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"HIV-PEPLOTION VACCINE" – A NOVEL APPROACH TO PROTECTION AGAINST AIDS BY TRANSEPITHELIAL TRANSPORT OF VIRAL PEPTIDES TO LANGERHANS CELLS FOR LONG-TERM ANTIVIRAL CTL RESPONSE*

(A REVIEW)

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Abstract

Viral vaccines which stimulate the humoral immune response in humans have been successful in preventing most of the known virus diseases except dengue fever, respiratory syncytial virus infections and HIV-1-related AIDS. Burke [1] raised a concern that anti-HIV-1 antibodies may add a risk factor to immunized individuals infected with HIV-1. An approach to develop HIV-1 vaccines capable of stimulating anti-HIV-1 cytotoxic T cells requires an

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understanding of the importance of epidermal and epithelial Langerhans cells (LC). These cells are professional antigen-presenting cells which express HLA class I and class II molecules. Epithelial LC are present in a specific layer in the skin, genitalia and gut and may be accessible to viral antigens by local application in a vehicle for transepithelial transport of viral proteins/peptides (designated "HIV-1 Peplotion vaccine"). This approach is supported by the reports that HIV-1 gp160 in ISCOM induced MHC class I CTL response [2], mixing of cationic lipids with viral proteins formed complexes which were delivered to cell cytoplasm and the degraded peptides stimulated CTLs by HLA class I mechanism [3] and viral proteins encapsulated in pH-sensitive liposomes administered to LC induced primary antiviral CTLs [4]. Current studies in our laboratory deal with (a) selection of the vehicle for transepidermal transport of peptides and the conditions for selective uptake by epidermal LC [5]; (b) computer analysis of HIV-1 proteins to detect the putative proteolytic cleavage peptides with amino acid motifs which allow association with different known HLA class I haplotype molecules on LCs and synthetic peptide uptake from "without" by LC.

The "HIV-1 Peplotion vaccine", when developed, will be useful for continual stimulation of antiviral CTLs in uninfected individuals and HIV-1 carriers by repetitive application to skin, genitalia and gut. The "Peplotion vaccine" will be applied by vaccinees, will be affordable for all human-populations and, hopefully, will be highly efficient.

Introduction

Infection of humans with human immunodeficiency virus (HIV-1) leads to stimulation of host cellular and humoral immune responses and synthesis of $CD8^+$ cytotoxic T cells, which react to epitopes on gp160 [6–8], *gag* [9, 10] and *pol* proteins [11], as well as antibodies to antigenic epitopes present on all the viral proteins. After a period of inapparent infection which may be as long as 5 to 10 years, the $CD8^+$ T cells decline and antiviral antibodies, reaching high titers, enhance virus replication, leading to the development of acquired immune deficiency syndrome (AIDS). At this stage, in addition to opportunistic bacterial and fungal infections and Kaposi's sarcoma [12], endogenous latent viruses of the herpesvirus family (CMV, HSV-1, EBV, HHV6) are activated and may lead to fatal diseases.

The current approach to vaccine development is to use: 1) inactivated, detergent-treated or formalin-treated HIV-1 virions, 2) multivalent HIV protein subunits: preparations of the viral gp160, gp120, gp41 structural glycoproteins, 3) subunit immunogens in live vectors, 4) gene therapy (e.g. $CD4^+$ cells resistant to HIV) or 5) direct immunization with viral cDNA capable of coding for HIV proteins [13].

Synthetic peptides, modeled according to the antigenic domains in the viral glycoprotein, are used to stimulate the immune system of uninfected and infected individuals to respond by synthesizing antiviral antibodies which have the ability to neutralize infectious virus. The basic assumption in vaccine development is that HIV-1 infection of an immunized person causes the memory cells to respond with a high level of virus-neutralizing antibodies which, in the immunized host, will eliminate the incoming virus and will protect the infected individual from HIV-1 infection and AIDS.

Dr. Jonas Salk [14] suggested in 1993 that "a prophylactic vaccine against human immunodeficiency virus (HIV) infection represents the best hope for controlling the

continuing and devastating worldwide AIDS epidemic" and that "a vaccination strategy which will induce a stable Th1 predominant memory state will favor the induction of a strong CTL response upon subsequent exposure to live HIV even if such responses are not induced by primary immunization". Salk et al. [14] anticipated that a vaccination strategy that induces stable Th1-predominant memory will lead to a protective response whether $CD4^+$ T cells, $CD8^+$ cells or both are required for protection.

A skeptical view on the efficacy of the approach to develop an anti-HIV-1 antibody-generating vaccine was taken by A. Sabin [15, 16] who suggested that anti-HIV-1 neutralizing antibodies will not protect a person against HIV-1 in infected $CD4^+$ T cells in semen or blood. Burke [17] summarized the current knowledge on the phenomenon of enhancement of HIV-1 replication in certain types of cultured human cells in vitro [18, 19] and asked whether antiviral antibodies may be a risk factor for immunized people. Ada et al. [20] argued "that 'first generation' vaccines, even though they may not possess all of the desirable properties, could prevent infection in a proportion of recipients, and much could be learnt to facilitate future vaccine development".

Thus, there is a need to evaluate the ability of viral proteins (inactivated virus, viral proteins subunit vaccines and synthetic viral peptides) to be taken up by antigen-presenting cells for proteolytic processing to generate peptides capable of presentation by HLA class I and class II peptide binding grooves to $CD8^+$ T helper and $CD4^+$ cytotoxic T cells, respectively. For this purpose it is necessary to understand the role of Langerhans/dendritic cells and macrophages, the "professional antigen-presenting cells", in the induction of humoral and cellular immune responses. Such knowledge may be useful in evaluation of the approach of Jonas Salk and his colleagues to anti-HIV vaccination strategies [14] and the statement by Albert Sabin that "the main challenge is to find a way to kill cells with chromosomally integrated HIV cDNA without harming normal cells".

Recently, Desrosiers [21] reported that an attenuated apathogenic HIV was able to immunize adult monkeys against challenge with HIV. Bolognesi [21], however, indicated that it is too early to use a live attenuated virus vaccine against HIV-1.

The impact of the HLA haplotypes on the ability of HIV to mutate in order to escape from the $CD8^+$ cytotoxic T cells was demonstrated by Phillips et al. [10]. Stott [22] indicated that immunization of monkeys with uninfected human cells protected the immunized monkeys against SIV which was grown in such cells. The possibility that induction of anti-HLA antibodies in uninfected humans may protect against infection by HIV-1 infected foreign cells will be discussed. The need to determine the major HLA haplotypes in a population with high risk for HIV infections will be evaluated.

The aim of the present study is to discuss the role of Langerhans cells (LC) in the skin and other epithelia in the human body as efficient antigen-presenting cells which can be utilized in transepithelial transport of peptides and viral DNA for processing by LC prior to antigen presentation by HLA class I molecules to $CD8^+$ cytotoxic T cells. Such a vaccine, named "HIV Peplotion vaccine", when developed may be useful in the protection of uninfected as well as HIV-1-infected individuals.

1. HLA haplotypes in HIV-1-infected individuals in relation to sensitivity to AIDS

Steel et al. [23] reported that HLA haplotype A1, B8, DR3 may be a risk factor for HIV-related disease in thirty-two patients who were exposed to a single batch of factor VIII contaminated with HIV. Of these infected individuals eighteen became antibody-positive while fourteen were seronegative. Of the eighteen seropositives two died and seven developed symptoms related to the HIV-1 infection. The authors suggested that HLA haplotype A2, B8, DR3 was weakly associated with an increased risk of developing disease by the seroconverted individuals and strongly associated with the rapid decline of T4 cells within four years of infection.

Kaslow et al. [24] examined 108 seropositive homosexual men for associations between HLA phenotype and the progression of HIV-1 infection. Forty-nine men of European ethnic origin showed very rapid two-year declines in the $CD4^+$ T cell counts, while fifty-nine men matched for ethnic background, but had no decline in $CD4^+$ T cells. The strongest relation was with the A1, Cw7, B8 haplotype, but associations between these antigen combinations and the development of AIDS were weaker. The authors suggested that humans with B8 and DR3 may experience a more rapid progression to fatal opportunistic infections and therefore have less opportunity of manifesting cancer.

Phillips et al. [10] reported that in a longitudinal study of HIV-seropositive patients there were fluctuations in the specificity of cytotoxic T cells for the virus which was matched by the variability in proviral *gag* cDNA in the patients' lymphocytes. Some of the virus variants were not recognized by autologous T cells. Most asymptomatic HIV-1 infected individuals had cytotoxic T cells (CTLs) that recognized viral antigens presented by HLA class I molecules, but when AIDS developed HIV-specific CTLs disappeared. The authors reported that a relatively high frequency of HIV mutations in the *gag* sequence was found in HLA B8 patients as compared to HLA B27 patients. As the result of mutations in HLA B8 patients CTLs did not recognize the *gag* peptides presented by the HLA B8 class I molecules, thus contributing to the poor outcome of the HIV-1nfected individuals. HLA B8 is relatively common in Caucasians. However, the effect of HLA B27 on the prognosis of HIV infection is not known.

Nixon et al. [9] reported that the HIV-1 *gag* peptide (aa265)KRWIILGLNKIVRMY(aa279)C is an HLA B27-restricted immunodominant epitope within the p25 *gag* polypeptide. This HLA class I epitope has been identified by the use of synthetic peptides to appropriate target cells. It was found by Meyerhans et al. [25] that the majority of infected peripheral blood mononuclear cells from patients with an HLA B27 haplotype bore viral peptides with the HLA B27 motif. Oldstone [26] summarized the sequences of HIV-1 peptide for several HLA class I haplotypes: 1) HLA A3, the nef peptide with the amino acid sequence (73)QVPLRPMTYK; 2) HLA B8, a *gag* p24 peptide with the amino acid sequence (253)NPPTPYGFTTKAWII; and 3) HLA B14, a *gag* p24 peptide with the amino acid sequence (140)GQMVHQAISPRTL.

These findings indicate that the mutations in the amino acids which constitute the walls of the peptide binding groove of the HLA class I molecules lead to the selection of viral peptides with amino acid sequences that allow their interaction and entry into the peptide binding groove. These peptides are proteolysis products of the viral *gag* and nef

proteins. The proteolysis occurs in the cytosol by the proteasomes and the resulting peptides are transported into the lumen of the ER, the site of interaction of a correct peptide with a specific HLA class I haplotype. The ability of an HLA class I haplotype to select a specific peptide from a mixture of viral and cellular peptides for presentation in the peptide binding groove to $CD8^+$ T cells is a key mechanism in CTL priming.

Buxton et al. [27] dissected the peptide binding grooves of seven subtypes of HLA B27 into the six specificity pockets defined by the 2.6Å structure of HLA A0201 and revealed that the B pocket is conserved among all the HLA B27 subtypes. Functional studies on residues which form the B pocket showed that the glutamine residue at position 45 of the HLA peptide binding groove plays a critical role in peptide binding. In HLA B8 the glutamine residue 45 is conserved, while in HLA B53, HLA B3501 the glutamine was mutated to threonine and in HLA B4401 the glutamine was mutated to lysine. A change in amino acid 45 undoubtedly leads to a change in the amino acid motifs of the nanopeptide which interacts with the HLA class I subtypes. Yet, as Phillips et al. [10] indicated, HLA class I B8 predisposes to HIV-related diseases and contributes to a poor outcome of HIV-1 infection, as well as giving rise to escape virus mutants.

2. Induction of $CD8^+$ cytotoxic T cells by HIV-1 proteins in infected individuals

Anti-HIV-specific cytotoxic T lymphocytes (CTLs) were detected in HIV-1-infected individuals [1]. In patients with lung disorders, the alveolar CTLs recognize and destroy HIV-1-infected alveolar macrophages under autologous conditions in vitro. The CTLs express CD3 and CD8, but not CD4, and are restricted by class I HLA transplantation antigen (101).

Using vaccinia virus recombinants expressing HIV-1 *gag* protein, it was found that HLA-class I-restricted *gag*-specific CTLs were identified in thirteen out of fifteen healthy HIV-seropositive individuals. Use was made of short HIV- 1 *gag* synthetic peptides in the lysis assay to screen for *gag* specific CTL epitopes. In one patient a peptide in p24 *gag* was recognized by CTLs in association with HLA B27. A *gag* peptide (265)KRWIILGLNKIVRMY(279)C induced specific lysis of the target lymphoblastoid cells [9].

$CD8^+$ cytotoxic T cells from an infected individual suppressed the replication of HIV in peripheral blood mononuclear cells [8]. The effect was dose-dependent and most apparent with autologous lymphocytes [28]. CTLs specific to HIV-1 were found in blood samples of HIV-1-infected individuals. CTLs directed against autologous B lymphoblasts expressing HIV-1 RT were detected in fresh blood samples from eight HIV-1 seropositive subjects, but not from HIV-1 seronegatives. The effector cells were HLA-restricted $CD3^+$ $CD8^+$ [28].

A peptide with residues aa203–219 of the HIV-1 RT in sera from HIV-1 seropositives was detected by CTLs.

Truncated HIV-1 envelope proteins expressed by vaccinia virus recombinants were detected by CTL clones isolated from HIV-1 seropositive individuals [10].

Littana et al. [29] described a conserved unique epitope on HIV-1 *gag* which was restricted by HLA Cw3 found in 20% of Caucasians and blacks, 62% of native Americans

and 47% of Asian populations. The authors indicated that HIV-1 *gag*-primed CTLs may have a biological role in the protection of HIV-1-infected individuals. The importance of virus-primed CTLs in the protection against virus infection was reported in animal systems [26]. The authors suggested that "it may be useful to combine several cross-reactive defined epitopes that may induce protection in the majority of individuals against HIV-1 disease" [29].

The studies on the isolation of CTLs from the blood of HIV-1-seropositive individuals which recognized HIV-1 *gag* RT and envelope indicate that MHC class I presentation of viral peptides to cytotoxic T cells takes place in HIV-1-infected individuals. Since antibodies against HIV-1 proteins enhance HIV-1 infections, the logical way to protect against HIV-1 is to use synthetic peptides modeled according to HLA class I motifs [30] for the priming of CTLs. The question is which antigen-presenting cell type in the body will be the efficient cell type for the presentation of synthetic peptides by HLA class I molecules.

3. Skin Langerhans LC function as antigen-presenting cells

The LC in the skin are generated in the bone marrow from stem cells defined as dendritic/LC colony-forming units [31]. It was found that macrophages and dendritic/LC share a common bone marrow stem cell [31]. The release of DC to the blood and their migration to the skin, first to the dermis and then to the epidermis by chemotaxis, was recently discussed by Knight et al. [32] and Bergstresser et al. [33]. LC are positioned among the keratinocytes in a single layer above the stratum granulosum. The involvement of LC in the regulation of the proliferation of the skin keratinocytes has been suggested [34].

Hapten-painting of the mouse skin was reported to stimulate LC migration from the skin epidermis, through the draining lymph nodes in the dermis to the regional lymph node [35]. Painting of human skin with dinitrochlorobenzene led to an increase in $CD1^+$ cells in the afferent lymph nodes draining the site [31]. Painting of mouse footpad skin with the contact sensitizer fluorescein isothiocyanate (FITC) and preparation of the epidermal sheets for observation under the UV microscope revealed that FITC is present only in association with LC while the keratinocytes were not stained by this hapten. Shortly after FITC treatment, fluorescein-labeled DC were found in the afferent lymph node cells (Becker Y., Sprecher E. and David D., unpublished). This observation is in agreement with Macatonia et al. [36] and Knight et al. [32] who studied the effect of a second exposure of mice to FITC one month after an initial exposure. It was found that a second exposure to FITC caused significantly lower numbers of DC carrying the antigen to the lymph nodes, as well as a reduced T cell proliferation.

The direct route of LC migration from the skin into the dermis was established in an organ culture system. Larsen et al. [37] reported that the majority of the migrating leukocytes were Ia^+ LC and the remainder of the cells comprised $Thy\text{-}1^+$, $CD3^+$, $CD4^-$, $CD8^-$, γ/δ^+ receptor epidermal dendritic T cells, which clustered with LC and a small population of macrophages. It was suggested that the maturation of LC commences in the epidermis and continues during LC migration.

Since LC are situated in their specific layer among keratinocytes, they may provide the vertebrate with a defence mechanism against foreign biological substances which enter the body through the skin. Such antigens may include proteins and infectious viruses entering through the damaged skin, as well as viruses and protozoa released into the skin with the saliva of biting insects. In studies on herpes simplex virus-1 (HSV-1) infection in the mouse skin [38, 39] it was demonstrated that in the absence of LC at the site of virus injection, HSV-1 strains that were not pathogenic to the mouse caused a lethal infection. However, when LC in the skin at the site of virus injection were treated with the immunomodulator OK-432 (a streptococcal cell wall preparation), even highly pathogenic HSV-1 strains did not cause a lethal infection. These results established a role for LC in the defence against HSV-1 infection. A similar observation was reported for vaccinia virus infection in mice [39]. The ability of viruses to interact with LC was recently reviewed [40, 41].

It was also reported that saliva proteins released during a tick bite into the skin are present only on LC in the skin [42]. indicating the ability of cell surface molecules on LC to interact with the foreign proteins. The ability of *Leishmania major* to infect LC was reported by Dompmartin et al. [43].

Dendritic cells express CDI (MHC class I-like) glycoproteins on the plasma membrane. The CDI antigen has a structure similar to HLA class I and is present on the cell membrane in association with a β_2-microglobulin molecule.

Hanau et al. [44, 45] used immunogold staining of human epidermal Langerhans cells by anti-Tc antigen to identify the LC membrane-associated antigen OKT6. These authors observed that the T6 molecules to which the antibody is attached were internalized via coated pits, coated vesicles and endosomes. Intracytoplasmic Birbeck granules which contain the T6 antigen may represent intracellular transport organelles carrying the T6 antigen from the Golgi apparatus to an unknown destination. Hanau et al. [44, 45] suggested that the T6 antigen on the plasma membrane of LC may be part of a receptor site. The T6 antigen may be a cell surface glycoprotein, CDI, associated with a β_2-microglobulin resembling HLA class I antigen of the major histocompatibility complex (MHC) [46, 47] which presents peptides to T cells with γ/δ receptors [48].

All the dendritic cells express HLA class I molecules [48] which consist of heterodimers of a highly polymorphic α chain associated with a β_2-microglobulin molecule [49]. The expression of the less polymorphic nonclassical murine class I molecules Qa-2.3 and T1a by dendritic cells has not yet been evaluated [48].

Knight et al. [50] reported that human blood dendritic cells are highly HLA class II-positive. The anti-HLA DR antibody labeled with 20 nm gold particles revealed that the MHC class II molecules can be seen on the plasma membrane, in cytoplasmic vacuoles near the plasma membrane and in cytoplasmic vacuoles near the nuclei (possibly the endoplasmic reticulum ER lumen), demonstrating the pathway of the HLA class II molecules from the ER to the plasma membrane.

Prue et al. [51] reported that antigen processing by LC correlates with the level of MHC class II and invariant chain (Ii) biosynthesis. Similarly, Kampgen et al. [52] reported that freshly isolated LCs synthesize large amounts of class II and invariant chain (Ii) polypeptides. The turnover of the MHC class II polypeptides is slow while the turnover of the Ii polypeptide is rapid. This result indicates that the MHC α and β chains and the Ii

polypeptides are efficiently transported from the membrane of the ER to the endosome pathway. At that site, the vesicle which transports the MHC class II molecules fuses with the endosomes which contain the proteolytic enzyme cathepsin. As the result of the fusion, the cathepsin proteolytically cleaves the Ii chain. The in vitro-cultured LC lose their capacity to process exogenous antigens, but retain their ability to prime T cells. Culturing LC resulted in the disappearance of endosomes and acidic granules under in vitro conditions which accompanied the loss of antigen processing [53].

Tang et al. [54] reported that it was possible by the PCR technique to demonstrate that mouse LC expressed the I-A gene. Expression of MHC class II antigen by cultured LC in vivo activated with haptens was reported by Aiba and Katz [55]. Using anti-I-A antibodies, a 2–3-fold increase in I-A and I-E expression by LC was shown, while CD45 antigen expression was not altered. Two subpopulations of LC were noted: LC with an unchanged Ia density, and 22–50% of LC with a markedly enhanced Ia density (10-fold increase in I-A and I-E). This change was caused only by haptens.

Epidermal LC possess, on their cell membrane, a formalin resistant ATPase activity [56, 57]. This ATPase is an ectoenzyme with an active site on the extracellular side of the plasma membrane and requires Mg^{++} or Ca^{++} for activation. Girolomoni et al. [58] reported that human LC are resistant to the permeabilizing effects of ATP and suggest that the membranal ATPase provides protection to LC.

The production of IL-1β and TNF-α by mouse skin LC was reported by Sprecher and Becker [59, 60] and Matsue et al. [61] also reported that LC from trypsinized mouse epidermal sheets contained PCR-detectable mRNA for the cytokines IL-1β and TNF-α. FACS-purified Ia^+ EC expressed IL-1β and macrophage inflammatory protein-1a (MIP-1a).

Normal human LC were integrin β1 (CD29)-positive by indirect immunogold labeling on transmission electron microscopy [62, 63]. By labeling with different VLA-α chain (CD49) monoclonal antibodies two subpopulations of LC were detected: about 40% had small amounts of VLA-1 and VLA-3, and 53% and 77% of LC expressed moderate amounts of VLA-2 and VLA-5, respectively. VLA-4 and VLA-6 were expressed by 67% and 90% of LC, respectively. VLA-6 the laminin receptor and VLA-5, the fibronectin receptor, are expressed by LC, possibly to help the LC during migration throughout the fibronectin network of the dermis before reaching the afferent lymphatics on the way to the regional lymph nodes. It was suggested by Staquet et al. [63] that the LC which attach to laminin and fibronectin in vitro mediated the passage of LC through the basement membrane and through the fibronectin network of the dermis by the a5β1 and a6β1 surface molecules.

Mouse keratinocytes were found to express E-cadherin, a member of the cadherin family [54] which is represented in epithelial and neural tissues and plays an important role in morphogenesis and maintenance of tissue integrity [64]. It was demonstrated that LC contain E-cadherin mRNA and were able to bind to cells which express E-cadherin on their surface (L-cells transfected with E-cadherin cDNA).

In healthy individuals 50% of CD1a-positive epidermal LC express the FcgRII/CDw32 receptor for IgG which has a molecular weight (M_r) of 40 KDa [65, 66]. In atopic dermatitis patients with elevated IgE in the serum about 50% of epidermal CD1a-positive cells present in lesional skin are IgE-bearing. It was found that IL-4 and/or

interferon-γ induce the expression of IgE receptors FcERII/CD23 on a percentage of normal human epidermal LC.

4. Dendritic cells prime cytotoxic CD8$^+$ T cells by MHC class I restriction

McKinney and Streilein [67] examined the relative alloimmunogenicity of epidermal Langerhans cells, Thy-1$^+$ dendritic epidermal cells and keratinocytes prepared from mouse skin. Each cell type was injected intravenously (IV) or subcutaneously (SC) into allogenic recipient mice. At 4 to 6 weeks spleens and lymph nodes of recipient mice were assayed for specific priming of cytotoxic T cells. LC were found to be powerful immunogens since only LC (at a dose of 10 LC) primed allospecific T cells of mice which received the injected cells.

Macatonia et al. [68] showed that DC promote CTL growth from unprimed lymphocytes by using viral peptides or from primed lymphocytes by using whole viral antigen. Cherrie et al. [69] used DC from peripheral blood to present respiratory syncytial virus (RSV) antigen to autologous peripheral blood monocytic cells which were obtained from normal donors. Reproducible CTL production in short-term cultures from all nine donors was found. These CTLs were tested against autologous Epstein-Barr virus-transformed lymphoblastoid cell lines, each infected with a different recombinant vaccinia virus, expressing one RSV gene only. It was found that CTLs recognized six of ten virus-coded proteins and that the recognition was restricted by major histocompatibility complex (MHC) class I specificity.

Nonacs et al. [70] reported that influenza virus-treated dendritic cells (DC) were 100 times more efficient than bulk spleen cells in stimulating CD8$^+$ CTL formation without exogenous lymphokines. The infectious virus entered the DC through intracellular acidic vacuoles (endosomes) and replicated in these cells.

Viral peptides derived from cytosol-processed viral proteins were presented by MHC class I molecules on the plasma membrane and generated CTLs. Dendritic cells pulsed with the MHC class I-restricted influenza virus nucleoprotein peptide NP(aa147–155) were poor stimulators in the absence of exogenous helper factors. It was concluded that viral protein synthesis in DC mediates the presentation of the viral peptides by the MHC class I molecules needed for the induction of influenza-specific CTLs. Langhoff and Steinman [71] showed that cloning of CD8$^+$ or CD4$^+$ T cells was possible in the presence of syngeneic or allogeneic DC.

5. Transepidermal transport of peptides through the skin

Banerjee and Ritschel reported [72] on the influence of skin pH, peptide concentration, sharing and surfactant on the in vitro transdermal permeation of vasopressin. They reported that a higher transdermal permeation of vasopressin was noted at pH 5.0 at a concentration of 49.5 mg/ml. It was also noted that treating the skin by 25 times of cellophane tape stripping caused a 70-times increase of transdermal permeation as

compared to untreated skin. Cellophane tape stripping of the skin is known to inactivate skin LC, indicating that the skin LC are a barrier to transdermal transport of peptides through the skin, which is the purpose of drug delivery through the skin into the skin dermal vascular network. For the purpose of transepidermal delivery of immunoreactive peptides to the skin LC, the method reported by Banerjee and Ritschel [72] may suffice.

Choi et al. [73] investigated the effects of the nonionic surfactant, n-decylmethyl sulfoxide (NDMS), and pH on the permeation of the pentapeptide enkephalin through hairless mouse skin. It was reported that permeation of the enkephalin peptide (YGGFL) was rapidly cleaved by a peptidase at the Tyr-Gly bond. At pH 5.0 the metabolic activity was significantly reduced and a substantial flux of YGGFL was observed. The authors concluded that a combination of a skin permeation enhancer, pH adjustment and inhibitors of the proteolytic activity in the skin can increase transdermal delivery of peptides.

6. "HIV-1 peplotion vaccine" – transepidermal transport of HLA class I-related synthetic viral immunogenic peptides targeted to the skin Langerhans cells

Aim: development of a "HIV peplotion vaccine" for application as a lotion containing HIV peptides/proteins/lipidic and nonpeptide antigens to LC in epithelial surfaces of skin, gut and genitalia which will prime the CTL responses in lymph nodes.

Based on the available knowledge on the transport of bioreactive peptides through the skin and the possible role of LC in inhibiting their transport from the epidermis to the dermis, it is possible to suggest that under appropriate conditions viral synthetic peptides can be introduced to Langerhans cells in the skin. LC, being professional antigen-presenting cells, will use the peptides for presentation to $CD8^+$ T cells in the lymph nodes. The following aspects of the "Peplotion" concept should be considered:

a) The optimal conditions for transepidermal transport of viral peptides in human skin since the available data are on mouse skin.

b) The HLA class I types and haplotypes of the human population must be determined prior to immunization and the motifs of the nonapeptides which bind to their HLA-class I peptide binding groove must be known.

c) Design of the viral nonapeptides to fit the peptide binding grooves of HLA class I molecules of the human population.

For each HIV-1 protein the sequence of the nonapeptide which induces CTLs must be determined by computer analysis. Also, this information was obtained by extracting the peptides from the surface HLA class I molecules of infected cells, as indicated above. Since different viral nonapeptides are selected by different HLA class I haplotypes of each individual, it is necessary to use computer modeling of the peptide binding grooves of different HLA subtypes [74], as well as modeling of the nonapeptides [75], to obtain the necessary information for designing of CTL-stimulating viral peptides. The concept of using skin LC for the priming of T cells may be expanded to the use of gut dendritic cells and lung dendritic cells to prime $CD8^+$ CTLs or cells in the gut or lung, respectively. Such an approach may provide enhancement of the immune responses in different organs of the body and may lead to more successful elimination of virus infections in humans and economically important animals [76, 77].

7. Experimental approach to the development of "HIV-1 peplotion vaccine"

1. Studies on the effects of immunomodulators and skin expanders on mouse skin LC in situ.

The compound OK-432 is an immunomodulator previously found to increase the number of Langerhans cells in mouse footpad skin. This is followed by a decrease in the pathogenicity of herpes simplex virus type 1 (HSV-1) [40]. Systemic administration of OK-432 prior to HSV-1 infection in mice was used as an experimental system to investigate and obtain information on the ability of this compound to protect against lethal HSV-1 infection.

The effect of systemic administration of the immunomodulator OK-432 to newborn C57BL/6 mice at various times both pre- and postinfection with HSV-1 was studied [78]. It was found that 0.5 mg of OK-432 injected at the same time as the virus is not protective, with survival in this group reaching 40% (P=0.96), virtually the same as that of the control group HSV-1 administered on day 0, mouse group 1, 41% survival. A 1 mg dose of OK-432 on day 0 is only slightly more effective in reducing mortality (53% survival, P=0.38). Mice treated only with OK-432 and not injected with the virus had a survival rate of 90% (P=0.0046). However, OK-432 administered four days before and at the time of virus injection resulted in a survival rate of 95% (P=0.0001). Finally, OK-432 administered at the time of, or one day after, injection of the virus (mouse group 6) has no protective effect (40% survival, P=0.96).

The effect of HSV-1 infection in mice following systemic administration of OK-432 was studied. The results presented above indicate that systemic administration of OK-432 to newborn mice prior to infection with HSV-1 provides a good level of protection (90%). OK-432 administered at the time of, or one day after, injection of the virus did not protect the mice against infection with HSV-1. The importance of the timing of OK-432 administration may be presumed to be a reflection of its mode of action, specifically the synthesis of IL-1 and the induction of maturation of macrophages and T helper cells and their antiviral effects [78].

2. Computer simulation of putative nonapeptides with motifs to fit known HLA class I haplotypes in HIV-1 proteins.

The aim of the present study is to use a computer program to predict, in HIV-1 proteins, the pattern of proteolysis by proteolytic enzymes and the availability of peptides with HLA allele-specific patterns of amino acid motifs that were reported [79] to restrict the binding of peptides to the peptide binding grooves of HLA class I haplotypes. Viral peptides with the correct HLA class I binding sequence must be selected by a complex of peptide transporter protein-HLA class Iα and β2 microglobulin molecules [80] from a pool of peptides. Such peptides arise from the proteolytic cleavage of the viral proteins by cytosolic proteasomes and possibly by cleavage of viral proteins by signal peptidases in the ER lumen. To obtain information on the availability of putative viral peptides with known motifs to fit certain HLA haplotypes a computer search [81] was made for nonapeptides with known HLA-specific motifs coupled with amino acid signals for proteolysis [82]. The prediction of the availability of putative peptides with HLA class I

motifs may provide information regarding the possible use of synthetic peptides to induce CTLs in uninfected individuals [83] and the availability of viral peptides in individuals with different HLA class I haplotypes to mount an antiviral CTL response.

Table I

Summary of the computer-simulated peptides with known HLA class I motifs. (From: Becker Y. Computer simulations to predict the availability of peptides with known HLA class I motifs possibly generated by proteolysis of HIV-1 proteins in infected cells, Virus Genes, **10**, 227, 1995)

	gag	pol	env	protease	vpu	nef	rev	tat
A2	7	5	18	0	1	0	1	0
A68	4	20	18	2	0	2	3	1
A24	0	0	0	0	0	0	0	0
B27	1	1	2	0	0	0	1	0
B8	0	0	1	0	0	0	0	0
B53	0	0	1	0	0	0	0	0
B35	0	1	0	0	0	1	0	0

The program "Findpatterns" in the GCG software [81] was used to search for, and identify in the amino acid sequence of each of the known HIV-1 proteins, the patterns of amino acids of putative nonapeptides (putative HLA class I antigens) with amino acids motifs capable of binding to peptide binding grooves with known HLA class I haplotypes. The amino acid patterns introduced into the computer program were defined using two parameters: a) the amino acid requirements for the proteolysis of proteins by cytosolic proteasomes and proteolytic amino acids (signals cleavage after amino acids L/E/F/R and processing proteinase (proteolytic cleavage after amino acids R/RK) [82], as well as signal peptidase present in the endoplasmic reticulum (ER) (proteolytic cleavage after amino acids A/C/G/S [82]); b) the amino acids in the putative nonapeptides which were found to be critical for the binding of nonapeptides to the peptide-binding grooves of various HLA class I haplotypes (detailed in Table I).

Cytotoxic T cells which recognize HIV-1 peptides generated from all viral proteins were reported. To predict the cleavage pattern of HIV-1 proteins by cytoplasmic proteasomes into peptides with motifs fitting known HLA class I molecules, the computer program Findpatterns was used. The combined amino acids patterns for proteolytic cleavages and the HLA class I haplotype-restricted peptides motifs were studied. It was noted that peptides with motifs of HLA class I A2 and A68 were abundant while no HLA class I A24 motifs were found. It is of interest that only five nonapeptides fitting HLA class I B27, one each in B8 and B53 and two fitting HLA class I B35 were detected.

The detailed analysis will be published in Virus Genes [83].

3. Summary of the computer-simulated nonapeptides with known HLA class I motifs.

The computer analysis provided a rational approach to the synthesis of viral peptides which will be incorporated by LC into the cytoplasm which, after proteolytic cleavage by cytosolic proteasomes will release the specific nonapeptides for presentation by the HLA class I molecules. These putative nonapeptides may be useful for populations which carry HLA class IA haplotypes, as well as HLA class I B haplotypes. These putative nonapeptides may suffice to induce anti-HIV-1 cytotoxic T cells.

The results of the computer analysis are presented in Table I. It can be seen that nonapeptides with motifs to fit HLA class I B27, B8, B53 and B35, as well as HLA class I A24 haplotypes were not detected in HIV-1 proteins. It is of interest that HLA B53, B35 and B8 haplotypes were reported as possible risk factors for enhanced HIV-1-related diseases.

4. The possibility for the design of synthetic viral peptides for transepidermal transport.

The results of the first phase of our studies on skin LCs and their behavior after treatment with immunomodulators required improvement of the technologies for detection and evaluation of the effects of solutions which enhance transepidermal transport of components in the epidermis. In further studies the effects of cationic lipids and liposomes on the transport of viral synthetic peptides and proteins will be investigated.

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REFERENCES

1. Burke,D.S.: Human HIV vaccine trials: does antibody-dependent enhancement pose a genuine risk? Perspect Biol Med **35**, 511 (1992).
2. Takahashi,M., Takeshita,T., Morein,B., Putney,S., Germain,R.N., Berzofsky,J.A.: Induction of $CD8^+$ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs [see comments]. Nature **344**, 873 (1990).
3. Walker,C., Selby,M., Erickson,A., Do D.C., Valensi,J-P., Van Nest,G.: Cationic lipids direct a viral glycoprotein into the class I major histocompatibility complex antigen-presentation pathway. Proc Natl Acad Sci USA **89**, 7915 (1992).
4. Nair,S., Babu,J.S., Dunham,R.G., Kanda,P., Burke,R.L., Rouse,B.T.: Induction of primary, antiviral cytotoxic, and proliferative responses with antigens administered via dendritic cells. J Virol **67**, 4062 (1993).
5. Becker,Y.: An analysis of the role of skin Langerhans cells (LC) in the cytoplasmic processing of HIV-1 peptides after "Peplotion" transepidermal transfer and HLA class I presentation to $CD8^+$ CTLS – An approach to immunization of humans. Virus Genes **9**, 133 (1995).

6. Takahashi,H., Cohen,J., Hosmalin,A., Cease,K.B., Houghton,R., Cornette,J.L., DeLisi,C., Moss,B., Germain,R., Berzofsky,J.A.: An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. Proc Natl Acad Sci USA **85**, 3105 (1988).
7. Takahashi,K., Dai,L-C., Fuerst,T.R., Biddson,W.E., Earl,P.L., Moss,B., Ennis,F.A.: Specific lysis of human immunodeficiency virus type 1-infected cells by a T HLA-A3.1-restricted $CD8^+$ cytotoxic T-lymphocyte clone that recognizes a conserved peptide sequence within the gp41 subunit of the envelope protein. Proc Natl Acad Sci USA **88**, 10277 (1991).
8. Takahashi,H., Nakagawa,Y., Pendelton,C.D., Houghten,R.A., Yokomuro,K., Germain,R.N., Berzofsky,J.A.: Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant. Science **255**, 333 (1992).
9. Nixon,D.F., Townsend,A.R.M., Elvin,J.G., Rizza,C.R., Gallwey,J., McMichael,A.J.: HIV-1 *gag*-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. Nature **336**, 484 (1988).
10. Phillips,R.E., Rowland-Jones,S., Nixon,D.F., Gotch,F.M., Edwards,J.P., Ogunlesi,A.O., Elvin,J.G., Rothbard,J.A., Bangham,C.R.M., Rizza,C.R.: Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition [see comments]. Nature **354**, 453 (1991).
11. Hosmalin,A., Clerici,M., Houghten,R., Pendleton,C.D., Flanxner,C., Lucey,D.R., Moss,B., Germain,R.N., Shearer,G.M., Berzofsky,J.A.: An epitope in human immunodeficiency virus 1 reverse transcriptase recognized by both mouse and human cytotoxic T lymphocytes. Proc Natl Acad Sci USA **87**, 2344 (1990).
12. Weiss,R.A.: How does HIV cause AIDS? Science **260**, 1273 (1993).
13. Haynes,B.F.: Scientific and social issues of human immunodeficiency virus vaccine development. Science **260**, 1279 (1993).
14. Salk,J., Bretscher,P.A., Salk,P.L., Clerici,M., Shearer,G.M.: A strategy for prophylactic vaccination against HIV. Science **260**, 1270 (1993).
15. Sabin,A.B.: Effectiveness of AIDS vaccines [letter] [published erratum appears in Science 1991 Apt 12; 252 (5003):192] [comment]. Science **251**, 1161 (1991).
16. Sabin,A.B.: Improbability of effective vaccination against human immunodeficiency virus because of its intracellular transmission and rectal portal of entry. Proc Natl Acad Sci USA **89**, 8852 (1992).
17. Burke,D.S.: Human HIV vaccine trials: does antibody-dependent enhancement pose a genuine risk? Perspective in Biol and Med **35**, 511 (1992).
18. Robinson,W.E., Kawamura,T., Gorny,M.K., Lake,D., Xu,J-Y., Matsumoto,Y., Sugano,T., Masuho,Y., Mitchell,W.M., Hersh,E., Zolla-Pazner,S.: Human monoclonal antibodies to the human immunodeficiency virus type 1 (HIV-1) transmembrane glycoprotein gp41 enhance HIV-1 infection in vitro. Proc Natl Acad Sci USA **87**, 3185 (1990).
19. Montefiori,D.C., Murphy-Corb,M., Desrosiers,R.C., Daniel,M.D.: TI: Complement-mediated, infection enhancing antibodies in plasma from vaccinated macaques before and after inoculation with live simian immunodeficiency virus. J Virol **64**, 5223 (1990).
20. Ada,G., Blanden,B., Mullbacher,A.: HIV: to vaccinate or not to vaccinate? Nature **359**, 572 (1992).
21. Desrosiers,R.C.: J NIH Res **6**, 54, 56 (1994) and Bolognesi,D.: J NIH Res **6**, 55, 59 (1994).
22. Stott,E.J.: Anti-cell antibody in macaques [letter]. Nature **353**, 393 (1991).
23. Steel,C.M., Ludlam,C.A., Beaston,D., Peutherer,J.F., Cuthvert,R.J.G., Simmonds,P., Morrison,H., Jones,M.: HLA haplotype A1 B8 DR3 as a risk factor for HIV-related disease. Lancet **i**, 1185 (1988).
24. Kaslow,R.A., Duquesnoy,R., Van Ruden,M., Kingsley,L., Marrari,M., Friedman,H., Su,S., Saah,A.J., Detels,R., Phair,J., Rinaldo,C.: A1, Cw7, B8, DR3 HLA antigen combination associated with rapid decline of T-helper lymphocytes in HIV-1 infection. A report from the Multicenter AIDS Cohort Study. Lancet **335**, 927 (1990).
25. Meyerhans,A., Dadaglio,G., Vartanian,J.P., Langelade-Demoyen,P., Frank,R., Asjo,B., Plata,F., Wain-Hobson,S.: In vivo persistence of a HIV-l-encoded HLA-B27-restricted cytotoxic T lymphocyte epitope despite specific in vitro reactivity. Eur J Immunol **21**, 2637 (1991).
26. Oldstone,M.B.A.: Molecular anatomy of viral persistence. J Virol **65**, 6381 (1991).

27. Buxton,S.E., Benjamin,R.J., Clayberger,C., Parham,P., Krensky,A.M.: Anchoring pockets in human histocompatibility complex leukocyte antigen (HLA) class I molecules: analysis of the conserved B ("45") pocket of HLA-B27. J Exp Med **175**, 809 (1992).
28. Walker,C.M., Moody,D.J., Stites,D.P., Levy,J.A.: $CD8^+$ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science **234**, 1563 (1986).
29. Littana,R.A., Oldstone,M.B.A., Takeda,A., Debouck,C., Wong,I.T., Tuazon,C.U., Moss,B., Kievits,F., Ennis,F.A.: An HLA-C-restricted $CD8^+$ cytotoxic T-lymphocyte clone recognizes a highly conserved epitope on human immunodeficiency virus type 1 *gag*. J Virol **65**, 4051 (1991).
30. Silver,M.L., Guo,H-C., Strominger,Jr., Wiley,D.C.: Atomic structure of a human MHC molecule presenting an influenza virus peptide. Nature **360**, 367 (1992).
31. Reid,C.D.L., Fryer,P.R., Clifford,C., Kirk,A., Tikerpae,J., Knight,S.C.: Identification of hematopoietic progenitors of macrophages and dendritic Langerhans cells (DL-CFU) in human bone marrow and peripheral blood. Blood **76**, 1139 (1990).
32. Knight,S.C., Stagg,A., Hill,S., Fryer,P., Griffiths,S.: Development and function of dendritic cells in health and disease. J Invest Dermatol **99**, 33S (1992).
33. Bergstresser,P.R., Cruz,P.D., Niederkorn,J.Y., Takashima,A.: TI: Third International Workshop on Langerhans cells: discussion overview. J Invest Dermatol **99**, 1S (1992).
34. Becker,Y.: Does radiation-induced abrogation of skin Langerhans cell functions lead to enhanced incidence of skin tumors in patients with genetic disorders of DNA repair? Cancer Invest **5**, 505 (1987).
35. Kripke,M.L., Munn,C.G., Jeevan,A., Tang,J.M., Bucan,C.: Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during ,contact sensitization. J Immunol **145**, 2833 (1990).
36. Macatonia,S.E., Knight,S.C., Edwards,A.J., Griffiths,S., Fryer,P.: Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. J Exp Med **166**, 1654 (1987).
37. Larsen,C.P., Steinman,R.M., Witmer-Pack,M., Hankins,D.F., Morris,P.J., Austyn,J.M.: Migration and maturation of Langerhans cells in skin transplants and explants. J Exp Med **172**, 1483 (1990).
38. Sprecher,E., Becker,Y.: Induction of interleukin-1 alpha and beta gene transcription in mouse peritoneal exudate cells after intraperitoneal infection with herpes simplex virus-1. Arch Virol **110**, 259 (1990).
39. Sprecher,E., Becker,Y.: Langerhans cell density and activity in mouse skin and lymph nodes affect herpes simplex type 1 (HSV-1) pathogenicity. Arch Virol **107**, 191 (1989).
40. Sprecher,E., Becker,Y.: Role of Langerhans cells and other dendritic cells in disease states. IN VIVO **7**, 217 (1993).
41. Sprecher,E., Becker,Y.: Role of Langerhans cells and other dendritic cells in viral diseases. Arch Virol **132**, 1 (1993).
42. Allen,J.R., Khalil,H.M., Wikel,S.K.: Langerhans cells trap tick salivary gland antigens in tick-resistant guinea pigs. J Immunol **122**, 563 (1979).
43. Dompmartin,A., Healy,A.T., Nancy,C.A., Hauser,C., Meltzer,M.S.: Leishmania major infects and replicates within murine epidermal Langerhans cells. J Invest Dermatol **91**, 404 (1988).
44. Hanau,D., Fabre,M., Scmitt,D.A., Stampf,J-L., Garaud,J.C., Bieber,T., Grosshaus,E.,. Benezra,C., Cazenave,J.P.: Human epidermal Langerhans cells internalize by receptor-mediated endocytosis T6 (CD1 "NA1/34") surface antigen. Birbeck granules are involved in the intracellular traffic of the T6 antigen. J Invest Dermatol **89**, 172 (1987).
45. Hanau,D., Fabre,M., Scmitt,D.A., Garaud,J.C., Pauly,G., Tongio,M.M., Mayer,S., Cazenave,J.P.: Human epidermal Langerhans cells cointernalize by receptor-mediated endocytosis "nonclassical" major histocompatibility complex class I molecules (T6 antigens) and class II molucules (HLA-DR antigens). Proc Natl Acad Sci USA **84**, 2901 (1987).
46. Lerch,P.G., Van de Rijn,M., Schrier,P., Terhorst,C.: Biochemical comparison of the T6 antigen and HLA-A,B antigens. Hum Immunol **6**, 13 (1983).
47. Van de Rijn,M., Lerch,P.G., Knowles,R.W., Terhorst,C.: The thymic differentiation markers T6 and M241 are two unusual MHC class I antigens. J Immunol **131**, 851 (1983).
48. Austyn,J.M.: Antigen uptake and presentation by dendritic leukocytes. Seminars in Immunol **4**, 227 (1992).

49. Barclay,A.N., Birkeland,M.L., Brown,M.H., Beyers,A.D., Davis,S.J., Somoza,C., Williams,A.F.: The Leukocyte Antigen Facts Book. Academic Press. 1993.
50. Knight,S.C., Fryer,P., Griffiths,S., Harding,B.: Class II histocompatibility antigens on human dendritic cells. Immunology **61**, 21 (1987).
51. Prue,E., Inaba,K., Crowley,M.T., Tardelli,L., Witmer,P.M., Ruberto,G., Fathman,G., Steinman,R.M.: Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. J Exp Med **172**, 1459 (1990).
52. Kampgen,E., Koch,N., Koch,F., Stoger,P., Schuler,G., Romani,N.: Class II major histocompatibility complex molecules of murine dendritic cells: synthesis, sialylation of invariant chain,, and antigen processing capacity are down-regulated upon culture. Proc Natl Acad Sci IISA **88**, 3014 (1991).
53. Stossel,H., Koch,F., Kampgen,E., Stoger,P., Lenz,A., Heufier,C., Romani,N., Schuler,G.: Disappearance of certain acidic organelles (endosomes and Langerhans cell granules) accompanies loss of antigen processing capacity upon culture of epidermal Langerhans cells. J Exp Med **172**, 1471 (1990).
54. Tang,A., Amagai,M., Granger,L.G., Stanley,J.R., Udey,M.C.: Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. Nature **361**, 82 (1993).
55. Aiba,S., Katz,S.I.: Phenotypic and functional characteristics of in vivo-activated Langerhans cells. J Immunol **145**, 2791 (1990).
56. Wolff,K., Winkelman,R.K.: Ultrastructural localization of nucleoside triphosphatase in Langerhans cells. J Invest Dermatol **48**, 50 (1967).
57. Chaker,M.B., Tharp,M.D., Bergstresser,P.R.: Rodent epidermal Langerhans cells demonstrate greater histochemical specificity for ADP than for ATP and AMP. J Invest Dermatol **82**, 496 (1984).
58. Girolomoni,G., Santantonio,M.L., Bergstresser,P.R., Gunz,P.D., Giannetti,A.: Membrane ecto-ATPase on epidermal Langerhans cells. J Invest Dermatol **99**, 185 (1992).
59. Sprecher,E., Becker,Y.: Induction of interleukin-1 alpha and beta gene transcription in mouse peritoneal exudate cells after intraperitoneal infection with herpes simplex virus-1. Arch Virol **110**, 259 (1990).
60. Sprecher,E., Becker,Y.: Detection of Interleukin-1β, tumor necrosis factor α and interleukin 6 gene transcription by polymerase chain reaction in keratocytes, Langerhans cells and peritoneal exudate cells during infection with herpes simplex virus-1. Arch Virol **124**, 83 (1992).
61. Matsue,H., Cruz,P.D., Bergstresser,P.R., Takashima,A.: Langerhans cells are the major source of mRNA for IL-1 beta and MIP-1 alpha among unstimulated mouse epidermal cells. J Invest Dermatol **99**, 537 (1992).
62. Le Vatlet,B., Dezutter-Dambuyant,C., Staguet,M.J., Delorme,P., Schmitt,D.: Human epidermal Langerhans cells express integrins of the beta 1 subfamily. J Invest Dermatol **96**, 518 (1991).
63. Staquet,M.J., Levarlet,B., Dezutter-Dambuyant,C., Schmitt,D.: Human epidermal Langerhans cells express beta 1 integrins that mediate their adhesion to laminin and fibronectin. J Invest Dermatol **99**, 125 (1992).
64. Takeichi,M.: Cadherin cell adhesion receptors as a morphogenetic regulator. Science **251**, 1451 (1991).
65. Schmitt,D.A., Bieber,T., Cazenave,J-P., Hanau,D.: Fc receptors of human Langerhans cells. J Invest Dermatol **94**,15S (1990a).
66. Schmitt,D.A., Hanau,D., Bieber,T., Dezutter-Dambuyant,C., Schmitt,D., Fabre,M., Pauley,G., Cazenave, J-P.: Human epidermal Langerhans cells express only the 40-kilodalton Fc gamma receptor (FcRII). J Immunol **144**, 4284 (1990).
67. McKinney,E.C., Streilein,J.W.: On the extraordinary capacity of allogeneic epidermal Langerhans cells to prime cytotoxic T cells in vivo. J Immunol **143**, 1560 (1989).
68. Macatonia,S.E., Taylor,P.M., Knight,S.C., Askonas,B.A.: Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. J Exp Med **169**, 1255 (1989).
69. Cherrie,A.H., Anderson,K., Wertz,G.W., Openshaw,P.J.M.: Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J Virol **66**, 2102 (1992).
70. Nonacs,R., Humborg,C., Tam,J.P., Steinman,R.M.: Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. J Exp Med **176**, 519 (1992).

71. Langhoff,E., Steinman,R.M.: Clonal expansion of human T lymphocytes initiated by dendritic cells. J Exp Med **169**, 315 (1989).
72. Banerjee,P.S., Ritschel,W.A.: Transdermal permiation of vasopressin I. Influence of pH, concentration, sharing and surfactant on in-vitro permeation. Int J Pharmaceutics **49**, 189 (1989).
73. Choi,H.K., Flynn,G.L., Amidon,G.L.: Transdermal delivery of bioactive peptides: the effect of n-decylmethyl sulfoxide, pH, and inhibitors on enkephalin metabolism and transport. Pharm Res **7**, 1099 (1990).
74. Sezerman,U., Vajda,S., Cornette,J., DeLisi,C.: Toward computational determination of peptide-receptor structure. Protein Science **2**, 1827 (1993).
75. Rosenfeld,R., Zheng,Q., Vajda,S., DeLisi,C.: Computing the structure of bound peptides. Application to antigen recognition by class I major histocompatibility complex receptors. J Mol Biol. **234**, 515 (1993).
76. Becket,Y.: HIV-1 proteins in infected cells determine the presentation of viral peptides by HLA class I and class II molecules and the nature of the cellular and humoral antiviral immune responses – a review. Virus Genes **8**, 249 (1994a).
77. Becket,Y. in: A novel approach to vaccination of humans against AIDS with an "HIV-1 peplotion vaccine" for transepithelial transport of HIV-1 proteins/peptides to epidermal Langerhans (dendritic) cells to prime antiviral $CD8^+$ cytotoxic T cells for long term protection. Scientific Reports from Members of the European Network "Man Against Virus". ROSTE pp.125-138. 1994.
78. Berkowitz-Balshayi,C., Rosen-Wolff,A., Darai,G., Becker,Y.: Immunosuppression of mice by cyclophosphamide (CyP) and cyclosporin A (CsA) changes the course of HSV-1 infection and the distribution of viral DNA in target organs. IN VIVO **9**, 155 (1995).
79. Rötzschke,O., Falk,K.: Naturally-occurring peptide antigens derived from the MHC class-I-restricted processing pathway. Immunol Today **12**, 447 (1991).
80. Suh,W-K., Cohen-Doyle,M.F., Fruh,K., Wang,K., Peterson,P.A., Williams,D.B.: Interaction of MHC class I molecules with the transporter associated with antigen processing. Science **264**, 1322 (1994).
81. Devereux,J., Haeberli,P., Smithies,O.: A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12**, 387 (1984).
82. Bond,J.S., Butler,P.E.: Intracellular proteases. Ann Rev Biochem **56**, 333 (1987).
83. Becker,Y.: Computer simulations to predict the availability of peptides with known HLA class I motifs generated by proteolysis of HIV-1 proteins in infected cells. Virus Genes **10**, 227 (1995).

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MICROBIAL PATHOGENICITY FACTORS AS PARTS OF GLOBAL REGULATORY NETWORKS*

(A SHORT REVIEW)

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Abstract

Pathogenic bacteria differ from non-pathogenic isolates by the expression of so-called virulence or pathogenicity factors, including adherence molecules, toxins, capsules and others. The majority of the genes encoding pathogenicity factors are not expressed constitutively, but rather undergo environmental regulation or random regulatory events. In enterobacteria, such virulence associated genes are often corregulated with determinants influencing metabolic properties. By analyzing the structure and regulation of genes which are essential for the urovirulence of pathogenic *Escherichia coli*, we were able to show that genes coding for alfa haemolysin, cytotoxic necrotizing factor I and P fimbriae are located on large instable DNA regions, termed "pathogenicity islands". These islands also comprise regulatory genes which are able to activate adherence specific genes that are not part of those islands. In addition, pathogenicity islands are associated with tRNA loci. One of these tRNA genes, which codes for a minor leucin tRNA and is therefore termed *leuX*, acts as a global regulator. It influences the expression of various genes of pathogenic *E. coli*, including adherence specific loci, enterobactin genes, flagella specific gene clusters and determinants involved in serum resistance.

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Pathogenicity factors and regulatory networks

Only 200 out of the 40 000 identified bacterial species are able to cause infectious diseases. The majority of pathogenic bacteria are characterized by so-called virulence or pathogenicity factors [1]. The se factors include adherence factors, toxins, invasins, capsules or enzymes which contribute to the manifestation of an infectious disease. While some of the metabolic genes of pathogenic and non-pathogenic bacteria are expressed constitutively, the majority of virulence associated genes exhibit a differential expression pattern [2]. Some of the virulence associated genes respond to environmental conditions. They are organized together with genes encoding metabolic enzymes and structural components in so-called regulatory networks. Genes, organized in such networks are regulated by so-called global regulators, such as the catabolite responder protein (Crp), the leucin responder protein (Lrp) and others [3]. The organization of virulence associated genes and genes coding for metabolic enzymes or structural components in global networks illustrate the strong interdependency between virulence associated trades and physiological markers of pathogenic bacteria.

Environmental response venus random regulation

As indicated in Fig. 1, the expression of virulence associated genes can vary due to various general mechanisms. On one hand, the majority if not all cells of a bacterial population respond to changes in the environmental conditions [3], including temperature, carbon hydrate sources, ion stringency, pH and others. It has been shown that in a number of pathogenic and non-pathogenic bacteria, a shift of growth temperature from 20 °C to 37 °C causes a strong variation in protein expression. Especially, the so-called two component systems are able to regulate the expression of virulence and non-virulence associated genes [4]. Such systems have been identified for pathogenic *E. coli* (e.g. OmpR, EnvZ) as well as for *Salmonella typhimurium* (PhoP/PhoQ), *Bordetella pertussis* (BvgS/BvgR), *Vibrio cholerae* (ToxR, ToxS), and many other microorganisms.

Certain strains of a given population, however, are capable of altering their pattern of expression of particular genes randomly, i.e. not in response to environmental stimuli. These processes have been designated "phase variation" and "antigenic variation". The best elucidated example of a phase variation system is the shift from type I fimbriated *E. coli* to non-fimbriated *E. coli* and vice versa [5]. It has been shown that a 314 base pair DNA fragment located in the front of the structural *fim*A specific gene carries the main promoter region of type I genes. If the fragment is inserted in the right orientation, the structural genes are transcribed, if it is inserted in the wrong orientation, the strains do not express type I fimbriae. Phase variation can be influenced by environmental conditions, but in general, in a certain population there is a "turn on – turn off" shift on the basis of single cells. Also the H1-H2 variation of flagella of *Salmonella* undergoes phase variation.

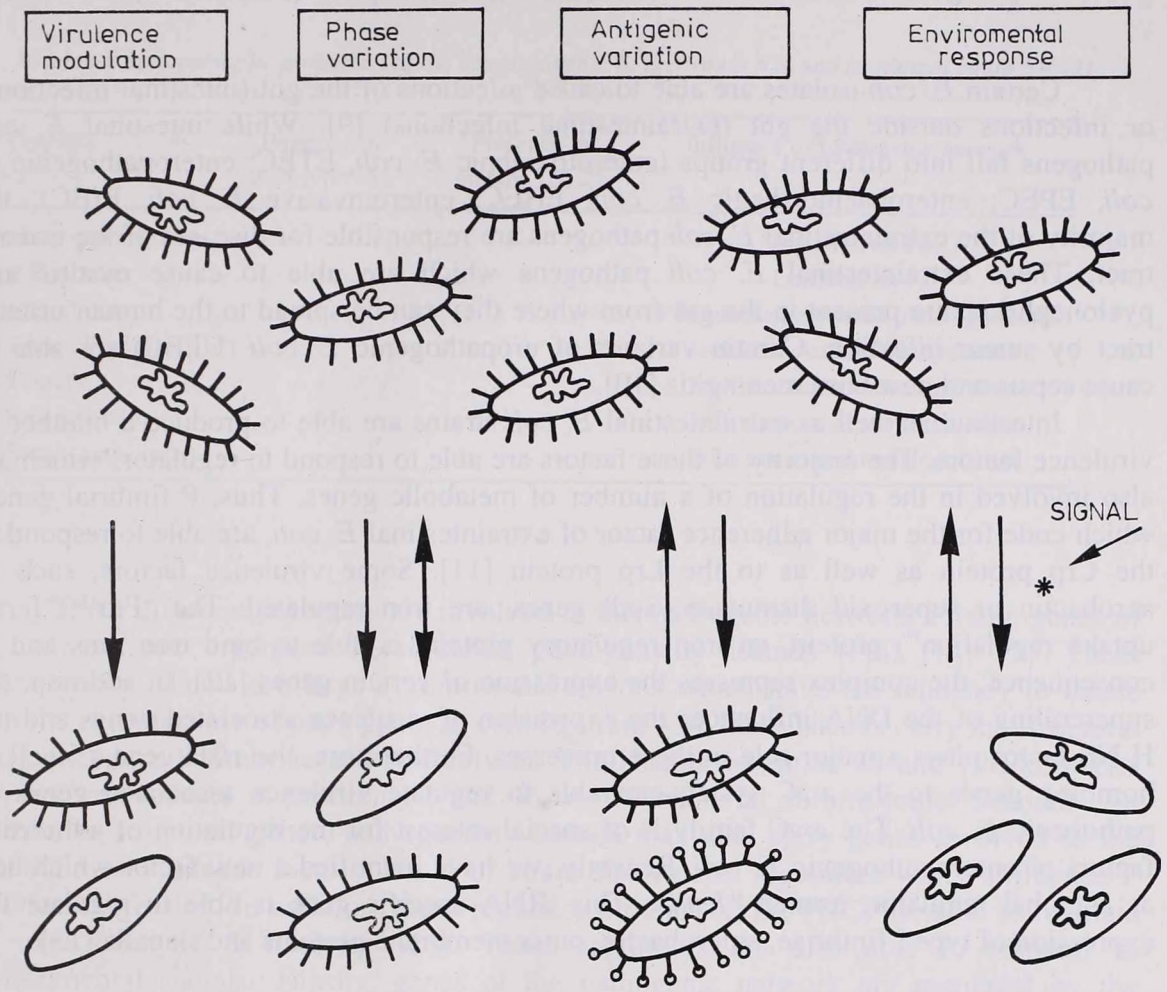

Fig. 1. Mechanisms leading to variation of virulence properties of pathogenic bacteria (for details see text)

In addition to phase variation, some bacteria are able to alter certain surface structures. This variation is termed "antigenic variation". The best elucidated system of such kind is the variation of pili of *Neisseria gonorrhoeae* [6]. Some silent copies of pilin specific genes are able to jump into expression sites and thereby cause a new composition of pili proteins. Similar processes have been shown for the variable surface protein (Vsp) of *Borrelia hermsi* [7]. While the phase variation of *E. coli* type I fimbriae is a reversible process, some of the antigenic variation processes are irreversible. In addition to phase variation and antigenic variation, so-called "virulence modulation" [8] can occur, which includes rearrangements, deletions or insertions of DNA fragments, and thereby results in a variation of surface proteins and other properties of microorganisms including pathogenic *E. coli.*

Regulation of virulence associated genes of pathogenic *E. coli*

Certain *E. coli* isolates are able to cause infections of the gut (intestinal infections) or infections outside the gut (extraintestinal infections) [9]. While intestinal *E. coli* pathogens fall into different groups (enterotoxigenic *E. coli*, ETEC; enteropathogenic *E. coli*, EPEC; enterohaemorrhagic *E. coli*, EHEC; enteroinvasive *E. coli*, EIEC), the majority of the extraintestinal *E. coli* pathogens are responsible for diseases of the urinary tract. These extraintestinal *E. coli* pathogens which are able to cause cystitis and pyelonephritis, are present in the gut from where they can be spread to the human urinary tract by smear infection. Certain variants of uropathogenic *E. coli* (UPEC) are able to cause sepsis and new born meningitis [10].

Intestinal as well as extraintestinal *E. coli* strains are able to produce a number of virulence factors. The majority of these factors are able to respond to regulators which are also involved in the regulation of a number of metabolic genes. Thus, P fimbrial genes, which code for the major adherence factor of extraintestinal *E. coli*, are able to respond to the Crp protein as well as to the Lrp protein [11]. Some virulence factors, such as aerobactin or superoxid dismutase (Sod) genes, are iron regulated. The "Fur" ("ferric uptake regulation") protein, an iron regulatory protein, is able to bind iron ions and in consequence, the complex represses the expression of certain genes [12]. In addition, the supercoiling of the DNA influences the expression of virulence associated genes and the H-NS factor plays a major role in these processes. Furthermore, the *rfa*H gene as well as homolog genes to the *ara*C system are able to regulate virulence associated genes of pathogenic *E. coli*. The *ara*C family is of special interest for the regulation of adherence factors of enteropathogenic *E. coli*. Recently, we have identified a new factor which acts as a global regulator, termed "*leu*X". This tRNA specific gene is able to regulate the expression of type I fimbriae, enterobactin, outer membrane proteins and flagella [13].

Analysis of a pathogenic network of virulent *E. coli*

In order to analyze the interplay of the regulation of virulence associated genes of pathogenic *E. coli*, the uropathogenic strain 536 has been used. As indicated in Table I, strain 536 is able to produce an O6 O antigen and a K15 capsule. In addition, the strain is able to produce flagella (H31), produces three types of adherence factors (P fimbriae, S fimbriae, type I fimbriae) and is able to secrete two α haemolysins [14]. Moreover, the strain is serum resistant, produces the iron uptake system enterobactin and is able to grow under anaerobic conditions. Mutants which were no longer able to express most of these virulence factors were isolated with relatively high frequencies (10^{-3}–10^{-4}). By analyzing the processes which lead to a shift from the virulent to the non-virulent phase, we were able to identify a so-called pathogenic network.

Table I

Virulence and metabolic properties of the uropathogenic E. coli strain 536 and its mutant strain 536-21

Property	Presence in wild type 536	Presence in mutant 536-21	Influence of pathogenic network
Haemolysin	+	–	Location on Pai
P Fimbriae	+	–	Location on Pai
S Fimbriae	+	–	P Fimbriae associated transregulation
Type I Fimbriae	+	–	*leu*X associated transregulation
Flagella	+	–	*leu*X associated transregulation
Enterobactin	+	–	*leu*X associated transregulation
Serum resistance	+	–	*leu*X associated transregulation

Three different processes are involved in this pathogenic network. Firstly, genes of the network are organized in so-called pathogenicity islands (Pai) [14, 15]. These pathogenicity islands are large chromosomal regions which have the tendency to delete from the chromosome of pathogenic *E. coli*. In strain 536, these islands carry the structural genes encoding P fimbriae and haemolysin. The islands comprise 70 and 190 kilobases (kb) and are located at position 82 and 96 on the bacterial chromosome. Secondly, in addition to structural genes, pathogenicity islands may also carry genes involved in the regulation of virulence factors. Pai II of strain 536 carries regulators which influence P fimbriae expression. In addition, these regulator proteins (PrfB, PrfC) are able to regulate the expression of S fimbriae [16]. These regulators are also able to respond to environmental signals. Thirdly, genes of the pathogenic network are regulated by the leucine specific tRNA *leu*X [13]. While most of the structural genes only carry very few *leu*X specific codons (TTG), some of the genes encoding regulatory proteins contain an increased number of *leu*X specific codons. For type I fimbria we have shown that the positive regulator FimB, which carries six *leu*X specific codons, is regulated on the translational level by the *leu*X specific tRNA (Olsen et al., in preparation). In addition, *leu*X is essential for full expression of enterobactin and serum resistance. Moreover, it affects the in vivo virulence of strain 536 [13]. It can therefore be concluded that the *leu*X system represents a new global regulatory system involved in the expression of virulence associated genes.

Conclusions

Virulence associated genes are organized in so-called pathogenic networks. The majority of virulence associated genes show a rather complicated mode of regulation. Activator and repressor molecules may be involved in the regulation of certain sets of genes. These regulators may represent the targets for signals from the environment or from

host organisms. While only very few data are available on the signaling of host cells to pathogenic bacteria, it is known that plant cells secrete phenolic compounds which are able to regulate the expression of virulence genes of *Agrobacterium tumifaciens* [2, 4]. Molecular analysis of pathogenic networks of pathogenic bacteria will be of help in the elucidation of host factors which have an impact on the regulation of virulence associated genes of human and animal pathogens. Moreover, the identification of these regulator proteins will aid in the development of new drugs which are essential for the treatment of infectious diseases.

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REFERENCES

1. Finlay,B., Falkow,S.: Common themes in microbial pathogenicity. Microbiol Rev **55**, 210 (1989).
2. Mekalanos,J.J.: Environmental signals controlling expression of virulence determinants in bacteria. J Bacteriol **174**, 1 (1992).
3. Parkinson,J., Kofold,E.: Communication modules in bacterial signaling proteins. Ann Rev Genet **26**, 71 (1992).
4. Gross,R.: Signal transduction and virulence regulation in human and animal pathogens. FEMS Microbiol Rev **104**, 301 (1993).
5. Klemm,P.: Two regulatory fim genes, fimB and fimE, control the phase variation of type 1 fimbriae in *Escherichia coli.* EMBO J **5**, 1389 (1986).
6. Meyer,T., Gibbs,C., Haas,R.: Variation and control of protein expression in *Neisseria.* Ann Rev Microbiol **44**, 451 (1990).
7. Barbour,A.G.: Antigenic variation of surface proteins of *Borrelia* species. Rev Infect Dis **11**, 5399 (1988).
8. Ott,M.: Dynamics of the bacterial genome: deletions and integrations as mechanisms of bacterial virulence modulation. Zbl Bakt **278**, 457 (1993).
9. Mühldorfer,J., Hacker,J.: Genetic aspects of *Escherichia coli* virulence. Microb Pathogen **16**, 171 (1994).
10. Virkola,R., Parkkinen,J., Hacker,J., Korhonen,T.K.: Sialyloligosaccharide chains of laminin as an extracellular matrix target for S fimbriae of *Escherichia coli.* Infect Immun **61**, 4480 (1993).
11. Hacker,J.: Genetic determinants coding for fimbriae and adhesins of extraintestinal *Escherichia coli.* Curr Top Microbiol Immunol **151**, 1 (1990).
12. Nelands,J.B.: Mechanism and regulation of synthesis of aerobactin in *Escherichia coli* K12 (pColV-K30). Can J Microbiol **30**, 728 (1992).
13. Ritter,A., Blum,G., Emödy,L., Kerenyi,M., Böck,A., Neuhierl,B., Rabsch,W., Scheutz,F., Hacker,J.: tRNA genes and pathogenicity islands: influence on virulence and metabolic properties of uropathogenic *Escherichia coli.* Mol Microbiol **17**, 109 (1995).
14. Blum,G., Ott,M., Lischewski,A., Ritter,A., Imrich,H., Tschäpe,H., Hacker,J.: Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect Immun **62**, 606 (1994).
15. Blum,G., Falbo,V., Caprioli,A., Hacker,J.: Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and alpha-hemolysin form the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. FEMS Microbiol Lett **126**, 189 (1995).
16. Morschhäuser,J., Vetter,V., Emödy,L., Hacker,J.: Adhesin regulatory genes within large, unstable DNA regions of pathogenic *Escherichia coli*: cross-talk between different adhesin gene clusters. Mol Microbiol **11**, 555 (1994).

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PASTEUR'S PLACE, ROLE AND IMPORTANCE IN THE HISTORY OF PHILOSOPHY*

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Philosophy is a science aimed at developing and determining our world concept and mind on the basis of our best scientific reasoning. In its history we can follow up the development of various science-historical eras, thus noting the parallel changes.

The permanent question of the origin of life and the development of the epidemic diseases, including the pandemic ones wiping out millions of living organisms, occupy a central position in our philosophy. Indeed, in the history of philosophy we find the names of the representatives of the various ideas and trends entwining both of these questions as well as the historical development of false doctrines and believes. However, Louis Pasteur's (1822–1895) name cannot be found even in the best works dealing with the history of philosophy, though he made his name immortal by disproving the false doctrine of abiogenesis, by proving the pathogenic role of bacteria and by introducing the entity and concept of infectious disease (Figs 1 and 2).

The present commemorative session of our Society held at the 100th anniversary of Pasteur's death offers an opportunity to meet this need and to ensure the right place in the "science of sciences" (i.e. philosophy) for the scientist who changed our world concept and mind concerning Nature.

Disproval of the doctrine of abiogenesis

Unlike other scientist, like Harvey, who formulated the principle "omne vivum e vivo"; Redi, Reaumur, Swammerdam, and Spallanzani, who called in doubt the abiogenesis principle, and Virchow, who formulated the principle "omnis cellula e cellula eiusdem generis", Pasteur, the chemist, when initiating investigation into the origin of living organisms, was not led by the curiosity of the researcher. Neither his

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Fig. 1. Portrait of Pasteur

religious conviction fronted him to oppose the false doctrine of abiogenesis, a principle which, from Aristotle's time on had been propagated by scientists like van Helmont and Needham and confirmed by Buffon. (Abiogenesis or spontaneous generation is mentioned in the Bible, in Samson's legends as well). In contrast with many successful researchers, even those who performed no systematic investigations, Pasteur was not supported in his discoveries by the grace of Fortune the goddess. He encountered this question by chance when he discovered fermentative bacteria. Being of quick temper, he wrote a letter in response to an essay published by Pouchet, director of the Museum of Natural History Rouen, a scientist of high authority on the "spontaneous generation" of "protobacteria" (1858). In his letter Pasteur illuminated the errors found by him in Pouchet's work. Without replying to Pasteur's letter Pouchet turned to the Academy of Sciences: he requested for inviting applications in order to re-examine the problem of abiogenesis. Despite the benevolent warnings received from his friends, Pasteur took part in the competition. Excluding the possibility of spontaneous generation and interpreting his own results with a brilliant logic, he won the Alhumbert-prize offered by the French Academy of Sciences. It should be noted that at the same time when Pasteur was disputing with Pouchet and other adherents of abiogenesis, the scientific world was full of disputes in the field of natural history. Followers of the Darwinian scientific naturalism were fighting against adherents of catholic idealism. Pasteur was attacked from both sides: by Renan and Bourget for atheist naturalism from the one side and by

Richet and his followers from the other for bigoted religiosity. In fact, Pasteur himself was a devout catholic who did not accept Darwin's evolution theory and for him the disproval of abiogenesis meant refusal neither the Creation, nor the rule of stability of species the rule in such an attractive manner by Linné: "Species tot numeramus quot ab initio creavit infinitum Ens". Pasteur was able to agree his scientific view with religiosity to such a degree that in co-operation with the French physicist Duhem he founded a Catholic Scientific Society in Brussels.

It is widely believed that Pasteur dealt at the false doctrine of abiogenesis a hard – moreover, a lethal thrust. However, a false doctrine resembles not only a chimera – as he said – but the seven-headed dragon of fairy tales: when one of its head has been cut off, seven new heads will grow out instead. Pouchet collected new arguments to prove his imaginary truth. Not much later, the English pathologist Bastian (1876) proposed further arguments for the existence of abiogenesis. All these attempts were weak in comparison to the efforts made by Engels to make this false idea acceptable.

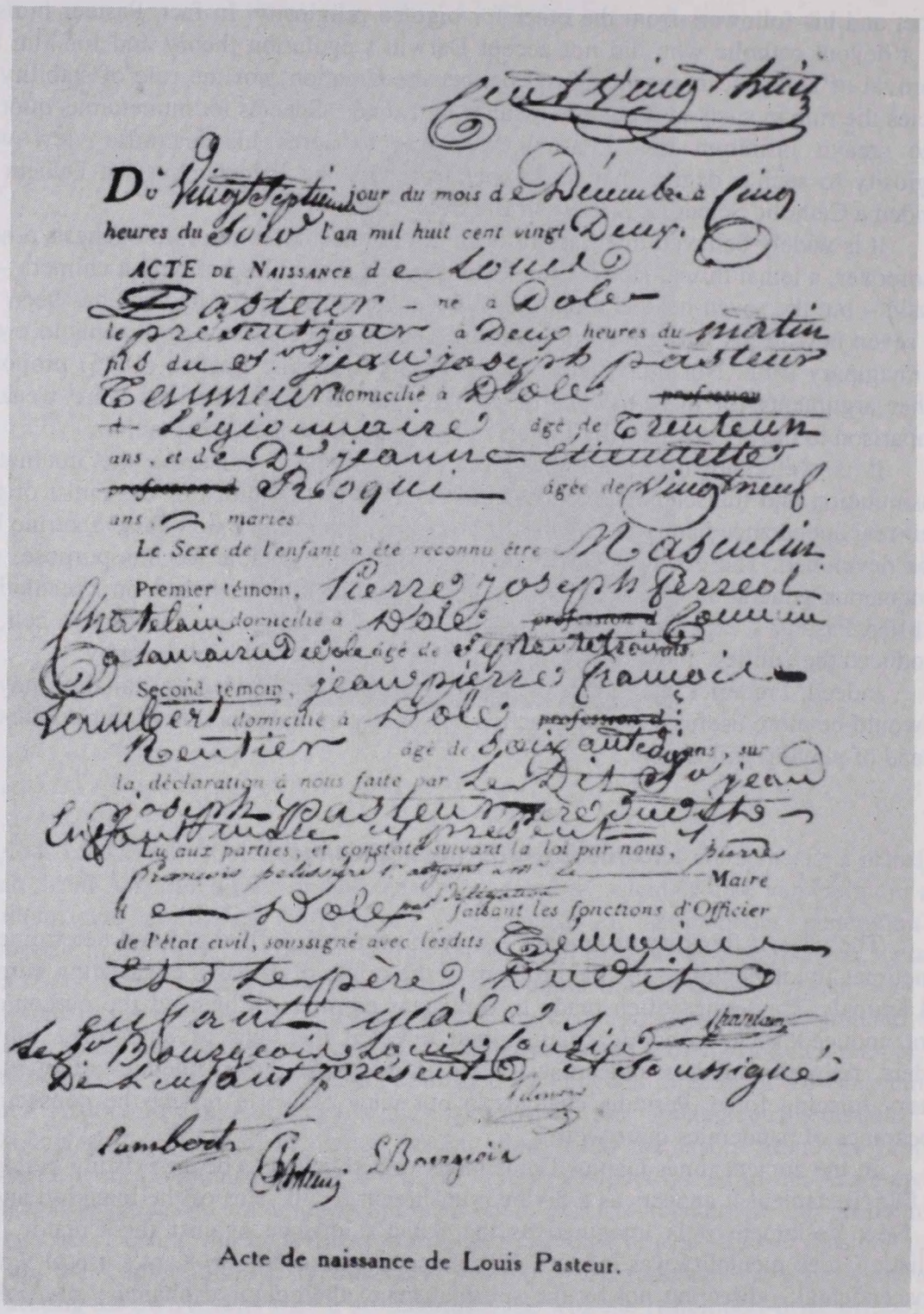
It is well known that in the great consecution in existence the qualitative transmutation and transelementation of the substance were entities of alchemist origin. Their real appearance had faded by the 19th century. Therefore a new false doctrine had to be developed. The evolution of species seemed to be suitable for this purpose, and spontaneous generation appeared within this doctrine. Engels, based on speculation, qualified Pasteur's experiments as being useless and valueless; instead of live cell, he introduced the entities "live protein" and "live protoplasma" in his philosophy.

Indeed, Frederick the Great was right in his letter addressed to Voltaire: may be we would be more useful if we were active personally in reproduction of our population instead of playing philosophy.

Revealing of Genius Epidemicus

The way of thinking of humans has been attracted, influenced and determined – sometimes fundamentally – by the problem of the origin of diseases devastating humans and animals. Pandemics often made to disappear empires, influenced the outcome of wars, induced socialeconomic changes and directed the fates of emperors, military leaders, religion founders and artists. There is no doubt that pandemics often had a history forming force. Periodic changes in our view of world reflect the consecutive appearance of pandemics quite well.

In the ancient times Genius Epidemicus was believed to be an existing being. In the Old Testament it appears as a divine punishment for the sins of the mankind and in the New Testament it is imagined as the Satan's intrigue against the Creator. The realistic Greek mentality was led to a metaphysical – according to Kant's use of words: transcendental – direction not by the spiritualism of the coloured phantasy of Arab or Christian Middle Ages, both inclining to mysticism, but by pandemics of plague, which could be overcome neither by religious ceremonies nor by the wonders of alchemy. Even astrologers failed to detect any relationship between celestial events and the appearance of pandemics. There is no doubt that the development of philosophy cannot



Acte de naissance de Louis Pasteur.

Fig. 2. Pasteur's name in the register of birth (December 22, 1822)

be attributed to the human intelligence growing generation by generation; it can be attributed – first of all – to untenability of the actually dominant world view as a result of devastating pandemics. The massacres due to plague and the raging of syphilis led Fracastoro (1478–1553) to describe the entity "contagion" and its spreading "per contactum", "per fomitem" and "per distantiam". The old concepts "mephitis" and "miasma" were followed by "contagium animatum" in both the medicine and the universal human way of thinking. The period in which the devastating pandemics were being attributed to decomposing rotted materials from which contagia were transmitted by the wind, lasted centuries.

These permanent speculative concepts all were to be overcome by Pasteur. The famous pathologist Osler characterized Pasteur with the following sentence: "Before him Egyptian darkness; with his advent light that brightens more and more as the years give us ever fuller knowledge". He was right.

Clearly, making people to accept the germ theory was not an easy task, though irrefutable arguments were available for Pasteur. Great authorities in science like Liebig the chemist, and good friend as the physiologist Claude Bernard, who was so difficult to convince and Koch, Pasteur's great rival were his opponents within profession. The universal acceptance of the germ theory and the victory against the Genius Epidemicus could not be achieved without the results published by Semmelweis, Lister and Koch and without the recognition of the economical importance of silkworm diseases and "pasteurization" and the resounding success of the vaccination against rabies. In addition, it had to be shown that the phantoms often seen by patients suffering from high fever were not ghosts in reality, they are not identical with a demon of the disease, instead, they are pareidolias, illusions, hypnagogic hallucinations during feverish delirium. Their appearance can be avoided and prevented by procedures successfully applied against infectious diseases. The recognition of all these indicated in fact the end of the Middle Ages – an age dominated by demon belief – and the beginning of a new era in human way of thought, in human mentality. It was Pasteur who opened a new aetiological epoche by introducing the "germ theory" and – by leading to the birth of bacteriological era – the concept of specific pathogens in the nosology.

The present changes in mode of life lead to a direct damage of health, to destruction of scientific view and the way of our thinking. Microbiologists should intensely fight against these harmful tendencies. This is a professional, moral and vocational responsibility of theirs. In his message addressed to the university students on the occasion of his 70th birthday celebration Pasteur emphasized: spend as much time as possible in the serene peace of laboratories and libraries. Say to yourselves first of all: what have I done for my instruction? and as you go on further, what have I done for my country? until the time comes when you have the happiness of thinking that you have contributed in some way the progress and to the welfare of humanity.

