamous cell carcinoma, 4 patients with CIN 3). However, no significant trend of association between HHV-8 positivity and severity of cervical lesion was observed ($p=0.6$, χ^2 test for linear trend).

Conclusions. Latent infection and/or shedding of HHV-8 in the uterine cervix occurs in a proportion of infected individuals and secretions of the female genital tract may be a source of transmission. The fact that HHV-8 is found in cervical samples of a proportion of women with high-grade cervical lesions warrant further studies on the pathogenic role of HHV-8 in cervical malignancy.

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Usefulness of an Epstein-Barr virus (EBV) IgG avidity assay in determining the EBV status of transplant patients

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Background. Although the EBV status of most patients (pts) can be correctly verified by parallel determination of VGA IgM, VCA IgG and EBNA-1 IgG, EBV serology is commonly unclear in pts under immune suppression.

Objectives. To evaluate the usefulness of an EBV IgG avidity assay in correctly determining the EBV status of transplant (TX) pts with unclear serology.

Methods. Inclusion criteria were: 1) TX-pts with negative results in at least 1 of 2 commercially available EBNA 1 IgG EIA, or 2-TX-pts with positive or borderline results in at least 1 of 2 commercially available EBV/VCA IgM EIA. All samples were tested with the EBV IgG avidity assay (Enzygnost anti EBV/IgG, DADE Behring).

Results. A total of 198 serum samples from 51 TX-pts were analyzed. Transplanted organs included: kidney (n=29), bone marrow (n=11), liver (n=6) and others (n=5). There were 49 pts with a past and 2 with an acute EBV infection. There were 25 pts in group 1, defined by EBNA-1 IgG negative results. Although IgM results were positive in 14/25 pts (56%) and borderline in 7/25 pts (28%), avidity testing clearly detected the only 2 pts with an acute EBV infection. There were 26 pts in group II, defined by EBNA-1 IgG positive results. Avidity testing confirmed that none of them had an acute EBV infection. Among all 49 pts of both groups with a past EBV infection, the EBV status was correctly identified by a positive EBNA 1 IgG result in 26/49 pts (53%). In contrast, avidity testing identified the past infection in 45/49 pts (91.8%, $p<0.01$).

Conclusion. EBV IgG avidity testing can improve the quality of EBV serology in TX-pts. In patients with EBNA-1 IgG negative results, the EBV IgG avidity assay can differentiate between acute and past infections avoiding the requirement of follow-up samples.

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A human endogenous retrovirus is expressed in particles in multiple sclerosis

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Previously, spontaneously formed B-lymphoblastoid cell lines were established by long-term cultivation of peripheral blood mononuclear cells from several MS patients. The cell lines produce a type C retrovirus which is reverse transcriptase (RT) positive and share a few antigenic determinants with HTLV-1, but is antigenically distinct from the known retroviruses.

We show here that intact retroviral particles can be purified from cell culture supernatants by ultracentrifugation in Optiprep gradients. The retroviral origin was confirmed by the co-localization in a few gradient fractions of retroviral particles as visualized by negative staining EM, RT activity, and RNA content.

To identify the retrovirus we performed RT-PCR with consensus *gag* and *env* primers on retroviral RNA templates obtained from lysed RT-positive retroviral particles purified as described above: sequence variants of the potentially functional subgroup RGH of the human endogenous type-C retrovirus family RTVL-H were found.

To determine if these endogenous retroviral sequences at particle level could also be associated with MS *in vivo*, we performed RT-PCR analyses on clinical specimens, i.e. RNA from cell-free, filtered, ultracentrifuged plasma samples from MS patients. Patients with autoimmune diseases and healthy individuals were controls. We demonstrated that expression of RGH sequences at particle level was specific for MS (70% of MS samples) and absent in the controls.

The infectivity of the retrovirus was assessed by cell-free infection of PHA-stimulated lymphocytes from healthy individuals. We have demonstrated RGH sequences in particles produced by the putatively infected cell cultures.

We suggest that MS is associated with replication of otherwise quiescent endogenous retroviruses. These retroviruses are putatively infectious.

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Prenatal diagnosis of congenital cytomegalovirus (CMV) infection

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Objectives. In developed countries, CMV infection is the leading cause of congenital viral infections. Primary infection during pregnancy is a risk for delivery of a child with severe sequelae. In order to establish preventive strategies to avoid the debilitating consequences of congenital CMV infections, evaluations of the prenatal diagnostic procedures are required.

Methods. From 1993 to 1998 systematic serological and virological surveys were carried out in four university hospitals for primary CMV infection during pregnancy. CMV serological status was established for all pregnant women and serological follow up was done for seronegative mothers. When seroconversion occurred and when required, amniocentesis was performed. Prenatal diagnosis of *in-utero* mother to fetus CMV-transmission was based on conjugated analysis of amniotic fluids by PCR and viral culture. All results were compared to those derived from early virus isolation attempts with urine of the newborns (or from analysis of fetuses in cases of termination of pregnancy).

Results. With a mean incidence of about 42% for CMV seropositivity as a background, more than 19,450 pregnancies were systematically analysed (details to be presented). 152 mothers seroconverted during pregnancy. Prenatal diagnosis was carried out for 97 cases. From these, 29 infants or fetuses were infected (29.9%), 66 infants or fetus were not infected (68%) and 2 miscarriages occurred. Results of viral culture and PCR diagnosis carried out on amniotic fluids were identical in 96 cases and one discrepancy occurred (PCR+ /cult–). As a whole, sensitivities, specificities, Positive Predictive Values and Negative Predictive Values for PCR and viral culture were: 72.4%, 72.4%; 96.9%, 98.4%; 91.3%, 95.5% and 88.8%, 89%, respectively. Two cases excepted,

most of «false» negative results are explained either by early and/or insufficiently delayed amniocentesis, i.e. before 21st week of gestation and/or less than 5 weeks after seroconversion.

Conclusions. This systematic survey carried out in different settings and with evolving procedures, demonstrates that prenatal diagnosis on amniotic fluids by both shell vial assays and PCR diagnosis is reliable and enables characterization of early transplacental infection.

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Seroprevalence of antibodies to hepatitis A and E viruses in pediatric age groups in Antalya-Turkey

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Hepatitis A and hepatitis E are enterically transmitted viral diseases occurring in epidemic and sporadic forms especially in developing countries. Previous studies in Turkey showed that most residents are infected with HAV by the second decade of life. Since HEV is generally transmitted by the same route as HAV we conducted a community-based seroprevalence study for HAV and HEV infection in Ahatli area in Antalya-Turkey where socioeconomic conditions are low.

Anti-HAV total immunoglobulin was tested by using a microparticle EIA (Axsym-Abbott Lab). Anti-HEV IgG, was assayed by a micro ELISA method (Genelabs-Singapore).

The results are shown in Table I.

Our data showed that seroprevalence of anti-HAV is high among children sampled but HEV infection appears to be relatively rare in pediatric age groups.

Table I

Seropositivity of anti-HAV total immunglobulin and anti-HEV IgG in pediatric age groups

Age groups (n)	Anti-HAV total Ab (%)	Anti-HEV IgG (%)
1–5 (151)	30 (19.9)	0 (0.0)
6–11 (187)	82 (43.9)	3 (1.6)
Total (338)	112 (33.1)	3 (0.89)

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EBV-specific cytotoxic T-lymphocytes in prophylaxis and treatment of PTLD

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Background. EBV-associated PTLD occurs in up to 10% of transplant recipients and carries a high mortality. Conventional treatment, involving reduction of immunosuppression, although initially successful in some cases, often leads to refractory recurrences. Cytotoxic T cell therapy using *in vitro* grown T cells specific for viral antigens was pioneered in the US by Riddell et al. for treatment of CMV1, and has since been used successfully for prophylaxis and treatment of PTLD in bone marrow transplant recipients using donor CTL2.

Methods. We carried out a pilot study in 3 solid organ transplant recipients where no donor cells are available. In this case autologous CTL grown from pretransplant samples were infused in a dose escalation study.

Results. CTL infusions caused reduction in EBV DNA and increase in CTL activity in peripheral blood, both of which were sustained beyond 4 weeks after the last infusion.

Conclusions. Although treatment with autologous CTL may be effective it is not a practical proposition on a large scale. Thus we now aim to carry out a multicentre trial on CTL treatment of PTLD in solid organ transplant recipients using allogeneic CTL grown from healthy blood donors on a best HLA match basis.

1. Riddell et al.: Science 257, 236–240 (1992). 2. Rooney et al.: Lancet 243, 9–12 (1995). 3. Heque et al.: J Immunol 160, 6204–6209 (1998).

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Reactivation of human Herpesvirus-6 (HHV-6) during pregnancy

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Reactivation of Human herpesvirus-6 (HHV-6) and Cytomegalovirus (CMV) during pregnancy and transmission of the virus to the foetus was investigated by polymerase chain reaction (PCR) and serology. Blood samples were drawn from 104 pregnant women at three occasions during pregnancy. From these and another 107 women blood samples were also drawn at delivery and examined together with cord-blood samples. HHV-6 DNA was detected in 41–44% of the samples from month 3–8 of

pregnancy, in 25% at delivery and in 24% of controls. HHV-6 DNA was found in 1% of the cord-blood samples. CMV DNA was found in 1.7% of leukocytes from 104 pregnant women but in no cord-blood sample. IgG antibodies to HHV-6 were found in 96% and CMV IgG in 62.5% of the women. HHV-6 IgG titers were significantly higher in HHV-6 PCR positive compared to negative women. HHV-6 reactivation seems common during pregnancy, and transfer of HHV-6 to the foetus may occur in around 1% of pregnancies.

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The prevalence of human papillomavirus in three different patient groups

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The human papillomaviruses (HPV), the most frequent sexually transmittable pathogens are regarded as biological precursors of cervical carcinoma. Three different patient groups were screened for HPV. Hybrid capture assay (HCA) was used for the detection of low- and high-risk types of HPV DNA.

In the group of asymptomatic women (n=1200), the average reported prevalence of HPV was 17.4%. In the HIV sentinel group (n=65) the prevalence of HPV was 27.7%. In the group of gynecological positive women (n=136) the HPV prevalence was 43.4% (n=136/313). This third group took part in a follow-up study. Forty-five originally positive women presented for the control examination. The current level of HPV positivity was only 45.5% in the initially HPV-positive women. HPV infection may therefore be transient. The parallel performance of cytology and the HPV HCA and the follow-up of HPV-positive women increases the certainty of the carcinoma prevention diagnostics and serves as a quality control of the cytological diagnosis. The very early beginning of sexual intercourse should be avoided. A monogamous sexual life and the avoidance of smoking may decrease the possibility of acquisition of HPV and cervical carcinoma. The relevant control and treatment of HPV infections decreases the rate of mortality from cervical carcinoma.

M. DEGRÉ

Interaction of viral and bacterial infections

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Humans and animals are constantly being inoculated with various microorganisms, including potentially pathogenic viruses and bacteria. Some of these microorganisms may interact directly with other microorganisms, and many of these can interact indirectly by exerting a direct or indirect effect on the various factors of the defence mechanism of the

host organism. Over the years we have studied such interactions both in the respiratory tract and in the intestinal tract in mouse model systems supplemented with *in vitro* studies. Several viruses, including Sendai virus, reduce the efficiency of some of the first line defence systems in the respiratory tract against superinfection by *Haemophilus influenzae*, such as the mucocilliary flow and the antibacterial effect of alveolar macrophages. A further effect on the macrophage activities is exerted indirectly by inducing production of interferons, which in turn also influence phagocytosis and bactericidal activities. Several different viruses influence the process of internalization of invasive bacteria into non-professional phagocytes. Both direct and indirect mechanisms are involved, and the effect is only partially dependent on replication of the viruses. Viral infections may also influence the haematopoiesis and reduce the development and differentiation of monocytes/macrophages. Similar effect can also be observed by some by-products of the viral infections, as interferons and cytokines.

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Epidemiology of herpes simplex virus type 2 infection (herpes genitalis) in Germany

Objective. HSV type 2 (HSV-2) infection is usually transmitted sexually and leads to recurrent genital diseases (herpes genitalis). Epidemiological and clinical studies from different parts of the world claim a dramatic increase of HSV 2 prevalence. The objective of our study was to evaluate if this is also the case in Germany.

Materials and methods. In a retrospective study we looked for the seroprevalence of HSV 2 infections in immunocompetent and immunocompromized people. Therefore sera of more than 9.000 patients were analysed for herpes simplex virus type-specific IgG antibodies.

Results. The seroprevalence of HSV 2 is highest in female sex workers (78.0%) and among HIV infected patients (33.5%), while in the control collective and in organ transplanted patients it was 14.2% and 13.6%, respectively. These data were in good agreement with an earlier study from our institute. In the control collective the rate of positives increases with the age and had its peak in the groups older than 40 years (25.0% in women and 15.6% in men). In the age groups of 30–39, 40–49 and >60 years this difference is statistically significant when considering the 95% confidence intervals.

Conclusion. So far, there is no indication for an increase of the HSV-2 seroprevalence in Germany.

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Anonymised prevalence study of human T-cell leukaemia virus types 1 and 2 antibody in antenatal clinic attendees at King's Healthcare NHS Trust in South London

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HTLV 1 is found worldwide with areas of high prevalence in Southern Japan, Central and West Africa and the Caribbean. Infection is associated with adult T-cell leukaemia and Tropical Spastic Paraparesis, with a lifetime risk of disease between 2 to 5%. HTLV infection is transmitted vertically via breastfeeding, sexually and by blood transfusion or needle-sharing. The overall rate of vertical transmission is around 20%.

The aim of the study was to assess the seroprevalence of HTLV antibody in women attending the antenatal clinic between January 1994 and December 1996. Sera from 7,662 women were tested anonymously for HTLV 1 and 2 antibody using the Murex ELISA (GE80/81). Initially reactive sera were confirmed using the Fujirebio Passive Particle Agglutination Test and Innogenetics HTLV 1/2 Inno-Lia, which allowed differentiation between types.

HTLV antibody was detected in 34 (0.44%) sera; HTLV 1 antibody accounted for 32, with 19 from black Caribbean patients (8 UK born), 10 from black African (2 UK born) and 3 from caucasian patients (2 UK born, 1 born in Jamaica, all 3 with black Caribbean partners); one black African lady was HTLV 2 antibody positive. There was one HTLV untypable result from a black African lady and one indeterminate result from a UK born caucasian lady with a caucasian partner. Of note, the unlinked anonymised HIV antibody prevalence study using dried blood spots collected from neonates for the same time period from the same hospital revealed that the prevalence of HIV antibody was between 0.31 and 0.42%.

These results suggest that HTLV antibody screening in the local antenatal population is as important an issue as HIV antibody screening in order to reduce the risks of vertical transmission.

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BKV DNA prevalence in lymphocytes from immunocompetent, healthy individuals

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Peripheral blood mononuclear cells (PBMC) of blood donors of two cities and of the personnel of a Blood Transfusion Center were analysed by nested PCR for BKV and JCV non-coding control region (NCCR) and VP1 DNA sequences. Twenty-two percent of 231 subjects had BKV-NCCR, but only 7% had also BKV-VP1; presence of the latter decreased with donor age. In both cities the BKV WW archetypal DDP strain, subtype 1, was found. Very few subjects contained JCV DNA, for both NCCR and VP1. Blood operators presented an increased prevalence of BKV-NCCR and BKV-VP1 DNA sequences, with respect to blood donors, suggesting the possibility of occupational risk of BKV reinfection or reactivation. Data suggest that: i) PBMC are not site of polyomavirus persistence in healthy individuals; ii) these cells loose BKV-VP1 sequences with time; iii) detection of BKV-VP1 DNA in PBMC is probably indicative of recent infection or of reactivation.

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Serodiagnosis of anti HSV-1 IgA antibodies in aids patients by using saliva and crevicular fluid

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Saliva and crevicular fluids contain antibodies of diagnostic value. The aim of our study was to test the possibility of their use for detection of anti HSV-1 IgA antibodies in patients with AIDS. Our investigation comprised 20 immunocompromised patients who suffer from AIDS with clinical symptoms of HSV-1 recurrent orolabial infection. The control group consisted of 15 immunocompetent individuals without manifestation of HSV-1 infection. The commercial ELISA test (Invitro dyagnostica GmbH) was used for detection of specific IgA antibodies against HSV-1.

Anti HSV-1 antibodies were found in serum, saliva and crevicular fluid sample of all immunocompromised patients. The average value of IgA antibodies level was 123.8±4 U/ml for patient's serum, 173.1±6 U/ml for saliva and 134.3±3 U/ml for patients crevicular fluid. We did not find anti HSV-1 IgA antibodies in samples of persons of the control group. For the reason of the high blood infectivity in AIDS patients, saliva and

crevicular fluid may be good replacement due to low viral content, for the detection of anti HSV-1 IgA antibodies.

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Molecular proof of the *in vitro* selection of *in vivo* wildtype or ganciclovir resistant mutant strains of cytomegalovirus in a bone marrow transplant recipient

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Only few data exist on ganciclovir (GCV) resistant HCMV strains in the bone marrow transplant setting. Using genotypic and phenotypic drug resistance screening we compared the UL97 mutant pattern of viral isolates with corresponding primary DNA extracts.

A 49-year-old HCMV seronegative woman suffering from AML received an allogeneous bone marrow transplant from a seropositive donor. Virological follow-up was performed for 7 months after transplantation. The screening for HCMV UL97 mutations included PCR-based RFLP analysis of relevant codons (460-603) and UL97 sequencing. Phenotypical drug resistance screening was done by a cell-associated plaque reduction assay. The patient underwent asymptomatic HCMV primary infection on day 46 after BMT. Initial genotypical detection of GCV-resistant UL97 mutants (S595 and W603) after 102 days of exposure to GCV preceeded the isolation of resistant HCMV strains (ID_{50}: 20μM) for 6 weeks. The patient developed HCMV retinitis and HCMV encephalitis and died. Molecular analysis of two viral isolates and the corresponding primary DNA extracts revealed *in vitro* selection of mixed HCMV strains *in vivo*. Either the wildtype UL 97 strain (L595, C603) or the mutant strain was detected after secondary cell culture passage. This phenomenon has strong implications for phenotypical diagnosis of HCMV drug resistance.

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Identification of an immunogenic protein of varicella zoster virus: pUL23

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The genome of the Varicella Zoster Virus (VZV) encodes seventy potential proteins. Only some of these have been recognized to induce immunoreactivity in infected individuals. These include the glycoproteins I-IV, the major-capsid-protein and the assembly-complex p32/36.

To identify further immunogenic proteins a genomic expression library was constructed and screened with a pool of 25 VZV-reactive human sera. We were able to detect a novel immunogenic protein in addition to the known immunogenic products of VZV. This protein is encoded by open reading frame 23. The pUL23 consists of 235 amino acids and is larger than the Herpes Simplex analog (VP16). After eukaryotic expression and separation by SDS-PAGE the protein exhibited a migration of 43 kDa that differed from that calculated molecular weight of 24.4 kDa. To examine the prevalence of pUL23-specific antibodies a protein fragment (12-235) was expressed in *E. coli*, purified by affinity chromatography and used as ELISA antigen.

62% of VZV-reactive human sera (primary infection or zoster patients) exhibited pUL23-specific IgM-titers. None of the control sera showed reactivity. 53% of IgG positive sera and none of the IgG negative sera were reactive with recombinant pUL23. On the basis of the distinct specificity of 100% for IgM and IgG tests, recombinant pUL23, possibly in combination with other recombinant VZV proteins, represents an adequate tool for a recombinant VZV-ELISA.

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Multiplex PCR for the diagnosis of viral and chlamydial conjunctivitis

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Objective. To develop a multiplex PCR for the detection of HSV, adenovirus and *C. trachomatis* in eye swabs and compare its performance to the single PCR, cell culture isolation, Amplicor PCR and immune dot blot test.

Methods. Single PCRs were first optimized and combined systematically to develop the multiplex PCR. The test was evaluated in eye swabs which were treated with a simple lysis buffer as a method of DNA preparation.

Results. The multiplex PCR was found to have detection limits of 400, 300 and 100 copies of adenovirus, HSV and *C. trachomatis* DNA, respectively. In a prospective study of 429 patients with conjunctivitis or keratitis, the test was evaluated against the single PCRs, cell culture isolation for adenovirus and HSV and the Amplicor PCR and immune dot blot test for *C. trachomatis*. A total of 95 patients were positive for one of these organisms (49 for adenovirus, 32 for HSV and 14 for *C. trachomatis).* Of the 49 adenovirus-positive patients, the multiplex PCR was positive in 48, the adenovirus single PCR in 48 and cell culture in 18 and was not tested in 12. Of the 32 patients positive for HSV, 29 were positive by the multiplex PCR, 30 by HSV single PCR and 29 by cell culture. *C. trachomatis* was detected in 12 patients by both the multiplex PCR and the single PCR, in 14 by the Amplicor PCR and in 8 by immune dot blot test which was reported equivocal in another 2 patients.

Conclusion. The newly developed multiplex PCR is sensitive, rapid, cost effective and can replace single PCRs, cell culture and immune dot blot for the diagnosis of viral and chlamydial conjunctivitis.

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Detection of adeno-associated viruses (AAV) in semen samples of infertile men

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Adeno-associated viruses (AAV) are human parvoviruses that require helper viruses for their replication. They are considered as non-pathogenic. However, experimental infection of pregnant mice with AAV-2 led to fetal death and early abortion. In humans, AAV could be detected in materials from spontaneous abortion in amniotic fluid and in the cervix uteri. To date, no detection of AAV in the male genital tract was reported. Thirty semen samples of infertile men and 8 control samples were separated into three fractions using a ficoll gradient: spermatozoa, non-spermatozoal cells and cell free supernatant. These fractions were examined by PCR for the presence of AAV-2 and HPV 16/18 because of the known helper function of HPV 16. In nine of 30 cases AAV-DNA could be detected in samples from patients, the control samples (8) were negative. Seven of nine AAV-positive patients showed an oligoasthenozoospermia, one patient an asthenozoospermia and one patient an azoospermia.

In addition, HPV 16/18 DNA could be detected in samples from eight patients and two control persons. In this case no association to the result of the spermatological examination could be found.

For the first time AAV-DNA could be detected in human semen samples. A sexual transmission of AAV as well as a role in male infertility seems to be possible.

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Does the suboptimal antibody level aggravate the course of tick-borne encephalitis?

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This report describes a case of tick-borne encephalitis (TBE) in a 42-years-old man. He was a healthy person before he fell ill with TBE. He suffered tick bites twice at different localities. One of them is a known endemic focus of TBE virus. After the tick bites he was administered twice with TBE specific hyperimmune gamma globulin, however the amount of the immunoglobulin was much less than the adequate one. The febrile illness started at the 12th day after the first tick bite, and respiratory insufficiency

occurred four days later. We could not detect specific antibodies by an in-house immunofluorescence test neither in the sera, nor in CSF at the time of onset of respiratory failure. Seven days later both specific IgG and IgM were detectable in serum sample and investigation of the CSF revealed intrathecal production of specific antibodies.

The short incubation period, the unusual slow development of the detectable specific antibodies and the severe course of the disease with fatal outcome suggest that this TBE case was an example for antibody dependent enhancement of infection, the well-known phenomenon in the family *Flaviviridae.*

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Epidemiological analysis of immunity to poliovirus in Germany

The objectives of the WHO is the global eradication of poliomyelitis by the year 2000. After a period of 37 years of vaccination with live oral poliovirus vaccine (OPV), in Germany since 1998 the inactivated poliovirus vaccine (IPV) is recommended.

The intention of this epidemiological analysis is to record immunity to poliovirus types 1, 2 and 3 in 4034 patients of the years 1993 to 1997 using a microneutralization assay. The evaluation of the non-age-specific total seroprevalence for antibodies against poliovirus 1 remains around 80%, similar the poliovirus type 2 around 80%, while the immunity to poliovirus type 3 decreased from 75% (1993) to 68% (1997). The prevalence of antibodies for poliovirus 3 is in 1997 45%, in the important group of the children and youth between 5–19 years clearly less than in 1993 (65%).

The relatively favourable seroprevalence of the antibodies in the year 1997 at the children between 1–4 years with 87% against poliovirus 2 indicates that the programs to vaccination are well accepted, but a good immunity to all 3 serotypes (antibody against polio 3 at only 58%) with the primary vaccination however is not reached. The objective to the global eradication of poliomyelitis is still not attained, there are especially in Africa and Asia great problems.

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Development of a polymerase chain reaction (PCR) and hybridization assay for the detection of adenovirus from respiratory specimens

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Objective. To develop and evaluate a PCR-hybridization-immunoenzymatic assay (PCR-HYB-EIA) using AdV genus-specific primers and probe defined in the VA RNA gene, and to compare it to IFA/VIT, and to one other PCR-HYB-EIA in the hexon gene.