Fig. 3. Bronze bust of Pasteur (by P. Dubois) in the Museum for history of the Veterinary Medicine, Budapest

In memoriam Pasteur

Having stressed the disproval of abiogenesis as Pasteur's main merit, I would be wrong not to mention his further ones such as the discovery of the microbial origin of infectious diseases, molecular dyssymmetry, fermentation and anaerobiosis. Besides, he invented "pasteurization", silkworm diseases, and developed vaccinations, the postinfectious immunization against rabies being the most important among them. For all of these results, Pasteur is honoured by experts engaged in medicine as well as by those working in the field of microbiology (in the wisest sense), immunology, agriculture and food industry. The whole mankind is grateful for his magnificent discoveries and his homeland is proud of his greatness. However, philosophy has not recognized the importance of Pasteur's scientific results by which he changed the fundamental features of our scientific reasoning. This situation should be changed. Are we right when we erect monuments above all on the memory of politician, revolutionists and military leaders and name streets, squares and gardens after them? Are we surprised when our government disregards the merits of our scientists if ourselves are inactive in this field?

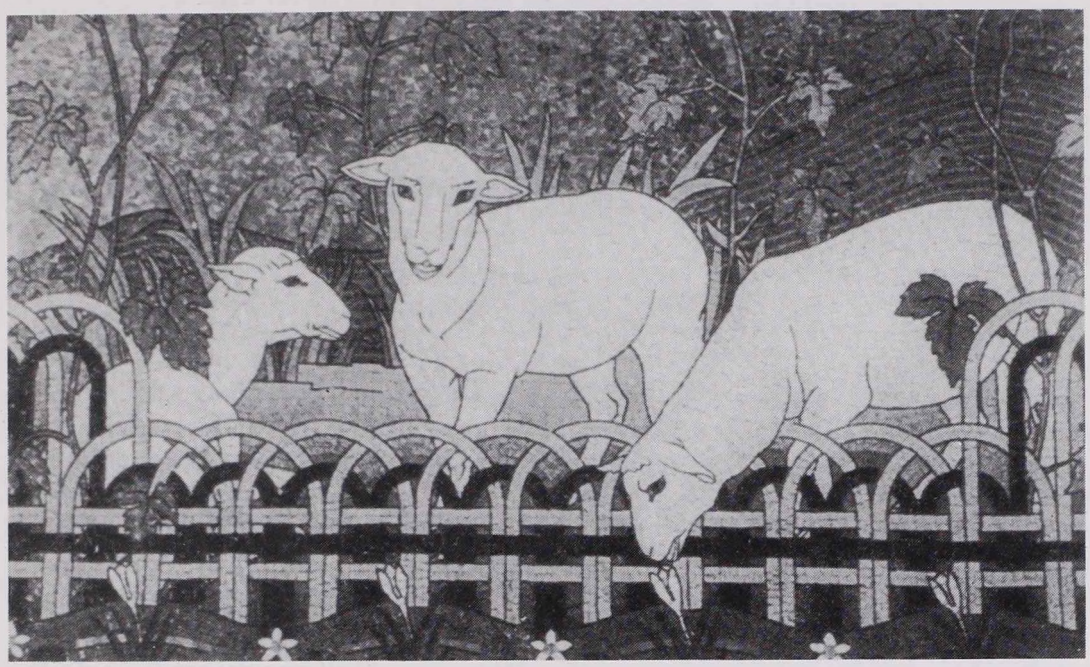


Fig. 4. Pasteur's sepulchre. A part of Mosaic picture representing a flock of sheep saved by preventive vaccination from anthrax (in the crypt of Pasteur Institute, Paris)

It was only professor Preisz (1860–1940) who raised the unique bust to Pasteur in the first National Institute of Bacteriology in Hungary, founded by him in Budapest (1900). This institute was soon incorporated into the Veterinary College, and the bust (Fig. 3) not long ago was placed in the Museum for Veterinary Medicine, founded in 1976. The misfortune of this bust represents well the present social appreciation of theoretical microbiological research in our Country. Therefore I would like to make a proposal: to keep Pasteur's memory green (Fig. 4) our Society should organize a Pasteur Commemorative Session year by year to help us in performing microbiological research in Pasteur's spirit.

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GLUTATHIONE–GLUTATHIONE REDUCTASE SYSTEM AND LIPID PEROXIDATION IN *SACCHAROMYCES CEREVISIAE* UNDER ALCOHOL STRESS

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The content of malondialdehyde (MDA) as well as diene conjugates increased in *Saccharomyces cerevisiae* under alcoholic conditions. These cells had lower content of water soluble antioxidant, i.e., glutathione. Ethanol-treated *S. cerevisiae* also exhibited lower activity of glutathione reductase (GR). The decrease in reduced glutathione (GSH) content cannot be attributed completely to lower GR activity. Not only the GSH content was lowered, but oxidized glutathione (GSSG) was also less in ethanol-treated yeast cells. The decrease in oxidized glutathione was much more (84%) as compared to that of reduced glutathione (40%).

Biological membrane lipids are usually rich in polyunsaturated fatty acids (PUFAs) which make them susceptible to peroxidation. Lipid peroxidation is initiated and propagated by free radicals and lead to the formation of malondialdehyde (MDA), conjugated dienes and lipid hydroperoxides [1]. Hydroperoxides are further oxidized to secondary autoxidation products. Hydroperoxides and other secondary autoxidation products are toxic and are closely associated with serious diseases and senescence in animals [2, 3]. The formation of lipid hydroperoxides in biomembranes results in damage of membrane structure and inactivation of membrane-bound enzymes [4]. So, lipid hydroperoxides are needed to be reduced to non-toxic lipid alcohols. This reduction is catalyzed by glutathione peroxidase at the expense of reduced glutathione [5]. But oxidized glutathione is also a toxic product [6]. Therefore, the reduction of GSSG is required in the cell for its proper functioning. GSSG is reduced back to GSH in GR-catalyzed reaction. The content of lipid peroxidation products has been reported to be higher in ethanol intoxicated organisms [7]. The present study was aimed to investigate the role of GSH-GR system in relation to lipid peroxidation in ethanol-treated *Saccharomyces cerevisiae*.

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Materials and methods

Yeast strain. S. cerevisiae var. *ellipsoides* was obtained from the University of California Davis, U.S.A.

Growth of yeast cells. Yeast cells were cultured in 500 ml Erlenmeyer flasks containing 100 ml basal synthetic medium [8] at 27±1 °C for 24 h. The composition of the medium per litre was glucose 20 g, yeast extract 1 g, ammonium sulphate 1 g, magnesium sulphate 0.5 g, potassium dihydrogen orthophosphate 0.875 g, dipotassium hydrogen phosphate 0.125 g, calcium chloride 0.1 g and sodium chloride 0.1 g. The cells were harvested by centrifugation at 5 000 *g* for 15 min and washed with 0.01 M phosphate buffer (pH 7.0) to remove the adhering metabolites and unused ingredients of the medium.

These cells (approximately 5 mg/ml) were treated with different concentrations of ethanol in 0.01 M phosphate buffer (pH 7.0) and 0.5% dextrose. During alcohol treatment, the cells were shaken on rotary shaker at 250 rpm. The control cells were suspended in the same phosphate buffer but without alcohol. After 12 hours of alcohol treatment, the cells were harvested, washed and used for various analyses.

Viability measurement. For estimating the growing capacity (viability) of the yeast cells, washed cells suspended in saline were diluted to 4×10^3 cells/ml and 0.05 ml aliquots of the sample was used to inoculate YPD agar plates. The plates were inoculated at 27±1 °C for 4 days. Number of colony forming units were counted in ethanol treated as compared to samples.

Measurement of lipid peroxidation. Lipid peroxidation in ethanol-treated yeast cells was estimated by assaying the contents of MDA as well as diene conjugates.

Assay of malondialdehyde. The method of Buege and Aust [9] was followed to assay MDA in yeast cells. MDA was extracted from yeast cells by homogenizing them in chilled saline (0.9% NaCl). Two ml of the extract was mixed with 4 ml of thiobarbituric acid reagent (15% TCA and 0.375% TBA in 0.25 N HCl), the tubes were covered with water ampoules and placed in boiling water bath for 20 min. After cooling, the contents were centrifuged and the absorbance of the supernatant was recorded at 535 nm in 1 cm cell against a blank prepared by taking saline in place of the homogenate. The MDA content was calculated by taking its molar extinction coefficient to be 1.56×10^{-5} M^{-1} cm^{-1}.

Diene conjugate assay. Conjugated dienes in ethanol-treated yeast cells were estimated in their lipids extracted according to the method of Folch et al. [10]. Lipid peroxidation results in rearrangement of double bond in fatty acids resulting in the formation of diene conjugates having absorption maxima at 233 nm [11].

Estimation of glutathione. Yeast cells were homogenized in 10% (w/v) chilled trichloroacetic acid (TCA) and centrifuged to have a clear supernatant for the estimation of glutathione. Reduced glutathione was measured by the method of Beutler et al. [12]. Total glutathione was measured after the reduction of GSSG using the methodology of Habeeb [13].

Glutathione reductase assay. The method of Tyson et al. [14] was followed to estimate the activity of GR. The protein content of the enzyme preparation was estimated by the method of Lowry et al. [15].

Results and Discussion

Ethanol tolerance of *S. cerevisiae* (i.e growing capacity after ethanol treatment) was studied (Table I) The viability of the yeast cells decreased after alcohol treatment. Ethanol treatment affects the structure and functions of plasma membrane which in turn may be responsible for decreased viability hence growing capacity of yeast cells.

Table I

Effect of ethanol treatment on growing capacity (colony forming units) of S. cerevisiae

Ethanol (% v/v)	Colony forming units
0	16.30×10^7
4	15.61×10^7
8	11.52×10^7
12	2.30×10^7

The results are mean of four independent experiments in triplicate

Yeast cells treated with ethanol showed higher levels of lipid peroxidation as indicated by their contents of malondialdehyde (MDA) and diene-conjugates (Table II). The increase in lipid peroxidation products in the presence of ethanol was concentration dependent. The increased lipid peroxidation might be the manifestation of increased oxygen centred free radical generation in these cells. Increased production of such reactive intermediates, i.e., superoxide radicals, hydrogen peroxide and hydroxyl anion in ethanol-treated neural crest cells has been reported [16] which lead to ethanol-induced toxicity in these cells. Ethanol might also have induced changes in structure as well as physical state of yeast cell membranes which make their lipids more susceptible to peroxidative attack.

Table II

Effect of ethanol treatment on lipid peroxidation in S. cerevisiae cells

Ethanol (% v/v)	MDA			Diene conjugates (nm/100 mg dw)
	nm/100 mg dw	nm/mg total lipids	nm/mg phospholipids	
0	5.02±0.09	1.21±0.05	2.60±0.09	121.20±4.41
4	6.21±0.09	2.40±0.10	4.73±0.11	148.76±3.81
8	6.66±0.10	3.92±0.08	11.92±0.33	168.67±4.21
12	6.96±0.12	5.20±0.10	29.24±0.27	186.42±5.89

Results are mean±SD of four independent experiments in triplicate. The values are statistically significantly different from the control at $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$

Reduced glutathione is a water-soluble chain breaking antioxidant that scavenges free radicals and suppresses their chain propagation and a protein cytosolic factor enhances radical scavenging properties of GSH [17]. Alcohol-treated *S. cerevisiae* cells showed a significant decrease in the levels of GSH (Table III). The decrease in GSH content was not only due to its oxidation as total glutathione content was also lowered significantly in 12% (v/v) ethanol-treated cells. Yeast cells treated with 12% ethanol had a total glutathione content half of that in the control cells. The mechanism whereby alcohol-treated cells lose their total glutathione is obscure. Furthermore, GSSG content decreased by approximately 84% while there was only about 40% decrease in the content of GSH. As a result, GSH/GSSG ratio increased from 2.50 in the control cells to 9.38 in 12% ethanol-treated yeast cells. Therefore, it can be said that the cells tried to maintain pools of GSH and it is because of decrease in total glutathione that GSH levels also decreased. Adams et al. [18] reported that there was no change in GSH content of alcoholics while GSSG content was decreased significantly. Zindenberg-Cherr et al. [19] proposed that rats taking chronic ethanol ingestion were at the risk of peroxidative damage with continued ethanol exposure because of decline in the protection afforded by glutathione system. The enhanced lipid peroxidation in rats exposed intratracheally to coalfish oil and polymorphonuclear leukocytes from hypertensives was also related to low level of GSH in them [20]. The decreased content of GSH in ethanol-treated *S. cerevisiae* might be responsible for higher levels of lipid peroxidation.

Table III

Effect of ethanol treatment on glutathione content of S. cerevisiae cells

Ethanol (% v/v)	Total glutathione (μg/100 mg dw)	Reduced glutathione (μg/100 mg dw)	Oxidized glutathione (μg/100 mg dw)	GSH/GSSG ratio
0	1076.02±12.90	768.75±9.28	307.64±4.28	2.50
4	901.16±10.41***	683.01±8.71***	218.15±5.54***	3.13
8	687.15±8.91***	571.63±6.37***	115.52±6.40***	4.95
12	513.20±6.71***	462.86±4.76***	49.33±1.62***	9.38

Results are mean±SD of four independent experiments in triplicate. The values are statistically significantly different from the control at ***$p<0.001$

Glutathione reductase activity in ethanol-treated *S. cerevisiae* cells decreased as the concentration of alcohol was raised (Table IV). GR activity was 65.25±2.87 units/mg protein in 12% ethanol-treated cells as compared to 110.35±3.96 units/mg protein in the control cells. The decreased GR activity in such cells might be catalyzing the reduction of GSSG to GSH less efficiently. However, this is not in agreement to the observation that GSH/GSSG ratio in ethanol-treated yeast cells was higher than in the control cells. No

doubt GSH content on dry weight basis decreased in yeast cells on alcohol treatment. To explain this further studies are required. As expected from the role of GR in antioxidant defence system, GR activity and lipid peroxidation were inversely related. A similar correlation between GSH content, GR activity and lipid peroxidation has also been reported in rats chronically exposed to vanadium and in those exposed intratracheally to coalfish oil [20].

Table IV

Effect of ethanol treatment on glutathione reductase activity of S. cerevisiae cells

Ethanol (% v/v)	Glutathione reductase activity (units*/mg protein)
0	110.35±3.96
4	87.41±2.66**
8	77.04±2.92***
12	65.29±2.87***

Results are mean±SD of four independent experiments in triplicate. The values are statistically significantly different from the control at **$p<0.005$, ***$p<0.001$

* One unit of the enzyme activity is defined as the enzyme required to generate one µg GSH/min under the assay conditions

Thus, it can be concluded that GSH-GR system was weakened in ethanol-treated *S. cerevisiae* which lead to raised levels of lipid peroxidation products in these cells.

REFERENCES

1. Pryor,W.A.: In Pryor,W.A (ed.): The role of free radical reactions in biological systems. Free radicals in biology Vol 1, Academic Press, New York 1976. pp. 1–49.
2. Sevanian,A., Stein,R.A., Mead,J.F.: Metabolism of epoxidized phosphatidylcholine by Phospholipase A_2 and epoxide hydrolase. Lipids **16**, 781 (1981).
3. Halliwell,B., Gutteridge,J.M.C.: Oxygen toxicity oxygen radicals, transition metals and diseases. Biochem J **219**, 1 (1984).
4. Tein,M., Svingen,B.A., Aust,B.A.: An investigation into the role of hydroxyl radicals in xanthine oxidase dependent lipid peroxidation. Arch Biochem Biophys **216**, 142 (1981).
5. Flohe,L.: In Pryor,W.A. (ed.): Free radicals in biology Vol 5, Academic Press, New York 1982. pp. 223–254.
6. Meister,A.: Metabolism and functions of glutathione. Trends Biochem Sci **6**, 221 (1981).

7. Uysal,M., Kutalp,G., Ozdemirler,G., Aykae,G.: Ethanol induced lipid peroxidation and glutathione content in rat brain. Drug Alc Depend **22**, 227 (1989).
8. Hayashida,S., Feng,D.D., Ohta,K., Chaitiumvong,S., Hongo,M.: Composition and role of *Aspergillus oryzae* proteolipid as a high concentration of ethanol producing factor. Agric Biol Chem **40**, 73 (1976).
9. Buege,J.A., Aust,S.D.: Microsomal lipid peroxidation. Methods Enz **52**, 302 (1978).
10. Folch,J., Lees,M., Sloane-Stanley,G.H.: A simple method for isolation and purification of total lipids from animal tissues. J Biol Chem **266**, 497 (1957).
11. Recknagel,R.O., Ghoshal,A.K.: Diene Conjugation assay. Methods Enz **52**, 308 (1966).
12. Beutler,E., Duron,O., Kelley,B.M.: J Lab Clin Med **61**, 882(1963).
13. Habeeb,A.F.S.A.: Reaction of protein sulfhydral groups with Ellman's reagent. Methods Enz **25**, 457 (1972).
14. Tyson,C.A., Lunall,K.D., Stephens,R.J.: Age related differences in GSH-Shuttle enzymes in NO_2^- or O_3^- exposed rat lungs. Arch Environ Health **37**, 167 (1982).
15. Lowry,O.H., Rosebrough,N.J., Farr,A.L., Randall,R.J.: Protein measurement with Folin-phenol reagent. J Biol Chem **193**, 265 (1951).
16. Davis,W.L., Crawford,L.A., Cooper,G.R., Thomas,D.L., Freeman,B.L.: Ethanol induces the generation of reactive free radicals by neutral crest cell in vitro. J Craniofac Genet Develop Biol **10**, 277 (1990).
17. Niki,E., Yamamoto,Y., Kamiya,Y.: Role of uric acid, Cysteine and glutathione as chain breaking antioxidant in aqueous phase. Chem Lett **8**, 1267 (1985).
18. Adams,J.D., Khaw,E., Jaresko,G., Noguchi,J.K.: Plasma glutathione and glutatione disulfide in alcoholics during detoxification. Res Commun Subst Abuse **11**, 153 (1990).
19. Zindenberg-Cherr,S., Halsted,C.H., Olin,K.L., Reisenauer,A.M.C.: The effect of chronic ethanol ingestion on free radical defence in the miniture pig. J Nutr **120**, 213 (1990).
20. Srivastava,P.K., Singh,Y., Misra,V.K.: Pulmonary and hepatic glutathione levels, glutathione shuttle enzymes and lipid peroxidation in rats exposed intrathecally to coal fish oil. Biochem Int **17**, 509 (1988).

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COAGULASE-NEGATIVE STAPHYLOCOCCI IN AIR SAMPLES FROM OPERATING THEATRES

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The aim of our study was to recognize coagulase-negative staphylococci (CNS) in the air of operating theatres. Out of the identification of 449 isolates, the most frequent species were *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. The strains were adherent to glass in 52.3%. Most of the *S. epidermidis* showed adherent growth, while the majority of the *S. haemolyticus* failed to adhere. The disk-diffusion antibiotic sensitivity tests showed great differences in sensitivity to penicillin, tetracycline and erythromycin between adherence-positive and negative isolates. On the whole, the species *S. haemolyticus* proved to be much more resistant than *S. epidermidis*. *Staphylococcus warneri* was the most, while *S. haemolyticus* was the least sensitive to phages.

In 1958 Smith and his colleagues called the attention to the potential pathogenic role of coagulase-negative staphylococci (CNS) [1]. Since that time according to different reports CNS have been one of the most problematic agents causing nosocomial infections. Among the CNS, *Staphylococcus epidermidis* is the most prominent one [2]. There are different reports about occurrence, spread and distribution of methicillin-resistant and multiresistant strains [2–5]. The ability of showing adherent growth can be considered as a virulence factor and also has a significant role in epidemiology [6–9].

According to our experience the CNS are the most frequent cause of bacterial air pollution in operating theatres. This group of bacteria was found in a high proportion in samples from operating theatres. The aim of our study was to detect the incidence of different CNS species in the air samples of operating theatres, their antibiotic sensitivity, adherence, phage-type and association of adherent growth with other properties.

Materials and methods

The air samples were collected in a period of three years between 1990–1992 from operating theatres of three hospitals of Hajdú-Bihar county. In the period of the experiments the samples were taken from 28 operating theatres in 48 occasions. The samples were taken according to instructions of

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the National Institute of Hygiene, Hungary [10]. In essence, blood agar plates were exposed to the sedimentation of bacteria from the air under standard condition. In more than 95% of the cases we took the samples before operation started.

Identification was done based on the methods of Akatov et al. [11].

Adherence to glass was tested according to Christensen et al. [6] modified by Barcs et al. [9].

Antibiotic sensitivity test. The antibiotic sensitivity tests of selected antibiotics were done by disk-diffusion method on Mueller-Hinton agar (Diagn. Pasteur, France), using penicillin-G, 3 U; oxacillin 10 μg; cefotaxime, 30 μg; erythromycin, 10 μg; vancomycin, 50 μg; trimethoprim/sulfamethoxazole, 25 μg; tetracycline, 30 μg; tobramycin, 10 μg; ofloxacin, 5 μg (Resistest disks, Human, Budapest, Hungary) and netilmicin, 30 μg (MAST Lab. LtD, Liverpool, England).

Phage typing. For CNS phage typing Pulverer's phage series were used [12].

Results

Incidence of species. As Table I shows in the air samples of the operating theatres the most common species was *S. epidermidis* (33.9%) followed by *Staphylococcus haemolyticus* (22.5%) and *Staphylococcus warneri* (8.7%). The unclassificable interspecies group formed 26.9% of the strains.

Table I

Incidence of different CNS species in the air of operating theatres

Staphylococcus	No.	%
S. epidermidis	152	33.9
S. haemolyticus	101	22.5
S. warneri	39	8.7
S. cohnii	12	2.7
S. saprophyticus	11	2.4
S. hominis	9	2.0
S. capitis	1	0.2
S. xylosus	3	0.7
Interspecies group	121	26.9
Total	449	100

Difference in antibiotic resistance between the two main species. Table II shows that a significantly higher portion of the *S. epidermidis* isolates was sensitive to penicillin-G, erythromycin and tetracycline, while more were resistant to oxacillin and trimethoprim/sulfamethoxazole compared to *S. haemolyticus.*

Table II

A comparison of the antibiotic susceptibility of S. epidermidis (n=125) and S. haemolyticus (n=47) strains cultured from the air of operating theatres

Antibiotic	Susceptible			
	S. epidermidis		*S. haemolyticus*	
	No.	%	No.	%
Penicillin	47	38	9	19
Oxacillin	101	81	42	89
Erythromycin	100	80	21	45
Tetracycline	33	26	3	6
Trimethoprim / Sulfamethoxazole	64	51	30	64
Tobramycin	117	94	45	96

Bacteriophage typability. A number of isolates of *S. epidermidis*, *S. haemolyticus* and *S. warneri* was randomly phage typed with the phage series of Pulverer and co-workers [12]. Table III shows that the isolates of *S. warneri* were more susceptible to the lytic effect of phages tested, except phage Ph:10 than isolates of the two other species. *S. epidermidis* and *S. haemolyticus* showed similar rates of lysis with the exception of lysis by phage Ph:5. There was seen a considerable difference in the number of the lytic phage receptors on the cell layers of the three species (Table IV). *S. warneri* possessed the highest numbers of the receptors followed by *S. epidermidis*, while *S. haemolyticus* exhibited the fewest receptor numbers indicating a notable difference in the cell surfaces of the three species.

Occurrence of adherent growth. All the 449 isolates were examined for adherence to glass and 235 showed adherent growth (52.3%). Among these, as Table V exhibits, the *S. epidermidis* species was predominant (40.8%) followed by the heterogenous interspecies group with 25.9% More detailed results are given in Table VI showing the incidence of the adherent growth within each species. One can see that the majority of *Staphylococcus hominis*, *S. epidermidis* and members of the interspecies group could adhere to glass. Adherent growth was hardly revealed in the species of *S. haemolyticus* and *S. warneri.*

Table III

Phage typability of the main coagulase-negative Staphylococcus species isolated from air samples of operating theatres

Phages	*S. epidermidis* n=50		*S. haemolyticus* n=28		*S. warneri* n=23	
	No. of isolates tested	Rate of lysis, %	No. of isolates tested	Rate of lysis, %	No. of isolates tested	Rate of lysis, %
Ph: 12	30	60	15	54	19	83
Ph: 13	29	58	17	61	17	74
Ph: 10	17	34	11	39	9	39
Ph: 5	13	26	12	43	17	74
U: 20	37	74	20	71	19	83
U: 16	34	68	19	68	17	74
U: 33	33	66	18	64	17	74
U: 14	26	52	16	57	16	70
U: 4	22	44	11	39	13	56
U: 46	19	38	12	43	12	52

Table IV

The frequency of the bacteriophage receptors on cultures of the main coagulase-negative Staphylococcus species

No. of receptor site	*S. epidermidis* n=50		*S. haemolyticus* n=28		*S. warneri* n=23	
	No. of strains	Percent lysis	No. of strains	Percent lysis	No. of strains	Percent lysis
1	8	16	6	21	2	9
2	4	8	2	7	1	4
3	3	6	3	11	2	9
4	7	14	–	–	–	–
5	2	4	–	–	–	–
6	1	2	2	7	2	9
7	5	10	2	7	1	4
8	5	10	4	14	2	9
9	1	2	1	4	2	9
10	1	2	2	7	2	9
11	2	4	–	–	4	17
12	5	10	1	4	2	9
13	2	4	3	11	1	4
15	–	–	–	–	1	4

Table V

Distribution of 235 adherence-positive isolates of CNS according to species

Staphylococcus	No.	%
S. epidermidis	96	40.8
S. haemolyticus	28	11.9
S. warneri	22	9.4
S. cohnii	11	4.8
S. saprophyticus	9	3.8
S. xylosus	8	3.4
Interspecies group	61	25.9
Total	235	100

Table VI

Frequency of adherence-positive and -negative strains of different CNS species isolated from the air of operating theatres

Staphylococcus	No. of isolates tested	Adherence			
		positive		negative	
		No.	%	No.	%
S. epidermidis	65	36	55.4	29	44.6
S. haemolyticus	27	8	29.6	19	70.4
S. warneri	18	6	33.3	12	66.7
S. saprophyticus	7	1	14.3	6	85.7
S. hominis	6	4	66.7	2	33.3
S. cohnii	1	1	100.0	0	0.0
Interspecies group	40	21	52.5	19	47.5

Association of adherent growth with antibiotic resistance. More erythromycin – and tetracycline – sensitive isolates were found among the adherence positives than among the adherence negatives, while the opposite was true for penicillin-G sensitivity (Table VII). All the tested CNS strains were susceptible to vancomycin, ofloxacin and netilmicin.

Table VII

The frequency of the antibiotic-sensitive strains of CNS in relation to adherent growth

Antibiotic	Percent of sensitive strains of	
	235 adherence-positives	87 adherence-negatives
Penicillin	6.5	13.0
Oxacillin	96.1	96.3
Cefotaxime	96.1	96.3
Erythromycin	71.4	66.4
Vancomycin	100.0	100.0
Trimethoprim / Sulfamethoxazole	62.3	65.8
Tetracycline	24.7	13.0
Tobramycin	94.8	94.7
Netilmicin	100.0	100.0
Ofloxacin	100.0	100.0

Discussion

The CNS are the most frequent members of normal skin flora [13]. One of the possibilities of spreading is loosening skin scales [14]. Concerning the hospital environment, beside the air [15], there are other passive ways for transmitting the bacteria, for example the skin and cloths of hospital staff. The amount of bacteria present in air of the operating theatres has an important role in healing of the wound. In hospital hygienic respect, the examination of the air of operating theatres is always problematic [16], which mainly concerns the CNS species.

In our study, although the species *S. epidermidis* proved to be relatively most frequent, the incidence of this species in the air samples of the operating theatres was much lower than has been published from pathological specimens [2, 9, 17–20].

Our results on antibiotic sensitivity patterns are in accordance with previous findings, namely isolates of *S. haemolyticus* showed a higher rate of resistance to a number of commonly used antibiotics than those of *S. epidermidis* [19–23]. The reason for this difference is unknown.

The occurrence of adherent growth has been found to be similar to those reported by Barcs [9] but much lower than that of isolates obtained from infected organs [6, 17, 19, 23] except from blood cultures [8];

These findings suggest that our isolates may not play a significant role as nosocomial infecting agents since they lack a number of properties characteristic of

hospital strains. This view is supported by the diversity in the numbers of the lytic phage receptors on the basis of which no large epidemic group of isolates was found.

Our work has confirmed a previous finding of Szűcs et al. [23] showing *S. haemolyticus* strains to be rare in adherence compared to S. *epidermidis*. The association between the property adherent growth and higher rate of resistance to erythromycin and tetracycline is in accordance with earlier report of Nagy et al. [24]. Further work is needed to examine the biochemical background of this phenomenon.

REFERENCES

1. Smith,I.M., Beals,P.D., Kingsbung,K.R., Hasenclever,N.F.: Observations on *Staphylococcus albus* septicemia in mice and men. Arch Intern Med **102**, 375 (1958). Kloss,E.W., Bannermann,T.L.: Update on clinical significance of coagulase-negative Staphylococci. Clin Microbiol Rev **7**, 117 (1994).
2. Hedin,G.: *Staphylococcus epidermidis* – Hospital epidemiology and the detection of methicillin resistance. Scand J Inf Dis Suppl **90**, 59 (1993).
3. Etienne,J.: Hospital dispersion of *Staphylococcus epidermidis* isolates resistant to fluoroquinolone, Pefloxacine. Epidem Inf **103**, 459 (1989).
4. Powell,J., Sanderson,P.J.: Resistant coagulase-negative Staphylococci in hospital patients. J Hosp Infect **9**, 48 (1987).
5. Pfaller,M.A., Herwaldt,L.A.: Laboratory, clinical and epidemiological aspects of coagulase-negative staphylococci. Clin Microbiol Rev **1**, 281 (1988).
6. Christensen,G.D., Simpson,W.A., Bisno, A.L.: Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Imun **37**, 318 (1982).
7. Drewry,T.D., Galvraithe,B., Wilkinson,J.: Staphylococcal slime: A cautionary tale. J Clin Microbiol **30**, 1292 (1990).
8. Kotialinen,P.: Association of coagulase-negative Staphylococcal slime production and adherence with the development and outcome of adult septicemias. J Clin Microbiol **28**, 12 (1990).
9. Barcs,I., Vályi-Nagy,T., Pánovics,J.: Clinical occurrence and virulence testing of coagulase-negative staphylococci. Acta Microbiol Hung **36**, 415 (1989).
10. Lányi,B. (ed.): Public Health and Clinical Bacteriology. Standard Methods (in Hungarian). National Institute of Hygiene, Budapest, Hungary 1980.
11. Akatov,A.K., Khatenever,N.L., Devriese,L.A.: Identification of coagulase-negative staphylococci isolated from clinical sources. Zbl Bakt Suppl **10**, 153 (1981).
12. Pulverer,G., Pillich,J., Klein,A.: New bacteriophages of *Staphylococcus epidermidis*. J Inf Dis **132** (1975).
13. Kloos,W.E.: Ecology of human skin. p. 37–50 1986 In: Kloss,E.W., Bannermann,T.L.: Update on clinical significance of coagulase-negative Staphylococci. Clin Microbiol Rev **7**, 117 (1994).
14. Davies,R.R., Noble,W.C.: Dispersion of bacteria on desquamated skin. Lancet **II**. 1295 (1962).
15. Oppenheim,B.A., Hartley,J.W., Lee,W.: Outbreak of coagulase-negative *Staphylococcus* highly resistant to ciprofloxacin in a leukaemia unit. Br Med J **299**, 294 (1990).
16. Editorial: Microbes in the air – When to count! J Med Microbiol **37**, 81 (1992).
17. Végh,Zs., Gacs,M.: A koaguláz-negativ staphylococcusok nyáktermelésének és kórokozó szerepének összefüggése. Orv Hetilap **131.5**, 231 (1990).
18. Barcs,I.: Koaguláz-negativ staphylococcusok nyáktermelő képessége: virulencia teszt és epidemiológiai bélyeg. Egészségtudomány **32**, 140 (1989).
19. Lenkei,G., Belicza,É., Sztroj,T., Rédai,I., Rozgonyi,F.: Distribution and antibiotic resistance of coagulase negative staphylococci isolated from human nosocomial infections. J Chemother Suppl **4**, 277 (1989).
20. Szabó,J., Belicza,É., Rédai,I., Rozgonyi,F.: Égési sebfertőzésekből izolált aerob baktériumok megoszlása és antibiotikum rezisztenciája. Orv Hetilap **132**, 687 (1991).

21. Schwalbe,R.S.: Selection for vancomycin resistance in clinical isolates of *Staphylococcus haemolyticus.* J Inf Dis **1**, 61 (1990).
22. Moller,J.K.: Antimicrobial usage and microbiological resistance in an university hospital during seven year period. J Antimicrob Chemother **24**, 983 (1989).
23. Szűcs,I., Sztroj,T., Pappné Falusi,E., Rédai,I., Rozgonyi,F.: A comparative study on growth in soft-agar, adherence to glass and haemolysis types of coagulase-negative staphylococci. Acta Microbiol Hung **40 (3)**, 181 (1993).
24. Nagy,I., Galgóczy,I., Ér,L., Rédai,I., Rozgonyi,F.: *Staphylococcus aureus* nyáktermelése és növekedése szérumos lágy agarban. Egészségtudomány **35**, 264 (1991).

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CHARACTERISTICS OF *STREPTOMYCES* STRAINS ISOLATED FROM SOILS IN TWO LANDFILL AREAS IN NORTH JORDAN

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A total of 114 *Streptomyces* strains were isolated and purified from soils collected from Al Akider (53) and from Al Kafeer (61) landfill areas in north Jordan. The Al Akider strains were classified into 5 colour series gray (27), white (18), yellow (3), violet (3) and green (2); while the Al Kafeer strains were classified into 6 colour series white (26), gray (16), yellow (9), red (2), green (7) and blue (1). Melanin pigment was produced by 21 and 25% of the Al Akider and Al Kafeer strains, respectively, while soluble pigment was produced by 15% of the A; Akider and 21% of the Al Kafeer strains; 17% of Al Akider strains and 23% of Al Kafeer strains showed distinctive reverse side pigment. Forty, 32, 19, and 9% of the Al Akider strains had spiral, rectus, flexous and retinaculum apertum spore-chains, respectively, while the distribution of the Al Kafeer strains in the same spore-chain forms was equally 25% each. Arabinose, xylose, inositol, fructose, rhamnose, sucrose, raffinose, and mannitol were utilized by 91, 53, 89, 60, 30, 25 and 79% of the Al Akider strains and by 84, 59, 62, 64, 70, 75, 41 and 26% of the Al Kafeer strains, respectively, while only 2% of the Al Kafeer strains were found to utilize cellulose. Thirty-six percent of the Al Akider strains showed, in vitro activity against *Staphylococcus aureus* followed by *Aspergillus niger* (26%), *Bacillus cereus* and *Saccharomyces cerevisiae* (25% each), *Fusarium monilliforme* (21%), *Candida albicans* (15%), *Escherichia coli* (8%) and *Pseudomonas aeruginosa* (4%), while 51, 49, 46, 34, 25, 15, 7 and 2% of the Al Kafeer strains exhibited activity against *A. niger*, *S. cerevisiae*, *B. cereus*, *S. aureus*, *F. monilliforme*, *C. albicans*, *P. aeruginosa* and *E. coli*, respectively.

Solid waste management problems are increasing in the world, in particular in the developing countries. Urban solid waste contains beside inert materials decomposable solid organic materials such as kitchen scraps and other garbage. The simplest way to handle solid waste disposal at the lowest direct cost is disposal in landfills. But, with increasing population and awareness of the people to pollution problems of our environment, this solution is no more satisfactory and therefore the problem is becoming more complex.

Streptomycetes appear to occupy all important position in the turnover and degradation of organic molecules such as chitin, starch and pectin [1]. Many Streptomycetes produce antibiotics, which, in addition to their medical importance, may

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play a role in ecological relationships among different microbial populations [2]. For these reasons special interest has been and still being directed towards the study of these microorganisms. Each developed country tries to raise up and develop its own collection of streptomycetes, where cultures are maintained, classified and evaluated. The numbers and types of streptomycetes present in a particular soil will be greatly influenced by geographical location, soil type and other edaphic factors. An understanding of microbial ecology allows us to reach acceptable solutions to current problems in agriculture, resource recovery, public health, and pollution control. The present paper deals with microbial count and the characteristics of *Streptomyces* flora of soils in two landfill areas in north Jordan.

Materials and methods

Area of study. Two landfills (open-dump sites), Al Akider and Al Kafeer, in North Jordan have been chosen to carry out this study. Al Akider landfill has been in use by Irbid and Remtha cities since 1980, it has an area of 60 ha and yearly rainfall amount of 200 mm, its ground water streams into Yarmouk river. Al Kafeer landfill has been in use by Jerash city since 1970, it has an area of 1 ha and yearly rainfall amount of 400 mm, its ground water streams towards King-Talal-Damm. Both landfills are lying in a slope position at hill side.

Collection of soil samples. Composite soil samples were collected from a depth of 10 cm in sterile clean polyethylene bags; Soil samples were mixed thoroughly and sieved in a siever (2 mm mesh).

Microbial counts. Soil dilutions were made in sterile distilled water, then 0.1 ml of the appropriate dilutions was pipetted and spread evenly over the surface of agar plates with a sterile L-shaped glass rod. Triplicate plates of standard plate count agar (Merck) and glycerol nitrate casein agar [3], were used for total viable bacterial count and streptomyces count, respectively. Plates were incubated at 27 °C for *Streptomyces* and at 37 °C for total viable bacteria and observed daily. The number of colonies was counted after 2–4 days for total bacteria and after 10 days for *Streptomyces.*

Isolation, purification and characterization of Streptomyces isolates. Colonies of *Streptomyces* which seemed to represent different types were transferred to oatmeal agar plates for further purification and characterization as described by Shirling and Gottlieb [4].

Production of antibacterial agents. The *Streptomyces* isolates were grown on oatmeal agar at 27 °C for 14 days. Then agar discs 9 mm in diameter were placed on nutrient agar plates inoculated with *S. aureus*, *E. coli*, *B. cereus*, *A. niger*, *P. aeruginosa*, *C. albicans*, *S. cerevisiae* and *F. monilliforme.* Inhibition zones were checked after 24 and 48 h incubation at the appropriate temperature [5].

Results and discussion

Soil microflora, especially bacteria, fungi and streptomycetes are affected by many biotic and abiotic factors such as plant type, plant age, plant exudate, soil type and mineral content of soil as well as soil moisture, organic matter content and temperature [6]. Estimates of microbial counts vary according to the means of determination. Plate counts usually give values ranging from few hundred thousands up to 200 million bacteria per gram of oven dried soil, the abundance being a reflection of many environmental factors affecting these inhabitants. Bacteria and other microorganisms may exhibit associations

with particular soil types. The environmental factors that characterize different soil types favour the development of particular microbial populations. Microbial counts in this study ranged between 10^5–10^6 for bacteria and 10^4–10^5 for streptomycetes. These counts are lower than the counts reported for Jordan Valley soils [5] but the percentage of *Streptomyces* (in relation to total viable bacteria) is higher.

Streptomyces colonize soil rich in organic matter more easier than other soils with low content of organic matter due to the nature of their hyphal growth. Jakubczyk [7] studied the effect of dead plant material decomposition on the number of bacteria, fungi and actinomycetes and found a rather strong variation in their numbers.

A total of 114 *Streptomyces* strains were isolated, purified, and characterized during this study. In general *Streptomyces* isolates were small, with discrete and leathery smooth surface but later developed a weft of aerial mycelium that appeared granular, powdery and velvety. The isolates were classified into six (Al Kafeer) and five (Al Akider) colour series according to the colour of their mature sporulated aerial mycelia. The characteristics and distribution of these isolates in the different colour series is shown in Tables I and II. Sixty-one and 53 were isolated from Al Kafeer and Al Akider, respectively. Forty-three percent of the Al Kafeer strains were white and 26, 15, 11, 3, 1.5% were grey, yellow, green, red and blue, respectively. Whereas 51, 34, 6 and 4% of the Al Akider strains were grey, white, yellow and violet, and green, respectively. This indicates that the grey serie and white serie represent the highest frequency of isolation in Al Akider and in Al Kafeer areas, respectively. In a previous study [5] it was shown that the grey serie represented the highest frequency of isolation in the Jordan Valley soils (50%). Twenty-one, 17 and 15% of the Al Akider strains; and 25, 23 and 21% of tile Al Kafeer strains produced melanin, distinctive reverse side pigment, and soluble pigment, respectively. The spiral form of spore bearing hyphae represent the highest frequency (40%) among the Al Akider strains; this is followed by rectus (32%), flexous (19%) and retinaculum apertum (9%). No dominant form of the spore chain was observed among the Al Kafeer strains. In the sugar utilization test arabinose was utilized by 84% of the Al Kafeer strains, followed by rhamnose (75%), fructose (70%), mannitol (64%), inositol (62%), xylose (59%), sucrose (41%), raffinose (26%), and cellulose (2%). Whereas 91, 89, 79, 60, 53, 30, and 25% of the Al Akider strains utilized arabinose, fructose, mannitol, rhamnose, inositol and xylose, sucrose, and raffinose, respectively; none utilized cellulose.

The results of the inhibitory effect of the *Streptomyces* strains against the tested organisms in-this study showed that 51, 49, 46, 34, 25, 15, 7, and 2% of the Al Kafeer *Streptomyces* strains were active against *A. niger*, *S. cerevisiae*, *B. cereus*, *S. aureus*, *F. monilliforme*, *C. albicans*, *P. aeruginosa*, and *E. coli*, respectively. Whereas 36, 26, 25, 21, 15, 8, and 4 percent of the Al Akider strains were active against *S. aureus*, *A. niger*, *B. cereus* and *S. cerevisiae*, *F. monilliforme*, *C. albicans*, *E. coli*, and *P. aeruginosa*, respectively. However, the percentage of active *Streptomyces* strains against the tested organisms as well as the other characteristics within each colour series was different. Ignoring *Streptomyces* colour series with less than three isolates, the characteristics of the individual *Streptomyces* colour series were as follows: the highest frequency of

Table I

Distribution and characteristics of Streptomyces strains isolated from Al Akider in 5 colour series

Streptomyces	Colour series					
	White	Gray	Yellow	Blue	Green	Total
Number of strains	18	27	3	3	2	53
Percent	34	51	6	6	4	100
Characteristics of the strains (percent)						
Production of pigments						
Melanin	17	19	33	–	100	21
Soluble pigment	11	22	–	–	–	15
Distinctive reverse side pigment	11	19	67	–	–	17
Spore bearing hyphae						
Spiral	22	59	33	–	–	40
Flexous	28	11	–	33	50	19
Rectus	39	22	33	67	50	32
Retinaculum apertum	11	7	33	–	–	9
Antimicrobial activity against						
E. coli	–	15	–	–	–	8
B. cereus	28	19	67	33	–	25
S. aureus	33	37	–	67	50	36
P. aeruginosa	6	–	33	–	–	4
A. niger	28	26	–	33	50	26
C. albicans	6	19	33	–	50	15
S. cerevisiae	44	11	33	–	50	25
F. monilliforme	17	26	–	–	50	21
Utilization of sugars						
Arabinose	78	96	100	100	100	91
Xylose	67	85	100	100	100	53
Inositol	39	74	33	–	–	53
Fructose	78	96	67	100	100	89
Rhamnose	44	70	67	100	–	60
Sucrose	28	37	33	–	–	30
Raffinose	6	41	33	–	–	25
Mannitol	61	85	100	100	100	79
Cellulose	–	–	–	–	–	–

Table II

Distribution and characteristics of Streptomyces strains isolated from Al Kafeer in 6 colour series

Streptomyces	Colour series						
	White	Gray	Yellow	Red	Blue	Green	Total
Number of strains	26	16	9	2	1	7	61
Percent	43	26	15	3	1.5	11	100
Characteristics of the strains (percent)							
Production of pigments							
Melanin	15	44	33	–	100	–	25
Soluble pigment	19	6	44	–	–	43	21
Distinctive reverse side pigment	12	13	56	100	–	29	23
Spore bearing hyphae							
Spiral	27	44	11	–	–	–	25
Flexous	12	31	44	50	–	43	25
Rectus	27	–	33	50	–	57	25
Retinaculum apertum	35	25	11	–	100	–	25
Antimicrobial activity against							
E. coli	–	6	–	–	–	–	2
S. aureus	27	50	33	–	100	29	34
B. cereus	42	50	67	–	100	29	46
P. aeruginosa	–	6	11	–	–	29	7
A. niger	58	63	33	–	100	29	51
C. albicans	15	13	22	–	–	14	5
S. cerevisiae	54	44	44	100	100	14	49
F. monilliforme	15	38	33	50	–	14	25
Utilization of sugars							
Arabinose	92	69	89	100	100	71	84
Xylose	54	56	44	100	100	86	59
Inositol	62	88	56	–	100	29	62
Mannitol	62	69	56	–	100	86	64
Fructose	69	94	44	–	100	71	70
Rhamnose	69	94	67	–	100	86	75
Sucrose	27	75	22	–	100	43	41
Raffinose	27	25	22	–	100	29	26
Cellulose	–	6	–	–	–	–	2

melanin, soluble pigment, and distinctive reverse side pigment positive isolates was in the yellow, gray, and yellow series of Al Akider strains, respectively; and in the grey, yellow and yellow series of Al Kareer strains, respectively. The highest frequency of spiral, flexous, rectus and retinaculum apertum spore bearing hyphae was in the grey, violet and yellow series of the Al Akider area, respectively; and in the grey, yellow, green, and white series of the Al Kafeer area, tested organisms in this study was in the grey and yellow series. Some actinomycete genera, such as *Streptomyces*, are widely distributed in soils throughout most of the world [8]. However, the distribution of *Streptomyces* colour series seems to differ from one area to other. Streptomycetes are known to produce antibiotics in synthetic media, it has been possible to demonstrate its production in sterilized and amended soils [9]. It was suggested [10] that the frequency of streptomycin resistant strains in some soils indicated that they are exposed to this antibiotic. Al-Rashidi [11] attributed the failure of *Rhizobium japonicum* serotype 135 to colonize in soils of Abou-Ghraib to the activity of two isolates of *Streptomyces* common in these soils.

Streptomyces strains may participate actively in establishing the microbiological equilibrium in soil, and may be a factor in affecting the incidence of certain soil-borne plant pathogens. The results indicate the possibility of using some *Streptomyces* strains as a source of antibiotic production or as a mean or biological control of some plant diseases in Jordan. However, this requires further screening of a large number of *Streptomyces* strains isolated from different regions in Jordan.

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REFERENCES

1. Williams,S.T., Lanning,S., Weltington,E.M.H.: Ecology of actinomycetes. In Goodfellow,M., Mordarski,M., Williams,S.T. (eds): The Biology of the Actinomycetes. Academic Press, London 1984. pp. 481–528.
2. Rothrock,C.S., Gottlieb,D.: Importance of antibiotic production in antagonism of selected Streptomyces species to two soil-borne plant pathogens. J Antibiot **34**, 830 (1981).
3. Kuster,E., Williams,S.T.: Selective media for tile isolation of *Streptomycetes.* Nature **202**, 928 (1964).
4. Shirling,E.B., Gottlieb,D.: Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol **16**, 313 (1966).
5. Abussaud,M.J., Saadoun,I.M.: *Streptomyces* flora of some Jordan Valley nurserie soils, characteristics and seasonal distribution. Dirasat **18(3)**, 66 (1991).
6. Davis,F.L., Williams,S.T.: Studies on the ecology of actinomycetes in soil. I. The occurrence and distribution of actinomycetes in a pine forest soil. Soil Biochem **2**, 227 (1970).
7. Jakubczyk,H.: Effect of dead plant material decomposition on the number of heterotrophic microorganisms. Roczn Nauk Poln **19**, 121 (1971).
8. Williams,S.T., Lanning,S., Weltington,E.M.H.: Ecology of actinomycetes. In Goodfellow,M., Mordarski,M., Williams,S.T. (eds): The Biology of the Actinomycetes.
9. Soulides,D.A.: Antibiotics in soil VII, production of streptomycin and tetracycline in soils. Soil Sci **100**, 200 (1965).

10. Soulides,D.A.: Antibiotic tolerance of the soil microflora in relation to type of clay mineral. Soil Sci **107**, 105 (1969).
11. Al-Rashidi,R.K.: Factors affecting the survival, establishment and symbiotic nitrogen fixation of Rhizobium japonicum serogroup in some Iraqi soils. M Sc Thesis, University of Bagdad, Iraq 1973.

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DETECTION OF POTENTIAL CYTOKINE GENE(S) IN AN ALU-PCR LIBRARY FROM THE HUMAN CHROMOSOME 4

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Jumping and linking libraries have been constructed in order to have more information of chromosome 4. Two types of jumping libraries (*Not* I and *Xma* III jumping) and one linking library were prepared. The jumping clones represent DNA regions between two rare-cutting enzymes sites (such is *Not* I or *Xma* III) whereas linking clones represent only the area which surrounds the rare-cutting site. These clones were used as target DNA in *Alu*-PCR. The *Alu*-PCR fragments were cloned into pGEM-T vector and transformed into *Escherichia coli* JM 109 competent cells. The colonies were screened for inserts by restriction analysis. More than 50% of the colonies were shown to contain inserts. Inserts from each library were screened by PCR. One of the jumping libraries appeared to have inserts containing CpG islands and AT-rich sequences.

In the last few years numerous members of a new family of cytokines have been identified [1]. These cytokines characteristically are basic, heparin-binding polypeptides. They exhibit from 20% to 50% homology in amino acid sequence, range from 8–10 kD in molecular weight and have proinflammatory and reparative activities. This family is called "intercrine" or "chemokine" family [2]. All the intercrines have four cysteine residues which form two disulfide bridges. This family of cytokines is divided into two subfamilies, depending on their amino acid sequence and chromosomal location. In the alpha subfamily, the first two cysteines are separated by only one aminoacid. Therefore it is called "C-X-C" subfamily. In the beta subfamily, the first two cysteines are in an adjacent position; that is why it is called "C-C" subfamily. The genes, for alpha subfamily are located close together in the q12-21 region of human chromosome 4, while genes for beta subfamily are located close to one another on the q11-21 region of chromosome 17. The cDNA for these cytokines have the characteristic conserved single open reading frames,

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typical signal sequences in the 5' region, AT rich sequences in their 3' untranslated regions and rapidly inducible mRNA expression [1].

To date less than 100 genes have been identified on chromosome 4 representing only 2% of the estimated genes on this area [3]. Just recently two different approaches have been proposed in order to map the genome. Both of them are based on using certain rare-cutting restriction enzymes, like *Not* I or *Xma* III. These approaches are called "jumping" and "linking" libraries [4–8]. Both enzymes have restriction sites in the CpG islands. The CpG islands can serve as "natural" landmarks for creating the mentioned libraries. It appears that most mammalian genes turn out to be associated with CpG islands [9, 10]. Equating the approximate number of islands (about 30 000) with the anticipated number of genes (20 000–50 000) in turn suggests that a high proportion of all islands are gene-associated [9]. In visualizing the relationship between CpG islands and genes it is important to realize that islands are large (between 500 bp and at least 2000 bp) and so cover an extended region of the 5' end, often including the first few exons as well as upstream sequences. The analysis of CpG islands and expressed sequences in the human genome revealed that over 20% of the CpG island-containing genes possess *Not* I site(s) in their sequences, and 65% have *Xma* III site(s) [8]. These data suggest that mammalian genome CpG islands are associated with functional genes and *Not* I (or *Xma* III) clones, as CpG island markers can be successfully used for joining physical and genetic mapping efforts.

In our experiments we combined the jumping and linking libraries with *Alu*-PCR [11–16] in order to prepare gene banks of chromosome 4 and find new genes on the mentioned area.

Materials and methods

Construction of Not I linking library. Human chromosome 4 DNA was isolated from the GM 11687 human–mouse hybrid cell line [17]. The DNA concentration was determined by spectrophotometer using 260 nm wavelength. The concentration was 130 µg/µl.

Four µg DNA was digested by 10 U *Bam*HI (Boehringer Mannheim) in a 50 µl reaction volume. The digestion was carried out at 37 °C for 1 h. The enzyme was inactivated by heating to 65 °C for 15 min. The fragments generated this way were ligated in a diluted reaction using T4 DNA ligase (Boehringer Mannheim). In this reaction the DNA concentration was adjusted to 200 ng/ml. This reaction condition does not lead to any significant intermolecular ligation. The nonligated, noncircularized fragments were prevented from cloning by destroying the *Bam*Hi sticky ends in a partial filling in reaction using 10 U Klenow enzyme (Boehringer Mannheim) together with 100 µM dGTP and dATP. The sample was incubated at room temperature for 30 min. An ethanol precipitation was performed and the DNA was resuspended in 80 µl TE buffer (pH 7.5). The circularized DNA was digested by 16 U *Not* I in a 100 µl reaction volume at 37 °C for 1 h and the enzyme was inactivated by heating to 65 °C for 15 min. The fragments were ligated into SuperCos vector (Stratagene) which was cut by *Bam*HI and *Not* I and purified by Geneclean (Bio101). The reaction volume consisted of 800 ng DNA, 400 ng vector and 10 U T4 DNA ligase (Boehringer Mannheim). The ligation was allowed to proceed overnight at room temperature. Figure 1 shows the whole procedure.

Construction of Not I and Xma III jumping clones. Six µg DNA was separately digested by *Not* I and *Xma* III. The reaction was carried out in 60 µl reaction volume using 16 U *Not* I and 8 U *Xma* III. In the case of *Xma* III the reaction had to be performed at 25 °C. In both case the reaction was allowed to proceed for 1 h. The *Not* I reaction was stopped by heating to 65 °C for 15 min while the *Xma* III

reaction was inactivated by adding EDTA. A diluted ligation was performed. The DNA concentration was 200 ng/ml in this reaction. T4 DNA ligase was used from Boehringer Mannheim and the ligation was allowed to proceed overnight at room temperature. The reaction was stopped by heating to 65 °C for 15 min. The DNA was precipitated with ethanol, washed with 70% ethanol and dissolved in 50 µl TE buffer (pH 7.5). The circularized DNA was digested by 10 U *Bam*HI (Boehringer Mannheim) in a 50 µl reaction volume. After 1 h the reaction was stopped by heating and the DNA was precipitated by ethanol, washed by 70% ethanol and dissolved in 50 µl TE buffer (pH 7.5). The estimated DNA concentration was 10–12 ng/ml. Another ligation was performed in which the DNA concentration was adjusted to 1 µg/ml. Ten units of T4 DNA ligase were used. The sample was incubated overnight at room temperature. Then the ligase was inactivated by heating and the cohesive ends were partially filling in by Klenow enzyme with 100 µM dGTP and dATP. A phenol–chloroform extraction was carried out followed by the ethanol precipitation. The precipitated DNA was dissolved in 25 µl TE buffer (pH 7.5). The circularized DNA was digested again by *Not* I and *Xma* III, respectively. The digestion was carried out in a 30 µl reaction volume, using 16 U *Not* I and 8 U *Xma* III separately. The digested DNA was precipitated by ethanol, washed by 70% ethanol and dissolved in 20 µl TE (pH 7.5). Thus the *Not* I and *Xma* III jumping fragments were generated. Then these fragments were ligated into SuperCos vector which previously was digested by *Not* I. The digested vector was prevented from self-ligation by dephosphorilation by CIP (calf intestinal alkaline phosphatase). The ligation reaction was performed in 10 µl volume overnight at room temperature using 10 U T4 DNA ligase. Figure 2 shows the whole procedure.

Alu-PCR of the linking and jumping clones. Three sets of primers were designed and synthesized. Two of these (pr.875 and pr.876) were *Alu*-primers while the third (pr.910) was for the SuperCos vector. The *Alu*-PCR reaction was done in 50 µl reaction volume containing 5 µl Bioline BioTaq 10×buffer, 1.5 mM $MgCl_2$, 8 µl New England Biolab's dNTP's (1.25 mM each), 2.5 U Bioline BioTaq Polymerase and 150 ng of each primer. One µl of the ligated DNA was used as a template. In the case of the linking clones two separate PCR reactions were carried out which varied only in the primers used. Reaction 1 contained primers 910 and 875 while reaction 2 contained primers 910 and 876. In the case of the jumping clones reaction 2 was used only. Conditions of *Alu*-PCR were as follows: 1 cycle of 95 °C 4 min, 55 °C 1.5 min, 72 °C 2 min followed by 35 cycles of 95 °C 1 min, 55 °C 1.5 min, 72 °C 2 min. The PCR products were purified by Wizard PCR Preps DNA Purification System (Promega) and then cloned into pGEM-T vector (Promega). Then the ligated products were transformed into *E. coli* JM 109 by means of heat-shock method and electroporation, respectively. The nucleotide sequence of the primers were the following:

Pr.910: 5'-GAT AAG CGG TCA AAC ATG AG-3'
Pr.875: 5'-GCG GAA TTC GAG CCA CCA CGC CGC CCA GCC-3'
Pr.876: 5'-GCG GAA TTC AGA GCG AGA CTC CGT CTC-3'

Screening of the transformants. Plasmid miniprep was used in order to screen the transformants for having inserts. The plasmid DNA was digested by *Apa* I (Boehringer Mannheim) and *Sac* I (Promega) because these enzymes had restriction sites on the opposite ends of the polycloning site of pGEM-T vector. Furthermore PCR reaction was used to screen the transformants for AT-rich sequences. Conditions of PCR were as follows: 94 °C 30 s, 53 °C 30 s, 72 °C 1 min 20 s, 30 cycles. Primer T7 and 91 were used. The nucleotide sequence of these primers were as follows:

Pr.91: 5'-GAT CGA ATT CTT AAT ATA TAT TAA ATA AAT-3'
Pr.T7: 5'-GTA ATA CGA CTC ACT ATA GGGC-3'

Results

The construction of linking and jumping libraries are quite similar. The difference is the order in which rare and frequently cutting enzymes are used. Linking libraries are merely a variation on jumping libraries and contain DNA fragments of the same physical structure, as in jumping libraries, but arranged in a different fashion. Jumping clones represent the DNA stretch between two *Not* I (or *Xma* III) sites while linking clones represent only the area around a *Not* I (or *Xma* III) site (Fig. 3).

Figure 4 shows one of the *Alu*-PCR reaction result. We used two types of vector-insert ratio for the ligation. The use of 1:3 ratio seemed better than the 1:6 ratio. Since *Alu* repeated sequences are interspersed throughout human DNA with an average spacing of 4 kb, accounting for some 3 to 6% of the total DNA, it was no surprise that we had "smear" on the gel instead of having discrete bands. Any particular DNA segment of 5 kb or longer has a high probability of containing *Alu* sequences. We had the same result in the case of the linking clones although the range of the smear was smaller than in jumping clones.

The *Alu*-PCR linking clones (APL) and the *Alu*-PCR jumping clones (APJN and APJX) were purified and then transformed into *E. coli* JM 109 using both heat-shock and electroporation methods. Table I shows the result of the transformations.

The transformation frequency was 10–15 times higher using the electroporation than using the heat-shock method despite the fact that in the second case commercial competent cells were used. The human genome is about 3×10^9 bp long having 3000 *Not* I sites. The size of chromosome 4 is 2×10^8 bp therefore it may have 300 *Not* I sites. Considering these facts we needed at least that many colonies containing fragments. Collecting more than 10 000 colonies in each library seemed to be essentially complete.

Plasmid miniprep and restriction enzyme analysis was used to estimate the number of insert containing clones. In all cases 10 independent colonies were chosen to make plasmid minipreps. *Apa* I and *Sac* I enzymes were used as both cut the pGEM-T vector at opposite ends of the polycloning site. In the case of APL 70% of the randomly chosen colonies had inserts ranging between 300 and 1900 bp. In the case of APJN, 40% of the plasmids had inserts whereas in APJX the ratio was 60%. Figure 5 shows the result of restriction analysis. In some cases the insert had an internal *Sac* I site which can be clearly seen on the photo.

Considering the fact that AU-rich motifs are found in the 3' untranslated region of mammalian mRNAs encoding cytokines, adhesion molecules and proto-oncogenes a PCR was performed using T7 and the AT-rich primers (Pr.91). 250 colonies were cultured in bulk from each library. Three plasmid minipreps were prepared (one from each library) and the miniprep DNA was used as target DNA for amplification. Results are shown in Fig. 6. No real product was observed in the case of APL. In the case of APJN an about 3 kb long fragment was observed while in the case of APJX the product we got seemed not specific enough to be evaluated. Control experiments were done using the primers alone in the PCR reaction to confirm this result. The control confirmed the result mentioned before.

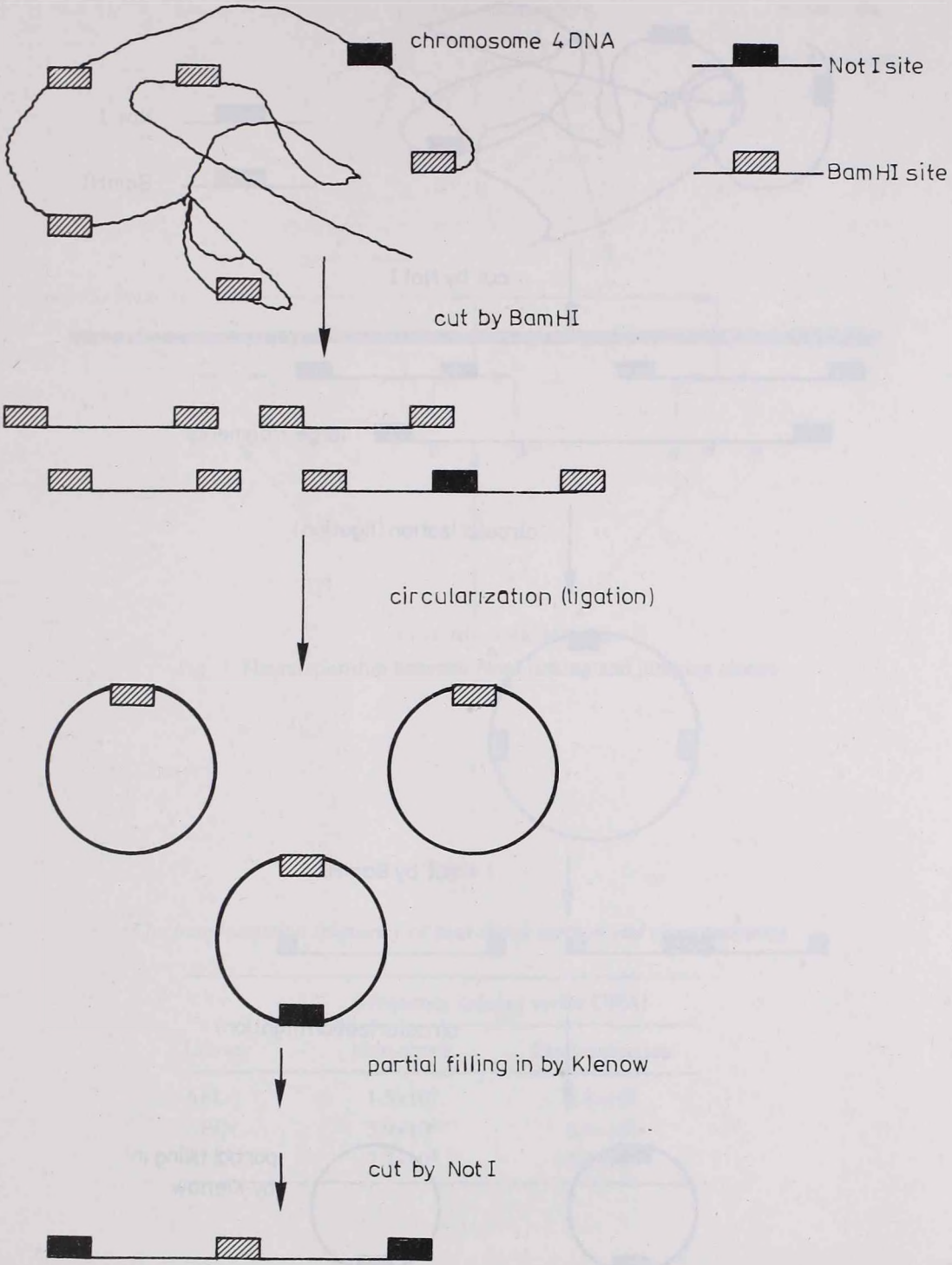

Fig. 1. Construction of *Not* I linking clones

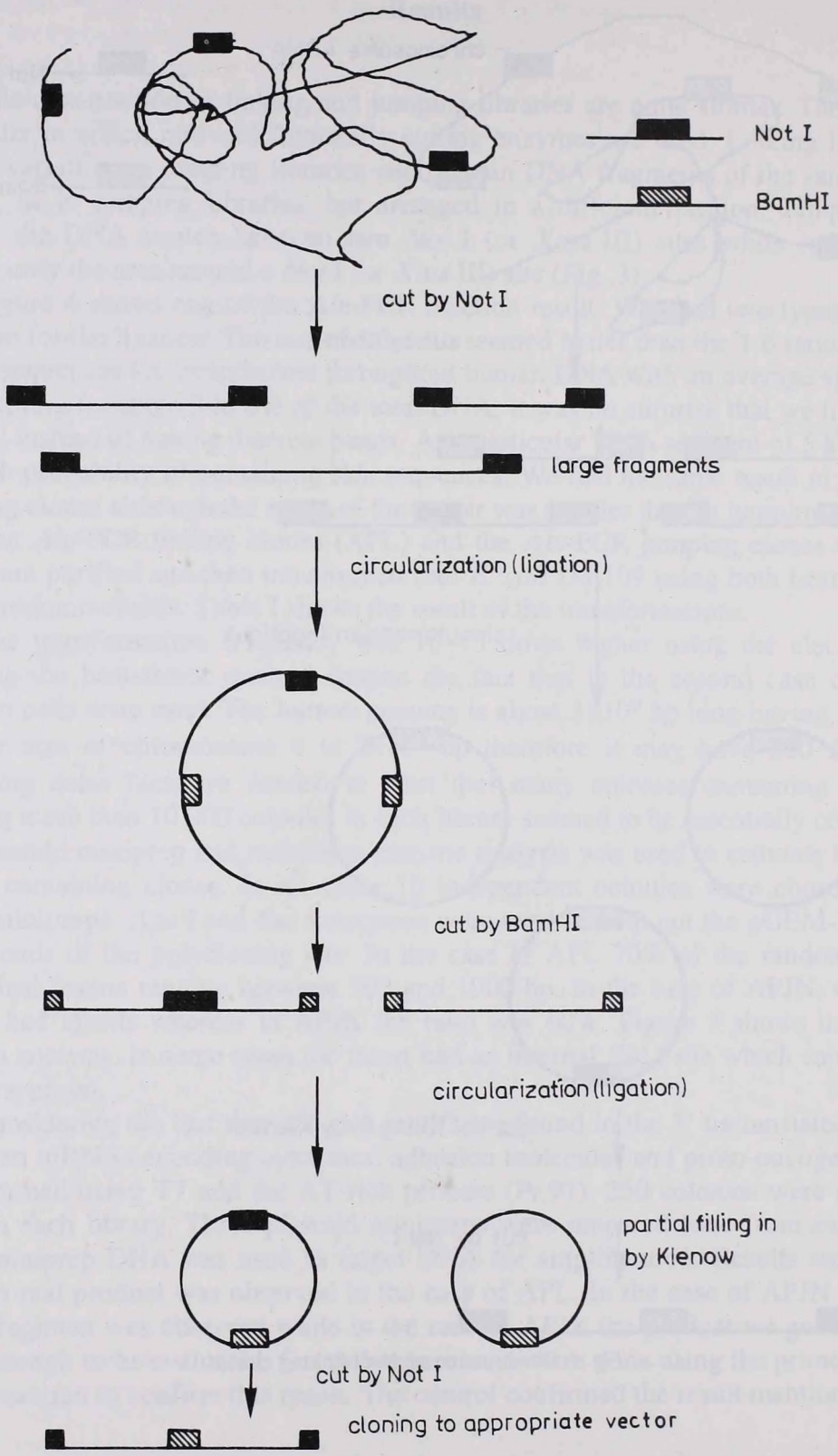

Fig. 2. Construction of *Not* I jumping clones

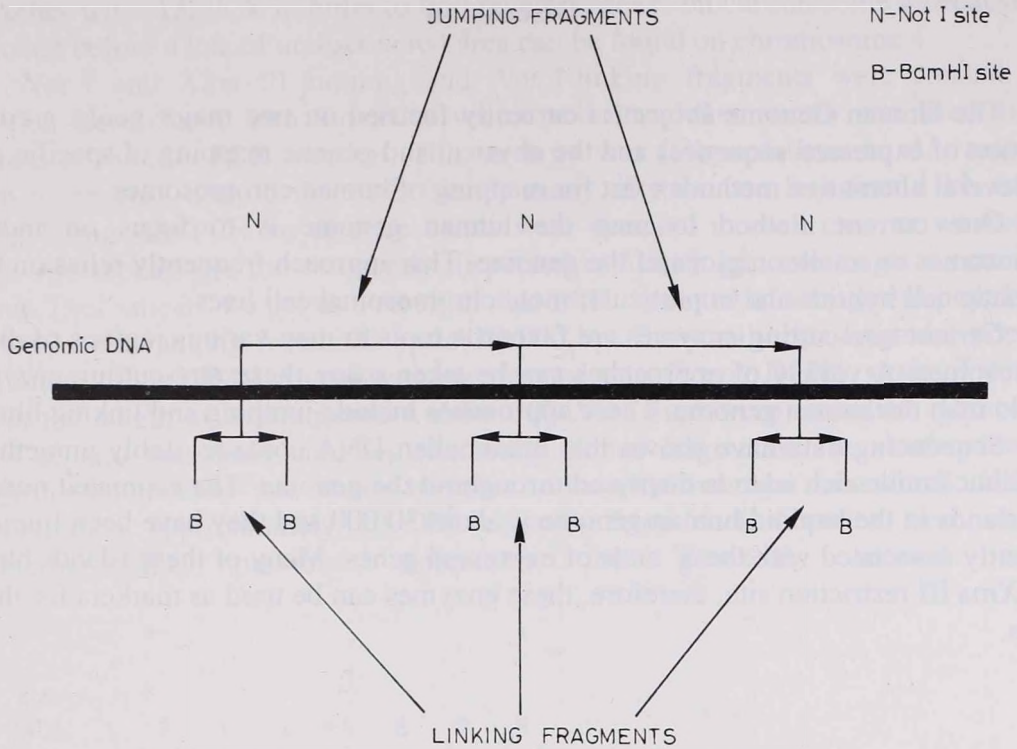

Fig. 3. The relationship between *Not* I linking and jumping clones

Table I

The transformation frequency of heat-shock method and electroporation

	Frequency (cfu/μg vector DNA)	
Library	Heat-shock	Electroporation
APL	1.5×10^4	8.8×10^4
APJN	5.9×10^3	6.4×10^4
APJX	7.7×10^3	2.0×10^5

Discussion

The Human Genome Project is currently focused on two major goals: a complete collection of expressed sequences and the physical and genetic mapping of specific genetic loci. Several alternative methods exist for mapping of human chromosomes.

One current method to map the human genome is to focus on individual chromosomes or smaller regions of the genome. This approach frequently relies on the use of somatic cell hybrids and in particular monochromosomal cell lines.

Certain rare-cutting enzymes are favourite tools to map various regions of different chromosomes. A variety of approaches can be taken using these rare-cutting enzymes in order to map the human genome. These approaches include jumping and linking libraries.

Sequencing data have shown that mammalian DNA contains stably unmethylated, CpG dinucleotide-rich islands dispersed throughout the genome. The estimated number of CpG islands in the haploid human genome is about 30 000 and they have been found to be frequently associated with the 5' ends of expressed genes. Many of these islands have *Not* I and *Xma* III restriction site, therefore, these enzymes can be used as markers for the CpG islands.

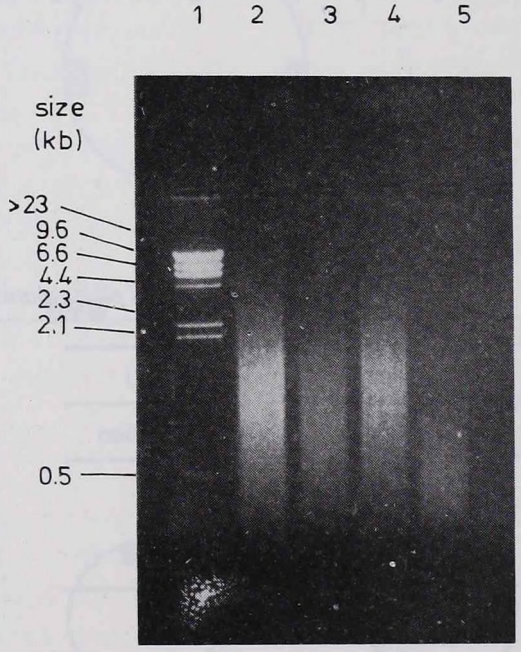

Fig. 4. Alu-PCR of the jumping clones. Lane 1: DNA size marker (λ/Hind III); lane 2: *Alu*-PCR of the *Not* I jumping clones (vector:insert= 1:3); lane 3: *Alu*-PCR of the *Not* I jumping clones (vector:insert = 1:6); lane 4: *Alu*-PCR of the *Xma* III jumping dones (vector:insert =1:3); lane 5: *Alu*-PCR of the *Xma* III jumping clones (vector:insert = 1:6)

In this study two types of rare-cutting enzymes (*Not* I and *Xma* III) were used to create jumping and linking libraries from human chromosome 4. We combined these

approaches with *Alu*-PCR in order to find potential genes on chromosome 4 because as we mentioned before a lots of undiscovered area can be found on chromosome 4.

Not I and *Xma* III-jumping and *Not* I-linking fragments were created. These fragments were cloned into SuperCos vector and the cloned vector was used as target molecule in the *Alu*-PCR reaction. This strategy was followed because *Alu* repeats are known to be very near to the CpG islands many of them are less than 1 kb from the islands. Using *Alu*-PCR no discrete bands were detected in each case as we suspected before. *Alu* repeats are too frequently dispersed in human genome to get a certain long product. The "smear" we got as a result of *Alu*-PCR consisted of a fragment population in different length ranging from 300 bp to 10 000 bp. Eventually this fragment population was cloned into pGEM-T vector and transformed into *E. coli* JM109 strain. Taking into account the length of chromosome 4 about 300 *Not* I sites were estimated. Therefore we needed at least that many colonies containing cloned fragments. The electroporation as transformation method was about 10-times more effective than heat-shock. Using the electroporation approximately 10 000 colonies from each libraries were collected. Considering the previous estimation the genebank seemed to be

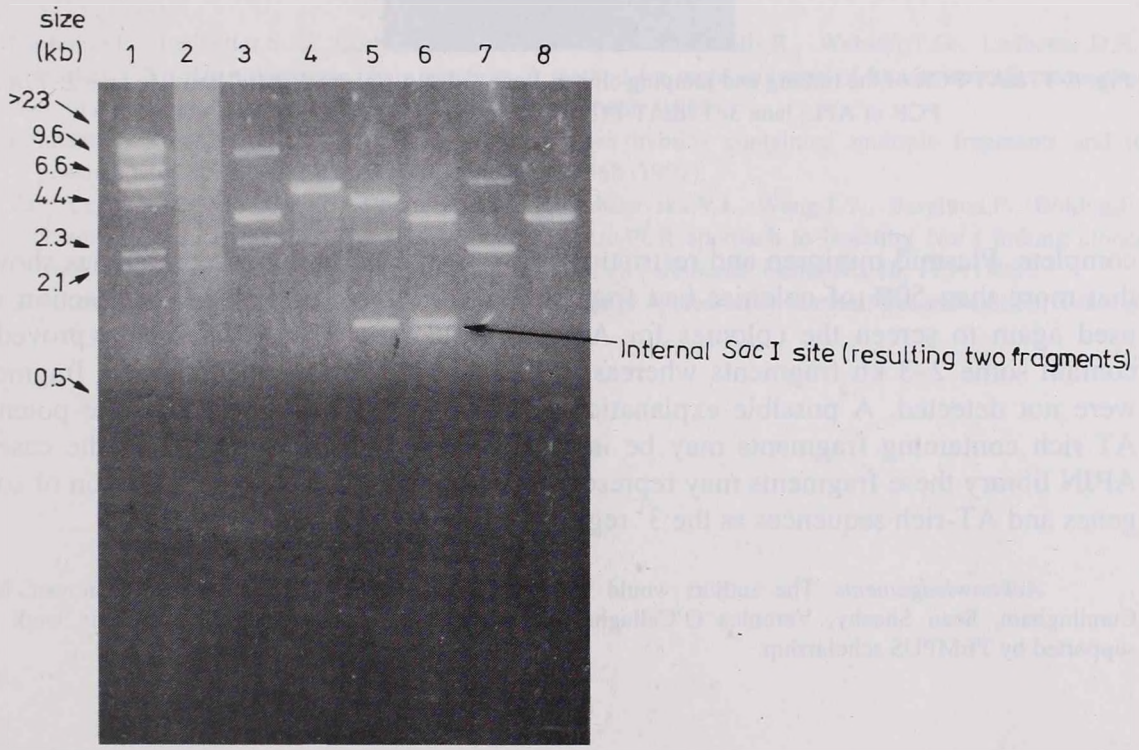

Fig. 5. Comparative restriction analysis. Lane 1: DNA size marker (λ/Hind III); lane 2: Insert amplified by PCR; lane 3: Uncut plasmid DNA; lane 4: Plasmid DNA cut by *Apa* I; lane 5: Plasmid DNA cut by *Sac* I; lane 6: Plasmid DNA cut by *Apa* I and *Sac* I; lane 7: Plasmid DNA from control colony; lane 8: Plasmid DNA from control colony cut by *Apa* I and *Sac* I

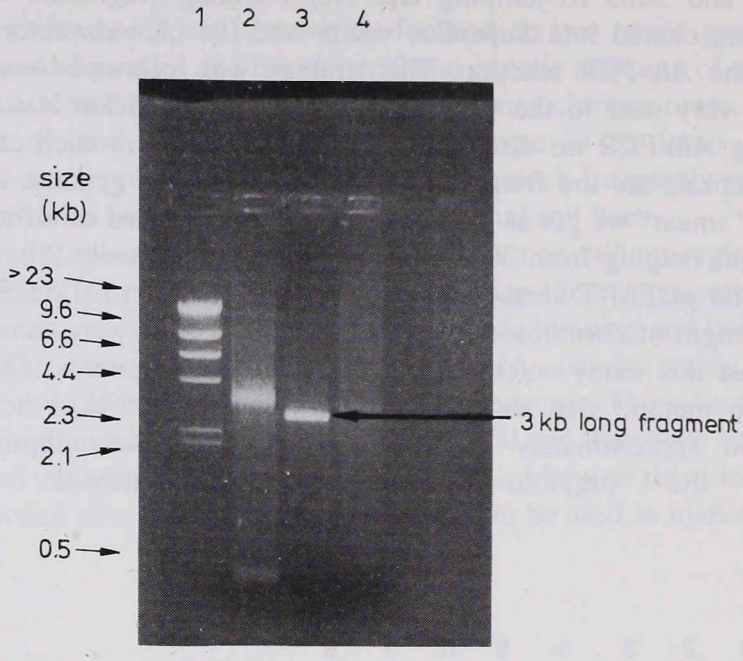

Fig. 6. T7&AT-PCR of the linking and jumping clones. Lane 1: DNA size marker (λ/Hind III); lane 2: T7&AT-PCR of APL; lane 3: T7&AT-PCR of APJN; lane 4: T7&AT-PCR of APJX

complete. Plasmid miniprep and restriction analysis of randomly chosen colonies showed that more than 50% of colonies had fragments in different length. A PCR reaction was used again to screen the colonies for AT-rich sequences. The APJN library proved to contain some 2–3 kb fragments whereas in the other two cases such specific fragments were not detected. A possible explanation is that preventing amplification the potential AT-rich containing fragments may be inserted in the opposite direction. In the case of APJN library these fragments may represent potential CpG island as the 5' region of some genes and AT-rich sequences as the 3' region of certain genes.

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REFERENCES

1. Oppenheim,J.J., Zacharie,C.O.C., Mukaida,N., Matsushima,K.: Properties of the novel proinflammatory supergene "intercrine" cytokine family. Ann Rev Immunol **9**, 617 (1991).
2. Balwit,J.M., Haak-Frendscho,M.: The chemokine. Promega Notes **44**, 34 (1994).
3. Riess,O., Siedlaczck,I., Kredtke,S., Melmer,G., Epplen,J.T., Deaven,L.L.: Characterization of a human chromosome 4 flow-sorted cosmid library. Cytogenet Cell Genet **65**, 238 (1994).

4. Poustka,A., Pohl,T.M., Barlow,D.P., Frischauf,A.M., Lehrach,H.: Construction and use of human chromosome jumping libraries from *Not* I digested DNA. Nature **325**, 353 (1987).
5. Collins,F.S., Drumm,M.L., Cole,J.L., Lockwood,W.K., Vande Woude,G.F., Ianuzzi,M.C.: Construction of a general human chromosome jumping library, with application to cystic fibrosis. Science **235**, 1046 (1987).
6. Zabarovsky,E.R., Boldog,F., Thompson,T., Scanlon,D., Winberg,G., Marcsek,Z., Erlandsson,R., Startbridge,E.J., Klein,G., Sümegi,J.: Construction of a human chromosome 3 specific *Not* I linking library using a novel cloning procedure. Nucleic Acid Research **18**, 6319 (1990).
7. Zabarovsky,E.R., Boldog,F., Edandsson,R., Kashuba,V.I., Allikmets,R.L., Marcsek,Z., Kisselev,L.L., Startbridge,E., Klein,G., Sümegi,J., Winberg,G.: New strategy for mapping the human genome based on a novel procedure for construction of jumping libraries. Genomics **11**, 1030 (1991).
8. Allikments,R.L., Kashuba,V.I., Pettersson,B., Gizatullin,R., Lebedeva,T., Kholodnyuk,I.D., Bannikov,V.M., Petrov,N., Zakharyev,M.V., Winberg,G., Modi,W., Dean,M., Uhlen,M., Kisselev,L.L., Klein,G., Zabarovsky,E.R.: *Not* I linking clones as a tool for joining physical and genetic maps of the human genome. Genomics **19**, 303 (1994).
9. Bird,A.P.: CpG islands as gene markers in the vertebrate nucleus. Trends Genet **3**, 342 (1987).
10. Bird,A.P.: CpG-rich islands and the function of DNA methylation. Nature **321**, 209 (1986).
11. Ullu,E., Tschudi,C.: *Alu* sequences are processed 7SL RNA genes. Nature **312**, 171 (1984).
12. Britten,R.J., Baron,W.F., Stout,D.B., Davidson,E.H.: Sources and evolution of human *Alu* repeated sequences. Proc Natl Acad Sci **85**, 4770 (1988).
13. Jurka,J., Smith,T.: A fundamental division in the *Alu* family of repeated sequences. Proc Natl Acad Sci **85**, 4775 (1988).
14. Nelson,D.L., Ledbetter,S.A., Corbo,L., Victoria,M.F., Ramirez-Solis,R., Webster,T.D., Ledbetter,D.H., Caskey,C.T.: *Alu* polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. Proc Natl Acad Sci **86**, 6686 (1988).
15. Benham,F., Rowe,P.: Use of *Alu*-PCR to characterize hybrids containing multiple fragments and to generate new Xp21.3-p22.2 markers. Genomics **12**, 368 (1992).
16. Zabarovsky,E.R., Kashuba,V.I., Pokrovskaya,E.S., Zabarovska,V.I., Wang,J.Y., Berglund,P., Boldog,F., Stanbridge,E.J., Sümegi,J., Klein,G., Winberg,G.: *Alu*-PCR approach to isolating *Not* I linking clones from the 3p14-p21 region frequently deleted in renal cell carcinoma. Genomics **16**, 713 (1993).
17. Sambrook,J., Fritsch,E.F., Maniatis,T.: Molecular Cloning. A laboratory manual. Second edition. 1989. p. 9.16 and 9.17

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A POSSIBLE ASSOCIATION OF HLA-DRβ1*04 SUBTYPES AND RHEUMATOID ARTHRITIS OF PEPTIDE BINDING REGION AMINO ACIDS

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HLA-DRβ1*04 subtypes associated with rheumatoid arthritis (RA) were studied by polymerase chain reaction with sequence specific primers and the 86th amino acid of the DRβ1 chain was analysed in RA patients vs healthy controls. The frequency of HLA-DRβ1*0401 and 0404/0407 alleles was significantly increased in RA patients vs the controls, HLA-DRβ1*0401 and 0404 being the dominant variants in RA. In most RA patients hydrophilic glycine proved to be the 86th amino acid of the peptide binding region thus it could have an effect on the development of RA.

The HLA-D region is implicated in the regulation of the immune response and susceptibility to the development of autoimmune diseases. Several studies have confirmed the association of HLA-DR4 antigen with rheumatoid arthritis (RA) in Caucasians [1] and in other populations. Most of the studies have reported that certain DR4 subtypes are strongly associated with RA, such as DRβ1*0401 and DRβ1*0404/0408 in Whites [1] and DRβ1*0405 in Japanese and in Israeli Jews [2, 3].

Although many different HLA-DR types and subtypes show the RA association, those which do so, share identical or similar sequences in a crucial part of the third hypervariable region of the β1 chain [4, 5]. Up to 85% of RA patients carry HLA II molecules containing one of these related sequences. This "shared epitope" forms part of

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the peptide binding groove, thus its association with RA contributes to the ability of binding some crucial peptides. Furthermore the analysis of the peptide binding to a set of HLA-DR alleles allows the proteins to segregate in function, depending on the amino acids at positions 57 and 86 of the β1 chain [6].

We studied the HLA risk factors of RA in Caucasians i.e. the genetic background (DNA) and the genetically determined 86th amino acid in HLA-DRβ1 chain in RA patients. We performed genomic typing of the DRβ1*04 and of DQβ1 alleles. In addition we analysed the amino acid at position of 86 of the DRβ1 chain in different HLA-DR4 subtype antigens (on the basis of a communication by Tiercy et al., Fig. 1).

Materials and methods

Thirty-one classical RA patients and sixty randomly selected healthy Caucasian controls were serotyped for HLA specificities. The study population comprised 19 (61.3%) Caucasian patients with RA and 20 (33%) healthy controls were genotyped for HLA-DR4 and HLA-DQ variants. High molecular weight DNA was isolated from peripheral blood leukocytes by a salting-out method [7] or by proteinase-K treatment. HLA-DR4 subtype antigens as well as DRβ1*0401-0412 allelic variants were analysed by using the polymerase chain reaction (PCR) amplification method with sequence-specific primers (SSP) based on the procedure of Zetterquist and Olerup [8]. The method makes a complete HLA-DR4 subtyping (DRβl*0401 to 0412) possible (Fig. 2). HLA-DR4 subtyping was performed 8+3 PCR reactions per person (Fig. 3), in case of DR4 homozygotes typing included an additional PCR step (nested PCR) in order to differentiate among certain DR4 subtype-combinations. HLA-DQ typing was performed by 10 PCR reactions per person, differentiating HLA-DQβ1*02 to 06 alleles. The absence or presence of PCR products was visualized by submarine agarose gel electrophoresis (Fig. 4).

Results

All the patients and the controls were tissue typed for HLA-DR and were genotyped for DRβ1*0401 to 0412 and DQβ1*02 to 06 variants. Significantly higher frequency of DR4 was detected in RA patients (RR=3.1) whereas none of the DQ types showed significant association with RA (Table I). DRβ1*0401 was the most common variant (36.8% in patients vs 10% in controls, RR=4.4). DRβ1*0404/0407 and DRβ1*0404 alleles were also increased in patients compared with controls (RR=3.3 and 1.9 respectively). DRβ1*0402,0409,0410,0411,0412 were absent. The frequency of DRβ1*0406 was low in both patients and controls. Having analysed the amino acid sequence in most RA patients hydrophilic glycine (65%) vs hydrophobic valine (35%) was found to be the 86th amino acid in the DRβ1 chain.

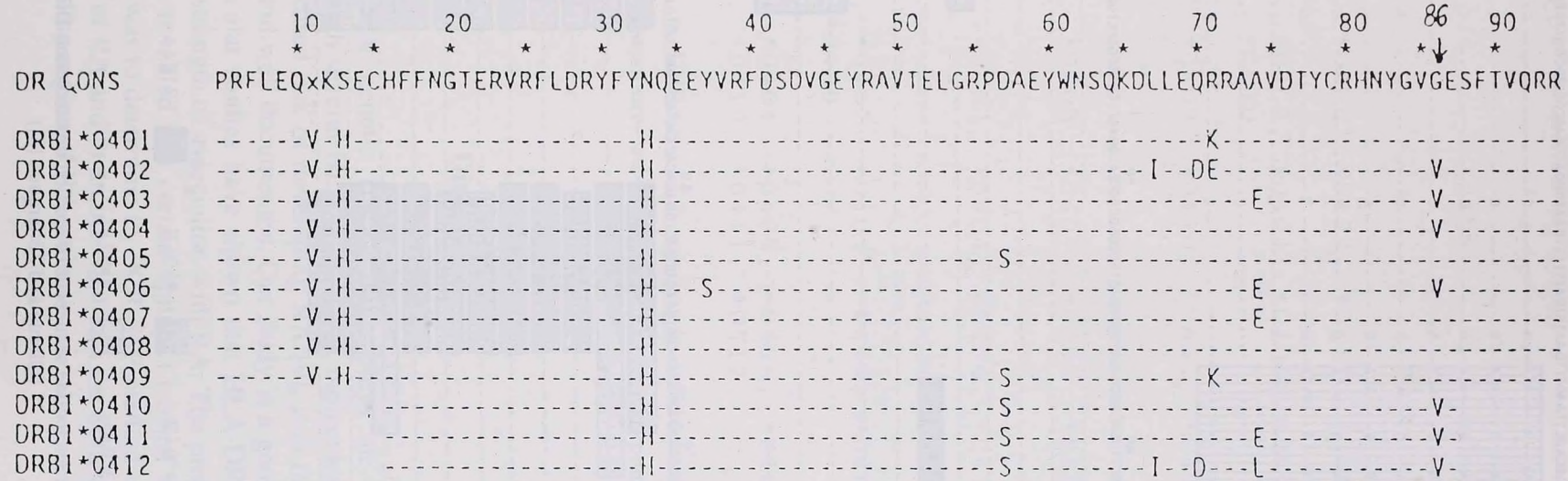


Fig. 1. Comparison of DRB1 first domain amino acid sequences of the DR4 subtypes (Tiercy et al. [6])

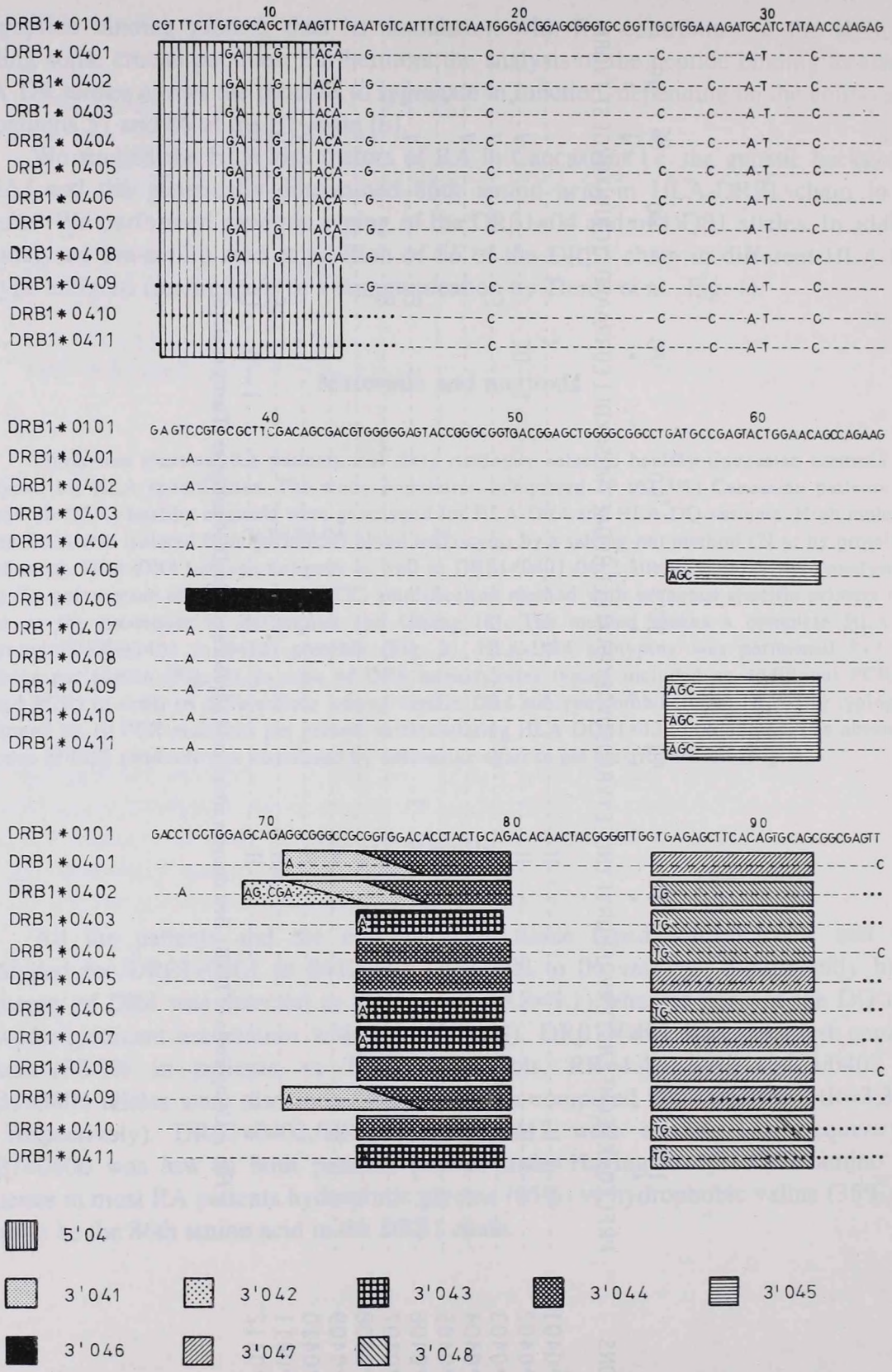


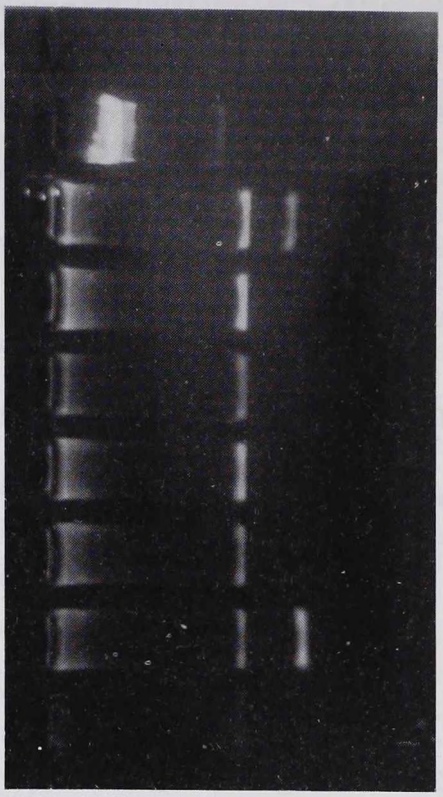
Fig. 2. Comparison of the nucleotide sequences of the first domain exon DRB1+0401-0411 alleles (Olerup and Zetterquist [8])

Mix	Specific for	Fragment size
4.1	DRB1*0401, *0409	212bp
4.2	DRB1*0402	206bp
4.3	DRB1*0403, *0406, *0407, *0411, *0412	222bp
4.4	DRB1*0401, *0402, *0404, *0405, *0408 DRB1*0409, *0410	223bp
4.5	DRB1*0405, *0409, *0410, *0411, *0412	171bp
4.6	DRB1*0406	111bp
4.7	DRB1*0401, *0405, *0407, *0408, *0409 DRB1*0412	260bp
4.8	DRB1*0402, *0403, *0404, *0406, *0410 DRB1*0411	260bp
4.9	DRB1*0401, *0402, *0403, *0404, *0406 DRB1*0407 *0408	171bp
4.10	DRB1*0403, *0404, *0405, *0406, *0407, DRB1*0408, *0410, *0411, *0412	212bp
4.11	DRB1*0401 - DRB1*0412 except DRB1*0406	111bp

Fig. 3. Fragment sizes and allele specificities for each primer mix

Discussion

It is well known that genetic susceptibility plays an important role in the pathogenesis of RA. Although several HLA class II DR molecules have been reported to be associated with an increased risk of developing RA [9], it is the DR4 association that has been well established and well documented. Our study is a good confirmation of these results. Cellular and molecular studies have shown that HLA-DR4 is heterogeneous, its subtypes differing in their strength of association with RA. The problem became still more complex when the studies revealed that certain epitopes carried an even higher risk for RA. The aim of our study was to determine by PCR-SSP method which of the HLA-DR4 subtypes were informative of RA and to define what was the 86th amino acid of the HLA-DRβ1 chain in peptide binding region of RA patients.



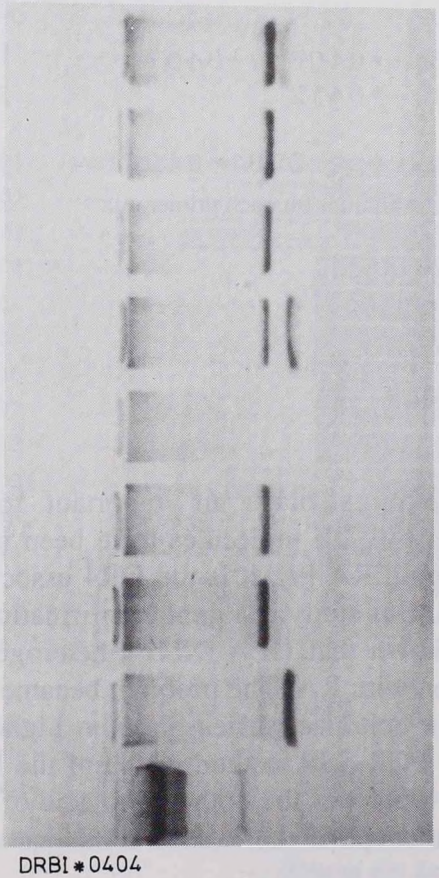


Fig. 4. Typing examples

Table I

The frequency of HLA-DR4 alleles in RA and the 86th amino acid of HLA-DRβ1 chain

	Controls		RA patients		RR	86th aa
	No.	%	No.	%		
Serological typing						
HLA-DR4	20/60	33	19/31	61	3.1	
HLA-DRβ1*04 alleles PCR-SSP typing						
0401	2/20	10	7/19	36.8	4.4	G
0403	2/20	10	1/19	5.2	–0.6	V
0403/0407	1/20	5	0/19	–	–0.33	V/G
0404	3/20	15	5/19	26.3	1.9	V
0404/0407	0/20	–	1/19	5.2	3.3	V/G
0405	6/20	30	1/19	5.2	–0.74	G
0406	1/20	5	0/19	–	–0.33	V
0407	4/20	20	2/19	10.5	–0.52	G
0408	1/20	5	2/19	10.5	1.86	G

RR = relative risk
aa = amino acid
V = valine
G = glycine

We found that 0401 was the main factor for susceptibility to RA and 0404/0407 allele-combination was characteristic of RA patients. The frequency of 0404 and 0408 alleles were also increased in RA but not significantly. The DRβ1*0401 and 0404 were prevalent variants in patients with RA (37%, 26.3%) compared to controls (10%, 15%). No differences in the distribution of DQβ1 alleles were found among patients and controls. The first conclusion of our study is that a major effect determining the susceptibility to RA is associated with DR4 subtype-alleles but not with any of the DQβ1 alleles.

When trying to find a common sequence for all DR4 alleles to be involved in RA pathogenesis, the hydrophilic glycine could be recognized at position 86 of the β1 chain in most of these alleles (0401, 0407, 0408), but not in the 0404 allele and in the nonsusceptible ones (0403, 0406), which contained the hydrophobic valine instead.

We suppose that the hydrophobic amino acid (i.e. valine) in the peptide binding region forms an epitope that is not suitable for binding rheumatoid peptides. On the other hand hydrophilic amino acid makes the rheumatoid peptide binding possible. In case of 0404 and hydrophobic valine we assume that another DR allele connected with

hydrophilic glycine results in developing RA or there might be additional factors to be associated with RA.

It should be kept in mind that besides the genetic background environmental factors, such as geographical localizations, could possibly determine the development of RA disease.

REFERENCES

1. Olliver,W., Carthy,D., Cutbush,S.: HLA-DR4 associated Dw types in rheumatoid arthritis. Tissue Antigens **33**, 30 (1988).
2. Otha,N., Nishimura,I.K., Tanimoto,K.: Association between HLA and Japanese patients with RA. Hum Immunol **5**, 123 (1982).
3. Gao,X., Brautbar,C., Gazit,E.: A variant of HLA-DR4 determine susceptibility to RA in a subset of Israeli Jews. Arthritis Rheum **34**, 547 (1991).
4. Lanchbury,J.S.: The HLA association with RA. Clin Exp Rheumatol **10**, 301 (1992).
5. Gregersen,P.K., Silver,J., Winchester,R.J.: The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to RA. Arthritis Rheum **30**, 1205 (1987).
6. Tiercy,J.M., Gebuhrer,L., Betuel,H., Mech,B., Jeannet,M.: A new HLA-DR4 allele with a DR11 alpha helix sequence. Tissue Antigens **41**, 97 (1993).
7. Miller,S.A., Dykes,D.D., Polesky,H.F.: A simple salting out procedure for extracting DNA from human nucleated cells. Nucl Ac Res **16**, 1215 (1988).
8. Olerup,O., Zetterquist,H.: HLA-DR typing by PCR amplification with sequence-specific primers (PCR SSP) in 2 hours: An alternative to serological DR typing in clinical practise including donor-recipient matching in cadaveric transplantations. Tissue Antigens **39**, 225 (1992).
9. De Vries,N., Ronningen,K.S., Tilanus,M.G.J., Rombouts,A., Segal,R., Egeland,T., Thorsby,T., Van De Pulte,L.B.A., Brautbar,C.: Sequencing HLA-DR1 in Israeli rheumatoid arthritis patients (abstract). Clin Exp Rheumatol **10**, 314 (1992).

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INTERTYPE SPECIFIC EPITOPES ON THE HEXONS OF ADENOVIRUS TYPES 1, 2, 5 AND 6

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The intertype specificities of the epitopes present on the surface of the hexon types belonging to human adenovirus (Ad h) subgenus C were studied. The reactivity pattern (RP) and the titres of ascitic fluids containing monoclonal antibodies (MAbs) were determined in ELISA. From three panels of MAbs 38 directed against Ad h1, 17 directed against Ad h 35 and 6 MAbs directed against bovine adenovirus type 2 were studied. Twelve of the MAbs proved to be genus or type specific. The rest of the MAbs showed numerous intertype specific RPs in different combinations on the different hexon types according to previous experiments with purified hexons of different Ad h subgenera and animal serotypes. With hexon types 1, 2, 5 and 6 of subgenus C all intertype specific MAbs gave identical RPs and the titres of the individual MAbs with all the four hexon types were generally high, and the most frequently found titres were evenly distributed among them. These results indicate the presence of numerous different intertype specific epitopes on the hexons of subgenus C in identical combination on the four hexon types.

The serotyping of adenoviruses is based on the different antigenic properties of their capsid proteins determined by polyclonal immune sera. The hexon antigen represents the bulk of these proteins and on its surface a great number of epitopes with different specificities exist. These specificities can be determined by means of monoclonal antibodies. The 49 human adenovirus (Ad h) serotypes are classified in 6 subgenera (A to F) according their structural, biochemical, biological, genetical and immunological characteristics [1]. Hexon antigens are known to have both type and genus specific determinants, detectable by different serological methods with polyclonal antibodies [2, 3]. Using monoclonal antibodies (MAbs) genus, intertype and type specific epitopes can be detected [1]. In previous experiments with more than twenty different hexon types we found numerous different intertype specific epitopes besides the type specific and genus specific ones [4, 5]. The hexons of subgenus D have shown diversity in the intertype specificities [6], thus it may be of interest to examine an other subgenus of human adenoviruses. For this purpose subgenus C, consisting of 4 serologically distinct serotypes was chosen and three panels of MAbs, consisting of 61 ascitic fluids were used in the experiments.

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Materials and methods

Antigens. Purified adenovirus hexons were prepared from the four subgenus C serotypes (1, 2, 5 and 6) as well as from 17 other types representing the all human adenovirus subgenera A to F and some animal adenovirus types. Viruses were propagated in Hep-2 or "293" cells [7] and the soluble hexon antigens were purified by repeated anion exchange chromatography on DEAE Sephadex A-25 or A-50 (Pharmacia, Sweden) columns and by exclusion chromatography, while hexon of Ad hi in addition was crystallized, too [8]. The protein concentration of the purified preparations was determined by Lowry's method [9].

Monoclonal antibodies. Three panels of hybridomas were produced by crystallized Ad h1, by purified Ad h35 hexons, while MAbs directed against bovine adenovirus (Ad bos2) were kindly supplied by M. Rusvai. Ascitic fluids were prepared by cultivation of antibody-producing hybridoma cells in Petri dishes. Cells in exponential growth phase ($1–5\times10^6$) were separated by centrifugation at 200 g, and injected in 0.5 ml PBS i.p. in mice. Ascitic fluids were harvested after 7 to 21 days, and stored at –20 °C after centrifugation. The specificities of the individual MAbs were determined in previous experiments with 21 different hexon types.

Indirect ELISA. Tests were carried out using 96-well polystyrene plates (Falcon Microtest II. T. C.). For coating 40 µg/ml concentration of purified hexon was used and MAbs were added to the wells in serial twofold dilution of ascites from 1:200. Binding of MAbs to hexons was detected by goat anti-mouse immunoglobulin, labelled with horse-radish peroxidase (Sigma). The substrate of enzyme was o-phenylen diamine and the color change was assessed by a Medicor-Multilab EPD-1 photometer at 492 nm wavelength. The titres were expressed as $\log_2$(reciprocal dilution of antibody$\times10^{-2}$), thus a titre of 1 means a dilution of 1:200 and a titre of 10 means a dilution of 1:102 400.

Results

Reactivity pattern of the ascitic fluids. Table I shows the reactivity patterns (RPs) of the 61 ascitic fluids with the hexons of the four subgenus C serotypes and the specificity of the individual MAbs or MAb groups determined in previous experiments by testing hexon types belonging to other human subgenera as well as to simian and bovine adenovirus types [5, 10–12]. Of the three panels MAbs 1A3, 2C2, 35H15 and IVF3 being genus specific reacted with all the four members of subgenus C. MAbs 5C1, 5D1 and 5D2 proved to be type specific to Ad h1, as they reacted only with this hexon type. Thirty-three MAbs directed to Ad h1 representing 12 different intertype specificities reacted with all hexon types of subgenus C. MAbs 35H10, 35H30 and 35H51, type specific to Ad h35 and MAbs IIA9 and IVF5, type specific to Ad bos2 gave no reactions as expected. The intertype specific MAb 35H26 directed to Ad h35 reacted with all, and on the contrary 12 MAbs from the same panel representing 4 different intertype specificities with none of the hexons of subgenus C. MAbs B11, A12 and CA12 raised against Ad bos2, representing two different intertype specificities reacted with all the four hexon types of subgenus C. It can be seen that all individual intertype specific MAb gave identical reactions, positive or negative with all hexon types of subgenus C. Data indicate the presence of numerous different intertype specific epitopes on the surface of the hexon types of subgenus C in identical combinations on the four hexon types which reflect a strong antigenic relationship within the subgenus. The only exceptions were the negative reactions with hexon types 2, 5 and 6 of the three type specific MAbs to Ad h1.

Table I

Reactivity pattern of 61 ascitic fluids with the 4 human adenovirus hexon types of subgenus C

MAb	Human hexon types of subgenus C				Specificity of MAbs
	1	2	5	6	
Directed to type: Ad h1 1A3, 2C2	+	+	+	+	genus
1A2, 1A5, 1A6, 1B1, 1B2, 1B5, 1B6, 1C4, 1C5, 1D4, 1D6, 2A1, 2A2, 2A3, 2A4, 2A5, 2A6, 2B1, 2B2, 2B3, 2B5, 2C1, 2C3, 2C4, 2C5, 2C6, 2D1, 2D2, 2D6, 5A1, 5A4, 5B4, H12	+	+	+	+	intertype
5C1, 5Dl, 5D2	+	–	–	–	type to Ad h1
Directed to type: Ad h35 35H15	+	+	+	+	genus
35H26	+	+	+	+	intertype
35H18, 35H43, 35H49, 35H50, 35H54, 35H55, 35H56, 35H57, 35H58, 35H59, 35H62, 35H63	–	–	–	–	intertype
35H10, 35H30, 35H51	–	–	–	–	type to Ad h35
Directed to type: Ad bos2 IVF3	+	+	+	+	genus
A12, B11, CA12	+	+	+	+	intertype
IIA9, IVF5	–	–	–	–	type to Ad bos2
Number of reacting/nonreacting MAbs	44/17	41/20	41/20	41/20	

Titres of the reacting MAbs. The titration of the individual MAbs was carried out in serial twofold dilutions of the ascitic fluids. Table II presents the titres of the individual MAbs and Table III shows the frequency distribution of titre values among the hexon types. MAbs not reacting with any hexon type of subgenus C and the type specific MAbs to Ad h1 were not involved in this evaluation. The titres of the individual MAbs with the hexon types of subgenus C were generally high. Only one MAb (B11) displayed a low titer of 3 (1:800), the titres of other MAbs varied from 7 (12 800) to 16 (13 105 200). The close antigenic homogeneity among the hexons of subgenus C was confirmed by the titres of the MAbs. Of the 41 reacting intertype specific MAbs 12 had identical titres with all the 4 hexon types. Fifteen MAbs have shown identical titres with 3 hexon types and one dilution step difference to the fourth one, and one MAb with two and one MAb with three dilution steps difference. Identical titres were given with 2 hexon types with one or two dilution step(s) difference to the titres of the other 2 types by 4 MAbs and 5 MAbs, respectively, and only 3 MAbs have shown four, five or six dilution steps difference

among the hexons of the four members of subgenus C. There were no significant differences in the mean values of the titres in the case of the individual hexon types. As Table III shows the most frequent titres were the values 9, 10 and 11, and their frequency distribution was similar among all hexon types of subgenus C.

Table II

Reactivity pattern and titres[a] of 3 panels of MAbs[b] in ELISA with adenovirus hexon types of subgenus C

MAb	hexon types of subgenus C			
	1	2	5	6
H12	12	12	12	12
1A3	11	11	11	11
2A6	11	11	11	11
2B5	11	11	11	11
2C5	11	11	11	11
ID6	10	10	10	10
2C6	10	10	10	10
1C4	9	9	9	9
1B1	8	8	8	8
2A4	8	8	8	8
A12	7	7	7	7
B11	3	3	3	3
2D6	14	13	13	13
1B2	11	11	11	12
1B5	11	11	11	12
2D2	11	11	11	12
2A2	11	11	10	11
2C3	11	11	10	11
1A6	10	10	9	10
2B3	10	10	9	10
2C4	10	10	9	10
2A3	10	9	9	9
2B2	9	9	8	9
2C1	9	9	8	9
2D1	9	9	8	9
2C2	8	9	9	9

Continued on p. 79.

Table II continued

1D4	9	8	8	8
1A5	10	11	10	11
1A2	10	10	9	9
1B6	9	10	9	10
2A1	9	9	10	10
2B1	11	10	9	10
1C5	11	11	10	9
5A4	10	9	9	8
5B4	10	9	9	8
35H26	9	11	11	10
2A5	10	8	8	8
5A1	11	11	11	8
35H15	7	11	11	10
CA12	13	16	10	10
IVF3	7	13	10	10
5D2	11	–	–	–
5C1	10	–	–	–
5D1	10	–	–	–
Mean[c]	9.93	10.02	9.51	9.65

[a] Titres are expressed as $\log_2$(reciprocal dilution of MAb $\times 10^{-2}$)

[b] MAbs not reacting with any hexon types of subgenus C are not included

[c] Titres of type specific MAbs are not included

Table III

Titre[a] ranges of reacting MAbs with hexon types of subgenus C

Hexon types of subgenus C	Number of MAbs showing the titres indicated									
	3	7	8	9	10	11	12	13	14	16
1[b]	1	3	3	8	11	12	1	1	1	–
2	1	1	4	9	8	14	1	2	–	1
5	1	1	7	11	9	10	1	1	–	–
6	1	1	7	8	12	7	4	1	–	–
Total number	4	6	21	36	40	43	7	5	1	1

[a] Titres are expressed as $\log_2$ (reciprocal dilution of antibody $\times 10^{-2}$)

[b] Titres of type specific MAbs to Ad h1 are not included

Discussion

The hexon types belonging to subgenus C have shown a remarkable homogeneity on the basis of the identical cross-reactivity pattern with the relatively large number of MAbs having different intertype specificities [6, 13] and according to the number of reacting and non reacting MAbs (Table I) as well as on the basis of similar level of titres (Table II). According to the three panels MAbs represented 12, 4 and 2 different intertype specificities, respectively. It is conceivable that using even more MAbs with different specificities and more hexon types possibly new specificities would be found. Because some of these intertype specificities proved to be identical in the two panels of MAbs raised against Ad hl and Ad h35, and several MAbs did not react at all with the hexon types 1, 2, 5 and 6 (not shown), the MAbs altogether represented 14 distinct intertype specificities on the hexon types of subgenus C. The different epitopes corresponding to these specificities all are present on the surfaces of all types of subgenus C but they are present or absent in different combinations on the different hexon types belonging to other subgenera. That means – in contrast to members of subgenus D [6] – a close antigenic homogeneity among all hexon types of subgenus C.

In the past few years recombinant adenoviruses (RAds) have emerged as promising technology in the gene therapy. RAds have been used for genetic modification of a variety of cells in vitro and in vivo [14, 15]. They offer several advantages over other vectors. Replication defective RAds can be produced in very high titres (1×10^{11} pfu/ml) thus allowing a substantially greater efficiency of direct gene transfer. They have the capacity to infect both replicating and nonreplicating cells, they are able to accept relatively large foreign genes up to 7.5 kb in length, the DNA functions in an extrachromosomal fashion, which eliminates the risk of insertional mutagenesis etc. [15, 16]. Several important limitations of adenovirus-mediated gene transfer are also known, such as the relatively short-term expression of foreign genes, the dose-related direct toxic effect, and that the host immune response – even in the case of the replication-defective recombinants – may substantially inhibit repeated infection with RAd [14].

Human adenovirus types 2 and 5 of subgenus C are used most frequently as vectors for direct gene transfer [17–19]. Yang and coworkers have shown in a mouse liver experiments with RAdL type 5 that not only the transgene was expressed but the newly synthesized hexon proteins were also detectable [20] and suggested that a virus specific cellular immune response was stimulated in the recipient that led to the destruction of the genetically modified hepatocytes and repopulation of the liver with nontransgene-containing hepatocytes. For improving RAd technology to avoid the host immune response several approaches could be considered such as to further cripple the adenovirus to diminish the viral protein expression [20], to modify the epitope composition of the virus especially on the hexon surface [21], or to use different RAd serotypes in the repeated infections [22] etc. To the successful realization of any of these approaches would need, however, the exact knowledge of the complete intertype specific epitope spectrum of RAd hexon types used for gene therapy determined and deemed by large number of standardized MAbs with different specificities. The data concerning the strong intrasubgenus antigenic homogeneity suggest that recombinant adenoviruses constructed from the types of subgenus C should not be replaced by each other to avoid the host

immune response in repeated infections in gene therapy because of their close antigenic relationship. For that purpose RAds should be constructed from those adenovirus serotypes which do not contain identical intertype specific epitopes, or at least only in a minimal quantity.

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REFERENCES

1. Russell,W.C., Bartha,A., de Jong,J.C., Fujinaga,K., Ginsberg,H., Hierholzer,J.C., Li,Q.G., Mautner,V., Nász,I., Wadell,G.: Adenoviridae. Fifth Report. Arch Virol Suppl **2**, 140 (1991).
2. Hierholzer,J.C., Stone,Y.D., Broderson,J.R.: Antigenic relationships among the 47 human adenoviruses determined in reference horse antisera. Arch Virol **121**, 179 (1991).
3. Wigand,R., Keller,D., Wetling,I.: Immunological relationship among human adenoviruses of subgenus-D. Arch Virol 72, 199 (1982).
4. Ádám,É., Erdei,J., Lengyel,A., Berencsi,G., Fachet,J., Nász,I: Delineation of antigenic determinants of adenovirus hexons by means of monoclonal antibodies. Intervirology **23**, 22 (1985).
5. Ádám,É., Erdei,J., Lengyel,A., Fejér,Gy., Fachet,J., Nász,I.: Monoclonal antibodies to adenovirus type 35 hexon. Intervirology **27**, 9 (1987).
6. Ádám,É., Nász,I., Lengyel,A.: Diversity in the antigenic structure of different hexon types among the members of adenovirus subgenus D. Acta Microbiol Immunol Hung **42**, 85 (1995).
7. Graham,F.L., Smiley,J., Russell,W.C., Nairu,R.: Characterization of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol **30**, 59 (1977).
8. Ádám,É., Nász,I.: Electron microscopic studies on the crystallization of adenovirus hexon capsomers. Intervirology **13**, 1 (1980).
9. Lowry,O.H., Rosebrough,N.J., Farr,A.L., Randall,N.J.: Protein measurement with the Folin phenol reagent. J Biol Chem **193**, 265 (1951).
10. Ádám,É., Rusvai,M., Lengyel,A., Belák,S., Nász,I.: Antigenic relationship between human and animal adenovirus hexons by means of monoclonal antibodies directed against bovine adenovirus type 2 hexons. Arch Virol **100**, 9 (1988).
11. Ádám,É., Nász,I., Lengyel,A.: Antigenic relationships among the members of adenovirus subgenera determined by monoclonal antibodies. Acta Microbiol Hung **39**, 309 (1992).
12. Nász,I., Ádám,É.: Characterization of the antigenic structure of adenovirus hexons by means of monoclonal antibodies. UNESCO-ROSTE Techn Rep **6**, 66 (1991).
13. Kaier,B., Wigand,R.: Antigenic homogeneity of adenovirus types 1, 2, 5 and 6. J Med Virol **18**, 283 (1986).
14. Gerard,R.D., Meidell,R.S.: Adenovirus-mediated gene transfer. Trends Cardiovasc Med **3**, 171 (1993).
15. Siegfried,W.: Perspectives in gene therapy with recombinant adenoviruses. Exp Clin Endocrinol **101**, 7 (1993).
16. Liu,T.-J., Zhang,W.-W., Taylor,D.L., Roth,J.A., Goepfert,H., Clayman,G.L.: Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. Cancer Res **54**, 3662 (1994).
17. Acsadi,Gy., Jani,A., Massie,B., Simoneau,M., Holland,P., Blaschuk,K. Karpati,G.: A differential efficiency of adenovirus-mediated in vivo gene transfer into skeletal muscle cells of different maturity. Hum Molec Genet **3**, 579 (1994).

18. Welsh,M.J., Smith,A.E., Zabner,J., Rich,D.P., Graham,S.M., Gregory,R.J., Pratt,B.M., Moscicki,R.A.: Clinical protocol. Cystic fibrosis gene therapy using an adenovirus vector: in vivo safety and efficacy in nasal epithelium. Hum Gene Ther **5**, 209 (1994).
19. Zatloukal,K., Cotten,M., Berger,M., Schmidt,W., Wagner,E., Birnstiel,M.L.: In vivo production of human factor VIII in mice after intrasplenic implantation of primary fibroblasts transfected by receptor mediated, adenovirus-augmented gene delivery. Proc Natl Acad Sci USA **91**, 5148 (1994).
20. Yang,Y., Nunes,F.A., Berencsi,K., Furth,E.E., Gönczöl,E., Wilson,J.M.: Cellular immunity to viral antigen limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA **91**, 4407 (1994).
21. Crompton,J., Toogood,C.I.A., Wallis,N., Hay,R.T.: Expression of a foreign epitope on the surface of the adenovirus hexon. J Gen Virol **75**, 133 (1994).
22. Natuk,R.J., Lubeck,M.D., Chanda,P.K., Chengalvala,M., Wade,M.S., Murthy,S.C.S., Wilhelm,J., Vernon,S.K., Dheer,S.K., Mizutani,S., Lee,S.-W., Murthy,K.K., Eichberg,S.W., Davis,A.R., Hung,P.P.: Immunogenicity of recombinant human adenovirus-human immunodeficiency virus vaccines in chimpanzees. AIDS Res Hum Retrov **9**, 395 (1993).

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DIFFERENT AND SELECTIVE CHEMOTACTIC RESPONSES OF *TETRAHYMENA PYRIFORMIS* TO TWO FAMILIES OF SIGNAL MOLECULES: LECTINS AND PEPTIDE HORMONES

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Tetrahymena chemotactically responds to the presence of different signal molecules. The response elicited is different to peptide hormones and to lectins however the cell distinguishes the individual members of the two groups. The negative chemotactic effect is characteristic of the hormones tested and it is especially true for oxytocin and vasopressin, however *Tetrahymena* distinguishes also these two hormones. While the effect of hormones was mainly independent to the concentration tested, lectins elicit an increasing chemoattractant effect above 10^{-9} M concentration. Below 10^{-9} M the sugar specific, receptor level effect of lectins is probable. The experiments draw attention to the perceptional selectivity of *Tetrahymena* embodied in chemotaxis.

Lectins, the mainly plant origin proteins are carbohydrate binding molecules and their sugar-specificity has a close relation to their receptor-specificity. Mostly two or more subunits compose the lectin molecule and this character is responsible for e.g. haemagglulatination, the characteristic function of the firstly described lectins, like ricin [1]. The capacity to recognize carbohydrates of the cell surface provides a very significant function of lectins in intervertebrates. However, in these organisms immunoglobulins are absent, they have a variety of agglutinins which molecules help in fine-tuning of immune recognition system based on carbohydrate specificities. This kind of role of lectins was demonstrated in molluscs [2] and tunicates [3] where lectins participate in recognition of particles to be phagocytized. Some evolutionary conclusions were also drawn as there are common determinants of lectins isolated from tunicates and immunoglobulins while mollusc lectins have no such homology [3]. The membrane associated glycosaminoglycanes of sponges are also suggested to have a role in immune recognition [4] and some endogenous lectins were detected in human peripheral mononuclear leukocytes [5] as well as in the unicellular *Tetrahymena* [6]. This way we can say that lectins are significant signal molecules at lower and higher ranks of evolution alike. Moreover this function, lectins have intracellular functions, too. Certain lectins e.g. PHA,

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Con A, Glycine max have mitogenic potency [7] and some other lectins are toxic e.g. a subunit of *Ricinus communis* penetrates the surface membrane and inhibits the protein synthesis at a ribosomal level [8]. Many of these functions are closely related to effects on different enzymes, however some lectins themselves have an enzymatic character, too [9].

Over these significant actions of lectins there are several unsolved problems. Among them the physiological actions of lectins are inspiring as these actions are also related to signal recognition but they are not well described.

In vertebrate and invertebrate systems hormones and hormone like substances represent an other significant and well-known group of signal molecules [10]. However, the hormone-receptor mediated signaling requires a highly specific recognition both at membrane or intracellular levels, the glycosyl groups of the receptor complexes – in our present knowledge – are not essential elements of this form in signal recognition.

Considering the action of peptide hormones not only the binding itself but the consecutive steps of action: action through the receptor linked systems (in the membrane and in the second messenger level in the cytoplasm) and the internalization of the receptor are also prerequisites of specific way of action [11]. It is worth to be mentioned that some second messengers e.g. cAMP, inositol phosphates or the internalization is involved in way of action of some lectins, too [12].

Chemotaxis is one of the most fundamental physiological reactions of the cell. This activity of cell is thought to be important in evolution of cell signalling. Finding and selection of the appropriate food – especially at unicellular level – is a chemotaxis related mechanism and some theories suggest that membrane components responsible for food recognition were the origin of membrane localized hormone-receptors [13].

Our unicellular model cell the eukaryote *Tetrahymena pyriformis* is a good tool to evaluate chemotaxis related effects [14]. Not only hormones [15] but other signal molecules released during immunological responses [16], volatile oils [17], some characteristic inorganic substances [18] are also able to induce specific chemotactic activity of this cell. Beside this the cell is well characterized, it possesses almost all of the essential biochemical pathways of intracellular signal transport (cAMP, cGMP, calmodulin-Ca^{2+}, inositol phospholipids) and hormone- and lectin-binding assays [18, 19] verified that there are specific binding structures in the surface membrane. Moreover the physiological responses (growth, phagocytosis, contractile vacuolar activity etc.) [20–22] are also pointing to that *Tetrahymena* is a suitable model for characterization of chemotactic activity to lectins and hormones.

In the present work there were two problems to be answered:

(i) According to concentration course study which are the chemotactic and repellent lectins and pituitary hormones acting at unicellular level?

(ii) Considering ligand specificity, is there any similarity of lectins and hormones which are eliciting chemotactic response?

Materials and methods

Cells and culturing. Logarithmic phase cultures of *Tetrahymena pyriformis* GL were applied grown in 1% Tryptone (Difco, Michigan, USA) medium, containing 0.1% yeast extract at 28 °C.

Hormones and lectins applied. The concentration course of lectin and peptide hormone induced chemotaxis was tested between 10^{-12}–10^{-6} M concentration. For final dilution of the substances we used always fresh culture medium, identical to the medium for culturing *Tetrahymena.* The following lectins were tested: Concanavalin A (Serva, Germany); Lens culinaris* [23]; Glycine max (Fluka, Swiss); Helix pomatia* [24]; Phytohemagglutinin M* [25]. Substances marked with asterisk were purified by us according the technique cited.

Peptide hormones tested were: Follicle stimulating hormone (FSH) (Peninsula, USA); Luteinizing hormone + Follicle stimulating hormone (HCG) (Serono, Italy); Thyroid stimulating hormone (TSH) (Organon, Oss, Holland); Growth hormone (STH) (Kio Vitrum, Denmark).

Assay of chemotaxis. To assay chemotaxis we applied a double-chamber test [26] modified by us [27] where the outer chamber was filled with the suspension of cells (cell density 10^4 cells/ml) while the inner chamber contained the test substance. A capillary served as connecting link between the two chambers. The assays were carried out at room temperature (22 °C), the incubation time was 15 min. The short time of incubation was chosen as our goal was to determine the unidirectional chemotactic movement of cells towards the attractant applied. Following incubation samples were taken from the inner chamber, the cells were fixed in 4% formaldehyde containing phosphate buffer (PBS). Determination of cell density of samples was done by Neubauer cytometer. Each experiment was repeated five times.

Statistical evaluation. For statistical evaluation of data SigmaPlot 4.0 and Origin 2.8 were used.

Results and discussion

Previous experiments demonstrated that the unicellular *Tetrahymena* possesses receptors for the endogenous signal molecules of its own or such signal molecules which are characteristic of higher evolutionary levels [28]. These receptors make possible the selection among the signal molecules in the binding of these substances. This activity of the cell induces a response which is specific at many times. Our previous binding studies demonstrated file presence of Con-A-insulin receptor overlap in *Tetrahymena*, which has been known in higher animals [18]. Chemotaxis experiments also pointed to the chemotactic or repellent effect of a wide range of hormones, e.g. ACTH, insulin, serotonin, histamine [15]. The present experiments support our previous observations as they show that *Tetrahymena* is able to recognize the signal family (lectin, hormone) specific moieties and selects the applied peptide molecules.

It seems to be group specific to lectins – except Con A – that the elicited chemotactic effect is dose dependent and increasing above 10^{-9} M concentration (Figs 1a and 1b). Below 10^{-9} M the effect (curve) is unbalanced and there are some peaks, e.g. PHA at 10^{-11} M or Glycine max at 10^{-10} M (Fig. 1a). Over these differences it is obvious that all of the lectins tested had positive chemoattractant effect. We explain this positive chemoattractant effect as the significant sugar specificity of the lectins applied considering that the plasma membrane contains high numbers of sugars and amino sugars in association with or independent to the receptors. The 50 000–120 000 dalton molecular mass proteins can serve as nourishment which fact could give an other possible

explanation. However we cannot reject the respect that sugar specificity dominates at the low concentrations and the protein character (consumption as a food) dominates at the higher concentration range.

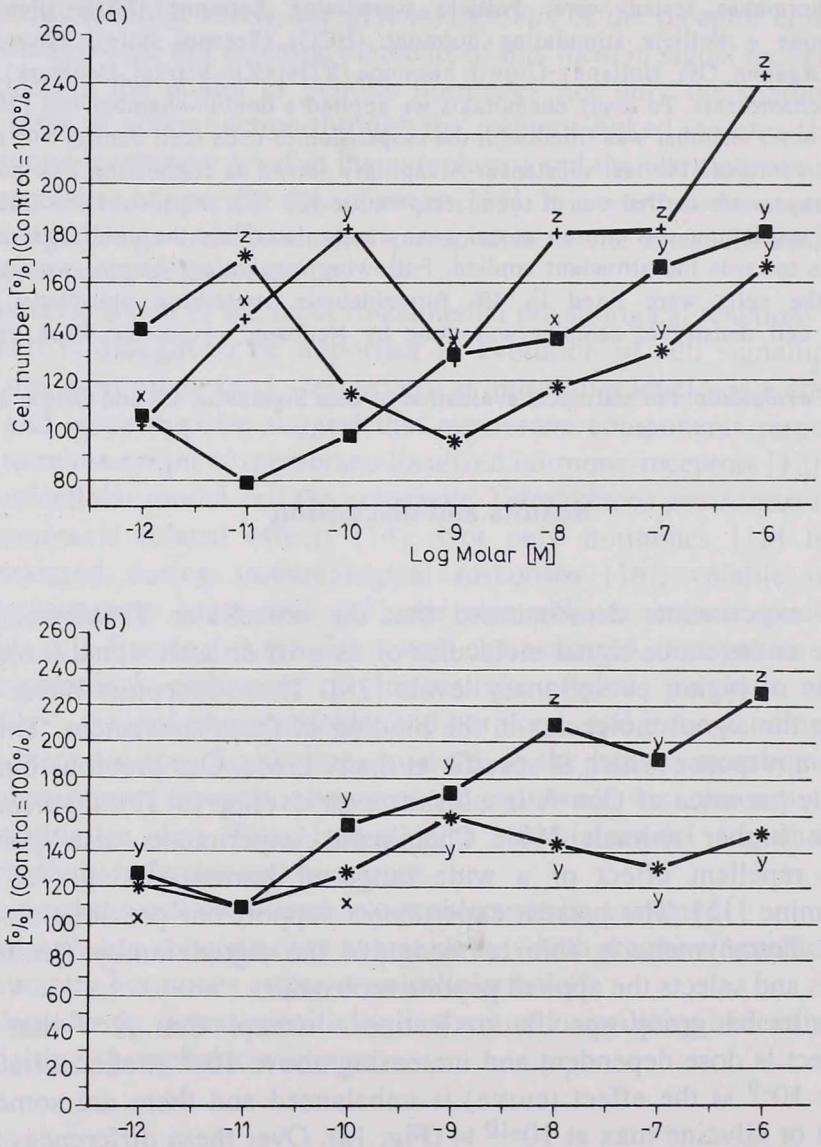

Fig. 1. Concentration course of chemotaxis elicited by (a) *Helix pomatia* (■), phytohemagglulinin (*) and Glycine max (+) and by (b) Lens culinaris (■) and Concanavalin A (*) lectins in *Tetrahymena.* (x – p>0.05; y – p>0.01; z – p>0.001)

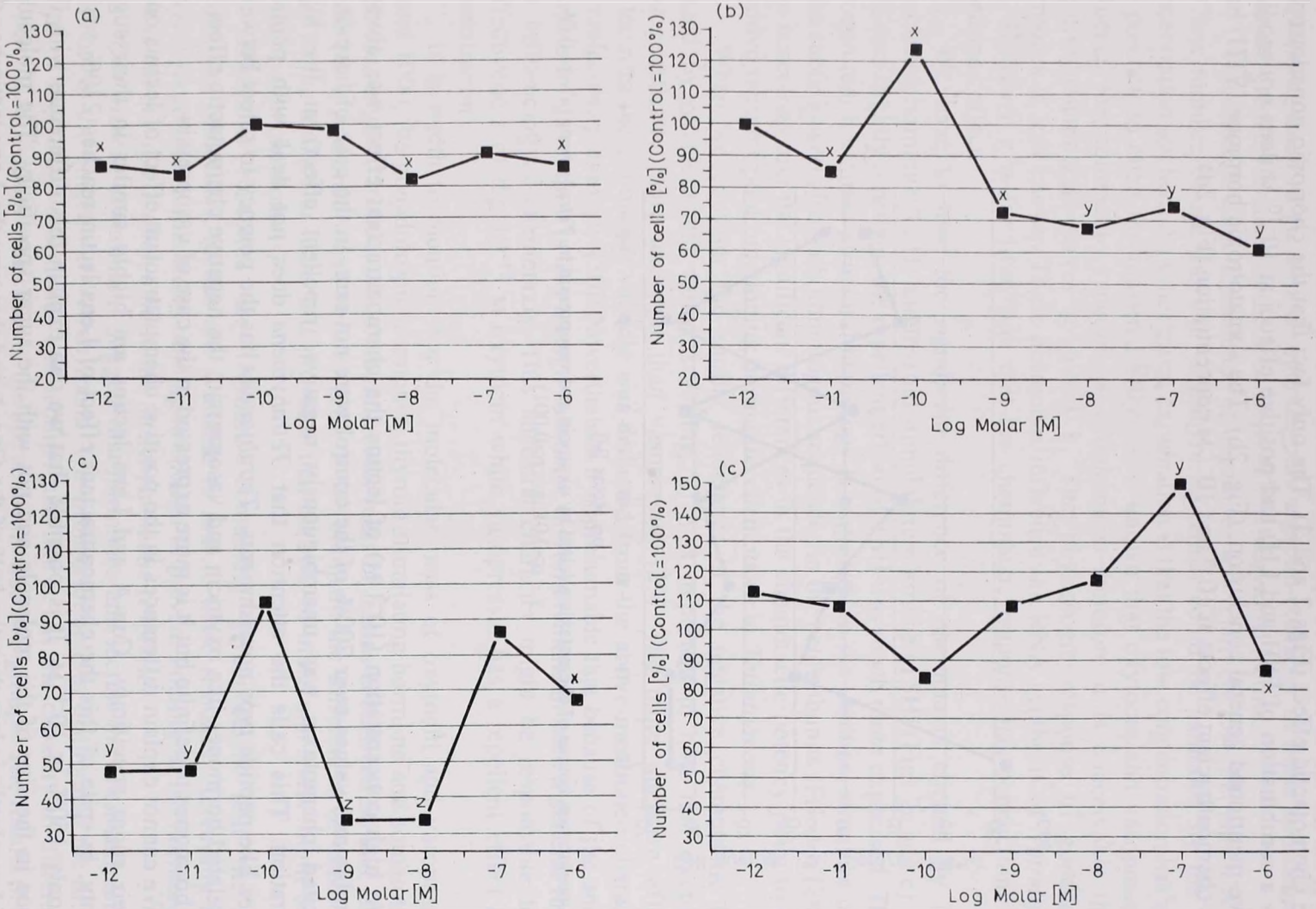

Fig. 2. Concentration course of chemotaxis elicited by (a) follicle stimulating hormone (FSH); (b) human chorion-gonadotropic hormone (HCG=FSH+LH); (c) thyrotropic hormone (TSH), and (d) somatotropic hormone (STH) in *Tetrahymena* (x – $p>0.05$; y – $p>0.01$; z – $p>0.001$)

Of the peptide hormones tested, oxytocin, vasopressin, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) had unambiguously a characteristic, negative chemotactic effect (Figs 2 and 3). The only fact that the chorionic gonadotropin (HCG = a combination of FSH and LH) had positive effects at 10^{-10} M does not modify the above-mentioned general conclusion. (Fig. 2b). The somatotropic hormone (STH) had positive chemoattractant effects at 10^{-8} and 10^{-7} M concentration (Fig. 2d).

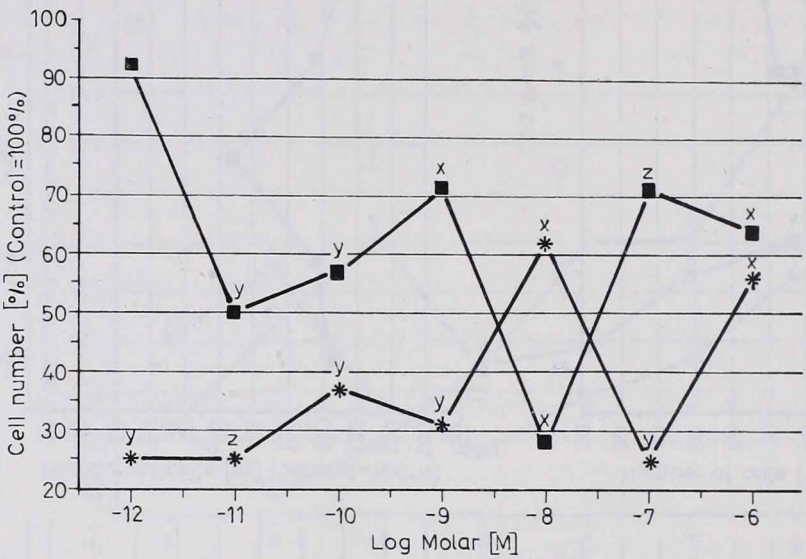

Fig. 3. Concentration course of chemotaxis elicited by oxytocin and vasopressin in *Tetrahymena* (x – p>0.05; y – p>0.01; z – p>0.001)

At high concentration (10^{-6} M) of lectins the chemoattractant effect was always considerable and values over 200% of the control were not rare. In the case of hormones the elicited chemotaxis was unambiguously negative (repellent effect) at the high concentration. This calls the attention that *Tetrahymena* does not deal with peptide hormones like peptide type nourishments. *Tetrahymena* has the potency to select between highly related hormones like oxytocin and vasopressin; the negative chemotactic effect of the two hormones is definite but it is more expressed in the case of vasopressin.

We cannot explain differences in the positive chemoattractant effect of lectins only with their sugar specificity. Con-A and Lens lectins are highly similar in their sugar specificity, in spite of this the chemoattractant effect of Lens lectin reaches 230% while Con-A only 150% at 10^{-6} M. It is possible that we can explain this difference with the difference in the size of the molecule and not with the sugar specificity. The molecular mass of Lens lectin is less than the half of the Con-A and this way the Lens lectin has the capacity to diffuse more fast and develop a more expressed effect on this way. Nevertheless at 10^{-12}–10^{-11} M range, which is the supposed effective concentration for the

receptors, the result of the two lectins is very similar. In the case of lectins (Glycine max, Helix and PHA) which are able to bind amino sugars the deviation of the curves is bigger. The binding itself to the amino sugars is only a group character of these substances, and there are great differences in the specificity of the individual lectins.

The concentration dependence of hormones was relatively low. Perhaps it is due to the low number of receptors of *Tetrahymena* which could be saturated by the lowest concentration applied. An other given explanation is that the low concentration has already the potency to elicit the maximal effect. It is known that oxytocin and vasopressin can influence the contractile vacuole of *Tetrahymena* therefore it is conceivable that the negative chemotaxis serves to avoid it. The physiologic response to gonadotropic hormones is less known. These hormones influence the RNA synthesis of *Tetrahymena*, too. However it is far from sure that the chemotactic response has connections to the mentioned effect.

We have to note the significant difference of chemotaxis elicited by follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) (Figs 2a and c). While FSH was slightly repellent, the repellent effect of TSH was much more expressed. The two glycoprotein hormones structural homology is expressed in the identical structure of their alpha subunits and also with homologous sequences in the beta subunits (Frieden [28, 29]). This homology and the significant differences in the chemotactic potency point to a very sensitive receptor pool functioning during the chemotaxis in *Tetrahymena*.

When we consider the similar tendencies of the negative chemotactic effects elicited by oxytocin and vasopressin (Fig. 3) it is important to note that oxytocin is phylogenetically more ancestral than vasopressin [29]. The more intensive effect of oxytocin to the contractile vacuole was deducted from the above-mentioned character of the molecule, previously [30]. Nevertheless it is presumable that because of the ancestral character of oxytocin it is less foreign substance to *Tetrahymena* in such a general function like influencing of chemotaxis. This ancestral character might be responsible for the ineffectiveness of the 10^{-12} M oxytocin while vasopressin has a repellent effect at this concentration.

It is worth to mention that the molecular mass of oxytocin and vasopressin is around 1000, the gonadotropic hormones, thyroid stimulating hormone and somatotropine reach the range of 36 000, while the molecular mass of lectins is between 50 and 120 000 dalton (as it was mentioned before) [28]. If we compare the differences of chemotactic effect to the molecular mass it is provable that the role of molecular mass might be only exceptional in chemotaxis.

Hormone receptors are regularly containing glycoproteins and the sugar recognizing molecules, lectins are available to link to these residues. According to our previous experiments this lectin binding is enough to prevent binding of the hormone [31] but it is not enough to elicit a hormone like chemotactic response.

Our present experiments suggest that hormones and lectins are chemoattractant or repellent for the unicellular *Tetrahymena*. The protozoan able to distinguish the different groups of molecules (hormones and lectins) just like it has the capacity to differentiate between the individual molecules of the above mentioned two groups. The results obtained by us at 10^{-12} M concentration, point to the high sensitivity of our model cell.

REFERENCES

1. Hartnut,F.: The ricin story, In Hartmut,F. (ed.): Advances in lectin research. Springer, Berlin 1988. p. 10.
2. Roitt,I., Brostoff,J., Male,D., Roitt,I., Brostoff,J., Male,D. (eds): Immunology. Gower Medical Publ, London–New York 1989. p. 15.2.
3. Roitt,I., Brostoff,J., Male,D., Roitt,I., Brostoff,J., Male,D. (eds): Immunology. Gower Medical Publ, London–New York 1989. p. 15.5.
4. Muller,W.E., Zahn,R.K., Muller,I., Kurelec,B., Uhlenbruck,G., Vaith,P.: Cell aggregation of the marine sponge *Geodia cydonium*. Identification of lectin-producing cells. Eur J Cell Biol **24**, 28 (1981).
5. Kieda,C., Monsigny,M., Waxdal,M.J.: Endogeneous lectins on human peripherial mononuclear leukocytes. In Bog-Hansen,T.C., Spengler,G.A. (eds): Lectins III. Vol. 3. Walter de Gruyter, Berlin–New York 1983. p. 427.
6. Csaba,G., Kovács,P.: lectins in the unicellular *Tetrahymena*. I. Lectin detection with FITC-labelled antilectins. Acta Histochem **73**, 53 (1983).
7. Stadier,B.M., Liechti,J., Bonnard,G.D.: T cell stimulating lectins are mitogen inducers but not mitogens. In: Bog-HansenT.C., Spengler,G.A. (eds): Lectins III. Walter de Gruyter, Berlin–New York 1983. p. 37.
8. Olsnes,S., Pihl,A.: Abrin and ricin – two toxic lectins. TIBS **3**, 7 (1978).
9. Gabins,H.J.: Non-carbohydrate binding partners/domains of animal lectins. Int J Biochem **26**, 469 (1994).
10. Barrington,E.J.W.: The phylogeny of the endocrine system. Experientia **42**, 775 (1986).
11. Csaba,G.: Evolution of receptors. In Bittar,E. (ed.): Fundamentals of medical cell biology – membrane dynamics and signalling Vol. 5A, JAI Press 1992. p. 99.
12. Berlot,C.: Identification of chemoattractant-elicited increases in protein phosphorylation. In Prescott,D.M. (ed.): Methods in cell biology Vol. 28. Academic Press, New York 1987. p. 333.
13. Lenhoff,H.M.: On the mechanism of action and evolution of receptors associated with feeding and digestion. In Muscatine,L., Lenhoff,H.M. (eds): Coelenterate Biology. Academic Press, New York 1974. p. 238.
14. Leick,V., Hellung-Larsen,P.: Chemosensory behaviour of *Tetrahymena*. Bioassays **14**, 61 (1992).
15. Kőhidai,L., Karsa,J., Csaba,G.: Effects of hormones on the chemotaxis in *Tetrahymena* - Investigations on receptor memory. Microbios **77**, 75 (1994).
16. Leick,V.: Chemotactic properties, cellular binding and uptake of peptides and peptide derivates: studies with *Tetrahymena thermophyla*. J Cell Sci **103**, 565 (1992).
17. Kőhidai,L., Lemberkovics,É., Csaba,G.: Molecule dependent chemotactic responses of *Tetrahymena pyriformis* elicited by volatile oils. Acta Protozool **34**, 181 (1995).
18. Csaba,G., Kovács,P.: Histamine-lectin and insulin-lectin binding site overlaps in *Tetrahymena*. Cell Mol Biol **28**, 153 (1982).
19. Tanabe,H., Kurihara,K., Kobatake,Y.: Sensitization of chemotactic response by changing the lipid composition of surface membrane in *Tetrahymena pyriformis*. Biochim Biophys Acta **553**, 396 (1979).
20. Hegyesi,H., Csaba,G.: Effect of pretreatments (imprinting) with different concentrations of insulin on insulin binding of nuclear envelope and plasma membrane in *Tetrahymena*. Cytobios **72**, 191 (1992).
21. Csaba,G., Darvas,Zs.: Insulin antagonizes the phagocytosis stimulating action of histamine in *Tetrahymena*. Biosci Rep **12**, 22 (1992).
22. Ghiladi,M., Christensen,S.T., Schousboe,P., Rasmussen,L.: Compounds stimulating growth and multiplication in ciliates. Do also free-living cells release growth factors ? Naturwissenschaften **79**, 234 (1992).
23. Kőhidai,L., Karsa,J., Csaba,G.: Effects of atrial natriuretic peptide (ANP) on the model *Tetrahymena* pyriformis. Microbios **82**, 27 (1995).
24. Howard,I.K., Sage,H.J., Stein,M.D., Young,N.M., Leon,M.A., Dyckes,D.F.: Studies on phytohemagglutinin from the lentil II. Multiple forms of Lens culinaris hemagglutinin. J Biol Chem **246**, 1590 (1971).
25. Hammarström,S., Kabat,E.A.: Purification and characterization of a blood group A reactive hemagglutinin from the snail helix pomatia and a study of its combining sites. Biochemistry **8**, 2696 (1969).

26. Entlicher,G., Kostir,J.V., Kocourek,J.: Studies on phytohemagglutinins III. Isolation and characterization of hemagglutinins from the pea, *Pisum sativum* L. Biochim Biophys Acta **221**, 272 (1970).
27. Leick,V., Helle,J.: A quantitative assay for ciliate chemotaxis. Anal Biochem **135**, 466 (1983).
28. Kőhidai,L., Csaba,G.: Effects of mammalian vasoconstrictor peptide - endothelin 1 - on the unicellular *Tetrahymena*. Comp Biochem Physiol **111C**, 311 (1995).
29. Frieden,E.H.: Hormones of the anterior pituitary. In Frieden, E.H. (ed.): Chemical endocrinology. Academic Press, New York 1976. p. 136.
30. LeRoith,D., Roberts,C.Jr., Lesniak,M.A., Roth,J.: Receptors for intercellular messenger molecules in microbes: similarities to vertebrate receptors and possible implications for diseases in man. Experientia **42**, 782 (1986).
31. Frieden,E.H.: Hormones of the anterior pituitary. In Frieden, E.H. (ed.): Chemical endocrinology. Academic Press, New York 1976. p. 173.
32. Csaba,G., Kovács,P.: Oxytocin and vasopressin change the activity of the contractile vacuole in *Tetrahymena*: newer contributions to the phylogeny of hormones and hormone receptors. Comp Biochem Physiol **102A**, 353 (1992).
33. Kovács,P., Csaba,G.: Is the saccharide component of the insulin receptor involved in hormonal imprinting? Cell Biochem Funct **4**, 111 (1986)
34. Csaba,G., Kovács,P.: Hormonal influence on lectin binding to *Tetrahymena*. Cell Mol Biol **28**, 509 (1982).

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MANNOSE RECEPTORS ARE IMPLICATED IN THE *CANDIDA ALBICANS* KILLING ACTIVITY OF EPIDERMAL CELLS

(A LETTER TO THE EDITOR)

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To the Editor:

Candida albicans infections constitute an important medical problem, but the mechanisms of the host defense against *Candida albicans* cells have not been completely understood. We earlier reported that freshly separated human epidermal cells are capable of the phagocytosis and killing of *Candida albicans* [1]. This activity of epidermal cells appeared to be greatly enhanced after stimulation with α-melanocyte stimulating hormone (α-MSH), interleukin-8 (IL-8), or ultraviolet light in vitro [2–4]. Although inflammatory mediators are implicated in the *Candida* killing activity of epidermal cells, the exact mechanism is not known [5]. Since recently mannose receptors have been shown to be involved in the phagocytosis and killing of *Candida albicans* cells of monocytes and monocyte-derived macrophages [6, 7], we were interested in whether similar mechanisms exist in epidermal cells.

The involvement of mannose receptors in the *Candida albicans* killing activity of epidermal cells was investigated by the inhibitory effect of mannan (an α-linked polymer of mannose) and mannose-bovine serum albumine (mannose-BSA, 23 mol mannose/mol BSA) on the killing activity. Mannan and mannose-BSA are the classical ligands of the mannose receptor, which substances were shown to inhibit *Candida* killing activity of macrophages by blocking specifically the mannose receptors [6]. Human epidermal cells were obtained from healthy individuals undergoing plastic surgery

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earlier [8]. In several experiments cultured immortalized HaCat human keratinocyte cell line were used. In the *Candida albicans* killing assay, freshly separated human epidermal cells or HaCat cells were incubated with *Candida albicans* cells (dissolved in PBS containing 0.25% BSA) in a ratio of 1:2 for 90 min, with or without the presence of 0.1–1 mg/ml mannan (Sigma, St. Louis, USA) or mannose-BSA (E/Y Labs, San Mateo, USA). Human cells were then lysed with 1% Triton X, and the percentage of dead *Candida albicans* cells was determined by staining with methylene blue. In several experiments, epidermal cells or HaCat cells were stimulated by 20 μg/ml of α-MSH (Sigma, St. Louis, U.S.A.) 1 h prior to the beginning of the killing assay.

Table I

The effect of mannan and mannose-BSA on the spontaneous and α-melanocyte stimulating hormone (α-MSH) induced Candida albicans killing activity of human epidermal cells (EC) in vitro

	Concentration of the principle	Candida killing index
unstimulated		
EC alone	–	8.67±2.00
EC + mannan	0.10 mg/ml	4.37±0.58
EC + mannan	0.50 mg/ml	4.67±2.00
EC + mannose-BSA	0.10 mg/ml	2.17±0.71
EC + mannose-BSA	0.50 mg/ml	3.17±0.71
α-MSH stimulated		
EC alone	–	24.33±2.08
EC + mannan	0.10 mg/ml	12.00±1.63
EC + mannan	0.50 mg/ml	8.67±3.61
EC + mannose-BSA	0.10 mg/ml	5.67±2.83
EC + mannose-BSA	0.50 mg/ml	3.67±1.41

The *Candida* killing activity of EC is expressed as the percentage of non-viable, killed yeast cells counted under a light microscope after staining with methylene blue; data represent mean±SD of four independent experiments. Mannan and Mannose-BSA induced a significant decrease in the *Candida* killing index at each tested concentrations ($p<0.05$, Student t-test)

Table I shows that both mannan and mannose-BSA dose-dependently inhibited the spontaneous *Candida* killing activity of freshly separated EC cells. Mannose-BSA proved to be more efficient in the inhibition of killing activity than mannan. α-MSH resulted in a 2.5-fold increase in the *Candida* killing activity, and this increased killing activity could be completely inhibited by incubation of cells with mannan or mannose-BSA. Similar results were obtained using the HaCat human epidermal cell line (not shown).

The major components of the cell wall of *Candida* yeasts are mannan. Binding of mannan or mannose-BSA to specific mannose-receptors on the surface of macrophages inhibit *Candida* phagocytosis and killing, suggesting that attachment of the yeast cells to macrophages is essential for the killing [6]. In the present study we could show that probably similar mechanisms are implicated in the *Candida albicans* killing activity of EC, since mannan and mannose-BSA dose-dependently inhibited the *Candida albicans* killing activity of EC. This suggest that the interaction of the *Candida* cell wall-mannan with mannose-binding structures on the surface of the EC are essential in the killing activity. This mannose-binding structures are probably the mannose receptors, which have been well characterized on macrophages. The further characterization and regulation of mannose receptors on EC might be important in the understanding of the physiological role of the epidermis in the defense to mycotic infections.

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REFERENCES

1. Csató,M., Bozóky,B., Hunyadi,J., Dobozy,A.: *Candida albicans* phagocytosis by separated human epidermal cells. Arch Dermatol Res **279**, 136 (1986).
2. Csató,M., Kenderessy,Sz.A., Dobozy,A.: Enhancement of *Candida albicans* killing activity of separated human epidermal cells by ultraviolet radiation. Br J Dermatol **116**, 469 (1987).
3. Csató,M., Kenderessy,Sz.A., Dobozy,A.: Enhancement of *Candida albicans* killing of separated human epidermal cells by α-melanocyte stimulating hormone. Br J Dermatol **121**, 145 (1989).
4. Kemény,L., Kenderessy,Sz.A., Arenberger,P., Peter,R.U., Dobozy,A., Ruzicka,T.: Interleukin-8 induced chemotaxis and increased *Candida albicans* killing activity of normal human epidermal cells. Arch Derm Res **284**, 26 (1992).
5. Csató,M., Kenderessy,Sz.A., Judák,R., Dobozy,A.: Inflammatory mediators are involved in the *Candida albicans* killing activity of human epidermal cells. Arch Derm Res **282**, 348 (1990).
6. Maródi,L., Korchak,H.M., Johnston,R.B.: Mechanisms of host defense against *Candida* species (I. Phagocytosis by Monocytes and Monocyte-Derived Macrophages). J Immunol **146**, 2783 (1991).
7. Maródi,L., Forehand,J.R., Johnston,R.B.: Mechanisms of host defense against *Candida* species (II. Biochemical Basis for the Killing of Candida by Mononuclear Phagocytes). J Immunol **146**, 2790 (1991).
8. Kemény,L. Kenderessy,Sz.A., Olasz,E., Michel,G., Ruzicka,T., Farkas,B., Dobozy,A.: The interleukin-8 receptor: a potential target for antipsoriatic therapy? Eur J Pharmacol **258**, 269 (1994).

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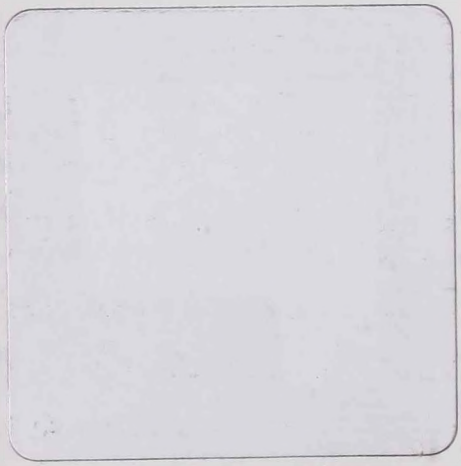

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EBV-B CELL INTERACTIONS: IMMORTALIZATION, RESCUE FROM APOPTOSIS, TUMORIGENICITY*

(A SHORT REVIEW)

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Translocation of the c-myc gene to an immunoglobulin locus is a rate-limiting step in the genesis of three B-cell derived tumors: Burkitt lymphoma (BL) in humans, mouse plasmacytoma (MPC) and rat immunocytoma. Its consequences have been best analyzed in BL. They involve a non-immunological and an immunological component. The former acts by preventing the B cell from leaving the cycling compartment and entering the resting stage when programmed to do so. The latter acts by the down regulation of certain HLA class I polymorphic specificities, adhesion molecules and EBV encoded proteins. According to our interpretation, the translocation fixes the BL cell in a phenotypic window that can be referred to as "a resting cell that is not resting". The linking of c-myc to immunoglobulin sequences in a B cell leads to constitutive myc expression. In spite of its switch to a "resting phenotype", the cell is therefore unable to leave the cycling compartment. Normally, EBV-carrying B-blasts face immune controls. They can only proliferate in immunodefectives, as in XLP or transplant lymphoma. Due to its "resting" rather than B-blast phenotype and the correlated defective expression of certain EBV, HLA and adhesion molecules, the BL cell is not rejected by the EBV-specific immune response, however.

The down-regulation of EBNA 2–6 in the BL cell may be also considered in relation to viral latency. The exclusive expression of EBNA 1 in the BL cell reflects the adaptation of the virus to prolonged persistence in long lived resting B cells, an important if not exclusive reservoir of the latent virus.

Normal B cells that fail to be activated by appropriate mitogens or antigens within a limited period of time undergo apoptosis, as a rule. EBV may protect resting B cells. The role of the only virally encoded protein they are known to express, EBNA 1, remains to be explored in this respect. EBV infected immunoblasts are protected from apoptosis by the LMP 1 induced elevation of bcl-2 expression. During the lytic cycle when cellular protein synthesis is switched off by the early viral proteins, a member of the early protein group, a bcl-2 homologous protein encoded by the BHRF1 gene, takes over the function to protect against untimely apoptosis.

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Burkitt's lymphoma: The discovery of Epstein–Barr virus and of proto-oncogene activation by chromosomal translocation

During the 1950s, Dennis Burkitt a surgeon working in Uganda, described an extremely agressive tumor occurring in African children [1]. He recognized that the same tumor could present as a jaw tumor or an abdominal mass frequently affecting the ovaries. These tumors have been diagnosed as sarcomas and dysgerminomas until O'Conor and Davies realized that they were lymphomas [2]. Subsequently, histologically identical tumors were identified in Europe and in the USA. Based on the geographic distribution of the tumor known today as Burkitt's lymphoma (BL), Burkitt proposed that the disease is caused by an arthropod-carried virus [3]. Although this hypothesis was not verified, it stimulated the studies that have led to the discovery of Epstein–Barr virus (EBV) in BL derived cell lines [4]. The viral DNA can be detected in the vast majority of "endemic" BLs (i.e. in tumors from Equatorial Africa and New Guinea), but only in a minority of the rare, "sporadic" BLs (occurring all over the world) (see [5] for a review). This suggested that EBV may contribute to the genesis of the high endemic, but not or only rarely of the sporadic BLs.

The discovery of a specific group of chromosomal translocations in both the endemic and sporadic BLs has led to a new, cytogenetical definition of the tumor and has significantly contributed to the understanding of its pathogenesis (see [6] for a review). The translocations involve one of the three immunoglobulin genes (14q32, 22q11 or 2p11–12) that are alternatively juxtaposed to the q24 region of chromosome 8, where the c-myc gene (the cellular homologue of the transforming element of the avian myelocytomatosis virus, MC29) is located. This rearrangement results in a deregulated, constitutive expression of c-myc, which is a critical component in the pathogenesis of BL.

The significance of these observations was corroborated by similar findings on two B cell derived rodent tumors: mouse plasmacytomas (MPC) and rat immunocytomas. The most frequent translocation in MPCs is 12;15 in which the c-myc gene is displaced from its position on chromosome 15 and juxtaposed to the immunoglobulin heavy chain locus on chromosome 12 [7]. In the spontaneous rat immunocytoma, a 6;7 translocation juxtaposes c-myc (located on chromosome 7 in the rat) to the IgH cluster on chromosome 6 [8].

Transgenic mice that carry the c-myc gene attached to an immunoglobulin heavy chain enhancer, a "facsimile" mimicking the most frequent chromosomal translocation in BLs and MPCs, develop lymphoid neoplasms within a few months after birth. This further supports the idea that abnormal expression of c-myc is a critical element in the pathogenesis of BL. The monoclonality of these tumors indicates, however, that additional genetic changes are also required for their development [9].

Consequences of c-myc deregulation and the role of EBV in the pathogenesis of endemic BLs

The c-myc gene encodes a nuclear phosphoprotein which is structurally and functionally similar to transcription factors (formation of hetero- and homodimers, DNA binding, transactivating capacity) [10–12]. The level of c-myc RNA is low in quiescent cells but mitogen stimulation leads to an immediate induction of c-myc, which declines slowly thereafter to the basal level [13]. It has been shown, however, that c-myc has not only a role in promoting proliferation, but it also triggers apoptosis (programmed cell death) if expressed in cells that do not have a set of appropriate growth factors at their disposal [14]. One may suggest that the apoptotic response represents a safeguard mechanism to avoid the detrimental effects of c-myc deregulation, including the generation of potentially malignant cells.

In the case of BL, the constitutive expression of c-myc keeps a cell type in the cycle in spite of the fact that its phenotype corresponds to noncycling ("resting" or memory) B cells. Part of the BL cells appears to avoid apoptosis by p53 mutations, whereas the rest remain unexplored [15].

The constitutive c-myc expression may also contribute to a reduced expression of HLA class I antigens and adhesion molecules (LFA–1, LFA 3 and ICAM–1) on the cell surface, potentially capable of facilitating the escape of immunogenic tumor cells from immune surveillance [16, 17].

In spite of the striking association of EBV with the endemic form of BL, its pathogenetic role is not clear. Conceivably, early EBV infection and such co-factors as tropical, hyper- or holoendemic malaria may increase the size of the target cell population in African children and thereby raise the probability of the chromosomal translocation accident that leads to constitutive c-myc activation [18]. EBNA 1, the only latent EBV protein expressed in BLs in vivo (see below) may also contribute to lymphoma development more directly, as suggested by the frequent development of B-cell lymphomas in E_{mu}-EBNA 1 transgenic mice [19].