Method. Specimens were 197 nasal aspirates from hospitalized infants during winter periods 1995–96 and 1997–98. Monoclonal antibody to AdV (Imagen®, Dako, UK) NCI-H292 and MRC5 cell cultures were used for IFA and VIT. Nucleic acids were extracted by a chelex procedure. The PCR-HYB-EIA1 used 2 primers and a probe defined in the hexon gene by Hierholzer et al. (J Clin Microbiol **31**, 1993). For the PCR-HYB-EIA2, we defined AdV genus-specific 2 primers and a probe from the reported sequences of the VA RNA gene, in the same gene region that described by Kidd et al. (J Clin Microbiol **34**, 622 1996). Amplification products were detected by agarose gel electrophoresis: band at 290–340 bp for 1 VA RNA gene (AdV of subgenera A, B2, F) and band at 460–560 bp for 2 VA RNA genes (AdV of subgenera B1, C, D, E) and by hybridization (HYBRIDOWELL™, Argene).

Results. The specificity was demonstrated with no amplified products from DNA extracted from non-adenoviral and bacterial agents commonly known to cause respiratory infections. The sensitivity of the PCR-HYB/EIA2 was carried out on four AdV prototype strains: AdV12, AdV3, AdV1, AdV8 from the main subgeneras: A, B1, C, and D, respectively. The number of equivalent-genomes per ml (EG/ml) and of infectious doses 50 per ml (ID50/ml) were 10^4 and 10^2, 10^6 and 10^4, 10^6 and $10^{4.9}$, 10^4 and $10^{2.9}$, for AdV12, AdV3, AdV1, AdV8, respectively. In the 197 nasal aspirates of infants, AdV were detected by IF-VIT in 19 (9.6%) cases, in 28 (14.2%) cases by the PCR-HYB/EIA1, and in 30 (15.2%) cases by the PCR-HYB/EIA2. PCR-HYB/EIA1 and PCR-HYB/EIA2 were positive in 10 and 11 of the 178 IFA/VIT-negative specimens, respectively.

Conclusion. The genus-reactive primer sets provided DNA amplification assays of better efficiency for the detection of AdV in nasal samples in children. The performance of the two PCR-HYB-EIA was very similar, but the VA RNA gene-PCR provided further helping in differentiating some subgenera according to the size of the PCR products.

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Quantification of hepatitis C virus (HCV) RNA in liver, peripheral blood mononuclear cells and serum from patients with chronic HCV infection

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Objective. Hepatitis C virus (HCV) RNA has been reproducibly found in the liver of patients with chronic HCV infections. However, no systematic effort has been made to comparatively quantitate viral RNA content in liver, serum and peripheral blood mononuclear cells (PBMC).

Methods. To this end HCV RNA was quantified by competitive rt-PCR (Grassi et al.: J Hepatol **23**, 403 1995) in liver and PBMC, and by bDNA (Chiron) in serum from 11 patients with chronic HCV infection and the findings were correlated with biochemical and histological data.

Results. Genomic HCV RNA was found in all the liver and serum specimens and in 64/70 (91.4%) PBMC specimens. Levels in the latter were lower compared with those found in liver extracts (median in PBMC: 5.9×10^3 Genomic Equivalents, Geq/μg RNA, range 0–2.5×10^5, vs median in liver: 1.6×10^5 Geq/μg RNA, range 1.6×10^3–1.2×10^6). Median serum levels expressed in Geq/ml were 8.9×10^6 (range 1.9×10^5–7.4×10^7). A relatively weak correlation was found between serum and liver HCV RNA levels (R=0.32; p=0.058), but not between PBMC and either serum or liver. However, there was no correlation between intrahepatic HCV RNA levels and liver enzymes (ALT, AST), HCV genotype or histological activity grade. Nevertheless, patients with high level of hepatic fibrosis (stages 3–4) showed a trend toward a lower viral burden in liver and serum compared with patients with a lower degree of fibrosis (stage 1).

Conclusions. These findings suggest that progression of HCV-related liver disease occurs independently of viral load and are consistent with previous data reporting an inverse relationship between severe and advanced liver disease and serum viral RNA level. Other host factors, including genetic background and immune response, may contribute to disease control or progression.

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Codon 1 and 28 mutants and HBV DNA levels – implications for health care workers

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Objectives. To measure BV DNA levels in hepatitis B carriers over time and relate these parameters to HBeAg status, pre-core mutations and to infectivity in the health care setting.

Methods. Retrospective longitudinal surveys of HBV carriers and outbreak clusters using PCR and Branch DNA assays for HBV DNA quantification, point mutation assays at codon 1, 15 and 28; direct consensus sequencing of surface and core genes; routine serology.

Results. Seroconversion for anti-HBe coincided with 10^5-fold HBV DNA reduction. HBV DNA could be measured (>400 copies/ml) in 130 of 200 HBeAg negative sera from 100 HBV carriers; of whom 8% have in excess of 10^5 copies/ml serum. Loads are not significantly influenced by pre-core codon 1 and 28 mutations. However, individuals' levels may fluctuate in excess of 100-fold, and are more likely to show downward trends in presence of codon 1 mutations and the reverse with codon 28 mutations. Transmission events from six negative carriers and one unusual cluster of non-surgical transmission from an e carrier were found.

Conclusions. Branch DNA and PCR commercial assays are robust but require re-calibration. PCR is necessary to investigate serum HBV DNA levels in anti-HBe carriers whose levels are often quite unstable. Pre-core mutations have surprisingly little influence on HBV DNA levels *in vivo*. HBV DNA quantification is likely to be a more secure way of assessing infectivity and should be considered in future.

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Molecular epidemiology of adenovirus type 8 isolated from patients with keratoconjunctivitis in Norway from 1991 to 1996

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Human adenoviruses (Ad) comprise 49 serotypes, which are classified into 6 subgenera (A–F), Serotypes 8, 19a, and 37, belonging to subgenus D, have been associated with epidemic keratoconjunctivitis (EKC). EKC is a severe, painful and highly contagious eye infection, which generally lasts for 2 to 6 weeks. The virus is transmitted from person to person by hands, or by instruments used in ophthalmological clinics. EKC can occur in outbreaks, and is predominantly caused by Ad8.

Genetic variability has been demonstrated with restriction enzyme analysis (REA) and nucleotide sequencing within each adenovirus serotype studied. However, only limited information is available about the genetic variability in Ad8.

Several local outbreaks of EKC caused by Ad8 have been reported from different parts of Norway during the winter 1995–96. Genetic variation was studied by sequencing the hypervariable region of the hexon gene and part of the fiber genes of Ad8 isolated from patients with EKC from different outbreaks. Some of the isolates were also analysed by REA.

The aim of this study was to investigate the genetic variability in Ad8 strains isolated from different outbreaks in Norway in 1995–96, and to compare them with Ad8 isolates from sporadic cases occurring during 1991–96. The results of the molecular epidemiological investigations will be presented.

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How much IPV and OPV is sufficient to eliminate poliomyelitis and prevent vaccine associated cases?

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In 1998, the United States Advisory Committee on Immunization Practices adopted a sequential combined program of IPV (2 doses) followed by OPV (2 doses) in order to eradicate Vaccine Associated Poliomyelitis (VAPP) as well as the wild polio virus. A combined program of OPV and IPV was initiated by us already in 1978 in Gaza and the West Bank, endemic areas when OPV alone had failed to control the disease (Tulchinsky et al.: American Journal of Public Health **79**, 1648 1989). Following a small epidemic of poliomyelitis in Israel in 1988, a combined program was adopted ("The Gaza program"). These IPV/OPV programs eliminated clinical cases of polio using 2 or 3 doses of IPV and 3–4 doses of OPV. The area has been free of clinical cases since 1988, despite introduction of wild poliovirus from neighboring areas (Goldblum et al.: Bulletin of the World Health Organization **72**, 783 1994).

With international progress in eradication of poliomyelitis, problems remain in areas with inadequate OPV coverage and with VAPP cases. The literature suggests that a modified combined program with an initial and early dose of IPV, followed by 3–4 doses of OPV during the first year of life would eliminate both VAPP and wild poliovirus cases, as well as providing the environmental reinforcement of the OPV coverage. A combined IPV/OPV program with 1 dose of IPV provides both benefits and should be attractive to developing areas with residual poliomyelitis because of its greater effectiveness and relatively low cost.

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Screening of reflex sympathetic dystrophy (RSD) patients for antecedent infections

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Introduction. RSD is a seriously disabling neuropathic syndrome with pain, swelling, autonomic dysfunctions, movement disorders and dystrophy or atrophy. Much is speculated about its etiology and often neurotropic infections are mentioned.

Design: In this study of 52 well-documented RSD patients we tried to find a connection between the presence of specific antibodies and predisposition to RSD.

Results. For Parvovirus B19 we found a higher seroprevalence in RSD and this most strikingly in lower extremity RSD patients. The seroprevalence for Parvovirus B19 is more than 94% in patients with lower extremity involvement and is significantly ($p<0.04$) higher than the 68% seroprevalence of Parvovirus B19 in upper extremity RSD patients. The seroprevalences for VZV (100%), EBV (96%), HSV (73%), CMV (40%), *Toxoplasma gondii* (23%) were not significantly different from an independent Dutch control group. Only four patients had detectable antibodies against *C. jejuni.* All patients were negative for *T. pallidum, B. burgdorferi* IgG and HTLV-1 antibodies.

Conclusions. No correlation was found between RSD and VZV, EBV, HSV, CMV, *T. gondii, C. jejuni, T. pallidum, B. burgdorferi* or HTLV-1. For Parvovirus B19, we found a significant, but yet unexplained higher seroprevalence in lower extremity RSD patients. However, it is too early to say that Parvovirus B19 is related to RSD. Further serological studies will give more necessary information.

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Virology isolation of influenza virus from a hepatitis C carrier

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A 47-year-old male hepatitis C carrier for ten years, with hypertension untreated and NIDDM treated by diet, was treated for the hepatitis with INFα for 6 months before being hospitalized during the 1997/8 influenza season due to shortness of breath. On admission, respiratory functions deteriorated rapidly and massive bilateral pneumonia was diagnosed. As the patient belongs to those at risk, he was previously immunized with inactive influenza vaccine by i.m. injection and was consequently immune to the three strains in the vaccine (<1:40). It was therefore of interest that despite the level of serum HI antibodies, considered to indicate immunity, a virus was isolated from a throat swab collected 19 days after admission. Isolation was carried out by passages in the amniotic-

allantoic sac of embryonated eggs. The 4th passage yielded fluid with titers of 1:1024 haemagglutination and $10^{9.0}$ EID_{50}/0.2ml infectivity. The isolate (A/Jerusalem/17/98) was identified by HI as A (H_1N_1). It was characterized in the CDC (by Dr. Klimov) by post-infection ferret antisera and was also found to be related to A/Bayern/07/97 (H_1N_1), the H_1N_1 component of the 1997/8 influenza vaccine.

These results may suggest that in immunocompromised patients, humoral immunity is not sufficient to assure prevention of respiratory infection. Local SIgA antibody presence may better predict immunity against influenza infection.

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The effect of interferon on HCC risk in post-HCV cirrhotics

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The influence of IFN on natural history of chronic hepatitis C and cirrhosis is still controversial. Over the last ten years, alfa-interferon has proved effective in attenuating the liver-cell necrosis and inflammatory changes associated with chronic hepatitis C, while its influence on HCC development is still under debate. We are analyzing the clinical histories of 228 patients (131 males, 97 females, aged 31–73 years; mean age 55.6±7.7 years) with cirrhosis and chronic hepatitis C, who were treated with a-IFN, in an attempt to identify factors predictive of HCC development. Enrollment criteria included histological diagnosis of cirrhosis with no evidence of hepatic nodules, at least one 3-month course of IFN administration and 12 months or more follow-up after termination. Treatment schedules and duration of therapy varied, but the total dose of IFN administered range from 72–2040 MU (mean: 566 MU). Follow-up is based on clinical data, blood-chemistry and iUS or TC scan evaluations every six months. At moment the median follow-up is currently 60 months (range 18–78 months) from the end of IFN treatment, or 74 months (14–143 months) from the initial histological diagnosis of hepatitis. HCC has been detected in 28 (12.2%) patients. In standard survival analysis, the probability of developing HCC is 4.9% 24 months after the beginning of IFN therapy, 7.3% at 36 months and 13% at 78 months. Using the initial histological diagnosis as the starting point, the risk is 3.1% at 24 months, 5.3% at 36 months, 12.2% at 78 months and 19.4% at 143 months. Male sex, age>55 years, ethanol abuse and long-lasting disease have emerged as risk factor for HCC in this population. Data on IFN therapy (duration, total dose, response) are still inconclusive, although increased doses of IFN seem to exert a protective effect on the development of HCC.

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An outbreak of West Nile fever among migrants in Kisangani, Democratic Republic of Congo

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In February 1998 an outbreak of acute febrile illness was reported from Kapalata military camp in Kisangani, the Democratic Republic of Congo. The illness was characterized by an acute onset of fever associated with severe headache, arthralgia, backache, neurological signs, abdominal pain and coughing. In one individual hemorrhagic manifestations were observed. The neurological signs included an altered level of consciousness, convulsions, and coma. Initially malaria was suspected but the patients showed negative blood films and failed to respond to antimicrobial drugs. A total of 35 sera collected from the military patients in the acute phase were tested for the presence of IgM against vector borne agents. IgM serum antibodies against West Nile fever virus were found in 23 patients (66%), against chikungunya virus in 12 patients (34%), against dengue virus in one patient (3%) and against *Rickettsia typhi* in one patient (3%). All sera were negative for IgM antibody against Rift Valley fever virus, Crimean Congo haemorrhagic fever virus and Sindbis virus. These data suggest that infections with West Nile fever virus have been the main cause of the outbreak.

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Immunization with a canarypox-cytomegalovirus (CMV)-phosphoprotein 65 (pp65) recombinant induces CMV-specific cytotoxic T lymphocytes and helper T cell responses in humans

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Canarypox-CMV-pp65 recombinant was examined for its ability to induce CMV-specific cytotoxic, helper T lymphocytes and antibodies in a phase I clinical trial. Twenty-one CMV seronegative healthy volunteers were inoculated intramuscularly with $10^{6.8}$ $TCID_{50}$ of canarypox-CMV-pp65 or placebo at months 0, 1, 3 and 6. Four CMV seropositive individuals were also included in the study as positive controls. Vaccines were well tolerated. Blood was drawn at months 0, 3, 4, 5, 6, 7 and 12. Peripheral blood mononuclear cells (PBMC) obtained from canarypox-CMV-pp65 immunized volunteers proliferated in response to stimulation with purified pp65 protein. CMV-specific CTLs

were demonstrated by restimulating PBMC *in vitro* with the canarypox-CMV-pp65 recombinant and testing the cytolytic activity of the PBMC on vaccinia-pp65 recombinant infected, EBV-transformed autologous and MHC-mismatched B lymphoblast target cells. CMV-specific CTL could be demonstrated in all immunized persons already after two or three vaccine doses and were still present at month 12. Cell depletion tests carried out at month 5 with PBMC of 12 CTL responders indicated that the phenotype of CTL was CD8+. A limiting dilution assay suggested similar pp65-specific CTL precursor frequency in vaccinated and naturally seropositive individuals. CMV-pp65-specific antibodies were also induced in all canarypox-CMV-pp65 immunized individuals. Volunteers receiving placebo did not show positive results at any time in any of the immunological tests. These results suggest that vaccination with a canarypox-CMV recombinant could be a useful approach for the prevention of CMV disease.

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Assessment of clinical sample integrity for RNA amplification testing – detection of human U1A MRNA by the Nuclisens® Basic Kit

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Objectives. To develop a RNA amplification assay for a low abundance mRNA encoding human U1 snRNP specific A protein (U1A mRNA) to critically monitor RNA integrity in a clinical sample.

Methods. For the detection of U1A mRNA the NucliSens® Basic Kit (Organon Teknika) was used as assay platform. This is a generic kit for RNA amplification testing. It provides standardized reagents far "Boom" silica based extraction of nucleic acid, isothermal NASBA amplification and Electrochemiluminescence (ECL) detection. The kit can be customized to measure any RNA target by designing analyte specific primers and probes.

Results. The U1A mRNA Basic Kit protocol (including primers and capture probe sequences) was downloaded from the application database hosted at an Extranet web site (http://lwww.teknika.nt.extranet/basickit). Use of the Basic Kit In combination with this protocol resulted in an assay with a high sensitivity (10–100 molecules of input RNA) and specificity for detection of U1A mRNA. U1A mRNA could be detected in whole blood, urine, plasma, cervical smears and nasopharyngeal aspirates. U1A mRNA analysis could be used to demonstrate that lack of CMV pp67 mRNA positivity in samples from patients clearly going through a CMV episode was due to degradation of sample RNA. In a simulation experiment clinical samples positive for HIV-I viral RNA and CCR5 mRNA were mishandled by heating and UV treatment: mishandled samples produced negative results for HIV-I, CCR5 and U1A.

Conclusions. Detection of human U1A mRNA seems a valuable tool for identifying false negative results from other RNA amplification assays, due to degradation

of sample RNA. The NucliSens® Basic Kit allowed for fast and easy development of an assay for this RNA integrity control.

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Cytomegalovirus reactivation during and lactation: risk factors for transmission to preterm infants

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The influence of breast-feeding on reactivation patterns and transmission of HCMV to preterm infants was analysed. We aimed to describe parameters having predictive value for postnatal virus transmission to breast-fed preterm infants.

Congenital and perinatal HCMV transmission were excluded. HCMV screening was done by serology, virus culture and PCR. Viral load was determined in milk whey and isolates from whey were characterized using HCMV glycoprotein gene regions. Levels of chemokines and lactoferrin in whey were determined longitudinally.

Nearly every seropositive mother showed HCMV DNAlactia in whey during lactation. HCMV reactivation was a local event. Neither duration of DNAlactia nor the presence of virolactia were correlated to transmission. However, an early onset of DNAlactia and the viral DNA load were predictive for HCMV transmission. In contrast, levels of lactoferrin, RANTES and GROα in whey seemed to be not different between the maternal transmitter and non-transmitter group.

HCMV transmission was linked in a minority of preterm infants (about 12%) to severe life-threatening sepsis-like symptoms. Making use of the benefits of breast-feeding, virus inactivation procedures have been evaluated.

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Comparison of molecular assay formats for detection of enteroviral RNA in stool and CSF

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Study aims. To evaluate an enterovirus-specific NASBA method based on the NucliSens® Basic Kit (Organon Teknika Ltd) and compare different detection systems for analysis of amplified products in a diagnostic setting.

Methods. Primers and probes were designed in the 5' untranslated region for detection of a wide range of sequenced enteroviruses. Samples were extracted using the "Boom" silica based method either manually or using an extractor machine (Organon

Teknika Ltd). More than 150 clinical samples and spiked controls were analysed as part of this study.

Results. The Nuclisens® Basic Kit assay was sensitive and specific for detection of enteroviral RNA in stool and CSF. The extractor machine gave comparable results to manual methods for preparation of nucleic acid from these samples. Results for use of an enterovirus-specific molecular beacon designed for "in tube" detection of NASBA products were encouraging but more work is needed to evaluate this approach on a large scale.

Conclusions. The NucliSens® Basic Kit reagents enabled simple development of an enterovirus-specific NASBA suitable for use in a diagnostic laboratory. Kit-based reagents, availability of an extractor machine and potential for "in tube" detection of amplified products will facilitate the widespread use of molecular amplification assays for detection of RNA targets.

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EBV-specific cytotoxic T cell therapy for post transplant lymphoproliferative disease (PTLD)

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Objectives. Donor derived, *in vitro* grown EBV-specific cytotoxic T cell lines (CTL) have been used successfully to prevent and treat PTLD in bone marrow transplant recipients. However, in solid organ transplantation donors are not usually available to provide CTL. The aim of this study is to establish a bank of HLA-typed EBV-specific CTL for *in vivo* use in the treatment of PTLD in solid organ transplant recipients. These *in vitro*-grown cells are now being used in a Phase I therapeutic trial.

Methods. CTL are grown *in vitro* from peripheral blood samples obtained from healthy blood donors by stimulation with the autologous EBV-immortalized lymphoblastoid cell line and IL2. Restimulation is continued until a high level of specific and low non-specific killing is achieved in chromium release assays. The CTL are then frozen in aliquots and stored in liquid nitrogen prior to *in vivo* use on a best HLA-match basis.

Results. In an initial pilot study 3 transplant recipients (without PTLD) received *in vitro* grown, EBV-specific autologous CTL in 3 escalating doses (5×10^7, 10^8, 2×10^8) given at monthly intervals starting 6 months after transplant. CTL remained detectable and function in peripheral blood for the whole study period (up to one month after the third infusion), and EBV DNA load in peripheral blood mononuclear cells was depressed below pre-infusion levels during the same time period.

Conclusions. Infusion of autologous EBV specific CTL causes enhanced cytotoxic activity against EBV infected cells and a reduction in viral load for a prolonged period of time. However, production of autologous CTL for all transplant recipients at risk of PTLD is not feasible. Thus we have developed a bank of HLA-typed, EBV specific CTL from

healthy blood donors which are being used in a phase I clinical trial. The results of the study will be presented.

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HIV-pheno- and genotyping – comparing of results

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The results of HIV-1 phenotyping (recombinant virus assay by Virco) were compared with the data of genotyping (sequencing) for four protease (53 patients) and two RT-Inhibitors (44 patients). For the PI's (protein inhibitors) the quantity of mutations corresponds obviously with the results of phenotyping. Up to 3 mutations no resistance was found (20.8%) in contrast to 9 and more mutations with resistance for all four drugs (39.6%). The patients with 7 or 8 mutations showed with one exception phenotypic resistance at least for 3 drugs. For Saquinavir and Ritonavir the primary mutations were more important for phenotypic resistance than for Nelfinavir and Indinavir. The primary mutation D30N was not found and 44% of the phenotypic Indinavir-non-resistant patients showed either the M46I/L or V82A mutation. None of the patient with a G48V mutation was phenotypic sensitive for Saquinavir. The V82A mutation was detected by 56% of the Ritonavir-resistant patients in contrast to 4.7% of the non-resistant patients.

The difference of frequency between AZT-resistant and non-resistant patients was much higher for D67N (50% versus 12.5%), K70R (25% versus 0%) and K219Q (41.6% versus 0%) than for M41L (88.8% versus 50%) and T215Y/F (94.4% versus 75%), because we were able to confirm that the M184V-mutation can remove the effect of M41L/T215Y/F. None of the phenotypic 3TC-sensitive patients had a M184V-mutation, but only 85% of the phenotypic 3TC-resistant patients showed the M184V-mutation.

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Serodiagnosis of Epstein-Barr virus infection by using novel VCA ELISA based on recombinant p23 and p18

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Using recombinant 15–30 kDa antigen fragments in fusion with glutathione S-transferase (GST) and a panel of 18 well-defined sera, we investigated the seroreactivity of three large structural proteins of Epstein-Barr virus (EBV), p150 (BcLF1, capsid), p143 (BNRF1, tegument) and gp125 (BALF4, membrane) in Western blots. None out of 13 fragments tested, however, was qualified for diagnostic application. In contrast, two small viral capsid antigens, p18 (BFRF3) and p23 (BLRF2), demonstrated very sensitive (100%) EBV-specific IgG reactivities. While p18 additionally showed maximum sensitivity for IgM detection, the IgM sensitivity of p23 was restricted (44%). An

autologous fusion protein, p23-18, was constructed, which N-terminally consists of full length p23, followed by the carboxy-half of p18. This antigen was applied for an indirect VCA IgG ELISA, as well as for an μ-capture IgM ELISA based on directly labelled antigen. Different serum panels, including detailed follow-ups (n=15), collected from mononucleosis patients (n=33), rheumatoid arthritis patients (n=23), other hospitalized patients (n=88), and healthy donors (n=194) have been tested in comparison with established reference assays. The VCA(p23-18)-IgG ELISA revealed a total sensitivity of 99.7% and a specificity of 100%. The sensitivity of the VCA(p23-13)-μc-IgM ELISA for acute primary infection was 97.0%, and the total specificity was 97.2%. The positivity rate of IgM declined to 14.3%, when sera from recently infected individuals taken 6 to 12 months after onset of symptoms were tested. This emphasizes rapid IgM seroreversion. In conclusion, the p23-18-IgG/-(μc)-IgM ELISA showed excellent performances and are promising new tests for diagnosing infectious mononucleosis and EBV seropositivity.