The coexistence of Epstein–Barr virus (EBV) and the human species – Two paradoxes

EBV, a herpesvirus widespread in human populations causes no disease in the vast majority of infected individuals. This fact is in apparent contrast with the observations made on B cells interacting with the virus in vitro, since EBV seems to be the most potent growth-transforming virus known so far: this is the first paradox. EBV carrying B cells grow as tumors only in hosts with a defective immune system (e.g. in patients with X-linked lymphoproliferative syndrome) and/or when they carry, in addition to EBV genomes, chromosomal changes resulting in altered oncogene expression as well (like the cells of endemic Burkitt lymphomas).

The second paradox arises from the fact that EBV-infected autologous B lymphocytes are highly immunogenic for autologous T cells in vitro. In the course of acute mononucleosis, cytotoxic T lymphocytes are generated, recognizing specific EBV-encoded protein epitopes. In spite of this powerful reaction, the virus is not eliminated from the organism. On the contrary, it persists in the B lymphocyte fraction itself.

How are these paradoxes resolved? It is reasonable to assume that the unlimited growth of EBV-transformed immunoblasts is curbed by immunological surveillance in vivo. This explains why infectious mononucleosis is a self-limiting disease. The latent virus persists in the "resting" B lymphocyte fraction where the expression of the highly immunogenic viral antigens is down-regulated.

Host cell dependent expression of latent EBV genes

EBV immortalized lymphoblastoid cell lines (LCLs) regularly express six nuclear antigens (EBNA 1–6) and three membrane proteins (LMP 1, 2a and 2b). In addition, two abundant, protein non-coding small RNA molecules (EBER 1 and 2) are also transcribed from the viral episomes [20].

A more restricted pattern of viral gene expression was observed in Burkitt lymphoma (BL) biopsies and phenotypically representative (group I) BL lines. Only a single viral protein, EBNA 1 could be detected [20] together with the EBERs [21].

There are clear phenotypic differences between LCLs and group I BL lines. The former express cellular antigens characteristic for "activated" immunoblasts whereas the latter look more like "resting", mainly memory B cells. The differential EBV protein expression is due to the alternative usage of viral promoters. In LCLs where all six EBNAs are expressed the transactivator EBNA 2 switches on the promoters for the genes of latent membrane proteins (LMP 1 and 2), and absence of EBNA 2 from group I BL cells explains why the LMPs are not expressed in these cells (see [22] for a review).

While the EBNA and LMP messages are transcribed by RNA polymerase II, the EBERs, which are invariably expressed both in LCLs and group I BL lines, are transcribed from internal promoters of their genes by RNA polymerase III [23].

Differential gene expression as a result of alternative promoter usage and alternative splicing

Three different promoters can be used for EBNA transcription in latently infected B cells. They are designated as Wp, Cp and Qp, based on their localization on the BamHI restriction map (Fig. 1).

Wp is activated early during in vitro infection of peripheral B cells while Cp is turned on at a later stage, with the concomitant silencing of Wp, and maintains its

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activity in the immortalized LCLs' [24]. Splicing of the giant RNA molecules initiated from Wp or Cp results in mRNAs coding for EBNA 1–6. This expression is an exclusive characteristic of the virally transformed immunoblasts.

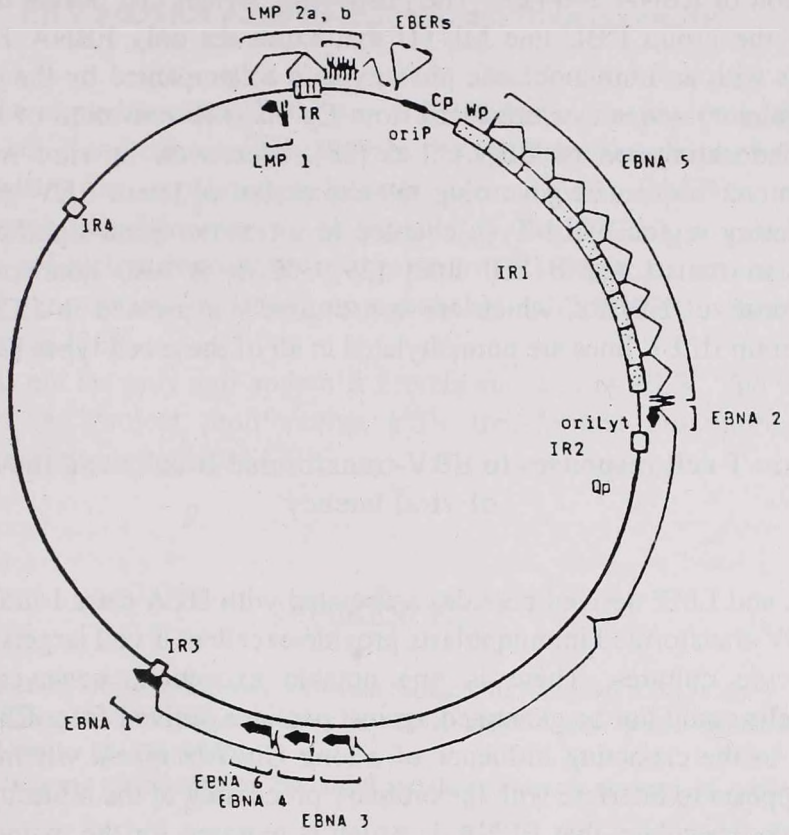

Fig. 1. Transcripts generated from latent episomal EBV genomes in lymphoblastoid cell lines. Latent promoters (Cp, Wp and Qp) of EBNA hnRNAs, internal repetitions (IR1-4) and (fused) terminal repeats (TR) are also indicated. Origins of latent (oriP) and lytic (oriLyt) replication are shown based on the prototype B95-8 sequence.

The alternative promoter, Qp [25], is inactive in LCLs but active in group I BL lines. The precursor RNA initiated at Qp gives only rise to a single message, coding for EBNA 1. EBNA 1 is expressed in all latently EBV infected cell types (B cells, nasopharyngeal carcinoma cells, Sternberg–Reed cells of Hodgkin's lymphomas, certain peripheral T cell lymphomas including midline granulomas, certain gastric carcinomas and EBV positive B cell–epithelial cell hybrids). This is probably due to the fact that EBNA 1, which binds to oriP, the latent origin of viral replication, is necessary for the maintenance of viral episomes in proliferating cells [26].

Cell-phenotype-dependent activation and silencing of latent EBV promoters correlates with methylation patterns of their regulatory regions

Group I BL cells that express EBNA 1 only can be induced to express all six EBNAs by 5-azacytidine, indicating that DNA methylation may be involved in the down-regulation of EBNA 2–6 [27]. The phenotypic switch that occurs during in vitro cultivation of the group I BL line MUTU that expresses only EBNA 1 to LCL-like, group III cells with an immunoblastic phenotype is accompanied by the demethylation of certain regulatory sequences upstream from Cp and the activation of this promoter, resulting in the expression of EBNA 1–6 [28]. Moreover, in vitro methylation of enhancer-promoter sequences governing the expression of latent EBV genes (Wp, Cp and the regulatory region of LMP 1) coupled to a reporter gene significantly reduces theft activity in transfected B cell lines [29, 30]. It is also noteworthy, that the transcription units of EBERs, which are constitutively expressed in LCLs as well as group I and group III BL lines are unmethylated in all of these cell types [21].

Cytotoxic T cell responses to EBV-transformed B cells and the strategy of viral latency

EBNA and LMP derived peptides associated with HLA class I molecules on the surface of EBV-transformed immunoblasts provide excellent T cell targets in autologous mixed leukocyte cultures. There is one notable exception, however. Autologous cytotoxic T cells could not be generated against peptides derived from EBNA 1. This is probably due to the cis-acting influence of a long Gly-Ala repeat within the EBNA 1 protein that appears to interfere with the ordinary processing of the molecule [31].

One may speculate, that EBNA 1, which is required for the maintenance of the viral episomes, was selectively modified during viral evolution to avoid recognition by T cells. During primary infection of B cells in vivo Wp and later Cp is switched on, resulting in expression of EBNAs and consequently LMPs in the transformed lymphoblasts. The resulting immunoblast proliferation is checked by the specific cytotoxic T cells. It is conceivable that only those infected B blasts that undergo a programmed phenotypic change (from "activated" to "resting") and switch off EBNA 2–6 and the LMPs, can persist as resting B cells. They may switch on Qp and express EBNA 1, although this is not necessarily so. In principle, nondividing cells might maintain the EBV genomes even in the absence of EBNA 1. There are no data concerning the expression of latent EBV proteins in resting B cells in vivo although RNAs transcribed from EBNA 1, EBER 1 and LMP2a could be detected in a subpopulation of peripheral B cells [32–34]. Following physiological activation the EBV-carrying B cells may switch on Cp and full EBNA 1–6 expression again. They

would be eliminated by cytotoxic T cells due to the up-regulation of EBNA 2–6 and LMP proteins. In the absence of activation signals, however, the infected resting B cells would be subject to the same control mechanisms as their noninfected counterparts and persist.

EBV encoded proteins may protect from apoptosis

One of the immediate early EBV genes, BHRF1, encodes a protein that is highly homologous to bcl-2 and can similarly elevate the apoptotic threshold of the cell [35]. The BHRF1 gene is not expressed in latently infected cells, however, and it is not required for growth transformation. In all probability, the BHRF1 protein protects the cells during the productive replication of the virus. This might be necessary since the synthesis of cellular proteins (including most probably bcl-2 as well) is inhibited during the productive cycle [36].

BHRF1 is not the only anti-apoptotic protein encoded by EBV. The transforming LMP 1 protein can protect proliferating, EBV transformed immunoblasts by up-regulating the transcription of bcl-2 [37].

REFERENCES

1. Burkitt,D.: A sarcoma involving the jaws in African children. Br J Surg **46**, 218 (1958).
2. O'Conor,G.T., Davies,J.N.P.: Malignant tumor in African children with special reference to malignant lymphoma. J Pediatr **56**, 526 (1960).
3. Burkitt,D.: Determining the climatic limitations of a children's cancer common in Africa. Br Med J **2**, 1019 (1962)
4. Epstein,M.A., Achong,B., Barr,Y.M.: Virus particles in cultured lymphoblasts from Burkitt lymphoma. Lancet **i**, 702 (1964).
5. Facet,C.A., Playfair,J.H.L.: Malaria, Epstein–Barr virus, and the genesis of lymphomas. Adv Cancer Res **53**, 33 (1989).
6. Magrath,J.: The pathogenesis of Burktitt's lymphoma. Adv Cancer Res **55**, 133 (1990).
7. Marcu,K.B., Hams,L.J., Stanton,L.W., Erickson,J., Watt,R., Croce,C.M.: Transcriptionally active c-myc oncogene is contained within NIARD, a DNA sequence associated with chromosome translocations in B-cell neoplasia. Proc Natl Acad Sci USA **80**, 519 (1983)-
8. Sümegi,J., Spira,J., Bazin,H., Szpirer,J., Levan,G., Klein,G.: Rat c-myc oncogene is located on chromosome 7 and rearranges in immunocytomas with t(6;7) chromosomal translocation. Nature **306**, 97 (1983).
9. Adams,J.M., Hams,A.W. Pinkeft,C.A., Corcoran,L.M., Alexander,W.S. Cory,S., Palmiter,R.D., Brinster,R.L.: The c-myc oncogene driven immunglobulin enhancer induces lymphoid malignancy in transgenic mice. Nature **318**, 533 (1985).
10. Hann,S.R. King,M.W., Bentley,D.L., Anderson,C.W., Eisenman,R.N.: A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt lymphomas. Cell **52**, 185 (1988).
11. Blackwood,E.M., Eisenman,R.N.: Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA complex with Myc. Science **251**, 1211 (1991).

12. Kato,G.J., Barrett,J., Villa-Garcia,M., Dang,C.V.: An aminoterminal c-myc domain required for neoplastic transformation activates transcription. Mol Cell Biol **10**, 5914 (1990).
13. Kelly,K., Cochran,B.H., Stiles,C.D., Leder,P.: Cell specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. Cell **35**, 603 (1983).
14. Evan,G.E., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z., Hancock,D.C.: Induction of apoptosis in fibroblasts by c-myc protein. Cell **69**, 119 (1992).
15. Wiman,K.G., Magnusson,K.P., Ramquist,T., Klein,G.: Mutant p53 detected in a majority of Burkitt lymphoma cell Vines by monoclonal antibody Pab240. Oncogene **6**, 1633 (1991).
16. Versteeg,R., Noordermeer,I.A., Kruse-Wolters,M., Ruiter,D., Jand Schrier,P.I.: C-myc down-regulates class I HLA expression in human melanomas. EMBO J **7**, 1023 (1988).
17. Inghirami,G., Grignani,F., Sternas,L., Lombardi,L., Knowles,D., Mand Dalla-Favera,R.: Down regulation of LFA-1 adhesion receptor by the c-myc oncogene in human B lymphoblastoid cells. Science **250**, 682 (1990).
18. Klein,G.: In defence of the "old" Burkitt lymphoma scenario. Advances in Viral Oncology **7**, 207 (1987).
19. Wilson,J.B., Levine,A.J.: The oncogenic potential of Epstein–Barr virus nuclear antigen 1 in transgenic mice. Current Topics in Microbiol and Immunol **182**, 375 (1992).
20. Rowe,M., Rowe,D.T., Gregory,C.D., Young,L.S., Farrell,P.J., Rupani,H., Rickinson,A.D.: Differences in B cell growth phenotype reflect novel patterns of Epstein–Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J **6**, 2743 (1987).
21. Minarovits,J., Hu,L.F., Marcsek,Z., Minarovits-Kormuta,S., Klein,G., Ernberg,I.: RNA polymerase III transcribed EBER 1 and 2 transcription, units are expressed and hypomethylated in the major Epstein–Barr virus-carrying cell types. Journal of General Virology **73**, 687 (1992).
22. Gregory,C.D.: Epstein–Barr virus and B cell survival. Reviews in Medical Virology **2**, 205 (1992).
23. Howe,J.G., Shu,M.D.: Epstein–Barr virus small RNA (EBER) genes: unique transcription units that combine both RNA polymerase II and III promoter elements. Cell **57**, 825 (1989).
24. Woisetschlaeger,M, Jin,X.W., Yandava,C.N., Fumarsky,L.A, Strominger,J.L., Speck,S.H.: Role for the Epstein–Barr virus nuclear antigen 2 in viral promoter switching during the initial stages of infection. Proc Natl Acad Sci USA **88**, 3942 (1991).
25. Schaefer,B.C., Strominger,J.L., Speck,S.H.: The Epstein–Barr virus BamHI F promoter is an early lyric promoter: lack of correlation with EBNA 1 gene transcription in group I Burkitt's lymphoma cell lines. J Virol **69**, 5039 (1995).
26. Yates,J., Warren,N., Reisman,D., Sugden,B.: A cis-acting element from the Epstein–Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc Natl Acad Sci USA **81**, 3806 (1984).
27. Masucci,M.G., Contreras-Salazar,B., Ragnar,E., Falk,K., Minarovits,J., Ernberg,I., Klein,G.: 5-aza-cytidine up regulates the expression of Epstein–Barr virus nuclear antigen 2 (EBNA-2) through EBNA 6 and latent membrane protein in the Burkitt's lymphoma line Rael. J Virol **63**, 3135 (1989).
28. Altiok,E., Minarovits,J., Hu,L.F., Contreras-Brodin,B., Klein,G., Ernberg,I.: Host-cell-phenotype-dependent control of the BCR2/BWR1 promoter complex regulates the expression of Epstein–Barr virus nuclear antigens 2–6. Proc Natl Acad Sci USA **89**, 905 (1992).
29. Jansson,A., Masucci,M., Rymo,L.: Methylation at discrete sites within the enhancer region regulate the activity of the Epstein–Barr virus BamHI W promoter in Burkitt lymphoma lines. J Virol **66**, 62 (1992).
30. Minarovits, J., Hu,L.F., Minarovits-Kormuta,S., Klein,G., Ernberg,I.: Sequence-specific methylation inhibits the activity of the Epstein–Barr virus LMP-1 and BCR2 enhancer-promoter regions. Virology **200**, 661 (1994).
31. Levitskaya,J., Coram,M., Levitsky,V., Imreh,S., Steigerwald-Mullen,P.M., Klein,G., Kurilla,M.G., Masucci,M.G.: Inhibition of antigen processing by the internal repeat region of the Epstein–Barr virus nuclear antigen-1. Nature **375**, 685 (1995).
32. Qu,L., Rowe,D.T.: Epstein–Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J Virol **66**, 3715 (1992).

33. Tieracy,R.J., Steven,N., Young,L.S., Rickinson,A.D.: Epstein–Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection in the carrier state. J Virol **68**, 7374 (1994).
34. Chen,F., Zou,J.Z., Di Renzo,L., Winberg,G., Hu,L.F., Klein,E., Klein,G., Ernberg,I.: A subpopulation of normal B cells latently infected with Epstein–Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. J Virol **69**, 3752 (1995).
35. Henderson,S., Huen,D., Rowe,M., Dawson,C., Johnson,G., Rickinson,A.D.: Epstein–Barr virus-encoded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc Natl Acad Sci USA **90**, 8479 (1993).
36. Gergely,L., Klein,G., Ernberg,I.: Host cell macromolecular synthesis in cells containing EBV induced early antigens, studied by combined immunofluorescence and radioautography. Virology **45**, 22 (1971).
37. Henderson,S., Rowe,M., Gregory,C., Croom-Carter,D. Wang,F., Longenecker,R., Rickinson,A.D.: Induction of bcl-2 expression by Epstein–Barr virus latent protein 1 protects infected B cells from programmed cell death. Cell **65**, 1107 (1991).

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ANTIBIOTICS AND THE HOST-PARASITE RELATIONSHIP*

(A SHORT REVIEW)

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The last twenty years have witnessed an increasing interest in the concomitant action that an antibiotic may have on cells other than its primary target, the bacterial or fungal cell. It is now recognized that some may exert direct immunomodulatory effects (positive and negative) on cells of the host's immune response (polymorphonuclear neutrophils, macrophages and T lymphocytes). Others may concentrate within phagocytic cells, remain bioactive therein and be transported involuntarily to sites of infection. In addition, there is evidence that a number of antibiotics, primarily those which act by inhibition of bacterial protein synthesis, interfere with the expression of structural virulence factors rendering the producer bacteria more or less susceptible to phagocytic ingestion and killing. It remains likely that some of all of these activities may occur in vivo during therapy.

The susceptibility of man and animals to a variety of microbial infections is determined largely by the outcome of the interaction between the host's reticuloendothelial cells and the invading microorganisms. Intervention by an appropriate antibiotic should swing the contest in favour of the host but a number of questions need to be addressed: (i) Do antibiotics influence phagocyte function? (ii) Do antibiotics make bacteria more or less susceptible to phagocytosis and killing? (iii) Do antibiotics penetrate phagocytic cells? (iv) Do antibiotics act on intracellular bacteria?

As will become clear, no single antibiotic fulfills these criteria. However, it is possible that any agent which exhibits one or other of the above-named may be classified as a Biological Response Modifier [1]. In so doing, it should be realized that any such effect will alter either positively or negatively the result of antibiotic therapy. Some examples of these effects both in vitro and in vivo will be described.

* Based on a guest lecture presented at the 12th Congress of the Hungarian Society of Microbiology, Budapest, August 23–25, 1995

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Each aspect of the phagocytic cell' s functional activities can be measured in vitro and by doing the measurements in the presence or absence of graded doses of an antibiotic, an estimate can be made of its possible immunomodulatory activity. In many of the published reports the drug concentrations which proved to be biologically active are above those which can be achieved in vivo during normal therapy. One drug which has been tested against each parameter of phagocytic cell function (adhesion, chemotaxis, phagocytosis and killing) is teicoplanin and a summary of the results obtained are described in Table I.

Table I

Teicoplanin: its effect on phagocytic cell function

Activity	Effect
adherence to nylon	50% decrease
directed migration	50% decrease
phagocytosis	50% decrease
killing	50% decrease

In each case the drug was tested at 500 µg/ml, adapted from [2]

At a dose in excess of those achievable therapeutically (500 µg/ml) each parameter was depressed by 50% [2]. Several other antibiotics display biological activity in this respect as well as other parameters of phagocyte function and the results are summarized in Fig. 1.

When one considers the target of the phagocyte's attentions, namely the bacterial pathogen and its interaction with various antibiotics to which it is susceptible, certain novel features of the bacterium's virulence potential are affected. In addition, to repression of various bacterial toxins and enzymes [3] antibiotics can also affect bacterial structure and expression of several important surface antigens [4, 5] when the bacteria are grown at sub-MIC levels of the antibiotics. Amongst the penicillins and cephalosporins, Lorian [6] has shown that sub-growth inhibitory concentrations are capable of either causing cellular filamentation or additional and thickened cell walls. More subtle changes in the surface topography can also occur. These changes affect the expression of various surface antigens such as M protein in *Streptococcus pyogenes* and K antigen in *Escherichia coli.* A fuller list of such changes is given in Tables II/a and II/b.

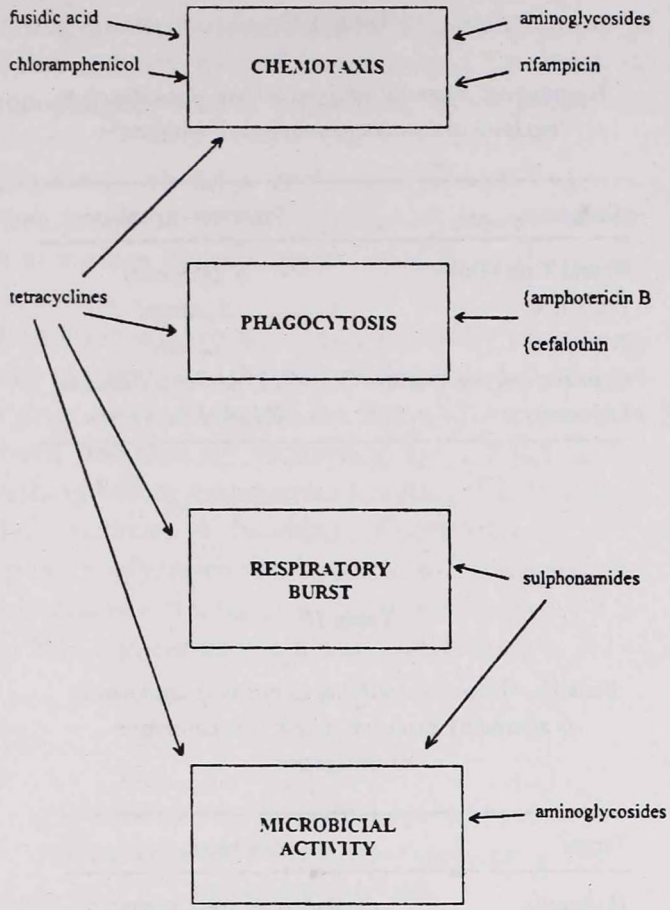

Table II/A

Examples of toxins whose biosynthesis is repressed by low concentrations of antibiotics

Toxin	Producer organism
α-Toxin Coagulase TSST-1	*S. aureus*
Streptolysin O Streptolysin S	*S. pyogenes*
Toxin B (cytotoxin)	*Clostridium difficile*
Protease	*Pseudomonas aeruginosa*
α-Haemolysin	*E. coli*

Table II/B

Examples of bacterial antigens whose biosynthesis is impaired by low concentrations of antibiotics

Antigen	Producer organism
M and T proteins	*S. pyogenes*
Protein A	*S. aureus*
K antigen	*E. coli*
Capsular polysaccharide	*Haemophilus influenzae*
Glycocalyx	*Bacteroides fragilis*

Table III

Bacteria whose susceptibility to opsonophagocytosis is altered by exposure to low concentrations of antibiotics

Target	Antibiotic
B. fragilis	clindamycin, spectinomycin
E. coli	carumonam, ceftriaxone
H. influenzae	ampicillin
P. aeruginosa	ceftazidime
S. aureus	clindamycin
S. pyogenes	clindamycin, erythromycin
S. agalactiae	penicillin

Several of these antigens serve as potential virulence factors and in particular may provide protection from opsonophagocytosis and killing. *S. pyogenes* grown in sub-MIC levels of clindamycin become susceptible to complement-mediated opsonisation [4, 7]. Less than 10% of normal *S. pyogenes* are ingested by polymorphonuclear neutrophils (PMN) whereas 40–50% of the same species grown in the presence of either 1/2, 1/4 or 1/10 MIC of the drug are ingested by the phagocytes. Evidence obtained by electron microscopy revealed that the drug-exposed bacteria were denuded of M protein–lipoteichoic acid complex. Similar changes were not caused by other antibiotics known to interfere with bacterial protein synthesis [8].

Similarly, ceftriaxone and netilmicin at sub-MIC levels reduce the expression of K surface antigen in *E. coli* and thereby potentiate its susceptibility to opsonophagocytosis [9]. Such potentiation only occurred in K-positive strains and not in K-negative strains.

Loss of K antigen in drug-treated bacteria exposed the underlying wall lipopolysaccharide in a similar way to that induced by cell carumonam [10]. Table III summarizes those bacteria whose opsonophagocytosis is potentiated by exposure to sub-MIC levels of certain antibiotics.

Several mechanisms whereby antibiotics modify bacterial susceptibility to phagocytosis have been proposed: (i) through loss of external barrier to serum opsonins; (ii) through changes in surface hydrophobicity; (iii) through increased exposure of new receptors for serum opsonins.

A number of antibiotics have also been shown to concentrate within phagocytic cells. The list includes clindamycin, the macrolides, the quinolones and rifampicin. The ratio of intracellular drug levels to that in the extracellular milieu ranges from 4 to 40. Such values have been obtained by incubating the phagocytic cells with the agent, followed by rapid washing before measurement of the amount of drug associated with the cell pellet by HPLC, radioactive labelling, fluorimetry or microbiologically [10]. Macrophages take up aminoglycosides and penicillins by pinocytosis whereas PMN lack this mechanism and hence penetration is much lower in this cell type. Some drugs are actively taken up by PMN, e.g. clindamycin where glycolysis is essential (see Table IV).

Table IV

Classes of antibiotics recognized to concentrate within phagocytic cells

Antibiotic class	Degree of concentration
Aminoglycosides	+
Azalides	+++
Lincosamines	++
Macrolides	++
Quinolones	++
Rifampicin	+

Intracellular localization is also important since it is recognized that some drags are only passively retained within the cytosol of the PMN whereas others preferentially migrate to regions of low pH within the cell such as the lysosome. Clindamycin being a weak base follows this route and appears to alkalinize the lysosome [11] but this only accounts for about 5% of intracellular drug. In contrast, almost one third of the intracellular concentration of the fluoroquinolones or the macrolides is lysosome associated [12]. These differences in intracellular distribution may account for the differences in bioactivity measured intracellularly. In the case of clindamycin it is equivocal whether the drug potentiates the normal intracellular killing process. However,

with PMN obtained from patients with chronic granulomatous disease it clearly takes over from the cell's poor bactericidal action against *S. aureus.*

In contrast, proven intracellular bactericidal activity of macrolides versus *Legionella pneumophila* (see Table V) and *Mycobacterium avium-intracellulare* undoubtedly aids recovery from infections with these agents. The fluoroquinolones too reduce the intracellular survival of *S. aureus.*

Table V

Intracellular survival of L. pneumophila in human PMN in the presence or absence of clarithromycin and its 14OH metabolite

Drug treatment	Percent survival
None	25.9
+ 2×MIC clarithromycin	14.7
+ 4×MIC clarithromycin	18.4
+ 2×MIC 14 OH-clarithromycin	14.9
+ 4×MIC 14 OH-clarithromycin	19.3

This review has sought to find answers to the four questions posed at the outset. Most of the evidence hitherto has been obtained from experiments in vitro which may not represent the real situation as it exists in a patient with ongoing infection. In only a limited way can it be stated that antibiotics can act as biological response modifiers in vivo. Ex vivo studies using phagocytic cells or serum from patients who have received the investigational drug have been used in an attempt to improve our understanding. For example, peritoneal dialysis fluid from patients with stable end-stage renal disease failed to alter the phagocytic killing function of their peritoneal macrophages. However, treatment with clindamycin stimulated intracellular killing of *Staphylococcus epidermidis* [13].

More recently attention has been directed to the recognition of drugs with direct immunomodulatory activity. One such drug has been cefodizine, a methoxyamino-2-thiazolyl cephalosporin, which has been shown to enhance both the killing and oxidative responses of PMN to opsonised particulate stimuli (zymosan and Staph. aureus). These effects are not seen with non-opsonised particles or soluble stimuli. The immunomodulatory activities of this drug are summarised in Table VI.

However, it should be borne in mind that the immunostimulatory activities seen in chronic haemodialysis patients [14] have not been demonstrated in other patient groups. Azithromycin, an azalide known to be concentrated within PMN, is transported

Table VI

Some biological properties of cefodizime

Binds to phagocytic cells
Does not influence chemotaxis
Enhances phagocytic ingestion of bacteria
Has no effect on oxidative respiratory burst
Augments O_2-independent killing
Releases IL-1 and interferon from macrophages

thereby to sites of infection [15]. It is likely that the greater clinical efficacy recognized with this drug, at least in animal models of infection, can be attributed to such facilitated transport.

Antibiotic action at sub-MIC levels probably also takes place in vivo. Patients with pneumonia treated with oxacillin were shown to have abnormal thick-walled staphylococci in their sputum [16]. Whether the host is capable of responding preferentially to such drug-damaged bacteria is uncertain but at least from some animal experiments, skin lesions induced by *S. aureus* or *S. pyogenes* were markedly reduced in severity when clindamycin-exposed bacteria were inoculated in murine skin [17]. Suppression of expression of surface and/or soluble virulence factors by the drug might have been responsible for the differences and suggests a promising new role for such drugs in the treatment of infection.

REFERENCES

1. Ritts,R.E.: Antibiotics as biological response modifiers. J Antimicrob Chemother **26** Suppl C: 31 (1990).
2. Capodicasa,E., Scaxingi,L., Rosati,E. et al.: In vitro effects of teicoplanin, teicoplanin derivative MDL62211 and vancomycin on human polymorphonuclear cell function. J Antimicrob Chemother **37**, 619 (1991).
3. Shibl,A.M.: Effect of antibiotics on production of enzymes and toxins by micro-organisms. Rev Infect Dis **2**, 865 (1983).
4. Gemmell,C.G., Schmeling,D., Peterson,P.K. et al.: Potentiation of opsonisation and phagocytosis of *Streptococcus pyogenes* following growth in the presence of clindamycin. J Clin Invest **67**, 1249 (1981).
5. Gemmell,C.G., O'Dowd,A.: Regulation of protein A biosynthesis in *Staphylococcus aureus* by certain antibiotics: its effect on phagocytosis by leukocytes. J Antimicrob Chemother **12**, 587 (1983).
6. Lorian,V.: Some effects of subinhibitory concentrations of antibiotics on bacteria. Bull NY Acad Med **51**, 1046 (1975).

7. Veringa,E.M., Verhoef,J.: Influence of subinhibitory concentrations of clindamycin on opsonophagocytosis of *Staphylococcus aureus*: a protein A-dependent process. Antimicrob Ag Chemother **311**, 796 (1986).
8. Gemmell,C.G., Peterson,P.K., Schmeling,P., Quie,P.G.: Studies on the potentiation of phagocytosis of *Streptococcus pyogenes* by treatment with various antibiotics. Drugs Exptl Clin Res **8**, 235 (1982).
9. Raponi,G., Vreede,R.W., Rozenberg-Arska,M. et al.: The influence of subminimal inhibitory concentrations of netilmicin and ceftriaxone on the interaction of *Escherichia coli* with host defences. J Antimicrob Chemother **23**, 565 (1989).
10. van der Auwera,P., Matsumoto,T., Husson,M.: Intraphagocytic penetration of antibiotics. J Antimicrob Chemother **22**, 185 (1988).
11. Klempner,M.S., Styrt,B.: Clindamycin uptake by human neutrophils. J Infect Dis **144**, 472 (1981).
12. Carlier,M.B., Zeneburgh,A., Tulkens,P.M.: Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. J Antimicrob Chemother **20**, Suppl. B: 47 (1987).
13. de Fijter,C.W.H., Verburgh,H.A., Heezius,H.C.J.M. et al.: Effect of clindamycin on the intracellular bactericidal capacity of human peritoneal macrophages. J Antimicrob Chemother **26**, 523 (1990).
14. Labro,M.T., el Benna,J.: Comparison of cefodizime with various cephalosporins for their indirect effect on the human neutrophil oxidative burst in vitro. J Antimicrob Chemother **26**, Suppl. C: 49 (1990).
15. Gladue,R.P., Snider,M.E.: Intracellular accumulation of azithromycin by cultured human fibroblasts. Antimicrob Ag Chemother **34**, 1056 (1990).
16. Lorian,V., Waluschka,A., Kim,Y.: Abnormal morphology of bacteria in the sputum of patients treated with antibiotics. J Clin Microbiol **16**, 382 (1982).
17. Gemmell,C.G.: Effect of subinhibitory concentrations of antibiotics on experimental pyogenic infections in mice. In: Current Chemotherapy. Acad. Press, New York. 1978. pp. 512–514.

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IDENTIFICATION OF DIFFERENT CIRCULATING AUTOANTIBODIES IN PATIENTS WITH BULLOUS PEMPHIGOID AND PEMPHIGUS VULGARIS BY MEANS OF IMMUNOBLOTTING

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Immunoblotting studies with salt-split human epidermis were performed on sera from 15 patients with pemphigus vulgaris and 20 patients with bullous pemphigoid by using peroxidase-labelled antihuman IgG and IgA. Eleven sera of pemphigus vulgaris patients yielded a 130 kD specific protein band against the pemphigus vulgaris antigen. Most of the sera gave additional specific bands at 210 and 80 kD, with lower intensity. The sera of 4 patients, 3 of them were in clinical remission, did not yield specific bands. Seventeen sera of the 20 bullous pemphigoid patients yielded a 220–230 kD protein band against the major bullous pemphigoid antigen and 4 of them gave another specific band at 160–180 kD. Five sera produced multiple bands (220, 130, 100 and 75 kD). IgA antibodies against the major bullous pemphigoid antigen were demonstrated in 2 cases with IgA deposits along their basement membrane, as revealed by direct immunofluorescence. The immunoblot patterns correlated only weakly with the clinical findings in bullous pemphigoid. There was a considerable diversity in both clinical findings and immunoblot patterns.

The diseases of the pemphigus group and bullous pemphigoid (BP) are characterized by autoantibodies to antigens expressed by keratinocytes or constituents of the dermal–epidermal junction, which can be demonstrated by direct immunofluorescence (DIF).

BP is a subepidermal blistering disease characterized by circulating autoantibodies which react with antigens in the lamina lucida of the basement membrane zone (BMZ) [1, 2]. BP antigens, which are produced by basal keratinocytes, have been shown to be synthesized by mouse and human epidermal cells in vitro [3, 4]. It has been reported that a 220–240 kD polypeptide is detectable by immunoprecipitation from keratinocyte cultures and by Western immunoblotting of human skin extracts [5, 6]. This polypeptide is recognized by the majority of BP sera and appears to be the major BP antigen. Some

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50–70% of BP sera recognize the major BP antigen (230 kD), but a minority of BP sera recognize other low molecular weight bands, most commonly the 180 kD band, and less frequently bands at 200, 97 and 77 kD [6–8].

Pemphigus is an intraepidermal blistering disease group characterized by autoantibodies reactive with antigens located in the intercellular spaces or on the surface of epidermal cells [9]. There are two clinically and immunologically distinct forms. In pemphigus vulgaris (PV), autoantibodies are directed against a 130 kD antigen that forms a complex with a protein of desmosomal and adherent junctions, called plakoglobin (85 kD) [10]. In pemphigus foliaceus (PF), the autoantibodies are specific for a 160 kD antigen, called desmoglein I, that forms a similar complex with plakoglobin [11, 12].

The present study included the determination of the target antigen specificity of the sera of 20 patients with BP and 15 patients with PV by immunoblotting methods, and the results were correlated with the immunofluorescence picture. In addition, the Western blot patterns of the patients with BP were correlated with certain clinical data.

Materials and methods

BP patients. Twenty patients (11 males and 9 females; mean age at onset: 69 years) with BP, as indicated by clinical, histological and immunohistological criteria, were studied. All BP patients exhibited the specific clinical symptoms. The clinical findings were documented according to the following characteristics [13]: *1. Lesion morphology*: typical (bullae and/or urticated erythema) or atypical (other lesion morphology). *2. Severity*: mild disease (less than 10 lesions at one time or localized to a single body site); severe disease (more than 50 individual lesions, affecting three or more body sites); moderate disease (between the two extremes). *3. The presence of mucosal lesions.*

PV patients. Fifteen patients with PV (not PF and not pemphigus erythematosus); (9 males and 6 females; mean age at onset: 53 years) were studied. Four of them were in remission at the time of serum sampling.

Sera were collected from these patients and stored at –20 °C for immunoblotting studies. Normal human sera (negative controls), and BP and PV sera known to be positive from immunofluorescence studies (a gift from Dr. M. Meurer, Department of Dermatology, Munich) were included in each immunoblotting run.

Immunofluorescence studies. For direct immunofluorescence (DIF) the standard technique was used. Each specimen was sectioned into 5 μm sections on a cryostat. Specimens were stained for IgG, IgA, IgM and C3 by incubation of the sections with monospecific FITC-labelled antibodies (Human Ltd., Budapest). For indirect immunofluorescence (IIF) all serum samples were examined by using cryostat sections of rabbit oesophagus and/or normal human skin. Sera were initially diluted 1:32 in phosphate-buffered saline. The sections were incubated with the patient's serum and then examined for the presence of IgG by using a FITC-labelled conjugate.

Immunoblotting. Immunoblotting studies were performed according to Hashimoto et al. [14] with slight modifications. Normal human skin was obtained from patients undergoing plastic surgery. The skin pieces were incubated in a mixture of 1 M NaCl, 1 mM EDTA and 10 μM phenylmethylsulfonyl-fluoride (PMSF) at 4 °C for 72 h. The epidermis was then easily separated from

the dermis and homogenized in a solution containing 31.2 mM Tris-HCl, 2% SDS, 1 mM PMSF, 2 mM EDTA and 0.1 M dithiotreitol. After a 24 h incubation at 4 °C, the homogenizate was centrifuged at 15 000 *g* and the supernatants were stored at –70 °C until use.

The constituent proteins of the epidermal extracts were separated out by SDS–PAGE [15] (with 6% separating gel) and then transferred to nitrocellulose [16] before probing with the test sera. All sera were used to probe immunoblots at a dilution of 1:40. Specific binding by the sera was detected by using peroxidase-linked class-specific second antibodies (goat antihuman IgG and IgA; Sigma) and visualized with diaminobenzidine.

Results

The Western blot patterns of a group of BP patients are demonstrated in Fig. 1. All sera (including those of healthy controls) yielded a diffuse reactivity in the 30–60 kD range. The strong specific bands show the reaction between the 230 kD BP antigen and the antibodies of the patients' sera. In this group of patients, specific reactivity can be demonstrated only against the major BP antigen.

In another group of BP patients, multiple specific bands were seen on Western blotting (Fig. 2). Specific reactions were demonstrated against the minor BP antigen (180 kD) and some other epidermal components (220, 130, 100 and 75 kD).

Fig. 1. Blots of BP sera showing one dominant band at 230 kD with peroxidase-labelled antihuman IgG. Lane A: blot of the epidermal extract stained with Coomassie brilliant blue; Lane B: normal serum; Lanes C–F: positive BP sera; Lane G: negative BP serum; Lane H: migration of standard molecular weight markers

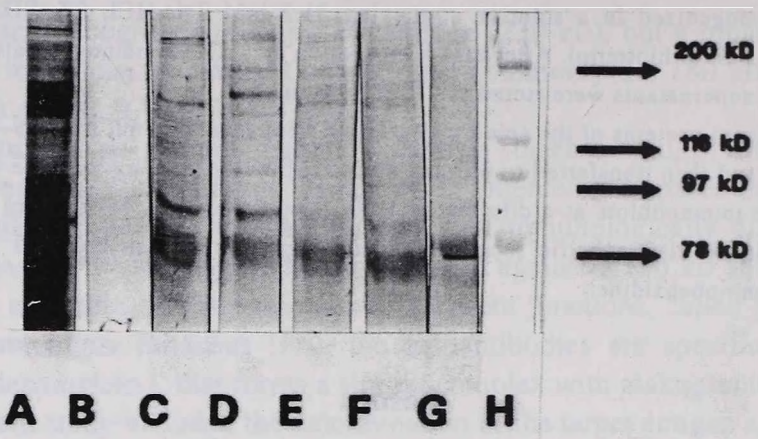

Fig. 2. Blots of BP sera showing multiple bands with peroxidase-labelled antihuman IgG. Lane A: blot of the epidermal extract stained with Coomassie brilliant blue; Lane B: normal serum; Lanes C–F: positive BP sera; Lane G: negative BP serum; Lane H: migration of standard molecular weight markers

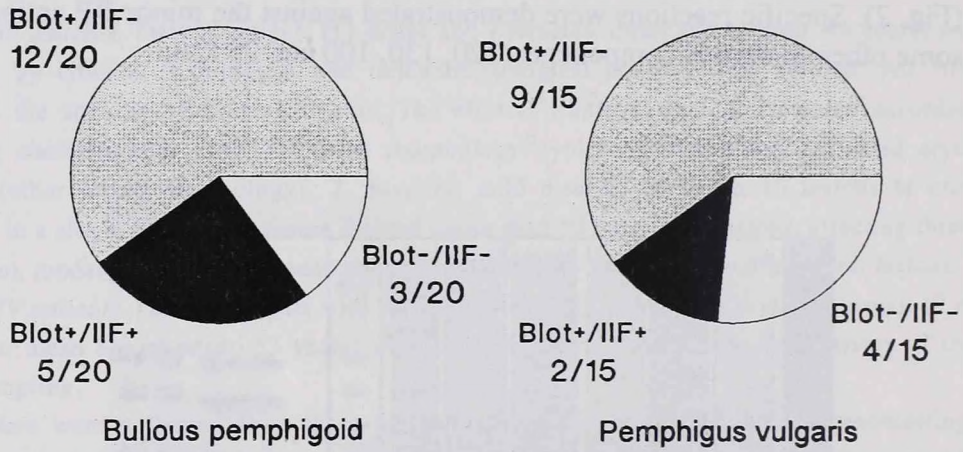

Fig. 3. Correlation between Western immunoblotting and IIF results

The Western blotting pictures were compared with the results of DIF and IIF investigations. In 16 BP patients, DIF revealed IgG and C3, and in 4 cases C3 and IgA deposition along the BMZ. In contrast, IIF positivity was found only in 5 patients. It is remarkable that, in spite of the negativity of the IIF examinations, specific BP reactions were revealed on Western blotting in 12 patients. Three cases were negative on both IIF and Western blotting (Fig. 3). The sera of 17 BP patients yielded a 230 kD protein band against the major BP antigen by on the use of peroxidase-labelled antihuman IgG, and 4 of them gave another specific band at 160–180 kD. Five sera produced multiple bands (220, 130, 100 and 75 kD). IgA antibodies against the major BP antigen were

demonstrated in 2 cases with IgA deposits along their basement membrane, as revealed by DIF.

The Western blotting patterns of the patients with BP were compared with certain clinical findings (Table I), but no specific correlation was found. The Western blot patterns of PV patients' sera are to be seen in Fig. 4. Among the PV patients, the sera of 11 yielded a specific 130 kD protein band against the PV antigen. Most of the sera gave additional specific bands at 220 and 80–90 kD, with lower intensity.

Among the PV patients, IIF positivity was found in only 2 cases. Specific reactions against PV antigen (130 kD) were revealed in 9 patients, in spite of the IIF negativity. Four cases proved negative by both methods (Fig. 3), and 3 of them were in clinical remission.

Discussion

IIF techniques are commonly used to demonstrate the presence of autoantibodies in the sera of patients with BP or PV. Western immunoblotting with its greater sensitivity provides a new possibility for the detection and specification of different antiepidermal antibodies in patients with bullous dermatoses.

Table I

Correlation between Western blotting patterns and clinical picture of patients with BP

Clinical findings	Single 230 kD (n=8)	Multiple bands (n=9)	Blot negative (n=3)
Age at onset (years)	60–82	48–82	60–72
Male/female	5/3	4/5	2/1
Lesion morphology	3 typical	5 typical	1 typical
	3 atypical	4 atypical	1 atypical
Severity	2 mild	6 moderate	2 moderate
	2 moderate	3 severe	
	2 severe		
Mucosal lesions	2	2	1

No clinical data available: 3 patients

Fig. 4. Blots of PV sera showing dominant bands at 130 kD and 85 kD with peroxidase-labelled antihuman IgG. Lane A: blot of the epidermal extract stained with Coomassie brilliant blue; Lane B: normal serum; Lanes C–F: positive PV sera; Lane G: negative PV serum; Lane H: migration of standard molecular weight markers

Fig. 5. Blots of BP sera; DIF revealed IgA deposition along the BMZ. IgA antibodies against the major BP antigen (230 kD) were demonstrated in 2 of them using peroxidase-linked class-specific second antibodies (goat antihuman IgA; Sigma) (Lanes D, E). Lane A: Blot of the epidermal extract stained with Coomassie brilliant blue; Lane B: normal serum; Lanes C and F: negative BP serum; Lane H: migration of standard molecular weight markers

In our study, Western immunoblotting proved to be a more sensitive assay than IIF for the identification of different antibodies. In 60% of our BP patients and 60% of our PV patients with negative IIF results, molecular weight specific antigen-antibody reactions were demonstrated by Western blotting on salt-split epidermis extracts. Our

results are in accordance with the findings of Bernard et al. [17], but there are some contradictionary data in the literature. In the study by Venning et al. [13], a number of BP sera produced negative immunoblots, despite having BMZ antibody detectable on IIF. Investigations of larger series of patients and standardization of different methods might yield an explanation for these discrepancies.

For PV patients, the results revealed that 11 sera yielded a specific 130 kD protein band against the PV antigen. Most of the sera gave additional specific bands at 210 and 80 kD, with lower intensity. The sera of 4 patients did not give any specific bands, and it is noteworthy that 3 of them were in clinical remission. This finding, together with experimental and clinical evidence, supports the hypothesis that pemphigus autoantibodies play a direct role in the pathogenesis of the disease [18, 19]. There was considerable diversity in the patients' immunoblotting patterns, especially in BP cases. The diffuse reactivity in all blots may correspond to cytokeratins [17, 20]. All patients with BP who displayed special reactivity against epidermal extracts yielded a sharp band at 230 kD. This has been considered to be the major BP antigen. However, 9 of them yielded multiple specific bands against different salt-split epidermis components (220, 180, 130, 100 and 75 kD). Although the number of our patients is limited, it seems that each patient yields an individual Western blot pattern besides the common reaction against the 230 kD protein component. A similar opinion has been reported by Shapiro et al. [21], who demonstrated that healthy individuals and patients with autoimmune diseases have antibodies, some tissue-specific and some not, which are present in a pattern that is unique to each individual.

In 4 patients with BP, DIF revealed IgA deposition besides C3 along the BMZ. IgA antibodies against the major BP antigen (230 kD) were demonstrated in 2 of them by means of immunoblotting (Fig. 5). It was interesting that IgG antibodies against the major BP antigen (230 kD) were also found on Western blots in these 4 patients, in spite of their negative IgG DIF results. Western immunoblotting provides a new possibility for the distinction of linear IgA dermatosis (LAD) and BP. LAD is characterized by circulating IgA antibodies against a 285 kD antigen of dermal extracts [22], but patients with LAD lack reactivity against the 230 kD BP epidermal antigen.

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REFERENCES

1. Holubar,K., Wolff,K., Konrad,K., Beutner,E.H.: Ultrastructural localization of immunoglobulins in bullous pemphigoid skin. J Invest Dermatol **64**, 220 (1975).
2. Schaumburg-Lever,G., Rule,A., Schmidt-Ullrich,B., Lever,W.F.: Ultrastructural localization of in vivo bound immunoglobulins in bullous pemphigoid: A preliminary report. J Invest Dermatol **64**, 47 (1975).

3. Woodley,D., Didierjean,L., Regnier,M., Saurat,J., Prunieras,M.: Bullous pemphigoid antigen synthesized in vitro by human epidermal cells. J Invest Dermatol **75**, 148 (1980).
4. Stanley,J.R., Hawley-Nelson,P., Yaar,P., Martin,S.R., Katz,S.I.: Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. J Invest Dermatol **82**, 456 (1982).
5. Domloge-Hultsch,N., Utecht,L., James,W., Yancey,K.B.: Autoantibodies from patients with localized and generalized bullous pemphigoid immunoprecipitate the same 230 kD keratinocyte antigen. Arch Dermatol **126**, 1337 (1990).
6. Labib,R.S., Anhalt,G.J., Patel,H.P., Mutasim,D.F., Diaz,L.A.: Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J Immunol **136**, 1231 (1986).
7. Ebihara,T., Hashimoto,T., Kudoh,J., Gamou,S., Shimizu,N., Nishikawa,T.: Detection of the 170-kDa bullous pemphigoid antigen by immunoprecipitation. J Invest Dermatol **100**, 176 (1993).
8. Robledo,M.A., Kim,S.-C., Korman,N.J., Stanley,J.R., Labib,R.S., Futamura,S., Anhalt,G.J.: Studies of the relationship of the 230-kD and 180-kD bullous pemphigoid antigens. J Invest Dermatol **94**, 793 (1990).
9. Shu,S.-Y., Beutner,E.H.: Isolation and characterization of antigens reactive with pemphigus antibodies. J Invest Dermatol **61**, 270 (1973).
10. Korman,N.J., Eyre,R.W., Klaus-Kovtun,V., Stanley,J.R.: Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. New Engl J Med **321**, 631 (1989).
11. Stanley,J.R., Koulu,I., Klaus-Kovtun,V., Steinberg,M.S.: A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human antibodies in pemphigus foliaceus. J Immunol **136**, 1227 (1986).
12. Eyre,R.W., Stanley,J.R.: Identification of pemphigus vulgaris antigen extracted from normal human epidermis and comparison with pemphigus foliaceus antigen. J Clin Invest **81**, 807 (1988).
13. Venning,V.A., Whitehead,P.H., Leigh,I.M., Allen,J., Wojnarowska,F.: The clinical expression of bullous pemphigoid is not determined by the specificity of the target antigen. Brit J Dermatol **125**, 561 (1991).
14. Hashimoto,T., Ogawa,M.M., Konohana,A., Nishikawa,T.: Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. J Invest Dermatol **94**, 327 (1990).
15. Laemmli,U.K.: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **277**, 680 (1970).
16. Towbin,H., Staehelin,T., Gordon,J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76**, 4350 (1979).
17. Bernard,P., Didierjean,L., Denis,F., Saurat,J.-H., Bonnetblanc,J.-M.: Heterogeneous bullous pemphigoid antibodies: Detection and characterization by immunoblotting when absent by indirect immunofluorescence. J Invest Dermatol **92**, 171 (1989).
18. Anhalt,G.J., LaSib,R.S., Voorhees,J.J., Beals,T.F., Diaz,L.A.: Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. New Engl J Med **306**, 1189 (1982).
19. David,M., Weissman-Katzenelson,V., Bon-Chetrit,A., Hazaz,B., Ingber,A., Sandbank,M.: The usefulness of immunofluorescent tests in pemphigus patients in clinical remission. Brit J Dermatol **120**, 391 (1989).

20. Krogh,H.K., Maeland,J.A., Tönder,O.: Indirect haemagglutination for demonstration of antibodies to stratum corneum of skin. Int Arch Allerg Appl Immunol **42**, 493 (1972).
21. Shapiro,P.E., McAllister,G., Lerner,E.A., Lerner,M.R.: Healthy individuals and patients with systemic lupus erythematosus have unique, person-specific spectra of antibodies detectable on immunoblots. Clin Immunol Immunopathol **59**, 129 (1991).
22. Wojnarowska,F., Whitehead,P., Leigh,I.M., Bhogal,B.S., Black,M.M.: Identification of the target antigen in chronic bullous disease of childhood and linear IgA dermatosis. Brit J Dermatol **124**, 157 (1991).

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ALTERNARIA MYCOTOXINS IN BLACK ROT LESION OF TOMATO FRUIT: CONDITIONS AND REGULATION OF THEIR PRODUCTION

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Alternaria represents the most colon decay organism of the post-harvest tomato fruit. The prevalent type of decay, black rot lesion, is caused by *Alternaria alternata* which may invade tomato tissue damaged by sun scald. The mainly natural mycotoxins produced in rotted tomato are alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TA). Whereas, altertoxin-I and -II (AT-I and -II), in addition to AOH , AME and TA were produced by local *A. alternata* in a synthetic medium. The optimum temperature for toxin production by *A. alternata* IMI 89344 was 28 °C for AOH and AME, 21 °C for TA and 14 °C for AT-I and -II. The growth and toxin were produced in a noticeable amount at 7 °C but drop at 35 °C. Significant inhibition in these toxins was attained at 500 ppm cinnamon oil in YES–Czapek's medium and in homogenate of tomato.

The spoilage of tomato by fungi is often accompanied by the production of mycotoxins which are a potential threat to human. Of these Alternaria moulds, which cause extensive deterioration results in economic loss to commercial marketers of these commodities. Also Alternaria spp. produce several toxic metabolites such as alternariol (AOH), alternariol monomethyl ether (AME), altertoxin-I (AT-I), altertoxin-II (AT-II) and tenuazonic acid (TA) which have been detected in a wide range of infected food [1–3]. These substances are toxic to animals and are responsible for human cell mutagenicity and carcinogenicity [4–6].

Consumption of tomato fruits containing these mycotoxins may represent a hazard to public health from either acute or chronic toxicity. The reported LD_{50}'s of the purified compounds are generally between 100 and 200 mg/kg for test animals [7]. The purpose of this investigation is to determine the predominant fungal species and mycotoxin that occur in infected tomato fruits. Also, an extensive study was carried to evaluate the effect

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of temperature and time on *Alternaria alternata* when grown in YES–Czapek's medium and tomato homogenate. Finally, I run an experiment for control of toxins by one of available essential oil which has flavour acceptability of foods.

Materials and methods

Infected samples. Naturally infected tomato fruits by black lesions were collected from commercial markets in Assiut city, Egypt.

Isolation and identification of fungi. The dilution and plating methods were used for isolation of the predominant fungi on glucose–Czapek's medium. The infected upper part and also the uninfected lower part of the same fruit were sampled. Five plates were used for each case and incubated at 28 °C for 1 week. The developing colonies were examined microscopically and identified.

Natural toxin. The infected fruits were sectioned into three portions, homogenized separately for 1 min in a Waring Blendor at top speed and adjusted to pH 2 by 2 N HCl. The chloroform was added to the homogenized tissue and rehomogenized for another 1 min. The organic phase containing mycotoxin was separated and concentrated for analysis.

Potentiality of *A. alternata* for toxin production. The *A. alternata* isolates were grown in YES–Czapek's agar medium at 21 °C for 10 days. The cultures were then homogenized as described above.

Conditions of toxin. *A. alternata* isolate IMI 89344 was grown on 50 ml YES–Czapek's medium composed of sucrose 30 g, yeast extract 5 g, NH_4NO_3 2 g, K_2HPO_4 1 g, $MgSO_4$ $7{\cdot}H_2O$ 0.5 g, KCl, 0.5 g in 1 litre distilled water. The fungus was also grown on 25 ml homogenate of tomato in Erlenmeyer flasks. The flasks were sterilized in autoclave, inoculated by one disc of 1 cm diameter, and incubated as stationary cultures at five temperatures ranging from 7 to 35 °C at 7-degree intervals. The mycelium growth and toxins production were determined periodically after days 5, 9, 14, 21 and 28. Triplicate flasks of each media were taken for each temperature.

Regulation of toxin. Three different concentrations 100, 250 and 500 ppm of cinnamon oil in ethanol were used for control of toxin production by *A. alternata* IMI 89344 in YES–Czapek's medium and in homogenate of tomato. The solvent was added into the control flasks. The flasks were inoculated and incubated at the optimum temperature for each toxin and analyzed at the adequate production time.

Analysis of toxin. TLC analysis was performed on silica gel plates with a fluorescence indicator (UV 6LC-12W-Vilber Lourmat, France) by using chloroform-acetone (93–7) as solvent system. AOH and AME were visualized under long wavelength UV light by their blue fluorescence at Rf 0.5 and 0.6, respectively. The spots of AME shifted to a greenish fluorescence after spraying with 50% sulfuric acid in ethanol and heating for 5 min at 100 °C, however the spots of AOH remained blue. AT-I and AT-II fluoresced yellow and orange at Rf 0.15 and 0.25, respectively. The spots were shifted to reddish fluorescence after spraying with ethanol–sulfuric acid mixture. TA was confirmed by TLC developed in toluene-ethyl acetate-formic acid (6:3:1). The Rf was 0.6 and identified by the formation of a deep reddish brown colour after spraying with 25% $FeCl_3$ in ethanol. The minimum detection was 0.1 µg for AOH, AME, AT-I and -II, and 0.7 µg for TA. Standard toxins were prepared by fermentation with *A. alternata* IMI 89344. The toxin quantified using Bausch–Lomb model 2000 fluorescence spectrophotometer in ethanol at 278–345 nm [8, 9].

Results and discussion

The predominant fungal flora and mycotoxins of rotted tomato fruits, the environmental factor influencing mycotoxin formation by *A. alternata* and were investigated in the present work.

Seven species belonging to four genera were isolated as the common fungal flora from the lesion and the remainder part of fruit on 1% glucose–Czapek's agar at 28 °C (Table I). The most prevalent fungi were *A. alternata*, followed by *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer*, *Aspergillus ochraceus*, *Aspergillus tamarii* and *Penicillium corylophilum*. Our results in agreement with Arnize [10] and Oladiran and Iwu [11] who mentioned that *A. solani*, *R. stolonifer*, *A. niger*, *A. flavus*, *Penicillium* sp. and *Fusarium* spp. are associated with the post-harvest disease of tomato fruits in Nigeria.

Table I

Incidence of predominant fungi in rotted tomato fruit

Fungal species	Black lesion part				Remainder part			
	C	NCI	I%	OR	C	NCI	I%	OR
Alternaria alternata (Fries) Keissler	4000	4	100	H	800	4	100	H
Aspergillus flavus Link	75	2	50	M	40	3	75	H
A. niger Van Tieghem	300	4	100	H	600	4	100	H
A. ochraceus Wilhelm	40	2	50	M	12	1	25	L
A. tamarii Kito	25	2	50	M	12	1	25	L
Penicillium corylophilum Dierckx	25	1	25	L	–	–	–	–
Rhizopus stolonifer (Ehrenb.) Lind	60	3	75	H	40	2	50	M
Total count of common (listed) and rare (unlisted) fungi	4600				1600			

C = Mean count of fungal colonies per gram fresh tomato
NCI = Number of isolation cases (out of 4 samples)
I% = percentage of incidence of species in samples
OR = Occurrence remarks: H = High occurrence (3–4 samples); M = Moderate occurrence (2 samples); L = Low occurrence (1 sample)

Table II

Natural toxin in rotted tomato fruits and potentiality of A. alternata for toxin production in YES–Czapek's agar medium

Toxin	Tomato 1			Tomato 2			Tomato 3			*A. alternata* strains		
	(µg/100 g fresh fruit)									(µg//20 g solid medium)		
	U	M	L	U	M	L	U	M	L	1	2	3
AOH	90	50	20	60	20	–	70	30	10	60	130	150
AME	–	–	–	80	–	–	30	–	–	–	100	120
AT-I	–	–	–	–	–	–	–	–	–	30	60	70
AT-II	–	–	–	–	–	–	–	–	–	30	40	50
TA	–	–	–	100	40	10	50	20	–	700	1000	1200

AOH = Alternariol; AME = Alternariol monomethyl ether; AT-I = Altertoxin-I; AT-II = Altertoxin-II; TA = Tenuazonic acid

U = Fruit part (Lesion); M = middle part (attachment to the lesion); L = Lower part (last third portion)

The natural mycotoxin in infected tomato fruits and potentiality of *A. alternata* strains for toxin production were given in Table II. The infected fruit was divided into three equal portions and the toxins were analyzed in each part. The three parts of fruit were polluted with AOH in different concentrations. The upper portion involved the lesion contains about 73 µg, the middle part followed the lesion contains about 33 µg and the last fruit part contains about 10 µg AOH per 100 g fresh weight. AME was detected in the lesion part only. TA was detected in small concentration in two lots of infected tomato, whereas AT-I and -II were not detected. The strains of *A. alternata* isolated from these lesions when grown on YES–Czapek's agar medium produced AOH, AME, TA in addition to AT-I and -II in reasonable concentrations. These results are in agreement with conclusion of Vinas et al. [3] who found that AOH and AME were produced in apples. Whereas, AT-I and TA production occurred only in YES medium by *A. tenuis* in addition to the AOH and AME. However Stinson et al. [1] found that TA was the main mycotoxin produced in *Alternaria*-infected tomatoes from commercial sources in New York.

Knowledge of the environmental factor influencing mycotoxin formation is important so that storage environments can be made unfavourable for fungal growth. Temperature plays one of the major environmental factor which affect the life of tomato fruits and their rate of deterioration by fungi. So, the results in Figs 1 and 2 show the effect of temperature and time on world isolate of *A. alternata* IMI 89344 (highly toxin producer) when cultured in liquid medium and homogenate of tomato.

Table III

Effect of cinnamon oil on toxins production by A. alternata IMI 89344 at optimum condition for each toxin

Conc.	Yes–Czapek's medium								Homogenate of tomato						
(ppm)	Temp. (°C)	Time (day)	Dry wt (mg/50 ml)	AOH	AME	AT-I	AT-II	TA	Temp. (°C)	Time (day)	AOH	AME	AT-I	AT-II	TA
					(µg/50 ml medium)							(µg/25 g medium)			
0	14	21	270	30	90	120	150	1000	14	9	60	60	60	40	1000
100			130*	150*	70	110	100*	400*			90*	120	30*	90*	800
250			90*	150*	60*	100	90*	200*			30*	45	15*	30	0*
500					No growth								No growth		
0	21	14	250	30	80	90	90	1200	21	9	60	100	30	30	1500
100		230	90*	80	60*	60*	800*				30*	40*	15*	15*	300*
250		110*	60*	60	30*	30*	600*				30	20*	10*	15*	150*
500		100*	45	50*	15*	20*	400*						No growth		
0	28	9	280	60	100	60	100	700	28	21	120	150	50	30	750
100			250	90	100	90*	100	550*			150	90*	35	30	300
250			20*	120*	65*	30*	25*	80*			30*	30*	20*	15*	0*
500					No growth								No growth		

* Means significant difference compared to the control at 5% level

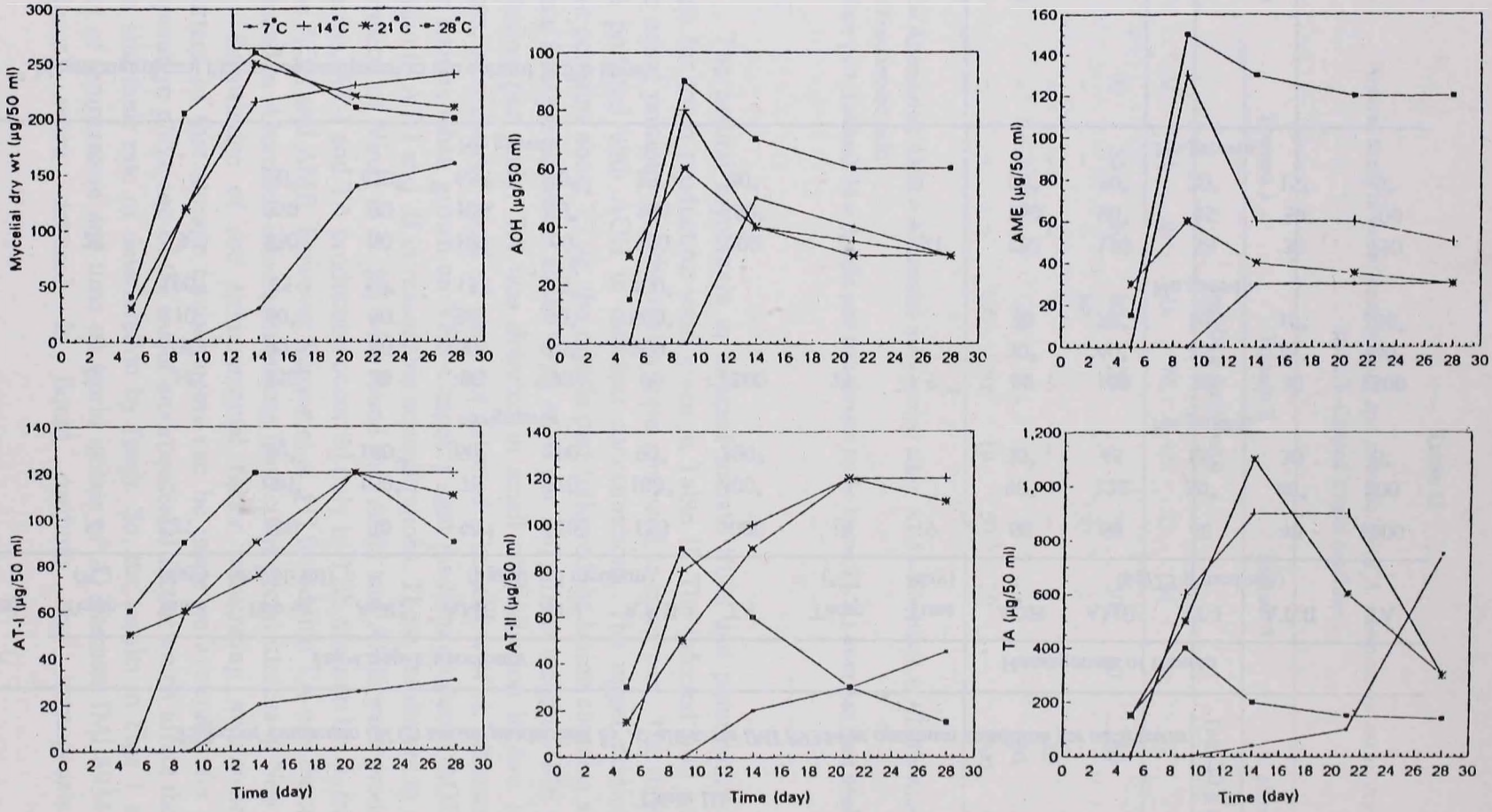

Fig. 1. Effect of time and temperature on growth of, and toxin production by *A. alternata* IMI 89344 cultured in liquid medium

No visual growth was detected on the 5th and 9th day at 7 °C, 5th day at 14 °C and all periods of incubation at 35 °C in both media. Five kinds of toxin viz: AOH, AME, AT-I and -II and TA were produced in both media. TA represents the main producing toxin. The maximum quantity of each one was favoured by a given temperature. The optimum AOH and AME were found at 28 °C on the 9th and 21st day in liquid and homogenate of tomato cultures, respectively. AT-I and -II were in high amounts detected at 14 °C on the 21st day in liquid medium and the 9th day in homogenate of tomato. However, TA was abundantly produced at 21 °C by the 14th day in liquid medium, and the 9th day in tomato culture. Also, our results indicated that *A. alternata* are capable of growing at 7 °C and of producing all previous toxins in reasonable amounts. For this reason it is often involved in spoilage during refrigerated storage of tomato fruits and tomato sauce. For this reason and because of these toxins represent a hazard to public health, I have therefore applied a food preservative, cinnamon oil, which was the most inhibitory to pathogenic yeasts, filamentous fungi and zearalenone production (Hasan [12] and unpublished data), for control *A. alternata* growth and toxin production (Table III). Cinnamon oil (100, 250 and 500 ppm) was tested in both liquid and tomato media inoculated with toxigenic *A. alternata* at the optimum temperature and time for each toxin. The growth and mycotoxins were significantly inhibited by 500 ppm at 21 °C in liquid medium and completely eliminated in the homogenate of tomato. No growth was recorded at 14 and 28 °C in both culture media. TA completely inhibited by 250 ppm in tomato at 14 and 28 °C. AT-I and -II were significantly decreased in both media by 250 ppm at all the optimum temperature and the proprietary time. Also, AOH and AME were significantly inhibited at this concentration in tomato medium, however, AOH was significantly increased in liquid medium. This result was agreed with Torres et al. [13] who found that AOH represents more resistance to chemical compounds than AME. In this field, cinnamon was one of the most oils used for controlling fungal growth of *Aspergillus* spp. as well as aflatoxin production [14, 15]. Some workers have identified a few ingredients of cinnamon oil like cinnamaldehyde (75%), eugenol (8%) and cinnamic acid.

Conclusion

The results running through this investigation indicate that the spoilage of tomato is accompanied by the production of *Alternaria* mycotoxins. Alternariol (AOH) represents the main toxin in the lesion and the far side of black rotted fruit. Moreover, the strains of *A. alternata* isolated from the lesions have potentiality for production of AME, AT-I and II, TA in addition to AOH in YES–Czapek's medium.

These toxins were produced at 7, 14, 21 and 28 °C during 4 weeks incubation in variable concentrations in both liquid and tomato homogenate media. To control the growth and toxin production in tomato fruits on the basis of temperature and time, the temperature degree must be maintained below 7 °C, and the storage period should not

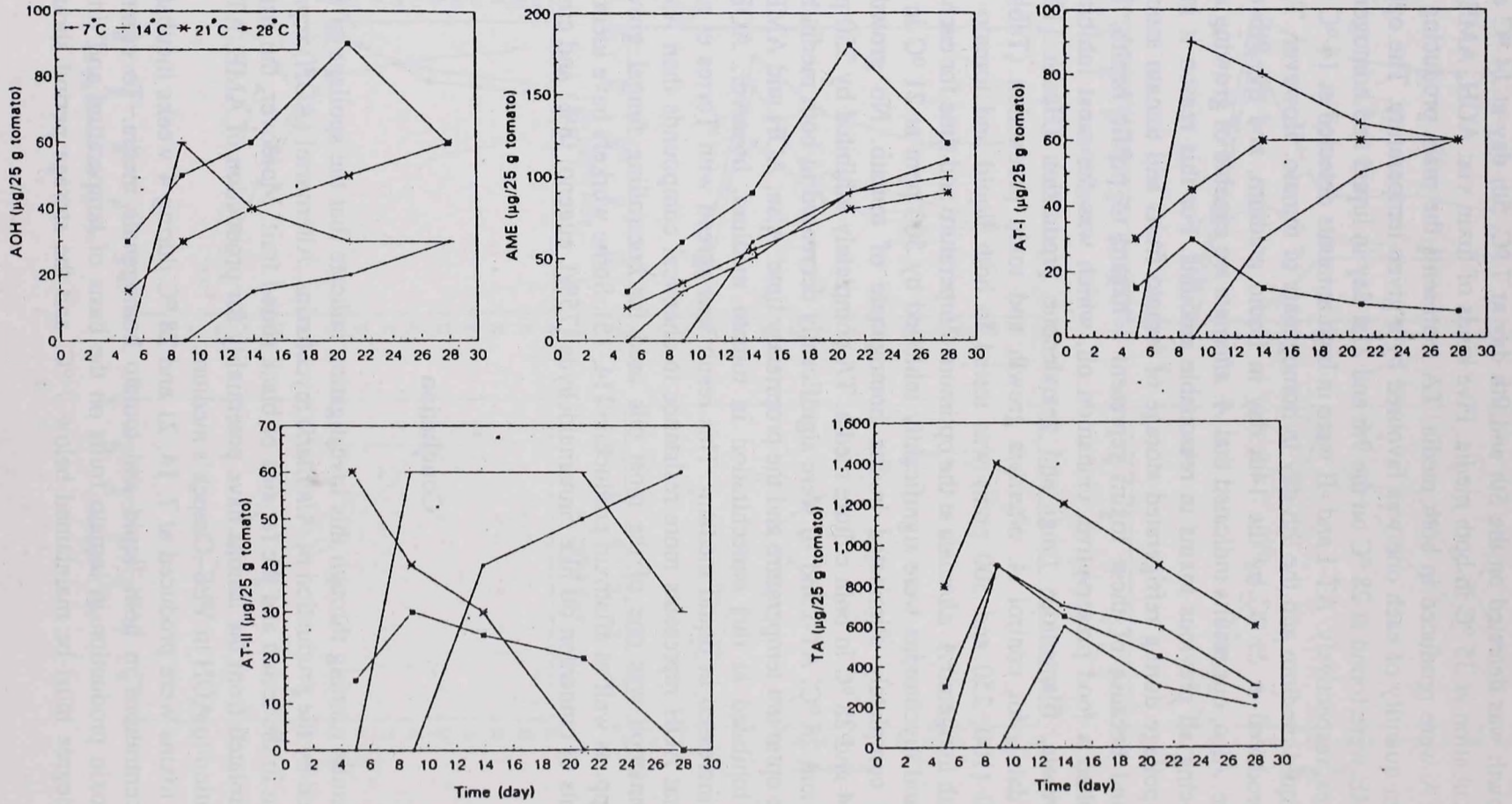

Fig. 2. . Effect of time and temperature on toxin production by *A. alternata* cultured in homogenate of tomato

exceed about 10 day. Also, these data indicate that cinnamon oil if used in sufficient amount (500 ppm) in certain manufactured products, such as tomato sauce, is fungistatic and provides therefore health protection against possible toxin development.

REFERENCES

1. Stinson,E.E., Osman,S.F., Heisler,E.G., Siciliano,J., Bills,D.D.: Mycotoxin production in whole tomatoes, apples, oranges and lemons. J Agric Food Chem **29**, 790 (1981).
2. Reiss,J.: Alternaria mycotoxins in grains and bread. Z Lebensm-Unter-Forschung **176**, 36 (1983).
3. Vinas,I., Bonet,J., Sanchis,V.: Incidence and mycotoxin production by Alternaria tenuis in decayed apples. Letters in Applied Microb **14**, 284 (1992).
4. Giambrone,J.J., Davis,N.D., Dienner,U.L.: Effect of tenuazonic acid on young chickens. Poultry Sci **57**, 1554 (1978).
5. Griffin,G.F., Chu,F.S.: Toxicity of the Alternaria metabolites alternariol, alternariol methyl ether, altenuene and tenuazonic acid in the chicken embryo assay. Applied and Environm Microb **46**, 1420 (1983).
6. Liu,G-T., Qian,Y-Z., Zhang,P., Dong,W-H., Q1,Y-M., Guo,H-T.: Etiological role of Alternaria alternata in human esophageal cancer. Chin Med J **105**, 394 (1992).
7. Sauer,D.B., Seitz,L.M., Burroughs,R., Mohr,H.E., West,J.L., Milleret,R.J., Anthony,H.D.: Toxicity of Alternaria metabolites found in weathered grain sorghum at harvest. J Agric Food Chem **26**, 1380 (1978).
8. Young,A.B., Davis,N.D., Diener,U.L.: Effect of temperature and moisture on tenuazonic acid production by Alternaria tenuissima. Phytopathology **70**, 607 (1980).
9. Coupland,K., Niehaus,W.G.: Stimulation of alternariol biosynthesis, by zinc and manganese ions. Exp Myco **11**, 60 (1987).
10. Arinze,A.E.: Post-harvest diseases of tomato fruits in Southern Nigeria. Fitopatol Brass **11**, 637 (1986).
11. Oladiran,A.O., Iwu,L.N.: Studies on the fungi associated with tomato fruit rots and effects of environment on storage. Mycopathologia **121**, 157 (1993).
12. Hasan,H.A.H.: Inhibition of mycoflora and zearalenone on rice by selected essential oils. Pak J Sci and Ind Res, in press (1994).
13. Torres,A., Chulze,S., Varsavasky,E., Rodriguez, M.: Alternaria metabolites in sunflower seeds: Incidence and effect of pesticides on their production. Mycopathologia **121**, 17 (1993).
14. Chatterjee,D.: An effective formulation for mould- and aflatoxin-free storage of corn. Letters in Applied Microb **9**, 25 (1989).
15. Patkar,K.L., Usha,C.M., Sherry,H.S., Paster,N., Lacey,J.: Effect of spice essential oils on growth and aflatoxin B1 production by Aspergillus flavus. Letters in Applied Microb **17**, 49 (1993).

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ACTIVITY OF NITROGEN FIXATION AND NITROGEN ASSIMILATION ENZYMES IN SOYBEAN PLANTS INOCULATED WITH *BRADYRHIZOBIUM JAPONICUM* STRAINS

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In studying the symbiotic associations of 8 *Bradyrhizobium japonicum* strains with two soybean varieties (NS-10 and NS-21), we determined the activity of nitrogen fixing (nitrogenase, NG) and nitrogen assimilation (glutamine synthetase, GS) and glutamate dehydrogenase (GDH) enzymes and the content of soluble proteins. The number of nodules was not in correlation with dry matter mass and nitrogen content. Dry matter mass and nitrogen content in the nodules of the soybean varieties in association with *Bradyrhizobium* strains 1, 1a, 2b, 2/1, 1003 and 8k turned out to be significantly higher than mass and nitrogen content of those in association with *B. japonicum* strains 17z and 2k. The results obtained indicate that the activity of the enzymes NG and GS in plants correlates with nitrogen fixation for both varieties on average, while the activity of GDH enzyme is conditioned by the specificity of variety and the *Bradyrhizobium japonicum* strains.

The suitability of *Bradyrhizobium japonicum* strain use for plant inoculation of soybean is determined by its ability to fix atmospheric nitrogen and its effect on the chemical composition (e.g. nitrogen content of plant) of the plant mass produced.

The differences between *B. japonicum* strains with respect to their effectiveness with different soybean genotypes were studied by several authors [1–4].

In the process of biologic nitrogen fixation both bacterial and plant genes are engaged [5]. The genes coded for nitrogenase–enzymic complex reducing N_2 into ammonia are limited to prokaryotic organisms but the energy essential for that process is provided by the host plant [6, 7].

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To increase nitrogen fixation, it is indispensable to select the strains of *B. japonicum* on the one hand, and improve the most important characters of the host plant which correlate with activities of N-fixing symbionts [8].

Several strains of *B. japonicum* and two soybean varieties were studied comparing different indicators to determine the quality of symbiotic association. The indicators are nitrogen fixation (dry matter mass and total nitrogen content of plants) on one hand, and biochemical indicators of the activity of the enzyme of nitrogen metabolism (nitrogenase, glutamine synthetase, glutamate dehydrogenase and the content of soluble proteins) on the other.

Materials and methods

Bacteria and inoculation. The bacteria used were *B. japonicum* strains 2/1, 1, 17z, 1a, 2b, 2K and 8K from the collection of the Department of Microbiology, of the Institute of Field and Vegetable Crops, University of Novi Sad (registrated in WDFF with acronym NSCNFN), and *B. japonicum* strain USDA 1003 obtained from Beltsville. The strains of *B. japonicum* were cultivated on liquid Demolon medium which contains: K_2HPO_4, 1 g; $K_2H_2PO_4$, 0.5 g; K_2SO_4, 0.25 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $FeSO_4$, 0.01 g; $MnSO_4$, 0.01 g; Na-borate (trace); Na-molybdate (trace); yeast extract, 2 g; distilled water, 1 l; 2 ml 3% alkaline solution bromthymolblue; pH=7.2. Incubation lasted for 96 h at 28 °C.

Plant material. In regard of the results of Mrkovački et al. [9], soybean varieties NS-10 and NS-21 were selected. These two soybean varieties had different capacity for nitrogen fixation as compared to most of the studied strains of *B. japonicum.* The plants were grown in a green house for 40 days in semi-sterile conditions with Jensen nutrient solution 1/4 strength [10].

Soybean seeds were sterilized with ethyl alcohol (95%) and 0.2% $HgCl_2$, washed with sterilized water and germinated in Petri dishes with 1% water agar for 4 days. After 4 days, the seedlings were transferred to pots (R=11 cm, H=9 cm). The pots were sterilized with 10% HCl, washed with sterilized water three times and filled with sterilized Jensen solution. Ground parts were protuded through the opening on the lid.

Four days old soybean seedlings were inoculated, separately, with 1 ml of liquid *B. japonicum* cultures in the logarithmic phase of growth (10^9 cells per ml). The number of cells was determined on solidified Demolon solution with the drop method.

The solution was added to the pots according to the following scheme: for the first 7 days the solution remained unchanged; after 7 days the solution was added to the pots with glass funnel to maintain the required quantity of the liquid; after 14 days the solution was discarded and changed with a new one twice a week up to the period of flowering, when the plants were manually harvested.

Non-inoculated plants were used for the control. Each treatment was done in six replications.

N assimilation enzymes activity. The activity of synthetized GS and NADH-dependent GDH was determined in an extract from leaves and nodules of 40 days old plants according to the methods described by Coombs and Hall [11]. Each measurement was performed in three replications.