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Clinical performance of AxSYM HAVAB-M™ 2.0

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AxSYM HAVAB-M 2.0 is an MEIA assay for the qualitative detection of IgM antibody to hepatitis A virus (HAV) in human serum or plasma. A test for IgM anti-HAV is indicated as an aid in the diagnosis of acute or recent HAV infection. Two hundred forty-seven specimens previously characterized as IgM anti-HAV reactive, including 40 specimens from a documented hepatitis A outbreak were tested with AxSYM HAVAB-M 2.0. The sensitivity of the AxSYM HAVAB-M 2.0 assay was 99.59% (244/245). Two specimens were determined to be nonreactive by supplemental assay. AxSYM HAVAB-M 2.0 detected IgM anti-HAV antibodies in 100% (40/40) of the hepatitis A outbreak specimens. Clinical utility of the AxSYM HAVAB-M 2.0 assay (as determined by transition from IgM anti-HAV reactive to gray zone reactive or nonreactive results within 6 to 9 months of first bleed date) was compared to the marketed AxSYM HAVAB-M assay using 20 serial bleed panels from patients with HAV infection. AxSYM HAVAB-M 2.0 showed improved performance compared to AxSYM HAVAB-M in 14 panels, comparable performance in 2 panels, and decreased performance in 1 panel. Three panels had inconclusive results. Five hundred eighty-nine serum and plasma specimens were tested with AxSYM HAVAB-M 2,0 including specimens from 300 volunteer whole blood donors, 200 random hospital patients, and 89 specimens from patients with infections other than HAV or containing potentially interfering substances. One specimen was repeatedly reactive and the remaining specimens were nonreactive for IgM anti-HAV by AxSYM HAVAB-M 2.0 and a comparator assay. The specificity of AxSYM HAVAB-M 2.0 was 100% (588/588). These results support the use of AxSYM HAVAB-M 2.0 as an aid in the diagnosis of acute or recent hepatitis A viral infection.

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Quantitative determination of hepatitis A virus antibodies in vaccinees: is it necessary?

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The inactivated hepatitis A vaccine has been demonstrated to be highly immunogenic inducing seroconversion rates in vaccinees of approximately 100%. Nevertheless, most of these studies have been conducted in relatively young and homogeneous study populations. The aim of the present study was to investigate the immune response to HAV in a random group of routinely vaccinated people in order to obtain information, whether and for whom a quantitative determination of HAV-specific antibodies should be recommended. For this purpose, quantitative levels of HAV-specific antibodies were determined in 1345 vaccinees ranging in age from 1 to >60 years, using a commercially available ELISA (Enzymun-test® anti-HAV, Boehringer Mannheim). The data obtained were analyzed with regard to antibody-level, age and the time interval between the 3rd vaccination and the collection of the serum samples (3 to 60 months with the majority drawn between 12 and 48 months post-vaccination). Although anti-HAV antibodies were detectable in nearly all of the vaccinees, a slight increase of seronegatives (≤20 ImU/ml) with increasing age was observed. In addition, the number of people exhibiting extremely high antibody levels was also growing with age. Since no prevaccination serum samples were available, this increasing number of vaccinees with extremely high antibody levels in the older age groups seemed to indicate immunity derived from a previous wild virus infection. A cut off level was calculated to differentiate with a high probability between vaccine-induced and wild virus infection-induced antibody levels. After excluding samples with antibody levels indicating a natural immunity, data were reanalyzed and demonstrate that quantitative HAV-antibody determination is not necessary in young vaccinees, but should be considered in those above the age of 60 with a risk of exposure 3 to 4 years after vaccination.

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Nosocomial epidemic of CMV conjunctivitis followed by systemic complications

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Background. CMV infection occurs in about 0.5–2.5% of newborns and is clinically manifested as CID. Severity of disease depends on the type of infection during pregnancy-primary infection or reactivation of endogenous infection.

Objective. In our work we describe the epidemic of conjunctivitis at the new-born ward in 1994.

Materials and methods. Virus isolation was performed from conjunctival and nasopharyngeal swabs and urine of 128 newborns (110 newborns and 18 twin newborns) in human diploid cells. Anti-CMV IgM and IgG Ab were detected by EIA (IMx, Abbott).

Results. CMV was isolated from conjunctival swabs in 32 newborns (15 boys and 17 girls). Positive isolations from both eyes were found in 7 newborns, repeated CMV positive isolations were found in 4 newborns. The course of infection was prolonged and lasted for 2–3 weeks. In 2 infants CMV was isolated both from conjunctival swab and urine or nasopharyngeal swab. In these v 2 infants systemic complications were diagnosed (hepatopathy, pneumopathy with presence of anti-CMV IgM Ab). Both children were followed during further 2 years. CMV from urine and sustained titre of specific IgM was detected.

Conclusion. In epidemic of conjunctivitis in 128 infants at newborn ward CMV was identified as the causative agent. CMV was isolated and specific IgM Ab were detected during the acute phase of infection and in 2 children as long as at the age of 2 years.

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HSV-1 and -2 seroprevalence among STD clinic attenders in Groningen, The Netherlands

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A total of 610 serum samples of STD clinic attenders of the university hospital was screened for the presence of HSV antibodies. Most patients visited the clinic for *T. pallidum* serology (n=566), only 44 patients visited for HSV-serology. ELISA was used for antibody detection and performed with the following antigens; detergent extract of HSV-1 infected cells, recombinant gD-1, recombinant gG-1 and a fragment of recombinant gG-2, together with all the appropriate controls. The recombinant proteins were produced in the baculovirus expression system. Sera were regarded as HSV-positive when either antibodies against HSV, gD-1 or both were present. Sera only positive for gG-1 and gG-2 and not for HSV or gD-1 were regarded as aspecific and were excluded. The prevalence of antibodies to HS V-1 was 68.1% in women and 72.1% in men; the prevalence of HSV-2 was 18.8% and 15.4%, respectively. HSV-negative were 20.5%. Antibodies against only HSV-1 were 55.6%, only against HSV-2 were 2.5%, and against both types were 14.6%. HSV-positive without gG antibodies was 6.9%. In the HSV patient group, the prevalence for HSV-2 and HSV-1 was 31.8% and 65.9%, respectively. The majority of persons acquired HSV-2 antibodies between 20 and 40 years of age.

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Human cytomegalovirus infection of hepatocytes

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Infection of the liver by human cytomegalovirus (HCMV) is a frequent complication after liver transplantation and also in the immunocompetent host, primary HCMV infection is present in a number of cases with signs of elevated liver enzymes.

The cytopathic potential of HCMV in human liver cells was analysed in cell culture and in tissue sections from patients with HCMV hepatitis. Liver cell cultures, consisting of hepatocytes, bile duct epithelial cells, and stromal cells were infected by various HCMV strains. Cytopathic effects, viral gene expression, and virus production were detected. Infected cell types were identified by immunocytochemical double labeling. Hepatocytes were the predominant target cells of HCMV infection in liver tissues as, well as in cell culture. They were permissive to the complete viral replication cycle. Late stage infected hepatocytes produced infectious progeny virus. Cytopathogenicity was pronounced with more recent clinical strains. HCMV infection in cultured liver cells closely resembled *in vivo* infection of the liver with regard to the target cell spectrum and the permissive course of infection. It is concluded that HCMV can cause direct liver parenchyma damage by efficient cytolytic infection of hepatocytes.

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Performance characteristics of the LCx HIV RNA quantitative assay

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Objective. Evaluate the performance characteristics of the LCx HIV RNA Quantitative Assay using two different specimen volumes: 1 ml and 0.2 ml.

Design. The lower limit of detection was determined by running dilutions of a quantitated stock obtained from the Virology Quality Assurance (VQA) Laboratory. Specificity was evaluated by running 496 negative donor specimens. Precision was evaluated with a dilution panel prepared by the VQA in a multi-site study. Subtype quantitation was evaluated by comparing the LCx assay and Roche Monitor quantitation results for 278 specimens from genetically diverse HIV-1 group M (A-G) and group O isolates.

Results. The lower limit of detection is 50 copies/ml with 1 ml of plasma, and 178 copies/ml with 0.2 ml of plasma. Specificity was 99.19% using the 1 ml protocol (n=496), or 100% using the 0.2 ml protocol (n=270). The total sd Log copies/ml for plasma panels 0.5 log above the lower limit of detection was <0.250 log copies/ml. The correlation coefficient was >0.91 when quantitation results from the LCx assay and Roche Monitor or

Roche Ultrasensitive assay were compared using either 1 ml or 0.2 ml of plasma. For the 278 specimens with different subtypes, 67.3% were positive by both assays, 30.2% were positive in the LCx assay and negative in the Roche Monitor v. 1.0 assay. When compared to the Roche Monitor v. 1.5 assay, 94.3% were positive in both assays and 3.2% were positive in the LCx assay and negative in the Roche Monitor v. 1.5 assay. All of the subtype O specimens (n=6) were positive in the LCx assay and negative in the Roche Monitor v. 1.5 assay.

Conclusion: The LCx HIV RNA Quantitative Assay is a highly sensitive assay with the ability to quantitate different HIV subtypes and is suitable for use in monitoring viral load in patients undergoing antiretroviral therapy.

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Virologic effect of immune stimulation with anti-CD3 and rhIL2 in HIV-infected patients on potent antiretroviral therapy

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Background. Multi-drug antiretroviral therapy results in HIV-RNA plasma concentrations to levels below the detection limit of ultrasensitive assays. Nevertheless, a reservoir of infected resting memory CD4+ T cells still exists. Immune stimulation of these cells under simultaneous antiretroviral treatment may result in activation, particle production and subsequent cell death, whilst infection of noninfected target CD4+ T cells is prevented by the antiretroviral drugs. The virologic effect of short course treatment with OKT3 and rhIL2 under combination treatment with five antiretroviral drugs was studied.

Methods. Three patients in whom HIV-RNA levels were suppressed to <5 copies/mL plasma following antiretroviral treatment with at least five drugs were treated with a combination of OKT3 and rhIL2. Plasma HIV-RNA levels, HIV-RNA and total and circular HIV DNA in PBMC and LNMC were measured.

Results. The percentage CD38+ CD4+ T cells in peripheral blood increased 100% and the number of Ki67+ cells in lymph nodes ten-fold. Plasma HIV-RNA peaked to 1500 copies/mL in one patient. Levels remained <5 copies/mL in the other two, but increased in the lymph nodes. Total HIV-DNA per PBMC did not change much and followed the disappearance and reappearance of CD4+ T cells. 1-LTR circular DNA levels in both PBMC and LNMC were lower after OKT3/IL2 treatment. CD4+ T cell numbers did not reach baseline levels up to 90 days after OKT3/IL2 treatment.

Conclusion. Treatment with OKT3/IL2 resulted in activation and proliferation of CD4+ T cells and subsequent cell death and provoked HIV-production. Simultaneous antiretroviral treatment resulted in decreased absolute numbers of HIV-DNA positive

CD4+ T cells in parallel with the total number of CD4+ T cells and decreased 1-LTR circular DNA in both PBMC and LNMC indicating reduction of the reservoir of infected cells.

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Accurate serodiagnosis of parvovirus B19 infections by 2nd-generation epitope type specificity EIA

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Background. We have recently detected in the B19 VP2 molecule an immunodominant heptapeptide epitope, against which IgG antibodies occur exclusively in acutely infected patients. Using this acute-phase specific epitope (KYVTGIN) we have set up a 2nd-generation epitope type specificity (ETS) EIA, which is based on the comparison of serum IgG binding to KYVTGIN vs. IgG binding to native VP2 capsids exhibiting conformational VP2 epitopes.

Objective. To assess the diagnostic performance (clinical sensitivity and specificity) of the 2nd-generation ETS-EIA.

Design. The study, representing diagnosis in "field conditions", included 444 serum samples from British, Danish and Finnish subjects with or without B19-related symptoms. The sera were studied for the time point (diagnosis) of B19 primary infection with the new 2nd-generation ETS-EIA and comparatively with various high-quality commercial and in-house reference IgM and IgG-based methods including the 1st-generation ETS-EIA.

Results. Among 284 sera with B19-IgG, 64 were from patients with B19 acute infection and 220 were drawn many years after B19 infection; 160 samples were devoid of B19 antibodies. In general, many samples showed atypical results and were unclassifiable with the conventional tests, suggesting immunological heterogeneity of infectious agents classified as human parvovirus B19. Among B19-seropositive subjects, the 2nd-generation ETS-EIA (performed alone) had a diagnostic specificity of 95% and a diagnostic sensitivity of 97%. If seronegative subjects were included, the diagnostic specificity of the new ETS-EIA rose to 97%.

Conclusion. The 2nd-generation ETS-EIA, telling both the immune status and the time of infection, is highly sensitive and specific. A combinatorial approach with conventional IgM and IgG-based (avidity or ETS) assays considerably increases the accuracy of B19 serodiagnosis.

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TT-virus prevalence in Finland; expression and characterisation of TTV proteins

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TT virus, found in 1997, is a nonenveloped small virus containing a genome of single-stranded DNA. This genome appears to be circular, thereby closely resembling that of circoviruses. TTV is globally prevalent, but has not been unequivocally associated with any particular disease.

We have studied serum samples by TTV-specific nested-PCR. The sera originated from 81 nonsymptomatic adults and three hepatitis patients. Among these subjects 74 were from Finland and 10 originated from other countries.

Of the three hepatitis patients one was positive for TTV DNA, whereas among the healthy adults TTV-DNA prevalence was 10%. Among the positive individuals 50% were of foreign origin. The obtained TTV amplicons were further sequenced and compared to the published sequences. In a phylogenetic tree these TTV sequences belonged to different subgroups.

We are also cloning and expressing the major open reading frames (ORFs) of TTV as immunological reagents.

Conclusions. We have found that the TT virus is prevalent in Finland. These viruses belong to published TTV subgroups. Data from protein expression and immunoblot experiments will be presented.

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Surveillance of respiratory virus circulation in Latvia (1996–1999)

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Nasopharyngeal swabs taken at an early stage of ILI are most appropriate specimens for virus detection. These materials are simultaneously processed for direct immunofluorescence assay (IFA) of influenza A and B, parainfluenza I, II, III, adenoviruses and RSV, and for virus isolation in tissue cultures. The last three influenza activity seasons in this country differed considerably in their beginning, duration and virological characteristics, different were also seasonal increases in other respiratory virus circulation. During the period of influenza virus activity there was a decrease in the activity of other respiratory viruses. Between these three respiratory virus activity seasons, the season of 1997–1998 was very special – with a very slow beginning, a very late (March–April) influenza virus peak of activity and with the change of the predominant influenza virus strain from H3N2/ Johannesburg33/94 to H3N2/ Sydney/5/97. Between 1998–1999 was noteworthy with a high parainfluenza III virus activity and a

corresponding ARD level in the population. The full spectrum of the respiratory virus circulation in Latvia will be presented. In spite of the epidemic potential of influenza viruses with their ability to cause a high incidence rate, the other respiratory viruses, such as respiratory syncytial virus (RSV) and parainfluenza viruses, as well as adenoviruses do cause widespread morbidity, particulary in certain groups of population and may be responsible for an overall burden of diseases that is comparable to that caused by influenza viruses.

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Are HHV-8 infections associated with HBV chronic infection?

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Background. Several studies consistently linked HBsAg carriers to a predisposition for infections with other microbial agents. This study analyses an assumption of a possible association between HHV-8 and HBV markers of infection.

Objectives. a) To estimate the prevalence rate of anti-HHV-8 (anti-LANA) among HBsAg chronic carriers. b) To study the association between HHV-8 and HBV markers of infection.

Methods. A historic cohort of 1648 blood donors detected as HBsAg positive between 1971–1975 were invited in 1992–1995 to attend a follow-up clinic for an interview, physical examination, and for serological re-testing for HBV markers of infection. 1074 (65%) out of the 1648 historic blood donors remained HBsAg+, 376 (23%) were found to have anti-HBs and anti-HBc, and 198 (12%) were negative for HBV markers of infection (by Abbott EIA). In 1998 all the sera were also tested for antibody response to HHV 8 using an indirect immunofluorescence assay (IFA) for the latent ORF-73 antigen (anti-LANA).

Results. Among the 1074 HBsAg chronic carriers, 22% (n=240) were also positive for HHV-8. Amid 376 subjects with anti HBV antibodies (anti-HBs and anti-HBc) 24% (n=89) were also seropositive for HHV-8, compared with only 14% (n=27) in the 198 blood donors with unconfirmed evidence of a former HBV infection. HHV-8 seroprevalence rates increased with age among HBV chronic carriers (p_v=0.019), were higher in males (23% vs. 13%), and in subjects of North American and Middle Eastern origin. HHV-8 prevalence rates were negatively associated with years of schooling among the HBsAg positives (OR=1.48{1.04; 2.1}). HHV-8 seroprevalence rates were not significantly associated with a history of blood transfusion, I.V. drug abuse and previous surgical procedures. The multiple logistic regression analysis found the independent variables: gender, origin of birth and education level to be significantly associated with HHV-8 positivity.

Conclusions. a) HHV-8 seroprevalence rate was associated with HBV chronic infection. b) HHV-8 seroprevalence rates are directly related to age. c) HHV-8

seroprevalence rates were significantly associated with: gender (males), place of birth (North Africa and Middle East) and with low level of education. d) This study does not support the assumption of common modes of transmission for HHV-8 and HBV.

P. E. KLAPPER, J. C. KLAPPER, A. S. BAILEY, P. TILSTON

Zen and the art of getting clinicians to understand and properly utilise viral load test results

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Conflict between laboratory staff and clinicians is commonplace yet much conflict might be avoided if laboratory staff would remember a few simple aphorisms:

(i) Medicine is an *Art*

(ii) Most artists do not understand *Science*

(iii) Artists understand pictures

We describe the development of an automated system for the production of individual patient cumulative viral load test results with graphical (pictorial!) representation of test results.

The introduction of this report format for our clinicians appears to have dramatically reduced inappropriate testing; improved the efficiency of selection of an appropriate antiviral initiation therapy; improved the speed of detection of emergence of antiviral drug resistance; allowed improved laboratory-clinician information interchange; and allowed clinicians to better manage and inform their patients with regard to the progress of their disease and the effects of antiviral therapy.

'And what is good, Phaedrus,
And what is not good –
*Need we ask anyone to tell us these things?'**

*'Pirsig S M (1974) 'Zen and the art of motorcycle maintenance', Bodley Head, Oxford.

M. V. KOKARAVA, T. V. SAVITSKAIA, V. A. TOKAREV

Features of current of a water flash enterovirus infection in one of the regions of the republic of Belarus

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The flash meningitis serosa was fixed by epidemiology service of the Republic of Belarus in the period of summer autumn 1997. Forty-five percent of the patients were registered among the children of preschool and school age. The analysis of the assembled material has allowed to exclude the bacterial factor in etiology of the flash. The conducted

complex of virological researches has shown that enteroviruses Coxsackie type B were released from water of external and internal sources, and also from the patients with symptoms of meningitis and colitic syndrome. A lot of positive find of viruses were marked in water from underground sources.

There was advanced a hypothesis about accumulation of viruses in base adjourment in internal lakes. Evidently, the virus was saved a long time in an external medium before hitting in a human organism The flash was continued for a long period. The diseases were observed also in winter period. We are of the opinion that low temperature of water of underground sources plays the significant role in preservation Enteroviruses at any time of the year. The degree of hitting of a virus from base adjourment depends on the volume of water consumption and the level of population's diseases.

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HTLV-I tax-"only positives" among blood donors and patients with hematological malignancies

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The presence of HTLV-I was tested using nested PCR with primer set targeted to tax gene. Positive samples were tested repeatedly using primers complementary to pol, env, gag genes and pX region of HTLV-I.

The presence of HTLV-I proviral sequences was detected in DNA from peripheral blood cells of 7 Latvian donors (4.6%) and 12 Swedish donors (8.0%). All positive cases were tax-"only positive", the presence of other HTLV-I genes sequences was not detected. Three samples were positive using primers complementary to larger part of HTLV-I pX region. All donors under investigation were seronegative using "Murex" test systems.

The presence of tax gene sequences was detected in 9 from 170 DNA samples from hematological patients (5.3%). No other genome regions sequences were found, therefore we consider these cases to be tax-"only positive". Diagnosis in these 9 positive cases are different.

No typical HTLV-I infection was found, but 6.3% of blood donors and 5.3% of hematological patients were found tax-"only positive". The role of tax-"only positivity" in normal and pathological cell processes is unclear so far. Our data show that tax-positivity is not associated with changes in T-cell proliferation activity, because no increased sIL-2 receptor level in plasma of all HTLV-I tax-positive cases were found. Since clinical diagnosis of positive patients are different, it is impossible to associate the tax-positivity with etiology of any concrete hematological malignancy, but we cannot exclude its possible role in severity and manifestations of pathological process.

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HHV-8 in peripheral blood of patients with multiple myeloma

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Multiple myeloma accounts for about 1% of all types of cancer and slightly more than 10% of hematological malignancies. The cause of multiple myeloma is unknown. Previously it was found that HHV-8 encode an IL-6 homologue that was capable of stimulating growth of a human myeloma cell line (Burger et al., 1998). We have studied the presence of HHV-8 in peripheral blood cells' DNA of patients with multiple myeloma by using PCR with HHV-8 specific primers (Chang et al., 1994).

Our preliminary studies showed that after 30 amplification cycles 2 of 14 patients with multiple myeloma were positive for HHV-8 DNA, however, when the number of cycles was increased up to 60 HHV-8 sequences were detected in 6 from 14 individuals. At the same time none of 17 patients with other hematological diseases as well as 150 healthy blood donors did harbour HHV-8 sequences. The results indicate that only 2 of myeloma patients (14.2%) harbour at least 5 copies of HHV-8 per 1.5×10^5 peripheral blood cells that correspond to the sensitivity of the method for 35 cycles. In other 4 positive cases (28.5%) the viral load in peripheral blood cells is lower (less than 5 copies of the viral genome per 1.5×10^5 cells) since HHV-8 sequences were detected only when the PCR sensitivity was increased (by increasing the number of amplification cycles to 60). Although peripheral blood cells from myeloma patients demonstrated amplified PCR product by using specific primers, our studies suggested the low viral load in multiple myeloma patients' peripheral blood cells. Enrichment of the cells for markers CD68 or CD83 or usage of bone marrow stromal cells are necessary for the final conclusion on association of HHV-8 with multiple myeloma.

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Frequency of specific markers of viral hepatitis G in patients and blood donors in the Czech Republic. Viraemia index and risk factors

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Objective. We attempted to elucidate the clinical and epidemiological role of the recently identified hepatitis G Virus (HGV) that remained controversial. Two HGV markers, i.e. HGV-RNA and anti-HGV, were investigated in 500 selected patients and blood donors with normal and elevated transaminase (ALT) levels. The viraemia frequency, HGV RNA positivity (GV) and positivity to antibodies against HGV (GAb)

are given in the table below. The viraemia index (VI), i.e. the GV to GAb ratio, may be indicative of etiological relatedness to viral hepatitis G (VHG). The total of GV + GAb positivity is suggestive of contact with VHG and may represent a transmission risk factor.

Methods. RT-PCR and DIG-ELISA were selected for HGV RNA and anti-HGV E2 antibodies detection, respectively. The Boehringer Mannheim kits (HGV 2nd gen. wit dU-DIG labeling and Anti-HGenvE2, respectively) were used for this purpose.

Results.

	No. (=100%)	GV, %	Gab, %	Both (No.) GV+GAb	Total (%) GV+GAb	VI
1. Blood donors, normal ALT	101	6	15	0	21	0.4
2. Blood donors, elevated ALT	118	23	17	0	40	1.3
3. Blood diseases, malignant	32	66	13	1	79	5.0
4. Blood diseases, benign	32	25	44	1	69	0.6
5. Kidney diseases	13	31	23	2	54	1.3
6. Suspected hospital VHG	16	50	38	0	8	1.3
7. Coinfection HGV+HCV	21	24	19	1	43	1.3
8. NonA-E VH	167	36	23	6	59	1.6

Conclusions. Surprisingly high GV (6%) and the total GV+GAb (21%) are in healthy blood donors (1). Elevated VI in ALT positive blood donors (2) compared to (1) and patients with malignant (3) compared to those with benign blood diseases (4), both requiring multiple blood transfusions, are indicative of etiological relatedness to HGV, which may also play the role of a co-factor. The elevated VI in patients with malignant blood diseases seems also due to immunomodulation therapy. Hospital transmission of VHG is suspected in surgical patients with postoperative complications (6). Patients with VH diagnosed as nonA-E (8) also showed an elevated VI. Higher number of both GV+GAb in patients with kidney diseases and nonA-E VH (5, 8) may be due to immunocomplexes.

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Diagnostics of herpesvirus and cytomegalovirus-infection

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Background. Last results show the increase of seropositivity of population of Russia to herpes simplex virus (HSV) and cytomegalovirus (CMV) infections. At the same time we had no own tests for serodiagnostics of HSV and CMV.

Objectives. 1. To develop immunoenzyme test-system for detecting the antibodies against CMV and HSV. 2. To determine the seropositivity to HSV in selected populations of people, which were investigated in diagnostic laboratories in Saint-Petersburg.

Methods. IgG and IgM antibodies to HSV were determined in sera from 318 pregnant women, 172 adults and 75 newborns by developing monoclonal immunoenzyme test system.

Results. 1. In our laboratory immunoenzyme tests for detecting of IgM and IgG against CMV and herpes simplex virus based on natural viral antigens derived from cell cultures and mouse monoclonal antibodies against human IgM and IgG were developed. These test-systems have high sensitivity (97%) and specificity (96%) in comparison with tests of "Abbott" and "Labsystems" (Finland). 2. 72% of 565 patients with different diagnosis were seropositive for HSV specific IgG, which were seen in 74–80% of adults and 44% of newborns. 13% of patients had high titer of specific IgMs. We found out IgMs to HSV in 22% of sera.

Conclusion. Seropositivity to HSV was high in groups of pregnant women, adults and newborns.

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HHV-6 reactivation is often associated with CMV infection in liver transplant patients

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Human herpesvirus 6 (HHV-6) infection has been recently reported in liver transplant patients. HHV-6 may cause fever and other clinical symptoms, such as neurological disorders and hepatitis. HHV-6 is closely related to cytomegalovirus (CMV) with large genomic overlapping, and some interaction between HHV-6 and CMV has also been suggested. In this study, we have investigated the post-transplant HHV-6 antigenemia in relation to symptomatic CMV infections in adult liver transplant patients.

Patients and methods. Consecutive 75 adult liver allograft recipients transplanted during 1996–98 were included in the study. CMV infections were diagnosed by the pp65-antigenemia test and by vital cultures. HHV-6 infections were demonstrated by the HHV-6 antigenemia test, which detects the virus specific antigens in blood mononuclear cells, and by serology.

Results. Clinically significant symptomatic CMV infection was diagnosed in 42 (56%) out of 75 patients during the first 6 months (median 26 days, range 5–150 days) post-transplantation. Only 8 of these were primary infections and 34 were reactivations. Antirejection treatment preceded CMV infection only in 8 out of 42 cases. The symptoms consisted of fever, graft dysfunction and pneumonia. CMV was also detected in 5 patients from bronchoalveolar lavage and in 15 patients from liver biopsy. All CMV infections were successfully treated with Ganciclovir, and the CMV-antigenemia subsided. Concurrent HHV-6 antigenemia was detected in 21 (50%) out of 42 patients with CMV infection, in 5/8 primary CMV infections and in 16/34 reactivations, respectively. No

crossreactivity between the CMV and HHV-6 detection methods was recorded. All HHV-6 infections were reactivations. HHV-6 also responded to the antiviral treatment, but the effect was less clear and slower than with CMV.

Conclusions. HHV-6 reactivation was often associated with CMV infection in liver transplant patients. The results support the suggestion that CMV and HHV-6 may have interactions. In addition to CMV infection, HHV-6 should also be monitored after liver transplantation.

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Influenza vaccine immunogenicity in the elderly in six consecutive winter seasons (1993–94/1998–99)

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Objective. To investigate the hemagglutination inhibiting (HI) antibody response of elderly people following influenza vaccination because of the common occurrence of impaired immunity that could influence vaccine efficacy.

Methods. HI antibody titers were measured in sera collected before and 30 days after administration of trivalent inactivated influenza vaccines in 1142 elderly volunteers (age ≥65 years) over years 1993–94 to 1998–99.

Results. Comparison of pre- and postvaccination HI titers according to the requirements of the European Commission of influenza vaccine for adults (postvaccination seroprotection rate ≥60%, mean fold increase of geometric mean titer (MFI) ≥2 and positive responses ≥30%) showed a not completely satisfactory response in the first three winter seasons studied (protection rate <60% against A(H1N1) and B antigens in the years 1993–94 and 1994–95 and against A(H3N2) in 1995–96; MFI <2 against A(H1N1) virus in 1993–94 and 1994–95; positive responses <30% against all three antigens in the winter 1993–94 and against A(H1N1) in the 1994–95). The results of the years 1996–97 to 1998–99 satisfied, in general, the requirements of the European Commission against all three vaccine antigens. The presence of prevaccination high-level immunity could justify the finding of MFI and seroconversion rate against A(H3N2) antigen slightly lower than those suggested in the winter 1997–98.

Comments. The data of the years 1993–94 to 1995–96 supported the possibility of a low antibody response to inactivated vaccines in the elderly, especially against A(H1N1) and B antigens. However, these observations were not confirmed in the winters 1996–97/1998–99. These different results could be due to: a) differences in vaccine antigenic composition, although the A(H1N1) component was the same in all the years studied, except for 1998–99; b) improvement in influenza vaccine production; c) presence of higher prevaccination serum level of immunity found in the population in the years 1996–97/1998–99, as compared with the first three winter seasons; d) differences in the health status of volunteers.

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Enterovirus infection and apoptosis markers in patients with various cardiac diseases

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The association between enterovirus (ETV) and cardiac pathologies was investigated in relation with markers of cell apoptosis: 26 cases were included, 11 from China with Keshan disease and 16 from France including 8 subacute myocarditis (SAM), 9 dilated cardiomyopathy (DCM), 7 ischemic cardiomyopathy (ICM), 1 alcoholic cardiopathy (ALC) and 1 myocardium failure (MF). For each case, a part of endomyocardial biopsy was examined for histopathology, detection of ETV infection by RT-PCR assay and VP1 immunostaining, lymphoid cells CD8 and TIA1, and markers of cell apoptosis including TUNEL assay, bax and bcl_2. The detection of ETV RNA and/or VP1 was positive in 7 out of 8 SAM, 9/9 DCM, 2/7 ICM and 1/1 MF samples, both of them being positive in 15 cases. In 6 cases, sequencing data led to the identification of 5 coxsackievirus B (CVB) 3 and 1 CVB-5. The TUNEL assay was positive in 18 samples (6/8 SAM, 7/9 DCM, 4/7 ICM and 1/1 ALC) and was associated to the presence of at least one ETV marker in 14 of them. In experiments testing in parallel VP1 and TUNEL markers, many myocytes were shown to exhibit a mixed coloration, located close to lymphoid cells foci. These results confirm the major involvement of ETV in various cardiac pathologies (without difference between Keshan and other inflammatory diseases) and suggest the occurrence of viral and/or lymphoid-induced apoptosis in the genesis of myocyte death.

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Amniotic fluid culture as a diagnostic aid for CMV infections

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Introduction. Cytomegalovirus is considered the most common cause of congenital infections, being responsible for neonatal mortality, morbidity and late sequelae in infants (1). Maternal infection is often asymptomatic or may present unspecific symptoms for CMV; however, in both cases, transplacental transmission can occur. CMV infection can be diagnosed prenatally by isolation of virus from amniotic fluid.

Aims. To evaluate the relevance of the amniotic fluid culture as an indicator in CMV transmission, as an aid to the diagnosis of CMV infections.

Methodology. Seven pregnant women were studied at the National Institute of Health, to detect transplacental CMV transmission by shell-vial assay for CMV isolation in amniotic fluid. Also IgG and IgM indirect immunofluorescence techniques were

performed in sera samples, when available; IgM positive results were confirmed by an immunoblot technique. Samples of amniotic fluid were inoculated in MRCS5 shell-vials tubes and CMV was detected by immunofluorescence technique.

Results. Of the 7 cases, 2 women had congenital malformation detected by echography, 4 women had not echographic alterations and in 1 case there was fetal death. CMV was isolated from amniotic fluid in 2 cases, both aged 33 years; in 5 cases we had a negative isolation result and the age range was 19–32 years. Five sera were positive for IgG and 2 of them were also positive for IgM, both by immunofluorescence and immunoblot techniques. The 2 positive amniotic fluid cultures correspond to the pregnant women with IgM positive serology.

Comments. Positive amniotic fluid culture is an indicator of congenital infection, but a negative amniotic fluid culture cannot exclude the presence of congenital CMV infection. So, children born from CMV amniotic fluid negative mothers, should be followed for further studies. CMV congenital infection is a major laboratory and medical problem (2) and a decisive evaluation of the best strategy to follow concerning the laboratory diagnostic techniques is needed.

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Cytomegalovirus glycoprotein B group

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Initiated by the assumption that the envelope glycoprotein B (gB) of human cytomegalovirus (HCMV) may have an influence on the outcome of prenatal infection, genotyping of strains from congenital infections was carried out. Eighty pregnant women were included in the study. Forty pregnancies were normal and in the other half of the examined group the fetuses or newborn babies were highly suspected to have viral infection.

The DNA was extracted from samples of amniotic fluid or urine and the gB genotype was determined by RFLP analysis of the fragment between bases 1319–1604 of gB gene amplified by nested PCR.

Of the samples from the patients suspected of having viral infection 12 showed HCMV. The amniotic fluids from 3 of the normal pregnancies had HCMV. All the strains were identified as gB type 1.

The association of gB genotype 1 of HCMV with prenatal infection provides further evidence that the gB variation may indeed influence the tropism and virulence of HCMV.

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Responses of CBV4-specific T cell lines to 2C protein

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Enteroviruses, especially coxsackie B4 virus (CBV4) have been suspected to be possible environmental triggers initiating autoimmune process. Molecular mimicry between CBV4 and glutamic acid decarboxylase 65 (GAD65) has been suggested to be responsible for autoimmune β-cell destruction. One possible homology area is an identical sequence of six aa (PEVKEK) which can be found both within CBV4 2C protein and GAD65.

CBV4-specific T cell lines were established from 11 healthy controls and 8 diabetic subjects. A panel of 33 partially overlapping synthetic peptides of 20 aa length covering the sequence of CBV4 2C protein was used to study the T cell epitopes of these lines. There was a correlation between T cell proliferation and INF-γ production to 2C peptides, even though, some T cell lines produced INF-γ in response to peptides which did not induce proliferation. The found epitopes associated very strongly with HLA-DR alleles of blood donors but the epitopes recognized by diabetic and healthy blood donors were similar. Peptide p4 which included the homology sequence PEVKEK contained a T cell epitope and its presentation was restricted with HLA-DR1 molecule. Three randomly selected T cell lines, which responded to peptide p4, did not recognize GAD65. GAD65 peptides containing homology region did neither stimulate these CBV4-specific T cell lines. In conclusion, this study shows that CBV4 2C protein is strongly immunogenic and the homology region with GAD65 is a T cell epitope but its role in pathogenesis of IDDM seems to be unimportant.

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Norwalk-like caliciviruses in food- and waterborne epidemics of gastroenteritis

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Norwalk-like caliciviruses (NLV) cause gastroenteritis both in children and adults. These nonenveloped viruses with linear ssRNA genome belong to the family *Caliciviridae*. The classification of the viruses has yet to be established, but they seem to form a very heterogeneous group. From the beginning of the year 1998 all suspected foodborne outbreaks were recommended to be reported to authorities in Finland. Stool samples (3–10 per epidemic) from 87 diarrheal epidemics were tested for viruses by electron microscopy and RT-PCR for astroviruses and NLV. Amplicons were confirmed

by plate hybridization with NLV amplicons several probes were used. Nucleotide sequences were determined from selected amplicons by direct sequencing. Between November 1997 and February 1999, NLV was found in 56 out of 87 (64%) epidemics. Most epidemics were suspected as foodborne, but also several waterborne, hospital and other outbreaks were included. The NLV epidemics occurred throughout the year, most in the winter and spring time. The NLV represented both genogroups I and II, the latter being more common. Also in these genogroups many different strains were found based on nucleotide sequences of the polymerase region. Frozen berries were suspected in 11 outbreaks as the virus containing food. Also the virus strains associated with these epidemics showed as much heterogeneity as the strains from all outbreaks. Based on these results Norwalk-like caliciviruses seem to be the most common viruses causing foodborne gastroenteritis outbreaks.

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Involvement of a p53-dependent pathway in rubella virus-induced apoptosis

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Objective. In light of the important role of apoptotic cell death in the pathogenesis of several viral infections, we asked whether the cytopathogenicity evoked by rubella virus (RV) might also involve apoptotic mechanisms.

Methods. In comparison with mock-infected cultures, Vero cells infected with the To-336 strain of RV were analyzed for the hallmarks of apoptosis by different methods, including TdT-mediated digoxigenin-dUTP end labeling (TUNEL) assay, DNA fragmentation assay, double-parameter cytofluorometric DNA analysis and ELISA. The steady-state levels of p53 and p21 proteins were examined by Western blot analysis. p53 and p21 mRNA levels were determined by a multi-probe RNase protection assay.

Results. The To-336 strain of RV induced apoptosis in Vero and RK-13 cells, but not in fibroblast cell lines. UV-inactivated RV virions did not elicit the apoptotic response, indicating that productive infection is required for the induction of cell death. Both p53 and p21 protein levels were highly elevated in RV-infected Vero cells. The level of p21 mRNA was increased, while expression of p53 gene was unaffected by RV infection. A dominant-negative p53 mutant ($p53^{W248}$) conferred partial protection from RV-induced apoptosis.

Conclusion. These data implicate a p53-dependent apoptotic pathway in the cytopathogenicity of RV, thereby suggesting a mechanism by which RV exerts its teratogenic effects.

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HIV-1 subtypes in Hungary

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The human immunodeficiency virus type 1 (HIV-1) genome is rapidly undergoing variations. Genetic variation is most pronounced in the viral envelope genet. Based on phylogenetic analysis of the envelope gene, HIV-1 strains can be separated into genetic subtypes A through J, which are designated as the M (major) group of HIV-1. Additional highly divergent variants have been found and these are termed group O (outlying) viruses. Investigation of the distribution of HIV-1 subtypes circulating geographically is important for understanding the epidemiology of HIV-1. HIV-1 subtype B is geographically the most widely distributed and is associated with AIDS epidemics in America, Europe and Asia.

HIV-1 genetic subtypes were determined in 20 adults from Hungary. Peripheral blood mononuclear cells (PBMCs) of HIV seropositive individuals and AIDS patients of the St. László Hospital, Budapest were cocultivated with PBMCs of HIV seronegative blood donors and PCR amplified *env* regions of HIV-1 proviral DNAs were analysed by heteroduplex mobility assay (HMA) and genomic sequencing. HMA results revealed that HIV-1 gp120 sequences from the big majority of patients were of subtype B. DNA sequencing confirmed the HMA results.

This study shows that subtype B is the predominant HIV-1 clade at present in Hungary.

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Bell's palsy – an unusual consequence of human parvovirus B19 infection

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Bell's palsy had developed in the case of a young woman 7 weeks after an acute exanthematous infection. The course involved the V, VII, and VIII cranial nerves of the right side. The patient had recovered completely within one month.

Microbiological tests excluded the etiology of *Borrelia burgdorferi* and several viral agents, such as rubella-, varicella-, zoster- and adenoviruses. IgM class antibody to Human Parvovirus B19 (HPV B19) has been detected by commercial microelisa test in the first serum and HPV B19 DNA amplifications were successful by PCR technique both

in the sera and cerebrospinal fluid. Our results suggest a possible causative role of the latter virus. No association of HPV B 19 infection with Bell's palsy have been reported until now.

I. MIHÁLY, A. LUKÁCS, E. RÉDEI, L. TELEGDY, E. IBRÁNYI, L. RÓKUSZ

Percutaneous exposure and viral infections in hospital personnel involved directly in health care

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Objectives. To determine the occupational risk of viral infections in health care.

Methods. Blood samples of 42 hospital workers (HWs) who suffered percutaneous exposure were examined for HIV, hepatitis A, B, C, E viruses (HAV, HBV, HCV, HEV), Cytomegalovirus (CMV), Epstein-Barr Virus (EBV).

Results: Ten of the HWs (24 twenty years old or younger. The source of exposure was HBV positive blood in thirteen, HIV positive blood in seven, HCV positive blood in five, acute hepatitis A in one, acute CMV infection in one and unknown in fifteen cases. Nobody has got occupational HIV infection. Six of eleven HWs (54%) seroconverted to HBV. One nurse seroconverted to HCV while she suffered acute hepatitis and she became chronic hepatitis within a year.

Another nurse suffered acute E hepatitis 3 months after a needlestick accident dealing with a patient with acute hepatitis of unknown origin. Two other nurses were temporarily HCV-RNA positive after exposure of HCV positive blood but did not seroconvert.

Conclusions. 1. The occupational risk of HIV infection among HWs is lower than of HBV or HCV infection. 2. The occupational HBV risk is a permanent factor despite the immunization among HWs. 3. The occupational risk of the HCV infection is as high as that of HBV infection, but due to the lack of the vaccine the clinical consequences are more serious than those of the HBV infection. 4. Hepatitis E infection can be contracted through blood exposure.

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Perinatal transmission of hepatitis C virus

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A total of 80 blood samples of 24 children aged from one day to 15 years old (m/f=9/15) and of 12 mothers were examined for hepatitis C virus (HCV) by 3rd generation HCV antibody EIA, different commercial Western Blot Assay, PCR (Amplicor and Monitor, Roche). The HCV genotype was determined by Innolipa (Innogenetics). Ten mothers were PCR positive in titer 7,000–600,000 genomcopies/ml, most of the maternal

HCV genome belonged to 1b genotype. The childrens' sera showed a very divergent test result pattern: Four children were HCV antibody (ab) and PCR positive under one year of age (17%) and afterwards three of them became negative. One girl with hepatomegaly and permanent abdominal complaint had a transient HCV ab and PCR positivity, her mother was seronegative to HCV. Altogether 15 children were ab and PCR negative above one year old age (62%). Two children older than one year were only HCV PCR positive without HCV ab (8%). One girl was ab and PCR negative in young infant age and later she became ab and PCR positive. Altogether 5 children had HCV genome and ab in their blood permanently by childhood (21%). Four of the children had symptoms of various liver injury (17%), two of them were treated with interferon alfa because of chronic C hepatitis without any response (8%). One boy has got chronic C hepatitis by transfusion at birth (4%).

Conclusion. 1. The negative HCV serostatus does not exclude the past HCV infection. 2. The children with risk of perinatal HCV infection must be monitorized regularly both for HCV antibody and by PCR. 3. The maintenance of clinical and virological follow-up of the children with risk of perinatal HCV infection is extremely important till the adult age. It can give opportunity for a deeper and more accurate understanding of the nature and consequences of HCV infection at early infant age, and the therapeutic effort for chronic hepatitis cases can be started as soon as possible.

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Evaluation of MABS specific to influenza A virus as probes for immunocytochemical staining

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The reactivity of eight influenza type A-specific monoclonal antibodies was tested in a rapid culture assay (RCA) by immunoperoxidase staining and correlated with their affinities with the aim to select antibodies suitable for influenza A typing in diagnostics. Seven MAbs were specific to influenza A nucleoprotein and one to membrane protein (290).

The affinities of MAbs were evaluated by a competitive radioimmunoassay. RCA was done on MDCK cell monolayer infected with influenza A virus, fixed with methanol and stained with each MAb and Swam-Px. We monitored cytoplasmic staining in infected cells.

According to the decreasing reactivity in RCA the MAbs could be divided into 4 groups: 1. MAbs with the best detection capabilities and the highest affinities: 1L3, IIB1 and 107L (5.6109, 4.9109, 1.4109 M-1); 2. 2L3 (1.3109); 3. 44 and IIH3 (7.8108, 6.0108); 4. IVE8, which was the worst in antigen detection (1.3109M-1). The reactivities of all MAbs in RCA correlated with their affinities except IVE8, which despite its relative high affinity was a rather weak detector. MAb 290 was evaluated separately because of its

different antigen specificity. Its reactivity could be compared to 2L3, though its affinity was the highest of all tested antibodies (1.51010 l/mol). From these results it follows that the MAbs of the first group seemed to be the best probes for typing in RCA.

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HFRS virus outbreaks in Wakayama Medical College Laboratory during 1976 to 1978

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Hemorrhagic fever with renal syndrome (HFRS) virus is carried by chronically infected rodents and causes serious human disease with fever, hemorrhage, and renal failure. The laboratory epidemics occurred in our college laboratory during 1976 to 1978. Serum samples collected from rats, mice, and laboratory workers were examined for the presence of antibodies to HFRS virus by immunofluorescence and immunoblotting tests. Percentages of antibody positive samples from rats, mice, and laboratory workers were 83.3% (368/442), 8.13% (921/1132), and 15.9% (17/107), respectively. Out of 17 antibody-positive workers, sixteen had contacted with rats or mice; only one worker was antibody positive without contacting. The epidemics among rats occurred at much higher incidence than mice. In addition, the titers of antibody positive rat sera were much higher than those of antibody-positive mouse sera. These results suggested that HFRS virus infected the laboratory workers mainly by contact with infected rats. Mice may also be infected with the virus from rats by accident without contacting. By immunoblotting test, antibody-positive sera from rats revealed an intense 48-Kd protein band from WKM (our isolate) and 76–118 (prototype) strains. This protein is most likely the nucleoprotein. In contrast, sera from mice recognized a 56-Kd band that is likely G2 antigen. The convalescent serum from a HFRS patient showed similar reaction to those of rats, but the serum from an asymptomatic worker showed similar to those of mice. This difference of antibody reactions to HFRS virus antigens could not readily be explained, but may be due to either sensitivity of host or amount of infecting virus. Western blotting test described here can be used for judging prognosis of persons with possible HFRS virus infection.

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Aciclovir-resistant (ACV-R) herpes viruses (HSV, VZV) in bone marrow transplant patients

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A two years prospective survey (1996–1997) of Herpes virus infections (HSV-VZV) has been conducted in 222 bone marrow graft recipients (60 children and 162 adults) who received a prophylactic ACV IV treatment. In 80% of these patients virological investigation has been performed including virus isolation and identification in MRC5 and Vero cells, measure of ACV IC 50 according to Langlois (HSV) and Berkowitz (VZV). The prevalence of HSV infection was 14% in adults and 8% in children, all due to HSV1. The prevalence of ACV-R was 9% of patients. The prevalence of VZV infection was lower (globally: 0.45%) but the ACV-R was also 9%. All ACV-R have been tested with Foscarnet, as were the ACV-R strains previously isolated from 1993 to 1996. Altogether 8 patients (3 adults and 5 children) excreted ACV-R HSV1 strains, of them 7 ACV-S and 13 ACV-R isolates have been genetically analysed (PCR + sequencing of the TK gene). We observed: 1. TK substitutions in ACV-S strains not linked with resistance. 2. TK substitutions in ACV-R strains, most probably linked to resistance, at positions 51, 83, 104, 105, 175 resulting in 1 amino acid change. 3. TK insertion/deletion frameshift mutations (linked to resistance) at positions 92 and 146. From the child excreting ACV-R VZV strains, 2 isolates have been checked for mutations: the first showed a substitution at position 427 (A→G) and the second one at position 253 (T→C) resulting in amino acid changes. Between factors favouring the emergence of R strains, we noticed ACV treatment, route of administration, delay after graft. All patients recovered after either increasing doses of ACV or change for Foscarnet. Recurrent infections could be due to ACV-R strains.

Conclusion. ACV has a beneficial preventive effect on the HSV and VZV infections in deeply immuno-suppressed patients, like B.M transplant. A virological systematic follow-up of Herpes virus excretion is needed to detect ACV-R strains and reconsider the antiviral treatment. More informations concerning the mutations responsible for antiviral R are needed before simple, quick and cheap genetic tests for detecting R strains could be established and validated.

K. NAGY, B. KEMÉNY, M. MARSCHALKÓ, A. HORVÁTH

Detection of HTLV-1 DNA in patients with eosinophilia

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Exogenous infection by the human T-cell leukemia virus (HTLV) induces eosinophilia with various forms of immunological disorders. Human genome contains C-

type related human endogenous retroviral elements with sequence homology to HTLV gag, pol and tax genes. We investigated the possibility of HTLV infection in our patients with dermatoses and eosinophilia (Eo) in Hungary, where exogenous HTLV infection has not yet been reported. DNA samples of skin and lymphocytes (ly) from 50 patients were analysed by PCR amplifying 159 bp of 3' *tax* and a 210 bp (13323-1442) of *gag* and cloned in *E. coli* XLI-Blue.

DNA analysis identified retroviral elements homologous (>95%) to *gag* in 70% of patients with lymphoreticular proliferations including Kimura disease, and in 60% of patients with bullous dermatoses with Eo. The majority of patients' ly were CD4+CD8. Blastogenic response to PHA, ConA and PWM were lowered. Elevated GM-CSF was measured in cultured ly stimulated by IL-2 of 40% of patients, while IL-5 could be detected in sera of 50% of patients with Eo. Neither of the patients had serum antibodies to HTLV-1/2 or HIV-1/2. We hypothesized that besides eosinophilic chemotactic factors, restricted expression of HTLV-related retroviral sequences contribute to the induction of eosinophilia in our patients.

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Human cytomegalovirus glycoprotein B genotypes in newborns under intensive care

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Genetic variation in glycoprotein B (gB) may play a role in human cytomegalovirus (HCMV) tropism and pathogenesis.

The gB genotype of HCMV strains derived from newborns with suspected virus infection under intensive care were determined.

The DNA from urine samples was extracted and the gB genotype was identified using nested PCR-based restriction fragment analysis.

In the samples of urine from 10 of 70 newborns gB genotype 1 strains were found. One of the newborns carried gB type 2 strain.

The association and prevalence of gB type 1 strains of HCMV with congenital infection suggests its role in the pathogenesis of prenatal infection. However, it seems that other virulence factors have also to be considered, since the outcome of these congenital HCMV infections were different.

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IL-12 production by PBMC in hepatitis C virus (HCV) infection: influence of genotype

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HCV infection develops frequently towards a chronic disease. The imbalance of T-Helper (Th) lymphocyte cytokine production is likely one of the mechanisms of the viral persistence.