GS activity was calculated from the produced hydroxamate content and expressed in μmole g^{-1} fresh weight h^{-1}. The GDH μmole activity was calculated from the consumption of NADH and expressed in μmole NADH g^{-1} fresh weight h^{-1}.

Nitrogenase activity. Nitrogen fixation activity of root nodules was assayed by the Acetylene Reduction Assay (ARA) of Hardy et al. [12] on a Hewlett Packard Gas Chromatograph 5840.

Nitrogenase activity was calculated from the concentration of the produced ethylene and was expressed in μmole C_2H_4 g^{-1} dry root h^{-1}.

The soluble proteins (mg/plant) were determined according to the method of Lowry et al. [13].

Statistical treatment. Standard errors of mean in the figures were calculated as Σ R $(n/x - x_i)$ 2/n (n–1). LSD (p=1%) statistical method was used for data processing.

Results

The results obtained are graphically presented separating the indicators in nodules and in the above-ground plant parts in the two used soybean varieties. The results of the variety NS-21 are presented in Fig. 1 (A, B, C and D) and of NS-10 in Fig. 2 (E, F, G and H).

According to the indicators of dry matter mass and nitrogen content in the nodules of NS-21, *B. japonicum* strains 1003, 2/1, 1, 1a, 2b and 8K achieved significantly higher mass (113–161 mg/plant) and nitrogen content (4.87–8.26 mg/plant) than *B. japonicum* strains 17z and 2K. The strain 17z had a nodule mass of 45 mg/plant and the strain 2K of 48 mg/plant. Plant specimens inoculated with *B. japonicum* strain 2K contained the lowest level of detectable fixed nitrogen (1.28 mg/plant) followed by 17z (2.05 mg/plant). Non-inoculated plants did not have nodules, so as to ensure their sterility. The number of nodules in the soybean variety NS-21 varied from 14 do 48 per plant. The largest number of nodules was formed by the plants inoculated with the *B. japonicum* strains 1003 (48 per plant) and 8k (44 per plant) and the lowest with 17z (14 per plant). The number of nodules is not in the correlation with dry matter mass and nitrogen content (Fig. 1C). However, the activity of GS, NG and protein content is in the correlation with the mass of dry matter and nitrogen content. The activity of GS enzyme ranged from 272 to 1019 in μmole g^{-1} h^{-1}. According to the activity of this enzyme, the strains can be arranged in this order: 2/1, 1003, 1, la, 2b, 8K and 2K. The strain 17z showed the lowest activity of this enzyme (Fig. 1A).

Different strains brought about different activity of NG enzyme in root nodules. In plants inoculated with strains *B. japonicum* 1003, 1, 2/1, 1a, 2b and 8K NG activity ranged from 4 to 12.5 μmole g^{-1} h^{-1}, while strains 17z and 2K had an activity of 0.8 and 2.3 μmole g^{-1} h^{-1} (Fig. 1A).

The activity of GDH enzyme in plant nodules is in opposite correlation with other indicators. *B. japonicum* strains 2K and 17z which are, according to other indicators, poorly efficient showed high activity of this enzyme: strain 2K achieved 48.50 and strain 17z 52.50 μmole g^{-1} h^{-1} (Fig. 1A).

The highest dry matter mass and nitrogen content in the above-ground plant parts were achieved with strains 1003, 1, 2/1 (Fig. 1D). These strains also gave rise to high values of GS and GDH activity and of the content of soluble proteins in the above ground plant part. Considering these indicators, other strains achieved different values (Fig. 1B).

In the above-ground plant inoculated with strain *B. japonicum* 17z the activity of GS enzyme was the lowest (94 μmole g^{-1} h^{-1}), whereas with the strain *B. japonicum* 2/1 it was the highest (192 μmole g^{-1} h^{-1}) (Fig. 1D).

According to the indicators obtained all studied strains of *B. japonicum* in symbiotic association with the variety NS-21 can be classified into two groups:

I. Efficient strains (1003, 2/1, 1, 1a, 2b and 8K);

II. Poorly efficient strains (2K and 17z).

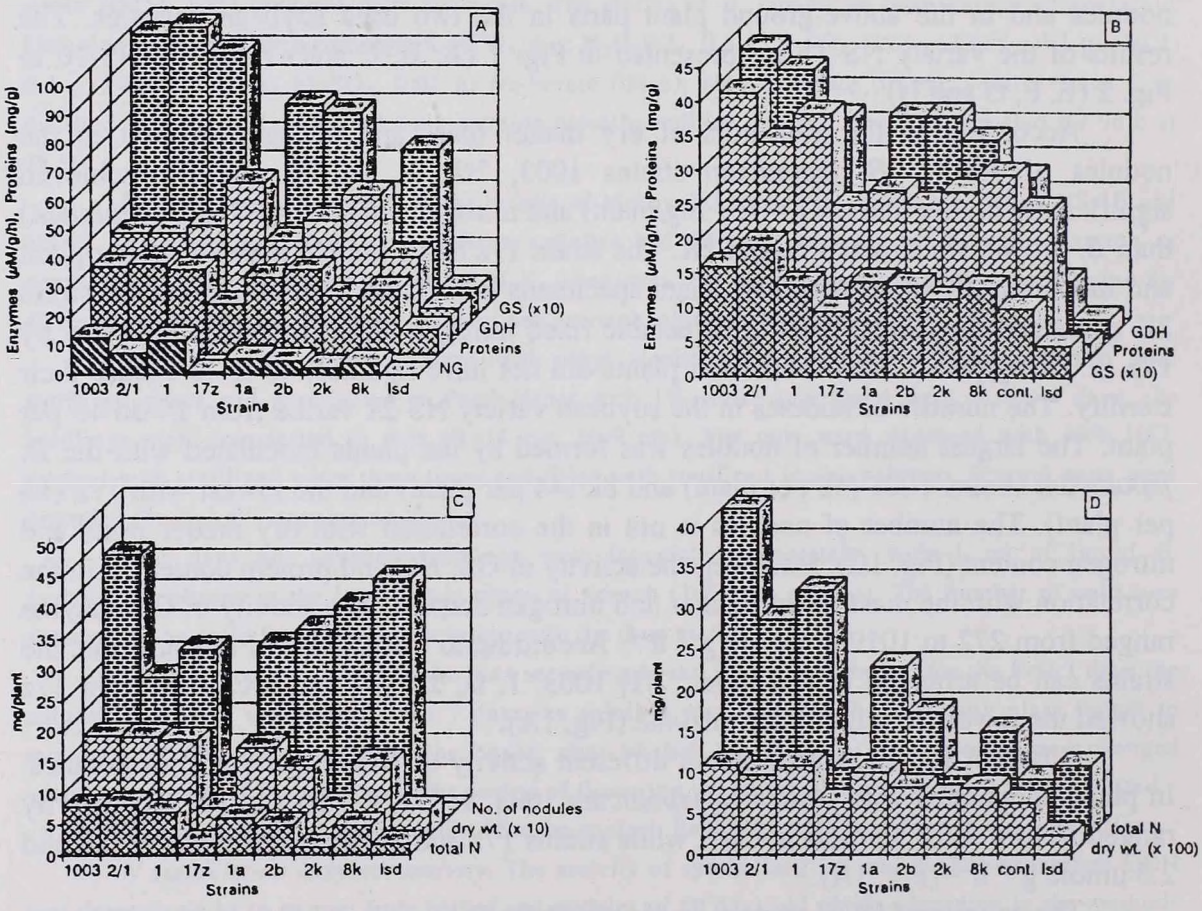

Fig. 1. Effect of inoculation with *B. japonicum* strains on the activity of enzymes (NG, GS, GDH), soluble proteins, number of nodules, dry weight and total N in nodules (A and C) and shoot (B and D) of soybean cultivar NS-21

With the studied *B. japonicum* strains we obtained higher mass and nitrogen content in nodules with the soybean variety NS-10. The strains 1003, 2/1 and 1 significantly differed from the other strains according to nitrogen content in nodules (from 6.87 to 8.94 mg/plant) while strain 17z achieved the lowest nodule mass (3.0 mg/plant) and the lowest nitrogen content (1.26 mg/plant). With these strains, the number of nodules formed ranged from 37 to 40 per plant. The largest number of nodules was observed in the root part of a plant inoculated with the *B. japonicum* strain 1 (60 nodules per plant) and the lowest with 17z (37 nodules per plant). The number of nodules of this soybean variety did not correlate with the mass and nitrogen content (Fig. 2G).

The highest activity of GS enzyme was achieved with *B. japonicum* strains (1a, 2b, 8K, 1003, 1 and 2/1) which achieved highest mass and nitrogen content in nodules of the plants. The GS activity ranged from 619 to 1047 μmole g^{-1} h^{-1}.

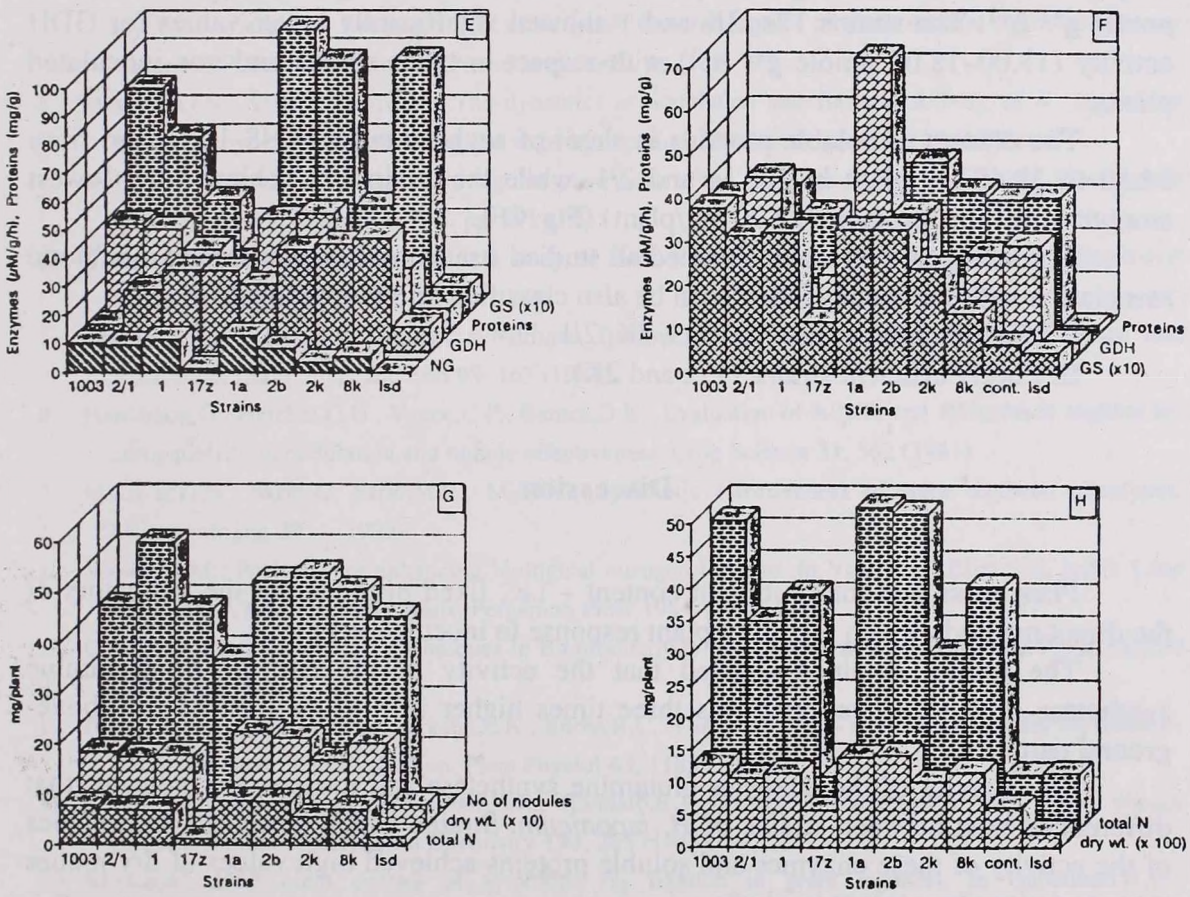

Fig. 2. Effect of inoculation with *B. japonicum* strains on the activity of enzymes (NG, GS, GDH), soluble proteins, number of nodules, dry weight and total N in nodules (E and G) and shoot (F and H) of soybean cultivar NS-10

The activity of GDH enzyme in the nodules of NS-10 soybean variety inoculated with the strains 8K, 2K and 2b was significantly higher than the activity in other associations (from 36.03 to 40.33 μmole g^{-1} h^{-1}).

According to the content of soluble proteins in plant nodules, the outstanding strains are 2b, 1a, 1003, 2/1 (from 36.30 to 70 mg/plant). However, strain 17z achieved the lowest protein content (12.86 mg/plant).

The activity of NG enzyme ranged from 5.6 to 12.8 μmole g^{-1} h^{-1}, and the order of *B. japonicum* strains was 1a, 1, 1003, 2/1, 2b, 8K, while the strains 17z and 2K showed the lowest value for this enzyme (1.2 and 3.4 μmole g^{-1} h^{-1}, respectively) (Fig. 2E).

The association of the soybean variety NS-10 with strains of *B. japonicum* 1a, 2b, 1003 achieved significantly higher mass (from 1282 to 1421 mg/plant) and nitrogen content (from 47.52 to 49.57 mg/plant) in the above-ground plant parts although all studied strains, except 17z, achieved higher mass and nitrogen content with respect to non-inoculated plants (Fig. 2H).

The strains 1003, 1a and 2b showed the highest activity of the enzyme GS and achieved the highest mass and nitrogen content in shoot. However, the activity of the enzyme GDH in the above-ground parts was different and ranged from 13.00 to 68.00 μmole g^{-1} h^{-1}. The strains 17z, 2K and 1 showed significantly lower values for GDH activity (13.00–18.00 μmole g^{-1} h^{-1}) with respect to other strains and non-inoculated plants.

The content of soluble proteins in shoot of soybean cultivar NS-10 ranged from 34.50 to 39.40 mg/plant in 2b, 1a and 2/1, while the strain 17z achieved the lowest amount of soluble proteins (22.90 mg/plant) (Fig. 2F).

According to the results obtained all studied strains of *B. japonicum* in symbiotic association with the variety NS-10 can be also classified into two groups:

I. Efficient strains (1a, 2b, 1003, 8K, 2/1, 1);

II. Poorly efficient strains (17z and 2K).

Discussion

Plant mass and total nitrogen content – i.e., fixed nitrogen are the indicators of the direct methods which represent plant response to inoculation [14].

The results obtained showed that the activity of the enzyme of glutamine synthetase of the two varieties was three times higher in nodules than in the above-ground part.

The activity of the enzymes glutamine synthetase and nitrogenase indicates that differences exist between strains of *B. japonicum*. Strains which presented high values of the activity of these enzymes and soluble proteins achieved high values of dry matter mass and nitrogen content in plants.

B. japonicum strains 17z and 2K achieved low values of dry matter mass and nitrogen content as well as the activity of the enzymes GS, NG and the content of

soluble proteins in both varieties. However, the activity of the enzyme GDH is conditioned by the specificity of variety and the strains of *B. japonicum* but is in opposite correlation with other indicators.

The results of the present study show that additionally to the indicators of nitrogen fixation, some biochemical indicators of the activity of the enzymes of glutamin synthetase, nitrogenase and the content of soluble proteins can also be used in the selection of *B. japonicum* strains.

REFERENCES

1. Kvien,C.S., Ham,G.E., Lambert,J.W.: Recovery of introduced *Rhizobium japonicum* strains by soybean genotypes. Agron J **73**, 900 (1981).
2. Sadowsky,M.J., Tully,R.E., Cregan,P.B., Keyser,H.H.: Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. Appl Environ Microbiol **53**, 2624 (1987).
3. Sarić,Z., Mrkovački,N., Milić,V.: N_2-fixation by *R. japonicum* strains during vegetation of different soybean varieties. In Bassam,N.E.L. et al. (eds): Genetic aspects of plant mineral nutrition. Cluwer Academic Publishers, Printed in Netherlands 1990. pp. 385–390.
4. Mrkovački,N., Sarić,Z., Milić,V.: The dynamics of nodulatien and fixation activity of *R. japonicum* strains in soybean varieties during the vegetation period. Mikrobiologija **26**, 123 (1990).
5. Holl,F.B., La Rue,T.A.: Genetics of legume plant hosts. Proc Ist Intern Symp N_2 fixation, Washington State University, Pullmann 1975. pp. 391–399.
6. Vincent,J.M.: Manual for the practical study of Root Nodule Bacteria, IBP Handbook 15, Blackwell Scientific Oxford, 1970.
7. Danson,S.K.A., Hera,C., Douka,C.: Nitrogen fixation in soybean as influenced by cultivar and *Rhizobium* strain. Plant and Soil **99**, 163 (1987).
8. Hardarson,G., Heichel,G.H., Vance,C.P., Barnes,D.K.: Evaluation of Alfalfa and *Rhizobium meliloti* for compatibility in nodulation and nodule effectiveness. Crop Science **21**, 562 (1981).
9. Mrkovački,N., Sarić,Z., Sarić,M.R., Milić,V.: Symbiotic effectiveness of some soybean genotypes. Mikrobiologija **29**, 1 (1992).
10. Vincent,J.M.: Potential for enhancing biological nitrogen fixation. In Vose,P.B., Blixt,S.G. (eds): Crop Breeding. A Contemporary Basis. Pergamon Press 1984. pp. 185–215.
11. Coombs,J., Hall,D.D. (eds): Techniques in Bioproductivity and Photosynthesis. Pergamon Press, Oxford 1982. pp. 118–141.
12. Hardy,R.W.F., Holsten,R.D., Jackson,E.K., Burns,R.C.: The Acetylene – Ethylene Assay for N_2 fixation: Laboratory and Field Evaluation. Plant Physiol **43**, 1185 (1968).
13. Lowry,O.H., Rosenbrough,N.J., Farr,L.A., Randalb,R.J.: Protein measurements with Folin phenol reagent. Journal of Biological Chemistry **193**, 265 (1951).
14. Bliss,F.A.: Host plant control of symbiotic N_2 fixation in grain legumes. In Gabelman,H.W., Longhman,B.C. (eds): Genetic aspects of plant mineral nutrition. Martinus Nijhoff Publishers, Dordrecht (Boston/Lancaster) 1987. pp. 479–493.

12TH CONGRESS

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PLENARY SESSION

G. KLEIN

EBV-B cell interactions: immortalization, rescue from apoptosis, tumorigenicity

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Translocation of c-myc to an immunoglobulin locus is a rate-limiting step in the genesis of three B-cell derived tumors: Burkitt lymphoma (BL) in humans, mouse plasmacytoma (MPC) and rat immunocytomas. Its consequences have been best analyzed in BL. They involve a non-immunological and an immunological component. The former acts by preventing the B cell from leaving the cycling compartment and entering the resting stage when programmed to do so. The latter acts by the down-regulation of certain HLA class I polymorphic specificities, adhesion molecules and EBV encoded proteins. According to our interpretation, the translocation fixes the BL cell in a phenotypic window that can be referred to as "a resting cell that is not resting".

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The linking of c-myc to immunoglobulin sequences in a B cell leads to constitutive myc expression. In spite of its switch to a "resting phenotype", the cell is therefore unable to leave the cycling compartment. Normally, EBV-carrying B-blasts face immune controls. They can only proliferate in immunodefectives, as in XLP or transplant lymphoma. Due to its "resting" rather than B-blast phenotype and the correlated defective expression of certain EBV, HLA and adhesion molecules, the BL cell is not opposed by immune response, however.

The down-regulation of EBNA 2–6 in the BL cell may be also considered in relation to viral latency. The exclusive expression of EBNA 1 in the BL cell reflects the adaptation of the virus to prolonged persistence in long lived resting B cells, an important if not exclusive reservoir of the latent virus.

Normal B cells that fail to be activated by appropriate mitogens or antigens within a limited period of time undergo apoptosis, as a rule. EBV may protect resting B cells. The role of the only virally encoded protein, EBNA 1, remains to be explored in this respect. EBV infected immunoblasts are protected from apoptosis by the LMP 1 induced elevation of bcl-2 expression. During the lytic cycle when cellular protein synthesis is switched off by the early vital proteins, one of the latter, a protein encoded by the bcl-2 like BHRF1 gene, itself a member of the viral early antigen complex, appears to overtake the protective function against untimely apoptosis.

About 60% of the BL lines carry mutated p53. Expression of wtp53 in such cells elicits apoptosis, probably as a result of "contradictory signals" by translocation-activated myc, versus growth-arresting p53. This can be count exacted by bcl-2 or by LMP 1 expression.

In freshly infected B cells, p53 is expressed at a high level. In the emerging LCLs, p53 is down-regulated. It may be presumed that only cells that can reduce contradictory signaling may escape apoptotic death.

HARLEY W. MONN

The *Escherichia coli* problem in pigs, cattle and people

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Escherichia coli strains carry different combinations of virulence attributes. Strains of the enterotoxigenic *E. coli* (CETEC) pathotype produce fimbriae (mediating intestinal colonization) and enterotoxins (mediating intestinal secretion), resulting in diarrhea. Another pathotype is verotoxigenic or Shiga-like toxin producing *E. coli* (VTEC or SLTEC). Shiga-like toxins are absorbed from the intestine, damage blood vessels, and cause systemic disease. Edema disease of swine and haemorrhagic colitis leading to the haemolytic uremic syndrome in man are diseases caused by SLTEC, and are the subjects of this report.

Edema disease is characterized by fimbriae mediated *E. coli* colonization of intestine and production of SLT-II_v resulting in systemic vascular damage and edema. We found that edema dim can be prevented by parenteral vaccination with a genetically engineered non-toxic variant of SLT-II_v [1]. Thus it may be practical to develop vaccines to control edema disease by antitoxic immunity. These result lead to the suggestion that it would also be possible to develop SLT based vaccines to protect people against SLT mediated disease.

Human outbreaks of haemorrhagic colitis and renal damage have been traced to consumption of improperly cooked ground beef contaminated with SLTEC of serotype O157:H7. Clinically healthy cattle are a potential source of contamination by O157:H7 SLTEC because they can carry the organism in their intestines. We found that nearly all of the *E. coli* O157:H7 isolates collected from U.S. dairy heifers in a national survey contained virulence genes characteristic of human pathogens (SLT, Attaching and Effacing Activity, EHEC Plasmid) [2]. However non-O157 *E. coli* that carried these virulence genes were more prevalent among the heifers than was O157:H7.

Experiments on the carrier/shedder state of O157:H7 SLTEC in cattle [3] gave the following results: (1) Fecal shedding of the agent varied widely among animals of the same age but persisted longer in calves than in adults. (2) *E. coli* O157:H7 did not spread from the intestine to other organs. (3) Recovered animals could be reinfected by the same strain of *E. coli* O157:H7. (4) The infectious dose for adult cattle ($>=10^7$) was higher than that calculated for people. (5) Most infected cattle remained clinically normal, and neither vascular nor intestinal lesions were detected in those that wife necropsied.

[1] Bosworth,B.T. et al.: Vaccination with an enzymatic mutant of Shiga-like toxin II variant protects against edema disease. In preperation. [2] Cray,W.H. et al.: Virulence attributes of *E. coli* from dairy heifer feces. Submitted for publication. [3] Cray,W.H. et al.: Applied and Environ Microbiol 61: 1586–1590 (1995).

J. HACKER

Microbial pathogenicity factors as parts of global regulatory networks

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Numerous prokaryotic genes are not expressed constitutively but rather respond to environmental conditions or internal signals. Thus, metabolic properties, structures of the coil wall and also pathogenicity factors are regulated by environmental signals via certain repressor and activator molecules. As shown for a number of serious pathogens, such as *Vibrio cholerae*, *Bordetella pertussis*, *Staphylococcus aureus*, *Salmonella typhimurium* and others, the expression of virulence factors is organized in so-called regulatory networks. We have been investigating the regulation of virulence factors

produced by pathogenic *Escherichia coli* strains. *E. coli*, which is a normal inhabitant of the gut of humans and animals, is also capable of causing infections of the intestine (intestinal *E. coli*) as well as infections outside the intestine (extraintestinal infections), such as of the urinary tract and the brain. Adherence factors (P-fimbriae, S-fimbriae, Type I-fimbriae), toxins (α-hemolysin, cytotoxic necrotising factor-CNF), serum resistance, iron uptake systems and other factors contribute to the pathogenicity of extraintestinal *E. coli.*

We were able to show tint some of the genetic determinants encoding these factors are located on large chromosomal regions, termed "pathogenicity islands" (Pais). These pathogenicity islands also include genes encoding regulatory factors. In addition, tRNA loci may be part of those pathogenicity islands. We have analyzed the structure and function of pathogenicity island of an *uropathogenic E. coli* isolate, termed 536. While one of the two Pais of strain 536 comprises to selenocysteine-specific tRNA gene *selC*, the gene *leu*X is part of a second Pais. It was demonstrated that the tRNA gene, *leu*X, which codes for a minor leucine specific tRNA, is involved in the regulation of a number of virulence associated genes. Thus, we were able to show that the expression of Type I-fimbriae, flagella, enterobactin and serum resistance highly depends on the presence of the tRNA gene *leu*X. In addition, an intact *leu*X gene is necessary for full in vivo virulence of pathogenic *E. coli.* There are indications that the product of the *leu*X gene regulates the expression of virulence associated genes on the translational level. Our data clearly illustrate the complexity of bacterial pathogenicity and the interdependence between pathogenicity factors and products involved in metabolic properties.

C. G. GEMMELL

Aspects of the antibiotic–phagocytic cell–bacterium interaction

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Recovery from infection in the immunocompetent host normally involves the conjoint activities of file hoses reticulo-endothelial system and the administration of an appropriate antibiotics). It is now clear that important interactions take place between certain antibiotics and the hoses phagocytic cells which may be direct or indirect. For the former, several antibiotics are capable of directly modulating several cellular activities such as chemotaxis, chemiluminescence and phagocytosis. We can include some of the cephalosporins and macrolides in this category. It is also known that some antibiotics are actively concentrated within the phagocytic cells; some to the cytoplasm and some to the phagosomes. By so doing, these antibiotics (clindamycin, the macrolides, the quinolones and rifampicin) display bioactivity within the phagocyte and thereby enhances the phagocyte's killing capacity. Thirdly, a number of antibiotics can indirectly affect the phagocytic ingestion and killing of pathogenic bacteria by altering their expression of

cell wall and soluble virulence factors. Several examples exist in which the presence of polysaccharide capsule or cell wall protein is reduced by growth in the presence of sub-growth inhibitory antibiotics such as clindamycin, erythromycin, carumonam and spectinomycin. These and other drugs can also inhibit the expression of several bacterial toxins.

In each of these ways, it is possible for the outcome of the host-parasite relationship to be affected by concomitant antibiotic therapy. However, most of the investigations have been in vitro but a number of examples exist in which phagocytic cell function in vivo has been similarly modulated. Such immunomodulation will be especially important when considering treatment of the immuno-compromised patient.

D. KARASSZON

Pasteur's place, role and importance in the history of philosophy

Societas Hungarica Historiae Artis Medicinae, Budapest, Hungary

From the point of view of the history of Philosophy, we should regard the spontaneous generation and the false doctrines of the etiology and spread of contagious diseases as especially important. For centuries they determined the world concept and word view of humanity, so those of Philosophy, too.

After much fantastic, speculative, moral philosophical ideas, a new period has been opened up by the profound changes operated by the scientific reasoning and irrefutable arguments of Pasteur, even though his name is not included in the history of Philosophy. The 100-year anniversary of his death offers itself as a good opportunity to fill this gap, and we consider the 12th Congress of the Hungarian Society for Microbiology to be an appropriate platform to assign a worthy place to Pasteur within the history of Philosophy who deserved this by virtue of his role played in the development of our modern world concept and world view in elaborating on their scientific basis.

GY. BERENCSI[1], E. FARKAS[1], L. GERGELY[2] and J. MINÁROVITS[1]

The seventieth birthday of 'second generation' Hungarian virologists educated at the National Institute of Hygiene – congratulation and biographics

[1] B. Johan National Institute of Hygiene, Budapest and [2] Hungarian Society for Microbiology, Budapest, Hungary

We have the pleasure to announce that five of our good friends, once virologist colleagues and coworkers of one of us (Dr. E. F.) at the National Institute of Hygiene, Budapest celebrated their 70th birthdays between January and June, 1995.

M. Simon, and *I. Hollós* both have retained virus research (diagnostic virology and hepatitis research, respectively) as main occupation, though – formally – *Simon* retired years ago and *Hollós* will do so in this year. *I. Dömök*, too, remained an enthusiastic virologists, though, recently as Deputy General Director of the National Institute of Hygiene, has little, if any, time for working at the bench of the virus laboratory, in which he was so busy and successful for many years, partly in close collaboration with WHO. *A. Koch's* interest turned from medical virology to virion morphology and membrane biology a few decades ago. Besides being active in research he has educated young people in an excellent scientific spirit. Unlike the above jubilees, all doctors of medicine, *D. Karasszon* is doctor of veterinary medicine. He worked in the Department of Viral Vaccines (directed by *Dr. F. Fornosi*) is an expert in histopathology of viral diseases, and in the last quarter of the century he has been highly productive in the history of medicine (human and veterinary).

Brief professional biographies of the jubilees

Dömök, István

Dr. Dömök began his career in the National Institute of Hygiene in 1950 immediately after graduation at the University Medical School, Budapest. He became an associate of the Chief of Department of Virology dealing with isolation and characterization of influenza virus strains eliciting epidemics in the early fifties. Later on he took an interest in enteroviruses testing their characteristics, aetiological role in different diseases and interactions in dual infections. In the late fifties he developed a laboratory for diagnostic virology within the Department, which served as a basis for organization of regional virus laboratories in the public health network. The Department of Virology is doing identification of enteroviruses even today with the collection of type-specific rabbit sera prepared and characterized by the team led by him in the early sixties. When the Sabin vaccine became available he contributed in its introduction in Hungary investigating its efficacy and safety. He provided experimental evidence that vaccine-associated poliomyelitis cases occur, and that poliovirus type 3 is responsible for the majority of such cases in Hungary.

A related topic was chosen for his studies carried out in Uganda between 1970 and 1973 as the Chief of Virus Study Team, Entebbe searching for the factors affecting adversely the efficacy of Sabin vaccine in warm climate countries. Since 1967 he has been a member of WHO Expert Advisory Panel on Virus Diseases and between 1972 and in 1986 he was appointed to contribute in the work of WHO Consultative Group on Poliomyelitis Vaccines. In connection with these memberships he participated in, and even organized on behalf of WHO, some international collaborations (*Dömök*, 1981; *Cockburn*, 1988).

Recently he initiated with his coworkers a complex programme for improvement of diagnostic, surveillance and preventive activities connected with viral hepatitis and took part actively in a policy for controlling HIV/AIDS epidemic in Hungary.

In the period between 1961 and 1970 he was associate editor of this periodical and from 1983 to 1988 he was the elected Secretary General of the Hungarian Society of Microbiology.

Between 1973 and 1988 he was the Chief of Department of Virology in this Institute, and from 1975 to 1984 in addition, he supervised the Division of Epidemiology and Microbiology. Since 1984 he is the Deputy General Director of the Institute.

He is a member of the AIDS Committee of the Ministry of Welfare, and in 1995 he was elected to become President of the Professional Committee in Public Health of the Hungarian Medical Chamber. One of his most important goal is to conserve and improve professional infrastructure of public health and preventive medicine in Hungary. We all wish good health, good partners, companions and good luck to proceed with this work as long as possible.

Hollós, Iván

Dr. Hollós was born in a middle class family. His father entered first the Medical School, however, he graduated at the Faculty of Economics. *Dr. Hollós* studied music and languages in the 'High School of Piarists'. He graduated in 1951 at the University Medical School, Budapest. He qualified as the winner of the students' competition in microbiology, and won the third place in the competition of preclinical subjects,and therefore, *Professor Ferenc Faragó* invited him to work at the Institute of Microbiology of the University. His research project was the development of a Shigella vaccine, as a member of the group of *Dr. Ernő Novák*. A vaccination accident in the early fifties provided opportunity to create a conceptional process against *Professor Faragó*, who has fallen victim to the political affair. The Institute of Microbiology became disintegrated, and in 1951 *Dr. Hollós* has entered the National Institute of Public Health.

He continued the work on the genus *Shigella* under the directorship of General Director *András Havas* as a member of the Department of Vaccine Control, led by *Dr. László Erdős* from 1951. He became familiar with the electron microscopic examination of bacteria, therefore, in 1953 the General Director decided to transfer him to the Department of Virology to work with the newly purchased Russian electron microscope.

The Head of the Department of Virology at that time was *Dr. Elek Farkas* teacher of most virologists in Hungary. *Dr. Gyula Takátsy* was the other leading personality in

the Department of Virology, who invented the spiral loop and the microplates, thus creating the first microtechnique of virological and serological research. Their help, and leadership enabled him to become concerned with the relatively new branch of science: virology.

His other challenge was the new electron microscope of the Institute, manufactured by a Russian factory on the basis of a US model (RCA). An ingenious technician *Árpád Barna* and the professionals of the Technical University, Budapest made it possible to rebuild the equipment. He prepared his Ph. D. Thesis ("Candidate Dissertation") on the replication of influenza viruses and their inhibition by the Francis inhibitor.

The global danger of smallpox epidemics induced him to introduce poxvirus diagnostic procedures upon the suggestion of *Dr. Farkas*. He had the opportunity to visit Moscow as a member of the Hungarian Smallpox Committee (1965). The hepatitis B virus research and diagnostic was introduced together with *Ernő Novák* (1969/70) in Hungary. Complement fixation test has been used first, and the ELISA microtechnique later on. Modern information on infectious hepatitis had been circulated in the Hungarian Medical Society by reviews published in 1965, 1978 and 1986.

The group led by him could quickly introduce the application of commercial kits when these became available for the detection of HBV-specific antigens and antibodies. Diagnostic virology of hepatitis A, B, C, D (delta) and E viruses is kept at the international level in Hungary by the laboratory led by him at the National Institute of Hygiene.

Karasszon, Dénes

His professional life has been committed to the comparative histopathology of diseases caused by neuroinfective viruses.

He was graduated at the University of Veterinary Medicine, Budapest. *Professor Gyula Sályi* invited him to work at the Institute of Pathology, and he successfully identified the pseudo-Negri bodies observed frequently in histological squash preparations prepared from horse brains, as artifacts. The material was found to be composed of lipofuscin, due to senescence of horses. Only a careless technique of the preparation resulted in their appearance on squash preparations stained by the Gerlach's method, which could be differentiated from true Negri bodies using appropriate staining procedures.

Professor István Környey, outstanding master of neuropathology of international reputation, accepted him as a member of the group examining the changes of the central nervous system in poliomyelitis of swine (Teschen's disease). Although he was not included among the authors of the published papers (1952) on the pathophysiology and clinicopathology of the disease, he gained a life-long paternal friendship and support of *Professor Környei* in addition to the training in techniques of neuro-histopathological research. His suggestion was to continue the work with the neurovirulence control of killed (Salk type) and attenuated (Sabin type) human poliomyelitis vaccines in monkeys. His recommendation supported his fellowship in the famous Collegium Hungaricum in

Vienna, when he could extend his neuro-histopathological knowledge in the Institute of Neurology.

After this fellowship he initiated an independent research project on the neurovirulence of polioviruses (1962) at the National Institute of Hygiene. Many interesting results have been obtained during this time. It has been observed that the encephalopathy of monkeys caused by *Shigella dysenteriae* interfered with the neurovirulence tests, and severely affected the viability of primary monkey kidney tissue cultures prepared from the animals (our late friend *Péter Ruzicska* contributed in these experiments).

It has been detected, that the type 3 attenuated vaccine strain produced histopathological changes in the brains of *Rhesus* monkeys resembling more to those of wild type viruses than alterations caused by the two other serotypes of the vaccine (1961).

Chromium-alum gallocyanine was shown to be the most suitable staining procedure for the routine histopathological control investigations of poliovirus vaccines (1965).

In connection with the neurovirulence tests the consequences of the trauma of inoculation were shown to be important, since on the monkeys occasionally paralyses, mental and motor disorders and disorientations have been observed due to mechanical damage (1968–1969).

In course of *Dr. Dömök's* research even lethal poliomyelitis has been observed associated with the Sabin type 3 strain. It has been proven that the increase of pathogenicity was not caused by mutations, but the selection of pathogenic variants from a mixed population of viruses. These results contributed to the decisions in connection with the Hungarian vaccination schedule.

Parallel to the neurovirulence tests inapparent virus infections have been examined together with *László Bodon* from 1963 using first immunofluorescence tests for the detection of the classical swine fever virus (hog cholera pestivirus) which was impossible earlier by serological methods. Neurohistopathological changes by an attenuated strain of the pseudorabies (Aujeszky herpesvirus) was examined, too (1965). The 'Budapest' strain of 'fixed' rabies virus has been examined and renamed as 'Hőgyes strain' after the Hungarian microbiologist who developed it. The neuropathological consequences of infection were compared in mice, hamsters, rabbits, sheep and monkeys (1968–1969). *Dr. Karasszon* clarified the role of hypothalamus in the pathogenesis of rabies (1970), and emphasized the importance of damages of the limbic system in the unusual tameness of rabid foxes (1971). The unaffectedness of visual pathways, but the damages in the extrapyramidal motor system by the fixed virus, and the spread of street-virus in the elements of vegetative nervous was published by him in the late seventies (1977).

In cooperation with *István Tariska* he examined subacute sclerotizing panencephalitis (SSPE) using immunofluorescence (1974). He reviewed vaccine associated complications, the role of immunofluorescence in the diagnosis of viral encephalitides, their public health and veterinary hygiene importance in addition to their geographical distribution (1974–1980).

Before he became the active member of Hungarian Society of the Medical History (he had been active as the Secretary General for several years, and then he was elected to be the President of HSMH) he had analysed a series of neurological diseases of animals (i.e. C avitaminosis, Encephalitozoon infection of rabbits, etc.) interfering with experimental work and industrial production of immune sera (1981–1984).

Members of the former working place and members of two Societies are celebrating his 70th birthday wishing successful further activities.

Koch, A. Sándor

The interest of *Professor Koch* for natural sciences is probably genetically determined. His grandfather studied organic chemistry in Germany, then he became the Head of Department at the University of Kolozsvár until the Peace Treaty of Trianon. The brother of his grandfather, *Antal Koch* had been the Member of the Hungarian Academy of Sciences and had earned the title 'Founder of Hungarian Geology'. His father (*Sándor Koch*) had been world-famous mineralogist, and the 100th anniversary of his birthday will be celebrated in 1996 at the International Congress of Mineralogy devoted to him in Budapest.

His maternal grandfather had been agronomist, and the grandmother helped *Ottó Hermann* (famous Hungarian zoologist) in the zoological preparatory work during his stay as guest in their house. His mother had been teacher of chemistry and natural science of medical interest, and the paternal home influenced him to choose the medical profession (by his late sister who had been organic chemist, and by the fantastic personality of his aunt's husband, *Arnold Winternitz* professor of lung surgery).

The lessons given by *Albert Szent-Györgyi, Győző Bruckner and Sándor Sik* remarkably impressed him even in 1943. He could continue graduate studies in 1945 as a released prisoner of war. The next year followed already under the leadership of his respected, outstanding Professor *György Iványovics* at the Institute of Microbiology, University Medical School, Szeged where the difficult post-war time was fleeting in progressive minded correct competition with outstanding colleagues *Ilona Béládi, Éva Cserey, Géza Dénes, István Horváth, István Koczka, Miklós Pintér, and Ervin Szöllősy.*

Under the stringent leadership of *Professor Iványovics* he began to work on lymphocytic choriomeningitis virus with *Pintér* and *Ábrahám* (the latter being now professor in Washington D.C.). In the fifties they cooperated with *Béládi* and *Cserey* working on Aujeszky virus. Here developed his commitment to virology, and they have realized only many years later the importance of being a member of the Iványovics School, which had been pulled down after 1953. Nevertheless, the group-members have given good account of themselves in their later professions.

Dr. Koch himself landed in the Human Institute of Vaccine Research and Production, Budapest where *István Joó* and *Richárd Backhausz* had been leading professional personalities. He himself manufactured smallpox and typhus vaccines, and developed the production of a vaccine against mumps together with *Aladár Benkő* upon the request of the army. At the National Institute of Hygiene the late, prominent *László Erdős* supported his professional struggles, and he learned much from discussions with the late, charming *Gyula Takátsy* and with *József Sinkovics* (being at present Professor

in Tampa, Florida) before his imprisonment due to a freigned action. In the meantime he developed the production of a freeze-dried typhus vaccine, which is still in use.

In the year of the National Uprising (1956) he visited the Institute of *Herdis von Magnus,* in Copenhagen together with *Elek Farkas* and *Gábor Veres* in order to learn the production technology of Salk's vaccine. The production of both Salk's and Sabin's vaccines was realized by him together with late *Péter Ruzicska*, and with his coworker for many years *Éva Csonka.* He had outstanding personal and professional connections to both *Albert B. Sabin*, and *Michail P. Chumakov* (both of them having their last farewell in 1994). The same time in 1960 he defended his Thesis on the mechanism of the virus-inactivating effect of formaldehyde.

The time of doing basic research arrived. He spent a year in the laboratory of *Professor André Lwoff* at the Institut Pasteur (1962/63), and continued the work on the initial interactions of poliovirus and host cell(s) together with *Emese György, Zoltán Nagy, Béla Lomniczi* and *Éva Csonka.* Then he spent four months in Isphahan, Iran as a WHO adviser (1968) and several months in Naples in the Institute led by *Professor Pietro Volpe* working on the replication of poliovirus. The necessity of basic research had not been realized by all leaders of the National Institute of Hygiene, therefore, he submitted his dissertation for the Academic Degree as the member of the 2nd Institute of Pathology of the Semmelweis University Medical School, Budapest.

Professor Harry Jellinek provided him the possibility to proceed with the independent theoretical research, since a few representatives of the basic research in Hungary did not realize the importance of theoretical studies in science, and the acknowledgement of his dissertation had been protracted and actively delayed until 1976.

Finally the support of *Professor István Mihály Szabó* resulted in his appointment as Associate Professor of the Institute of Microbiology at the Faculty of Natural Sciences of the L. Eötvös University, Budapest. This position provided him the possibility to teach general virology for biology students.

The eighties brought him the brightest period of theoretical research. He worked together with *Alfréd Rényi, Pál Juhász-Nagy* (died in 1994), *Elemér Lábos, István Lukovits* and *György Fehér.* In close cooperation with the Computer Center of the University of Münster (*R. Nienhaus*) a dynamic model of the cytoplasmic membrane of eukaryotes has been developed. He spent several years with the Origami theory of embryogenesis, i.e. with the modelling of three-dimensional structures 'folded' from the plain (flat) together with *Tibor Tarnai* and *László Orzó.*

He should have been retired in 1990, nevertheless, his graduate students, members of the Federation of Young Democrats, elected him to become Deputy-Mayor of the 2nd District of Budapest. He has retired from this position in 1994. His 70th birthday, the birthday of a descendant of predecessors interested in Natural Sciences, a student of *Professor Ivánovics* and *Lwoff* is celebrated today by many Hungarian professionals in virology, molecular biology and by representatives of natural sciences.

Simon, Miklós

Dr. Miklós Simon was born on the 18th of February 1925, exactly on the 100th anniversary of one of the most famous writer of romances in the Hungarian Literature, *Mór Jókai.* He became no writer fortunately, but one of the most talented experimental virologist of the country. His father was an official clerk at the tax office, and he did his best in order to provide the best education and training of his four children. The family lived in beautiful towns (Celldömölk, Zalaszentgrót, and Pápa) of the western part of Hungary. Then they moved to Szeged, where *Miklós Simon* finished the high school.

He graduated in May 1949 at the University Medical School, Szeged which is now named Albert Szent-Györgyi University Medical School. In those times he had not too much possibilities to think about his future. He had been recruited by the 1st of June into the Hungarian Army, and he was compelled to work as an army surgeon. Several years passed until he could enter the Scientific Health Research Institute of the ancient Army Medical Corps, Budapest, in 1955.

He started in bacteriology, and in 1956 passed the clinical laboratory examination usual in Hungary for medical graduates.

He was appointed in 1959 an independent scientific research fellow of the army in the Department of Virology of the National Institute of Hygiene (the Institute has been named recently after its first director '*Béla Johan*' M.D. who had been nominated by Rockefeller in 1924 founder of this Institute among several similar institutions all over Eastern Europe after the 1st World War). *Dr. Simon* is working continuously in the same department. More than 20 years as an Army officer up to 1980, and then as a part time senior virologist even today.

At the Department of Virology he met the persons celebrated in this script, and authors of this paper. *Dr. Simon* has emphasized several times, that he is a very lucky person, since he could work in a developing field of the medical profession. His position provided him the freedom not to fight for titles and higher salary. He could follow his experimenting temper using simple, but frequently brilliant ideas and methodology, since the virological methodology had been less sophisticated that time. When he defended the Academic Thesis this fact has been emphasized by *Professor Iványovics* acknowledged as teacher by *Dr. Simon*, too.

He worked with members of most virus groups being of medical importance in Hungary. He introduced numerous young professionals into the methodologies applied, improved and found efficient by him in clinical or diagnostic virology. He served for many years as member of the Presidium of the Hungarian Society for Microbiology and contributed regularly in its scientific meetings and congresses. Reviews and papers compiled by him for the Hungarian textbooks of virology cited below have been used by many physicians, and professionals in microbiology.

Dr. Simon is a very smart personality. He has acknowledged always openly the help, advice of his teachers (*Dr. E. Farkas* among the authors) and the contributions and help of his coauthors (*Drs. I. Dömök, S. Koch and M. Koller*). He published more than 60 papers during his career in a clinical diagnostic laboratory of virology. We are all very glad to have an opportunity to celebrate his birthday together with him.

References with contributions of the jubilees
Bakács, Tibor and Farkas, Elek (eds): Orvosi Virológia. (Medical Virology, in Hungarian), Medicina, Budapest, 1965; Cockburn, W. Chas: The work of WHO Consultative Group on Poliomyelitis Vaccines. Bull. WHO **66**, 143, 1988; Dömök, István.: Report on a WHO collaborative study. Markers of poliovirus strains isolated from cases temporally associated with the use of live poliovirus vaccine. J Biol Standard **9**, 163, 1981; Hargitai, Rezső and Kiss, Ákosné (eds): A gyermekbénulás elleni küzdelem (The challenge to poliomyelitis, in Hungarian). Literatura Medica, Budapest, 1994; Hollós, Iván, Kulcsár, Gizella, Ongrádi, József, Nász, István, László, Barnabás: Hepatitis vírusok, vírushepatitisek (Hepatitis viruses, viral hepatites, in Hungarian), Aesculap Medicina, Budapest, 1986; Lapis, Károly (ed.) Az orvosi virológia aktuális kérdései (Actual problems of the medical virology, in Hungarian Medicina, Budapest, 1978.

SYMPOSIUM ON HOST–PARASITE INTERACTIONS
I. BACTERIAL VIRULENCE FACTORS

L. EMŐDY

Alpha-haemolysin and virulence in *Escherichia coli*

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Escherichia coli alpha-haemolysin is the firstly recognized member of the so called repeat toxin (RTX) family. Alpha-haemolysin producing *Escherichia coli* strains frequently occur in the normal human intestinal flora without causing any harm to the host. They range, however, among the most commonly identified bacterial pathogens in extraintestinal infections.

While in strains of animal origin the genetic information for alpha-haemolysin is located on transmissible plasmids, the same function in human isolates is encoded by the bacterial chromosome. Alpha-haemolysin is secreted across both membranes of *Escherichia coli*, and in this way it exerts its effect also at body sites far from the localization of the infection. It is a pore forming cytotoxin with a wide range of target cells including erythrocytes, cells of the immune system, and mucosal epithelial cells.

Using isogenic haemolytic and non-haemolytic clone pairs we have shown the superiority in virulence of the haemolytic clones in various animal models. Haemolytic counterparts exhibited a special tissue tropism to the kidney in both urinary and haematogenous mouse models. The presence of intravasal haemolysis was indicated by intensive haemoglobinuria. The haemolytic clones elicited a rapid haemorrhagic lung edema when instilled intranasally to mice. The rapid death in the mouse models suggest a direct in vivo toxic effect for alpha-haemolysin.

The pathogenetic role of alpha-haemolysin in human infections has been supported by numerous statistical data. Here we present direct evidence for this role analysing cases of infections by alpha-haemolysin producing *Escherichia coli*. In these cases the pathogen has been identified as the causative agent of haemolytic-uraemic syndrome and acute haemolytic crisis. The appearance of specific anti-haemolytic antibodies, and their quantitative relationship with progression of the infectious process is demonstrated.

Finally, it is necessary to mention that the actual virulence of extraintestinal *Escherichia coli* strains appear, as the outcome of a concerted action of a set of bacterial factors acting in a given host. Alpha-haemolysin is only one of these factors but certainly a decisive one when present.

T. PÁL

The role of the lipopolysaccharide molecule in the pathogenesis of invasive enteric bacterial infections

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The lipopolysaccharide (LPS) antigen is one of the major constituents of the surface of Gram-negative bacteria. Lipid A, the endotoxin of the cell wall, is part of the LPS molecule. Although the role of the lipid A has been well known in inducing a set of pathological changes in the host, recently a broader role of the LPS molecule in the pathogenesis of some enteric infections has also been envisaged. These studies involved mutants and hybrid strains of *Salmonella Yersinia* and *Shigella* expressing LPS antigens in different backgrounds. It has been known that in some *Salmonella* strains the chemical composition of the O antigen-specific side chain of the LPS influences the interaction with the complement cascade, thus influencing the characteristics, and the outcome of the infections. Ra mutant of *Salmonella choleraesuis*, while retaining virulence when given i.v., was avirulent for mice when administered per os. This, taken together with the observation that the same Ra mutant was unable to infect polarised epithelial cells, suggests, that the LPS molecule may have a role in the interaction with the target cell, e.g. the intestinal epithelium. Whether this is a direct interaction involving the LPS molecule, or just a contribution of the LPS to the surface presentation of other interacting bacterial components, remains to be seen. Recently it was proposed for *Yersinia*, and for *Shigella flexneri* LPS that they are necessary to secure the proper membrane insertion and surface expression of proteins necessary for infecting, or in case of Shigella, for moving within host cells. The recent data concerning this broader role of the LPS antigen in the pathogenesis of invasive enteric infections will be reviewed.

B. NAGY[1], G. SZMOLÉNYI[1], Z. BITAY[2] and I. GADÓ[3]

Chances to control *Salmonella* colonization of poultry

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The significance of colonization as the first logical step in the pathogenesis of salmonellosis of chicken has been assessed in oral infection experiments using salmonellae most frequently occurring in chicks in Hungary (*S. enteritidis*, *S. typhimurium*, *S. hadar*, *S. infantis*). Virulence attributes have been searched for by immunofluorescence, electron microscopy, bacteriology and DNA analysis without any clearcut relationship between phenotypic or genotypic character and virulence. In trials using competitive exclusion products (Broilact, Aviguard) and different probiotics, none of them were consequently and significantly effective during colonization of experimental infection by the most *S. enteritidis* (PT1). Significance of the results in terms of host parasite relationship is discussed.

T. WADSTRÖM and P. ALELJUNG

Helicobacter pylori in pathogenesis of chronical gastritis and stomach cancer

Department of Medical Microbiology, Lund University, Sweden

Helicobacter pylori is a gastric pathogen in human and other *Helicobacter* species causes gastric infections in the stomach of various animal species. *H. pylori* cause gastritis in human volunteers and in specific animal models such as the young pig. Most recently *H. pylori* has also been proven to infect nude mice and Balb C mice, and to cause gastritis in these animal models. Virulence determinants of *H. pylori* assayed with gastric tissue damage and tissue inflammation can be grouped as follows:

(1) Urease and other enzymes with toxic metabolites (including phospholipase A_2 and C), (2) Vacuolating (VAC) toxin and other cytolytic toxins such as haemolysins; (3) Chemotactic factors recruiting inflammatory cells and (4) Gastric tissue adhesins. The complex gastric colonization process will be discussed with *H. pylori* binding to components of the gastric mucin layer and binding to blood group antigens and specific cell surface glycoproteins and glycolipids such as GM 3 ganglioside and glycosaminoglycans (or GAGs). How sucralphate and sulphated carbohydrate structures interfere with this colonization process will be discussed.

A model on how *H. pylori* may deplete growth factors and delay tissue repair in gastric and peptic ulcer disease will be described. Finally, various mechanisms how

chronic gastritis with *H. pylori* as a key primary carcinogen interplay with other carcinogen and co-carcinogens will be discussed. Various strategies to develop an oral immunoprophylaxis to prevent gastritis and cancer will be updated and summarized.

L. MAJOROS, E. PAPP-FALUSI, I. ANDIRKÓ and F. ROZGONYI

A comparative study on the virulence of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* strains

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The pathogenicity and virulence of the highly methicillin-resistant *Staphylococcus aureus* (MRSA) mutant 1190R and its congenic methicillin sensitive *S. aureus* (MSSA) counterpart 1190S selected from the heteroresistant wild type *S. aureus* 1190 isolated from the blood of a burn patient were examined in BALB/c mice by intraperitoneal challenge using graded suspensions containing different numbers of colony forming units (CFU) of each strain. On the basis of the lethality rates the MSSA strain proved to be more virulent ($LD_{50}=3.2\times10^7$ CFU/g body wt) than the MRSA mutant ($LD_{50}=10\times10^7$ CFU/g body wt). The survivors were sacrificed and examined on the tenth day of infection. There were seen much more splenomegaly and kidney abscess in the MRSA infected mice than those in the MSSA infected ones, while peritoneal abscesses were caused in a higher number by the MSSA strain. The persistence of the MRSA mutant in the kidneys, liver and spleen was significantly more frequent than that of the MSSA strain. These results indicate that both bacterial populations are virulent for mice, the difference in virulence between MRSA and MSSA strains is dose dependent, however, the mechanism of the pathogenesis of the MRSA and MSSA infections appears to be basically different.

SYMPOSIUM ON HOST–PARASITE INTERACTIONS
II. INFECTIONS IN IMMUNOCOMPROMISED HOSTS

GY. PRINZ

Infections in neutropenic patients

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Infections are major complications of drug induced neutropenia during treatment of cancer. The profound and prolonged neutropenia predispose to bacterial, fungal and viral infectious. The febrile episodes of neutropenic patients are mainly due to infectious. In recent years the number of documented infections decreased, the number of Gram-positive and fungal infections increased. The current preventive and therapeutic strategies – selective gut decontamination, empiric antibacterial therapy, antifungal prophylaxis, empiric antifungal therapy are widely used – but in many points are debated. The infections are due to barrier injuries e.g.: colonizations, resistance, mucositis due to irradiation and cytotoxic therapy and the use of intravascular devices. The pathomechanism of the development of infections is colonization, translocation, finally invasions by pathogenic microorganisms. The microbiological laboratory provides a continuos support in the selection of antiinfective strategies.

P. ANDERLIK

Bacterial translocation in healthy and immunocompromised host

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In certain bacteriological conditions, e.g. enhanced permeability of the intestinal mucosa, excess of some bacterial species in the gut of impaired immune function, members of the intestinal flora may permeate the intestinal wall and be translocated into mesenteric lymph nodes and other organs. The name of this process in medical literature is Bacterial Translocation (BT). The possible situations in which the BT appears, the consequences of BT, comparison of germfree and conventional host; septic state, and the quality and quantity of translocating bacteria will be discussed.

É. CZIRÓK[1], GY. PRINCZ[2], R. DÉNES[3] and P. REMÉNYI[3]

Predictive value of surveillance cultures in a bone marrow transplantation unit

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Bacterial infection is a frequent complication of marrow transplantation as patients require immunosuppression, which can predispose to serious infections. Accordingly, infection control for them is necessary. Surveillance culture may be useful especially during the absolute neutropenic periods, although in the literature the value of surveillance remains controversial. Our aim was to analyse results of surveillance cultures among bone marrow transplantation patients between January 1, 1992 and May 15, 1995. Among 26 transplanted patients 14 had febrile episodes in the course of which potentially pathogenic bacteria (PPB) could be isolated from blood cultures. In 49 febrile episodes of them 27 had positive blood cultures: 18 with one kind of bacteria (in 13 Gram-positive, in 2 Gram-negative or anaerobic bacteria and in one Candida) and 9 with two or three kinds of bacteria (in 3, two kinds of Gram-positive in 6, beside Gram-positive, also Gram negative–2, anaerobic bacteria–2 or Candida–2). In 16 episodes, beside blood culture, PPB could also be detected in one or the other of surveillance cultures. In three of them identical bacteria could be isolated from blood and from surveillance cultures. In two of these cases surveillance cultures were positive before bacteria could be detected from blood (*Enterococcus faecium* one week earlier from vaginal swab and urine, *Xanthomonas maltophilia* and *Streptococcus mitis* one day earlier from throat) in one case blood culture was positive before throat became positive. In 12 episodes blood cultures were negative while surveillance cultures yielded one or more PPB (coagulase negative staphylococci (CNS) in 5, CNS with enterobacteria–1, or with two kinds of Gram-negatives–2, or with *Candida*–1, enterobacteria–2, enterobacteria with Gram-positive–1). In one patient 10 kinds of microorganisms were isolated in different times of observation (enterobacteria-3, Gram-positive coccus–4, *Corynebacterium*–1, *Pseudomonas aeruginosa*–1, *Candida*–1) in 10 episodes blood culture was positive, surveillance cultures were negative in one case there was no surveillance culture. Blood and surveillance cultures were negative (8) or beside blood culture negativity there were no surveillance cultures (2). On the basis of the above results it can be stated that surveillance cultures can indicate the effectiveness of isolation and selective decontamination, and also help to study epidemiology of infections on the unit, however, to predict the invasive infection are of limited value.

J. ZALA[1], R. DÉNES[3], GY. PRINZ[4], É. CZIRÓK[2], M. TARPATAKI[5], Á. HERENDI[2] and CS. BOGNÁR[2]

Trichosporon species as emerging pathogens in immuncompromised patients; the first *Trichosporon capitatum* infection in Hungary

[1] Department of Mycology, [2] Department of Bacteriology, B. Johan National Institute of Hygiene, [3] National Institute of Haematology, Blood Transfusion and Immunology, [4] St. László Hospital for Infectious Diseases, [5] Budapest Institute, National Public Health Service, Budapest, Hungary

The rapidly expanding population of immunocompromised patients nowadays results the increased occurrence of fungal infections. Although most of these infections were due to *Candida* and *Aspergillus* species, several other fungi have recently emerged as clinically important pathogens.

Among them the species of the *Trichosporon* genus can be recognized as agents of invasive mycoses. Mainly two of these species namely *T. beigelii* (formerly *T. cutaneum*) and *T. capitatum* (formerly *Geotrichium capitatum*, now properly *Blastoschizomyces capitatus*) have important role to cause systemic infection. These fungi are members of the human skin-microflora and may colonize the respiratory, urinary or gastrointestinal tract of hospitalized patients *(T. capitatum* can be more often isolated from the sputum in Europe than in the other pan of the world).

Why can these fungi become more and more dangerous? 1. Trichosporons have unique mode of reproduction. They can bud, produce hyphae, blastoconidia and arthroconidia at the same time making possible a rapid spread of this fungus in the tissues. 2. The different strains of these species may have variable sensitivity to the available antimycotic drugs. 3. The phagocytic and killing effect of PMN and monocytes against the trichosporon cells has been found significantly less efficient than against the other yeast cells (*C. albicans*). This insufficient response may be an important factor in developing disease in patient having granulocytopenia. 4. On the one hand there are not enough laboratory facilities to support the clinical diagnosis yet, on the other hand so far these fungi have not been taken into consideration as a possible risk factor with a sufficient gravity.

The first case of a fatal disseminated trichosporonosis after bone marrow transplantation caused by *T. capitatum* in Hungary, drew our attention to the importance of the presence of this fungus in urine or in other sites of the body.

M. VARGA[1], JIURU XIE[2], E. HÜTTER[1] and GY. BERENCSI[1]

Laboratory diagnosis of human cytomegalovirus infection before and after organ transplantation

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Markers of human cytomegalovirus (HCMV) infection were examined in samples of 316 transplant recipients. The virus serological examinations are of essential importance during the pretransplantation screening of donors and recipients. ELISA reagents of 4 different manufacturers specific for HCMV IgG and IgM have been compared in order to reduce the number of false serological findings. The IgG-specific reagents were shown to give reliable results of comparable sensitivity, and specificity in the case of all products. Nevertheless, the IgM-specific reagents were found to give variable results. The reason for the heterogeneous results might be the limited number of monoclonal antibodies used by the manufacturers for the anchorage of HCMV antigens to the bottom of the microplates. The suggestion of the user diagnostic laboratory is to combine all available monoclonal antibodies of different specificity, in order to prevent false negative results caused by strain-specific molecular heterogeneity of the HCMV proteins of different patients.

About one-fourth of the transplant recipients had episodes of acute HCMV infection with or without clinical symptoms. Antibody detection was found to be of the lowest diagnostic value in the recognition of HCMV reactivation or primary infection. The detection of virus-coded immediately early (IE) antigens in the patient's granulocytes (HCMV antigenaemia test), was shown to be the most reliable procedure. The so-called "accelerated" virus isolation detected using monoclonal antibodies developed by Dr. Koller was shown to be a reliable procedure, nevertheless, only a small proportion of the samples was found to be positive in the tests, 3 to 4 days after the appearance of HCMV antigen carrying leukocytes.

The polymerase chain reaction (PCR) was used to detect HCMV specific DNA in the serum samples, and leukocytes of the patients. Primers specific for the IE region of the virus were used. On the basis of the semiquantitative results it can be concluded, that the HCMV particles are not simply taken up by phagocytosis, but the DNA is actively replicating within the leukocytes. In some cases when clinical symptoms of HCMV reactivation were observed, no specific HCMV IE amplimer was detected, but the appearance of aspecific heterogeneous amplimers indicated the reactivation of other lymphocyte-associated herpesviruses, i.e. HHV-6, or 7, Epstein–Barr virus or probably the recently discovered Kaposi's sarcoma herpesvirus (HHV-8). The verification of the PCR results using herpesvirus specific primers is continued.

BACTERIOLOGY

M. KONKOLY THEGE[1], I. PULAY[2] and M. ÁRKOSY[3]

Relevance of bacteriological results in infected necrotizing pancreatitis

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Infected necrosis is one of the most dangerous complications in acute pancreatitis. Infected necrotizing pancreatitis has a considerably worse prognosis than in case of sterility. Therefore bacteriological examinations have a great importance in diagnosis and therapeutic management of this complication. The 1st Department of Surgery, Semmelweis University Medical School, Budapest, Hungary is specialized in treating patients with acute pancreatitis. A retrospective study on 20 patients with proven necrotizing pancreatitis has shown that the bacteriological results gained by culturing postoperative drain samples were not relevant: 3.7 bacteria per patient, all but 3 cases polymicrobial, lack of predominance of *Enterobacteriaceae*. As a consequence of these findings, samples for bacteriological examinations have been taken at operation this year. Bacteriological results of 63 perioperative samples were analysed. Corresponding to the literary data two-third of the samples (42) proved sterile, the average number of bacteria per positive patient was 1.5, the infection was monomicrobial in the majority of the patients (13 out of 21 positive), enterobacteria predominated, the isolation frequency of anaerobes was low (5).

A. GEVORGJÁN[1], E. PAPP-FALUSI[1], I. ANDIRKÓ[1], I. ERDEI[2] and F. ROZGONYI[1]

Distribution and antibiotic resistance of aerobic bacteria isolated from wounds and bloods of burned patients

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The distribution according to genus and species as well as the susceptibility to antimicrobial agents were studied with 606 bacterial isolates cultured from 112 blood cultures and 385 wound swabs of burned patients. 38.4% of blood cultures and 95.4% swabs resulted in a positive culture. Of the 606 isolates 66.1% was Gram-positive and 33.9% was Gram-negative. Of the total isolates 30% was *Staphylococcus aureus*, 21.6% coagulase-negative *Staphylococcus* (CNS), 15.8% *Pseudomonas* species, 6.1% *Streptococcus* species, and 5.1% *Enterobacter* species. More than 50% of the total

Gram-negatives belonged to the *Enterobacteriaceae* indicating faecal contaminations of the wounds. Most of the *S. aureus* isolates were resistant to penicillin and piperacillin, while about 60% showed cross-resistance to oxacillin, cefazoline, cefaclor, cefoperazone and ceftriaxone. There was a lack of a complete cross-resistance to oxacillin (60%), imipenem (40%) and cefamandole (20%) in vitro, suggesting different mechanisms in maintaining resistance to these agents. The antibiotic resistance patterns of CNS isolates showed a great variety indicating the individual non-hospital sources of these isolates. A considerably high degree of cross-resistance was noticed to all macrolides. The most effective drugs were vancomycin, mupirocin, chloramphenicol and netilmicin against both *S. aureus* and CNS. Most of the *Pseudomonas* isolates were resistant to the old antipseudomonas agents such as azlocillin, mezlocillin, carbenicillin, chloramphenicol and neomycin, while the newer agents amikacin, ciprofloxacin, cefoperazone, ceftazidime and imipenem were effective in 50–90%.

P. SOTIROVA and Y. TYAGUNENKO

Resistance of clinical isolates *Acinetobacter baumannii* to different generations of beta-lactams

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Acinetobacter baumannii has emerged during the last two decades as one of the most significant opportunistic pathogens causing severe nosocomial infections and representing a very difficult therapeutic problem, due mainly to the progressive increase of its resistance to commonly used antimicrobial drugs. In the present communication, the resistance of hospital strains, isolated in the intensive care units of the Higher Medical Institute, Stara Zagora, during 1991–1992 to cephalothin (CF), cefazolin (CZ), cefamandole (MA), cefotiam (FOT), cefuroxime (CXM), ceftriaxone (CRO), ceftazidime (CAZ) and imipenem (IPM) was studied. The frequency of resistance to cephalosporins was very high in a generation dependent manner: resistant to the first generation (CF, CZ) were more than 90% of the isolates, to the second generation (MA, FOT, CXM) more than 80%; however resistance to the third generation was between 50% (CAZ) and 80% (CRO) of the isolates. Very indicative were the MICs geometric means (in mg/l) as follows: CF – 414, CZ – 335, MA – 184, CXM – 85, CRO – 32, CAZ – 13. The strains were most sensitive (more than 80%) to imipenem. The patterns of resistance to the cephalosporins fell into 3 predominant groups: (1) resistant to all cephalosporins tested – 42.5%, (2) resistant to all cephalosporins except ceftazidime – 25.9%, (3) sensitive to all cephalosporins used – 10.0%. Other patterns were exceptions. These data are a good illustration of the multiresistance of clinical isolates of *A. baumannii*. In addition, a considerable part of our isolates multiresistant to several penicillins, aminoglycosides and fluoroquinolones. The very high resistance of the strains tested could be also explained by the fact that they are isolates from intensive care units.

I. MAYER and E. NAGY

Postantibiotic effect of quinolones against three clinical isolates of *Pseudomonas* – A new method for checking growth curve

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The postantibiotic effect (PAE) of quinolones (ciprofloxacin, ofloxacin and pefloxacin) determined for three *Pseudomonas* strains isolated from severe nosocomial infections. The MICs of quinolones were different for the three strains (0.125–1 µg/ml for ciprofloxacin, 2–16 µg/ml for ofloxacin and 2–16 µg/ml for pefloxacin) and they were highly or moderately resistant to netilmycin, amikacin, ceftriaxon, cefoperazone and ceftazidime.

PAE was induced by 0.5×, 1×, 5× and 10× MIC of quinolones for 2 h and the antibiotic was then removed by washing. The growth curves of the cultures were followed for 15 h by continuos monitoring of the optical density in an ELISA reader (Anthos HT III) every half an hour.

Ciprofloxacin, ofloxacin and pefloxacin induced a PAE of 0.1–6.0 h, 0.1–12.8 h and 0.6–15.0 h for all three strains depending on the concentration of the antibiotics.

We found rather big difference in the values of PAE depending on the isolates, the resistance of isolates, the antibiotics and the concentration of the drug. The pefloxacin had longer PAE at 10× MIC concentration while the ciprofloxacin acted longer at 0.5× MIC drug concentration. Ofloxacin showed no PAE when it was used in sub-MIC or MIC concentration. The most sensitive strain had longer PAE against ciprofoxacin, but the most resistant isolate had the longest PAE when we studied pefloxacin.

K. ANTMANN and P. ANDERLIK

Pharmacological effect of simultaneous treatment with *Bordetella pertussis* vaccine and chlorpromazine in mouse experiments

Semmelweis University Medical School, Budapest, Hungary

In acute toxicity experiments we observed increased mortality on the first day following 75 mg/kg chlorpromazine (CPZ) treatment in mice pretreated with *Bordetella pertussis* vaccine (9×10^9 killed bacteria/mouse) compared to control animals treated with CPZ alone. Initially, the increased drug sensitivity observed after combined treatment was attributed to summation of the toxic effects. However, the cumulation of mortality did not cease on the following days; furthermore, an increase of bacterial translocation

(translocation index of P-CPZ group: 4,5) was observed on days 6 and 7, i.e. when the lymphocytosis, splenic hypertrophy and shrinkage of thymus – changes consequent to the vaccination – were at their maximum levels. On the basis of all these and on literary data it is supposed that the early cumulation of deaths after combined treatment may be in connection with an interaction between the two agent/and that the side-effects following vaccination of humans may be induced by undesirable pharmacological interactions.

I. TÓTH[1,2], M. TRINEI[1], P. FREESTONE[1], S. GRANT[1] and V. NORRIS[1]

Eukaryotic-like protein phosphorylation in *Escherichia coli*

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Eukaryotic-like protein kinase activities as defined by phosphorylation on serine, threonine and tyrosine residues are responsible for the majority of the protein phosphorylation observed in *Escherichia coli.* To identify these phosphoproteins, we have performed in vivo and in vitro experiments on laboratory strains and on a clinical isolate using ^{32}P. Proteins are purified by a variety of chromatographic techniques and separated on one- or two-dimensional SDS-PAGE. N-terminal sequences are then obtained and immunoblots carried out. In collaboration with numerous groups, we have obtained preliminary identifications of more than twenty phosphoproteins involved in many important aspects of cell physiology including transcription and translation (0591, S1 and S6), chromosome structure (H-NS and HU), virulence (FanB), metabolism (enolase, SucD) and stress (DnaK, Dps, UspA).

L. FODOR, Á. CSIVINCSIK and J. VARGA

Characterization of *Rhodococcus equi* strains isolated from foals

Department of Microbiology and Infectious Diseases, University of Veterinary Science, Budapest, Hungary

The cultural, biochemical and serological characteristics of 27 *Rhodococcus equi* strains isolated from pneumonic lungs of foals in Hungary were examined. All the strains produced catalase and urease, 4 of them were slightly positive in the oxidase test. None of the strains could split glucose, lactose, mannitol and sucrose. They were gelatinase and ONGP negative and apart from 5 strains they reduced nitrate. One strain

could grow at pH 4.5, 4 at pH 5.0, 12 at pH 5.5, while all the strains could be cultured at pH 8.5 and pH 9.0. Using the passive haemagglutination test they belonged to serotype 1 described by Prescot et al.

J. NACSA[1], L. NAGY[2] and J. MOLNÁR[3]

Effect of some metal complexes of tricyclic compounds on extrachromosomal genetic elements

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The effect of various metal complexes of trifluoperazone (TFP) and chlorpromazine (CPZ) in two bacterial systems was studied. The plasmid elimination and antibacterial effect of Cu, Ni, Pd complexes of TFP and CPZ-Pt complex were examined on *E. coli* F' lac culture. The effect of some derivatives did not exceed the potency of parent compounds. We have found that the antiplasmid action of TFP-Cu complex was four times more effective compared to the well-known antiplasmid promethazine. The minimal inhibitory concentration of this compound was significantly lower than that of TFP. The megacin induction as an indicator S.O.S. function was studied in presence of coordination complexes of two phenothiazines as well. *Bacillus megaterium* strain 216 was induced by mitomycin and Pd-, Cu-, Ni-TFP and CPZ-Pt complexes were given 10 min before or after induction. These metal coordination complexes reduced the megacine induction by mitomycin similarly to that of the parent compounds. The MIC values and the acute toxicity of the complexes were not higher than that of TFP and CPZ. Studying the DNA-compound interaction we measured the differences between the polarization degrees of DNA-compound and poly-A-compound systems.

We can conclude that the genotoxic effects are not involved in the biological action of the compounds, because the S.O.S. induction was reduced or cancelled in a dose dependent manner by the phenothiazine complexes.

B. HORKAY and N. LENDVAI

Tests regarding virulence factors of *Bordetella pertussis* strains

HUMAN Serum Production and Medicine Manufacturing Co., Budapest, Hungary

Four *Bordetella pertussis* strains of different origin were examined. Virulence investigations were performed by intracerebral injection of mice giving 100 000 germs/0.03 ml volume. In addition, from the supernatant of the strains, the haemagglutination and the heparin inhibited haemagglutination assays were carried out, demonstrating the FHA activity. The lysis of the strains was done by sonication treatment. The DNT activity was tested examining the spleen atrophy of mice and the skin reactions in guinea-pigs. The LPF activity of the strains was determined as well.

A. LAKOS

Platelia Lyme ELISA kit – Clinical and Laboratory testing

Lyme Disease Clinic, Nagykovácsi, Hungary

Platelia Lyme ELISA kit showed an excellent quality as a factory product. The only but – considering the consequences – very serious problem is in the interpretation of the results. That is especially the low level of the factory set cut-off. This results – contradictory to the factory description – not a 5 but a 24% false positivity ratio, at least in the Hungarian population. The factory instruction is incomplete because there are no suggestions for CSF tests and it does not help to distinct indeterminate, weak and strong positive results. Our modification in the calculation of the cut-off resulted a sensitive but extremely specific test. The Platelia Lyme ELISA kit with the above complements in the calculation and the modification of the cut-off level will be an excellent test for detection of *Borrelia burgdorferi* antibodies.

M. KERÉNYI[1], I. BÁTAI[1,2], V. TÓTH[1,3] and L. EMŐDY[1]

Neutralization of the lung toxic effect of *Escherichia coli* and *Morganella morganii* by human and rabbit sera

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Alpha-haemolytic *Escherichia coli, Morganella morganii* and *Proteus penneri* strains exhibit a strong toxic effect in the lung when instilled intranasally to mice. This toxic effect is characterized by the development of haemorrhagic lung edema and death within a couple of hours after instillation. In contrast to the extracellular alpha-haemolysin lung toxin is cell associated, and its activity can be evoked by bacterial cells or sonicates thereof.

Immune sera raised with live lung toxic bacterial cells showed high neutralizing capacity when preincubated with serologically homologous or heterologous toxic bacteria. Immune sera to boiled bacteria exhibited a neutralizing capacity only to serologically homologous bacteria. These results indicate the existence of 1. high level cross-neutralizing antitoxic antibodies, and 2. lower level neutralizing antibacterial antibodies working only in a homologous system. The antitoxic antibodies are cross-acting not only within the *Escherichia* genus but also in the *Escherichia–Morganella* system.

Sera of patients with infection by alpha-haemolysin producing *E. coli* exerted a high level neutralizing effect in the mouse lung toxicity assay. These sera were effective in the heterologous system with *M. morganii* indicating the presence of cross-reacting antitoxic antibodies also in human cases. During the course of infection changes in titres for the individual patients could be observed. Preimmune rabbit sera or control sera from healthy humans showed no neutralizing capacity.

The above results suggest that the lung toxic substance is expressed also in natural and experimental in vivo conditions, and it elicits the synthesis of antitoxic antibodies.

Further studies are needed to exactly characterize the nature of tire lung toxic substance, and to evaluate its role in the pathogenesis and immunology of human infections.

V. TÓTH[1,2], M. KERÉNYI[1], T. TOMCSÁNYI[3] and L. EMŐDY[1]

Virulence of *Proteus penneri* strain 357 and its non-haemolytic mutants

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Proteus penneri, formerly known as "*Proteus vulgaris* biogroup 1" is a pathogen isolated most frequently from urine. It has also been implicated as the cause of wound infection. Most of the clinical isolates produce an extracellular haemolysin resembling to alpha-haemolysins of other genera of *Enterobacteriaceae*. The aim of this study is to assess the role of alpha-haemolysin in virulence of *P. penneri* by comparing the effect of the wild type wound isolate *P. penneri* strain 357 with its various mutants in several in vitro and in vivo model systems.

Similarly to *Escherichia coli* and *P. mirabilis* spontaneous mutations affecting the haemolytic region were used. No difference between the nutritive and biochemical properties of the wild type strain and its mutants could be detected. Serum resistance was not abolished by the mutations. In tissue culture assays with various cell lines only the original haemolytic strain exerted a toxic effect.

In mouse assays the difference between the virulence of the wild type strain and its derivatives was remarkable. Intraperitoneal and intravenous LD_{50} values indicate a marked decrease in lethality of the mutants. When applying identical infectious doses death occurred much earlier in the case of the haemolytic strain indicating an acute in vivo toxic effect of alpha-haemolysin. Similarly to alpha-haemolytic strains of other genera, haemolytic *P. penneri* caused an acute haemorrhagic lung edema and death within a couple of hours after intranasal instillation to mice. The mutants produced no symptoms in this assay either.

In case of spontaneous mutants not only the haemolytic capacity and related virulence functions were handicapped. Motility in soft agar and swarming on the surface of agar plates were also impaired but not entirely abolished. This suggests that a DNA sequence responsible for regulation of motility and swarm cell differentiation might be deleted. Recently global regulators affecting flagellar functions have been identified in *Escherichia coli* and *P. mirabilis*. Further experiments should be devoted to the elucidation of the question if a global regulator is involved in these functions in *P. penneri*.

M. KÁLMÁN, B. CZERMANN and E. SZÖLLŐSY

Arcobacter butzleri strains in Csongrád county and their cytotoxin production in different cell lines

Csongrád County Institute, National Public Heath Service, Szeged, Hungary

In 1994 we isolated 12 *Arcobacter butzleri* strains partly from clinical (4) and partly from non-human (8) sources. The non-human strains were delivered from random raw milk and chicken. The chicken samples were transported from a farm, where the house-wife could be infected by contamination.

We examined the cytotoxin production of the strains and compared the cytopathogenic effect in Vero, Hep-2 and HeLa cells. Reference toxigenic VT *E. coli* O157 was included as control. As arcobacters from the only species of *Campylobacteriaceae* that tolerates air, the handling of *A. butzleri* strains before toxin detection was the same as that of VT *E. coli* O157.

One of the human strains (associated with enteritis) showed a characteristic [+ + + +] cytopathogenic effect by rounding of the cells in Veto cell-lines, similar to the VT *E. coli* O157. Three other strains (from human enteritis, chicken, milk) seemed to produce cytotoxin too, but the cytopathogenic effect was weaker [+ +]. In HeLa and Hep-2 cell lines both the control and the *A. butzleri* strains showed a weaker [+ + or +] cytopathogenic effect.

H. MILCH, J. PÁSZTI, I. GADÓ, I. TÓTH, É. CZIRÓK and M. HERPAY

Phage typing plasmid profile determination and genetic probing for Shiga-like toxins of *Escherichia coli* O157

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Complex typing (sero-, phage-, colicin typing and plasmid profile determination) of *Escherichia coli* O157:H7 and NM strains derived from different countries was carried out. By the use of gene probes SLT I, and SLT II efforts were made to discover the incidence of *E. coli* strains causing haemorrhagic colitis or nonbloody diarrhoea in Hungary. Complex typing was examined of 8 *E. coli* O157:H7 strains isolated from a patient suffering from haemorrhagic colitis, one O157:HNT strain, caused food poisoning with symptoms of haemorrhagic colitis, 6 O157:NM strains isolated from enteritis of piglets, 11 O157:H3, O157:H16, O157:H38, O157:H39, O157:H45 and O157:HNT strains, originated from nonbloody diarrhoea in Hungary. Out of the different serotypes isolated in Hungary O157:H7, O157:HNT were SLT I and SLT II positive. 88 O157:H7, NM and other H antigen possessing strains isolated from the USA, UK, Germany and Russia were examined. The verotoxin producing 9 O157:NM

strains belonged to 4 different phage-groups and 5 kinds of plasmid profiles. The 82 O157:H7 strains belonged to 6 phage-groups and 16 plasmid profiles (4 were frequent). Out of 19 colicinogenic strains colicin type D was produced by 10 strains. Strains possessing other H antigens than H7 were characterized by 8 phage-groups and 10 plasmid profiles; the phage-group of only one strain was identical with that of an NM strain, out of the plasmid profiles 2 identical ones were observed among the NM or H7 strains. In the USA, Canada and UK Khakhria et al. (1990) phage typing method is used for typing *E. coli* O157:H7 strains. The comparison of our scheme with the above mentioned typing method gives besides the coincidence in certain cases a better possibility for the differentiation. The examinations proved furthermore, that the differentiation of *E. coli* O157 strains of different serotypes according to phage-group, plasmid profile and colicin type might be more reliable than one of the typing methods.