To investigate whether such an inadequate immune response is present in HCV infected patients, we evaluated the *in vitro* production of IL-12 by PBMC isolated from a cohort of 36 patients prior to IFN-α/Ribavirin treatment, 50% of them being infected with genotype 1a or 1b and the others with genotype 2a/2c, 3a or 4c/4d, and 38 healthy donors.

The amount of cytokine released in the supernatant of PBMC after 72 hours of culture was determined by a specific ELISA (Total IL-12 p40&p70, Endogen).

Low levels of spontaneous production of IL-12 were detected in cell cultures from 17/36 patients (mean: 6.12 pg/ml) and 17/38 of control persons (4.3). Following LPS stimulation, IL-12 production was higher in patients (368.8±147.7) than in controls (347.68±109). Interestingly, PBMC from patients infected with HCV 1a/1b released lower amounts of IL-12 (335.8±152.1) than PBMC from patients infected with other HCV genotypes (401.8±202.5). There was no correlation between the viral load and the amount of cytokine produced.

These data indicate that the ability to secrete IL-12 is reduced in PBMC from patients infected with HCV 1a/1b. These results suggest that the genotype might influence the severity and outcome of the disease.

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The quality of vaccine-induced rubella immunity

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To evaluate the effectiveness of rubella vaccine we have characterized the specificity and avidity of the humoral immune response after rubella immunization.

Serum samples of 18 vaccinees (MORUPAR, RA 27/3), aged 12–24 months, obtained after 2 weeks and 1 and 24 months, and sera of 62 vaccinees, aged 9–14 years, obtained after 1, 2, 6 and 12 months, were studied for anti-RV IgM, IgG, HI antibodies and anti-RV avidity. In some sera, the specificity and avidity of IgG antibodies for individual RV proteins (E1, E2 and C) were further examined with immunoblot assays with recombinant RV proteins as antigens.

Primary immune responses detected in 61 vaccinees were characterized with IgM responses and low avidity IgG antibodies, appearing within 4 weeks. Immune response to E1 protein was dominant. Prolonged maturation of IgG response specific to whole virus, paralleled with maturation of anti-E1 antibodies. Two persons failed to respond to RV vaccination and a few developed transient response to E1 protein after natural as well as after vaccinal RV infection. Children naturally immune before vaccination had a higher mean-titer both before and after vaccination than the children seronegative before vaccination.

This results show that determination of specificity and avidity of IgG antibodies to individual RV proteins contributes to a better assessment of vaccine induced immunity.

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Comparison of six commercial IgG-ELISA kits for the detection of TBEV-antibodies

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Six commercial ELISA-Kits for the detection of tick-borne encephalitis virus (TBEV) specific antibodies are compared, using serum panels (n=139) of subjects with known and documented clinical history (109 sera from TBEV infected patients, 30 from people vaccinated against TBEV). For analysis of the cross-reactivity 24 sera from yellow fever vaccinated people and 13 sera positive for Dengue virus were also included. All sera were analysed for TBE specific antibodies by immunoblot.

The tests show marked differences in sensitivity and specificity, correctly detecting 75% and 98% of TBEV positive sera. Regarding the cross-reactivity, the situation is even worse: YF sera were correctly detected at a range between 4% and 96%, Dengue virus positive sera at a range between 0% and 54%. "Critical" sera showing different results in more than three commercial test kits were sent to the manufacturers for further analysis.

The results of this study show the need for further improvement of the existing TBE test systems.

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The development of a high-throughput real-time quantitative assay for the detection of Epstein-Barr virus DNA

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With the recent introduction of real time PCR analysis or TaqMan technology, we were able to develop and evaluate a rapid and very reproducible method for the detection

of EBV viral DNA in plasma samples. This method enables us to screen in a quantitative manner, over a range between 100 and 10 million copies per ml a great number of patients for this virus, using two sample preparation methods based on silica absorption. The limit of detection was determined using a half log serial dilution of an EM quantified standard. Both methods were able to detect 100 EBV DNA copies per ml of plasma. A precision study yielded an average coefficient of variation for both isolation methods of less than 12%, with a coefficient of regression for the standard curve of minimal 0.98.

In order to estimate the EBV viral load in a number of patient groups and healthy individuals, we measured with this assay EBV DNA in a cohort of 150 asymptomatic immunosuppressed kidney transplant patients, 24 healthy donors, 10 patients with diagnosis of post-transplant lymphoproliferative disorder or PTLD. In all the healthy immunocompetent individuals, we were unable to detect any viral EBV DNA. In the cohort of kidney transplant patients, we could detect in 14 out of 150 patients EBV DNA with a mean of 370 copies per ml. In the group diagnosed with PTLD, the mean EBV viral load was 125.000 copies per ml, which is significant higher than in the previous groups ($p<0.0001$).

Our studies indicate that this technique can be used to rapidly screen patients for EBV replication. In a transplant setting, simultaneous quantitative screening for different viruses is possible.

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Comparison of cytokine induction by HHV-6 and HHV-7

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Acute and chronic viral infections can alter normal cytokine profile, which can contribute to viral pathogenesis. Here we report a comparative analysis of human herpesviruses 6 and 7 (HHV-6, -7; causative agents of exanthematous skin disorders and severe immunosuppression upon reactivation) on cytokine synthesis. We quantitated the production of key cytokines in infected PBMC and permanent lymphoid cultures by ELISA and bioassays. HHV-6A (GS) induced no IL-2 in PBMC and HSB-2 cultures, diminished cell proliferation and IL-2 production induced by PHA, PMA, OKT-3 MAb in both PBMC and Jurkat cultures. It elicited IL-1β, TNF-α, but not-IL-6 production by PBMC. In HSB-2 cultures, it induced TNF-β, but suppressed IFN-γ release induced by bacterial LPS. Heat- and UV-inactivated viruses had similar, but reduced effects. HHV-7 (KHR), live or inactivated preparations, in combinations with PHA, LPS, OKT-3 MAb augmented IL-2 and IFN-γ production in primary, but diminished in secondary infection. HHV-7 induced parallel TNF-α and IL-1β production, and its combination with mitogens favoured IL-1β release. Production of IL-4 and IL-6 was not affected, and that of TGF-β was augmented by HHV-7. HHV-7 also induced IL-10, which is known to inhibit helper T cells and play a role in skin disorders. Maximal release of each cytokine occurred at different time points after HHV-6A or HHV-7 infections; the first peaks were seen at 12

to 48h. Alterations of the cytokine balance by HHV-6A were more profound corresponding to severe clinical consequences, while the effect of HHV-7 was mild which has a known tendency of spontaneous recovery.

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Parvovirus B19 infection: evaluation of the laboratory of virology casuistic: 1995–1997

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Introduction. Human Parvovirus B19 is the aetiological agent of erythema infectiosum ("fifth disease"), transient aplastic crisis, chronic anaemia, arthritis, hydrops fetalis and fetal death. B19 infection can occur in any age group but diseases are age related.

Aims. Retrospective evaluation of the cases of possible B19 infection received for laboratory diagnosis at NIH during 1995–1997.

Material and methods. Between 1995 and 1997, a total of 1061 cases were received at NIH for laboratory diagnosis of Parvovirus B19 infection. An IgG ELISA and an IgM IFA were performed in all sera sample. The borderline IgG and the positive IgM results were confirmed by immunoblot.

Results. The distribution of the 1061 cases according the serological results and age were as follows: 453 cases (42.7%) were IgG and IgM negative, with age<1 year: 39 cases (8.6%), age≤10 years: 145 (32%), age=11–18 years: 31 (6.8%), age=19–35 years: 120 (26.5%), age>36 years: 89 (19.7%) and 29 (6.4%) unknown. 557 cases (52.4%) were IgG positive and IgM negative, with age<1 year: 45 cases (8.1%), age≤10 years: 54 (9.7%), age=11–18 years: 57 (10.3%), age=19–35 years: 167 (30%), age>36 years: 172 (30.9%) and 61 (11%) unknown. 3 cases (0.3%) were IgG negative and IgM positive were aged 5 months, 12 years and 42 years old. 49 cases (4.6%) were IgG and IgM positive, with age≤10 years: 14 (28.6%), age=11–18 years: 4 (8.2%), age=19–35 years: 19 (38.8%), age>36 years: 11 (22.4%) and 1 (2%) unknown.

The 52 cases IgM positive were from patients with *erythema infectiosum* (22), arthritis (12), aplastic crisis (8), flu-like illness (6), fetal death (2), organ transplant (1), blood transfusion (1). 39 (75%) of the IgM positive cases have occurred between March and August.

Comment. In spite the low number of cases presented, we have detected 52 (4.9%) of Parvovirus B19 acute infections and 38 (73%) of them are in accordance with the clinical diagnosis and the pattern of Parvovirus B19 seasonal incidence.

C. PAYAN[1], M.-C. ROUSSELET[2], F. OBERTI[3], I. FOUCHART-HUBERT[3], P. FIALAIRE[4], P. CALES[3], F. LUNEL ET LE RÉSEAU HÉPATITE C DES PAYS DE LOIRE-EST

Genotype evolution of hepatitis C virus in western part of France from 1993 to 1998

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The aim of this study was to evaluate the distribution and the evolution of HCV genotypes in the last 5 years in our region.

Methods. 541 HCV infected patients were genotyped in relation with the region "réseau hépatice C 49" hepatologists from Angers city and different hospitals: Angers, Saumur, Cholet, Bressuire. The LiPA system (Innogeneticd/InGen) was used with PCR products from Amplicor (Roche) or in-house Nested-PCR.

Result. The global genotype distribution was: 21% of 1a, 38% of 1b, 4% of 1a+1b, 3% of 1 non a/b, 7% of 2a/2c, 0.5% of 2b, 18% of 3a, 2% of 4a, 3% of 4c/4d, 0.2% of 4f 'and 4h, 1.1% of 5a and 2% of mixed types. Type 1b was found increasing: (i) in women (49/31%), (ii) with age (64% after 60 years old vs 22% before 40), (iii) with transfusion contamination (62 vs 15% with IV drugs), (iv) with a Knodell score > 6 (39 vs 24% if k<6) and (v) with poor interferon response (91 vs 9%). Type 3a was associated with: (i) young adults (25 vs 6%) and (ii) with IV drug use (41 vs 10%). Type 4 was also correlated to: (i) young adults (10 vs 2%), (ii) HIV coinfected patients (19 vs 4%), (iii) recent discovery (8% after 1995 vs 1% before) and (iv) no interferon response (100%).

Conclusion. HCV genotyping is a good epidemiologic and therapeutic marker.

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Detection and subtyping of influenza A virus subtype H9N2

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Influenza A virus subtype H9N2 is being recognized as widespread in poultry in Asia. Its recent isolation from pigs and humans has highlighted its pandemic potential. Awareness of this virus and laboratory methods for its recognition in clinical specimens are therefore essential. The disease caused by influenza H9N2 iu two children was that of a mild "flu-like" illness. Commercially available immunofluorescent reagents (DAKO Imagen™) and the DirectigenFluA (Becton-Dickinson™) test for influenza A antigen detection are reactive with H9N2 isolates from diverse hosts. Same reagents used for subtyping human H1 and H3 viruses by immunofluorescence and haemagglutination inhibition tests cross-react with avian H subtypes (including H9). Unawareness of this may lead to non-recognition, of H9-subtype viruses. An RT-PCR based on primers conserved among the avian, porcine and human H9N2 isolates has good sensitivity and specificity for H9N2 isolates from a range of hosts including humans.

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Parvovirus B19 infected staff in an obstetric unit represents a low risk to exposed patients

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Parvovirus infection during pregnancy can lead to adverse obstetric outcome. Nosocomial outbreaks of parvovirus B19 have been reported. However, during an "outbreak" of B19 infection in the community, it may be difficult to differentiate transmission within the hospital from that acquired in the community. Following the diagnosis of B19 infection in two symptomatic staff members in an obstetric service, we investigated 127 other exposed staff members and 131 exposed patients for evidence of recent infection by tests for IgM and IgG antibody (using immunofluorescence; Biotrin International, Ireland) and viral DNA (using PCR). 68% of patient and 73% of staff contacts were susceptible to B19 infection (IgG negative). One other asymptomatically infected staff member was identified (IgM positive, virus DNA positive) during the investigation. None of the patients at-risk had evidence of recent B19 infection. PCR amplified B19 viral DNA from the VP1/2 and NS-1 regions were sequenced. Phylogenetic analysis revealed that only two staff members had a virus strain that was epidemiologically related. The third staff member acquired his infection independently, from the community (his spouse). These findings indicate that parvovirus infection is not highly infectious to immunocompetent patients in a nosocomial setting. It also indicates that all infections documented during an outbreak investigation may not be epidemiologically related.

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The introduction of multiplex RT-PCR techniques improves the diagnosis of respiratory viral diseases in relation to isolation in cell cultures and immunofluorescence in clinical specimens

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Along the last three epidemic periods of respiratory viral illness we have introduced different multiplex RT-PCR assays for diagnosing respiratory syncytial virus (RSV), parainfluenza (PIV) and influenza viruses (IV). Clinical specimens taken during the maximum circulation period of each virus were used to prepare the panels for the evaluation of the respective PCR methods. For diagnosis of RSV, 173 samples were assayed, 61.8% proved to be positives by PCR, 60.8% by IFI and 49.4% by isolation in cell cultures. When PCR was used to RSV subtyping (A or B), the technique allowed to

type 97.8% of the viruses versus only 59.5% using IF. Of the 230 samples assayed for PIV, 28.3% were positives by PCR, 12.6% by IF and 19.6% in cell cultures. Besides, PIV4 was detected in 10 specimens by PCR and only in 1 by cell culture. In the case of influenza, 171 samples were tested being 30.4% positives by PCR and 23.4% in cell cultures (evaluation still in progress; provisional data). IF was not performed in some of these specimens and when it was performed proved to be slightly less sensitive than cell culture. Currently we consider RT-PCR the most sensitive technique for PIV and RSV. Moreover, these multiplex RT-PCRs permitted a simple and rapid way of typing in one single assay. This is especially important for epidemiological purposes, representing a good alternative to more cumbersome techniques or expensive reagents.

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Genotypic classification of respiratory syncitial virus (RSV) and the possible relations with the clinico-epidemiological characteristics of the infections produced in children

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The main objective of the study was to determine whether the existence of two types (RSV-A and RSV-B) or different lineages/genotypes of RSV could explain the clinical and epidemiological differences found among the infections produced by RSV. The population consisted of children attending the Emergency Room or hospitalized at the Hospital Severo Ochoa (Madrid) because of viral respiratory infections, between 1990 and 1997.

The methodology used was based in the RFLP patterns obtained when analysing fragments of the genes F and N, amplified by RT-PCR. Seventeen "genotypes" were differentiated, 11 among the RSV type A and 6 on the type B.

Clinical and epidemiological variables were recorded and were analysed in relation to the type of respiratory viruses detected. In the cases where RSV infection was demonstrated a comparative analysis was made trying to establish a relationship between the types of RSV and the clinical or epidemiological features. It is suggested that the type A is mare virulent and involved in higher number of reinfections than type B. The prevalence of the different genotypes and the characteristics of their circulation will be discussed in more detail.

The phylogenetic tree obtained from the RFLP data shows the relation among the types/genotypes and their distribution along the time.

A. PISTA, N. VERDASCA, F. RIBEIRO

Typing of human papillomavirus (HPV) by PCR among young women

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Background. HPV is strongly implicated in the etiology of cervical neoplasia; however the frequency, rate, and predictors of neoplastic progression are unknown (1).

Aims. The main purpose of this study is to evaluate the proportion of HPV infection in young sexually active women attending in private gynecologists' offices and STD clinics.

Methods. HPV DNA analysis was carried out by PCR using MY09/MY11 primers and RFLP typing.

Results. HPV DNA was detected in 36/160 (22.5%) women studied (16–30 years old; mean age 24.4); 56 (35.0%) women had symptoms and 104 (65.0%) were asymptomatic. HPV DNA was detected in 18 (32.1%) patients with symptoms and 18 (17.3%) patients without symptoms. The most prevalent HPV were 6/11 (25.0%), followed by HPV16 : (19.4%) and HPVX (13.9%). Multiple infections were found in 5 (13.9%) women, 3 of them with an uncharacterized HPV.

Comments. HPV infection occurs in many young women soon after their sexual debuts, even without clinical manifestation. Furthermore, these results show the need for a real policy to provide an early screening programme and information on STD.

1. Ho,G.Y.F., Bierman,R., Beardsley,L. et al.: N Engl J Med **338**, 423–428 (1998).

L. PLIŠKOVÁ, V. ŠTEPÁNOVÁ, S. PLIŠEK, V. PALIČKA, J. HORÁČEK

Detection of HSV 1 and HSV 2 in cerebrospinal fluid by PCR in patients with CNS infections

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Background. HSV 1 and HSV 2 are responsible for life-threatening infections of CNS. Their rapid, specific and sensitive detection is required to introduce immediate therapy with acyclovir. Application of PCR for HSV detection in CSF leads to rapid diagnosis of HSV infections of CNS without. the need of invasive procedures such as brain biopsy.

Objective. To optimize PCR for HSV 1 and HSV 2 detection in CSF. To use nested PCR for HSV 1 and HSV 2 identification in CSF in patients with CNS infections. To compare the positive findings with the course of infections.

Design. During 1997–1999 we examined 210 CSF samples from patients of all age groups with suspected CNS infections. For a nested PCR for HSV 1 two pairs of primers selected from the glycoprotein D and for HSV 2 from the glycoprotein G gene regions

were used. The isolation procedure and the reaction was optimized for our lab conditions. To reduce the possible contamination we performed "in one tube" PCR and used UNG.

Results. HSV 1 DNA was detected in 7 CSF samples (3.3%) and HSV 2 DNA was identified in 6 CSF (2.8%) of the total of 210 CSF samples. All patients were hospitalized with the diagnosis of viral meningitis, encephalitis and meningoencephalitis and the successful therapy with acyclovir was introduced. There was no fatal case of CNS infection caused by HSV 1 or HSV 2.

Conclusion. Our results proved the reliability of "in one tube" nested PCR for HSV 1 and HSV 2 DNA detection in CSF in a routine laboratory practice.

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Prevalence of HIV-1 serological subtypes types in HIV-1 infected patients from Slovenia

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Different subtypes of HIV-1 are prevalent in various geographical regions. Knowledge of HIV-1 subtype distribution in a particular country is of importance with respect to possible differences in biological properties as well as to diagnostic problems that may arise when specific subtypes are not recognized by standard screening serological assays or molecular assays used for the detection of viral load.

To investigate the prevalence of HIV-1 serotypes A-E in Slovenia, 82 HIV-1 infected individuals (approximately half of all HIV-1 infected individuals from Slovenia recognized to date) were tested for the presence of HIV-1 subtype specific antibodies for the five major HIV-1 subtypes (A-E) using a research gp120/V3 loop HIV-1 specific peptide-based competitive enzyme immuno assay (Boehringer Mannheim, Germany).

Unambiguous HIV-1 subtyping results were obtained in 74 out of 82 individuals tested (57 males and 17 females, ranging in age from 5 to 74, the mean age was 39.6 years). 64 out of 74 (86.5%) Slovenian HIV-1 infected individuals were infected with subtype B. Subtypes C, A, D and E were detected in 6 (8.1%), 2 (2.7%), 1 (1.3%) and 1 (1.3%) individuals, respectively. The majority of subtype non-B infected individuals were found to be epidemiologically linked to Africa or Thailand.

Twelve individuals with clear epidemiological relationships were included in the study (three hemophiliacs and their wives, two homosexuals with known HIV-1 infected partners, and a mother who transmitted HIV-1 to her child). In ail 12 cases, serologically determined HIV-1 subtypes matched each other, thus confirming the specificity of the assay used. Eight HIV-1 positive samples among the 82 tested (9.7%) did not react with any of the subtype mixtures, indicating that the use of such peptide-based serotyping assays for routine screening is still limited.

Our data indicate that HIV-1 subtypes, other than B, originating from Africa and Thailand are circulating in the Slovenian population. Although the number of non-B

infections in Slovenia is, at present, not as high as in Western European countries, it will certainly increase in the next few years and should be carefully monitored.

M. POLZ

Prevalence of HBs Ag and anti-HCV in hospital patients in district Lublin in 1994–1998

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Background. Hospital patients belong to the group of increased risk for exposure to infections diseases, especially with parenteral mechanism of transmission.

Design. To analyse the prevalence of HBV and HCV infection among hospitalized patients.

Objective. Serum samples from patients with various diseases hospitalized in hospitals in Lublin during 1994–1998 were tested for HBsAg and antibody to HCV.

Results. We revealed the decrease of prevalence of HBsAg from 26.7% in 1994 to 5.2% in 1998. Decrease of prevalence of HCV infection at the same time was lower; 17.2% in 1994 to 12.9% in 1998.

Conclusion.

1. Between 1994 and 1998 prevalence of HBV infection decreased; these results support that introduction on immunization against hepatitis B influenced the observed trend.

2. Now, HCV infection is more frequently than HBV.

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Improvement of the differential diagnosis of EBV-associated diseases using the NucliSense® Basic Kit

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Primary infection with EBV is usually a self-limiting disease leading to a life-long persistence of the virus in peripheral resting B-cells. However, in rare cases the immune surveillance is insufficient and virus-associated cell-proliferative diseases or chronic active courses of Infection (CAEBV) develop.

We recently confirmed, that in healthy individuals with serologically proven but otherwise undetectable reactivation of EBV, early antigen (EA) mRNA was detected but no expression of viral capsid antigen mRNA (VCA), Thus, no uncontrolled active replication of EBV (indicative for CAEBV) could be shown. The results indicated a partially impaired but sufficient immune surveillance of cells capable of EBV reactivation in these patients. In contrast, in a case of severe CAEBV, lytic replication was clearly

confirmed by the demonstration of EA- and VCA-mRNA in the peripheral B-lymphocytes.

Here we report the detection of EA and VCA mRNAs using the new NucliSens® Basic Kit (Organon Teknika), a generic kit for RNA amplification testing. It provides standardized reagents for RNA extraction, NASBA amplification and ECL detection. Assays were developed with the help of the Extranet-hosted Basic Kit Helpdesk. The assays were at least as sensitive as the previously described "in-house" nested-RT-PCR protocols. Moreover, the contamination risks reduced significantly and the turn around time was reduced by one full day. In experiments with blood samples from patients with infectious mononucleosis we were able to detect mRNA derived from lytic cycle genes in most of the cases. In contrast, the acknowledged serum PCR for the detection of viral genomes gave mainly negative results. Therefore, the detection of mRNA in peripheral blood cells seems to be more sensitive than the "gold standard". In summary, the detection of EA- and VCA-mRNA via the Basic Kit Technology offers a suitable tool to prove lytic cycle gene activity in the peripheral blood and will help to improve the differential diagnosis and treatment of clinical forms of EBV infection. The commercial availability of the NucliSens Basic Kit will allow NASBA to be used much more widely in diagnostic and research settings.

W. PREISER, U. AYLIFFE, S. KAYE, J. A. GARSON, N. S. BRINK, R. S. TEDDER

Development of a fully controlled quantitative cytomegalovirus (HCMV) DNA PCR

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Objective. To develop a quantitative HCMV DNA PCR controlled for efficiency of extraction, amplification and detection, using murine CMV (MCMV) virions added to the clinical sample as an internal control ("spike").

Methods. ~100 virions from MCMV cell culture supernatant were added to 1 ml of EDTA plasma. Viruses were pelleted by centrifugation at 24,000 g for 1 hour at 4 °C and resuspended in PBS before DNA extraction by Qiagen™ spin columns and elution into 50 µl. PCR primers (one biotinylated) located in HCMV gene UL50 recognize both HCMV and MCMV. Amplicons were captured onto a streptavidin-coated solid phase, denatured and hybridized separately with AP-labelled probes specific for HCMV and MCMV. A chemiluminescent substrate and an external standard curve using an HCMV dilution series allowed quantification over a dynamic range in excess of ~ 4 $\log_{10}$.

Results. We were able to quantify extracellular HCMV, reflecting active viral replication, from clinical samples with a theoretical sensitivity of 5 copies/ml.

Conclusions. MCMV virions behave like free HCMV during extraction and can be amplified by PCR using common primers but detected separately. The MCMV "spike" controls for efficiency of sample preparation and DNA extraction as well as amplification and detection, making this a potentially ideal method in clinical settings.

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HIV-1 subtypes present in the Czech Republic

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Objectives. Presence of different HIV-1 subtypes in the CR was investigated. Epidemiological background of HIV infected patients was studied regarding their HIV-1 subtypes infection.

Methods: Subtyping of isolated HIV-1 strains was performed by genomic analysis. Epidemiological investigation was done with the use of personal interviews and laboratory and clinical records.

Results. Altogether 45 HIV-1 strains were studied by sequence analysis of *gag* and *env* region fragments. Majority of the isolates belonged to subtype B cluster (n=36). However, subtypes A (n=2), C (n=4), E (n=1) and F (n=2) were also identified. The "exotic" strains were isolated from persons with a short- or long-term sojourn in countries with high prevalence of these subtypes.

Conclusion. The genomic analysis of HIV-1 strains isolated in the CR demonstrated that subtype B was the most dominant subtype similarly as in other European countries.

Other subtypes prevalent in far distance regions (subtypes C, E; and F) were also registered in the CR. Epidemiological tracing of these "exotic" subtypes infections revealed in all cases connection to countries with high prevalence of these subtypes. It seems that these strains were imported.

M. G. REVELLO, M. ZAVATTONI, M. FURIONE, F. BALDANTI, M. TORSELLINI, G. GERNA

Prognostic value of quantitation of human cytomegalovirus (HCMV) in fetal blood and amniotic fluid (AF) of congenitally infected fetuses

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Objectives. To assess the prognostic significance of quantitation of different virologic parameters and IgM antibody in fetuses with HCMV congenital infection.

Methods. pp65 antigenemia, viremia, leukoDNAemia (L-DNAemia) and virus-specific IgM in fetal blood as well as viral DNA in AF were quantitated in 21 fetuses with HCMV infection diagnosed by virus isolation from and/or DNA detection in AF. Fetuses were divided into 2 groups: group A included 7 fetuses presenting ultrasound abnormalities at the time of prenatal diagnosis (4 fetuses), or with symptomatic HCMV infection at birth (3 fetuses); group B included 14 fetuses with normal findings at the time of prenatal diagnosis and subclinical HCMV infection at birth.

Results. Compared to HCMV isolation and/or DNA detection in AF, sensitivities of antigenemia, viremia, L-DNAemia and IgM were 60.0%, 52.6%, 83.3% and 60.0%, respectively, whereas specificities were 100.0% for all assays. Higher levels of all virologic parameters were detected in blood of fetuses with abnormal ultrasound findings compared to asymptomatic fetuses. In detail, median values were 13 (range, 0–146) and 0.5 (range, 0–49) pp65-positive peripheral blood leukocytes for antigenemia, 3 (range, 0–10) and 0 (range, 0–89) p72-positive fibroblasts for viremia, 480 (range, 5–1000) and 80 (range, 0–30000) genome equivalents (GE) for L-DNAemia, and 10.3 (range, 6.4–11.8) and <1 (range, <1–10.8) IgM ratio value for IgM antibody in group A and B, respectively. Antigenemia and IgM antibody levels were significantly different between the 2 groups ($p=0.03$ and <0.01, respectively). As for DNA quantitation in AF, median GE levels were 1.25×10^8 (range, 1.25×10^5–1.25×10^9) and 3.15×10^6 (range, 16–1.25×10^8) in group A and B, respectively. The difference was not statistically significant ($p=0.09$).

Conclusions. Virologic and ultrasound findings may contribute to a better prognostic definition of the fetal infection. However, a reliable prenatal marker of symptomatic congenital HCMV infection remains to be identified.

F. ROELES, P. VAN AARLE, P. HAIMA, H. FOOLEN, M. PEETERS, P. SILLEKENS, F. CROMME

NucliSens™ basic kit: a new tool for developing home made amplification based (viral) diagnostics

Organon Teknika b.v., Boxtel, The Netherlands

For a number of viral analytes e.g. HIV, HCV, CMV, amplification based diagnostics are commercially available. These assays are often well-standardized, quality-controlled and, at least partially, automated. However for a large number of analytes a research and/or (routine) clinical interest is present but assays are not available. As a consequence institutes start to develop their own "home-made" assays. By constructing these assays only a limited number of target-specific reagents, e.g. primers and probes, are needed while the majority of reagents are generic. Much of the assay development time is used to purchase, adjust and aliquot these reagents. To facilitate this work we developed a set of generic reagents with detailed assay development instructions, available in a easy-to-use format, that was used to construct several RNA-based qualitative amplification assays using the "BOOM" method for RNA extraction, NASBA for amplification and ECL technology for detection of amplicons.

NASBA is an enzymatic amplification process, which is able to amplify under isothermal conditions. Specificity of the reaction is determined by two oligo nucleotide primers that are specific for the RNA target of interest and a capture oligo for hybridization of the amplicons. Standardization of the assay can be achieved by using the NucliSens reader with Basic Kit specific software with which ECL detection and data reduction is fully automated. With the above-described procedure NASBA based amplification assays for several viral targets like CMV, EBV, Dengue virus, Entero virus,

HHV-8 etc. have been developed. Using Basic Kit reagents also gives access to an Internet based application data base where many assays of interest can be copied for own purposes. This also contributes to standardization.

In conclusion, generic reagents for nucleic acid release, isolation, amplification and detection have been developed. Use of these reagents simplified creation of amplification based home made assays, as was shown for several viral targets, while ascertaining a high level of standardization and quality control. The Internet based application data base gives access to a large number of ready-to-use protocols that can easily be copied for own purposes. If needed support in setting up home made Basic Kit assays can be given by the I Basic Kit help desk.

F. ROELES, P. SILLEKENS, N. TACKEN, N. OLDENBURG, J. MIDDELDORP, J. LUNENBERG, F. CROMME

Detection of viral mRNAs by NASBA in CMV infected and immunocompromised patients

Organon Teknika b.v., Boxtel, The Netherlands

Expression of CMV mRNAs, especially those with late kinetics, is a reflection of the virus replication status. Therefore, detection of viral mRNAs in blood could be of valid support in diagnosing active disseminating CMV infection.

Assays were developed for the qualitative detection of the viral immediate early (IE) 1 mRNA and of a late mRNA encoding a structural protein of 67 kDa (pp67). The assays are based on the NASBA amplification technology, a sensitive and specific method to directly amplify RNA in background of genomic DNA. Primers and probes were designed to recognize clinical strains of CMV without cross-reacting to other herpes viruses. The specificity with specimens obtained from random CMV seropositive (n=50) and seronegative (n=50) whole blood donors was 100%. Using whole blood, sensitivity was approximately 10^2–10^3 RNA molecules per ml. Anticoagulants did not influence the performance of the assays. Neither did storage of whole blood specimens for 24 hours at room temperature or at 2–8 C. Comparison of late mRNA detection with other diagnostic methods on a per test basis revealed a sensitivity that was higher than for CMV culture but slightly lower than for pp65 antigenemia. However, comparison on a per patient basis showed that most patient samples that were positive in the pp65 antigenemia assay but negative when tested for the presence of pp67 mRNA, originated from episodes of clinically irrelevant CMV activity. In contrast, episodes of CMV infection, as confirmed by several viral markers, were all recognized by the pp67 mRNA assay as well.

L. ROJKÓ[1], A. PÁL[2], R. PUSZTAI[1]

Strain-specific neutralization antibodies to human cytomegalovirus in pregnant women

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The major target for neutralizing antibodies of human cytomegalovirus (HCMV) is the envelope glycoprotein B (gB). Strains can be classified into four genotypes of the gB. Our previous studies have shown that gB type 1 is the predominant strain in congenital HCMV infections.

The present study was carried out to investigate the influence of gB strain-variation on neutralizing antibody titers.

The study population consisted of 30 women with normal pregnancy, 30 women with fetus suspected viral infection and 30 age-matched healthy not pregnant blood donors. Neutralizing antibody activity against Towne (gB type 1) and AD169 (gB type 2) reference strains was measured in sera or plasmas by a microneutralization assay.

The study revealed high prevalence of neutralizing antibodies against both gB genotypes (96.6% and 73% for pregnant women and blood donors, respectively) and an equal neutralizing capacity against AD169. However, pregnant women had significantly higher titer of neutralizing antibody to Towne than to AD169 ($p<0.05$). No significant differences were found in the same respect between the two groups of pregnant women ($p=0.485$).

Our data demonstrate differences in the prevalence and capacity of gB1 strain-specific neutralizing antibodies in pregnant women and age-matched blood donors, which might have important implication for the course of prenatal HCMV infection and development of anti-HCMV vaccines.

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Anti-gB116(UL55)-IgG A HCMV-specific seromarker assists differentiation of primary and secondary infection

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The glycoprotein B (gB) of HCMV, encoded by UL55, also known as gp58/116, is one of the major targets for neutralizing antibodies. In particular, the N-terminally located antigenic domain 2 (AD2) of gp116 contains mapped epitopes for neutralisation and is highly immunogenic in man. While other regions of gp58/116 show homologies within different herpesviruses, AD2 is HCMV specific. According to our development of an anti-gB IgG ELISA, highest serological reactivity was mediated by a recombinant inter-strain fusion antigen (CG3) comprising homologous AD2 regions from the divergent HCMV

strains AD169 and Towne. The supplementary use of AD2-Towne together with AD2-AD169 resulted in an increased sensitivity of about 10–12%. The total CG3-ELISA specificity in HCMV-seronegatives was 99.4%. The sensitivity in reactivated acute HCMV-infection (transplant- and AIDS patients) was 95–96%. A lower prevalence of about 82% in healthy blood-donors suggests a secondary decline of anti-gB in some individuals. Besides its correlation to neutralising-bioassays, the anti-CG3-ELISA showed a prominent delay of seroconversion of 80–120 days after primary infection. In contrast, secondary acute infection is characterised by an immediate serological response. In conclusion, the presented anti-CG3 IgG ELISA provides the method to detect a strongly delayed IgG-marker, which can be advantageously used for the differentiation of primary and secondary HCMV-infections. The major benefit for routine application should be diagnosis in pregnancy. Here, IgM positivity in the majority of cases, is related to subclinical HCMV reactivations in pregnants. Therefore, testing for anti-CG3 IgG will support the identification of clinically more relevant primary infections.

V. RUSSEV

Biological characteristics and antigenic title: structure of the influenza viruses isolated during 1998–1999

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With modern methods for studying antigenic structure and some biological properties 15 isolated influenza type A strains were examined for their haemadsorption, haemagglutination, inhibitor-susceptible, eluent, pathogenic, taxic and immunogenic activity. The examined viruses demonstrated various adaptation and isolation ability on 10–12 day chicken embryos. The results indicated that the 7 strains from 1998–1999 have been isolated only after the first-second passage with haemagglutination titre 1:32, 1:64, 1:128. The majority of the examined strains demonstrated a well-expressed haemadsorptive and haemagglutinative activity at both temperatures. The investigated isolated strains demonstrated both high and low inhibitor-sensitivity. The studies showed continuing active circulation of influenza type A viruses with formula H_3N_2, antigenically similar to standard strains A/Beijing/32/92, A/Johannesburg/33/95, A/Uhan/359/95 with the respective biological properties.

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Cross-reacting antibodies to human hepatitis E virus among swine and wild pigs in Hungary

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Cross-reacting antibodies against human viral antigens have been detected in animal sera. A commercially available ELISA kit was modified by replacing the original conjugate to ProteinA-conjugate.

Using this method antibodies cross-reacting to the human hepatitis E virus capsid antigens were identified in pigs and wildpigs all over the country.

11 out of 62 serum samples drown from wild pigs and 8 out of 96 domestic pigs hold in differently located herds were found reactive.

25 piglets kept in a separated room were seronegative at the first bleeding. Cross-reacting antibodies were detected in 7 of them later on. The latter ones remained reactive during our observation period.

Our results point out the potential risk of zoonosis or xenozoonosis following the use of organs (xenografts) or biologically active compounds (insulin) derived from pigs.

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De novo DNA methylation at nonrandom founder sites 5' from an unmethylated minimal origin of DNA replication in latent Epstein-Barr virus genomes

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Latent episomal (extrachromosomal) genomes of Epstein-Barr virus, a human gammaherpesvirus, represent a suitable model system for replication and methylation of chromosomal DNA in mammals. We analyzed the methylation patterns of CpG dinucleotides in the latent origin of DNA replication of Epstein-Barr virus using automated fluorescent genomic sequencing of bisulfite-modified DNA samples. We observed that the minimal origin of DNA replication (which contains a dyad symmetry sequence) was unmethylated in 8 well-characterized human cell lines or clones carrying latent Epstein-Barr virus genomes as well as in a prototype virus producer marmoset cell line. This observation suggests that unmethylated DNA domains can function as initiation sites or zones of DNA replication in human cells. Furthermore, 5' from this unmethylated region we observed focal points of *de novo* DNA methylation in nonrandom positions in

the majority of Burkitt's lymphoma cell lines or clones studied while the corresponding CpG dinucleotides in viral genomes carried by lymphoblastoid cell lines and marmoset cells were completely unmethylated. Clustering of highly methylated CpG dinucleotides in lymphoma cells suggests that de novo methylation of unmethylated double stranded episomal viral genomes starts at discrete founder sites *in vivo* and can spread to adjacent CpG sites in *cis*.

T. V. SAVITSKAIA, N. D. KOLOMIETS, O. V. ALEJNIKOVA, A. A. ZBOROVSKAYA, S. E. BUGLOVA

Detection of CMV and HSV antigenemia using flow cytometry

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The method of flow cytometry was developed for quantitative determination of cytomegalovirus (CMV) and herpes simplex virus (HSV) antigenemia in mononuclear cells (MNC) of peripheral blood among children with hemoblastosis.

Design. MNC samples were investigated from 32 children. For detection antigenemia we used monoclonal antibodies, immediate early protein – p72 CMV and common structural protein gp 60 of HSV-1 and HSV-2. At the same time MNC samples were examined on cellular culture.

Result. p72 CMV was found in 23 patients in an amount from 0.1% up to 18.8% (average 6.8%) positive cells on 5000 MNC, gp 60 HSV was revealed in 22 children in an amount from 0.1% up to 26.0% (average 4.8%). In the group of 12 patients with the low contents p72 CMV positive MNC (<5%) the virus was detected from 2 (16.7%) children. The group with the high contents (>5%) p72 CMV positive MNC consisted of 11 patients. Viremia was confirmed by selection of CMV at 8 (72.7%, $P<0.05$) children. In the group of 11 patients with the low contents gp60 HSV positive MNC virus was isolated from 4 (36.4%) patients. The high contents gp60 HSV positive cells was observed at 11 children. Virological evidence of HSV infection was proven by 7 (63.6%) persons.

Conclusion. The flow cytometry can be used as a highly sensitive method of laboratory diagnosis of herpesviruses.

E. SCHALLER, T. SCHEPERS, D. GRACEY, S. MASON, P. GREEN

Performance of AxSYM® HAVAB® 2.0 Quantitative vs. Boehringer Mannheim anti-HAV Enzymun-Test

Abbott Laboratories, North Chicago, Illinois, USA

AxSYM HAVAB 2.0 Quantitative (AxSYM) is an MEIA assay for the quantitative determination of total antibodies to hepatitis A virus (anti-HAV) in human serum or

plasma. A test for anti-HAV is used as an aid in the diagnosis of previous or ongoing hepatitis A virus (HAV) infection and for the detection of anti-HAV after vaccination. Boehringer Mannheim Anti-HAV Enzymun-Test (BM) is an ELISA assay for the qualitative and quantitative determination of total anti-HAV. An external study was conducted to compare AxSYM toBM for the detection of anti-HAV in 731 specimens from the following categories: random hospital patients, random volunteer whole blood donors, specimens from patients with disease states other than HAV, previously characterized IgM reactive specimens, characterized total anti-HAV reactive specimens, and HAV vaccine recipients. Using criteria from the BM package insert, specimens with <12 mIU/mL anti-HAV were considered nonreactive (cutoff sensitivity 12–27 mIU/mL) and specimens with results of 12 mIU/mL or greater were interpreted as demonstrating immune response to HAV. Overall agreement on immune response from the 731 clinical specimens was 96.17% (703/731). To determine the ability of the AxSYM and BM assays to detect anti-HAV after vaccination, single and serial bleed specimens from HAV vaccine recipients were tested. Agreement on immune response was 98.46% (64/65) in the single bleed vaccine recipients. In the serial bleed specimens, both assays determined the vaccinees to have achieved immune response in the same bleed. The AxSYM assay was determined to be effective for the quantitative determination of total anti-HAV.

T. SCHEPERS, E. SCHALLER, D. GRACEY, S. MASON, P. GREEN

Performance of AxSYM® HAVAB® 2.0 in external clinical evaluations

Abbott Laboratories, North Chicago, Illinois, USA

AxSYM HAVAB 2.0 (HAVAB 2.0) is an MEIA assay for the detection of total antibody to hepatitis A virus (anti-HAV) in human serum or plasma. A test for anti-HAV is indicated as an aid in the diagnosis of previous or ongoing hepatitis A virus (HAV) infection or for the detection of anti-HAV after vaccination. An external study was conducted to evaluate the performance of HAVAB 2.0. Assay sensitivity was compared to the marketed AxSYM HAVAB assay using specimens from the following categories: pedigreed anti-HAV and IgM anti-HAV reactive, HAV outbreak specimens, populations at increased risk for HAV infection, single bleed and serially collected specimens from HAV vaccine recipients, and HAV infection serial bleeds. Assay sensitivity was calculated using the results of the pedigreed anti-HAV and IgM anti-HAV reactive, and the HAV outbreak specimens. Assay specificity was compared to the marketed HAVAB EIA assay using the results from random hospital patients, blood donors, patients with infections other than HAV, and specimens containing potentially interfering substances. Analytical sensitivity of HAVAB 2.0 was also evaluated. Assay sensitivity of HAVAB 2.0 was 99.74% (383/384) vs. 99.22% (381/384) for AxSYM HAVAB. Assay specificity of HAVAB 2.0 was 98.69% (378/383) vs. 100.00% (383/383) for HAVAB EIA. Analytical sensitivity ranged from 9.53 to 10.84 W.H.O. mIU/mL for AxSYM HAVAB

2.0. AxSYM HAVAB 2.0 was determined to be effective for the detection of total anti-HAV and more sensitive than AxSYM HAVAB.

J. SCHIRM, A. M. VAN LOON, P. KLAPPER, J. REID, E. VALENTINE-THON, G. CLEATOR

Proficiency panels for quality control of nucleic acid amplification of HIV-RNA, HCV-RNA and HBV-DNA

Blood Borne Virus (BBV) Working Party, EU Concerted Action for Quality Control of Nucleic Acid Amplification in Diagnostic Virology

The program of the "European Union Concerted Action for Quality Control of Nucleic Acid Amplification in Diagnostic Virology (QCCA)" includes proficiency panels for human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C Virus (HCV). These panels are specifically designed to assess the ability of diagnostic laboratories to diagnose and/or monitor viral infection. They are suitable for evaluating both qualitative and quantitative amplification technologies and, for HIV, the new ultra-sensitive amplification tests.

In May 1999 the first panels for HIV, HBV and HCV were distributed to laboratories throughout Europe. A second set of panels will be sent out in February 2000. All panels consist of 8 vials containing various amounts of each virus in addition to negative controls. To minimize the costs of transportation – all panels had to be kept at dry ice – the panels for all three viruses were sent simultaneously.

The panels were designed by the QCCA BBV Working Party and produced on their behalf by Boston Biomedica Inc. (BBI). The HIV-panels were prepared from cultured virus of Clades A, B and C, and contained different quantities of HIV-RNA in the range of 200–200,000 copies/ml. The HCV-panels were prepared from HCV-RNA positive plasma; and contained HCV-RNA of genotypes 1, 2 and 3 in the range of 200–500,000 IU/ml. The HBV-panels were prepared from HBV positive plasma, and contained HBV-DNA of subtypes ad and ay varying from 1000–10,000,000 copies/ml. In addition to the QC employed within the production facility, all panels were evaluated prior to release by QCCA appointed independent QC facilities, and by each of the major manufacturers of nucleic acid amplification procedures.

The results of the participating laboratories will be anonymized by the neutral office – at the University of Manchester – and evaluated just prior to the ESCV Summer Meeting 1999. At this meeting, some of the preliminary results will be presented.

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A panel for quality control of nucleic acid amplification of herpes simplex virus (HSV)

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Aim. External quality control (EQC) of nucleic acid amplification (NAA) for HSV1 and -2, using a well-characterized panel containing samples with known amounts of HSV1 and -2 genomes.

Materials and methods. Within the "the European Union Concerted Action for Quality Control of Nucleic Acid Amplification in Diagnostic Virology", a working party (WP) for EQC of amplification of HSV1 and -2 DNA was created. A EQC panel was produced at the Swedish Institute for Infectious Disease Control (SIIDC). The Neutral Office was at the University of Manchester. The well-characterized MacIntyre strain (HSV1) and MS strain (HSV2) were purchased from ATCC and cultivated at the SIIDC. Freeze-dried cultures were sent to all laboratories of the WP, for verification that they were HSV1 and -2. PCR before and after freeze-drying and inactivation established that the HSV DNA remained stable. The viruses were thereafter cultivated in large batches, inactivated and diluted tenfold from –2 to –8. PCR at the SIIDC identified a likely cut off for detectability. Eight dilutions around the cut off for each virus were dispersed into 300 vials each. A test panel of all dilutions was freeze-dried, and sent to the laboratories of the WP. The last PCR-positive dilution for all laboratories was identified. The quantity of viral DNA at this endpoint was determined both by end-point-titration together with materials containing known amounts of HSV DNA, and in a real-time PCR. Dilutions around the end-point were chosen, freeze-dried, re-examined at the SIIDC, and panels containing 12 samples each were distributed to 63 laboratories in Europe.

Results. All members of the WP identified the HSV-strains correctly. The cut off for detection of HSV1 was 500–3000 genomes/mL in most laboratories, and mostly tenfold higher for HSV2. The variation in quantification of genomes between end-point titration and real-time PCR was less then fourfold. The material that was finally included in the panel, and the results from the 63 participating laboratories will be presented at the ESCV Budapest meeting.

Conclusion. Many panels for various QC purposes are presently produced by various organizations. The QC of the panel production itself is a matter of concern. Rules with respect to panel production for commercial use should be agreed upon, but initial careful consideration on what precautions that are necessary must precede the creation of rules.

J. SCHUBERT, B. WEISSBRICH

Diagnosis of acute hantavirus infections and with a recombinant immunoblot assay

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Nephropathia epidemica (NE) caused by the hantavirus serotype Puumala is endemic in large parts of Europe. The prognosis of this disease is usually good. However, a serological diagnosis is important to differentiate NE from more severe renal conditions.

We selected serum samples of 40 NE patients from Under-franconia (Bavaria) and control samples for analysis with a commercially available immunoblot (Mikrogen, Germany). The assay is based on recombinant N proteins of the Puumala and the Hantaan serotypes. By enzyme immunoassays and immunofluorescence assays, all NE cases had been classified as infections with the Puumala serotype. Most of the serum samples from the acute phase of NE reacted strongly in the IgG and IgM immunoblot with both hantavirus antigens. However, three NE patients showed only a weak IgM response, which was below the cut-off of the immunoblot. In general, follow-up samples of the NE patients reacted clearly stronger with the Puumala antigen than with the Hantaan antigen in the IgG assay. Of 50 blood donor samples, only two showed weak reactions with hantavirus antigens.

The immunoblot assay appears to be suitable for the diagnosis of NE. Redefinition of the IgM cut-off criteria may further improve the assay.

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Detection of caliciviruses in pediatric hospitalized patients: a French prospective case-control study (1998–99)

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Viral gastroenteritis (GE) is a major cause for hospitalization in pediatrics. Increasing evidence suggests that human caliciviruses (HuCV) represent the second etiological agent associated with GE. In France, little is known about the frequency of HuCV in hospitalized young children or the carriage in pediatric population.

To address these questions, we conducted a prospective case-control study and evaluated stool samples of patients and age-sex matched controls for the presence of 5 enteric viruses (HuCVs, rotaviruses, adenoviruses, astroviruses, enteroviruses). Stool specimens (40 per month) were collected in children under five years of age within 48 hours after hospitalization. Patients with GE were sex and age-matched with controls. The diagnostic procedures comprized ELISA detection for rotaviruses (RV), adenoviruses (ADV) and astroviruses (AST) (IDEIA, DAKO) and RT-PCR detection for HuCV and

enteroviruses (EV). Genotyping of caliciviruses was done either by hybridization (Norwalk-like viruses) or sequencing (Sapporo-like viruses).

We detected a viral agent in 56% of the patients and 24% of the controls. In 4 patients, we found both RV and EV. HuCV were detected in 5.5% of the patients. According to hybridization results, we identified 4 HuCVs of Norwalk-like genus, one related to Norwalk virus and 3 to Snow Mountain virus. Based on sequence analysis, we identified also 2 Sapporo-like viruses, closely related to Plymouth/92/UK.

No. of samples	Total	with				
		caliciviruses	rotaviruses	adenoviruses	astroviruses	enteroviruses
Patients	109	6	33	4	0	18
Controls	96	0	3	0	0	20

Preliminary results of our study suggest that HuCV are significantly associated with GE in pediatric patients hospitalized for diarrhoea, with cocirculation of both Sapporo and Norwalk-like strains. EV were found frequently in patients' faecal samples; however, EV were detected also in 20.8% of the controls, suggesting frequent carriage.

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Incidence of human astrovirus isolates from hospitalized children in north-East Germany

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Astroviruses are agents of human gastroenteritis. Although they are commonly associated with mild diseases of short duration, symptoms are often as severe as those observed with rotavirus diarrhoe and lead to hospitalization. The incidence of astrovirus in children hospitalized for acute gastroenteritis during august 1998 to March 1999 was analyzed. The virus was isolated during the winter month mainly in the age of 2 and 5 month.