I. GADÓ[1], V. G. LÁSZLÓ[1], B. NAGY[2], H. MILCH[1], I. DRIN[1] and J. HORVÁTH[3]

Phage restriction and aspecific hybridization by small plasmids in *Salmonella enteritidis*

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Plasmid content of 78 *Salmonella enteritidis* strains was determined and DNA-hybridization was performed with a gene probe of *E. coli* heat-stable enterotoxin STb consisting of 460 bp. A small (2.5–4.5 Md) plasmid was present in the majority of strains of phage type (PT) 1b, 1c, 6b and 6c strains besides the serotype-specific (38 Md) plasmid. Among the small plasmid carrier strains hybridization was positive in 77% in "high stringency" conditions. The appearance of STb sequence in the small plasmids was proved by Southern blot technique. The swine intestine loop test, performed with random selected six STb positive *S. enteritidis* strains did not exhibit STb production. Correlation was found between the presence of small plasmid and phage restriction to two additional phages (which were used to subdivide *S. enteritidis* strains of PT1 and PT6), by plasmid transfer experiments and epidemiological observation, but it seems, that the restriction did not depend on the sequence of STb. The correlation between the small plasmid content and the STb-positive hybridization result could be verified in case of 137 *Salmonella* strains belonging to 28 various serotypes. Our results called the attention to the possibility of aspecific reactions in connection with the application of DNA probes.

J. DEÁK[1], I. VERÉB[2], E. NAGY[1], J. SZÖLLŐSI[3], L. TÖRÖK[4] and M. FÖLDES[1]

Detection of *Chlamydia trachomatis* by polymerase chain reaction, culturing and nucleic acid hybridization methods

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Chlamydia trachomatis is the leading cause of nongonococcal urethritis and epididymitis in men and cervicitis in women. Because of the increasing number of new cases and the severe consequences, there is an urgent demand to introduce sensitive and specific rapid diagnostic methods.

A molecular diagnostic method, the polymerase chain reaction (PCR) (Amplicor *C. trachomatis* test) was compared with a culturing method in the McCoy cell line in a high-risk population. The PCR was compared with the nucleic acid hybridization method (PACE 2 Gen-Probe) in a low-risk population.

The Amplicor *C. trachomatis* test is based on three major processes: PCR target amplification, hybridization of the amplified product to a specific nucleic acid probe and detection by biotin-avidin horseradish peroxidase ELISA. To avoid aerosol contamination, the test contains AmpErase (uracil N-glycosylase), which recognizes and catalyzes the construction of uracil-containing DNA, but not thymine-containing DNA. The samples were amplified in a GeneAmp PCR System 9600 thermal cycler.

In the high-risk population, 40 urethral samples were cultivated on the McCoy cell line, and determined with PCR. Sixteen samples were positive and 20 samples were negative with both methods. Three samples were positive only with PCR, and one sample was positive only with cultivation.

Of 45 samples, 28 were negative in both tests in the low-risk population; 11 PCR borderline cases were negative with the Gen-Probe, and 1 PCR borderline case was positive with Gen-Probe; 4 PCR positive cases were negative with the Gen-Probe, and there was no PCR and Gen-Probe positive case. One Gen-Probe positive case was negative with PCR.

The advantages of PCR are the possibility of non-invasive sampling, and the detection of bacteria in urine samples. Our results suggest, the high sensitivity and specificity of PCR. The good correlation between PCR and cultivation, which is regarded as gold standard, indicates that PCR is equivalent to the culturing method. The *C. trachomatis* Gen-Probe is a nucleic acid hybridization method without amplification, but exhibited a relatively good correlation with PCR.

A. LIPCSEY[1] and C. G. DOWSON[2]

The role of amino acid alterations of penicillin binding protein (PBP) 2b in the penicillin resistance of *Streptococcus pneumoniae*

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Streptococcus pneumoniae has a PBP mediated resistance to beta-lactam antibiotics. Pneumococci possess 5 high-Mr PBPs (1A,1B,2A,2B,2X). The penicillin resistant isolates produce altered forms of PBPs and therefore they can re-model the active centre of the transpeptidase domain of the high-Mr PBPs so they can decrease their affinity for penicillin but still function with natural substrates.

As donor species in the natural transformation *Streptococcus mitis* and probably other oral streptococci are responsible for the evolution of low affinity PBPs in *S. pneumoniae.*

The pneumococcus needs at least three altered PBPs to achieve high resistance against penicillin. Though the PBP 2B is not the primary target of penicillin the 2B gene plays a very important role in the resistance mechanism.

Comparing the sequences of the PBP 2B genes of penicillin resistant and penicillin sensitive isolates one of the most interesting changes is the alteration of seven consecutive amino acids between Thr 425 and Phe 434 and a second alteration which seems to be within the penicillin binding site as well. The latter one is the substitution of Thr 445 to Ala following the crucial Ser-X-Asn motif.

Using oligonucleotide mutagenesis kit, PCR technique and specially designed oligonucleotide (which contains the alteration of Thr→Ala) we were able to transform the mutated, cloned PBP 2B gene into penicillin sensitive competent cells. Consequently the MICs of the transformants have increased significantly, proving the importance of this single amino acid alteration within the active site.

J. DEÁK[1], I. VERÉB[1], A. SOMFAY[2], P. KRASZKÓ[2] and E. NAGY[1]

Detection of *Mycobacterium tuberculosis* complex with cultivation and amplified nucleic acid hybridization probe

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There are three different levels for the diagnosis of the *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis, Mycobacterium bovis* BCG, *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti*) in different countries: direct detection of the bacterium by acid-fast Ziehl–Neelsen staining,

cultivation of the bacterium in regional centres, and identification and antibiotics sensitivity testing methods in the national centres. New radiometric liquid cultivation methods [BACTEC], and new, rapid diagnostic methods have been introduced in recent years, e.g. chromatographic methods [HPLC, GLC], nucleic acid hybridization and amplification methods for diagnosis of the *M. tuberculosis* complex.

The conventional cultivation method, which is regarded as the "gold standard" (Löwenstein–Jensen), and the Gen-Probe an amplified mycobacterium direct test, were compared in our laboratories. The Gen-Probe method utilizes a proprietary isothermal enzymatic amplification of target rRNA via DNA intermediates. The detection of amplified product is achieved by using an acridine ester-labeled DNA probe.

A total of 107 different clinical samples (sputum, bronchoalveolar lavage, thorax puncture, CSF and skin) were tested by the Gen-Probe and cultivation methods, and in pan samples were stained by Ziehl–Neelsen staining. From each patient 1–4 samples were tested. Ten samples were positive and 34 samples were negative by the cultivation and Gen-Probe methods. One sample was positive only by cultivation, and 11 samples were positive by the Gen-Probe method. The parallels of Gen-Probe positive samples were negative on cultivation in a higher procent, whereas the clinical symptoms and Gen-Probe results generally correlated.

The introduction of amplification and hybridization methods has opened up new dimensions in microbiological diagnostic methods. The conventional cultivation methods are time-consuming. Cultivation, identification and antituberculotic drug sensitivity testing require months. The Gen-Probe method requires hours after decontamination. Detection of the *M. tuberculosis* complex does not necessitate the presence of live bacteria in clinical samples. The rRNA sequence of the *M. tuberculosis* complex is detectable after the beginning of antituberculotic therapy. The procedure is suitable for detection of the *M. tuberculosis* complex from immune complexes and white blood cells.

The culture method is not indispensable because of drug sensitivity testing. The Gen-Probe method is highly sensitive and specific and will be useful as a rapid mycobacterium diagnostic method. The cost of the test is apparently high, but the benefit is really much higher, considering the advantages of rapid diagnosis: the beginning of immolate therapy, the prevention of further infections and the lower hospital hotel costs.

Zs. Senoner[1], J. Sáradi[1], D. Bánhegyi[2] and Cs. Bognár[3]

Rhodococcus equi caused lung abscess and empyema in a HIV-1 infected patient

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Rhodococcus equi has been rarely isolated from human specimens. We present a case report and describe the microscopic, macroscopic, and biochemical characteristics and antibiotic susceptibility of the agent. It may assumed that *R. equi* may be an indicator of HIV infection.

Z. Tigyi[1], T. Pál[1,2], and T. Tomcsányi[3]

Identification of the plasmid responsible for the colicin type 7 production in *Shigella sonnei*

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The genetic background of colicin type 7 production in *Shigella sonnei* (Horák's scheme) was studied. Data in the literature referring to conjugation experiments mention the plasmid coded nature of this phenotype without the proper identification and characterization of this presumably non-conjugative extrachromosomal element. Our goal was to identify the colicin type 7 plasmid. Plasmid profile analysis of colicin producer and non-producer strains did not provide any help. Due to the possible clonal dissemination, all the isolates harboured several plasmids of equal or similar size. Beyond colicin production, no other selectable or detectable markers could have been associated with colicin producer strains, and thus probably with the colicin plasmid. First; we extracted the plasmids from a colicin type 7 producer strain, and this mixture of plasmids was electroporated into a sensitive *Shigella sonnei* strain under circumstances optimised for the recipient. The basis of selection for the proper transformants was the assumption that, similarly to other colicin plasmids, this plasmid may code for the colicin-immunity of the host strain. Thus, on plates containing colicin type 7, beyond the tolerant (*tol*) mutants deriving from the sensitive recipient strain with a frequency of c.a. 10^{-4}, only those clones having acquired the colicin type 7 plasmid would grow. Some 1.2×10^4 clones obtained in this way were replica-plated and tested for colicin type 7 production; 48 positive clones were found. These transformants harboured a plasmid (pTZ1) of 50 Md, while pTZ1 was absent from the *tol* mutants tested. Plasmid pTZ1, when reisolated from the total plasmid mixture of one of the transformants (ZT100), and transformed again into the recipient also yielded colicin producing clones. These

experiments prove that in *S. sonnei* the production of colicin type 7 is coded for by a plasmid of c.a. 50 Md, which, at the same time, is also responsible for the development of colicin-immunity.

A. HEVÉR[1], J. CHEVALIER[2], A. CREMIEUX[2], J. BARBE[3] and J. MOLNAR[1]

Effects of antitrypanosomal tetracridines on the extrachromosomal genetic elements of bacteria and tumour cells

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A wide variety of tricyclic compounds have been shown to inhibit the antibiotic resistance of bacteria, protozoa and multidrug resistance of tumour cells. The resistance is often related with interaction of the compounds with the DNA or enzymes, e.g. gyrase or topoisomerase. Therefore it was promising to study the effect of DNA complexing acridines (synthesized for antitrypanosomal action) on bacterial antibiotic resistance and multidrug resistance of tumour cells.

In our experiments effects of some new acridines were studied on the plasmid elimination and the inhibition of conjugal transfer of antibiotic resistance in *Escherichia coli*. In addition the effect of new acridine derivatives was measured on the expression of multidrug resistance function in the mouse lymphoma cell line transfected with human Mdr-1 gene and its parental cell line. The fluorescent substrate rhodamine 123 was the test compound, which shows the highest differential fluorescence between Mdr and parent cells.

The majority of tested tetracridines exhibited weak antibacterial effect with MIC values above 200 pg/ml. Some compounds had high plasmid curing frequency as BG-549 and BG-609 and inhibition action on DNA gyrase. In case of the most effective compound BG-549 the two effects were found simultaneously. BG-609 was an exception having high plasmid elimination without remarkable effect on gyrase activity. Transconjugal plasmid transfer was reduced by the majority of compounds having antibacterial action.

R. KISS[1], J. BILLE[2], E. BANNERMAN[2] and M. RODLER[1]

Comparison of results of antibiotic susceptibility examinations on *Listeria* strains carried out by various methods

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We were using the NCCLS procedure for disk diffusion tests, the ATB Expression (BioMérieux) microbiological analyser, and the E test (AB BIODISK).

A total of 108 *Listeria monocytogenes* strains were examined with the first method. They were susceptible to ampicillin, penicillin, gentamicin, cotrimoxazole, ticarcillin, oxacillin, carbenicillin, azlocillin, teichoplanin and to ciprofloxacin in 91.7%. To cefaclor, ceftriaxon, ceftazidime, cefoperazone, all strains were resistant.

With ATB susceptibility of 113 strains was determined. They were susceptible to ampicillin in 96%, vancomycin 88%, erythromycin 61%, riphampycin 94%, kanamycin 94%, tobramycin 65%, gentamicin 72%, tetracyclin 53%, pristinamycin 61%, teicoplanin 100%, cephalotine 86%; 75% of the strains showed intermediate susceptibility to penicillin. The examined strains were resistant to oxacillin in 94%, lincomycin 89%, pefloxacin 100%, cotrimoxazole 76%, fosfomycin 93%, nitrofurantoin 90%, cefuroxim 100 %.

E test was as follows: ampicillin, teicoplanin, cefalothin susceptible (100%), benzylpenicillin 98.9%, cefaclor 82%, cefotaxime 42.8%. Against teicoplanin all *Listeria* strains proved be sensitive with all the three methods.

R. KISS[1], P. T. BORSÓS[2], M. SZEMENYEI[2], and M. RODLER[1]

Listeria monocytogenes strains isolated from ice-cream

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Listeria monocytogenes contamination was detected out of 66 ice-cream samples in 7, out of 29 environmental samples in 1, furthermore in the tube connected to the ice-cream feeding spoon and in the rinsed cooking cauldron. The shops were closed and disinfectant cleaning was performed. After this, control of the producing and marketing places was repeated and examinations were made from throat swabs of the workers. Listeriae could not be cultured either from the environmental or the throat samples.

In the course of the identification of the strains, Palcam agar (MERCK) and API LISTERIA test (BioMérieux) were applied. The isolated strains were serotyped with the

following results: 2 vanilla ice-creams, 1 marble ice-cream, 1 chocolate ice-cream samples 1/2a, 1 chocolate ice-cream and 2 vanilla ice-creams samples 4b, 1 feeding spoon and 1 cooking cauldron samples 1/2a.

Zs. Langó[1] and L. G. Tóth[2]

Data on microbial communities in mud of Lake Caldera (Spain)

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The actinomycete communities of mud in Lake Caldera (Spain) were studied. Lake Caldera, located at high altitude (2600 m), has a very special water chemistry and an interesting nutrient cycling. The aim of our work was to identify the members of these populations at species level by collecting data on their morphological, biochemical and ecological activities. The species compositions were compared in the autumn and spring period. Isolates from samples collected in the autumn and identified by standard methods, all proved to be typical members of the species *Streptomyces arenae*. This species otherwise is common in the lakes and it has been shown from Lake Fertő (Hungary), too. Our strains have wide biochemical activities and ecological tolerance (utilization of carbohydrates, gelatinase activity, nitrate reduction, temperature tolerance, etc.) From mud samples collected in the spring period *Micromonospora*-type strains were isolated. *Micromonospora* species are very frequent in mud of lakes and 10–20% but sometimes 40–50% of the actinomycete communities in lake sediments belong to the genus *Micromonospora*. Concerning their biochemical and ecological features of our strains are very heterogeneous. It seems that the species composition of *Micromonospora* population is more complex then the *Streptomyces* population in Lake Caldera.

J. Molnár[1], K. Szalay[1], A. Hevér[1] and A. Aszalós[2]

The effect of phenothiazines on the antibiotic resistant bacteria and multidrug resistant tumour cells

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We have studied the effect of substituted chlorpromazine (CPZ) and promazines (PZ) in two different systems. First we established that various plasmids were eliminated by some substituted phenothiazines from *Escherichia coli*. The transfer of kanamycin resistance plasmid was inhibited either by pretreatment of donor cells or by the treatment

at transconjugal plasmid transfer on the preformed mating pair. Second, we studied the effect of the same CPZ and PZ derivatives on P-glycoprotein responsible for multidrug resistance in tumour cells. Murine lymphoma cell line and its human MDR1 gene transfected form were treated with various CPZ derivatives and accumulation of the fluorescent rhodamine 123 was measured by flow cytometry. The 3,7,8-trihydroxy-CPZ and 7,8-dihydroxy-CPZ were effective P-glycoprotein blockers. 7,8-dihydroxy-, dimethoxy- and 7,8-dioxo-CPZ-7-semicarbazone had moderate activity, as compared to verapamil (5 μM). 7,8-diacetoxy and 5-oxo-CPZ were without effect. There is a possible correlation between the biological activity and the electrostatic potential of the studied molecules. CPZ affects the binding of anti-actin, however, binding of antitubulin and antivinculin was not disturbed. Our results suggest that the inhibition of plasmid transfer and P-glycoprotein function may depend on similar electronic properties of the studied CPZ derivatives, that induce enzyme inhibition and alteration in cytoskeleton.

MINISYMPOSIUM: INHIBITORS OF HIV PROTEASE

S. OROSZLAN

The proteinase of human immunodeficiency virus (HIV) and its role in virus replication: an overview

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HIV is a causative agent of acquired immunodeficiency syndrome (AIDS) a fatal disease occurring worldwide in epidemic proportions. The proteinase (PR) of HIV and other retroviruses is a virally encoded enzyme that is essential for virus replication. HIV PR is well characterized biochemically and structurally, and its function in the late phase of replication is well established. The proteolytic processing of Gag and Gag-Pol polyproteins that are the primary translational products of the genome size mRNA into' smaller functional units is crucial for the production of mature infectious virus. Therefore, the PR is a potential target for chemotherapy of virus infection and associated disease. HIV PR inhibitors have been extensively studied in many laboratories and several of them are currently in advanced clinical trials. We have also suggested the possible involvement of PR in the early stages of the vital replication cycle on the basis of in vitro experiments with purified capsids of equine infectious anaemia virus (EIAV), a lentivirus related to HIV. We reported the in situ fragmentation of the nucleocapsid (NC) protein inside intact EIAV capsids. Furthermore, we have found that substrate-based HIV PR inhibitors of various chemical structures have antiviral effect in a single cycle of infection. HIV-1 NC protein is also cleaved by the PR into three fragments. The first cleavage occurs between Phe16 and Asn17 in the first Zn-finger. Mutations

replacing the Asn with various amino acids resulted in mutant proteins which were either better substrates or resistant to cleavage depending on the particular amino acid, but fully corresponding to our predictions based on the knowledge of the substrate specificity of HIV-1 PR. Mutations were introduced into pMM4HXB2, an infectious provirus of HIV-1_{IIIB} and HLtat cells were transfected with wild-type and mutant DNAs. Viruses containing the mutations produced particles and packaged RNA at about the wild-type level. The mutant and wild-type viruses were then used to infect H9 cells. Of the two mutants showing no apparent defect in RNA packaging, the infectivity of the Asn17→Ala mutant was, as expected, the same as wild-type. However, the virus with the Asn17→Gly mutation had a much lower and delayed infectivity. These data provide evidence that the PR has a crucial role in the early phase of HIV infection, since NC cleavage by the PR appears to be required for subsequent integration of the viral DNA. These studies uncovered hereto unknown biochemical pathways in the replication of HIV-1 and provide the basis for the rational design of PR inhibitors capable of blocking infection before integration.

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Proteolytic processing mechanisms of human immunodeficiency virus

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We have investigated the mechanism of autoprocessing of the human immunodeficiency virus, type 1, protease from a polyprotein that contains the protease coding sequence linked to short native flanking sequences fused to the maltose binding protein [MBP-TF-PR-Pol]. The dimeric MBP-TF-PR-Pol protein has an active site that can bind inhibitors and substrates similar to that of the mature protease, although its catalytic activity is distinctly lower than the mature protease. The N-terminal strands are cleaved first via an intramolecular process leading to the transient protease intermediate, PR-Pol, which has enzymatic activity comparable to that of the mature protease (Louis et al. Proc. Natl. Acad. Sci. USA 91, 7970–7974, 1994). The reaction intermediate (PR-Pol) was purified to near homogeneity by chromatography on a size exclusion column under denaturing conditions. Both the kinetic parameters for the hydrolysis of the peptide substrate Lys-Ala-Arg-Val-Nle(NO2)Phe-Glu-Ala-Nle-NH2 catalyzed by the PR-Pol and the inhibition constant determined with a pseudopeptide inhibitor are indistinguishable from those for the mature enzyme at pH 5. However, the pH rate profile for k_{cat}/K_m, is different from that of the mature protease with apparent pK_a's of 4.69 and 4.74. Notably, the PR-Pol also exhibits a higher sensitivity to urea than the mature protease. Finally, the spontaneous conversion of the PR-Pol to the mature PR is second-order in protein concentration. Both the enzymatic activity and the intrinsic

fluorescence of PR-Pol are linearly dependent on protein concentration (between 50–500 nM) indicating that the protein is largly in its dimeric form. These results are discussed in terms of a mechanism in which the Pol strands are cleaved intermolecularly to release the mature protease.

L. POLGÁR and Z. SZELTNER

Stability and activity of HIV-1 protease

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The activity of HIV-1 protease is markedly increased with increasing ionic strength. The structural basis of this phenomenon has been explored by several independent methods, including urea and pH denaturation. The enzyme exhibited higher stability at 1.0 M than at 0.1 M NaCl concentration, which is interpreted in terms of preferential hydration of the protein. A mutated form of the enzyme, designed to better resist autolysis, displayed higher conformational stability than the native protease at the same ionic strength. The mechanism of action of HIV-1 protease was studied by using Lys-Ala-Arg-Val-Leu*Phe(NO2)-Glu-Ala-Nle (substrate E) and its counterpart (substrate Q) containing the neutral Gln in place of the P2' Glu, which is the most preferred residue of the substrates of HIV protease. The importance of the charged residue was apparent from the much higher k_{cat}/K_m with substrate E than with substrate Q. The pH dependencies of k_{cat} and K_m exhibited different profiles for the two substrates, indicating differences in the ground state and transition state stabilizations of their reactions. Solvent kinetic deuterium isotope effects have shown that the rate-limiting catalytic step may be a chemical or physical process depending on the substrate, i.e. the nature of P2' residue can control the mechanism of action of HIV-1 protease.

K. NAGY[1,3], M. M. RAYNER[2] and S. OROSZLAN[3]

The role of viral protease (PR) in the early phase of HIV-1 replication: antiviral activity of C-2 symmetric inhibitors of PR

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The involvement of HIV PR in the early phase of viral replication was studied in connection with the antiviral activity of C-2 symmetric inhibitors of HIV PR during a single cycle of infection.

Antiviral activity of HIV-1 PR inhibitors designated Q8024 and P9941 was quantitatively determined in a single cycle of infection using the MAGI assay (Kimpton, J. Virol. 66, 2232, 1992) on HeLaCD4-LTR/β-gal cells. Extracellular, mature virus preparation was also treated with 0.1–6.5 μM of Q8024 for up to 60 min and after a 50-fold dilution infectivity was assayed.

Q8024 inhibited effectively primary infection by HIV-1_{IIIB} when it was added to the cells 1 h prior to infection (IC_{50}=1.151 μM, IC_{90}= 4.48 μM); antiviral effect of P9941 was not dose dependent, however treatment of cells with 13 μM of P9941 resulted in >50 per cent inhibition of infectivity. No cytotoxicity was seen at the highest concentration of inhibitor used (13 μM). TC50 of Q8024 was 83 μM. When HIV-1_{IIIB} (10^5 IU/ml)was incubated in medium containing 2 μM (~ IC_{50} on cell-base assay) of inhibitor, than diluted 50-fold as added to cells (0.1 m.o.i., 0.04 μM) Q8024 gave >90% inhibition. This inhibition was partially reversible by allowing the inhibitor taken up by the virus to leach out (>65% of infectivity was regained after 60 min leaching).

The results demonstrate that PR inhibitors were capable of penetrating the virus and that tightly bound inhibitor (to the PR in the particle) entered the cells rapidly, together with the virus. Pretreatment of cells was not necessary to obtain nearly complete (>90%) inhibition. We found that uptake of the PR inhibitor by the cells (when treated) is both dose and time dependent. These and our results with other PR inhibitors (Nagy et al. J. Virol. 68, 757, 1994) suggest that the target of PR in early phase of viral replication is the nucleocapsid (NC) protein. It is likely that PR and NC protein itself and/or its proteolytic fragments may have an active role in the early phase of virus replication contributing to the formation of the properly assembled cDNA protein preintegration complex and/or its transport to the nucleus.

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DMP 450: an orally bioavailable cyclic urea inhibitor of the HIV protease

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An effective therapy for AIDS must combine potency towards wild-type and mutant variants of HIV with oral bioavailability such that blood levels of inhibitor exceed that required for significant inhibition of virus replication at all times between doses. DMP 450 is a water soluble cyclic urea inhibitor of the HIV-1 protease and is a potent inhibitor of virus replication in cell culture, including HIV-2 and AZT-resistant patient isolates of HIV-1. Inhibition results in production of non-infectious, immature virions in both lymphoid and monocytoid cells. Studies assessing the potential maturation of these immature virions suggest that this virus may not readily mature and become infectious. DMP 450 also inhibits several mutant variants which contain single amino acid substitutions in the S1/S1' pocket of the protease, although up to 9-fold more

inhibitor is required for 90% inhibition of replication. DMP 450 is highly selective for the HIV protease compared to cellular proteases, consistent with its displacement of the retrovirus-specific structural water molecule in the X-ray crystal structures. More important, DMP 450 shows substantial oral bioavailability from solid dosage forms, such that single PO doses of 10 mg/kg in man result in plasma levels in excess of that required to inhibit both wild type and several mutant HIVs.

VIROLOGY

W. DOERFLER, R. SCHUBBERT, H. HELLER, C. KÄMMER, M. KNOBLAUCH and K. FECHTELER

Integration of foreign DNA into mammalian genomes: mechanism and consequences

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We have been interested in the fate of foreign DNA in mammalian cells. The DNA of adenovirus type 12 (Ad12), of bacteriophage λ or M13 have been used in these investigations. We report on integration patterns in mammalian cells, on studies in a cell-free system with highly purified proteins from nuclear extracts of hamster cells, and on the de novo methylation of integrated foreign DNA. This de novo methylation has been interpreted as an ancient cellular defense mechanism against the activity of foreign genes. Ad12 transformation and/or the insertion of foreign DNA, possibly by itself, elicit striking changes in the patterns of methylation of cellular DNA segments far remote from the site of foreign DNA insertion. Foreign (M13mp18) DNA survives transiently and in fragments the digestive regimen of the gastrointestinal tract (GI) in mice. M13mp18 DNA is taken up by epithelia and leukocytes in Peyer's plaques in the intestinal wall, and is transported by peripheral blood leukocytes to various organ systems of the animals. Is this foreign DNA ingested by the GI tract integrated into the cellular genome?

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Analysis of methylation patterns in the regulatory region of a latent Epstein–Barr virus (EBV) promoter (BNLF1) by genomic sequencing

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We studied the methylation patterns in the regulatory region of the latent EBV promoter BNLF1, where transcripts for LMP-1 (a growth-transformation-associated membrane protein) are initiated in a clone derived from the Burkitt lymphoma Mutu, and in another clone (derived from the same tumor) which does not express LMP-1.

We used the method of automated genomic sequencing (Myöhänen et al., *DNA Sequence* 5, 1–8, 1994) after bisulphite-induced modification of DNA. The modified target sequence was amplified in a nested PCR where one of the inner primers was biotinylated. This permitted the separation of strands with the aid of streptavidin coated magnetic beads and subsequent sequencing with a fluorescent primer and automated DNA sequencer.

We found that in the Mutu clone expressing LMP-1 the regulatory sequences are completely unmethylated. On the contrary, there are methylated CpG sequences upstream and downstream of a potential CBF1-binding site (EBNA 2 responsive element) in the Mutu clone which does not express LMP-1.

D. BÜCHNER, G. GLASER, H. WOLF and H. H. NILLER

Nucleoprotein complexes and DNA 5'-ends at *oriP* of Epstein–Barr virus

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Visualizing protein-DNA interactions at origins of DNA replication throughout the cell cycle and mapping of initiation points at nucleotide resolution sheds further insight on the mechanism of initiation and replication control The Burkitt's lymphoma cell line Raji harbours multiple copies of latent Epstein–Barr virus (EBV). The origin of plasmid replication (*oriP*) of EBV together with the vital protein EBNA1 and the cellular replication machinery provides replication function for the viral DNA once per cell cycle. Therefore, we asked, if the nucleoprotein complexes on *oriP* would undergo an alteration in dependence of the cell cycle.

Raji cells were synchronously growth arrested by the drugs mimosine and colchicine at G_1S and mitosis of the cell cycle respectively. The association of DS with proteins was visualized by in vivo footprinting with dimethylsulphate. Methylation protection patterns indicated a novel binding activity within 'each element of a 9bp

sequence 5'-TTAGGGTTA-3' repeated three times at DS. The binding activity was strongly diminished in mitotic cells.

In order to correlate more the protein binding data at *ori*P with initiation function, we tried to locate the initiation points of DNA replication within DS at nucleotide resolution. 5'-ends of Epstein–Barr virus DNA were mapped to the nonamers by ligation mediated polymerase chain reaction (LMPCR) on purified genomic DNA from untreated Raji cells. These 5'-ends strongly accumulated under aphidicolin treatment, suggesting potential initiation sites for replication from DS. We conclude that the nonamer binding activity may be involved in the initiation of DNA replication at *ori*P. In order to characterize this novel binding activity, electrophoretic mobility shift assays were conducted. The nonamer specifically bound nuclear protein from Raji cells that was not identical with EBNA1.

This line of research may lead towards the detection of a unique and novel mechanism for the plasmid replication of EBV or of a general mechanism for the controlled replication of chromosomal DNA.

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The adenovirus E1A enhancer-binding protein, E1A-F; isolation, structure and function

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The adenovirus early region 1A (E1A) gene plays a central role in productive infection. The E1A gene is transcribed immediately after infection through an action of multiple enhancer elements located upstream of the E1A gene. E1A gene products then stimulate transcription from its own gene by an auto-activation mechanism, and activate transcription from other early vital genes. Focusing on host cell factors which modulate E1A gene expression, we identified the E1A enhancer element-binding protein, E1A-F. A cDNA clone encoding E1AF protein was recently isolated by the sequence-specific DNA-binding activity and found to be a new human member of the *ets* oncogene family. Here, We show structural properties of the gene regulatory protein E1AF, effects of E1A-F on viral E1A gene as well as cellular target genes, and possible roles of E1A-F in adenovirus infection and tumor cell invasion.

E1A-F has a DNA-binding domain at the C-terminal region, that is conserved among the *ets* oncogene members. Potential activation domains are located at the acidic amino acid-rich and glutamine-rich regions of the N-terminal half. E1A-F moderately stimulated promoter activity of the E1A gene, although the effect of auto-activation by E1A proteins was significantly high. We assume that E1A-F participates in an initial activation of the E1A gene in the absence of a potent viral activator at very early times of infection. By introducing the intact E1A-F gene and the dominant-negative mutants

lacking activation domains into host cells, we are currently investigating whether E1A-F has effects on E1A gene transcription during adenovirus infection.

Evidence has been accumulated that deregulated expression of extracellular matrix-degrading metalloproteinase (MMP) genes in neoplastic tissues may lead to tumor invasion and metastasis. Transcription control region of MMP genes often contains binding sites for gene regulatory proteins, *ets* and AP1 families. E1A-F highly stimulated promoter activity of three different subclasses of MMP genes: type I collagenase, stromelysin, and 92 kD type IV collagenase. Stable transfectants of human non-invasive cancer cells with the E1A-F expression plasmid acquired invasive phenotype, accompanied by induction of 92 kD type IV collagenase. Tumors derived from E1A-F transfectants were highly invasive and secreted MMP proteins in intercellular spaces. The result indicates that the *ets*-related E1A-F positively regulates tumor cell invasion activity through transcriptional activation of MMP genes.

Y. BECKER

"HIV-1 peplotion vaccine" – a novel approach to protection against AIDS by transepithelial transport of viral peptides to Langerhans cells for long-term antiviral CTL response

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Viral vaccines which stimulate the humoral immune response in humans have been successful in preventing most of the known virus diseases except dengue fever, respiratory syncytial virus infections and HIV-1-related AIDS. Burke [1] raised a concern that anti-HIV-1 antibodies may add a risk factor to immunized individuals infected with HIV-1. An approach to develop HIV-1 vaccines capable of stimulating anti-HIV-1 cytotoxic T cells requires an understanding of the importance of epidermal and epithelial Langerhans cells (LC). These cells are professional antigen-presenting cells which express HLA class I and class II molecules. Epithelial LC are present in a specific layer in the skin, genitalia and gut and may be accessible to viral antigens by local application in a vehicle for transepithelial transport of viral proteins/peptides (designated "HIV-1 Peplotion vaccine"). This approach is supported by the reports that HIV-1 gp160 in ISCOM induced MHC class I CTL response [2], mixing of cationic lipids with viral proteins formed complexes which were delivered to cell cytoplasm and the degraded peptides stimulated CTLs by HLA class I mechanism [3] and viral proteins encapsulated in pH-sensitive liposomes administered to LC-induced primary antiviral CTLs [4]. Current studies in our laboratory deal with (a) selection of the vehicle for transepidermal transport of peptides and the conditions for selective uptake by epidermal LC [5] (b) computer analysis of HIV-1 proteins to detect the putative proteolytic cleavage peptides with amino acid motifs which allow association with different known

HLA class I haplotype molecules on LCs and synthetic peptide uptake from "without" by LC.

The "HIV-1 Peplotion vaccine", when developed, will be useful for continual stimulation of antiviral CTLs in uninfected individuals and HIV-1 carriers by repetitive application to skin, genitalia and gut. The Peplotion vaccine will be applied by vaccines and will be affordable for all human populations, hopefully with high efficiency.

[1] Burke DS, Perspect Biol Med 35, 511–530, 1992; [2] Takahashi M, Takeshita T, Morein B, Putney S, Germain RN and Berzofsky JA, Nature 344, 873–875, 1990; [3] Walker C, Selby M, Erickson A, Do DC, Valensi J-P and Van nest G, PNAS 89, 7915–7918, 1992; [4] Nair S, Babu JS, Dunham RG, Kanda P, Burke RL and Rouse BTJ, Virol 67, 4062–4069, 1993; [5] Becker Y, Virus Genes 99, 133–147, 1995.

E. M. FENYŐ

The role of human immunodeficiency virus (HIV) biological phenotype in transmission and pathogenesis

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Early observations showed that slowly replicating viruses were usually isolated from HIV infected individuals with no or mild symptoms, whereas viruses with high replicative capacity were isolated from immunodeficient patients. This suggested that the virus biological phenotype, slow/low or rapid/high, is a marker for the severity of HIV infection. In addition to replication in peripheral blood; mononuclear cells, these two groups of viruses can be distinguished by syncytia induction and by replication in cell lines.

Studies on mother–child pairs and on discordant couples, where seroconversion of the sexual partner could be documented, showed that both rapid/high and slow/low viruses can be transmitted, from mother to child as well as sexually. Individuals infected with slow/low virus may retain this virus, in some cases also during development of overt immunodeficiency and AIDS.

In fact, in one-half of AIDS patients HIV-1 remains slow/low. Even if viral phenotype does not evolve to rapid/high, an increase in replicative capacity can be demonstrated in these subjects over time. In the other half of the patients with progressive HIV-1 related disease the phenotype of isolated virus changes from slow/low to rapid/high. Sometime before this change a mixture of viruses can be detected. Individuals infected with rapid/high virus may also retain this phenotype. Follow-up of such cases for 6–36 months shows that infection with a rapid/high virus leads to a significantly faster decrease in CD4+ cell counts than infection with slow/low virus. Thus transmission of a rapid/high virus apparently increases the severity of HIV-1 infection. In addition, mothers or index cases carrying virus with rapid/high phenotype

show an increased risk of transmission as compared to those carrying slow/low virus. These results indicate that virus with high replicative capacity is indeed more virulent and increase in the virus in vitro replicative capacity signals a more severe disease.

J. RAJČÁNI

Herpes simplex virus (HSV-1) glycoproteins as tools for neuroinvasivity and their efficacy as protective antigens

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The role of HSV-1 glycoproteins for virulence and neuroinvasivity was studied by following the pathogenesis of deletion mutants and recombinants. The gE minus deletion mutant from the pathogenic ANGpath (derived from ANG) showed remarkable loss of neuroinvasivity in DBA/2 mice and in rabbits after peripheral inoculation. In addition, the gE^{DEL} virus exhibited decreased ability to establish latency in regional sensory ganglion as result of quick axonal spread. Furthermore, ANGpath was rendered nonpathogenic by replacement of its gB gene with the corresponding sequence from gB^{KOS} (UL27) which resides in the *BamHI* G fragment of HSV-1 DNA. Using several recombinants with replaced shorter subfragments and by comparison of the syn^3 sequence of several HSV strains such as 17 (path, nonsyn), KOS (nonpath, nonsyn), HSZP (nonpath, syn) and ANGpath (path, syn) we found that gB related pathogenicity of HSV-1 resides in the entry locus (between aa. #515–525) rather than in the syn^3 locus (am. #853–857) of the gB molecule. The third important glycoprotein which affects virulence is gD as proved by the behaviour of viruses constructed by transferring the *BamHI* J fragment from KOS to ANGpath $gD^{DEL}/LacZ^+$ DNA prepared from genetically engineered virus propagated in BSC-1 cells expressing gD. The subunit DNA free HSV-1 vaccine which contains gB, gC, gD, gE and at least three additional glycoproteins as minor components, protects mice and cross-protects guinea pigs against lethal challenge with HSV-1 and HSV-2, respectively. In addition, vaccination protects rabbits from establishing latent infection and under certain conditions it may even limit the frequency of epinephrine induced recurrent virus shedding.

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Proline 137 is critical for adenovirus endoprotease trafficking but not enzyme activity

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Infection with the adenovirus type 2 ts1 mutant at the nonpermissive temperature results in the production of noninfectious virions [1]. This has been ascribed to the P137L mutation in the virus encoded cysteine protease which causes a defect in protease activity [2, 3]. Here we have examined the ts1 defect in detail as a means of learning more about the viral protease. The ts1 protease accumulated in the nucleus normally and was found associated with incomplete particles as was the case with wt. This enzyme was active in both wt and ts1 incomplete particles produced at 39 °C (ts1-39 TCs), provided they were dissociated with 4 M urea. While the wt protease was packaged into complete particles the tsl-39C particles were totally devoid of protease. This defect was nearly completely corrected by addition of the eleven residue activating peptide pVIc (GVQSLKRRRCF) to the medium late in infection. Rescue of ts1 occurred via restoration of enzyme activity and packaging of the ts1 enzyme into complete virions. Recombinant ts1 enzyme expressed in *Escherichia coli* was not temperature sensitive. The P137L mutation responsible for the ts1 defect appeared therefore to be an in vivo phenotype involving apparently linked events of protease packaging and activation mediated by the PVI protein.

[1] Weber, J. Virol. 17:462–471, 1976; [2] Weber, J. Curr. Top. Micro. Immunol. in press, 1995; [3] Weber, J., Tihanyi, K. Methods in Enzymol. 244:595–604, 1994

M. M. MEDVECZKY, E. HORVATH, T. LUND, P. GECK, C-S. CHOU and P. G. MEDVECZKY

Two proteins and small RNAs of herpesvirus saimiri involved in induction of lymphokines in transformed T cells

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Members of the gamma or lymphotropic herpesviruses including Epstein–Barr virus and Herpesvirus salmiri (HVS) can induce lymphoproliferative diseases. HVS causes acute T cell lymphomas in monkeys and rabbits and the virus can infect a variety of different cells yet only T cells are immortalized by the virus suggesting that viral gone products interact with T cell-specific elements.

It is consistent with this hypothesis that lymphotoxin alpha (tumor necrosis factor bets, TNF-beta), IL-2 and IL-4 are secreted by CD-8 lymphoid tumor cells transformed by HVS. Deletion analysis of a highly oncogenic strain 484-77 shows that two proteins (orf-1 and orf-2) are required for oncogenesis in rabbits, for IL-2 independent growth of infected T cells, and for IL-2 and IL-4 expression suggesting that these proteins are involved in lymphokine induction. The orf-2 product is a transmembrane protein and is strongly associated with a lymphocyte-specific tyrosine kinase pick56. Analysis of proteins associated with orf-1 is under way by using the two hybrid systems.

Two small RNAs with AUUUA repeats are also involved in induction of lymphokines as suggested by several experiments. Primer extension experiments indicate high level expression of IL-4 and TNF beta mRNAs in rabbit tumor cells. Actinomycin D inhibition of RNA synthesis demonstrates that the normally unstable mRNAs (half life < 1 h) are greatly stabilized in these cells (> 8 h). Viral small RNAs appear to have a function in cellular mRNA stabilization, since mutant viruses lacking two small RNAs (HSUR1 and HSUR2) failed to express increased levels of IL-4 mRNA and cytotoxicity. Highly conserved AUUUA repeats on viral small transcripts as well as IL-4 mRNA bind and compete for lectin inducible 70 kD factor. We also report here that antisense oligonucleotides against the AUUUA repeat region in vital small RNAs can partially reconstitute rapid IL-4 and TNF-beta mRNA turnover.

In summary, the results strongly suggest a role of orf-1 and orf-2 proteins and viral small RNAs in the upregulation of lymphokines.

J. BURGYÁN[1] and M. RUSSO[2]

Localization of tombusvirus gene(s) responsible for targeting to subcellular compartments

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It is known from earlier study [Russo et al. 1987] that the genome replication of cymbidium ringspot (CyRSV) and Carnation Italian Ringspot (CIRV) tombusviruses occur in cytopathic inclusions known as multivesicular bodies (MVB). However, MVBs can be derived from different cell organelles. CyRSV replication is localized in MVBs derived from peroxisomes while in the case of CIRV MBVs are formed exclusively from mitochondria. Hybrid viruses were prepared from the available biologically active cDNA clones of CyRSV and CIRV [Burgyán et al.1990; Burgyán, unpublished results] and they were used to localize vital gene(s) responsible for subcellular sites of viral genome replication.

Burgyán, J., Nagy, P. and Russo, M.J. Gen. Virol. 71, 1857–1960, 1990; Russo, M., Di Franco, A. and Martelli, G. P. Intervirology 28, 134–143, 1987

Z. HAVELDA, T. DALMAY and J. BURGYÁN

The mechanism of generation of cymbidium tombusvirus defective interfering RNA ringspot

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Defective interfering (DI) RNAs are deletion mutants of viral genomes. They cannot replicate in the absence of the helper virus since they have lost all essential genes required for viral functions. DI RNAs are heterogeneous in size, ranging from c. 400 to 700 nt. The sequence of the flanking regions of recombination points was analyzed but it was failed to detect any homology or complementarity with each other.

It was noticed that a new smaller DI RNA (400 nt) was formed from the known largest DI RNA (DI-13, 700 nt) in planta one month after inoculation and a subsequent passage. Since the analysis of the flanking sequences did not give any positive result our attention was focused on the deleted sequence itself. Folding this sequence with MFOLD programme, the computer predicted a rather strong base-paired structure. This structure can be found if the whole C block (the last 400 nt) of the DI-13 RNA is folded and the recombination points are located up- and downstream of this 18 nt long stem region. To prove that the locally occurred highly base paired structure in the longer DI RNA is responsible for the generation of the smaller DI RNA the stem was strengthened by in vitro mutagenesis of the full length cDNA clone of DI-13. As it was expected the frequency of recombination was increased when plants were inoculated with the mutant DI RNA. The obtained result suggests that the viral replicate is not always able to unwind this base paired region and copies it, but circumvents it without releasing and produces colinear deletion mutants. This deletion event is not sequence but rather structure specific since it can be reproduced if the appropriate DI RNA sequence is substituted with foreign sequence carrying strong secondary structure.

GY. SZITTYA, T. DALMAY and J. BURGYÁN

Rapid accumulation of de novo generated defective interfering RNA dimer of cymbidium ringspot tombusvirus and its role in the cell-to-cell movement

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Defective interfering (DI) RNAs are deletion mutants of vital genomes that have frequently lost all essential vital genes required for movement, replicates and encapsidation. They need the presence of helper virus to produce transacting factors for replication. DI RNAs accumulate at the expense of helper virus from which they are derived and they frequently associated with animal and plant viruses.

The cymbidium ringspot tombusvirus (CyRSV) DI RNA and its helper virus is one of the most extensively studied DI RNA system. CyRSV is a member of the tombusvirus group, with positive-sense, single-stranded RNA genome (4733 nt). It encodes at least four nonstructural (33K, 92K, 22K and 19K) and the coat proteins (CP). CyRSV DI RNAs are mosaic molecules made up of noncontiguous regions of genomic RNA [Burgyán et al., 1989] that are generated de novo during viral replication [Burgyán et al., 1991]. The presence of DI RNA in CyRSV-infected plants attenuates symptom severity and prevents death of plants, as ascertained in experiments with in vitro synthesized DI RHA from a cDNA cleric coinoculated with in vitro transcripts of genomic RNA [Burgyán et al., 1992) and with transgenic plants expressing D1 RNA sequences [Kollar et al., 1993].

Inoculation of *Nicotiana clevelandii* plants with biologically active in vitro transcripts of both genomic and defective interfering (DI) RNAs of cymbidium ringspot tombusvirus (CyRSV) results a rapid accumulation of a new DI like RNA species. These RNAs are head to tail diners as was clearly demonstrated by cloning and sequencing of the putative junction regions. The high accuracy of junction sequences suggests that the formation of diner DI RNA is a usual step of replication of the small DI RNA molecules. It was also demonstrated that unit length DI RNAs are formed in both plants and protoplasts inoculated with in vitro transcribed diner DI RNAs.

However only the smaller DI RNAs produced diners, while the largest DI-13 RNA (679 nt) was unable to accumulate diners during vital infection. Analysis of deletion and insertion mutant DI RNAs revealed that the accumulation of diners depends only on the size of the monomer molecule used for inoculation.

The accumulation of DI RNA diners was not associated with replication, since diner DI RNAs failed to accumulate in inoculated such plant cells where the movement does not take place at all (protoplasts) or has less importance (DI RNA expressing transgenic plant). However, it is very likely that diner DI RNA is more efficient in cell-to-cell movement than its monomer counterpart, since it accumulates more rapidly (3–5 days post inoculation) in the inoculated leaves where the most important vital movement is the cell-to-cell spread.

As far as we know, this is the tint report about a closely related viral RNAs having different ability to move cell-to-cell in inoculated plant.

Burgyán,J., Grieco,F., and Russo,M. J Gen Virol 69, 235–239 (1989); Burgyán,J. Nagy,P.D., and Russo,M. J Gen Virol 71,1857–1860 (1990); Burgyán,J. Rubino,L. and Russo, M. J Gen Virol 72, 505–509 (1991); Burgyán,J., Dalmay,T., Rubino,L. and Russo, M. Virology 190, 579–586 (1992); Kollar,A., Dalmay,T., Burgyán,J. Virology 193, 313–318 (1993)

J. F. SZILÁGYI

Herpes simplex virus light particles

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Tissue culture cells infected with HSV-1 release into the medium approximately equal numbers of infectious virions and non-infectious "light particles" (L particles) which can be separated by Ficoll gradient centrifugation [1].

Unlike the virions which consist of four distinct morphological components (the packaged DNA, the icosahedral capsid which surrounds the DNA, the amorphous tegument and the vital envelope, consisting of a bilaminar membrane and glycoprotein spikes projecting from its surface) the L particles are composed only of the tegument and the surrounding viral membrane. Since they lack the vital capsid and DNA they are unable to reproduce themselves after infection of the host cells. Furthermore, cryoelectron microscopy revealed the presence of spherical membrane-enclosed inclusion vesicles (IVs) embedded in the tegument in a large proportion (60 to 80%) of L particles but not in the tegument of the virions [2]. SDS-polyacrylamide gel electrophoresis confirmed that L particles contain the membrane and tegument proteins of the virions, but lack the capsid and capsid-associated proteins. However, L particles contain five additional phosphopolypeptides (175K, 134K, 92K, 60K and 55K) which are absent from the virions [1]. Thus the presence of these unique phosphopolypeptides as well as the IVs clearly indicates that the L particles are not merely defective virions but are distinct entities produced by the infected cells.

Subsequent experiments indicated that all alpha herpes viruses produce L particles [3], that infected cells can release L particles even when virion production is inhibited [4], and that L particles may supply functions which facilitate infection by the virions [5].

The roles of the five unique phosphopolypeptides and their possible correlation with the IVs will be discussed.

[1] Szilágyi,J.F., Cunningham,C. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. Journal of General Virology 72, 661–668 (1991); [2] Szilágyi,J.F., Berriman,J. Herpes simplex virus L particles contain spherical membrane enclosed inclusion vesicles. Journal of General Virology 75, 1749–1753 (1994); [3] McLauchlan,J., Rixon,F.J. Characterization of enveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. Journal of General Virology 73, 269–276 (1992); [4] Rixon,F.J., Addison,C., McLauchlan,J. Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells. Journal of General Virology 73, 277–284 (1992); [5] McLauchlan,J., Addison,C., Craigie,M.C., Rixon,F.J. Noninfectious L particles supply functions which can facilitate infection by HSV-1. Virology 190, 682–688 (1992)

GY. SZŰCS and M. ÚJ

Detection of enteric adenoviruses by PCR and Taq I restriction enzyme analysis of amplimer

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There are 49 known human adenovirus serotypes and of them, only the enteric adenoviruses (EAds), types 40 and 41, are considered as the second causative agents after rotaviruses of gastroenteritis in infants and young children. So far, to detect these fastidious enteric adenovirus types, isolation in Graham 293 cells, subsequent typing of restriction enzyme analysis, and type specific immunoassays have been used in our laboratory. These specific methods, unfortunately, are time-consuming and unsuccessful many times. Recently, a procedure originally developed by Dr. A. Allard et al. [Progr in Clin Virol, Stockholm, Sweden, 1994. Abstract, p. 142] was introduced for the specific discrimination and typing of EArls after direct detection by PCR amplification of a highly conserved sequence in the hexon gene. By this PCR, thirty-six of viral DNAs extracted from cells inoculated with 60 stool samples were found positive. Subsequently, Taq I restriction of the 301 bp amplimers were carried out. DNA of EAds (subgenus F) could be discriminated by this enzyme digestion from DNAs of adenoviruses belonging to all other subgenera. With the use of Taq I, 14 EAds were detected of the 36 PCR positive samples. Thus, PCR detection of adenoviral DNA combined with restriction analysis of amplified products provides a valuable tool for use in diagnosis of diarrhoeal diseases associated with enteric adenoviruses.

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Bronchioalveolar carcinoma: a retroviral disease?

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Bronchioalveolar carcinoma (BAC) is a fore of undifferentiated lung adenocarcinoma. It is not associated with smoking. Its incidence is rapidly rising. Originating from the bronchiolar epithelium, its cells form sheets growing without stroma on the alveolar walls. BAC is occasionally observed with or following treated Hodgkin's disease (HI)). After surgical removal of the primary tumor(s), relapses are frequent. Recurring tumors are radio- and chemotherapy-resistant. BAC cells contain tubuloreticular filaments, inclusion bodies and other structures suggestive of vital origin. In one study [Coalson et al. Labor Invest 28:38, 1973] cytopathic effects were transferable from BAC cells to indicator cells, but no viral isolate(s) resulted. Retrolentiviral and/or immunosuppressive ethiology is further suggested for BAC associated with HD or AIDS.

Ovine infectious pulmonary adenomatosis (jaagziekte or sheep BAC) is a horizontally spreading infectious disease resembling human BAC. It is transmissible with the respiratory discharge of sick animals. A transforming retrovirus has recently been found in this entity and its genomic sequences have been cloned [York et al. J Virol 66:4930, 1992].

In our laboratory we establish cell lines from human BAC and test immune reactivity of patients and controls against first explanted and established BAC cells. We are most interested in humoral responses of married couples one with, one without the disease. Cross-reactivities would indicate shared and probably vitally encoded antigens in BAC cells.

Primers derived from a highly conserved region of retroviruses were used to amplify retroviral sequences from genomic DNA of BAC cells and other tumors. Some of the unique amplified fragments were cloned and studied. To detect similar sequences from possible vital particles, cell-free tissue culture supernatants of BAC cells and other tumors were treated with DNAse I (to eliminate cellular DNA contamination) and were precipitated with PEG. RNA, than cDNA was prepared from the pellet and used as template. Again in some instances retroviral sequences were amplified from the tissue culture supernatants of BAC cells and of the T-lymphoblastoid cell line, Jurkat. We attempt to clone and identify the alleged vital agent and compare it to other known human retroviruses. Whether such a virus plays a role in the pathogenesis of BAC or is present as a "passenger", it has to be determined.

H. ZUR HAUSEN

Human papillomaviruses and cancer

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During the past years a number of virus infections have been linked to specific human cancers. Papillomavirus infections have been shown to be etiologically related to cancer of the cervix, additional anogenital cancers and cancers of the skin. Epstein–Barr Virus, hepatitis B and possibly also C-viruses and the human T-lymphotropic retrovirus represent members of other virus groups also linked to specific cancers. The mechanism by which these virus infections contribute to cancer development seems to vary even within the same virus group. None of the virus infections is sufficient per se to induce cancer. In every instance additional mutations in host cell DNA appear to be required. This appears to lead to the disruption of specific signalling pathways engaged in normal cells in the control of potential viral oncoprotein functions or of viral oncogene transcription. Attempts are presently being made to analyze the cellular genes involved in detail.

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Correlation of human papillomavirus 16 and 18 with prognostic factors of invasive cervical neoplasias

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Forty-seven patients with cervical carcinoma were examined in order to correlate human papillomavirus (HPV) types with prognostic factors of invasive cervical neoplasias. Age, clinical stage, histological type and grade and parity were analysed with respect to HPV status as determined by a general primer mediated polymerase chain reaction (PCR) or a type specific PCR. All but one samples (98%) harboured HPV sequences: HPV 16 was found in 26 cases (55 %), HPV 18 in nineteen cases (40%) and HPV 31 in one case. The presence of HPV 18 DNA was significantly associated with cancers developed below 40 years of age (p=0.029). HPV 18 detection was associated with poor differentiation grade (p=0.045) and histological types of poor prognosis (adenocarcinoma or non-differentiated carcinoma; p=0.006). HPV 18 positivity was also correlated with advanced clinical stages (FIGO II and III; p=0.032). Parity and HPV status proved to be independent of each other (p=0.99). Eighty-seven percent (27/31) of pelvic lymph nodes from HPV positive patients contained HPV DNA. The virus types found in lymph nodes were identical to those of the primary tumours in all cases. Virological results were compared to those obtained by routine histological examination. Only 6 of 27 patients with HPV positive lymph nodes had any histological evidence of metastasis. Nevertheless, the lack of metastasis as detected by histology does not exclude the possibility of relapses. Following the clinical course of the disease is required to assess the prognostic significance of PCR detection of HPV in the possible sites of early metastases.

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Transformation of human peripheral blood T-cells by coinfection with human herpesvirus-6 and Epstein–Barr virus

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Recent studies linked the presence of EBV genomes not only to B-cell lymphomas, but also to nasal T-cell lymphoma, angioimmunoblastic lymphadenopathy (AILD) like T-cell lymphoma, peripheral T-cell lymphoma of immunocompetent host, and T-cell lymphoma in AIDS patients. These reports indicate that infection of T-cells by EBV is relatively frequent and EBV may be an important factor in some T-cell malignancies. Several recent studies also indicated that some T-cell lines may be

infected directly with EBV. In these cell lines, however, EBV exists as linear molecule associated with lytic cycle but not in episomal form and does not express EBNA-1. To date, no in vitro model existed were EBV could infect T-cells and establish transformation and latency. Human Herpesvirus-6 (HHV-6) is a relatively new Herpesvirus which is topic for T-cells. Infection of T-cell lines and T-cells, isolated from cord blood (CB) or peripheral blood (PB), with Human Herpesvirus-6 (HHV-6) bring on the expression of new cellular markers and lymphokines in the infected cells. Within 24 hours the infected cells are expressing CD1, CD4 and CD21 (which is the cellular receptor for the Epstein–Barr virus) molecules on their surface. We identified several T-cell lines which can be infected with EBV in the presence of HHV-6 and also demonstrated that T-lymphocytes, from cord blood or peripheral blood, can be transformed with EBV and HHV-6 into immortalized cell lines. These cell lines were grown in tissue culture for three years without losing the EBV genome. While all cell lines expressed EBNA-1 in 100% of the cells, the EBNA-2, EBNA-3 and LMP expression varied, and in some cell lines EBER expression was absent. The majority of T-cell lines were strictly non-producers while a few cell lines expressed EBV viral antigens associated with the lyric cycle in 8 to 15 % of the cells. Some of the cell lines will retain both EBV and HHV-6 genome, however the majority of the established cell lines (over 80%) will lose the HHV-6 genome after longer time in tissue culture. These studies suggest that HHV-6 may be a helper factor for EBV for the infection of T-cells in vivo, and both EBV and HHV-6 may play a role in the development of some T-cell malignancies and in development of immunodysfunctions in humans. Data will be presented for the possible role of HHV-6 and EBV co-infections in AIDS patients. Analysis of these in vitro established T-cell lines and the use of our method, which allows the introduction of EBV into normal T-cells, may lead to a better understanding of the role of EBV in the T-cell malignancies and immune processes in immunocompromised patients.

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Cellular control of Epstein–Barr virus latency

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Epstein–Barr virus (EBV) can establish two different types of latency in B-lymphocytes. In lymphoblastoid cell lines (LCL) six nuclear antigens (EBNA 1 to 6) are transcribed from BCR2 or BWRI promoters. In the EBV carrying BL cell lines and in normal resting B cells only one nuclear antigen, EBNA 1 is expressed however from another promoter, Qp. We have investigated the role of transcription factors in the establishment of distinct latency programs in different B cell phenotypes. NFkB is a transcription factor that has a key role in the regulation of many genes including those

involved in B cell activation and differentiation. We have observed several fold differences in the amount of NFkB subunits between cells of activated and resting phenotypes. This suggested that the levels of NFkB can be a marker of EBV latency in B cells. Origin of latent replication (oriP) family of repeats (FR) act as transactivator or repressor of BCR2 activity depending on the presence of EBNA1 in reporter constructs. However in group I BL lines, the oriP is at much lower activity than LCLs despite the presence of EBNA1. This raised the possibility that other factors interacting with oriP in activated cells contribute to the regulation of oriP, hence BCR2 activity. We studied the oriP FR binding transcription factors in EBV host cell types by electrophoretic mobility shift assay (EMSA). We found that octamer transcription factors (OTF1 and -2) and EBNA1 form complex with oriP. OTF2 joins the complex only in blastic cell lines. OTF factors are essential for the activity of many promoters and enhancers. OTF2 is crucial for the activity of immunoglobulin promoters in blastic cells. These results implicate the role of EBNAI-OTF interaction in the regulation of viral latency and the potential of EBNA1 in activating the cellular promoters and enhancers that contain octamer sequences. The development of different latency programs in B-lymphoid cells seems to be mediated through the interaction of stage specific cellular and viral factors.

L. SZÉKELY

EBNA-5 in the Epstein–Barr virus mediated cell transformation

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Epstein–Barr virus (EBV) very efficiently transforms human B-lymphocytes. Five virus encoded nuclear antigens (EBNA-s) and a membrane protein (LMP) are necessary for the transforming function. EBNA-5 together with EBNA-2 are the earliest expressed EBNA-s. These two proteins are sufficient to drive resting B-cells into GI phase of the cell cycle. EBNA-5 is a nuclear matrix associated phosphoprotein that accumulates in nuclear bodies at the later stages of latent infection and co-localizes with the retinoblastoma (Rb) tumour suppressor protein, heat shock protein hsp70 and the PML protein. This later protein is involved in the genesis of acute promyelocytic leukemia where the PML gene translocates to the gene of retinoic acid receptor alfa forming a fusion gene.

EBNA-5 is expressed at the highest levels during the early phase of latent infection and its expression maximum coincides with the increased expression of p53 and Rb proteins. EBNA-5 is able to form molecular complexes with these two proteins in vitro.

Székely,L., Üzvölgyi,E., Jiang,W-Q., Durko,M., Wiman,K.G., Klein,G. and Sumegi.J. Subcellular localization of the retinoblastoma protein. Cell Growth and Diff 2, 287–295 (1991); Jiang,W-Q., Székely,L., Wendel-Hansen,V., Ringertz,N., Klein,G. and

Rosen,A. Co-localization of the retinoblastoma protein (Rb) and the Epstein–Barr virus encoded nuclear antigen EBNA-5. Exp Cell Research 197, 314–318 (1991); Székely,L., Selivanova,G., Magnusson,K., Klein,G. and Wiman,K.G. EBNA-5, an EBV encoded nuclear antigen binds to the Rb and p53 proteins. Proc Natl Acad Sci USA 90, 5455–5459 (1993); Székely,L., Pokrovskaja,K., Jiang,W-Q., Selivanova,G., Löwber,M., Ringertz,N., Klein,G. and Wiman,K.G. Epstein–Barr virus induced B-blasts and established lymphoblastoid lines differ in their Rb, p53 and EBNA-5 expression. Oncogene 10, 1869–1874 (1995); Székely,L., Jiang,W-Q., Pokrovskaja,K., Klein,G., Wiman,K.G. and Ringertz,N. Reversible nucleolar translocation of the EBV-encoded EBNA5 and Hsp70 proteins after exposure to heat shock or saturation conditions. J of General Virol (in press)

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Differential expression of D-type cyclins in B cell lines in relation to cell phenotype

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EBV infects human B cells in vivo and efficiently immortalizes peripheral blood lymphocytes in vitro. Lymphoblastoid cell lines (LCLs) raising after EBV infection of B cells display activated immunoblastic phenotype. Burkitt lymphoma (BL) phenotypically resembles B centrocytes from germinal center. EBV+BL (BL group I) often drift spontaneously towards activated, LCL-like phenotype (BL group III) starting to express EBV latent genes and LMPs.

Key regulators of G1 progression in mammalian cells include three D-type cyclins (D1, D2 and D3), and cyclin E. The three D-type cyclins are induced in a lineage specific manner as part of the response to mitogens, and it is unclear whether they carry out redundant functions or their roles are different. In haematopoetic cells cyclin D1 is normally not expressed unless it is activated by the translocation t(11;14) in the centrocytic lymphomas. Cyclins D2 and D3 are differentially expressed in haematopoetic cells.

We studied the cyclin D2/D3 expression in LCLs, BL cell lines and in purified freshly EBV-infected CD19+ B lymphocytes at the mRNA and protein level. We found that cyclin D2 is expressed in the LCLs and BLs with activated LCL-like phenotype, whereas only cyclin D3 is expressed in the BL cell lines that retain original tumor cell phenotype (BLs group I) and in the EBV-negative BLs in vitro converted by EBV. Interestingly, in some of the BL cell lines (Namalva, Jijoye M13, Raji) cyclin D2 is not expressed or is expressed at the low level (BL16, BL36 and E95-B-BL28). These cell lines partially display some of the activation markers (CD39, CD23) characteristic for LCLs, but retain to the different extend BL-specific cell surface antigens CD10 and

CD77. The level of cyclin D2 expression in BL16, BL36 and E95-B-BL28 correlates with the partial acquisition of an LCL-like phenotype.

Cyclin D2 is detectable by immunostaining in the nuclei of the EBV-activated blasts. Immunoblotting showed that the level of cyclin D3 protein is 5–7 times lower in LCLs compared to BLs group I. Cyclin D3 protein is not detectable in LCLs by immunostaining with anti-cyclin D3 specific antibody. Neither D2 nor D3 are expressed in resting B cells. However, cyclin D3 is detectable in 20–50% of the activated blasts early after EBV infection of primary peripheral blood B cells. Thus, in addition to cyclin D2, cyclin D3 is induced upon EBV infection of rating B cells. The level of cyclin D3 48 hr post-infection detected by immunoblotting with the specific antibody is similar to the level of this protein in exponentially growing LCL.

Immunocytochemistry for cyclin D2 in LCLs and cyclin D3 in BLs group I showed that about 5–20% of the exponentially growing cells displayed positive nuclear staining, probably due to the fact that these cyclins act at the restricted time point in G1 of the cell cycle.

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Anti-herpesvirus activity of different acridine derivatives

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The antiviral effect of eight acridine derivatives was studied on the multiplication of herpes simplex virus type 2 (HSV-2) in Vero cells. Two derivatives namely 2,7-dimethoxy-9-thioacridinone and 2,7-dihydroxy-9(3'-aminopropyl)-thioacridine had potent inhibitory effect on virus yield while another compounds had moderate activity or only slightly affected the virus multiplication. The inhibitory effect of compounds on the virus infectivity was also tested and two of them showed virucidal activity. 2,7-dihydroxy-9(3'-aminopropyl)-thioacridine inhibited the virus replication without any direct effect on the virus infectivity.

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Polymerase chain reaction assay for the detection of chicken anaemia virus

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An AmpliWax-mediated hot start polymerase chain reaction assay (PCR) was developed for the detection of chicken anaemia virus (CAV) DNA in various biological samples. The detection limit of CAV DNA from MSB1 cells infected with CAV increased by 50 times if wax mediated hot-start PCR was performed instead of using mineral oil overlay. When the DNA templates were extracted from CAV infected MSB1 cells or from cell-free CAV containing tissue culture fluid by the guanidine thiocyanate (GuSCN) and nucleic acid-binding silica particles as described by McCaustland et al. [1991], we were able to detect CAV equivalent to 1 infected cell or 1 $TCID_{50}$ respectively. Furthermore, the construction of a spike is described which can be used as an internal control to validate negative PCR results and to estimate the number of CAV DNA copies present in the test material.

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Comparison of the structure of adenovirus hexon antigens belonging to two different subgenera

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Antigenic relationships of hexons within human adenovirus (Ad h) subgenera C and D were studied using 61 monoclonal antibodies (MAbs) raised against Ad h1, Ad h35 and bovine type 2 hexons. The reactivity pattern (RP) and titres of the MAbs were determined in indirect ELISA. According to previous experiments with hexons of the six human subgenera and three animal serotypes, 45 of the MAbs displayed at least 15 different intertype specific RPs besides the genus specific and type specific ones. With the four types of subgenus C (1, 2, 5, 6) all MAbs gave identical RPs except three, which were type specific to Ad h1. The titres of the individual MAbs with all hexon types were generally high and the most frequently found titre values were evenly distributed among them. Thus this results reveal the existence of a remarkable homogeneity in the antigenic structure among the types of subgenus C defined by the presence of identical or closely related intertype specific epitopes on the surface of hexons.

Of subgenus D hexons of 8 types (8, 9, 10, 13, 10, 26, 27 and intertype 9/13 {I13}) eight different RPs could be distinguished. The RP of hexons of types 8, 9, 10,

I13 and 19 were identical or nearly identical and the occurrence of the eight different RPs were based mainly on the combination of positive or negative reaction of different MAbs with hexons of types 13, 26 and 27. According to these data three groups of hexon types with diverse antigenic structure could be distinguished: types 8, 9, 10, I13 and 19 differ remarkably from type 27, while types 13 and 26 represent an intermediate position between the two groups. Thus – in contrast to subgenus C – a remarkable diversity exists in the antigenic structure of subgenus D hexons.

GY. FEJÉR, ZS. RUZSICS, A. KISS, CS. JENEY, T. TAKÁCS and M. BENCZIK

Characterization of adenoviral early promoters in different biological situations

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The early promoters of adenoviruses are known to be induced by the immediate early E1A proteins. This is mediated by the direct transactivational effect of the 13S E1A product and also by the activation of cellular transcriptional factors by both the 13S and 12S E1A products. Since the distribution and the inducibility of these transcription activators can be tissue specific, it seems likely that cell type specific factors can modify the general transactivator function of the E1A gene.

During our presented work we wanted to study the possible effect of tissue specific factors on the activation of the adenovirus early region 2 (E2) and early region 3 (E3) promoters, the expression of the E3 and E2 genes was measured in vivo using mice infected intraperitoneally with CsC1 purified adenoviruses. The amount of different mRNAs in different organs was quantified with RNase protection and quantitative RT-PCR experiments. Transgenic mice containing the entire adenovirus E3 region were treated with bacterial lipopolysaccharide (LPS) and the inducibility of the E3 promoter was demonstrated. This is probably carried out by the LPS induced cytokine liberation which can activate different signal transduction pathways. In order to study this, the activity of the cloned E2 and E3 promoters was measured in vitro using luciferase expressing vectors driven by either the E2 or E3 promoters transfected transiently into different cell lines.

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DNA sequencing and phylogenetic analysis of bovine adenovirus types 1, 2, 3, 4, 7, 9, and 10 justify the existence of two subgroups

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Bovine adenoviruses (BAVs) are divided into two subgroups based upon their biological properties. DNA restriction enzyme analysis and hybridization studies further support this subgrouping. We started to sequence the genome of different bovine adenovirus types. First, we obtained the complete DNA sequence encoding the proteinase (23 kD protein) of bovine adenovirus type 1, 2 and 4. Other genes have been partially sequenced. Analysis of the sequence data showed that the AT content of the DNA of subgroup 2 BAVs is significantly higher (around 61–67%) than that of other mastadenoviruses (39–52%). Since the sequence of the proteinase gene had been determined for a large number of different adenoviruses, a detailed phylogenetic analysis could be performed based on this gene. Other (partial) sequences were similarly analyzed to prove our findings. Based on parsimony and distance matrix or maximum likelihood analysis, the subgroup 2 BAVs were found to be genetically distant from subgroup 1 BAVs and from other known members of the *Mastadenovirus* genus, while all the mastadenoviruses (excluding subgroup 2 BAVs) were found to cluster together.

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Surprising genetic relatedness between the egg drop syndrome virus and the subgroup 2 bovine adenoviruses

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The egg drop syndrome (EDS) virus is a member of the *Aviadenovirus* genus, however, it seems to differ from other aviadenoviruses. The complete DNA sequence of the proteinase (23 kD protein) gene, and partial DNA sequence of the hexon gene of the EDS virus were determined. These sequences showed a relatively high AT content of approximately 59%, while the average AT content of the genomes of the so far examined other aviadenoviruses had been estimated to be around 45–46%. Using the available sequences of the proteinase and the hexon genes of different adenoviruses, a detailed phylogenetic analysis was performed. EDS virus was found (by parsimony and

distance matrix or maximum likelihood analysis) to cluster together with the subgroup 2 bovine adenoviruses (BAVs). EDS virus seemed to be genetically more closely related to subgroup 2 BAVs, than subgroup 2 BAVs are to other mastadenoviruses, or EDS virus is to other aviadenoviruses, respectively. Because of the limited DNA sequence data of EDS virus, further study is needed for a final conclusion.

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Detection of bovine adenovirus type 10 by in situ DNA hybridization

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Bovine adenovirus (BAV) type 10, was first isolated in New Zealand from a heifer which died from systemic adenovirus infection. Four further BAV-10 strains were isolated in Northern Ireland, all of them from fatal cases of enterocolitis characterized by adenovirus-associated nuclear inclusion bodies in vascular endothelium of the intestine. There have been several reports from other countries of similar cases of fatal enterocolitis in cattle. In most cases, the type of the BAV involved could not be determined, but BAV-4 and BAV-7 were recovered respectively from 2 separate cases. In sire DNA hybridization tests were performed on stored (or archival) paraffin embedded organ samples from twelve cases of this disease using type (BAV-10) specific and subgroup 2 specific probes. All cases hybridized with the BAV-10 probe. None hybridized with the subgroup 2 specific probe. This provides further evidence for the role of BAV-10 in fatal enterocolitis. However, the study needs to be extended to include more cases of the disease in order to determine if BAV-10 alone or certain other serotypes have significant role in provoking this disease.

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Mapping of naturally occurring deletions in the genome of bovine adenovirus type 10

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Four bovine adenovirus strains isolated from fatal enterocolitis cases of cattle and typed by virus neutralization test as BAV-10, were compared to the prototype strain of BAV-10. The isolates exhibited considerable variation in their restriction enzyme patterns. The genome sizes of the strains also varied between 24 and 28 kb. After cleavage with *Hin*dIIIenzyme, four out of the five BAV-10 strains differed from each

other in the size of fragment A only, while the RE patterns of the fifth strain looked very divergent with each enzyme. In order to localize the variable fragment, and determine its genetic importance, we have started to clone and map the genomes of the BAV-10 strains. Physical maps and conclusions will be presented.

VIROLOGY, IMMUNOLOGY AND PARASITOLOGY

K. NAGY, V. VÁRKONYI and A. HORVÁTH

Sequence analysis of V-3 loop and replication capacity of HIV strains isolated in Hungary

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The delayed occurrence and the still low rate of the AIDS epidemic in Hungary raise the possibility of the circulation of HIV subtypes with altered replication capacity and pathogenicity compared to those strains of HIV detected in Western Europe and the USA.

As predominant mode of transmission of HIV in Hungary is the homosexual transmission, we isolated and characterized HIVs to analyze transmission of HIV in this high risk population.

HIV detection and virus isolation were carried out from the peripheral mononuclear cells (PMC). The presence of HIV provirus in the patients' PMCs were directly detected by PCR amplification using *gag* primers, followed by DNA hybridization. HIV was isolated from PMCs as well as by cocultivation techniques. Permanent cell lines of lymphoid and mononuclear/macrophage origin were productively infected by cell-free virus preparations from the patients. Biological (syntitium induction) and replication characterization of the isolates have been determined.

DNA sequencing of HIV-1 *env* V3 loop encoding region was carried out by the chain termination method: with PCR amplification of a 211 nucleotid fragment (7063-7274) of HIV-1 *env*, containing the entire V3 region and surrounding sequences, using appropriate synthetic oligonucleotides as primers. Alignment comparison was made with HXB-2, HIV-1 BRU (LAV-1) and HIV-1 NL43 (Ny5).

Replication analysis shows that the HIVs isolated from homosexual persons are of the rapid/high (R/H or SI) phenotype. High similarity (96.9%) to HIV-1 BRU *env* V3 region, containing the CPGR sequence motif at the highly immunogenic tip of V3 loop suggest that these isolates belong to the B subtype of HIV similarly to LAV-1.

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Studies on the mechanism of complement-mediated antibody-dependent enhancement of HIV infection

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We have recently demonstrated that complement (C')-dependent infection enhancing antibodies correlate with immunosuppression and disease in HIV-1 infected individuals. The aim of the present study was to reveal the mechanism, by which these antibodies exert their effect(s). Three human anti-gp41 monoclonal antibodies were found to enhance infection of MT-4 cells by HIV-1 in the presence of human C'. Using sera deficient for various C' components (C2, Bf, MBP), we could demonstrate that the activation of C' necessary for the enhancing activity proceeded by the classical C' pathway. Blocking of the type 2 C' receptor (CR2) by a monoclonal anti-CD21 antibody inhibited the C'-dependent enhancing effect of all anti-gp41 antibodies. Significant enhancement of HIV-1 infection was also found when purified C1q and anti-gp41 were applied to HIV before infection. However, the rate of enhancement mediated by the complete C' system was higher than that mediated by C1q alone. These data suggest that the C'-mediated infection enhancing effect of gp41 antibodies cannot be explained exclusively by the interaction between C3b bound to HIV and CR2. Especially in the presence of high levels of these antibodies C1q is also bound to the virus particles and may facilitate their uptake by the cells through the collectin (C1q) receptors.

L. DI RENZO

Complement factor 3 molecules act as autocrine growth factor in Raji cells

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Burkitt lymphoma (BL) derived cell lines and lymphoblastoid cell lines obtained by transformation of normal B cells with EBV in vitro grow in an autonomous fashion. It has been shown that they release growth factors in their supernatants. It is known that fragments derived from complement factor 3 (C3) modulate the growth of B cells through their binding to complement receptor type 2 (CR2).

The work presented here was aimed to verify whether Raji cells, a BL derived cell line, synthesize C3 molecules and to study their eventual function on cell growth.

Biosynthetic labelling studies demonstrated that Raji cells synthesize C3 molecules, which undergo fragmentation, as detectable by Western blot analysis. Northern blot analysis of total RNA from Raji cells hybridized with a human C3 cDNA

probe determined that they expressed the 5.2 Kb mRNA for C3. The addition of anti C3d antibodies to Raji cells, cultured in serum free medium, caused a 35% inhibition of cell proliferation.

Taken together, these results indicate that endogenous C3 molecules act as autocrine growth factor in Raji cells. In tumour cell lines the inappropriate expression of oncogenes and suppressor genes may contribute to cause the cells to bypass control mechanisms, such as the dependence on external stimuli: whether the synthesis of C3 molecules represents an advantage of transformed B cells or is common to all B cells, is presently unknown and deserves further investigation. The possibility that BL cell lines synthesize also a C3 related molecule will be discussed.

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Interactions between human T-cell leukemia-lymphoma virus type I and human cytomegalovirus in human term syncytiotrophoblast cells cultured in vitro

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The placental syncytiotrophoblast (ST) layer serves as the first line of defense of the fetus against viruses. We analyzed the patterns of replication of HTLV-I and HCMV in singly and dually infected ST cells. HCMV was capable of establishing a latent infection in ST cells, with restriction of gene expression to immediate early (IE) and early (E) proteins. Although ST could be infected with cell-free HTLV-I, no viral protein expression was found in the singly infected cells. Coinfection of the cells with HTLV-I and HCMV resulted in simultaneous replication of both viruses. Bidirectional enhancing activities between HTLV-I and HCMV were mediated primarily by the Tax and IE proteins, respectively. The stimulatory effect of HTLV-I Tax on HCMV replication appeared to be mediated partly by tumour necrosis factor β and transforming growth factor β-1. We observed formation of pseudotypes with HTLV-I nucleocapsids within HCMV envelopes. Our results suggest that in vivo dual infection of ST cells with HTLV-I and HCMV may contribute to the transplacental transmission of both viruses.

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Activation of HIV-1 by HHV-6 in vivo and in vitro

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Progression of HIV-1 infection to AIDS might depend on the virus activation by external factors, e.g. soluble mediators and heterologous viruses, as in vitro data suggest. Although both pathways act by binding cellular transcriptional factors to HIV-1 LTR consensus sequences, no data are available on the activation of HIV-1 by mediators induced by heterologous viruses. Human herpesvirus type 6 (HHV-6) can either accelerate or diminish in vitro HIV-1 replication, but its in vivo role in AIDS development has not been revealed yet. We studied the frequency of both latent infection (by *env*-specific PCR) and expression of HHV-6 in PBMCs at mRNA level (by RT-PCR) in different groups of HIV-1 infected patients (haemophiliacs, IVDAs) during successive stages of AIDS. HHV-6 expression was shown to enhance mortality of AIDS patients by approx. 35%. Expression of carried HHV-6 in HIV-1 seropositive patients was found 6.1 times more frequent than in HIV-1 seronegative subjects. From early stages onwards, HIV-1 expression has been stably associated with HHV-6 expression through disease progression, but is not associated with silent HHV-6 carriage. To assess if HHV-6 induced humoral factors may alter HIV-1 replication, HSB-2 lymphoid cells were infected by HHV-6 (strain GS) and/or treated with bacterial endotoxin CLPS). At different time intervals supernatant of these cultures were filtered virus free. ^{35}S-Met/CYs labelled proteins and cytokin content were analysed. Supernatant aliquots were mixed to CEM-ss cultures infected with a range of HIV-1 inocula to quantitate syncytia, RT and p24 antigen production. Although HHV-6 CPE developed from day 10 to day 15, TNF release showed twin peaks on days 1 and 4, it peaked at 12 with LPS treatment. HV-6 did not induce WN release, but it delayed WN induction by LPS between days 1 and 4. No IL-2 was produced by any HSB-2 culture. Virus free supernatants of HHV-6 infected cells collected between 12 h and 4 days p.i. gradually increased all three parameters of HIV-1 replication upto 30-fold, while supernatants at full blown HV-6 replication decreased HIV-1 production. It is concluded that TNF-D contributed to the continuous, basal level HIV-1 replication, while WN induced by LPS decreased HIV-1 activation. The very efficient, but transient HIV-1 activation is attributed to another diffusible product possibly a 38 kD polypeptide demonstrated in early supernatants between days 1 and 5, of HHV-6 infected cells. These data demonstrate that virokines might have an important role in the activation of HIV-1 in vitro and in vivo.

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Prevention by TP-4 of activation induced apoptosis of T-cells from HIV infected individuals

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Activation induced programmed cell death (apoptosis) accounts for functional defect and selective loss of CD4+ T-helper cells in HIV infected patients, and inappropriate induction of a form of apoptosis in Th cells is involved in the pathogenesis of AIDS.

We have investigated the possible regulating effect of Thymocartin (TP-4) – a synthetic tetrapeptide, representing the active site of the thymus hormone thymopoietin – on the apoptosis of in vitro cultured lymphocytes of HIV infected patients.

Separated peripheral blood mononuclear cells (PBMC) from 10 asymptomatic HIV-1 infected patients with CD4 cell counts <500/µl, as well from 7 healthy individuals were plated on 24-well trays. For in vitro activation/stimulation human recombinant IL2 (20 IU/ml) was used; 24 h after activation TP-4 treatment (0.1 and 0.001 µg/ml) was performed.

Progress of apoptosis in the stimulated, stimulated and treated as well as non-stimulated and non-treated cells was evaluated by (a) morphological analysis, (b) immunoperoxidase (Apoptag) detection of fragmented DNA, (c) quantitative flow cytometric assay and (d) agarose DNA fragmentation assay.

The detailed quantitative analysis revealed an advanced progress of apoptosis in cultured cells of HIV infected patients compared to uninfected individuals (d=50); however TP-4 treatment of stimulated cells resulted prevention of progression of apoptosis in 62% of the cases. The most remarkable recovery was observed in cells from those with higher CD4 counts, independently of the duration of the HIV infection.

These findings suggest that in vivo CD4+ T-cell apoptosis may account – independently of HIV mediated cytopathic effect – for the progression of AIDS and thymic hormones may play an important role to control this process, which might have therapeutic importance.

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Feline immunodeficiency virus (FIV) infection of cats – a seroepidemiological survey

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Serum samples taken from 150 clinically healthy and 45 sick cats originating from different pads of Budapest were tested for the presence of antibodies to feline immunodeficiency virus (FIV). We used an indirect ELISA, employing a FIV antigen clarified by gradient ultracentrifugation, and the results were validated by using Western blot.

FIV infection could not be diagnosed in healthy cats, while one third of the sick cats proved to be FIV positive. The overall incidence of FIV among clinical samples was approx. 7%.The FIV-infected cats were generally domestic males older than two years of age, and they had regular access to outdoors. Feline urologic syndrome occurred frequently among the affected cats, while other clinical signs considered typical for FIV or FIV-RC were underrepresented in our study. Follow-up study was not done, because all of the FIV positive sick cats died within 6 months after initial testing.

It has to be pointed out that – solely on the basis of the anamnestic data, the course of infection or the clinical manifestation – FIV infection can neither be diagnosed nor differentiated from the feline leukemia virus (FeLV) and other primary or secondary infections.

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The dominant T-helper function of lymphocytes of patients with HIV infection

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The clinically asymptomatic phase of the HIV infection is characterized by the increased activity of the immune system, which contributes to the spread of the virus in vivo, and the progression of the disease.

The aim of our study was to analyse the progression of the immunopathological processes in connection to the cytokine expression of the Th1 and Th2 phenotype of the CD4+ cells, as well as to determine the possible phenotypic dominancy.

Selected patients were grouped into four categories: (a) 10 patients with HIV infection, (b) 10 persons who were seronegative sexual contacts of those in group (a), (c) 10 persons with atopia, and (d) 10 healthy control persons. Expression of mRNA of

IFNγ as well as IL-10 have been determined from the lysates of the separated lymphocytes of the patients by semiquantitative rPCR. Total IgE and specific IgE antibodies for common allergens have been detected from all of sera.

Results show that both the IFNγ and IL-10 expression were increased in those with HIV infection compared to the healthy control. In the HIV-infected group a dominancy of the expression of IFNγ could be observed in those with >450 CD4+ cells/μl, while IL-10 expression was characteristic in the patients with <450 CD4+ cells/μl.

As a conclusion, our results do not support: (1) a TH2 as well as a TH1 dominancy; (2) a connection between IL-10 level and CD4+ cell count; (3) a connection between serum total IgE antibodies and IL-10 transcription; (4) a TH1-TH2 subset fenotype switch in asymptomatic HIV infected people.

Our results support a general activation of immune system, because increased expression of cytokines characteristic of both Th1 and Th2 functions have been detected.

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In vitro decrease of natural killer activity of chicken granulocytes against adenovirus infected target cells

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We described that in the peripheral blood of chicken the granulocytes are responsible for the natural cytotoxicity against spontaneously transformed chick fibroblasts (LSCC-H32) as target cells. The human adenovirus infected LSCC-H32 cells are not producing interferon as the normal chick fibroblasts, however, their sensitivity to the cytotoxic activity of granulocytes is decreased. With the use of adenovirus mutants that lack the region E1A, E3 or E4 the role of these regions in the impairment of sensitivity has been excluded. All human adenoviruses tested so far induce the synthesis of the heat shock protein 70 in the target cells. Target cells in the presence of antibody against the heat shock protein 70 regain their sensitivity to the cytotoxic effect of granulocytes. These findings may provide explanation how adenoviruses result in persistent infection through their ability to avoid the first line host defense.

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Study of pentoxifylline induced modulation of cytokine secretion and ICAM-I expression in septic patients following surgical infections

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Tumour necrosis factor (TNF) has a pivotal role in the pathogenesis of sepsis and septic shock. Suppression of its biosynthesis might therefore be one of the strategies in the treatment of sepsis.

When peripheral white blood cells were stimulated with either LPS or *Staphylococcus aureus*, pentoxifylline (PTX) inhibited TNF production. In contrast, no inhibitory effect was observed on the induction of interleukine-6 (IL-6). PTX inhibited not only the TNF production of monocytes, but also the TNF secretion of both granulocytes and unseparated whole blood. The in vitro TNF and IL-6 producing capacities in septic patients (n=31) were higher than in healthy blood donors (n=15). Administration of PTX (400 mg/day) to 20 of the septic patients resulted in TNF and IL-6 productions similar to those found in healthy controls. It also subsequently led to an improvement of the clinical status classified by the APACHE II score. The ICAM-I level in the sera of septic patients before PTX treatment was significantly higher (800–1200 ng/ml) than in normal individuals (50–150 ng/ml), but it decreased following PTX therapy. Mononuclear cells of septic patients (n=5) exerted a higher ICAM-I expression; the intensity of fluorescence after anti-ICAM-I staining was 1.5±0.25 times higher than normal controls even without in vitro stimuli, which might be a consequence of their in vivo primed conditions. FACS analysis revealed that the expression of ICAM-I on stimulated mononuclear cells was inhibited by PTX. It is presumed that the suppressive effect of pentoxifylline on TNF production may be of clinical importance, improving the therapeutic strategies in septic syndrome.

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Experiences gained on screening for Hepatitis B surface antigen (HbsAg) among pregnant women

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It is well known that Hepatitis B virus (HBV) infection is commonly transmitted from HBsAg positive mothers to their infants. Almost all infants who are infected neo- or perinatally become carriers. These cases develop at a rate of about 90% into the chronic form of hepatitis and some decades later a quarter of them may progress to cirrhosis, hepatocellular carcinoma and death.

Specific immunoglobulin (HBIG) applied within 12 h of birth and the concomitant initiation of a tree-dose Engerix B vaccine series prevents the vertically transmitted infection with more than 90% effectiveness.

In a three and half-year period (September 1991–April 1995) 34 HBsAg positive women (0.3%) were discovered among the 10 848 pregnants tested by the virological laboratory of the Institute. The effectiveness of the immunization among the vaccined infants was controlled by HBsAg and anti-HBs microelisa tests at the age of 15 months. The results showed no HBsAg positivity in the 11 children checked. The antibody titres were also mostly satisfactory with the exception of two cases when a fourth dose of vaccine administration was suggested.

Considering that before the mentioned period no screenings were carried out, HBsAg positivity was not always revealed during the first pregnancy, so the investigation was focused on children born earlier in these families. In the case of four families former HBV infection with maternal origin could be detected. These data will also be detailed.

On the basis of the results it is clear that screening of pregnant women for HBsAg is beneficial. With the help of immunization the health of the affected offsprings could be protected and the formerly infected, asymptomatic children revealed as HBV carriers could get into infectological care. Furthermore interferon therapy could be applied when it was necessary.

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Sporadic Hepatitis E virus (HEV) infections in Hungary

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Hungary does not belong to the HEV endemic territory of the world and no HEV epidemics have been registered yet. Nevertheless, sporadic occurrence of the infection was detected in different non-endemic regions of the world using the recently developed recombinant reagents (ABBOTT Ltd). Sera of hepatitis patients negative for non-A, non-B, non-C (NABNC) markers, health care workers and different globulin preparations were tested in order to detect sporadic cases of HEV in Hungary. The HEV reagents used detected both IgG, and IgM type antibodies.

Two groups of blood donors were tested, and 48 of 957 (0.05), and 8 of 274 (0.03). The latter group consisted exclusively from young soldiers at the beginning of their compulsory service period.

Twenty NANBNC patients of 404 were found to be HEV positive including several chronic hepatitis cases, and 5 proven acute hepatitis cases verified by the

increase of antibody titres in paired sera. Surprisingly the mean age of the HEV positive patients was found to be 48 years of age.

Only 1 of 33 haemodialysis patient was found to be seropositive for HEV, indicating that this treatment is not a risk factor in the department tested.

Three persons of 77 health care workers (one of them of Arabic origin from endemic geographical region) were positive indicating, that longer than 10 years professional exposition (diagnostic virology or haemodialysis ward) has not been associated with elevated frequency of HEV seropositivity (2/77–0.028).

All Hungarian gamma globulin preparations prepared in 1994 were found to be positive for HEV antibodies. In contrast to this, several products, manufactured from blood donations from 1992 were found to be seronegative.

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Immuno-potentiating effect of attenuated viruses and chemical biological response modifiers

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The authors investigated the natural-defence-mechanism increasing activity of attenuated Newcastle Disease virus-, Gumboro virus- and Avian Infectious Bronchitis virus vaccines, as well as the one of following BRM-s of chemical origin:

Quaternary ammonium base QAB: ARQUAD 2 HT, DDA-QUAB preparation both kindly supplied from Utrecht, a lipo-polyamine preparation signed LPA.

The main target of these investigations was to define the manipulative potential of ahead substances influencing the natural resistance of mice infested with Ehrlich Ascites tumour and S180 mouse sarcoma strain (Lewis lung tumour was also applied but without valuable results).

Evaluation of the efficacy of applied substances has been based on the survival-prolonging activity in tumour-infested mice. The appropriate supply of applied substances was described already in our previous publications concerning the dosage and timing of substances' supplies. The degrees of natural-defence-increasing activities, i.e. the differences between survivals' periods were evaluated statistically, applying the T-statistic, defining the skewness and kurtosis coefficients with standardized values of each, the polynomial smoothing. Simple and multifactor ANOVA analyses were always applied.

In regard to the life prolonging activity of the investigated viruses and chemical compounds the applied tumour strain did not influence the experiment even in statistical sense. So, a statistically and biologically reasonably supported efficacy-hierarchy was confirmed between the "antitumour", i.e. natural-resistance-increasing activity of the materials applied throughout the experiments.

Dominant survival prolonging products were: Gumboro virus vaccine and the two quaternary ammonium bases, the Arquad-QUAB and DDA-QUAB. Mediocre effect exhibited (in succession of activity) Avian Bronchitis vaccine, LPA and Newcastle disease virus vaccines. None of the investigated substances exhibited non-protective activity.

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Examination of chickens for presence of *Mycoplasma gallisepticum* antibodies by immunoblot

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Sera of chickens infected with *Mycoplasma gallisepticum* in natural conditions as well as infected with *M. gallisepticum* intra airsac were tested by immunoblot for presence of specific *M. gallisepticum* antibodies. Antigen from *M. gallisepticum* strain 1226 was run in 12.5% polyacrylamide gel (200 V, for 4 h), proteins were blotted onto nitrocellulose membrane (Sigma) at 30 V overnight. The membrane was treated with chicken sera collected 1–8 weeks after natural infection or 106 weeks after experimental infection. Binding of antibodies was detected with anti chicken IgG peroxidase conjugate (Sigma) and developed by 4 chloro-1 naphtol (Sigma). Due to natural infection the most important antibodies binding to the following polypeptides were detected: p110, p64, p56, p43, p33-35, p30 and p26. One week after experimental challenge antibodies reacting with antigen of p160, p66, p64, p56, p50, p45, p41 were developed. Two weeks later antibodies reacting with p39 and p24 were seen too. All these antibodies were existing during 6 weeks post infection.

According to the literature antibodies to p64 are responsible for haemagglutination inhibition and antibodies to p56 for reaction in MyGa test.

L. A. RÉTHY and L. RÉTHY

Genetic aspects of safe immunization in senescence

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Current theories invoke that aging consists of many programmed intrinsic and stochastic (extrinsic) processes characterized by progressive declines in tissue function and that these age-associated characteristics occur in the absence of disease.

The main intrinsic causes of senescence are (i) the cessation of cell divisions, i.e. the decrease of replicative potential, (ii) the low or no expression of somatomedins

[IGF-I gene], (iii-iv) the amplification of both, the growth suppressor SDI-1, find senescence SEN6 gene, (v) the decrease in responsiveness to platelet-derived, basic fibroblast, and epidermal growth factors.

We were dealing with human immunization without exerting any influence on the stochastic-extrinsic processes. We tried to manipulate the age-specific homeostatic regulation by giving different doses of lipopolysaccharides (LPS) of various origin, as immuno-amplifiers.

As already published by us, we immunized people of different ages by supplying tetanus toxoid alone or associated with LPS prepared from *Salmonella typhi*, *S. flexneri* and *S. sonnei*, as well as one extracted from Vibrio cholerae (Inaba and Ogawa strains).

The immuno-amplifying effect of LPS – as already claimed by us – was always and significantly present and exhibited dose dependent activity.

We demonstrate that the molecular basis of the immuno-amplifying mechanism of LPS depends upon its beneficial effect exerted on responses of natural defence mechanisms, to any kind of injury. The LPS provokes a kind of "acute phase" host response with a cascade of reactions. Triggering factors are released as a result of inflammation, caused by the introduction of endotoxin or its functionally detoxified de-derivatives.

The beneficial "intrinsic" activity of endotoxins can be traced back mainly to subcellular, genetic events, as mentioned ahead. In spite of fair achievements, the aging process seems to be inexorable. Any beneficial intervention like provoking a cascade of stress responsible gene activities, cannot prevent the senium.

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Prevention of puerperal tetanus

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Maternal tetanus, defined as tetanus occurring during pregnancy or shortly after delivery has to be divided into two groups because of the differences in the way of tetanus infection. We propose to use the terms "tetanus during pregnancy", usually resulting from infection through a non-genital portal of entry, while the term "puerperal tetanus" is to be applied provided tetanus occurs within six weeks after any termination of pregnancy.

Death of women during pregnancy, delivery and puerperium is a major public health problem in developing countries. Nearly 500 000 of young mothers die every year following complications from a total of births amounting 130–150 millions. Ninety-nine percent of puerperal deaths occurs in developing countries and ten percent of already mentioned amount of deaths is caused by tetanus infection acquired during or shortly after termination of-pregnancy: delivery, spontaneous, legal, or – mainly illegal – abortion. According to the data of Mother Care Corporation (Arlington, Va., USA), the

life time risk of death from pregnancy and child birth for woman in Africa corresponds to 1 in 18, while this risk is 1 in 10 000 for a woman in Europe.

As known, the only possibility of preventing both the tetanus during pregnancy, and puerperal tetanus is the specific anti-tetanus immunization with tetanus toxoid.

According to the immunization results achieved in volunteers we defined the efficacy of anti-tetanus immunization performed during pregnancy with accent on the time of immunization as related to the "age"/period of pregnancy, the dosage of vaccine and the possibility of evoking full protection in immunizees.

Immunization started or not yet completed before pregnancy, if repeated or finished shortly post partum, decreases the risk to acquire lethal tetanus infection following delivery. We present antitoxin titres as threshold of safe immunity against puerperal tetanus and propose a fair immunization schedule for general practice among "bush" circumstances.

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Down-regulation of HLA A11 in Burkitt's lymphoma

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Burkitt lymphomas that arise in HLA A11 positive individuals are characterized by selective loss/down-regulation of the HLA A11 polypeptide. We have investigated the molecular basis of such down-regulation by comparing five pairs of BL lines and Epstein–Barr virus (EBV) transformed lymphoblastoid cell lines (LCL) derived from the normal B cells of the same individuals.

The presence of apparently intact HLA A11 genes was confirmed in all five BL/LCL pairs by polymerase chain reaction (PCR) typing and by Southern blot hybridization with HLA A locus specific probes. Northern blot analysis with locus and allele specific probes revealed a significantly lower expression or absence of A11-specific mRNA in all five BL lines compared to the corresponding LCLs. Upregulation of A11-specific mRNA was achieved by IFNa treatment of two BL lines with low HLA A11 expression while the treatment had no effect in three BL lines that did not express the endogenous gene. HLA A11 expression was restored by transfection of the gene in WW1-BL whereas transfectants of BL-41 remained A11-negative. An HLA A11 promoter driven chloramphenicol acetyl transferase reporter gene (pA11CAT) was active in WW1-BL but not in BL-41. HLA-A11 was expressed in hybrids of BL-41 with an A11-positive LCL while expression of the endogenous HLA A11 gene could not be restored by fusion of BL-41 with an A11 negative LCL although an adequate set

of transcription factors was present in the hybrid. The results suggest that genetic defects and lack of transcription factors may contribute to the selective down-regulation of HLA A11 in BL cells.

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Heterogeneity of mycobacteriophage D29

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Centrifugation of bacteriophage D29 (a phage of *Mycobacterium smegmatis* ATCC 607) on linear CsCl gradient resulted in two phage-containing bands: one at the density of 1.53 g/cm^3 and the other at the density of 1.56 g/cm^3. These phages proved to be different regarding their DNA patterns (based on restriction enzyme analysis) and their morphological properties.

The lower band contained phages with isometric heads, the upper band phages with elongated heads. Both types of phages had long, noncontractile mils.

While the DNA of D29 phage with isometric head differed from that of other *M. smegmatis* phages having isometric heads (By, V72, D4, 33D) the patterns of Kpn I-, Bgl II-, BamH I-, EcoR I-, and Sac I digests of D29 with elongated head were indistinguishable from that of Bo1 or By:SN2 (phages of *M. smegmatis* strains having elongated heads).

Phages purified from single plaque were propagated, concentrated and analysed again on CsCl gradient. Restriction enzyme analysis of the fractions was also carried out. On the basis of these observations we try to interprete the contradicting data published previously by different laboratories.

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PM activity and antibody formation by piglets immunized with an *Actinobacillus pleuropneumoniae* extract vaccine

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Three groups of piglets each consisting of 15 animals were treated with an extract vaccine (group I), an extract plus bacterial material vaccine (group II), and with a placebo (group III). Prior to application of vaccines or placebo as well as 2, 3, 4, 5, and 6 weeks thereafter, blood samples were drown from each animal and sera were tested on specific antibodies by means of ELISA and activity of PM by bacterial killing and cytochrome-C reduction test.

Up to the 4th week after vaccination, bacterial killing by PM of piglets of groups I and II was about 20–30%, whereas activity of piglets of group III was measured at about 10–15%. In contrast to these results, there have been no significant differences between the three groups in cytochrome-C reduction test. However, concerning antibody formation, vaccinated animals developed significant elevations of antibody titres in contrast to piglets of placebo group.

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Combined suppression of mouse natural killer cells by retrovirus and marijuana

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Both marijuana and retroviruses impair natural killer (NK) cell functions. No data on their simultaneous effects are available. Similarities to human AIDS induced early by Friend leukaemia complex (FLC) and its replication competent helper Rowson–Parr virus (RPV) provides a mouse model to study drug-virus action. Leukaemia susceptible BALB/c and resistant C57BL/6 mice were infected, then at time intervals their nylon wool separated splenocytes were exposed to tetrahydrocannabinol (THC) for 3 h. NK activity against Yac-1 cells was assayed by ^{51}Cr-release for 4 and 18 h. Recovery of splenocytes was suppressed by FLC, but in BALB/c only by RPV with time. After a temporary enhancement in C57BL/6 animals, only by FLC, the NK cell activity of both mice became suppressed early (2 to 4 days p.i.), normalized subsequently and enhanced late (11 to 14 days p.i.). A moderate effect in BALB/c, no change in C57BL/6 were induced by low (1–2.5 μg/ml) THC doses. Activity of NK cells of BALB/c mice became suppressed exponentially by higher (5–10 μg/ml) THC doses in 18 h tests as compared to 4 h assays, while proportional and moderate impairment was seen in C57BL/6 mice. THC effects determined magnitude of NK cell activity of infected mice, primarily: pattern of enhancement or impairment followed those of untreated counterparts, but on the level of THC-treated cells. Low THC doses hardly, high doses additively influenced NK cells of infected BALB/c animals. THC minimally affected the very early and late NK cell enhancement of FLC infected C57BL/6 mice, but augmented RPV induced suppression late in 18 h assays. Genetic factors similar to endotoxin resistance and altered cytokine profile as well as cannabinoid receptor expression might determine these effects. Similar phenomena in humans results in anergy of immune cells with consequent earlier manifestation of AIDS.

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Enhanced lymphoblastogenesis of HIV-1 infected persons upon combined treatment with immunostimulants

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Restoration of immune functions through promoting cell cycle might delay AIDS development with maintaining HIV-1 latency. Therefore, stimulation of peripheral lymphocytes obtained from HIV-1 infected patients in successive clinical stages by immunostimulants with different effects was studied. In vitro blastogenesis was quantitated by ^{3}H-thymidine uptake. The response to PHA stimulation decreased to 63.1% in ARC patients, to 13.6% in AIDS patients as compared to healthy subjects. Minimal amount of rIL-2 or indomethacin solely not promoting lymphocytes, improved PHA response. In a dose-dependent manner radio-detoxified endotoxin (RD-LPS) alone increased stimulation of lymphocytes from ARC patients approx. 2.5-fold, did not alter that of controls and AIDS patients. RD-LPS plus PHA combination synergically stimulated lymphoblastogenesis of ARC patients to the level of single PHA stimulation of controls, while no boosting effect was observed in cases of controls and AIDS patients. Indomethacin or rIL-2 increased the RD-LPS boosted responses in an additive manner. Alone ineffective methyl-ester and methyl-phosphonate inosine derivates augmented PHA response of controls and ARC patients by 1.5-fold, but the same response of AIDS patients while statistic, ally significant was minimal. The effect on PHA response of inosines was additive with rIL-2 and indomethacin, while neither inosines had any effect on RD-LPS boosting. Anergy due to HIV-1 infection damages synchronisation of secondary messenger systems induced on cell surface receptors, therefore this selective influence by minimal rIL-2 or indomethacin is less efficient. In the early stages of virus infection, RD-LPS might bind to the altered receptors of immature T cells and facilitate cell cycle through cyclic guanosine 3',5'-monophosphate production. Inosine derivates promote cell cycle by inhibiting cyclic adenosine 3',5'-monophosphate production. These results indicate that, combinations of the biological response modifiers may have therapeutic value for patients with early HIV-1 infections.

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Cytokines activate feline immunodeficiency virus (FIV) in macrophages

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Monocytes are thought the first cells interacting with HIV-1, then the virus establishes latent infection. By evading immune surveillance, macrophage migration disseminates HIV-1 in different tissues. Replication of HIV-1 in immune cells depends on their differentiation and activation by soluble mediators. FIV, another member of Lentiviruses also replicates in macrophages. Differences in its cell surface receptor binding and regulation of gene expression do not alter the clinical course of disease. Factors able to accelerate F-AIDS progression through virus activation are not known. We studied the effect of 4 different monocyte stimulants on the early phase of FIV infection.

Monocytes were separated from peripheral blood of SPF cats, subsequently infected with FIV strain Pisa-M2, finally treated with TPA, IL-1α, M-CSF or GM-CSF. At different time intervals macrophages were collected and lysed. Their FIV content was determined by RT and p24 antigen ELISA. Integrated proviral DNA was shown by *gag*-specific PCR, semiquantitated by Southern-blotting and autoradiography. Cell-cycle stage was analysed by flow cytometry.

FIV infection resulted in no CPE during the first 36 h of observation, and the majority of cells remained in the G_1 phase of cell cycle. FIV infection induced early apoptosis in 2/3 of unstimulated cells, while TPA or IL-1α treatment decreased the ratio of cell death to approx. 1/3. Few percent of treated cells entered G_2 and S phases. Intracellular RT activity increased between 6 and 24 h, p24 antigen increased until 6 h, and after a declination increased until 24 h, finally both decreased. In contrast, cell stimulants enhanced RT activity from 6 h onwards. TPA facilitated RT 3.5-fold by 9 h, increased p24 content by 70%. M-CSF enhanced RT activity, while GM-CSF increased p24 content. IL-1α doubled both intracellular RT and p24 by 9h. Already at 3 h p.i. a minimal amount of proviral DNA was found in each infected culture. All stimulants, especially TPA augmented its production.

It is concluded that TPA might facilitate FIV absorption and transcription, M-CSF increased gene expression and translation, GM-CSF facilitated absorption and transcription, while IL-1α prevented cell death and augmented virus absorption. In vivo, co-operation of these cytokines might result in simultaneous macrophage differentiation and virus activation. Early appearance of proviral DNA suggests that FIV integration might be faster than that of I-HV-1. Already bone marrow precursors stimulated by cytokines can become early catalyzators to absorb, amplify and transmit HIV-1 or FIV to other cells.

G. G. KOVÁCS

Immunocytochemical investigations in prion diseases

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Human prion diseases are the following: Creutzfeldt–Jakob Disease (CID), Gerstmann–Straussler–Scheinker Syndrome (GSS), Fatal Familial Insomnia (FFI) and kuru. In these diseases an abnormal form of the physiological prion protein can be found, which has a probable role in the transmission and pathological courses. Three immunocytochemical methods (GFAP-glial fibrillar acid protein, Ubiquitin, IA8-antibody against prion protein) were used to estimate the pathological changes in Creutzfeldt–Jakob Disease and Gerstmann–Straussler–Scheinker Syndrome. Investigation of three brain samples revealed that IA8 antibody proved to be specific in prion diseases showing also the morphological differences.

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Scaling-up of an anti-D (IgM) antibody producing human/mouse heterohybridoma cultivation from t-flask to bioreactor

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Identification of the Rh(D) antigen has a particularly importance in blood group serology. Traditional reagents routinely used for this purpose were made of human serum which is getting less and less available. Reagents based on the appropriate monoclonal antibodies are able to substitute the human serum based ones. For this reason the production of monoclonal antibodies in bioreactors is increasing all over the world.

A human/mouse heterohybridoma cell line was established by the fusion of Epstein–Barr Virus (EBV) transformed human B lymphocytes and human/mouse heteromyelomas. The mother cell line was cultivated in static culture (m T-flasks), it was recloned several times and now it is stored in liquid nitrogen.

Static cultivation was performed in Iscove Modified Dulbecco's Medium supplemented with 15% Fetal Calf Serum (FCS) and with a special metabolic additive (FM).

During scaling-up the cell line was first adapted to suspension culture by Roller Bottle cultivation, then the original culture medium was gradually changed to Dulbecco Modified Eagle's Medium (DMEM) supplemented with 7.5% FCS without FM additive. This composition is more economical in higher-scale production.

Main characteristics of the cell line were determined as follows:

(i) the growth rate is highly oxygen-dependent; (ii) the oxygen consumption rate is relatively high; (iii) Dissolved Oxygen (DO) level higher than 10% is toxic to the cells; (iv) maximum specific growth rate is μ_{max}=0.052 h^{-1} (corresponds to doubling time of 13.3 h); (v) average specific growth rate is μ_{ave}=0.025 h^{-1} (corresponds to doubling time of 27.7 h).

The adapted cell line has kept its multiplication and antibody secreting characteristics also in the new medium. The master-seed was stored in liquid nitrogen.

Parameters of batch cultivation in bioreactor (Celligen, New Brunswick Sci) were optimized: (i) optimal inoculum cell count: 3×10^5 cell/ml; (ii) optimal DO concentration: 10%; (iii) a gentle Marine Blade Impeller agitation system was chosen, for supporting the requested oxygen transfer. Using these optimized parameters we are producing high-titre (1:128–1:256) basic material of the Monoclonal Anti-D (IgM) reagent, which has been registered in 1995 and is distributed by HUMAN Co. Ltd.

Á. GYÖNGYÖSI-HORVÁTH, I. BARNA-VETRÓ and L. SOLTI

A new monoclonal antibody against ochratoxin A

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Ochratoxin A is a secondary metabolite of various fungal species in the *Aspergillus* and *Penicillium* genera. This storage mycotoxin is a dihydrocumarin linked to L-β-phenylalanine. Chemically it is a very stable compound, which can be consumpted either directly by different cereals: barley, oat, wheat, maize and foods prepared from them or indirectly with meat from animals fed by mycotoxin contaminated feed. Ochratoxin A is causative agent of nephrotoxic, immunotoxic, carcinogenic and teratogenic effects.

The preparation and characterization of monoclonal antibodies with high sensitivity to ochratoxin A is presented in this lecture. Haptene-carrier type antigens (OA-BSA) and a rarely used immunization and testing technique were applied for antibody production. The monoclonal antibody was of the IgG_1 heavy chain subclass with a kappa type light chain. The 50% inhibition value was 0.45 ng/ml in a direct competitive ELISA and the detection limit was 42 pg. This antibody is very specific, cross-reacting only with ochratoxin B (9.3%).

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HLA-A2.1 binding peptides in EBNA-1 EBNA-2 and BZLF1-derived overlapping peptide series

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EBNA-1 is the only transformation associated EBV-encoded protein which was found not to induce CTL in human lymphocyte populations. To test the possibility that this would be due to lack of peptides which could bind to MHC class I molecules we compared the proportion binding motifs to the frequent A2.1 allele in all the transformation related EBV proteins. EBNA-1 had the lowest proportion. We have tested the HLA-A2.1 binding capacity of 41 and 31 overlapping peptides covering EBNA-1 and the immediate early protein of the EBV lyric cycle, BZLF-1, respectively, by cytofluorimetric measurement of their effect on A2.1 expression on the surface of 721.174/T2 (T2) cells. None of the overlapping EBNA-I peptides bound to A2.1 although five of them included A2.1 binding motifs. Four of the five nonamers including these motifs were however detected as binders. Among the 31 BZLF-1 peptides 7 showed considerable binding. The four peptides with the strongest effect included A2.1 binding motifs. Four peptides with motifs were weak binders and three binding peptides did not include motifs. Thus, the postulated inability of EBNA-1 to induce CTL is not due to lack of A2.1 binding peptides in its sequence. It is more probable that these peptides are not processed naturally or that they may not include side chains recognizable by TCR.

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Acanthamoeba keratitis

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Infections caused by small free-living amoebae are generally not very well known or recognized clinically. The methods for laboratory diagnosis are unfamiliar in most laboratories. The most recently revealed group of infections caused by these organisms is *Acanthamoeba* keratitis, uveitis and corneal ulceration related primarily to poor lens care. The taxonomy of these small amoeba species has not been well established. Consequently, little is known about the relationship between pathogenic and nonpathogenic strains. Several studies report on the possible use of the variations in mitochondrial DNA (mtDNA) to study the taxonomy of *Acanthamoeba*.

We used the restriction fragment length polymorphism (RFLP) analysis for the typing of *Acanthamoeba* isolate from the contact lens container of a patient with keratitis and for typing and comparing isolates from the environment. The Acanthamoebae were isolated on nonnutrient agar plates covered with the suspension of the *Enterobacter aerogenes*. The isolates were cloned by micromanipulation, cultured and maintained on agar plates spread with a lawn of heat-treated *Escherichia coli* DH1. The cloned isolates were adapted and cultured axenically. The mtDNA-s isolated by alkaline lysis method were digested by Bgl II and EcoR I endonucleases. The clinical isolate has a RFLP phenotype partly similar to that of the isolate from the roof of a moss, but totally different from that of the isolate from the river Danube.

All strains of *Acanthamoeba* may have pathogenic potential, but additional studies are needed to improve the classification of them. The mtDNA RFLP phenotype may be a useful aid in the taxonomy of free-living amoeba species and in the definition of their pathogenic potential.

MYCOLOGY

K. SWART

Transmission of nuclear genes and extra-nuclear genetic elements in *Aspergillus*

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Transmission of nuclear genes has well been studied in many eukaryotes including several fungi. Asexual propagation by conidiospores is common in *Aspergilli* and thus an isogenic clone of progeny is produced. Nuclear genes are transmitted properly in this way and members of such a clone can in principle interact by anastomoses to form a common mycelium. Eventually, nuclei in a mycelial cell can fuse and form a diploid that can subsequently haploidise. Among the haploids produced in this so-called parasexual process, recombinants may show up if some genetic differences exist between the fusing nuclei. This parasexual cycle can be useful to study gene transmission and recombination in the asexual life cycle.

Some *Aspergilli*, e.g. *A. nidulans*, additionally make use of a sexual cycle to produce offspring. The essential features of the sexual cycle are fusion of two haploid nuclei, meiosis, and the formation of sexual spores. During meiosis genetic recombination is required to ensure proper chromosome segregation and new combinations of genes arise in the offspring. Although the meiotic cytology of *A. nidulans* is still poor, this fungus is well suited to study several aspects of meiosis and recombination at a molecular level because of the availability of a variety of molecular genetic tools. Currently, we are investigating mutants that are impaired in meiosis at a

microscopical and molecular level. The cytology is described and the respective genes are picked up from a library by complementation of the mutant phenotype. The next step will involve the characterization of these genes and an analysis of gene function by re-introduction of new alleles obtained by in vitro mutagenesis.

The transmission of two essentially different extra-nuclear genetic elements, i.e. mitochondria and mycoviruses, was studied in *A. nidulans* and *A. niger*. Several isolates of *A. niger* contain dsRNA viruses and these were shown to be efficiently transmitted to the vegetative progeny (conidiospores). At least one strain could be cured from its virus and a direct comparison of a virus-containing strain with its virus-free "sister" was possible. In this case the virus had a clear effect on sporulation and growth of the host strain. In many other cases hardly any phenotypic effect of the virus on the host could be demonstrated. Horizontal transfer of viruses between different isolates of *A. niger* occurred in only very few cases, apparently due to the inability to form inter-isolate heterokaryons. Forced transmission was tried by protoplast fusion. Virus transfer could now be obtained in more cases, but was not successful in all cases, suggesting resistance to viruses in part of the *A. niger* isolates.

Protoplast fusion was also used to transfer virus from *A. niger* to *A. nidulans*. This appeared to be possible in many cases. Vegetatively, viruses were transmitted efficiently in *A. nidulans*, whereas in the sexual cycle viruses were lost. Mitochondria are transmitted uniparental in the sexual cycle of *A. nidulans* and currently we are investigating the inheritance of mitochondria and viruses in the same crosses in order to find similarities and differences of the transfer of these extranuclear genetic elements.

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Extrachromosomal genetic elements in *Phaffia rhodozyma*

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Phaffia rhodozyma, the only known yeast species in the *Phaffia* genus, was isolated from exudates of deciduous trees in cold climatic areas. One of the striking characteristics of this species is its astaxanthin production. Astaxanthin is an important component of fish food as it provides the pigmentation of the flesh of salmon and other fish. The use of *P. rhodozyma* as a potential astaxanthin source in the aquaculture industry has made it the subject of extensive biotechnological research for many years.

The present study was directed at the complex extrachromosomal genetics of this species. The existence of dsRNA viruses in *P. rhodozyma* has recently been demonstrated [1, 2]. Our study revealed a considerable variability among the six examined strains.

Although the strains harbour different sorts of dsRNA, only one type of VLP was detected by electron microscopy and gel electrophoresis, suggesting that this VLP is a member of the *Totiviridae* genus. Despite the results published earlier [2] none of the dsRNA bands could be eliminated by UV irradiation. The inheritance of the VLPs was

followed by crossing a virus-containing (lysine-requiring mutant of strain ATCC 24203) and a virus-free (leucine-requiring mutant of strain CBS 6938) strain and analysing the dsRNA pattern of the basidiospores of the prototrof hybrids.

Several dsDNA plasmids were demonstrated in the crude mitochondrial fraction of all six strains. These plasmids were protected against DNase when the mitochondria were treated with DNase in the presence of an osmotic stabilizer. Though yeast linear DNA plasmids are generally insensitive to ethidium bromide, *P. rhodozyma* strains were efficiently cured by ethidium bromide treatment. A DNA hybridization study revealed a strong sequence homology among the plasmids, while only a weak homology was detected to some chromosomal DNA bands. No homology was found between the *P. rhodozyma* and *K. lactis* linear DNA plasmids. No killer activity due to the presence of dsRNA virus or dsDNA plasmids could he detected. Further studies are in progress to establish the structure and explain the function of these extrachromosomal elements in *P. rhodozyma.*

M. M. EL NAGGAR[1] and A. L. ÁDÁM[2]

In vitro differences in extracellular polygalacturonase (Ec-PG) activities of six isolates of *Botrytis cinerea* (pers.): a possible factor in their differential pathogenicity in cabbage leaves

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There was found a good correlation between in vitro extracellular polygalacturonase (Ec-PG) activity and pathogenicity on cabbage leaves of six different isolates of pathogenic gray mould fungus (*Botrytis cinerea* Pers.) isolated from tomato, lettuce, grape, cucumber, pepper and sunflower. In this study, the native technique developed by Cruickshank and Wade in 1980, was suitable to study the isoenzyme pattern of different pectic enzymes, pectin methylesterase (PME), pectatelyase (PL), and polygalacturonase (PG), acting simultaneously in the same gel on the same substrate. PME activities were not detectable in the isolates of *Botrytis* investigated. All isolates of *B. cinerea* tested showed only two PG bands. The anodic isoform in most cases showed much higher activity than the cathodic one. Especially tomato, grape and pepper isolates showed a greater amount of an anodic isoform.

The possible in planta significance of increased PG activity in pathogenicity of gray mould fungus is discussed. *Botrytis* isolates showed variability in the virulence on cabbage leaves. The tomato isolate showed the highest disease degree (95.5%) followed by grape, pepper and sunflower isolates with disease degrees of 65, 58 and 10%, respectively. On the other hand, lettuce and cucumber isolates were practically unable to infect cabbage leaves.

J. ŠANDULA

Structural characteristics and immunomodulatory activity of β-glucans isolated from *Saccharomyces cerevisiae* and *Aspergillus niger*

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High molecular weight water-insoluble β-glucan is a typical skeletal constituent and consists of β-glucose units mutually linked by β-1,3- and β-1,6-glucosidic bonds. β-Glucans were isolated from the yeast *Saccharomyces cerevisiae* and filamentous fungus *Aspergillus* niger. For their physicochemical characterization ^{13}C-NMR spectroscopy and HPLC were applied. In order to achieve water solubility of glucans various derivates were prepared: carboxymethyl-, hydroxymethyl-, sulfoethyl-glucan [1, 2].

The immunomodulatory activity of these compounds, investigated with mitogenic and comitogenic tests on rat thymocytes [2], was antimutagenic and radioprotective [3, 4]. Conjugation of biologically active low molecular weight compounds with these macromolecular carrier may have a positive influence onto their pharmacokinetic and pharmacodynamic properties [5]].

[1] Šoltés L. et al. J Appl Polym Sci 48, 1313 (1993); [2] Šandula J. et al.: Int J Biol Macromol in press; [3] Chorvatovicová D., Šandula. J.: Mutation Res 346, 43 (1995); [4] Hofer M. et al.: Int J Pharmacol 97, 167 (1995); [5] Kéry V. et al.: Int J Biochem 22, 1203 (1990).

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Application of yeasts for secondary fermentations in wine making

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The alcoholic fermentation of grape juice as the primary role of yeasts in wine technology has been widely studied and well elucidated. Beside this task yeasts can be applied in secondary fermentation processes either to produce special wines or to improve wine harmony. These processes, which can be sorted into three separate groups, have been studied at the University of Horticulture and Food Industry for several years. The report aims to give an overview of the present state of these applications focusing on the recent progress.

1. Sparkling wine production by secondary alcoholic fermentation using appropriate starter yeasts is a classical branch of biotechnology. To avoid the difficult clarification process of bottle-fermented sparkling wines, the immobilized cell systems

are getting to be introduced. An improved Ca-alginate entrapment technique was elaborated using cell-free coat on the surface of the gel beads.

2. Biological aging process by film-forming wine yeasts is a crucial step in the technology of some famous special wine like "flor" sherry (Spain), "vin jaune"(France) and also applied in Tokaj "szamorodni" aging (Hungary).The traditional methods rely on the activity of the indigenous yeast populations. For production of sherry-type wines a complex technology was developed using Hungarian base wines as well as yeast strains of Tokaj origin. The metabolic activity of pure ad mixed cultures of several strains in film-phase was thoroughly studied. In a separate project the possible control of Tokaj szamorodni aging was studied using selected film-forming *Saccharomyces* strains of the local microflora.

3. Decomposition of malic acid in wines by malo-alcoholic fermentation might be an alternative to the bacterial malo-lactic fermentation. *Schizosaccharomyces pombe* strains show excellent potential for this application but the most strains are apt to produce off-flavour, primarily hydrogen-sulphide. Diversity and correlation of beneficial and disadvantageous properties were tested in numerous strains. Sulphide formation was found effectively suppressed by using immobilized yeasts in wine, rather than in must. In order to eliminate the risk of off-flavour production perfectly, strain selection and optimization of the immobilized cell fermentation are continued as well as a strain improvement program are under elaboration

J. ZALA

Some possible fungal answers to antimycotic therapy

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Antifungal therapy gains more and more important role in the treatment of immunocompromised patients. The rising number of the patients having some kind of immunodeficiency cause an increase of the number of serious fungal diseases in advanced cases. The long-term hospitalization of these patients needs a satisfactory prevention policy in order to avoid the possible risk factors, among them the fungal colonization.

However, fungi may respond unfavourably to prophylactic measures. They may change their susceptibility against the used drugs. The azole derivatives namely fluconazole, ketoconazole, miconazole create the opportunity on the one hand of the selection of the less sensitive species (e.g. *Candida krusei, C. glabrata, Trichosporon* spp.) on the other hand the induction of the development of resistance (e.g. *C. albicans, C. tropicalis*). Polyene derivatives (amphotericin B, nystatin) are much less active in this field except *C. lusitaniae* and *C. guilliermondii.* Moreover, as a possible consequence of the above, quite "new" species can colonize the body of the patients when the treatment is modified (e.g. azole to polyene).

These findings call the attention to the importance of changes of the mycoflora during the therapy.

Furthermore, we must not neglect the possibility that the antifungal drugs may influence the effect of one another in producing cross-resistance within one chemical group and even between different chemical groups, too (e.g. a long-continued fluconazole treatment theoretically may lead to an insensitivity to amphotericin B).

T. NAGY, E. K. NOVÁK, J. ZALA and ZS. HORVÁTH

Computer aided yeast identification in mycology

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A computer aided yeast identification for 590 program species referring 91 biochemical tests, 15 other important morphological tests and an additional "test" for selecting human pathogen species has been developed on the basis of the descriptions yeast species [J. A. Barnett et al., 1990].

The computer program (FUNID, FUNGAL IDENTIFICATION) with other database can also be used to identify other fungal taxa (e.g. yeasts). This software is also good at validating yeast identification test kits (API20C Aux, Auxacolor, Mycotube, etc.), commercially available, or examining some practical or theoretical questions.

The coding system (code/meaning): 1 –/–; 2 +/+; 3 D/Delayed; 4 W/Weak; 5 V/Variable; 6 ?/Result not known; 7 C/D,W; 8 d/D,+; 9 w/W,+; 0 c/D,W,+; q/– and (+ or D or W)

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Molecular genotyping of yeasts with PCR fingerprinting using primers of arbitrary nucleotide sequence

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Yeasts are becoming increasingly important organisms in both industry (as main producing organisms or causes of contaminations) and medicine (as disease agents in humans). Quick and reliable methods for yeast identification/differentiation are highly appreciated in both mentioned areas. In addition to phenotypic methods, based on morphological and biochemical tests (suffering from possible variations under varying environmental conditions), many molecular genetic techniques have been developed for yeast characterization: DNA–DNA and DNA–RNA reassociation studies, RFLP, i.e. rDNA and mtDNA restriction analysis, partial or complete rRNA sequencing and

electrophoretic karyotyping with subsequent DNA–DNA hybridization with isolated genes or whole chromosomes as templates for probes preparation etc. Despite of sensitivity and reproducibility, these methods are laborious, time and material-consuming, require special equipment and prior knowledge of target nucleotide sequences and in this way are hardly applicable to non-specialized laboratories. In the last few years new methods for DNA fingerprinting have been developed on the basis of PCR amplification of nonspecific genomic DNA fragments with single or multiple arbitrary primers (AP–PCR fingerprinting). The differences in appearance of RAPD markers, resulting from priming sites mutations or insertions/deletions all over the genome may be useful in main identification/differentiation as well as in defining the genetic relationships between organisms in evolutionary, phylogenetic and taxonomic studies [1, 2].

The results of application of arbitrary primed polymerase chain reaction (AP–PCR) on several industrially important yeasts from *Saccharomyces*, *Torulaspora*, *Debaryomyces*, *Schwanniomyces* and some other closely related yeasts genera will be presented. Polymorphisms in genomic fingerprints generated by several decamer oligonucleotide primers (50–80% G+C content, Promega, Operon) and microsatellite DNA primers (i.e. $(GTG)_{5x}$, $(GACA)_{4x}$, MWG-Biotech) have been studied for intra- and interspecific RAPD markers for differentiation of 30 yeast strains from 18 species. Genotypic relatedness of yeasts, evaluated on the basis of totally more than 350 stable polymorphic RAPD markers, is expressed as calculated genetic similarity coefficients and duster analysis based on UPGMA method (NTSYS – pc program [3]).

In comparison to some other molecular-genetic methods of yeast characterization, it is shown, that PCR fingerprinting with arbitrary chosen oligonucleotide primers is a useful tool for classification of industrial yeasts as well as for differentiation of very closely related mains of the same species. Reproducibility difficulties which sometimes make AP–PCR fingerprinting problematic with regard to standardization between laboratories, were largely eliminated by high purity of template DNA, isolated from CsCl gradients in our experiments. For even wider applicability AP–PCR fingerprinting in quick yeast differentiation tests simple miniprep DNA isolation methods should be further evaluated.

[1] Williams, J.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. Tingey, S.V.: Nucl Acids Res 18, 6531–6535 (1990); [2] Caetano-Anoles, G.: PCR Methods and Application, Cold Spring Harbor Laboratory Press, N.Y. (1993); [3] Rohlf, F.J.: NTSYS – pc numerical taxonomy and multivariate analysis system, version 1.80. Exeter Software, Applied Biostatistics Inc., Setauket, New York (1993).

A. MARÁZ[1], É. BALLA[2] and GY. VÉGH[1]

Construction of *Candida glabrata* genomic cosmid library and screening for killer genes in yeasts

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Genomic library was constructed from the CBS 138 killer strain of *Candida glabrata* in *Escherichia coli* using the yeast – *E. coli* shuttle cosmid vector BHB 3000 which contained the *HIS3* gene as selectable marker in yeast. Chromosomal DNA was isolated by phenol–ethanol extraction and partially digested with *Sau3A* restriction endonuclease. Yeast DNA fragments 35–50 kb were ligated with the modified BHB 3030 cosmid containing two *cos* sites ("arms"). Recombinant cosmids were packed in vitro into the λ phage proteins (Gigapack, Stratagene) and *E. coli* VCS 257 strain was infected with the phage particles. As much as 3000 ampicillin-resistant clones were isolated and stored in the wells of microtiter plates at –70 °C.

Two *C. glabrata* non-killer strains (222 and 6) were selected as possible hosts for screening killer genes from the gene library. Expression of the killer phenotype in the somatic hybrids of these two and the CBS 138 strains was checked. Hybrids were selected by prototroph complementation which proved diploids and polyploids but only the hybrids of the strain 6 expressed the killer phenotype. Screening for killer genes in the library is in progress using *S. cerevisiae* AH 220 *his3* and *C. glabrata* 6 strains.

I. BALOGH and A. MARÁZ

Effect of chromosomal copy numbers of *STA* genes on glucoamylase production in recombinant yeast strains

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The *STA* genes are responsible for producing glucoamylase enzyme in *Saccharomyces cerevisiae* race *diastaticus*. These genes exist in three forms named *STA1*, *STA2* and *STA3* locating on three different chromosomes. The nucleotide sequence of the *STA* genes are highly homologous. A sporulation-specific gene called *SGA* exist in every *S. cerevisiae* strain having also partly homologous DNA sequence with the *STA* genes (see the review of Vihinen and Mantsala).

Recombinant yeast strains were prepared by sexual hybridization using *S. cerevisiae* r. *diastaticus* and laboratory *S. cerevisiae* strains aiming to get hybrids containing more than one copy of the *STA* gene. The hybrids were screened for starch digestion and characterized by pulsed-field gel-electrophoresis using the ROTAPHOR system. A general gene probe for all these *STA* genes was prepared by PCR using a part of the *STA1* gene as target DNA. The probe was labelled by ^{32}P and hybridized with the

separated chromosomes of the hybrids using the Southern-hybridization method. Hybrids having different type and copy number of *STA* genes were detected. The glucoamylase activity of the hybrids was measured by enzymatic test. Significant differences of glucoamylase activity were found among the hybrids suggesting gene dosage effect of *STA* genes.

Vihinen, M., Mantsala, P.: Critical Reviews in Biochemistry and Molecular Biology, 24, 329–418 (1989).

A. GELETA, A. MARÁZ and E. VARGA

Ultrastructural studies of flocculation in *Schizosaccharomyces pombe*

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Flocculation is a relatively widespread phenomenon among different yeast species. It takes place at the end of the logarithmic growth phase in the presence of calcium ions.

Schizosaccharomyces pombe R.4.2.1. strain was selected in our laboratory which had strong flocculation character in the presence of ethanol and calcium ions. Flocculation occurs both under aerobic and anaerobic growth conditions.

Cell wall lytic enzymes, β-galactanase and proteases are able to eliminate floc-forming ability of cells, while β-glucosidase, β-glucuronidase and β-galactosidase have little effect.

According to these results it seems probable that lectin-type protein molecules are responsible for flocculation. For testing the sugar specificity of lectin deflocculation effect of mono- and disaccharides was checked. Lectin was specified by that sugar what was effective in the lowest concentration. D-mannose, D-mannitol and fructose had no effect on flocculation, while D-glucose, D-galactose, lactose, maltose and saccharose dispersed the flocs within some seconds in high concentration. D-galactose and its derivatives bearing galactose in a terminal, non-reducing position had deflocculation effect as low as 10 mM concentration.

The ultrastructure of the outer cell surface of the flocculated and dispersed cells was studied by scanning (SEM) and transmission electron microscopy (TEM). For SEM preparation cells were fixed with glutaraldehyde, postfixed with osmium tetroxide and specimens were prepared by splash freeze lyophilization. SEM and TEM studies revealed the presence of slimelike amorphic material on the surface of the R.4.2.1. cells independently whether flocculation was induced by ethanol or not. Close contact of the adjacent cells was found, however only in the case of floc-formation. Our hypothesis is that ethanol induces changes in the galactomannan structure to act as receptor for lectins.

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Yeast diversity in Slovene wine-producing region "Primorska"

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Yeasts have been isolated from variety of natural environments, such as soil, plant surfaces (leaves, tilts) etc. The increased interest is paid to wine-making yeasts since wine production has turned to a well-controlled process with extended microbiological studies on the alcoholic fermentation. The quality of wine is also a consequence of a diversity of microorganisms involved in wine fermentation process. Yeast studies in wine regions world-wide show variations in yeast population kinetics, which are mainly related to differences in ecosystems (soil composition, climate conditions, viticultural and enological practices). The aim of our study is the evaluation of diversity of natural yeast population in Slovene wine regions (Primorska) and its correlation with yeast population kinetics during spontaneous fermentation of some typical Slovene wines.

Samples of soft and grapes of four vine varieties were taken from the vineyards in three subsequent weeks before vintage in autumn 1994 at 25 locations in Primorska region. Musts and young wines after completion of fermentation were also studied. After a direct or enrichment technique of isolation, 460 pure yeast cultures were included in ZIM-Culture Collection of Industrial Microorganisms, Ljubljana. Classical microbiological tests were made according to Barnett (1990) and associated PC-program. In addition, karyotyping of yeasts with Pulsed Field Gel Electrophoresis (CHEF-PFGE, Pulsaphor System, Pharmacia LKB Biotechnology) was made for intraspecific differentiation of closely related strains (mainly Saccharomyces) which can not be distinguished with classical methods of yeast identification.

Yeasts were negligible in all samples of soil and also on grapes 3 and 2 weeks before vintage. Total number of yeasts increased in the last week before technological ripeness of grapes and reached the concentration 1.3×10^3–3.5×10^5 CFU/ml. All yeast strains isolated from the vineyards, which are already biochemically and genetically characterized are non-saccharomyces yeasts. 15% of isolates were identified as *Hanseniaspora/Kloeckera* yeasts with specific apiculate cells and specific karyotypes after PFGE. At least five separate fragments (size range 650–2500 kb) can be recognized The majority of the other isolates possess much longer chromosome fragments. Biochemical tests confirmed *Candida*, *Metschnikowia* and *Rhodotorula* species. The same strains were identified also in musts and young wines, but the concentration of *Saccharomyces* strains strongly increased. Great heterogeneity was found among *Saccharomyces* strains in musts and wines, particularly due to polymorphisms and/or different numbers of copies of short (I, VI, HI, IX) and middle long (X, XIV, H, XIIL, XVI) chromosomes. The results support the idea, that not only one specific strain is

responsible for fermentation, but heterogeneous populations of yeasts (from *Saccharomyces* and also non-saccharomyces genera) are included in wine fermentations. This is particularly obvious for "Kra ki teran" with specific yeast population, which is different from, for example, "Refo k", produced from the same vine variety (refo k) but in different climate conditions (Kras). The correlation between specific chemical and sensory characteristics of "Kra ki teran" and microbial association responsible for its fermentation remains to be evaluated.

Možina, S. S., Podjavoršek, J., Vodopivec, M., and Raspor, P.: Diversity of yeasts in some Slovene vineyards and spontaneous wine fermentations. International Biodiversity Seminar Abstract Book, Gozd Martuljek, 30. 6-3.7. 1995, Slovenia.

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RFE karyotyping and RAPD analysis of yeast strains

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The results of deoxyribonucleic acid relatedness studies (Vaughan Martini and Kurtzman 1985; Vaughan Martini and Martini 1987; Vaughan Martini 1989) the species *Saccharomyces cerevisiae* (sensu Yarrow, 1984) was shown to include four different species i.e. *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*. In addition to DNA reassociation, other methods of molecular biology, such as electrophoretic karyotyping (Naumov et al. 1992, Cardinali and Martini 1994), mitochondrial DNA restriction pattern (Guillamón et al. 1994) have been also suggested for species separation within the group of *Saccharomyces* sensu stricto. Another molecular typing technique developed more recently is the Random Amplified Polymorphic DNA (RAPD) analysis. This method has been successfully used for characterization of yeast species. The present study investigates the significance of intact chromosomal pattern and RAPD analysis in identification and characterization of Saccharomyces sensu stricto species. A total of 72 Hungarian wine and brewery yeast strains were examined by RFE (rotating field electrophoresis). Karyotypes were analysed by scanning densitometry and evaluated statistically. For RAPD assay were using selected 10-mer oligonucleotides.

Chromosome length polymorphisms were found within the wine yeast strains belonging to *S. cerevisiae*. Their electrophoretic karyotypes were very similar to that of *S. paradoxus*. *S. bayanus* showed the most species-specific chromosomal pattern of the four examined species. When, on the basis of scanning densitometry, strains seemed to be heterozygote, monosporic subcultures were prepared from one solitary tetrad. These subcultures showed different electrophoretic profiles. Karyograms of brewing yeast strains displayed five closely packed, intensively lighting bands that may be due to polyploidy. Their electrophoretic karyograms did not seem to be similar to that of the

type strain of *S. pastorianus*, which showed 21 well distinct bands. Our results demonstrated the presence of different RAPD patterns within the *Saccharomyces* sensu stricto group.

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Can yeasts accumulate chromium (Cr^{3+}) into the cell?

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Yeasts *Hormoascus platypodis*, *Pichia kluyveri*, *Yarrowia lipolytica* and *Schizoblastosporion starkeyi-henricii* grew in media loaded with Cr^{3+} ion. The growth was followed spectrophotometrically at 690 nm wavelength, and at the end of the process chromium was determined in yeast biomass by ETA-AAS. Concentrations added in the media were in the range from 3.7 to 7.6 mM Yeasts which grew in chromium loaded media capable of Cr^{3+} accumulation in the cell. Concentration of accumulated Cr^{3+} among yeasts depends on concentrations in growth media. Accumulation of chromium in yeasts *P. kluyveri*, *Y. lipolytica* and *S. starkeyi-henricii* reached values 0.94–1.02 mgCr^{3+}/g, 1.62–2.93 mgCr^{3+}/g and 1.68–1.96 mgCr^{3+}/g. The most valuable yeast with greater accumulation ability of Cr^{3+} among yeasts tested was *H. platypodis* with 1.90 and 4.14 mgCr^{3+}/g. In yeasts Cr^{3+} was distributed in different organic compounds in the yeast cell what was represented almost half of the total accumulated Cr^{3+} (26.6% to 47%). The rate of organically bound chromium (OBC) increased among yeasts with its concentration in the media, except in yeast *H. platypodis* which showed reversal behaviour. The highest concentration of OBC Cr^{3+} (1.34 mgCr^{3+}/g) is observed in yeast *Y. lipolytica*.

Among yeasts *H. platypodis*, *P. kluyveri*, *Y. lipolytica* and *S. starkeyi-henricii* the addition of chromium (Cr^{3+}) in higher concentrations to the media almost always effected cell growth and proliferation. The chromium accumulation and distribution in OBC always influenced by chromium (Cr^{3+}) concentration in the media.

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Effect of chromium(VI) on yeast protoplasts studied by electron spin resonance spectroscopy

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The effect of $K_2Cr_2O_7$ on the wild-type strain ATCC 44829 (33 erg+) and an ergosterol-less ATCC 44831 (erg-2) of the unicellar, petit-negativ, human pathogen eukaryotic microbe, the yeast *Candida albicans* has been studied using electron spin resonance (ESR) spectroscopy both as a means to detect paramagnetic forms of chromium and to monitor the fluidity of the plasma membrane by the spin labelling method. ESR spectra of 5-SASL (stearic acid labelled at the 5th carbon atom from the head group), and those originating from Cr(V) or Cr(111) were recorded in the temperature range of 0–30 °C.

Minimal inhibitory concentration of chromium(VI) in liquid culture containing minimal medium was 0.5 mM for booth strains. Protoplasts were produced by snail enzyme treatment using overnight cultures. For osmotic stabilization of protoplasts 0.9 M mannitol or 0.6 M KCl were applied.

Nitroxide hyperfine splitting parameters report that the membrane fluidity increases in the order of strain erg-2 in 0.9 M mannitol, strain erg-2 in 0.6 M KCl, and strain 33 erg+ in 0.9 M mannit. While 0.5 mM $K_2Cr_2O_7$ has no effect on or slightly increases the fluidity of erg-2 it makes the plasma membrane of 33 erg+ significantly more rigid. ESR signal decay at 0 °C, indicating spin label reduction by redox processes, has been observed to be slower in the presence of $K_2Cr_2O_7$.

An unexpectedly strong ESR signal of chromium has been explored in the absence of spin label and only in erg-2 yet. This signal can be observed even at room temperature. Based on the agreement with literature data we interpret this signal, characterised by a g-value of 1.980 and peak-to-peak linewidth of 2–8 Gauss, as Cr(V) complex within the call or in the plasma membrane as it is only detectable in the presence of both the protoplast and $K_2Cr_2O_7$ in the osmotic stabilizer and it is known to be impermeable to biological membranes. The signal is similar in g-value, but different in sense of the hyperfine structure from the one obtained from 0.5 mM $K_2Cr_2O_7$ in 0.9 M mannitol. It is also different from the signal originating from 60 mM $CrCl_3$ in 0.6 M KCl as this latter gives g=1.965 and a linewidth of 250 Gauss.

Our results indicate that spin labelled *C. albicans* strains of different membrane composition and fluidity is an ideal model system for studying the processes of the uptake of Cr(VI) and its intracellular reduction to Cr(V) and possibly Cr(111) and to explore the effect of these processes on the plasma membrane.

J. KUCSERA, I. PFEIFFER and L. FERENCZY

Basidiosporulation induction by nitrogen deprivation in *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*)

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Teleomorphic state of *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) has been described recently [1]. It was suggested, that exogenous polyols, especially ribitol, are the main triggers for sporulation. We have investigated the sexual activity in *Phaffia rhodozyma* at the level of response to nutritional factors. *Phaffia rhodozyma* strains sporulated as a consequence to deprivation of nitrogen, although the efficiency was lower than using polyols as carbon source [1].

The sexual activity involved each mating between two yeast cells, transient nuclear fusion, meiosis and formation of basidiospores. Mating is possibly started by G1 phase arrest of the cell cycle like in the case of other yeasts. The life cycle showed homothallic features as the four spores isolated from the apex of holobasidia sporulated again on nitrogen limited medium. Mating factors important in conjugation of a number of yeasts could not be found.

[1] Golubev, W.I.: Yeast 11,101–110 (1995)

ZS. PALÁGYI[1], CS. VÁGVÖLGYI[1], Á. NAGY[2] and L. FERENCZY[1]

Efficient method of mutant isolation from the yeast *Phaffia rhodozyma*

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Phaffia rhodozyma synthesizes astaxanthin as its principal pigment. This carotenoid is an important component of fish food in the aquaculture industry. Wild-type strains produce astaxanthin only at a low level, and there is therefore continuous interest in elevating the pigment production to an economic level. Here, a mutation strategy is described for the production of *P. rhodozyma* mutants suitable for further strain improvement studies.

Six *P. rhodozyma* strains (CBS 5905, CBS 5908, CBS 6938, ATCC 24203, ATCC 24229 and ATCC 24261) were used in the experiments. Cells were subjected to γ-radiation until approximately 90% of them had been killed. Different colour mutants were isolated on the basis of their appearance. Drug-resistant mutants were obtained by positive selection. For auxotrophic mutant enrichment, a modification of the selective cell-wall lysis method was applied.

This mutation strategy re-suited in various types of *Phaffia* mutants: 388 colour mutants, 7 drug-resistant mutants (6 benomyl and I cycloheximide-resistant), and 14 auxotrophs were isolated. Double auxotrophs were also isolated following a repeated 3,-irradiation cycle. These results could accelerate efficient protoplast fusion-based strain improvement work on *P. rhodozyma.*

Á. NAGY[1], ZS. PALÁGYI[2], CS. VÁGVÖLGYI[2], T. PAPP[2] and L. FERENCZY[2]

Chromosomal rearrangements observed in auxotrophic and hybrid strains of *Phaffia rhodozyma*

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Phaffia rhodozyma synthesizes astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) as its principal carotenoid. There is increasing interest in the utilization of this red pigmented yeast as a microbial source of astaxanthin for the aquaculture industry.

Eighty-eight mutants obtained from two *P. rhodozyma* strains (ATCC 24203 and ATCC 24229) by a recently described method were investigated. Protoplast fusion experiments were carried out between mutants with auxotrophic and morphologic (colour) markers and the resulting fusion products were isolated. Contour clamped homogeneous electric field (CHEF) separation of the chromosomal size DNAs was used to detect changes in the chromosomal pattern in the course of both mutant production and hybrid formation.

Electrophoretic karyotyping revealed a substantial degree of chromosomal rearrangement after mutagen treatment. A very characteristic phenomenon, the disappearance of certain high molecular weight chromosomal bands, could be explained in terms of a DNA chain break followed by a translocation event. No definite correlation was found between a given type of chromosomal aberration and a phenotypic character. When hybrid strains resulting from protoplast fusion were analysed, both the disappearance of certain bands and the appearance of new bands were detected. These results, together with the chromosomal length polymorphisms observed in wild-type strains, suggest a high plasticity of the fungal genome in accepting substantial changes in the original chromosomal arrangement.

M. PESTI and Y. PINTÉR

A scanning electronmicroscopy investigation of the structure of colonies of *Candida albicans* morphological mutants

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Candida albicans ATTC 64,385 was used to receive colony morphology mutants. A mutant designated MCCP 0073 was selected from a population after UV treatment resulted 5% survival.

It was segregated spontaneously into two colony morphology variants named irregular wrinkle (W) and fuzzy (F). They were recultured five times using single colony descendent method. The genetically stable lines were further studied and compared with the smooth (S) colonies of the wild type strain. The percentage of different cell types within the colonies was determined by light microscope. Ultrastructure of colonies was revealed by scanning electron microscope (Type: JEOL 6300).

S colonies consisted of blastospores. Some pseudohyphae have appeared in the bottom of the colonies near to the agar surface. W and F colonies contained 1.2% and 17.0% pseudohypha and hypha, respectively.

The diameter of blastospores was heterogen in W colonies and they produced chains within the colonies. Hypha and pseudohypha were the characteristic cell types of F colonies not only inside but also on the surface resulting spongy structure. Aerial hyphae were observed, too.

Independently from the observed three colony morphologies under the colonies in the agar medium true hyphae was the dominant cell type. It means that regulatory processes of bud-hypha transition overcame the effect of these mutations.

J. ZALA, M. HENCZ, ZS. HORVÁTH AND T. NAGY

The survival of *Candida albicans* on inanimate vehicles

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The surface of the objects of the everyday life are continuously exposed to fungi. Settling of human pathogenic fungi on these places and surviving the unfavourable conditions may be dangerous. We examined the survival of the most frequent pathogenic yeast *Candida albicans* on different types of vehicles, e.g. lastic (tooth brush, nipple, floor covers made of "white" and "green" polyvinylchloride), loose materials (cotton, textiles, swab of cotton wool, tissue, paper napkin, intime-towel, wipe).

A given number of the yeast cells was put on the surface in a suspension. The number of the living cells was examined at different time intervals (0, 20, 40 min, 1, 2,

4, 24 h, 13 days) at different places of the surface. Sabouraud agar slides were used for the cultivation. The result was a definite (four scales) decreased after 1 and after 24 h in the case of the toot brush and the nipple, respectively.

There was no considerable decrease in the number of the living cells at the loose cellulose-type materials within 24 h.

After 13 days the number of colonies on the napkin decreased to 0 while on the other cellulose materials there was only very little change compared with the start.

These shows the long/good surviving ability of the *C. albicans* cells as well as the relevance of the speed of the drying up in the survival.

J. ZALA, ZS. HORVÁTH, and T. NAGY

New sampling method suggestion based on some theoretical consideration and experiments on quantitative and qualitative examination of fungal elements in air

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It is well known that the purpose of the air fungal examinations are hospital hygiene and the examination indoor and outdoor allergens. Examinations may be of the direct method showing the presence (as well as in the Burkard-trap and personal-sampler), and the cultivation (as Koch exposition and the using of centrifugal air sampler, etc.). The latter being based on the volumetric principle as well as the Burkard-trap method.

The direct microscopic examination of fungal elements in the Burkard-trap could only be based on the morphology of the fungus sedimented to the film layer which significantly limits the possibility of identification.

The count of the total fungal elements is underestimated more than ten times according to our examinations by means of cultivation based on volumetric method in comparison to using the Burkard-trap. The reason for this is that those of the Burkard-trap can show the dead fungal elements and by suppressed other or quicker growing species and the special supply-dependent growing ones as well as tile fungal elements existing in groups in the air – usually adsorbed at the surface of organic pollution.

And on top of all that according to our earlier study (E. K. Novak et al.) we can not find coherence in the colony forming units between the different cultivation methods as well as the different exposition time by the Koch method and the volumetric ones. The reason of the latter is that using the Koch method results depend on the sedimental circumstances of the fungal elements and other aerodynamical parameters of the air – consequently the Koch method is not suitable for the quantitative content of fungal elements in air.

In conclusion we suggest a new method for the determination of the total number of the fungal elements in air, the principle of which is that, by replacing the sampling container filled with vaselined film layer in the centrifugal air sampler, each fungal element will be trapped. Parallel examinations with the cultivation method (by means of different nutrient substrates as well) give more comparable results for further evaluation.

Zs. Horváth and J. Zala

Aspergillus fumigatus mycelial antigen preparation for immunofluorescence purpose and experience with it

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There are requirements developing the more sensitive methods in the immunological techniques for the examination of *Aspergillus fumigatus*, *A. nidulans* and other medically important aspergilli. Applying the sensitive immunofluorescence method to show the presence of antibodies requires a special bonding of antigens because the pathogenic state of these moulds usually is mycelial. It is well known that mycelial antigen could be sourced first of all from liquid culture of the moulds.

The method of preparation of mycelial antigen is as follows. The *A. fumigatus* strain isolated from the patient's bronchoscopic leavage and then subjected to shaken cultivation in Sabouraud broth until 21 days. The propagated fungus was filtered and thoroughly washed with non-ionic distilled water then lyophilized without protecting supplement. This lyophilizate after being homogenized according to Potter was suspended in non-ionic distilled water. Then it was fixed in the microscopic slide, and used as antigen.

By means of this method the positivity rate from the bronchial diseases of 112 patients' sera was increased from 16.7% to 31.2% when compared with the counter-immunoelectrophoretic method. Surprisingly the vesicula and phialids and moreover the conidia of the *A. fumigatus* were shown in the fluorescence as well. Practical disadvantages of this method are that it is time-consuming and requires an immunofluorescence microscope.

E. RINYU, J. VARGA and L. FERENCZY

Detection and typing of *Aspergillus fumigatus* isolates by RAPD technique

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Aspergillus fumigatus Fresenius is an opportunistic human pathogenic fungus, which may cause several diseases, such as allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis. Several methods have been developed for the detection of *A. fumigatus* in body fluids; typing of these isolates is at the centre of interest of epidemiological studies. We have tested the applicability of a PCR-based method for both purposes. Sixty-one isolates and collection strains of *A. fumigatus* were compared by the random amplified polymorphic DNA (RAPD) technique. Although the patterns of the strains were very similar for most of the primers, the application of three primers (OPC-06, OPC-07, and OPC-10) made it possible to cluster the 61 *A. fumigatus* isolates into 47 groups. The results allowed the RAPD technique to be used more efficiently for typing *A. fumigatus* isolates than isoenzyme analysis, or restriction enzyme analysis of the mitochondrial DNA and the ribosomal gene cluster of these strains. The application of another primer (OPC-08) permitted the distinction of *A. fumigatus* from other potentially pathogenic aspergilli (*A. niger*, *A. flavus* and *A. terreus*) and also from closely related species (species of section *Fumigati*, e.g. *Neosartorya fennelliae* and *N. pseudofischeri*). The only cross-reacting species was *Neosartorya fischeri*, which is the species most closely related to *A. fumigatus*. Hybridization analysis revealed that the amplified fragment came from the mitochondrial DNA. Further studies are in progress to evaluate the feasibility of this approach to detect *A. fumigatus* in diseased tissue and body fluids.

J. VARGA and K. KESZTYÚS

Assignment of RAPD, RFLP and isoenzyme markers to *Aspergillus nidulans* and *Aspergillus quadrilineatus* chromosomes

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Chromosome-substituted haploid segregants were selected from the benomyl-induced progeny of an interspecific allodiploid hybrid produced by polyethylene glycol-induced fusion of protoplasts of an *Aspergillus nidulans* "master strain" and an *A. quadrilineatus* auxotrophic mutant. These segregants were used to allocate several random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and isoenzyme markers to parental chromosomes. With the use of 14 primers, 25 RAPD fragments were allocated to *A. nidulans* and/or *A.*

quadrilineatus chromosomes. The activities of 12 isoenzymes, including cellulase, lactate dehydrogenase, phosphatase, malate dehydrogenase, superoxide dismutase and α- and β-arylesterase isoenzymes, were also assigned to parental chromosomes. The *A. nidulans* ribosomal repeat unit was assigned to chromosome V, while the *ben*A and the *pyr*G genes were allocated to linkage groups VIII and I, respectively, of *A. nidulans.* Chromosome-specific RAPD, RFLP and isoenzyme markers were found for all *A. nidulans* and *A. quadrilineatus* chromosomes. Attempts were also made to allocate extracellular enzyme activities to parental chromosomes, mostly without success. The isoenzyme and DNA sequences assigned to chromosomes could be used to saturate the genetic map of *A. nidulans*, or could serve as starting points for the construction of a genetic map of *A. quadrilineatus.* No method providing the same possibilities has been described so far for *Aspergilli.*

B. TÓTH, ZS. HAMARI and F. KEVEI

Recombination of mitochondria among compatible strains of *Aspergillus niger* aggregate without selection pressure

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Considering the banding patterns of *Hae*III-*Bgl*II digested mitochondrial DNA (mtDNA) of the strains belonging to *Aspergillus niger* species aggregate can be divided into three main groups (1, and 2, groups consist of several subgroups). The mtDNA groups 1, 2, and 3, corresponding to nuclear ribosomal RNA genes groups I, II, and III [1]. Successful mitochondrial transfer were performed by protoplast fusion using 1a mtDNA type of mitochondrial oligomycin resistant mutant as a donor. The recombination of mtDNA occurred among incompatible strains belonging to *A. niger* aggregate under strong selection pressure. Mitochondrial resistant progenies could be recovered after the fusion selecting for the nuclear phenotypes of the recipient partners in presence of oligomycin. The most extensively studied transfer 1a→2b resulted a great variety of RFLP profiles of the partners. Beside the recombinants an other type of resistant clone occurred from this cross bearing unchanged donor mitochondria with the recipient nuclear background. Resistant strains carrying either recombinant or substituted mitochondria were crossed with isogenic origin of compatible sensitive parent by anastomosis. Heterokaryons possessing mixed mitochondrial populations showed different growth ability. When conidia were harvested from the prolonged growth of heterokaryons both parental nucleo-types of progenies could be recovered most of them bearing recombined mitochondria. This result suggests that the mitochondrial recombination may occur without any selection. This model is a useful tool for the study of mitochondrial inheritance and the mechanism of mtDNA recombination in asexual conditions.

F. SOMOGYVÁRI, CS. VÁGVÖLGYI, T. PAPP and L. FERENCZY

Electrofusion of *Mucor circinelloides* protoplasts

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Electrofusion provides an efficient and sample method for obtaining fusion products from fungal protoplasts. This technique has several advantages, e.g. it avoids the need for chemical fusion inducers, or the fusion process is more controllable and can also be monitored microscopically.

In the present work, electrical parameters were determined and quantified for induction of the optimum fusion of *Mucor circinelloides* protoplasts. Protoplasts were formed from two auxotrophic strains (M. *circinelloides* leu^- and ade^-) and subjected to various physical parameters with a Krüss CFA 400 electrofusion unit in a "Pipetten" chamber. The alignment field frequencies necessary for the most effective pearl chain formation and for the highest fusion frequency were found to be different: 1 MHz and 200 kHz, respectively. The optimum parameters for the fusion of *Mucor* protoplasts were 60 V pulse voltage, 66 μs pulse duration and six pulses at intervals of 1.0 s. Nutritionally complementing colonies were obtained at an average frequency of 12.4%, which is substantially higher than that achieved earlier with the PEG-Ca^{2+} method (1.7%) with *M. circinelloides* protoplasts.

B. SZÖŐR[1], ZS. FEHÉR[2], G. SZABÓ[2], P. GERGELY[1] and V. DOMBRÁDI[1]

Purification and cloning of protein phosphatases 1 and 2a 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 inhibitor sensitivity and metal ion dependence. We found that all the four main classes of Ser/Thr protein phosphatases are present in *N. crassa*. The catalytic subunits of PP2A and PP1 ($PP2A_C$ and $PP1_C$) were successfully purified to near homogeneity by using ammonium sulphate–ethanol precipitation, DEAE Sephacel, Heparin–Sepharose and MonoQ FPLC chromatography. The apparent molecular mass of $PP2A_C$ proved to be 35 kD in gel filtration experiment. $PP2A_C$ was completely inhibited by 1 nM okadaic acid, was insensitive to rabbit muscle inhibitor-2, and was specific for the α-subunit of rabbit muscle phosphorylase kinase as substrate. The catalytic subunit of PP1 was found to be 33 kD in size, was inhibited by okadaic

acid and inhibitor-2. It could be established that PP1 and PP2A *of N. crassa* showed striking similarity to their mammalian counterparts with respect to biochemical characteristics. The pSV50 cosmid library of *N. crassa* was screened using a *Drosophila* PP1 cDNA probe. Four recombinant cosmids of different restriction patterns were isolated. The heterologous cDNA probe hybridized to 4–5 BamHI and HindIII fragments in a Southern hybridization experiment. Subclones from two recombinant cosmids were partially sequenced with primers homologous to the conserved regions of PP1. One of the sequences proved to be a truncated version of the other. The obtained sequence showed high degree of homology to *Aspergillus nidulans bimG* gene and other known yeast, *Drosophila* and mammalian protein phosphatase 1 genes.

C. FEKETE[1], T. WESZELY[1] and L. HORNOK[2]

Cloning, sequencing and chromosomal mapping of an endochitinase gene from *Trichoderma hamatum*

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Antagonistic and hyperparasitic fungi have increasingly been applied in integrated pest management to reduce the use of pesticides. Members of the genus *Trichoderma* are among the most potent biocontrol agents applied against soil inhabiting plant pathogens. Extracellular mycolytic enzymes, mainly chitinases are considered to be major factors in the hyperparasitic attack but volatile and non-volatile antibiotic compounds are also involved in the biocontrol ability of these fungi.

Several lines of evidence suggest that there is a negative correlation between mycolytic activity and antibiotic activity of the different strains. Improved biocontrol strains are likely to be produced by combining these two desirable traits by introducing a powerful chitinase gene into a strong antibiotic isolate. In order to promote such a strain-breeding programme an endochitinase gene was isolated from *T. hamatum* by means of polymerase chain reaction. This PCR-generated sequence, 1.6 kb in size contains three short (50–60 bp) introns inserted in between the potential coding regions. The deduced polypeptide sequence of this clone shows a >90 % homology to the endochitinase gene from *T. harzianum* and 66 % homology to the chitinase gene of *Aphanocladium album.*

Integral fragments of the chitinase gene cloned from *T. hamatum* were hybridized to Southern blots of electrophoretically separated chromosomes-sized DNAs of other *Trichoderma* species, *T. atroviride*, *T. harzianum*, *T. koningii*, *T. longipilis*, *T. minutisporum* and *T. viride* in order to map this important sequence on representative members of the genus.

T. PAPP[1], CS. VÁGVÖLGYI[1], K. MAGYAR[1], T. J. MICHAILIDES[2] and L. FERENCZY[1]

Use of randomly amplified polymorphic DNA to distinguish isolates of *Mucor piriformis*

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Mucor piriformis is an important postharvest pathogen of different fruits and vegetables. This fungus can cause the substantial decay of such agricultural products if they are stored or transported at low temperature for a prolonged time.

The twenty-nine *M. piriformis* isolates used in this study were from infected pear (cv. d'Anjou) fruit. Six different 10-bp primers were used to generate random amplified polymorphic DNA (RAPD) markers from the isolates; each was analysed in three separate reactions. These primers revealed different levels of diversity; three to nine different amplification patterns were detected. The results led to the definition of 26 composite amplification types, indicating a higher degree of variability than that found in previous isoenzyme studies. The dendrogram produced by the unweighted pair group method from the RAPD data revealed a correlation between the two clusters obtained and the mating potency of the isolates. These results demonstrate that RAPD analysis provides an efficient approach for strain identification and genetic studies of *M. piriformis.*

G. GICZEY[1] and L. HORNOK[2]

Random DNA clones for the identification of fungal endophytes

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Asymptomatic, systemic fungal endophytes are frequently found in association with various grass species. There is a mutualistic symbiotic interaction between these fungi and their hosts. The endophyte benefits from this association by nutrition, protection and dissemination. On the other hand, the fungus stimulates the growth of the plant and enhances its tolerance to phytophagous insects. Ergot and loline alkaloids produced by the fungus are responsible for this protection. These and other alkaloids of the endophytes are implicated in toxicoses of domestic grazing animals.

Conidial forms of these fungi belong to the genus *Acremonium*, some of them are anamorphs of *Epichloë typhina* or related *Ascomycetes.* Most endophytes grow very slowly on artificial medium and are rapidly overgrown by other fungi, therefore they are not easy to bring into pure culture and difficulties are arisen in their identification as well.

In order to find a rapid and reliable method for the detection of fungal endophytes random DNA clones were isolated from *A. coenophialum*. Sizes of these clones ranged from 0.7 to 10 kb. Two of them, E9414 and E9423 were tested for specificity on *Eco*RI and *Pst*I digested tDNA extracts from endophytes (*A. coenophialum*, *A. uncinatum* and *Acremonium* sp. strain 8907) as well as from a free-living *Acremonium* species, *A. cerealis*. E 9414 gave strong hybridization signals on *A. coenophialum* and *A. uncinatum*, but negative reactions were observed on *Acremonium* sp. 8907 and on *A. cerealis*. Probe E9423 hybridized to DNA extracts of all the three endophytes and gave no signal on *A. cerealis*. Restriction fragment length polymorphisms were also detected in the positive cases allowing further differentiation within endophytes.

E. RAHMANI and CS. DOBOLYI

Effect of benomyl on the mycorrhizas of different alfalfa species

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The development of arbuscular mycorrhiza (AM) symbiosis of different alfalfa species was investigated in the presence of the fungicid benomyl in pot experiments.

The effect of the agronomically applied concentrations of benomyl proved to be significant both on the morphological modifications and the colonization kinetics in the root tissue of four alfalfa species (*Medicago orbicularis*, *M. sativa*, *M. scutellata*, *M. truncatula*). Fungal elements were detected with tripan blue staining method, at time of 2, 4, 6, 8 and 10 weeks after sowing.