Diagnosis of astrovirus disease was carried out from faeces by antigen-ELISA and by culture in the continuous cell line CaCo-2. The isolates were characterized by RT-PCR and sequencing.

Results. The incidence was calculated to be 1.5% (14 of 813 samples) compared with rates of 9.4% for rotavirus and 6% for adenovirus. One diseased patient was found to have 4 weeks earlier infection with rotavirus, a nosocomial transmission could not be excluded. In the other 13 positive samples no accompanying viral or bacterial pathogen could be identified. Nucleic acid sequence analysis showed the typical structure of astrovirus. Few mutations were found between the 7 isolates analyzed.

Conclusion. This analysis shows that the incidence of astrovirus infection was low, but the virus is also an important causative agent in severe gastroenteritis of children.

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Viral and bacterial coinfections in cervicitis

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Different sexually transmitted viral and bacterial infections in women often occur concomitantly and, generally, signs, symptoms as well as routine clinical investigation allows to formulate only a diagnostic hypothesis. In cervicitis, microorganisms can establish subclinical and latent infections and the detection of etiological agents is almost neglected. In this study, cervical samples of women with past and present symptoms of inflammation were tested for the presence of viruses and bacteria commonly involved in urogenital tract infections. By molecular and routine diagnostic procedures, we looked for 45 genital human papillomaviruses (HPV), herpes simplex viruses (HSV), adenoviruses, *C. trachomatis*, *M. hominis*, *U. urealyticum* and *M. genitalum*. A low correlation between colposcopy and HPV genomic DNA detection was observed. Double and triple infections were evidenced in symptomatic women with flogosis or abnormal transformation zones, whereas in women with negative colposcopic findings none or only one agent was discovered. HPV DNA was always present in coinfections with bacteria, adenovirus or HSV. *In vitro* assays carried out to investigate a possible interaction between HPV and HSV, both associated with carcinoma of human cervix, showed in HSV2 infected HeLa cells a modulation of mRNA transcription of HPV18 constitutively expressed. Results obtained emphasize the pivotal role of the microbiology laboratory in cervical multiple infections detection.

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Prevalence of hepatitis C and G virus infection among Slovenian and Croatian intravenous drug users

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Blood borne viruses pose major risks to the health of people who inject illicit drugs. Previous studies clearly showed that the prevalence of human immunodeficiency viruses, human T cell leukemia/lymphoma virus type I and hepatitis B virus infections among intravenous drug users (IVDUs) in Slovenia and Croatia was still luckily significantly below the Western European average (Folia Biologica **44**, 23 1998). However, the prevalence rates for two most recently discovered blood borne hepatitis viruses, hepatitis C virus (HCV) and hepatitis G virus (HGV) among IVDUs are still missing for both neighbour countries.

To determine the prevalence of HCV and HGV infection among IVDUs in Slovenia and Croatia, we have conducted a study on 115 Slovenian and 102 Croatian IVDUs from both state capitals. The prevalence of HCV and HGV infections was assessed by the detection of both anti-viral antibodies and viral genomes in serum samples.

HCV and HGV infections were detected in 60 (52.2%) and 61 (53.0%) Slovenian IVDUs, and in 70 (68.6%) and 39 (38.2%) Croatian IVDUs, respectively. HCV positive Slovenian and Croatian IVDUs were significantly older, and reported longer duration of the intravenous drug use in comparison to HCV negative IVDUs. In contrast, no significant differences in both parameters were found among HGV positive and negative IVDUs. The prevalence of HGV RNA (9.5% and 7.8%) and the overall prevalence of HGV infection (53.0% and 38.2%) found in our study is the lowest found to date among IVDUs. In cohort of Slovenian anti-HCV negative IVDUs who have been followed for three successive years the incidence rate for HCV was 11.5% per person-year of follow-up.

In conclusion, HCV and HGV infections are quite frequent among Slovenian and Croatian IVDUs, although the established prevalence rates are lower in comparison with those found among IVDUs from the majority of other European countries.

C. SERRA, G. MAMELI, A. BIOLCHINI, S. CURRELI, A. DOLEI

Characterization of an HIV-1 strain that preferentially replicates in adherent cells

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Various call types may be sites of HIV variant selection. We observed in the past years productive HIV infection in a variety of adherent cells. A T-tropic HIV-1 strain (HIV-1P1) was selected from epithelial Hela-T4 cells persistently infected for >40 passages (HIV-E) and compared to the original T cell-derived virus (HIV-T).

Both viruses are produced in high titres, and have approximately the same p24/SFU ratio, indicating similar proportions of uninfectious particles. Through binding to mAb-conted wells, call molecules incorporated in the envelope were evaluated. Both viruses had HLA-ABC and CD44. HIV-T had some CXCR4 and CCR5, while HIV-E was CXCR4(–), CCR5(+) and EMA(+). They have identical env V3 loops, containing the nucleotides associated to the syncytium-inducing phenotype, but HIV-E presents a TAR duplication in the LTR region. When given at the same multiplicity of infection, both viruses bind preferentially to calls of the same type of the parental one. In T cells, virus yields are proportional to the extent of adsorption; in epithelial cells, instead, provided that similar amounts of virus are bound, HIV-E yields are >3 Log10 higher, suggesting that the selection of a tissue-specific HIV variant, adapted to the epithelial cell machinery, occurred.

C. SIMS

HCV genome screening for blood transfusion

National Blood Service in England

Objective. To develop validated procedures allowing national implementation of minipool screening for HCV RNA by PCR for c.2.4MM blood donations p.a. to the National Blood Service.

Methods. Stability studies of HCV RNA before and after pooling; minipooling using Robotic Sample Processing; RNA extraction; PCR amplification and amplicon detection; sensitivity and specificity of the test method; resolution and confirmation of positive minipools; data handling.

Results. Peripheral centres pool donations and send plasma to be tested at a single national laboratory. High throughput testing with the necessary IT links has been successfully achieved with the Qiagen BioRobot 9604 and Roche Amplicor Cobas technologies. The 2D resolution algorithm allows confirmed positive pools to be resolved down to single viraemic donors. To date a total of about 600,000 donations have been tested with 36 HCV viraemic donors identified, all of whom were seropositive for HCV. The rate of unconfirmed positives is around 0.14%.

Conclusions. It is possible to introduce HCV RNA testing into transfusion practice using commercially available options for nucleic acid extraction, amplification and detection. This procedure is robust and capable of identifying correctly the individual HCV RNA positive donors. It will be possible to increase the commitment of the system to allow for real time screening for component release and to incorporate other viral targets in the future.

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Tick-borne encephalitis in Eastern Bohemia in 1995–1998

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Objective. To follow the occurrence of tick-borne encephalitis (TBE) in patients with CNS infections in Eastern Bohemian region of Czech Republic.

Materials and methods. Serum and CSF samples were obtained from patients with suspected CNS infection, anti-TBE IgM and IgG were detected by Elisa (Test Line, CR). The total number of examined patients increased from 165 in 1995 up to 480 in 1998.

Result. Of the total number of 1383 patients with suspected CNS infection TBE was diagnosed in 115 patients (77 males and 38 females; age 3–72; 17 children younger than 15) according to positive detection of anti-TBE IgM and IgG in CSF and serum samples. TBE manifested as viral meningitis in 51% encephalitis or meningoencephalitis in 25%, 20% of patients suffered from nonspecified symptoms. One case was fatal. Following the seasonal occurrence TBE was diagnosed from May till November with the

maximum number of cases in August and September. About 3/4 of cases informed about the tick bite. There were 3 regions in Eastern Bohemia with the maximum occurrence of TBE after the tick bite – Pardubice, Chrudim and Rychnov regions.

Conclusion. Opposite to TBE occurrence at the end of the eighties and the beginning of the nineties – when only single cases were diagnosed, during the period of 1995 to 1998 the number of cases increased markedly. As the possible reason of this fact we can admit the improvement of laboratory diagnosis (detection of anti-TBE IgM and IgG by Elisa in CSF and serum) but also the increase of tick population in the recent years in our region.

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Earlier detection of HCV infection using an automated chemiluminescence assay to detect HCV core antigen

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We are currently developing an immunoassay to detect HCV core antigen for use on the Vitros^{TM} Immunodiagnostic System. The Vitros HCV Antigen assay is a solid phase enzyme immunoassay with a detection system based on chemiluminescence. The system pipettes 80 µl of sample together with 60 µl of assay reagent into a single plastic well coated with monoclonal antibody to HCV core antigen. After a 30 minute incubation, the well is washed and 140 µl of horseradish peroxidase-labelled monoclonal antibody to HCV core antigen is added. After a second incubation of 15 minutes, the well is washed, luminogenic reagent added and the light emission measured. Sample and reagent pipetting, incubation, washing, and chemiluminescence detection take place within an incubation and processing ring which is thermostatically controlled at 37 °C.

The Vitros HCV Antigen assay detects HCV infection significantly earlier than conventional anti-HCV assays. Specificity in a population of blood donors was >99.5%.

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Molecular techniques for the diagnosis of CMV disease in BMT recipients

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Study aims. To evaluate four molecular detection techniques for the early diagnosis of CMV reactivation in recipients of bone marrow and peripheral blood stem cell transplants.

Methods. More than 200 blood samples from 35 transplant recipients were monitored for CMV reactivation. Each sample was tested by two nucleic acid

amplification assays using modifications of the PCR, together with a NASBA assay. PCR techniques were based upon the detection of low or high level CMV DNA in whole blood extracts and upon the detection of cell-free CMV DNA in plasma. CMV late mRNA transcripts (*pp*67) from nucleic acid extracted from whole blood were detected using NASBA. All results produced by the molecular assays were compared against those obtained from the non-molecular *pp*65 antigenaemia test.

Results. Results presented show discordance in 5% of samples. Results from whole blood PCR and NASBA compared well good, but qualitative plasma PCR results did not.

Conclusions. Early data suggest that results obtained from cell-free plasma PCR should be interpreted with caution. However, in monitoring these patients we will determine which molecular based assay is the most useful for the prediction of CMV disease in this group of post transplant patients.

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Early detection of post-transplant lymphoproliferative disorders: the need for weekly monitoring of Epstein-Barr virus load dynamics in peripheral blood

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Epstein-Barr virus (EBV) is the major predisposing factor for the development of post-transplant lymphoproliferative disorders (PTLD). EBV load in peripheral blood is a putative diagnostic or prognostic marker for the development of PTLD and may be a valuable tool for clinical management of the patient. Therefore, we developed a quantitative PCR assay (Q-PCR) which enables EBV load measurement in unfractionated whole blood. This Q-PCR is based on competitive co-amplification of a wild-type target sequence with an internal standard of known amount. We applied this assay in a primary EBV infected lung transplant recipient with two PTLD episodes. Q-PCR became positive at four weeks before both diagnoses (7,000 and 8,600 EBV genomes/ml blood, respectively) and reached peak levels at PTLD (67,000 and 95,000 EBV genomes/ml blood). EBV load decreased dramatically upon reduction of immune suppression and increased upon rejection treatment. As EBV load was shown to vary dramatically within a week, frequent sampling is essential for early detection of PTLD. At present, we are extending our study to other PTLD and non-PTLD patients with primary EBV infection or virus reactivation.

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IgG antibodies to human Herpesvirus-6 in pregnant women

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The prevalence and the level of human herpesvirus-6 (HHV-6) specific IgG were examined in pregnant women and aged-matched blood donors.

The study group consisted of 60 women with normal pregnancy, 60 women with fetus suspected of virus infection and 60 blood donors. Plasma or serum samples were examined for IgG antibodies to HHV-6 by an immunofluorescence assay. Antibody titers ≥10 were considered positive.

Ninety-eight percent of blood donors and 97% of pregnant women had antibodies to HHV-6. The rate of seropositivity of normal and diseased pregnancies was the same. Pregnant women had significantly lower antibody titer then blood donors ($p=0.001$). No significant differences were found in the same respect between the two groups of pregnant women ($p=0.134$).

The high seroprevalence of HHV-6 specific IgG in women of childbearing age suggests that primary infection is expected to be very low. However, there is uncertainty about the role of possible reactivation of the latent virus when the immunity surveillance decreases during pregnancy. Furthermore, antibody to HHV-6 present may not prevent infection with a different genotype of HHV-6.

A. SUCHÁNKOVÁ[1], H. MACHOVÁ[2], L.GLOSOVÁ[3]

Intrathecal antibody synthesis against herpetic viruses in patients with some neurological disorders

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Objectives. Serum and cerebrospinal fluid (CSF) samples from 60 patients with aseptic encephalitis or meningitis, multiple sclerosis and polyneuropathy were investigated to detect intrathecal synthesis of virus specific IgG antibodies against herpes simplex (HSV), varicella zoster (VZV), Epstein-Barr (EBV) and cytomegalovirus (CMV).

Material and methods. Serum and CSF samples from 26 patients with aseptic encephalitis or meningitis, 22 patients with multiple sclerosis, 12 patients with polyneuropathy and 30 controls with dorsopathy were diluted to the same IgG concentration. Antibodies to HSV, VZV, EBV and CMV were detected by indirect ELISA tests. If the Antibody Index AL reaches 1.5 or more, this is indicative of local antibody synthesis in the central nervous system CNS (Reiber and Lange 1991).

Results and conclusions. Intrathecal synthesis of IgG antibodies against herpes viruses was detected in two groups of patients: in 6 (23%) patients with acute CNS

infection – aseptic encephalitis or meningitis – and 7 (32%) patients with chronic disease – multiple sclerosis. Most frequent was intrathecal synthesis of IgG antibodies against CMV which was found in four patients with aseptic encephalitis and three patients with multiple sclerosis. In another four patients with multiple sclerosis, intrathecal synthesis of IgG antibodies against EBNA-1 antigen of EBV was evidenced. Diagnostic value of our results will be discussed.

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Coinfection of enterocyte-like cells by rotavirus and Yersinia spp.

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Cell modifications induced by viruses whose normal habitat is the intestinal tract can alter the adherence and invasiveness of bacterial pathogens, favouring the establishment of more severe infections. In this study, a mixed infection with rotavirus and either *Y. pseudotuberculosis* or *Y. enterocolitica* was analysed in Caco-2 cells, an enterocyte-like cell line highly susceptible to these pathogens. Results obtained showed an increase of bacterial adhesion and internalization in rotavirus infected cells. This enhancement was dependent upon the time of rotavirus infection and the maximal increase was observed 5 h postviral infection. Similar results were obtained with *E. coli* HB101 (pRI203) strain, harbouring the inv gene from *Y. pseudotuberculosis*, known to be involved in the invasion process of host cells. In contrast, the superinfection with bacteria of Caco-2 cells preinfected with rotavirus resulted in a decreased viral antigen synthesis. Transmission electron microscopy observations confirm these findings. Results obtained suggested that the rotavirus infection may induce alterations at the level of enterocyte-like cell surfaces which modulate and favour the early interaction of *Y. enterocolitica* and *Y. pseudotuberculosis* with target cells.

A. SVAHN, H. GAINES, A. LINDE

Cell-mediated immunity against varicella zoster virus

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Aim. To evaluate the measurement of cell-mediated immunity (CMI) against varicella zoster virus (VZV) using identification of blast-transformation in whole-blood cultures as detected by flow cytometry (WB/FC) compared to peripheral blood mononuclear cell (PBMC)/DNA synthesis assay.

Methods. CMI to VZV was determined by the measurement of *in vitro* lymphocyte proliferation induced by antigen: by detection of incorporated tritiated thymidine in

PBMC cultures (conventional lymphocyte stimulation); and by WB/FC. The proliferative response was monitored as development of lymphoblasts by the FC assay. The cpm obtained in the DNA synthesis assay was compared with the development of lymphoblasts measured as % of lymphocytes in the FC.

Results. Ten patients have been analysed regarding CMI against VZV with both methods. Eight patients were seropositive and also displayed CMI by both assays. Two patients were seronegative and no stimulation indices by the PBMC/DNA assay and no development of lymphoblasts, as detected by the WB/FC assay, could be measured.

Conclusion. The two methods, PBMC/DNA synthesis and WB/FC assay, are in concordance in the initial part of the study. Further samples will be examined to compare the two assays. The optimal method will be used for examination of specific CMI in VZV antibody-negative patients, and also in vaccinated persons, to evaluate whether some of these may display a measurable lymphoproliferative response.

C. M. A. SWANINK, F. E. STRIJKS, N. P. M. SCHAAP

Cytomegalovirus encephalitis in a patient after bone marrow transplantation

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A 35-year-old patient with a diagnosis of chronic myeloid leukaemia underwent a bone marrow transplantation from a matched unrelated donor. Two months after transplantation she developed fever and interstitial pneumonia. A bronchoalveolar lavage (BAL) was strongly positive for cytomegalovirus (CMV) and the antigenaemia test (pp65) was positive, too. She was treated with ganciclovir 5 mg/kg b.i.d. i.v. for 14 days. In addition, she received CMV-immunoglobulin once per week. Shortly after discontinuation of ganciclovir, she had a relapse that was initially treated with ganciclovir, followed by foscarnet 6 gram b.i.d. intravenously for 3 weeks. One month later she had another episode of CMV pneumonitis that was treated with foscarnet and CMV-immunoglobulin. After clinical improvement she went home with oral ganciclovir 1 gram t.i.d. One week later she presented with neurological signs: diplopia, nystagmus, vomiting, cerebellar ataxia, and intention tremor. A CT-scan was normal. In the spinal fluid 2 leukocytes, normal protein and glucose level was found. Because the pp65 was positive again a polymerase chain reaction on cerebrospinal fluid was performed that was positive for CMV. She was treated with ganciclovir i.v. b.i.d. for 3 weeks. After clinical improvement she went home. Ganciclovir treatment was continued 3/week i.v. Consecutive cerebrospinal fluid samples showed oligoclonal immunoglobulin response with high affinity for cytomegalovirus, which confirmed the diagnosis cytomegalovirus encephalitis.

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Molecular comparison of an echovirus type 11' (prime) Hungarian epidemic strain with other European, Japanese and Israelian strains from sporadic cases

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An epidemic provoked by an echovirus 11 prime (EV11') strain occurred in 1989 in Hungary causing hemorrhagic hepatitis of lethal outcome in 13 newborn babies. The molecular comparison of the Hungarian isolates with the prototype EV11 (strain Gregory) and with other EV11 strains (Finnish, Dutch, Romanian, Russian, Japanese and Israelian) isolated from sporadic cases was the aim of this study. Four hundred and forty nucleotides (nucleotide position 163-603) of the 5'-untranslated region (5'-UTR) were amplified by RT-PCR and sequenced. Moreover, 250 nucleotides of the VP1 capsid coding region (3001-3260 nt.) and 477 nucleotides of the 3Dpolymerase/3'-UTR end region (6995-7372 nt.) were determined. For phylogenetic reconstructions, groups of nucleotide sequences were aligned with the Clustal W program. Phylogenetic relationships were inferred with the programs Neighbor and DNApars (Phylip package 3.5).

When the VP1 coding region was considered, most of the analysed EV11 strains including the EV11' epidemic isolate clustered in the same subgroup of the enterovirus phylogenetic group B (Coxsackie B-like group). This suggests that the VP1 coding genomic region contains serotype-specific information that can be used for enterovirus identification. The Hungarian isolate was closely related to some Romanian and Finnish strains but differed from the other EV11 strains, in particular, from the EV11 strain Gregory. When the 5'-UTR and 3D/3'-UTR genomic regions were considered, the Hungarian EV11' strain was related to its closest European strains, and to some other EV11 strains. Relationship to the human echovirus 9 and/or the swine vesicular disease virus (SVDV) strains was also observed suggesting that intertypic recombination perhaps contributes to the evolution of these enteroviruses. These results prepare the way for the detailed analysis of the determinants which differentiate the epidemic EV11' from its neighbouring strains isolated from sporadic cases.

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Detection of unidentified and recently discovered hepatitis viruses in Hungary

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Sera of blood donors with elevated transaminases, sera of polytransfused patients and sera of patients suffering from non-A-E hepatitis were tested. 51 of 2076 were found

to be reactive to reagents prepared from the yellow fever flavivirus vaccine strain. Nested PCR products were obtained with GBV-B primers from double-stranded DNA in 20 of 391 of non-A-E hepatitis cases. No GBV-A product was obtained. According our data, HGV is rare (2 of 119) among the patients suffering from non-A-E hepatitis and it does not seem to be the main cause of cryptogenic hepatitis. The newly discovered single stranded DNA virus, TTV is common (9 of 19) in polytransfused patients having non-A-E hepatitis.

R. TEDDER, W. QUINT FOR THE HPV STUDY GROUP

Detection and typing of HPV DNA in cervical cells, implications for patient management

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Objectives. To compare the MY09/11 PCR with the newly developed SPF10 PCR assay for detection of HPV DNA in cervical cells and to investigate the implications of HPV typing for the management of women with cervical intraepithelial neoplasia (CIN).

Methods. Cytology, MY09/11 PCR and SPF10 PCR and viral genotyping were performed on cervical cells collected from 98 consecutive women attending the colposcopy clinic of UCLH. Amplicons were detected in a microtiter-based hybridization assay and typed by a Line Probe Assay for 25 known genotypes.

Cytology (n)	MY09/11 positive	SPF10 positive
Normal (54)	12 (22%)	39 (72%)
ASCUS (11)	5 (45%)	10 (91%)
Mild dyskaryosis (17)	12 (71%)	16 (94%)
Moderate dyskaryosis (9)	6 (67%)	6 (67%)
Severe dyskaryosis (7)	6 (86%)	7 (100%)
Total (98)	41 (42%)	78 (80%)

Results. MY09/11 detected HPV DNA in 42% and SPF10 in 80% of specimens (Table). High risk HPV DNA, often in multiple infections, was detected in 18 (33%) of 54 samples with normal cytology and 30 (66%) of 44 samples with abnormal cytology and may have been influenced by prior treatment.

Conclusions. The new SPF10 amplification system was more sensitive than MY09/11 in detection of HPV DNA. Typing can be helpful in planning the treatment and follow-up of women with CIN. Introducing HPV typing as an adjunct to cytology in colposcopy follow-up will allow better use of clinical resources.

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Evaluation of Copalis CMV multiplex versus plasma PCR authors for diagnosis of CMV viremia

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Objective. To evaluate the Copalis CMV Multiplex assay (Diasorin) for the detection of antibodies to CMV in response to CMV infection with the CMV plasma PCR (Roche) for the detection of CMV viraemia in renal-transplant and bone marrow transplant recipients (RTR & BMT).

Method. Plasma samples were collected weekly from 20 RTRs and 20 BMT patients post-transplant. All samples were tested for CMV using the CMV plasma PCR and Copalis CMV Muliplex. The Copalis CMV Multiplex assay uses polystyrene microparticles with three different antigens specific for the infection phase so the reactivity pattern in the patient sample allows dating of infection. The CMV Multiplex assay takes 20 minutes for results to be available.

Results. CMV viraemia was detected by at least one test in 121/240 (50.4%) samples from 40 patients. Plasma PCR detected CMV in 108/240 (45%) of patients, compared with CMV Multiplex, which detected viraemia in 71/240 patients (29.5%).

The plasma PCR was more sensitive than the CMV Multiplex and detected CMV viraemia 1–2 weeks before plasma PCR. Although CMV Multiplex did not detect infection in three out of four CMV infections in BMT patients, it was able to detect all CMV infections in renal transplant patients.

Conclusions. The CMV Multiplex is a rapid means of detecting CMV viraemia. Although less sensitive than plasma PCR, it may be more useful and cheaper in detecting clinically relevant CMV infection in renal transplant recipients.

D. THAKKAR

Impact of high throughput DNA technologies on genomics

High throughput screening systems exemplified by DNA chips, are poised to significantly impact drug discovery processes. DNA microarray systems coupled with high throughput robotics will enable parallel analysis of a large population of genetic targets. DNA arrays will shift the paradigm of drug development by impacting the traditional bottlenecks of drug discovery. Pharmacogenetics will help identify novel targets for the drug development process by pairing gene expression to the onset and progression of disease, thereby providing increased small and macromolecule drug targets. Pharmacogenomics on the other hand will identify individual genetic variations and their potential impact on drug activity. These technologies will expedite the drug

development process and not only reduce the overall cost of development but also bring safer, more effective drugs to the market sooner.

A general overview of these automated approaches will be provided, along with a glimpse of technologies on the horizon of development.

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TT virus infection in Turkish patients with fulminant hepatic failure and cryptogenic liver diseases

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A novel single-stranded DNA virus (TTV) has been reported in patients with post-transfusion non-A-G hepatitis. A high prevalence of TTV has been described in patients with chronic liver diseases and fulminant hepatitis of unknown etiology. The aim of this study was to determine the prevalence of the virus among different populations in Turkey. We have analysed the prevalence of TTV in sera from patients with liver disease of different etiology and from patients with a high risk for blood-borne viruses, HIV seropositive and thalassemia patients. The results are shown in the table below. TTV DNA was detected in 20 (13%) of 148 patients with liver disease not significantly different as in 3 (10%) of 31 normal controls, but significantly different in HIV seropositive patients and thalassemia patients which means that TTV is mainly parenterally transmitted.

Among patients with chronic liver disease TTV DNA tended to be more frequent in those with liver disease of unknown etiology than in those with chronic hepatitis B or C. This results indicate that TTV infection is highly prevalent in Turkish population. The association of this novel virus with a particular disease requires further investigation.

Patients	No.	TTV-DNA positive (%)
Chronic B hepatitis	22	3 (13.6)
Chronic C hepatitis	69	5 (7.2)
Cryptogenic chronic active hepatitis	13	5 (38.4)
Cryptogenic liver cirrhosis	34	7 (20.5)
Fulminant hepatitis	3	0
Acute viral hepatitis (non-A-G)	7	0
HIV seropositive	34	17 (50)
Thalassemia	10	8 (80)
Normal controls	32	3 (9.3)
Total	224	48 (21.4)

A. UZUNOVA

An attempt in obtaining resistant variants of influenza virus under the influence of oxadin

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Our previous studies have revealed the antiviral effect of oxadin (oxadiazine derivative) on the reproduction of some influenza A viruses. Resistant variants of influenza A (H_3N_2) virus obtained following the 7th passages were evaluated. 30% of virus populations remained resistant between the 7th and 13th passages when the oxadin was not present in the milieu. Some relationship between the resistant and sensitive virus populations were observed after 13th passages. The resistant variants obtained under the influence of oxadin prove its specific antiviral action.

E. VALENTINE-THON

Amplicor HCV test version 2.0 compared to Sharp Signal System^{tm} for HCV RNA detection

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Purpose. The sensitivity and reproducibility of the recently introduced "Amplicor hepatitis C Virus Test, Version 2.0" (Roche Boehringer) for detection of HCV RNA in patient sera was evaluated and compared to our standard HCV RNA assay system "SHARP". Both systems are RT-PCR based but utilize different RNA isolation and amplicon detection methods.

Methods. In AMPLICOR, HCV RNA was isolated with the kit-provided lysis reagent (GITC, DTT, glycogen), amplified in a 1-tube RT-PCR, and detected by solid-phase hybridization to a DNA probe. In SHARP, HCV RNA was isolated with the QIAamp Viral Kit (Qiagen), amplified in a 2-step RT-PCR, and detected by liquid hybridization to an RNA probe in the SHARP Signal System^{TM} (Digene/Abbott).

80 samples were tested: 53 patient sera (tested in duplicate in AMPLICOR and singlet in SHARP) and 27 samples (tested in duplicate in both assays) from 5 dilution series of 3 HCV standards (EUROHEP genotype 1, VQC genotype 1, INSTAND genotype 1b) and 2 HCV-quantitated patient samples.

Results. Of 53 patient samples tested in both assays, 52 (98.1%) were concordant, with the 1 exception AMPLICOR-pos. and SHARP-neg. but HCV seropos. and thus presumably weakly viremic. The reproducibility of AMPLICOR was 98.1%, with the 1 discrepant sample SHARP-neg. but HCV seropos. and likewise presumably weakly viremic. Of the 5 dilution series (range: 10^0 to 3.3×10^4 copies/ml), AMPLICOR was consistently the more sensitive assay, detecting 3.3 copies/ml while SHARP detected 33 copies/ml. The average sensitivity level with 100% reproducibility was 117 copies/ml for AMPLICOR and 1168 copies/ml for SHARP.

Conclusions. 1. The new Amplicor HCV Test, Version 2.0 detects HCV RNA in patient samples with high reproducibility (98.1%) and high sensitivity (117 copies/ml with 100% reproducibility). 2. The new Amplicor HCV Test, Version 2.0 is ca. 10× more sensitive than the SHARP Signal System™.

D. W. VAN DAM, C. J. SCHINKEL, L. C. VAN DINTEN, W. J. M. SPAAN, A. C. M. KROES

Persistence of TT virus (TTV) coinfection among chronic hepatitis C patients before and after interferon treatment

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Recently, by representational difference analysis a new human unenveloped single-stranded DNA virus named TT virus (TTV) has been discovered in Japan. TT represents the initials of the first patient, who suffered from a post-transfusion non-A to G hepatitis. By pure coincidence TT could also stand for Transfusion Transmitted, and Transfusion Transmitted Virus is actually used by some authors, also indicating one possible route of transmission. TTV shares several characteristics with Parvoviridae and Circoviridae. The viral genome contains at least 3700 bases, and most likely two open reading frames (ORF). There are at least two different genotypes. TTV in serum has only been detected by nested PCR. At this moment a serological assay is not available.

We retrospectively analyzed serum samples of 49 chronic hepatitis C (HCV) patients before and after interferon (IFN) treatment for the prevalence of and influence on TTV. TTV was present in 9 (18%) before and 7 (14%) patients after treatment. There was clearance in 6 patients, persistent TTV infection in 3 patients. Four patients were TTV positive only after treatment. There was no significant relationship between persistent TTV infection and clearance of TTV regarding to age, gender, HCV clearance after therapy, and alanine aminotransferase (ALT) levels.

We conclude that TTV is widely present among chronic HCV carriers and that this infection may respond to IFN therapy, although not invariably. Patient characteristics related to viral persistence could not yet be identified. Quantitative aspects of this infection should be included in further studies.

H. G. A. M. VAN DER AVOORT, A. RAS, J. W. DORIGO-ZETSMA

Enterovirus surveillance in The Netherlands: evidence for the absence of wild poliovirus circulation

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Vaccination, surveillance and certification are the cornerstones of the programme to reach the goal of global polio eradication by the year 2000. Certification of regions as polio free zones can only be obtained when intensive surveillance activities coupled to

correct laboratory data have shown the absence of wild poliovirus circulation in all countries of the region during at least three years. Gold standard for the surveillance of poliomyelitis is the surveillance of acute flaccid paralysis (AFP). A minimum rate of 1 case of non-poliovirus caused AFP in 100,000 children below 15 years of age is required for surveillance to be considered as sufficient, provided for each AFP case appropriate viral analysis of two stool specimens has been performed in a WHO-accredited laboratory. Non-polio enteroviruses have many characteristics in common with polioviruses, such as site of replication, disease manifestations and patterns of virus shedding and transmission, and can therefore be detected by the same diagnostic procedures in the same type of patients and materials. To be useful as proof for the absence of wild poliovirus circulation, a system of enterovirus surveillance needs to contain: 1. collective data on the number of all faecal specimens that were cultivated on poliovirus-sensitive cells obtained from children below the age of 15 years. 2. collective data on the number of enterovirus-positive isolates from these specimens that were proven not to be wild polioviruses (by growth characteristics, typing, intratypic differentiation or genetic characterization), and 3. a quality control system guaranteeing the optimal performance of all diagnostic procedures.

In The Netherlands, data on enterovirus surveillance were obtained from 14 (74%) of the 19 virological labs. In 1996, 1997 and 1998 over 15,000 stool samples received for detection of viruses were subjected to viral culture in these labs. 980 (6.5%) samples were enterovirus positive. Eight enteroviruses were Sabin-derived polioviruses, isolated from asymptomatic patients. In all cases the patients were recently vaccinated in OPV using countries outside The Netherlands. 90% of all virological laboratories passed the yearly proficiency test for isolation and typing of enteroviruses in stool samples. The system of enterovirus surveillance in The Netherlands has shown the proven absence of wild polioviruses in the past three years.

A. M. VAN LOON, K. E. VAN VLIET, P. MUIR, J. M. ECHEVARRIA, P. KLAPPER, G. CLEATOR FOR THE EU CONCERTED ACTION ON QUALITY CONTROL OF NUCLEIC ACID AMPLIFICATION IN DIAGNOSTIC VIROLOGY

Multicenter proficiency testing for the molecular detection of enteroviruses

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Introduction. The aim of the European Union (EU) Concerted Action (CA) on Quality Control of Nucleic Acid Amplification in Diagnostic Virology is to establish a Quality Control (QC) programme to assess and evaluate existing and evolving nucleic acid amplification procedures used in diagnostic virology.

Approach. The programme started in 1998 and includes distribution of proficiency panels of six different (groups of) viruses within the next three years. The first panel was distributed in December 1998 and focused on enteroviruses. The panel consisted of 12 samples with various concentrations of heat-inactivated, freeze-dried enteroviruses, and

negative controls. Positive samples included poliovirus type 2, echovirus 9 (2×), echovirus 22 and a dilution series of Coxsackievirus A9.

Results. A panel and a questionnaire were sent to 63 laboratories in the European region. Transport time was 3 days or less for 85% of the participants. Fifty-nine laboratories reported a total of 71 data sets; 4 did not submit results. Of the reports, 19 were obtained within 3 weeks after distribution of panels, 32 between 3 and 6 weeks, and the remaining 10 were received within one week after sending a reminder. One of the data sets concerned results of an echovirus 22 and 23-specific PCR only and was excluded from analysis.

Three laboratories reported a false-positive result in one of the two negative samples; one laboratory in both. Classifying positive samples as strongly, intermediately and weakly positive, over 90% of laboratories detected the strongly positive samples correctly and over 80% the intermediately positive samples; 43% and 15% of participants correctly reported one or both of the weakly positive samples, respectively. An in-house PCR was used for 52 data sets, a single PCR in 17 labs, a seminested in 8 and a nested PCR in 27 labs. The Roche Amplicor assay was used 16 times. A scoring system was used to compare performance of laboratories and PCR's. Results of approximately 70% of data sets were as good or better than those from the two reference laboratories. The use of a nested format was significantly associated with a better performance. The performance of the Roche Amplicor assay was comparable to that of single in-house PCR's.

Conclusion. Most laboratories performed satisfactorily on the first EU-CA enterovirus proficiency panel. The majority used an in-house PCR; a nested format was significantly associated with better performance. The next distributions are planned for September 1999 and May 2000.

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European hantaviruses: epidemiology and diagnostics

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Only two hantaviruses, Puumala (PUU) and Dobrava (DOB) were seen to cause human disease in Europe according to reliable typing methods (focus-reduction neutralization assays using appropriate hantavirus serotypes or RT-PCR with subsequent sequencing). In addition, Tula (TUL) virus can infect man but is not associated with human disease.

We have developed sensitive and specific IgM and IgG EIAs based on baculovirus-expressed PUU, DOB and HTN nucleocapsid proteins. The IgM test is applicable also using peroxidase-conjugated N antigen in a μ-capture EIA format and further development towards a rapid immuno-chromatography test is in progress. Our data suggest that baculovirus-expressed N is antigenically indistinguishable from the native protein and the whole N protein is more sensitive than a truncated N-terminal antigen. Furthermore, although during the first days of the disease IgM and often also IgG

antibodies are present, in rare cases (<2%) PUU IgM antibodies may be negative up to 5 days after onset of illness.

We detected 1989–1996 7000 PUU infections in Finland (957/year) resulting in an incidence of 19/100 000 (in the Etelä-Savo district with highest incidence 90/100 000) with a mortality of less than 0.1%. Local epidemics mirrored bank vole densities with 3–4 year cycles. Women contracted the disease at 44 (mean), men at 40 years (male:female ratio 2:1). The PUU antibody prevalence for women entering the maternity clinics nationwide was 3%, but far the whole population in various panels from endemic areas, 10–20%.

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Four year follow-up of patients from a BMT UMT by determination of EBV viremia

Epstein-Barr virus (EBV) is implicated in B lymphoproliferative disorders, which occur in deeply immunosuppressed patients. A convenient semi-quantitative determination of EBV viremia has been devised. PBMC recovered by ficoll gradient were counted. Aliquots of 5µL of cell suspension and dilutions (containing 500 and 100 PBMC) were submitted to a nested PCR.

From September 1995 to September 1998, 45 patients attending the BMT unit were followed. Thirteen were PCR positive at least once during this period. Eleven patients received a BMT, one had a lymphohistiocytosis and one newborn had a SCID syndrome. Four patients presented with a moderate EBV proliferation (PCR positive for 500 cells or more), three had a favorable outcome. Among patients with high viral load (PCR positive with 100 cells) two deaths were related to graft failure and one to a fatal evolution of a neuroblastoma. One patient had a transient EBV reactivation. For the 5 others a lymphoma was diagnosed 4 times and one died of lymphoproliferation.

The follow-up of patients is useful as the positivity of EBV-PCR for a long period is of poor prognosis and should bring to investigate for a lymphoproliferative syndrome.

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Chlamydia pneumoniae and various herpesvirus DNA in human atherosclerotic plaques

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Certain pathogens may be involved in the initiation and progression of atherosclerosis (AT). Some seroepidemiological data have indicated that the anti-human cytomegalovirus (HCMV) and anti-*Chlamydia pneumoniae* (*C. pneumoniae*) antibody

titer is correlated with AT, but other data have not confirmed such a correlation. Additionally, either HCMV or *C. pneumoniae* have been detected in various percentages in AT plaques by polymerase chain reaction (PCR), immunocytochemistry, *in situ* hybridization or electron microscopy. To investigate the potential involvement of multiple infectious agents in the development of AT, we tested, by nested PCR, the presence of both HCMV and *C. pneumoniae* and other ubiquitous lymphotropic herpes-viruses, e.g. EBV and HHV-6, in the same carotid plaque, or aortic or mitral valve samples, obtained from carotis endarterectomy or valve-replacement surgery, respectively. Eighty percent of 35 AT samples contained at least one and some samples contained more than one of these pathogens. No significant correlation was observed between the level of antibodies specific to HCMV or *C. pneumoniae* and the presence of these pathogens in the plaques of the patients. These studies are important in the assessment of the infectious theory and possible prevention and therapy of AT.

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First experiments with an accelerated cytomegalovirus antigen detection system: CMV Brite Turbo

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Introduction. Detection of cytomegalovirus (CMV) pp65 antigen in leukocytes is widely used for early detection and monitoring of CMV in patients at risk. The CMV Brite test (IQ Products) is a 4 hour indirect fluorescent test kit which is based on isolation of leukocytes by dextran. In this study we evaluated an accelerated version of the Brite test, called "CMV Brite Turbo". This test kit employs a modified leukocyte isolation procedure using lysis of erythrocytes and has a reduced incubation time schedule.

Methods. Blood samples of transplantation patients were prospectively collected and both the classical Brite test and the Turbo Brite test were performed on each sample.

Results. Hundred and seven blood samples were collected. Twenty-six samples had a positive test result for both the classical and the accelerated Brite assay. Discordant results occurred in 8 samples and were restricted to samples with low numbers of positive cells (range 1–2). The Brite Turbo test counted more positive cells than the classical Brite test (221 versus 120, $P<0.05$). This could be explained by a 25% higher input of granulocytes on the cytocentrifuge preparations. The sensitivity of the Turbo Brite test was not increased. The test performances of the Turbo Brite test compared favourably to the classical test. The time span required for the completion of the test was reduced 1 h and 40 min to 2 h and 20 min (75 min less hands-on time), and the morphological aspects of the cell preparations appeared to be better.

Conclusions. Implementation of the Turbo Brite kit can save considerable hands-on time and will reduce the turn-around time for CMV antigenemia detection with 35%. The sensitivity and specificity of the accelerated test appear unchanged.

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Quantitative method to determine the coreceptor usage of human and simian immunodeficiency viruses, HIV and SIV

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The human osteosarcoma cell line, HOS, has been engineered to stably express CD4, the primary receptor used by HIV and SIV, and each of the coreceptors (CCR3, CCRS, CXCR4, BOB /gpr15/ or Bonzo /STRL33/). The cells carry the gene of the green fluorescence protein (GFP) driven by the HIV-2_{ROD} long terminal repeat (LTR). Upon infection of these cells, designated GHOST(3), the viral Tat protein – which is one of the earliest viral proteins expressed by infected cells – transactivates the GFP gene through the HIV-2 LTR. Consequently, infected cells produce GFP and become green when observed in UV microscope. The method is easy, fast and cheap; involves a simple microscopic observation of the outcome of virus infection within three days.

Quantitative instrumental measurements are also possible by using a flow cytometer (FACS). Quantitative data about the proportion of fluorescing cells, and fluorescence intensity can be obtained. We calculated for each sample the fold difference to uninfected control cells by taking into account the percentage of fluorescence positive cells and the fluorescence intensity. The value thus obtained is characteristic for the virus and allows comparison of the results of experiments carried out on different occasions.

To determine whether productive infection has taken place, the viral protein p24 (HIV) or p26 (SIV) in cell supernatants was measured six days after infection. For this purpose we used an enzyme linked immunosorbent assay (ELISA).

Taken together, with the help of the GHOST(3) cell system we could extend biological characterization of HIV and SIV to include co-receptor usage. Results were obtained in three different ways; by UV microscopy and flow cytometry we tested events immediately preceeding virus entry, by ELISA we measured virus production.

With this experimental system we tested the coreceptor usage of

1. HIV-1 (mainly subtype A) isolates obtained from pregnant women in Cameroon; and 2. sequential isolates from monkeys experimentally infected with different SIVsm (of sooty mangabey origin) isolates.

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Surveillance of respiratory pathogens and influenza-like illnesses in general practices in The Netherlands in winter 1998-99

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Introduction. NIVEL is running a surveillance network of 43 sentinel general practices (GP), covering 1% of the Dutch population and representative with respect to region, urbanisation, age and sex. The incidence of influenza-like illnesses (ILI) is calculated weekly during the winter season by NIVEL from the data of the network. On NIVEL request the system was extended with virus detection from nose/throat swabs by the RIVM. Swabs are obtained from patients with an acute respiratory, infection (ARI), of whom about 70% are registered with ILI. The aim of the study was to provide insight in the etiology of ARI in the primary care, population.

Materials and methods. A total of 405 nose/throat swabs were examined by cell culture and PCR, using standard procedures. PCR was performed for respiratory syncytial virus, rhinovirus, enterovirus, coronavirus OC43 and 229E and *Mycoplasma pneumoniae.*

Results. In 58% of the samples at least one micro-organism was detected. 28% of the pathogens were recognized by PCR only. In 5% of the samples a double-infection was observed. Influenza viruses were detected most often (25%), followed by rhinoviruses (13%). Of the rhinoviruses, 77% was detected by PCR only, whereas 23% was detected by isolation and PCR. Registration of ILI and isolation of influenza viruses were in accordance with each other. However, in 26% of the patients registered with ILI other respiratory pathogens than influenza virus were detected (44% rhinovirus) and in 47% no pathogen was detected.

Conclusions. 1. Influenza viruses and rhinovirus were the predominant viruses detected in patients with ARI from a GP sentinel system. 2. Application of the PCR improved the detection of respiratory pathogens in nose/throat swabs considerably. 3. Registration of ILI by GP's was in accordance with isolation of influenza viruses in the laboratory.

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Parvovirus B19 persistent infection in haemophiliacs with progressive arthropathy

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Parvovirus B13 infection may cause arthralgia and acute arthritis mainly in adults. In a subset of these patients the arthropathy may persist for months or years. A progressive arthropathy is a common complication which occurs in haemophiliacs. Its etiology can be only partially explained by the intra-articular bleeding and it has been hypothesized that other factors such as infections may contribute to the appearance and progression of this disorder. B19 infection is frequently transmitted by clotting factor concentrates prepared from large plasma pools and is highly prevalent in haemophiliacs. This study has been performed with the aim to assess if parvovirus B19 infection is involved in haemophilic arthritis. We have analysed synovial tissues from 60 haemophiliacs and from 20 patients with arthrosis as controls for the detection of B19 DNA sequences. After DNA extraction and quantification from the bioptic tissue, two nested PCR have been performed to amplify two B19 genomic regions. Moreover B19 antibody detection in sera from patients and controls has been done; IgM and IgG against VP1 and VP2, the two B19 capsid proteins, have been detected by commercial ELISAs (Biotrin), whereas for IgG against the non-structural viral protein NS1 a commercial Western blot assay (Mikrogen) has been employed.

Anti-NS1 antibody, in fact, could be related with persistent B19 infections. The prevalence of B19 DNA in synovial tissues was significantly higher in haemophiliacs than in controls. In addition a significantly higher prevalence of anti-NS1 antibody was shown in haemophiliacs than in controls. A strong relationship was observed between B19 DNA presence in synovial tissues and anti-NS1 antibody presence in sera. Further investigations are required to better assess the pathogenetic role of B19 virus in haemophilic arthritis. If confirmed, the association of B19 infection with haemophilic arthritis could allow to assess diagnostic, therapeutic and preventive measures.

E. ZUSINAITE, R. SALUPERE, E. RAUKAS, K. OTT, K. KIIVER, V. USTINA, J. SCHMIDT, L. SIZEMSKI, T. KRISPIN, M. USTAV

Hepatitis C virus genotypes in Estonia

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Hepatitis C virus (HCV) is one of the major causes of the chronic damage of the liver, which can lead to liver cirrhosis and hepatocellular carcinoma. HCV is characterized by a high degree of heterogeneity; there are six major genotypes and many subtypes,

which are playing different role in pathogenesis of the disease, response to antiviral treatment and transformation into hepatocellular carcinoma.

We have analyzed serum samples from 169 consecutive HCV RNA positive patients to determine genotype of HCV. For genotyping there was used a genotyping system recently described by T. Ohno et al., based on polymerase chain reaction (PCR) of the 5' non-coding (5' NCR) – core region of the HCV genome with genotype-specific primers, that allows for identification of genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a.

Results. HCV genotype was determined in 159 patients (94.1%). Different genotypes were distributed as follow: 1a – 1 patient (0.6%); 1b – 76 (56.6%); 2a – 17 (10.7%); 3a – 24 (15.1%); 4 – 1 (0.6%); mixed genotype – 26 (16.4%). To control the high presence of mixed genotypes we use another typing method, based on restriction fragment length polymorphism (RLFP) of the amplified DNA from the 5' NCR region.

Conclusion. HCV genotype 1b was the most prevalent in Estonia.

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HCV genotype as predictor of response to INF therapy

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Aim. To evaluate the relations between HCV genotype and variations of RNA in the patients with chronic HCV hepatitis treated with interferon.

Patients and methods. 120 patients with chronic HCV hepatitis treated with INF alfa 2a, trice weekly with 3MU, for at least 6 months, 63.33% M, 36.66% F, mean age 37.31 y (range 16–68). Serum HCV RNA was measured by PCR (Roche HCV Amplicor and Monitoring) before, during and after treatment. Mean follow-up period after therapy was 9.41 months (up to 23). Genotyping was performed after amplification by line-probe assay (Inno-Line, Inogenetics).

Results. At the beginning of the study 13 patients (10.83%) had very low or undetectable concentration of RNA and stayed as that, so the virus genotype could not be determined. Genotyping was performed in 107 patients. Among them type 1 had 69 patients (64.48%, type 1 without subtype in 7=6.54%, 1a in 7=6.48%, 1b in 55=51.4%), type 3 had 35 patients (32.71%) and type 4 had 3 patients (2.8%). Response to INF therapy had 47 patients (39.16%, with complete response up to negativisation in 33.33%) and their genotypes were: type 1 without subtype in 14.89%, 1a in 14.89%, 1b in 19.74% and type 3 in 51.06%. Transient response with relapse after therapy had 9 patients (7.5%) and in all of them virus genotype was 1b (100%). Concentration of HCV RNA increased during the therapy in 13 patients (10.83%) and they also had genotype 1b in 100% of cases. In the group of nonresponders were 38 patients (31.66%) and they had subtype 1b in 63.15%, type 3 in 28.94% and type 4 in 7.89%.

Conclusions. In our study group the most prevalent was subtype 1b (51.4%). Differences in the presence of subtype 1b between responders (19.74%) and nonresponders (63.15%) or transient responders (100%) is statistically important

($p<0.001$). This supports our hypothesis that response on therapy strongly depends on virus genotype, i.e. subtype 1b of HCV is bad predictor.

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Comparison of plasma HIV-1 RNA quantification assays in a cross-sectional study involving individuals believed to have been infected in Africa and Western Europe

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Four commercial assays for plasma HIV-1 RNA quantification NASBA QT (Organon-Technika), Quantiplex Version 2.0 (Chiron) and Amplicor Monitor Versions 1.0 and 1.5 (Roche) were evaluated in a cross-sectional study. The first three assays were optimized for subtype B virus strains and the Amplicor 1.5 assay included a new primer set. Increasing numbers of individuals with non-subtype B virus infections are being reported and it has been recognized that some assays may underestimate HIV RNA load due to mismatches between the probes used in the assay and the target sequence.

Plasma HIV-1 RNA levels were determined by the four assays in a cross-sectional study including samples from 107 individuals believed to be infected in Africa (Group 1) with non-B virus strains and a control group of 102 individuals believed to be infected with subtype B virus strains (Group 2). Comparison of the Amplicor Version 1.5 with NASBA, Quantiplex Version 2.0, and Amplicor Version 1.0 in samples from Group 1 revealed >1.0 log differences between assays in 15, 13, and 14 samples respectively and for group 2, 6, 2, and 5 samples respectively. Moreover, HIV RNA levels below or at the limit of assay detection in either 1, 2, 3 or all 4 assays were found in 44 antiretroviral naive HIV-1 positive individuals. These results suggest that significant differences in HIV RNA levels may be attributed to assay performance especially in individuals infected with non-B virus strains.

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