The colonization rate in *M. scutellata* decreased at 75% after 4 weeks, at 56% after 6 weeks, and at more than 40% even after 10 weeks on the effect of the dose of 2×10^{-3} g/g of the drug. The dose of 3×10^{-3} g/g proved to be fungicidal against the AM fungal population: no detectable colonization developed in any investigated species even in the 10 weeks of experiment.

The effect of benomyl, in general, was much more expressed on the AM colonization in the case of the annual than the perennial species. The mode of application of benomyl in agronomical practice should be considered depending on the soil types and the alfalfa species.

T. PUSZTAHELYI, I. PÓCSI and A. SZENTIRMAI

In vitro and in vivo inhibition of the *Penicillium chrysogenum* N-acetyl-β-D-hexosaminidase

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In *Penicillium chrysogenum* NCAIM 00237 strain N-acetyl-β-D-hexosaminidase accumulated inside the cells in the stationary phase of growth and was secreted later into the culture fluid. It was demonstrated that the formation of the enzyme was controlled by glucose repression [1].

To elucidate the physiological function and the enzymological characteristics of the enzyme we investigated the in vitro and in vivo inhibitory effect of the transition-state analogue O-(2-acetamido-2-deoxy-D-glucopyranosylidenamino)-N-phenylcarbamate (GlcNAc-PC-oxime) [2] and two substrate analogue inhibitors, p-nitrothiophenyl-2-acetamido-2-deoxy--β-D-glucopyranoside (PNTP-GlcNAc) and thiophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (TP-GlcNAc) [3].

In the in vitro inhibitory experiments all the transition and substrate analogues were found to be competitive inhibitors of the extracellular N-acetyl-β-D-hexosaminidase.

The K_I values for the inhibitors were: GlcNAc-PC-oxime: $(6.82\pm0.14)\times10^{-8}$ M; TP-GlcNAc: $(4.13\pm0.27)\times10^{-3}$ M; PNTP-GlcNAc: $(1.064\pm0.04)\times10^{-3}$ M.

Although both TP-GlcNAc and GlcNAc-PC-oxime were potent inhibitors of the enzyme in vitro no change in the mycelial dry weight, the morphology and the production and secretion of the N-acetyl-β-D-hexosaminidase could be observed at an inhibitor concentration of 0.6 mM and 2 μM respectively.

More recently Rast et al. [4] suggested that multienzyme complexes consisting of chitin synthase, chitinase and N-acetyl-β-D-hexosaminidase may play a crucial role in the biosynthesis of the chitin fibres in the cell wall of different fungi. Regarding these findings we can assume that the *P. chrysogenum* N-acetyl-β-D-hexosaminidase is a typical vacuolar/cytosolic enzyme [5], which takes part in the degradation of different glycoprotein and polysaccharide reserves during prolonged starvation. Any possible participation in the cell wall biosynthesis has remained yet to be shown.

[1] Pócsi, I., Pusztahelyi, T., Sz. Bogáti, M. and Szentirmai, A.: J Basic Microbiol 33, 259–267 (1993); [2] Beer, D., Maloisel, J.-L., Rast, D. M. and Vasella, A.: Helv Chim Acta 73, 1918–1922 (1990); [3] Pécsi, I., Kiss, L., Zsoldos-Mády, V. and Pintér, I.: Biochim Biophys Acta 1039, 119–122 (1990); [4] Rast, D. M., Horsch, M., Furter, R. and Geeday, G.W.: J Gen Microbiol 137, 2797–2810 (1991); [5] Mayer, C., Horsch, M., Linder, C. and Rast, D. M.: In Muzzarelli, R. A. A. (ed.): Chitin Enzymology, pp. 485–492. Ancona, European Chitin Society 1993.

T. VARGA and I. MIKLÓS

Analysis of chromosome-segregation in a yeast by pulsed-field gel electrophoresis

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The pulsed-field gel electrophoresis offers a powerful tool to show chromosomes of eukaryotic microorganism, where the traditional cytochemical methods cannot be used.

We carried out the analysis of chromosomal DNA patterns of a distillery's yeast strain of *Saccharomyces cerevisiae*, as this strata showed different abnormalities in its cell division. It produced abnormal asci with 2–3 spores and the viability of the spores was very low. These features might be caused by aneuploidy.

However, the analysis revealed that there are differences between the laboratory and the distillery's strains in their electrophoretic karyotypes. This can more easily be explained by chromosome polymorphism than by aneuploidy.

The examination of mitotic and meiotic segregation of chromosomes in the distillery's strain suggest that the mitosis is regular but the meiosis is highly biassed. As the chromosomal patterns of the meiotic segregants differ from that of the original strain and from each other, we conclude that the found abnormal sporulation cannot come from aneuploidy. It is most probable that the original strain studied is a hybrid of different varieties with different chromosomal sets.

I. MIKLÓS[1] and M. SIPICZKI[1,2]

Characterization of a novel temperature-sensitive cell cycle mutant in *Schizosaccharomyces pombe*

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The cylindrical cells of the unicellular yeast *Schizosaccharomyces pombe* provides a great possibility to study the cell cycle. We isolated a new cycle mutant, which seems to be defective in a cell cycle gene (spl1), which is different from the known genes. It is a thermosensitive mutant, whose cells undergo morphological changes (bending and producing short mycelia) when shifted to 35 °C and gradually stop growing. The mycelia are the results of the fact that the cells are unable to split their septa (no cell separation). The fluorescent staining of septa and the electron microscopic examination suggest that these septa might have normal structure and width.

Mitosis also seems to be regular according to the DAPI-stained image of nuclei. It is interesting that the inhibitory effect is not observable when stationary phase cells are

shifted to 35 °C, because they can grow at this temperature and do not show any morphological aberration. So this mutation might affect a gene encoding a protein which is specific for the logarithmic phase cells. Cloning of the gene is in progress.

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A *Schizosaccharomyces pombe* mutant defective in cytokinesis

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A number of factors required for the regulation of DNA synthesis and mitosis have been identified. However, much less is known of cytokinesis, how cell divide and how this process is coupled to nuclear division. In order to learn more about the genetic regulation of cytokinesis we isolated spontaneous mutants in *Schizosaccharomyces pombe* defective in cytokinesis. Hybridization experiments revealed that they can be classified into several complementation groups. Here we present the characterization of a mutant belonging to the group *sep2*.

The fission yeast *Schizosaccharomyces pombe* is a unicellular fungus that is haploid under natural conditions. As a result of a mutation in the *sep2* gene complementation group considerable number of the cells form mycelia consisting 2–4 compartments. In addition, the position, the structure and the number of the septa are also abnormal. The cells show resistance against enzymatic dissolution and the electron microscopic image also shows abnormalities in the structure of the septa. This phenotype might result from various defects. Interestingly, the actin and tubulin cytoskeleton is not abnormal.

Nuclear division does not appear defective in the mutant. However, the mutant cells form spores with low germination rate. The germination process is often abnormal and slow, and many of the spores are diploid. A possible explanation is that spontaneous endodiploidisation occurs quite often in the *sep2* mutant. This is consistent with the finding, that with flow cytometric analysis a remarkable 4C peak was detected which corresponds to diploid cells in the G2 phase.

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Growth pattern of cell length in the fission yeast cell cycle

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Mitchison and Nurse (J Cell Sci 75, 357–376, 1985) found that growth in the cell length during the cell cycle of *Schizosaccharomyces pombe* is linear with a rate change point (RCP) at 0.3–0.35 cell cycle position. The RCP is coincident with the so-called NETO event (new end take off). At RCP the growth rate is not doubling, but increasing by 35% only. This means that there must be another RCP at cell division with a further 65% increase in rate. On the contrary, they could not find NETO in *wee* mutants of fission yeast.

Using time-lapse microphotography we measured the cell length of 40 cells from birth to division on a film of wild type (WT) fission yeast, and found an avenge position of RCP at 0.36 with a 30% increase in growth rate. By analysing a number of *wee* mutants' films (*wee1.6*, *wee1.50*, *δwee1*, *cdc2.33*) we have established that there is an RCP even in these cells. The position is somewhat earlier in *wee* mutants than in WT cells (0.21–0.26 cell cycle position), but here the rate is nearly doubling (70–80% increase).

From our results we suggest that there are two RCPs in the cycle, one of them is connected to S-phase or START-event (this is the stronger RCP), the other one is connected to NETO (this is the weaker RCP). In WT cells the position of S-phase is 0.0, which is connected to a 70% RCP, and the position of NETO is 0.3–0.35 with a 30% RCP. In *wee* mutants the position of S-phase is 0.3, which is connected to a 70–80% RCP, and the position of NETO is 0.0 with a 20–30% RCP. So we suppose that in *wee* mutants the NETO event occurs at cell division (logically it must be true).

By analysing the largest cells from an induction synchronized *cdc2.33* mutant (the first cycle after temperature shift-down), we have not found an RCP in the cycle, but found a 60% RCP at cell division (there is no doubling in rate because this population is not homeostatic). Our conclusion is that RCP (and even NETO which is connected to it) is a size-controlled event in the fission yeast cell cycle. By artificially enlarging the size of the cells (*cdc2* block and release experiment), the position of RCP goes back from the normal 0.3–0.35 position to earlier in the cycle. In the largest cells it reaches 0 position, therefore there is no RCP in the cycle except at cell division.

INDUSTRIAL MICROBIOLOGY

I. BARTHÓ[1], S. KOVÁTS[1], K. VAJDA[1], L. MÓZES[1], Á. ZENTAI[2], B. KŐVÁRI[3] and Á. BUDA[3]

Supervisor computer system to monitor the fermentation laboratory and pilot plant

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In the optimization and scale-up of fermentation processes there is an obvious need for a monitoring system that covers the entire process cycle from laboratory scale up to pilot scale.

After a long term development we completed the supervisor and monitor computer system in our fermentation laboratory and pilot plant. The system was developed to monitor the local network of the stand alone controllers, called bioprocessors that were developed and manufactured by INEL Ltd. The supervisor computer system consists of four computers in a local network. The supervisor software developed by ASK Ltd. is running under QNX operation system. The system offers continuous monitoring of our laboratory and pilot scale fermenters and also the monitoring of our base energy system. With its special data storage, print, off-line data input and display options it supports the on-line comparison of different batches and assures their complete documentation.

G. SZAKÁCS[1], J. FEKETE[2], G. MORAVIÁN[2] and R. P. TENGERDY[3]

Monitoring of selected metabolites from fermentation broths by high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) coupled with UV-diode array detector and personal computer have been used for detection of metabolites in *Aspergillus terreus* and *Aspergillus fumigatus* fermentation broths. The target compounds were mevinolin and fumagillin, respectively. The medium composition was optimized on a shake-flask scale for production of mevinolin and fumagillin using the Graeco-Latin square technique.

In screening for microorganisms capable of degrading xenobiotic compounds (e. g. chlorinated phenols), HPLC was also the method of choice.

K. KÖNCZÖL and S. KOVÁTS

Practical aspects of fermentation process development

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The R and D activity can be divided into three consecutive stages: (i) basic research – aiming the enrichment of our undemanding on natural phenomena; (ii) applied research – targeting the possible advantages for mankind; and (iii) process development – dealing with the industrial exploitation of scientific results.

In the past decades scientific efforts were concentrated on the upstream parts of fermentation processes in contrast with downstream processing. Highly sophisticated and really effective fermentation technologies were developed concerning the upstream part, but their commercialization has failed in many cases because of the problems associated with downstream processing.

In the period of extensive industrialization, environmental protection was ignored. The cost calculations were frequently made on a single process basis, without the consideration of the consecutive steps towards the final product. Nowadays, however a systems-approach must be accepted in fermentation process development, considering the technological and economical implications of the present situation. The various processes have to be integrated as far as it is possible, no matter whether they are of microbiological or chemical nature. Development and evaluation of such whole technological chains give inevitable advantages during the industrial realization.

Process development needs a pool of complex expertise, that can only be provided by project teams. Knowledge of legal restrictions, environmental regulations, patent rights must also be present beyond the special expertise in the areas of biotechnology and chemistry. The materials used in a given process have to be selected considering not only the efficiency, but also their toxicity, inflammability, biodegradability, possible recovery and some special features, such as tendency to getting electrically charged or to forming azeotropic mixtures. Multipurpose equipment and methods provide substantial flexibility both in the upstream and the downstream part of fermentation processes. The full exploitation of existing capacities is of crucial importance just like the minimalization of investment demand. Running the processes economically is not a question of lime but rather a question of timing: avoiding delay is more significant than such traditional factors as volumetric productivity of a single process. Volumetric profitability is the right concept.

The trend of and demand for "clean technologies" require the application of enzymes instead of fermentation broths, and adsorption or membrane techniques instead of organic solvent extraction. Our lecture deals with the details of the above-mentioned practical aspects of process development, including some illustrative examples.

S. KOVÁTS[1], B. CSUKÁS[2], L. TERDY[1] and I. JAKSA[1]

Modelling aspects for scale-up and optimization of packed-bed columns in adsorption

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In the scale-up and optimization of adsorption processes there is an obvious need for models that cover the entire process cycle rather than a single step. Many models are only concerned with the adsorption step and simulating breakthrough curves. However, as a unit operation in biotechnology, it is necessary to also model the other steps involved.

The batch-type adsorption processes are more or less complicated networks of multicomponent two phase columns with a definite mixing of both phases. The small scale batch measurement of isotherm and kinetic parameters cannot be exactly fitted to the column experiments, because there may be major contaminants that determine the process basically, but cannot be isolated and/or measured at all.

We propose a new modelling methodology for the direct mapping of the studied conservation processes onto a descriptive computer program that can be executed with the help of a general purpose simulator directly. In the present study the involvement of the computer assisted modelling tool ADSIM in the work of our laboratory is discussed.

Small scale batch experiments have been discussed, which provide estimates of the isotherm and kinetic parameters for the model and these will be compared with values fitted to experimental data from small column.

L. TERDY, I. JAKSAR and S. KOVÁTS

Recovery of products from fermentation broth by using adsorption resins

Chemical Works of Gedeon Richter Ltd., Budapest, Hungary

Pharmaceutical industry obtains some of its active ingredients or intermediates by fermentation processes. Frequently the synthetic and biotechnological steps are combined to prepare the drug, e.g. in manufacturing steroid preparations.

One of the most widely used method in down-stream processes of fermentation is extraction. It provides usually good results when concentration of the product is relatively high in the fermentation broth. In case of low product concentration – which occurs in the majority of cases (e.g. fermentation broth of vitamin B_{12} preparation) – the solvent requirement can be extremely high. The method can be objected from the point of view of environmental protection too.

Recently other methods, like ion-exchange and adsorption chromatography seem to replace some extraction methods. Adsorption chromatography is especially useful in the recovery of bioconversion products from low-concentration fermentation broth.

Macroreticular, non-ionic synthetic adsorbents can be successfully applied for isolation and purification of compounds with low polarity from more polar solvents. The adsorbed products can be selectively eluted from the adsorbent by low-molecular weight alcanols or ketones or their aqueous solutions. This method has been used and developed at Gedeon Richter Ltd. for almost 10 years. Our contribution gives some insight into the development and scale-up problems and results of this technique.

G. KOVÁCS[1], M. LÁSZLÓ[2], B. BARNABÁS[1] and GY. RAJKAI[1]

Regulatory effects of medium pH during fermentation of haploid maize cell suspensions

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Establishment and maintenance of embryogenic cell suspensions of maize, and nearly all other species, have relied primarily on manipulation of added growth regulators. Another, not well studied possibility is the maintenance of preglobular stage proembryos at a uniform developmental stage and their continued development into later embryo stages can be controlled by the pH of the medium pH as it had been shown using carrot cell suspensions. For the detailed study of this possibility in monocots maize (*Zea mays* L.) haploid cells were cultivated in 1500 ml aerated and stirred batch bioreactors using modified BM media. Cell growth was highly affected by pH and dissolved oxygen, and we observed two fairly distinct growth phases. During the first two days after inoculation at pH 5.8 oxygen consumption is high and the cells are actively lowering the pH to a value around 4.3. After this period the pH stabilizes at 4.1 and dissolved oxygen reaches a steady level. As long as the pH remained below 4.5 no embryoid formation was observed and the cell aggregates remained small (containing around 30-30 cells/aggregates). Fresh weight was approximately doubled over a week period. If the pH was buffered at or above 4.5 later embryoid formation were observed. Embryoids continued developing to mature stage when the medium was buffered to pH 5.5 or higher after two successive culture periods. When the initial medium pH was externally controlled at 5.5 using NaOH as a base titrant we observed some initial activity in the culture but little or no growth at all.

L. DENCSŐ[1], T. SAAD EL-DIN[2] and J. REZESSY-SZABÓ[2]

Cis-acting regions of the *Saccharomyces cerevisiae* ADHI promote

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The ADHI encodes a glycolytic enzyme which converts acetaldehyde to ethanol, it is a very important enzyme for fermentation industry. To characterize the regulation mechanism of ADHI expression in yeast, the complete promoter and its various deleted derivatives were fused to the LacZ gene of *Escherichia coli* encoding the β-galactosidase enzyme in plasmid YEp-type. Transcription activity of different constructions was calculated from the β-galactosidase enzyme activity in presence of both fermentable and non-fermentable carbon sources.

The deletion analysis of the ADHI promoter revealed additional sequences above the well-known UAS_{RPG}-box and the CTTCC pentamer which were associated with UAS_{RPG}-box for the maximal transcription activity. One is the poly(dA-dT) sequence between the AluI and SphI site. It is a putative binding site for a regulatory protein that can interact with the RAP-1 and/or GRC-1 regulatory proteins. The other important sequence is situated between the EcoRV and Ava II site.

We also studied the effects of different environmental conditions with the various promoter regions for the optimization of the high rate fermentation system. We can conclude that the transcription of ADHI intact promoter is highly related to cell growth phase.

T. EMRI and A. SZENTIRMAI

Effect of the nitrogen source on the regulation of the oxidative pentose phosphate shunt in *Penicillium chrysogenum*

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It has been known for a long time that the regulation of oxidative pentose phosphate shunt depends on the nitrogen source in many microorganisms. Namely, the specific glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are higher in the presence of inorganic nitrogen sources than in the presence of organic ones.

Here we show how the specific glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities changed when *Penicillium chrysogenum* was grown on different nitrogen sources. When ammonia was added to the media the specific activities of the two NADPH producing enzymes were higher than in the

presence of yeast extract, glutamate or in the absence of any nitrogen source. Using methylamine we demonstrated that an increase in ammonia concentration was responsible for the observed changes in the enzyme activities. Studying the effect of the carbon source on the nitrogen source depending regulation of the oxidative pentose phosphate shunt we found that the ammonia methylamine was effective only in the presence of glucose or fructose and it had no effect in the presence of caffeine or in media containing lactose as carbon source. Since a lower cAMP concentration was found with glucose and fructose than with lactose as well as when glucose or fructose and caffeine were added together to the media we assume that the elevated cAMP concentration could reverse the effect of ammonia on the regulation of the oxidative pentose phosphate shunt.

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Investigation of production of aromatic compounds by White-rot fungi

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One area where biotechnology-based processes are likely to replace and improve on existing production methods is in flavours and fragrances. However, most of the work in this industry is still at the research and development stage.

White-rot fungi (*Pleurotus*, *Trametes*, *Lentinus*) synthesize a wide range of aromatic compounds with a methoxygroup at the para-position. It is anticipated that these kinds of compounds are produced via the Shikimate pathway. *Pleurotus chrysosporium* biosynthesizes veratryl alcohol via L-phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratrylglycerol [1]. Many white-rot fungi also produce anisyl alcohol and aldehyde as well as their chlorinated derivatives [2]. The production of secondary metabolites is very much dependent on the strain, the growth substrate and the growth conditions.

The submerged fermentation of several white-rot mushroom and the recovery of mycelium cultivated by submerged fermentation to obtain fungal flavour is discussed.

Quantity of aromatic compounds depends upon the mycelium being subjected to shear stress or cell disruption. After cell disruption (by means of DINO MILL) the formation of flavours can be increased. The disrupted mycelia of white-rot fungi contained more than 1000 ppm 1-octen-3ol based on solids, because the cell linoleic acid comes into contact with linoleic acid oxidising enzymes and oxygen.

[1] Shimada, M., Nakatsubo, F., Kirk, T. K., Higuchi, T. (1981): Arch Microbiol 129, 321–324 (1981); [2] Kamaya, Y., Higuchi, F.: FEMS Microbiol Lett 24, 225–229 (1984)

G. VERECZKEY[1], I. PARLAGH[1], M. TEN WOLDE[2] and M. HOUTMANN[2]

Benzenediol oxidoreductase fermentation and purification from the White-rot basidiomycete *Pleurotus sajor caju*

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Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) a copper-containing oxidase [1] has been shown able to perform the C_α-C_β-cleavage of a phenolic β-1 lignin substructure compound (phenolic diarylpropane) [2] with a mechanism analogous but not identical to that proposed for manganase peroxidase. Laccase is a potentially useful enzyme in waste treatment because this oxidoreductase is able to degrade a broad spectrum of structurally diverse organic compounds or polymerize them [2].

In this paper we present the results of an extended study of the fermentation and purification of the laccase enzyme produced by *Pleurotus sajor caju* (MUCL 29757). After optimization of the laccase production, fermentation were carried out in Bioflo III fermenter. After 8 days 600 LCU/ml activity was achieved. The medium was separated from mycelium, purified by Hollow Fibre (Amicon H1P30-43) the enzyme was precipitated by $(NH_4)_2SO_4$, dialysed and purified by FPLC and Rotofor preparative IEF cell. For determination of molecular mass SDS-PAGE Phast System was used. Two isoenzymes have been found. The molecular masses of the isoenzymes were 65 and 70 kD with 33 and 3.9 PI values. The specific activity was 8.5 LCU/μg.

[1] Fahraeus, G., Lindeberg, G.: Physiol Plant 6, 150–158 (1967); [2] Kawai, S., Umezawa, T., Higuchi, T.: Arch Biochem Biophys 262, 99–110 (1988); [3] Bollag, J. M., Schuttleworth, Andersson, D. M.: Appl and Environm Microb 54, 3086–3091 (1988)

G. HALMOS[1], B. JANZSÓ[1] and K. OLASZ[2]

Optimization of the 11α-hydroxylation of levodion by *Aspergillus awamori*

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The 11α-hydroxylation of steroid by fungi is used extensively as an intermediary step in the synthesis of new steroids since the chemical synthesis of these compounds is often impossible or far too complicated for industrial production. It has been observed that the formation of side products and product yield can be profoundly affected by the composition of the growth medium.

The aim of this work is to determine an optimum growth medium for the 11α-hydroxylation of the steroid levodion (13-ethyl-gon-4-en-3,17-dion), with submerged culture of the *Aspergillus awamori* species. Three constituents are investigated simultaneously by varying the concentration of each so that all possible combinations of constituent concentrations are considered for the experiment. The method used in this procedure is commonly known as Factorial Experiment. From the analysis of the results a mathematical model was constructed for product yield with respect to the three variable constituents.

B. LENKEY[1] and L. SOMOGYI[2]

The metabolism of 3-acetyl-2,2-diphenyl-5-methyl-1,3,4-oxadiazoline by *Alternaria alternata*

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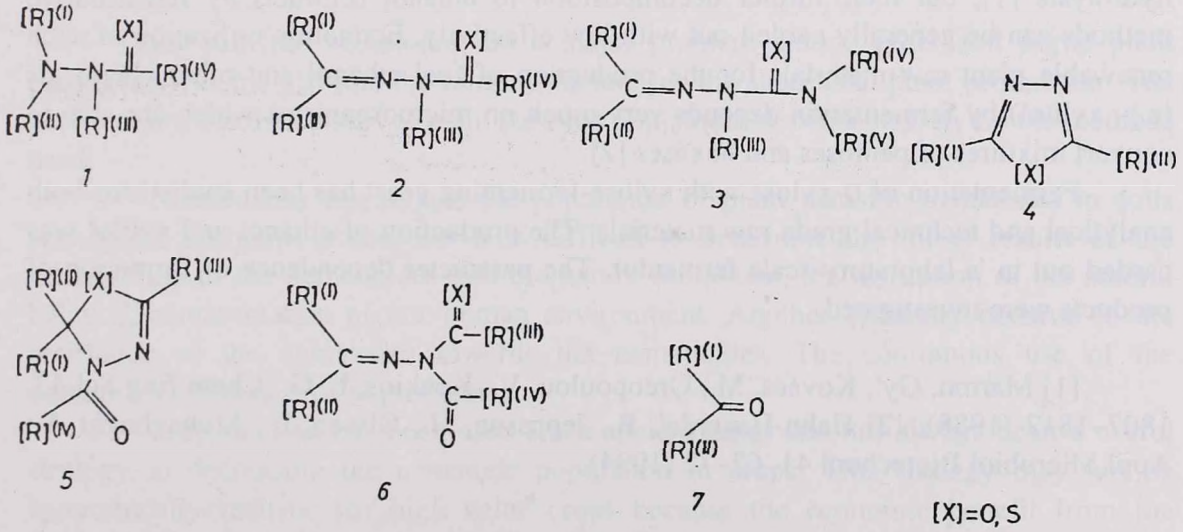
Several hydrazine derivatives, antibiotics and synthetic products (e.g. the antidepressant drug Iproniazid Me_2CH-NH-NH-CO-C_5H_4N-(4)), (tio)acid hydrazides (*1*), (tio)acylhydrazones [1] (*2*), e.g. (tio)semicarbazones (*3*) [2] and cyclization products: 1,3,4-oxa(thia)diazoles (*4*) and dihydro derivatives [3] as well as their metal complexes [4] exhibit biological activity.

The heterocycle of 3-acyl-1,3,4-oxa(thia)diazolines (*5*), cyclic isomers of the corresponding aldehyde or ketone diacylhydrazones (*6*) is stabilized by the presence of the acyl group at position 3.



As various acylated compounds can be partially or fully deacylated enzymatically (among others in the liver or kidney) the above heterocyclic compounds they themselves or their (partially deacylated) metabolites may act as human or veterinary pharmaceuticals and agrochemicals, respectively.

With respect to the above chances we aimed at the investigation of the effect of *Alternaria alternata* on 3-acetyl-2,2-diphenyl-5-methyl-1,3,4-oxadiazoline (*5*) [5] a cyclic isomer of benzophenone diacetylhydrazone [5] (*6*), R^1, R^2 = Ph, R^3, R^4 = Me, X = O) as the model substrate.

The metabolites of this substrate by *A. alternata* are 2 (R^1, R^2 = Ph, R^3 = H, R^4 = Me, X = O) and benzophenone (7) (R^1, R^2 = Ph).

[1] E. Piscopo, M. V. Diurno, R. Gagliardi, O. Mazzoni, F. M. De Francesco, C. Parrilli: Boll-Soc Ital Biol Sper 65, 311–316 (1989); [2] S. N. Pandeya, J. R. Dimmock: Pharmazie 48, 659–666 (1993); [3] L. Somogyi: Liebigs Ann Chem 931–934 (1993); [4] I. H. Hall, K. G. Rajendran, D. X. West, A. E. Liberta: Anti-Cancer Drugs 4, 231–240 (1993); [5] L. Somogyi: Tetrahedron 41, 5187–5190 (1985).

L. GALLÓ[1] and CS. SISAK[2]

Fermentation of D-xylose by yeast

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Pentoses of hemicellulosic origin, especially D-xylose are important constituents of agricultural biomass. They can be produced from hemicellulose by a simple acidic hydrolysis [1], but their further decomposition to ethanol (biofuel) by fermentative methods can be generally carded out with low effectivity. Economic utilization of such renewable plant raw materials for the production of fuel ethanol and other chemicals (e.g. xylitol) by fermentation depends very much on microorganisms which are able to convert mixtures of pentoses and hexoses [2].

Fermentation of D-xylose with xylose-fermenting yeast has been studied for both analytical and technical grade raw materials. The production of ethanol and xylitol was carded out in 'a laboratory scale fermentor. The parameter dependence of formation of products were investigated.

[1] Marton, Gy., Kovács, M., Oreopoulou, V., Koukios, E. G.: Chem Eng Sci 43, 1807–1812 (1988); [2] Hahn-Hagerdal, B., Jeppsson, H., Olsson, L., Mohagheghi A.: Appl Microbiol Biotechnol 41, 62–72 (1994).

K. BÉLAFI-BAKÓ and E. NAGY

In situ product recovery in ethanol fermentation

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Integration of traditional separation techniques and bioconversions in order to get higher yield, productivity and effectivity has been intensively studied for a couple of years all over the world. Application of simultaneous fermentation and product separation is especially useful if the end product(s) has inhibitory or even toxic effect on the bioconversion, for example in case of ethanol fermentation.

Several methods have utilized the volatile character of ethanol for its traditional separation as well as in-situ removal (e.g. evaporation, gas stripping, vacuum fermentation, etc.). Application of membranes combined with the techniques mentioned may enhance the conventional processes considerably. For example gas stripping combined with special membranes – called pervaporation – has given rather selective, excellent method for ethanol removal. This paper presents our results on ethanol fermentation integrated with product removal by pervaporation.

M. DUDÁS-SZABADY

Biological control of nematodes by nematode trapping fungi

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Plant parasitic nematodes are a major problem among other soil borne plant pathogens which constitute the limiting factors for the successful plant production. The performance for the control of plant parasitic nematodes depends heavily on the methods used:

– Nematicides can reduce the population of plant parasitic nematodes in soils with great economic value, but it is difficult to determine the direct results of the introduction of the nematicides into the nature ecosystem, the disruption of the natural balance, contamination of our human environment. Another difficulty consists in the resistance of the nematodes towards the nematicides. The continuous use of the nematicides makes several problems.

– Crop rotation has been used since ancient times and has always been a useful strategy in decreasing the nematode population in crops. This strategy may not be economically realistic for high value crops because the economic benefit from the alternative crops is always lower than that of the main protected crops.

– The large number of organisms in soil (fungi, bacteria etc.) can predate or parasitize the plant parasitic nematodes. That gives the hope that biological control of

nematodes can be realized. Modern agriculture demands nematode management strategies that are reliable, effective and economically viable and which can be satisfactorily integrated with overall production systems.

Naturally occurring fungi can maintain population of plant parasitic nematodes below economic threshold. The aim of our research work was to find the best circumstances of maintain of *Arthrobotrys irregularis* nematode trapping fungus and its fermentation in research fermenter to get the highest biomass production. The change of different parameters during the fermentation process as pH, biomass production, substrate consumption was measured. The effect of the different substrates was also investigated. The total biomass concentration became 1.6 g/100 g at 192 h.

L. KARAFFA, E. SÁNDOR and J. KOZMA

Studies on the morphology and physiology of *Acremonium chrysogenum* in continuous flow culture

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In our previous studies we have examined the properties of the respiration of a filamentous fungus *Acremonium chrysogenum* together with the influencing factors like NADH/NAD content, oxygen tension, and ADP-limitation. We have established that the fungus possesses a cyanide-resistant alternative respiration in addition to the cytochromic one, and it may have some role in the overproduction of the cephalosporin-C antibiotics. The fungus also undergoes morphological changes during cultivation, showing filamentous form followed by an arthrosporic one. However it was not possible to avoid the effects of changes of the milieu, especially the effect of nutrient depletion.

Because of the reasons mentioned above we have tried to examine the morphological changes and the intensity of respiratory pathways and antibiotics production rate under constant conditions, which can be achieved in continuous culture (chemostate). Applying different dilution rates (different generation tunes) we studied the changes of the capacity and the activity of alternative respiration. We have established that the intensity of the respiration periodically changes, similarly to the capacity of alternative respiration. The length of periods depends on the generation time.

I. LÁNG, A. ANDOR, N. MAKK, I. ILKŐY and A. JEKKEL

Microbial transformation of sitosterol by mutant *Mycobacterium* sp. strain

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Nowadays there is an increasing demand on the steroid drugs all over the world. As it is known 9α-hydroxy-4-androstene-3,17-dione is used for the producing of corticosteroid drugs, 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) are the starting materials of the androgen hormones. and contraceptives, respectively. For the economical reason steroid compounds are produced by microbiological degradation of natural sterols in the pharmaceutical industry.

We have used an efficient mutagen method for developing of such kind of *Mycobacterium* strains which can convert sitosterol to ADD with a high yield. The efficiency of the mutagen agent has been increased by means of sodiumdodecyl-sulfate which solves the lipid-rich outer layer of the *Mycobacterium* cells. Screening the sitosterol converting ability of the isolates after the mutagenic treatment we have got a strain which accumulates 22-hydroxy-23,24-dinor-1,4,17(20)-cholatrien-3-one and 3-oxo-23,24-dinor-1,4,17(20)-cholatrien-22-al in the fermentation broth which have never described in the literature. Among the several thousand isolates there were bacterial cells which produce ADD at high level (80%). One of these strains was applied for the optimization of the conditions of the ADD fermentation.

M. NAGY[1], Á. SZÉCSI[2] and K. OLASZ[1]

Stimulation of sporulation and preservation of fusaria

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Fusaria have a very important role in the industrial bioconversion of steroids. Therefore the long-term maintenance of these useful strains is required in artificial environment.

Preservation of original characteristics of Fusaria is not possible on agar media containing sugar. The sugar stimulates mutation which may appear as a change of conidial morphology, cultural characteristics and/or bioconversion capability.

We would like to report our observations related to the *Fusarium semitectum*. This strain has been preserved on potato-dextrose agar for many years and then freeze-dried with protection of sugar. Regenerating after 3 years, the strain lost its sporulation capability, the culture grew poorly and changed its colour. These observations induced the search for a more reliable preservation method.

The successful preservation is based on regaining of sporulation capability. The sporulation was stimulated by Bilay solution (this is a sugar deficient medium) and monosporic culture was prepared from the spores. Then the spore suspension was transferred onto SNA media containing carnation leaves. After 10 day-incubation at 24 °C the pieces of carnation leaves were placed into ampoules and lyophilized with Skim milk. The survival of the strain after this procedure met the requirements.

N. NÓGRÁDY, I. PÓCSI and A. SZENTIRMAI

Isolation and characterization of the intra- and extracellular cyclodextrin glycosyltransferases of *Bacillus macerans*

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Cyclodextrin glycosyltransferase (CGT-ase) of *Bacillus macerans* catalyses the degradation of starch into cyclodextrins. Although CGT-ases are among the best-known hydrolyses their physiological function has yet remained to be elucidated. Summing up the results of the previous physiological studies we could set up the following hypotheses [1]: (i) CGT-ase production took place in the exponential phase of growth of the microorganism and the enzyme was fully responsible for the degradation of the starch present in the culture medium, (ii) the CGT-ase was probably anchored to the cell membrane in the exponential phase (it is measurable as non-soluble intracellular enzyme activity), was released and entrapped between the cell membrane and the cell wall in the early stationary phase (soluble intracellular activity), and was released into the culture medium in the late. stationary phase and during cell lysis (extracellular activity).

In order to verify this hypotheses we isolated, pitied and characterised the intra- and extracellular CGT-ases of *B. macerans*. The intra- and extracellular CGT-ases were pitied to homogenity by a subsequent application of (1) adsorption on starch, (2) ion exchange chromatography on DEAE-Sephacel and (3) gel filtration on Sephacryl S-200 HR. The two forms of the enzyme showed identical molecular characteristics. They could be eluted from the DEAE-Sephacel column at the same NaCl concentration: c_{NaCl}=0.05–0.08 mol/dm^3 and the molecular weights of the enzymes determined by SDS-PAGE were also found to be the same: M_r=75000±3000. Regarding these findings we concluded that the enzyme was released from the bacterial cell without any modification.

Now we are raising polyclonal and monoclonal antibodies against both forms of the enzyme. Using immunogold electronmicroscopy techniques we plan to elucidate the exact subcellular localization of the enzyme.

[1] Nógrády, N., Pócsi, I., Szentirmai, A.: Biotechnol Appl Biochem 21, 233–243 (1995)

GY. RAJKAI[1], F. HOLLÓSI[2], M. LÁSZLÓ[2] and G. KOVÁCS[1]

Dynamics of pH changes in *Rubia tinctorum* cell suspension

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In the case of nearly all studied plant species medium pH is found to be one of the most important factors of cell suspension cultures. Similar pH profiles were observed during different plant's cell cultivations. Controlling medium pH effects cell growth, differentiation and even secondary metabolite synthesis.

In the present study *Rubia tinctorum* leaf originated cell suspensions were cultured in modified Murashige–Skoog medium in shaking flasks and in bioreactor experiments to study the stress reactions caused by the periodic changes of medium pH during subculturing. After each subculture inoculation at pH 5.8 the medium pH rapidly drops to 4.4 both in shaking flask and bioreactor studies. The most part of this pH decline occurs already in the first hours after inoculation. After this, the pH stabilizes at a somewhat higher pH value. This stress condition caused by pH fluctuation can be eliminated by decreasing the pH of the medium to 4.6 before inoculation which results in higher cell production efficiency.

N. SZŰCS, VAN THU CAO, L. SZIGETI and B. SEVELLA

Simulation of lactic acid cultivation

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The aim of this simulation research is to prepare the optimization of the lactic acid cultivation and develop non-conventional models.

The simulation tests are based on some submerged batch cultivations. The developed model contains equations for the cell growth, the substrate consumption, the product formation and the product inhibition. Also, it is taken into consideration how to avoid the inhibition effect.

The MATLAB program package was used for carrying out the simulation. This program is an interpreter that makes sure an easy and user-friend environment for model construction and development. Its graphical facility results in illustrative model output. Our simulation results support the assumed model and make sure an initial point to the further examinations.

M. KECSKÉS JR.

Examination of anaerobes isolated from the sediment of a small stream

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Our main aim was to work out a preliminary method for the isolation and identification of anaerobic bacteria present in the streambed of Szilas stream. Just like many other streams in Budapest, the bottom of Szilas stream is partly covered by concrete and its present state is far from natural.

The sediments of waters take important part in the removal of toxic agents,. organic or inorganic chemicals. Due to the increased load received from settlements in the form of industrial and municipal waste the anaerobic environments and their microbes cannot be neglected. There are plans for rehabilitation works in Budapest which aims to get nearer to the natural states of small waterflows.

Samples were collected at three sites (from more or less natural and canalized stream reaches) and from two different depth levels (0–3.5 cm; 3.5–7 cm). A few simple technics were applied to maintain anaerobic conditions from sampling to laboratory work. Several different media were tried to isolate bacteria and an anaerobic glovebox system was used to provide maximum opportunity for oxygen-free growth.

Routine morphological and biochemical tests were carried out and, by using gas-chromatography, fermentation end products of microbes were analysed to help identification and to discover possible taxonomic relations.

Among the isolates less than half proved to be obligate anaerobic bacteria in most cases Gram-positive rods. Pleomorphic sporeforming species could also be detected together with clostridia. From the most polluted region of the Szilas stream, as a first trial, sulphate reducers could also be isolated on selective media.

GY. HRUBY[1], M. LANGER[1], P. KOMÁROMY[2], M. BUDAI[1], CS. SISAK[2] and K. ZALAI[1]

Intensification of anaerobic waste water treatment in a three-stage fluidized-bed reactor

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The efficiency of a three-stage fluidized-bed pilot reactor has been studied for anaerobic waste water treatment. In the reactor an activated and adapted inoculum from sewage sludge was used which contained mixed population of methanogenic microorganisms.

It was found that applying a well-defined range of low frequency and amplitude of rectangular waves form pulses for the activation and adaption of the inoculum the biomethanation process improved significantly.

In the progress of anaerobic waste water treatment experiments, biofilm was grown on the surface of natural zeolite support particles. The growth of biofilm was investigated and the protein content of biofilm was determined. The effect of increasing COD loading model waste water on the conversion up to steady state was studied.

R. P. TENGERDY[1], G. SZAKÁCS[2], J. SCHMIDT[3] and J. SIPŐCZ[3]

Bioprocessing of sweet sorghum with enzymes produced by solid substrate fermentation (SSF)

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Enzyme assisted ensiling (ENLAC) with in situ produced enzymes was developed for a novel bioprocessing of sweet sorghum to ethanol and protein enriched animal feed. The process is based on the ensiling of freshly harvested sweet sorghum with in sire produced enzymes and lactic acid bacteria additives. ENLAC increases the sugar yield and the storability of the crop for year round continuous processing. The ensiled material was extracted in sugar industry type countercurrent diffusers, and the extract was fermented with hexose and pentose fermenting yeasts to produce ethanol. The extracted pulp was recycled as in situ enzyme source, after solid substrate fermentation (SSF) with cellulolytic fungi, such as *Gliocladium* and *Trichoderma* spp. In a 96 h SSF, the fermented pulp had 4–8 IU/g DW cellulase and 1000–1500 IU/g DW xylanase activity. About 2% of this fermented substrate was used for inoculating the fresh sorghum, equivalent to 20–40000 IU cellulase/MT green material. The use of in situ enzymes results in about 30% saving of processing costs, compared to the use of commercial enzymes. The rest of enzyme enriched, partially digested substrate was used as animal feed.

G. SZAKÁCS[1], J. FEKETE[2], J. HOLLÓ[3], G. MORAVIÁN[2], E. BÉKÁSI[1] and K. KORDAS[1]

Microbial and enzymatic transformation of taxol and 10-deacetylbaccatin-III (DAB)

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Taxol is an antitumour and antileukemia agent isolated from the bark of the Pacific yew tree, *Taxus brevifolia.* 10-deacetylbaccatin-III (DAB), a related compound that may be converted to taxol or to taxol analogs through a semi-synthetic route, can be extracted from the needles and twigs of *Taxus baccata*, a European yew bush. Both compounds are slightly soluble in water. There is an increasing effort to develop taxol analogs, some of which might be more effective or less toxic than taxol itself.

Microbial transformation is a principal approach which is widely practiced for structural modifications of complex natural products, Moreover, enzymes (especially immobilized enzymes) in organic solvents may also be used to create new derivatives through acylation, esterification, or hydrolysis. Sixty microorganisms were screened for the ability to form new derivatives from taxol or from DAB. Five microbes have been shown to produce new derivatives with better water solubility. Isolation and characterization of these products are in progress.

Also, acylation of taxol was performed, utilizing an immobilized lipase enzyme, an acyl donor, and a dry, non-hydrolytic organic solvent. The enzyme is absorbed onto a solid support and the enzyme/support catalyzes the acylation. The enzyme/support complex can then be filtered from the reaction mixture and recycled for use.

Metabolic products were separated and identified by using high-performance liquid chromatography (HPLC).

AGRICULTURAL AND FOOD MICROBIOLOGY

K. MÁRIALIGETI[1], J. KHOGA[1] and R. FARKAS[2]

Skin bacterial population shift as a predisposing factor of flystrike disease in sheep

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The sarcophagid fly, *Wohlfahrtia magnifica* (Schiner) is one of the serious pests of livestock in Afroeurasia. In Hungary – according to recent investigations – 35–50% of sheep in a flock can be infested by the larvae of this obligatory parasite fly. Similar

figures of infestation, together with 20% of lamb mortality seems to be true in the whole developing region of Eurasia.

The bacteriological aspect of cutaneous myiasis caused by *W. magnifica* was investigated to reveal the ethiology of the disease. The bacterial populations of healthy breech skin and vulval myiafic lesions in sheep as well as *Wohlfahrtia* maggot hindgut were analysed by numerical taxonomic methods. Moreover, bacterial communities of *Achillea* sp. flowers, the favourite resting and feeding plant of male *Wohlfahrtia* adults were also analysed. Fly attractive property of strains was checked, and the chemical composition of bacterial odours was analysed by gas chromatography. The suspected volatile fly attractant compounds were controlled in field experiments.

Our results suggest that skin injuries lead to characteristic rearrangement of skin bacterial populations which is a predisposing factor of *Wohlfahrtia* flystrike disease. In skin bacterial populations, surrounding lesions, the population density of *Brevibacterium* spp. is increasing. As compared to healthy breech skin, the incidence of brevibacteria in *W. magnifica* hind gut communities, is also higher. *Brevibacterium* strains isolated from different environments (skin, wound, etc.) produce volatiles, first of all thiols. In field experiments plate cultures of thiol producing *Brevibacterium* strains attract significantly more *W. magnifica* adults than control bacterial cultures. Similarly the determined attractant used as a pure chemical, selectively attracted *W. magnifica* flies. In the bacterial community of *Achillea flowers* brevibacteria seem to be also present.

It is supposed that the increase of germ numbers of cutaneous brevibacteria due to skin injuries can lead to a fly attractant odour production. Similarly, presence of brevibacteria in *Achillea* flowers exerts attractivity to *Wohlfahrtia* adults. The attractant belongs to the compound family of thiols, being most possibly methane-thiol. Unfortunately we do not know yet why wounds at the same time serve with ovipository stimuli to females, on contrary to *Achillea* flowers.

H.E.A.F. BAYOUMI HAMUDA[1], Z. KISS[2], GY. VÁRADY[2], S. BALÁZSY[1], N. KUCSMA[2] and M. KECSKÉS[2]

Nitrification and respiratory process inhibitors influencing growth and respiration of some *Rhizobium* species

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In vitro, addition of different concentrations of nitrapyrin (0.1, 1, 10 and 100 mg l^{-1}) and sodium azide (10, 20, 30, 40 and 50 mM in the growth media of *Rhizobium* strains representing four species (*R. leguminosarum*, *R. phaseoli*, *R. trifolii* and *R. loti*) influenced the growth of these strains within 48 h at 28 °C incubation on a rotary shaker (150 rpm).

All nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] doses inhibited the growth of these strains. In contrast, 0.1 mg l^{-1} enhanced the respiration rate of the strains using

Warburg's respirometer. The doses 10 and 100 mg l^{-1} were significantly high toxic and depressed the respiration rates of *Rhizobium* within 180 min. Application of 10 mM NaN_3 enhanced the growth and respiration rates of the strains. The growth of *R. leguminosarum* strains (Lóbab Z and Bükköny 75/4) was stimulated by 20 mM and also, *R. phaseoli* (Bab 5/3 strain) by 30 mM NaN_3. The growth and respiration rates of the *Rhizobium* strains were significantly depressed by 50 mM NaN_3, whereas the respiration rates of the strains Lóbab Z and Bükköny 75/4 and Bab 5/3 at 30 mM NaN_3 were higher than the control. However, 50 mM NaN_3 was significantly high and depressed the respiration rates of *Rhizobium* strains at P=0.05.

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Ecological factors influencing the saprophytic competence of endosymbiont *Rhizobium leguminosarum* introduced to brown forest soil

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This report describes, in sterile glass vials, the saprophytic competence and survival of different *Rhizobium leguminosarum* (10^6 cell capacity/ml/g soil) strains inoculated to 50 g non-, short- and long-autoclaved brown forest soil (Gödöllő) under different soil conditions (4.66, 5.1, 6.4, 7.0 and 8.4 pH values, 0, 25, 50, 75 and 100% equivalent percent moisture content (EPMC) and 5, 15, 28, 35 and 45 °C) for four weeks incubation. Significant differences between the initial and final populations of the inocula were determined at P=5% level every week. The population of *Rhizobium* strains was determined by rhizobial viable counting method on the Congo red yeast extract mannitol agar medium. Data were expressed as log10 values of the average of triplicates.

It was established that a lowest population of *Rhizobium* strains was obtained when soft pH was 4.66, EPMC (0, 75 and 100%), in non-autoclaved soft, when incubated at temperatures 5 and 45 °C. The most effective ecological parameters supporting the saprophytic competence of these strains were at pH 6.4–8.4, EPMC (25–50%), short autoclaved soil and at the incubation temperature 28 °C. Temperatures between 15 and 28 °C were suitable for both E1012 (British) and Bükköny 75/4 (Hungarian) strains. HB-3841 (Libyan) strain was preferred 28 °C. Bükköny 75/4 strain was significantly the most tolerant one to the investigated experimental conditions. It is important that the rate of survival of these strains rapidly declined in non-sterile soil below and above 28 °C, with moisture content less than 25% and more than 50%, and other varying under the investigated factors within the incubation time.

G. KOVÁCS and A. HALBRITTER

Rhizoplane bacterial communities of *Typha angustifolia* of a floating wetland ecosystem

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In Hungary there had been an enormous abundance of different wetland ecosystems, both terrestrial and floating ones. By the 60s of our century most of them have been destroyed mainly due to human activities (first of all agriculture), neglecting their important role in self-purification processes of natural waters. We have extremely scanty knowledge on plant associated microbial communities of floating mats, though the development of wetlands strongly depends on plants living at these sites. The growth conditions of these plants are definitely influenced by the co-operative microbial partners of their roots.

The aim of our work was to investigate bacterial communities of a floating mat dominated by *Typha angustifolia*. Root region mat block samples were collected three times (in summer, autumn and spring) in a reedbed of the Danube-arm of Soroksár. In the laboratory *Typha* rhizomes were aseptically collected, then adequate zones of the roots were cut off, adequately washed and macerated for preparing sample suspensions. More than 200 bacterial strains were characterized by their morphological features, biochemical and ecophysiological properties. Based on these phenotypical data computer aided numerical analysis was performed. Clusters were generated by using the UPGMA-method.

In contrast to previous publications referring to microbial communities (dominated by e.g. *Pseudomonas* spp.) of rhizoplane of different plants, in our case there was a significant predominance of root surface bacteria belonging to genus *Bacillus*. Although the root environment is strictly anaerobic (due to the waterlogged pseudo-soil with huge hydrogen-sulphide production) nearly all of the strains were strict aerobes, indicating the presence of aerobic microsites at least on root surfaces. According to an additional investigation these Bacillus strains may have a capability to oxidise hydrogen-sulphide. This result suggests that microbes protect Typha (and perhaps other wetland plants) from the toxic effect of this bacterial metabolic end-product. There is only a weak seasonal variation in the population density of the predominant species, indicating an obligate interaction between *T. angustifolia* and its *Bacillus* community.

Up till now only one endospore forming *Bacillus* has been described to be able to carry out the oxidation of hydrogen-sulphide. Further investigations are needed to determine the phylogenetically correct taxonomic position of these *Bacillus* strains.

Z. KISS

Frequency of inorganic phosphate-solubilizing microbes in Hungarian soil types

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The insoluble and hardly soluble inorganic forms of phosphorus can be found in the agricultural soils in relatively high quantity which is difficult to be taken up by the plants. Nowadays, the study of inorganic phosphate-solubilizing microorganisms (IPSM) is one of the most important tasks of soil microbiology as well as soil biotechnology. Our aim to make a survey of the distribution and frequency of these microbes in the different Hungarian soils and select the best IPSM strains for plant inoculation on the basis of their ecophysiological characters.

Using Pikovskaya as well as Razumovskaya media, ten bacterial strains were isolated from 24 heat-treated sod samples of 21 different Hungarian soil types which proved to be PO_4^{3-} solubilizing. Thirteen PO_4^{3-} mobilizing bacterial and fungal strains were isolated from brown forest soil, medow and medow chemozem soils. Fifty-one strains of 26 species of *Azomonas*, *Azotobacter*, *Bacillus*, *Rhizobium* and *Trichoderma* genus, as well as other 57 *Trichoderma* strains (isolated from the 21 different Hungarian soil types) were tested for their inorganic PO_4^{3-} solubilizing capacity. The examination of IPSM in long term field experiment which is carried out in slightly acidic brown forest soil "Nyírség" is going on.

I. NAGY[1], F. COMPERNOLLE[2], K. GHYS[1], A. DE SCHRIJVER[1], J. VANDERLEYDEN[1] and R. DE MOT[1]

Involvement of the thiocarbamate-inducible cytochrome P-450 system in atrazine degradation by *Rhodococcus* sp. NI86/21

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Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a major herbicide of the *s*-triazine family which has been heavily used throughout the world, especially in corn growing areas. Because of the detection of the herbicide in surface and ground water, its degradation and metabolism are the subjects of much attention. The apparent recalcitrance of these compounds to biodegradation is in line with the observations that very few micro-organisms capable of complete atrazine mineralization are known, and in most cases microbial consortia of unknown complexity are required to effect this mineralization.

The maize rhizosphere isolate *Rhodococcus* sp. NI86/21 degrades thiocarbamate herbicides by an inducible cytochrome P-450 system, and produces N-depropyl EPTC, and EPTC sulfoxide as major metabolites. This nocardioform actinomycete is also capable of partial degradation of atrazine. During atrazine degradation by *Rhodococcus* sp. NI86/21, N-dealkylated metabolites and the hydroxyisopropyl derivative are produced. The thiocarbamate-degrading cytochrome P-450 system is also induced in atrazine-containing media. Atrazine-degrading activity was conferred to two strains that do not degrade thiocarbamates or atrazine (mutant FAJ2027 of *Rhodococcus* sp. NI86/21 and *Rhodococcus erythropolis* SQ1), by transformation with the genes encoding the cytochrome P-450 system.

Nagy, I., Schoofs, G., Compernolle, F., Proost, P., Vanderleyden, J. and De Mot, R.: J Bacteriol 177:676–687 (1995); Nagy, I., Compernolle, F., Ghys, K., Vanderleyden, J. and De Mot, R.: Appl. Environ Microbiol 61:2056–2060 (1995).

P. OUDIN[1], J. A. TÓTH[2], S. BALÁZSY[3] and O. REISINGER[1]

Bioelimination of low concentrated PCB's wastes by biological filtration

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PCB's are a large chemical family. As they are not very biodegradable, the main problem is their bioaccumulation in environment and into food chains. Today, the only way of complete elimination is incineration. But this technic cannot be used for low concentration wastes for economical reasons. That is, for this kind of wastes that we will try to elaborate biofilters able to make accumulation and/or degradation of those substances.

So the goal of our research is to study the interactions between PCB's and microorganisms and especially yeast.

Two yeast strains (*Rhodotorula glutinis* and *Saccharomyces cerevisiae*) have been chosen by the three teams because of their experience in those two strains and to study two kinds of yeast.

The first strain: *R. glutinis* is a yeast which produces extracellular lipids that might be able to enhance PCB's solubility and so enhance bioaccumulation. The second strain: *S. cerevisiae* is the best known yeast and can be produced at low cost in high quantity.

We studied first the direct effects of PCB's on cells: physiological effects by spectrometry following growth, and morphological effects by direct electron microscopy observation.

Bioaccumulation by intracellular PCB's titration was investigated with our own method and we tried to enhance this bioaccumulation by culture medium modification.

We have now results which prove the PCB's bioaccumulation into living cells. Today, we are trying to elaborate biofilters for economically interesting view: we use Brewery's wastes accumulation biomass.

S. SZABÓ[1], S. BALÁZSY[1] and O. REISINGER[2]

The influence of five dominant wastewater bacteria on the bioproduction of duckweed cultures (*Lemna gibba*)

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Five various dominant strains of meat industrial waste water bacteria (*Aeromonas* sp., two strains of *Pseudomonas* sp., *Micrococcus* sp., *Vibrionaceae* spp.) were used in combination to inoculate cultures of axenic duckweed (*Lemna gibba*) grown on autoclaved wastewater for 7 days. The experiment was carded out by full factorial design (32 combination of 5 bacterial strains). The total chlorophyll production of the duckweed cultures was the indicator of the influence of bacteria strains.

Inoculation with *Pseudomonas*, *Micrococcus* and *Vibrionaceae* spp. strains caused the total chlorophyll production to be significantly greater as a direct cause by the effects of the treatments rather than of axenic control. The total chlorophyll production of the cultures increased an average more than 50% by the influence of *Vibrionaceae* spp. The interactions between strains of *Pseudomonas* sp. – *Vibrionaceae* spp., *Micrococcus* sp. – *Vibrionaceae* spp., *Aeromonas* sp. – *Pseudomonas* sp. – *Micrococcus* sp. *Vibrionaceae* spp. also significantly promoted the bioproduction of *L. gibba* cultures. The bioproduction of *L. gibba* cultures were significantly prevented by the interactions between strains of *Pseudomonas* sp. – *Micrococcus* sp. , *Aeromonas* sp. *Micrococcus* sp. – *Vibrionaceae* spp., *Pseudomonas* sp. *Micrococcus* sp. *Vibrionaceae* spp. The results showed that although some of the treatments which used a single strain had a preventive influence, the same effects of these strains were not detectable in mixed population.

T. DEÁK

Molecular methods in food microbiology

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Current practices in microbiological analysis of foods rely on conventional cultivation techniques based on the determination of colony counts. Though simple, convenient and widely accepted, colony count techniques have a number of disadvantages, in that they are labour and material intensive, give a low reproducibility, and, most importantly, plate counting is slow and provides data only for retrospective evaluation. A primary need in food microbiology is to detect viable cells by rapid, accurate, sensitive and reproducible procedures. In recent years, there have been substantial developments in molecular techniques which are widely used in microbial taxonomy, genetics, and have already found application in biotechnology. Numerous approaches have also been made to use these techniques in food microbiology as well. These comprise analysis of electrophoretic protein patterns, fatty acid composition, and most importantly, various ways of nucleic acid fingerprinting, such as DNA sequence polymorphisms, chromosomal DNA patterns, and ribosomal RNA sequence comparisons. Requirements for sophisticated equipment and special personal skills are precluding widespread application of these techniques in industrial setting. Recent modifications made some molecular methods more simpler and more rapid, such as the PCR-based random amplified polymorphic DNA technique. Examples are given on the application of molecular techniques for the detection and identification of important foodborne microorganisms.

P. Á. BIACS and Á. KARDOS

Safety of food enzymes

Central Food Research Institute, Budapest, Hungary

Biotechnology provides a great variety of the possible applications for the food processing. Among these biotechnological applications, the enzyme technology with a history of about two decades has a remarkable importance.

Enzymes can be found throughout living nature, from a simple microorganism to the most complicated being, man. Enzymes are natural substances, but the fact alone does, of course, not guarantee that they can be regarded as always harmless. Knowing the nature of enzymes, being large proteins, it is not surprising that enzymes have never been reported to be mutagenic or carcinogenic, and no teratogenic or other reproductive effects resulting from enzymes have ever been established.

It is foreseen that in the near future microbial enzymes will completely replace all existing industrial uses of animal and plants enzymes: not only because of the low price of the microbial enzymes but also because with the help of gene-technology the properties of the enzyme, like stability, heat resistance, pH tolerance etc. can be modified.

The amount of enzymes used in food processing relative to the total mass of the food is very small. As during further processsing the enzymatic activity is most of time destroyed or removed at the time of consumption of treated food.

From a safety point of view a few parameters may be distinguished: (i) safety of the source organism; (ii) raw materials used for enzyme production; (iii) processing techniques used; (iv) additives to the final products; (v) safety of the final product; (vi) safety of the products resulting from the enzymatic action; (vii) safety of immobilized enzyme preparations; (viii) safety of enzymes from genetically modified microorganisms.

Strict international requirements fix the safety of food enzymes. As a consequence of the safety of enzymes, legislation should not be unnecessary restricting and allow the development of new fields of enzyme biotechnology.

J. FARKAS and É. ANDRÁSSY

Luminometry of a bioluminescent *Bacillus subtilis* strain for real-time monitoring spore germination and vegetative growth

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A bioluminescent derivative of *Bacillus subtilis* containing a plasmid encoding a luxAB fusion under the control of a vegetative promoter (P45) was investigated by a Bio-Orbit 1251 Luminometer for its utility in studying effects of food preservation treatments on spore-formers and the regeneration of their damaged cells. This test bacterium gives bioluminescence upon the luciferase-catalysed oxidation of its FMNH2 and an exogenous aldehyde substrate such as nonanal. We have found a strong correlation ($r>=0.99$) between the traditional viable cell count of suspensions of vegetative cells and the light emission measured as a 10-sec integral value of the luminescence intensity.

The luminometric measurement showed good reproducibility (C.V.=<3%) and a detection limit for viable cell counts lower (hundred to thousand viable cells per ml) than that of most other rapid instrumental methods. Kinetics of germination and outgrowth of spores of the bioluminescent test bacterium was investigated luminometrically as compared to turbidimetry based on changes of refractivity and absorbancy of spore suspensions. Changes in cell-state distribution were followed by phase-contrast microscopy. It was found that the bioluminescence intensity appears

abruptly during spore germination, remains constant during outgrowth of germinated spores, and increases afterwards in relation to vegetative growth. Kinetical curves drawn from luminescence data showed more clearly individual phases of the spore vegetative cell transformation than turbidimetric measurements. A sub-lethal heat shock (50 min at 60 °C) which enchanted the rate of spore germination did not inactivate the luciferase system of spores. Results of these preliminary investigations are promising for the utility of this test organism and luminometry in studying complex processes of aerobic spore-forming bacteria, and applying the strain as a bio-indicator of efficacy of food preservation treatments.

G. PÉTER and O. REICHART

The effect of lyophilization parameters on the survival of some microorganisms

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The effect of some lyophilization parameters (growth phase, cryoprotectant, freezing temperature, duration of secondary drying) on the survival of some microorganisms (*Bacillus cereus*, *Lactobacillus plantarum*, *Escherichia coli*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Candida utilis*, *Mucor racemosus* and *Aspergillus niger*) was studied. The surviving rate was determined directly after the lyophilization procedure as well as half and two years later. The decrease of viability was within the range of 0–3.27 and 0.07–4.86 logarithmic value directly after lyophilization and two years later respectively, depending on the microbial strains and the combinations of treatments.

The effect of growth phases (exponential and stationary) on the survival was different among the strains. When the effect of cryoprotectants [8% skim milk powder ± 4% inositol and inactivated horse serum (100 ml) + inositol (5 g)] on the survival was significant, the serum + inositol combination always proved to be superior.

Among the three freeing temperatures (freeing on the freeze drying equipment, –25 °C and –196 °C) studied in case of bacteria, freezing on the freeze dryer proved to be the worst, while –196 °C was often the best. In case of yeasts and moulds, except for *S. cerevisiae*, –196 °C caused the biggest decrease in survival. The effect of the duration of secondary drying (3 h and 18 h) never proved to be significant. In several cases interactions between treatments were also observed.

K. KÖVES-PÉCHY, B. BITÓ, E. OSZTOICS and I. VÖRÖS

Effect of microsymbionts (*Rhizobium*, AMF) on the macroelement-content of pea and maize

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Macronutrient (N, P, K) dynamics of a mono-cotyledonous and a di-cotyledonous (leguminous) plant species (*Zea mays* L. and *Pisum sativum* L.) were compared in a pot experiment using calcareous sanely loam of Őrbottyán (Hungary).

Maize and pea were cultivated in steam-sterilized or original soil samples (as controls) and in pots inoculated with an Arbuscular Mycorrhizal Fungi (AMF, *Glomus* sp.) or a *Rhizobium leguminosarum* bv. *viceae*, respectively, in 3 replicates. Strain *Glomus* sp. Vm 5 was isolated from a luvisol-like brown forest dump spoil disturbed by coal-mining activities (soil characteristics [1]).

After eight weeks of cultivation and the very beginning of experiments macro-element content (N, P, K) of the soil was determined [2]. Dry weight of pea and maize biomass (shoot and root); N, P, K-uptake; root colonization by AMF (F %) and by rhizobia (nodule number) were determined [3, 4].

We established that mycorrhizal inocula strain (*Glomus* sp. Vm 5, as being adapted to poor ecological environment) enhanced the uptake of phosphorous and potassium elements in the shoot biomass of maize, but had no significant effect on the nitrogen content.

In case of pea both mycorrhizal and rhizobial inoculation increased the dry weight of total biomass. Not only the uptake of P and K elements, but the N-content was enhanced by AMF symbiosis, as well, suggesting the importance of biofertilization with microsymbionts (other than rhizobia) and the necessity of strain selection.

[1] Bíró, B., Vörös, I., Köves-Péchy, K., Szegi J.: Geomicrobiol J 11:275–283 (1993); [2] Thamm, F., Kramer, M., Sarkadi, J.: Agrokémia Talajtan 17:145–156 (1968); [3] Krjuger, L., Selivanov, I., Nozadze, L., Capayeva, I., Sjuzeva, J.: Uchonije Zapiski Permskovo Gos, ped. info. 69–70 (1968); [4] Trovelot, A., Kough, J. L., Gianazzi-Pearson, V.: In V. Gianinazzi-Pearson, S. Gianinazzi (eds): Physiological and genetical aspects of mycorrhizae. INRA, pp. 217–221 1986

A. A. ZALMUM[1], K. MÁRIALIGETI[1] and J. HEINZ[3]

Water quality of bank wall filtered wells as a function of bacterial community changes in the biofilm of Danube river

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The Szentendre island, lying north of Budapest towards the Danube bend, with a length of 33 km and an area of 56 km^2, is the most important source of drinking water for the capital of Hungary. Wells and rows of wells had been located here by the Water Works of Budapest since 1897 and the utilization of riverside sections suitable for water gaining was completed in 1980. The granulation and permeability of the layer used for water gaining are rather favourable. The water gained here can be pumped into the network without any treatment. However the water quality of bank wall filtered wells is influenced by the destruction of the biological filter layer of the river bed.

A bank wall filtered well located directly on the Danube riverside was chosen for continuous sampling. Well water samples and rolling gravel samples from the Danube in the filtration area of the well were collected and examined at monthly intervals for bacteriological parameters. Water samples were membrane filtered, whereas biofilm samples were plated on 4 different agar media. Adequate numbers of isolates were purified, and the strains obtained were subjected to morphological characterisation and metabolic fingerprinting.

Hygienic microbiological parameters (coliform numbers, *Enterococcus faecalis*, *Clostridium* counts, *Pseudomonas aeruginosa* numbers, bacterial counts at 37 °C and 22 °C, algal counts) and physico-chemical characteristics (water conductivity, temperature, pH, TDS, DOC, NO_3^-, NO_2^-, NH3, PO_4^{3-}, SO_4^{2-}, Ca, Mg, Fe, Mn, particle size distribution, etc.) were determined.

Changes in the dominance relations of bacterial populations in well-water and gravel biofilm are discussed as a function of fluctuations of different physico-chemical parameters.

B. TÓTH[1] and I. NÉMETH[2]

The effect of the organic and mineral fertilizers on the yield and on the soil's biological activity in a long-term (34 years) experiment at Keszthely

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The field experiment focusing on the effects of the livestock manure and artificial fertilizers has been established in 1960.

The soil is a slightly leached Ramann brown forest soil, has a poor humus and soluble P content and moderate K content.

Thirteen different treatments were distinguished and after a 34-year-long monotonous exposition changes in the total number of aerobic bacteria, the activity of the invertase and urease enzymes, the cellulolitic activity in the soils of sugar beet and wheat the humus content and the yield were examined.

The livestock manure increases the crop productivity less than the mineral fertilizers with the same amount of active ingredients. The livestock manure in 69.4 t/ha dose increases significantly with the humus content of the soil.

The examination of the parameters showing the soil's biological activity indicates that the livestock manure has larger effect on the intensity of microbiological–biochemical courses than the mineral fertilizers.

S. BALÁZSY[1], M. TÓTH[1], J. A. TÓTH[2], P. OUDIN[3] and O. REISINGER[3]

Microbial uptake of nickel from pure cultures

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The metal nickel is naturally widespread in many components of the terrestrial environment. The main sources of this metal caused contamination, in regard of mining and associated activities, automobile traffic and fertilizer applications. Metal toxicity has become a major cause of concern owing to the increased of trace elements in the environment. Metal pollutants have shown an effect to the diversity of the microorganisms.

Metal removal from the contaminated soil is important. Conventional chemical treatment methods, which include precipitation–filtration, oxidation–reduction, membrane separation and other techniques may be ineffective or uneconomical when the trace element concentrations in the polluted environment are in the range of

10–100 mg/kg. An alternative method the microbial uptake of metals could be much more economical than chemical methods.

We have examined the influence of nickel on soil bacteria that we isolated from polluted environment. The aim to find bacterial strains which are able to absorb heavy metal and could be used for treatment of the polluted environment.

We isolated more than 50 different bacterial strains from contaminated soils. Nickel pollution of soil ranged from 33 to 504 mg/kg. Nickel uptake of bacterial strains was measured photometrically at 540 nm with dimethyl-glioxime reagent, at 28 °C in three estimations during 96 h at 3.5–6.5 pH. Our results have shown that the bacteria survived the high metal concentration of 200 mg/kg and could take up the nickel. The pH optima of the elimination are from 4.5 to 5.5 pH. Heavy metal uptakes ranged from 13 to 49% of the cell dry weight.

P. G. OTT, Z. BOZSÓ and Z. KLEMENT

Effect of heat treatment of plant on the interaction between tobacco leaves and *hrp* mutants of *Pseudomonas syringae* pv. *syringae*

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Non-host plants respond to necrogenic phytopathogenic bacteria with a rapid death of the infected plant tissue, called hypersensitive reaction (HR). This is an active process revolving newly synthesized proteins, For the bacterial part of HR (induction) several genes, called *hrp* genes are responsible. The *hrp* mutants are characterized by their inability to cause HR and reduced pathogenicity. However, under special circumstances, some mutants can induce HR. We have examined the effect of a heat treatment (51 °C, 15 seconds) of plant leaf tissue on the interaction between tobacco leaf and Tn5-*gusA1 hrp* mutants of *Pseudomonas syringae* pv. *syringae* strain 61 (Pss). One mutant (*hrpK*) caused HR in heat-treated tissue, albeit slower than the wild type. Further unique characteristic of *hrpK* mutant was the ability to grow in heat treated tissue at a similar rate than the wild type. *hrpK* mutant required longer time to induce HR, which was reflected by the delayed leakage of electrolytes from the hypersensitive tissue. A common feature of the mutants was the more rapid decline of cell number in tissues without heat treatment. In 6 out of 7 mutants presence of a heat-treated plant tissue induced expression of the respective *hrp* genes several fold (compared to the induction level in the control tissue). One likely mechanism of HR in heat treated plant tissue by the *hrpK* mutant is that heat treatment temporarily inhibits the plant's inducible response which would otherwise be inhibitory to the already delayed HR inducing ability of the mutant bacterium.

K. MANI, G. PÉTER, D. DLAUCHY and J. TORNAI LEHOCZKI

Molecular characterization of yeast isolated from some Indian foods

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Yeast play an important role in production processes in brewing industry as well as spoilage of food. Yeast are traditionally characterized, classified and identified by morphological and physiological criteria. But the whole process involves 2–3 weeks and consumes valuable time in preparing various media necessary for the identification. On the other hand molecular technics for identification such as karyotyping consume less time and are more accurate.

Beer, wine and commercial bakers yeast were obtained from India and several yeast strains were isolated from them using YPGA (Yeast extract - 1%, peptone - 2%, dextrose – 2%, agar – 2%) media. The isolated strains were observed under a light microscope for their morphological characteristics such as shape of cells, mode of sexual and asexual reproduction, formation of hyphae and pseudohyphae.

The various physiological characteristics were also observed; fermentation of various carbohydrates; aerobic assimilation of various carbon and nitrogen sources, growth at different temperatures, growth in vitamin-free medium, growth in presence of 0.01% cycloheximide and growth in medium containing 50% glucose.

Subsequent to identification based on the above tests, intact chromosomal DNAs were isolated and purified by Kerin's method (1992), and subjected to karyotyping by pulsed field gel electrophoresis. The chromosomal DNA banding pattern was obtained and comparisons made with known type strains. The results obtained either support or contradict identification by the conventional method.

P. C. TEIXEIRA[1] and CS. MOHÁCSI-FARKAS[2]

DSC studies on heat denaturation of whole cells and crude ribosomes of *Lactobacillus bulgaricus*

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Following previous microcalorimetric studies of thermal inactivation of vegetative bacterial cells reported at the 1994 Annual Meeting of the Hungarian Society for Microbiology, further investigations by differential scanning calorimetry using Shimadzu DSC-50 apparatus attempted to identify those cellular structures which may be responsible for thermal death. DSC thermograms of wet biomass of whole cells of *Lactobacillus bulgaricus* in the temperature range of 10 to 120 °C were compared with

that of crude ribosome preparation obtained by disruption of stationary phase cells by sonification followed by deoxiribonudease treatment and high speed centrifugation.

While whole cells exhibited a multipeaked thermogram, that of crude ribosomes showed only two significant transitions, the first one starting at about 56 °C, which is approximately equal to the lowest temperature, where measurable loss of viability of cells starts. These preliminary observations confirm former British studies with *Escherichia coli* demonstrating that denaturation of certain ribosomal subunits and/or ribosomal proteins, loss of higher order structure and melting of helical regions of the rRNA might be major contributors of thermal death.

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THE DEVELOPMENT OF A *BACILLUS SUBTILIS* 168 CULTURE CONDITION FOR ENHANCED AND ACCELERATED β-MANNANASE PRODUCTION

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A shaken flask cultivation condition for enhanced and accelerated β-mannanase formation by *Bacillus subtilis* 168 was achieved. Among five examined fermentation media a formula that supported enzyme generation and retarded biomass yield and sporulation was selected. The deficiency of biomass production in this medium was mastered by choosing a seed culture medium that accelerated growth and initiation of β-mannanase synthesis. With respect to enzyme production, the optimum pH and temperature were 7 and 37 °C, respectively. The biosynthesis of the enzyme was extremely influenced by the cell growth state as a modulator, glucose as a catabolite repressor, and galactomannan as an inducer. A galactomannan concentration of 4 g l^{-1} induced a β-mannanase activity level of 17.5 U ml^{-1} after 24 h of incubation at the experimental condition. Higher inducer concentrations supported growth rather than enzyme production. The influence of inoculum size was so remarkable that, at optimum, a crude filtrate with an enzyme activity of 33U ml^{-1} was yielded within 4 hours. It appears that this is among the highest rates reported for β-mannanase production. We have also demonstrated that blocking of the sporulation process at stage II do not affect enzyme production significantly. This would allow an extended enzyme production phase especially in a continuous culture.

β-Mannanases refer to a group of degradative enzymes capable of hydrolysing the (1-4)-β-D-mannopyranosyl linkages of the (1-4)-β-D-mannans, namely, mannan, galactomannan, glucomannan and galactoglucomannan [1]. Mannanases of this type have assigned EC 3.2.1.78 and are called mannan endo-l,4-β-mannosidase, (1-4)-β-D-mannan mannohydrolases, or endo-mannanases [2]. They have wide potential applications in paper manufacturing, food and feed industry, and waste treatment [3].

Various microorganisms have been reported as β-mannanases producers [4, 5]. Among those is *Bacillus subtilis* which is recommended because of its safety, fast growth, and ability to secrete a wide variety of hydrolytic enzymes before the commitment to sporulate as a result of carbon, nitrogen, or phosphorous starvation [6]. Many of these

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enzymes are encoded by genes under the control of temporal activation, catabolite repression (CR), and substrate induction (SI) [7, 8].

In the present work, we have investigated some of the growth factors that control the biosynthesis of β-mannanase by *B. subtilis*. The main objective was to formulate a growth condition that enhances and accelerates β-mannanase formation by the bacterium, mainly, by means of minimizing CR, maximizing SI, controlling biomass production, and delaying or blocking the development of the cells into mature spores.

Materials and methods

Bacterial strains. The *B. subtilis* strains used in this study were obtained from Leighton's laboratory (Department of Biochemistry and Molecular Biology, University of California at Berkeley, CA, USA). These included: a prototrophic strain of the 168 genetic background (RS8) and two isogenic sporulation deficient strains numbered as RS908 (*spoIIGB55*) and RS960 (*lysI spoIIJ::Tn917ΩHU19*).

Growth media. Five different media were tested for their ability to support *B. subtilis* growth, mannanase production, and bacterial sporulation. They included: (g l^{-1})

Medium I: Tryptone, 16; yeast extract, 10; and NaCl, 5 [9].

Medium II: Glucose, 1; KH_2PO_4, 2; NH_4NO_3, 2; $MgSO_4.7H_2O$, 0.2; and yeast extract, 2.5 (modified after Panbangred et al. [10]).

Medium III: Glucose, 1; beef extract, 1.5; yeast extract, 1.5; peptone, 5; NaCl, 3.5; K_2HPO_4, 3.68; and KH_2PO_4, 1.32 (Difco Penassay broth).

Medium IV: Glucose, 1; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.05; yeast extract, 0.5; K_2HPO_4, 7.54; and KH_2PO_4, 2.33 (modified after Jones and Ballou [11]).

Medium V: Beef extract, 6; peptone, 10; $MgSO_4.7H_2O$, 0.5; KCl, 2 and stock solutions: 10%glucose, 10; 1 mol^{-1} $Ca(NO_3)_2$, 1; 0.1 mol^{-1} $MnSO_4$, 1; and 0.001 mol^{-1} $FeSO_4.7H_2O$, 1 ml l^{-1} (Schaeffer [13]).

Mineral medium: carbon source, 1; NH_4Cl, 1; KH_2PO_4, 0.9; K_2HPO_4, 0.9; NaCl, 0.04 g l^{-1} and 1% (v/v) of a mineral stock solution containing, $CaCl_2.6H_2O$, 2; $MgCl_2.6H_2O$, 2; $MnCl_2.4H_2O$, 1; $FeSO_4.7H_2O$, 0.4; $CuSO_4.5H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1; and $COCl_2.6H_2O$, 0.01 g l^{-1} (Williams [12]).

Maintenance of bacterial strains. The *B. subtilis* wild-type strain was maintained as a spore stock. Sporulation deficient strains were maintained as frozen permanent cultures in 15% glycerol.

Spore counting. After 48 h of growth, 3 ml of each culture suspension were placed in a 5 ml screw capped sterile bottle. Vegetative cells were killed by the addition of 0.1 ml chloroform. Spore yields were determined by plating serial dilutions onto nutrient agar plates. Sporulation efficiency was calculated as the number of spores (mg biomass)$^{-1}$.

Bacterial growth and enzyme production. Throughout the work, enzyme fermentation experiments were carried out in triplicates and the averages of assay results are shown. Seed culture media were inoculated from overnight MI agar slants that were cultivated from the spore suspension or frozen permanents. Pre-warmed 50 ml aliquots of liquid media dispensed in 250 ml Erlenmeyer flasks were inoculated by 1 ml (unless stated otherwise) exponentially growing seed cultures. The composition of the fermentation medium was progressively modified and this will be specified in the text. Cultures were shaken at fixed temperatures. Growth rate was monitored by measuring culture absorbance at 550 nm. Biomass dry weights corresponding to different absorbance values were routinely determined from a standard curve.

For enzyme assay, samples of 1.5 ml were transferred from the cultures into Eppendorf tubes at times of interest and centrifuged under cooling. Clear supernatants were then frozen and stored at –20 °C.

Determination of enzyme activity. The activity of β-mannanase in cell free supernatants was assayed according to Araujo and Ward [5]. Locust bean gum (galactomannan from Sigma Biochemicals) containing a mannose:galactose ratio of 4:1 was used as a substrate. Galactomannan was suspended by homogenizing and heating to 80 °C in 0.1 mol l^{-1} sodium acetate buffer, pH 5.8. The mannanase assay mixture contained 0.5 ml of 0.5% (w/v) galactomannan and 0.5 ml of suitably diluted culture fluid. The reaction mixture was incubated at 50 °C for 30 min. Reducing sugars produced as a result of enzyme activity were determined as mannose reducing equivalents using the dinitrosalicylic acid (DNS) method of Miller [13]. One activity unit (U) is defined as the amount of enzyme producing 1 μmol product in 1 min. In some experiments, it was of interest to take account of variations in biomass levels exerted by the factor under study. Therefore, another parameter called enzyme productivity was calculated as U (mg biomass)$^{-1}$ in such cases.

Results

Suitability of the fermentation medium. A seed culture of the experimental wild-type bacterium was prepared using the MI formula described in the section Materials and methods. Homogeneous suspensions were used to inoculate five different pre-warmed liquid media including MI, MII, MIII, MIV, and MV. Cultures were initially adjusted to pH 7.0 and incubated at 30 °C for 48 h. Growth rate and enzyme activity were determined at different time intervals and spores were counted at the end of the fermentation period.

The primary objective of this experiment was to screen for a medium which stimulates the cells to synthesize β-mannanase more than to divide or to sporulate. Therefore, activity results were expressed as productivity which reflects the amount of enzyme produced per unit weight biomass. The results given in Fig. 1 (a, b, c) show that the bacterium responded differently to the composition of the used media. However, expression of the enzyme was commonly low at the early exponential growth phase. The most effective medium for enhancing the biosynthesis of β-mannanase by *B. subtilis* cells was MIV broth (Fig. 1b) although it attended poor biomass yields throughout the experiment (Fig. 1a). The cells grown on this medium expressed about 3.9 U (mg biomass)$^{-1}$ within 24 h. Longer incubation periods were not significantly effective. On the other hand, highest growth yields were obtained by MI followed by MIII with maximum initiation of β-mannanase expression [1.4 U (mg biomass)$^{-1}$] by the latter after 8 h of incubation (late exponential phase of growth). Hereafter, the MIII culture supported biomass yield rather than β-mannanase production.

The results of the third parameter studied in this experiment, sporulation efficiency, proved that MIV did not strongly promote spore formation (Fig. 1c). It inhibited the development of more than 90% of the *B. subtilis* cells into mature spores. Therefore, MIV was selected as a basal fermentation medium for enzyme production whilst MIII was chosen for the preparation of seed cultures.

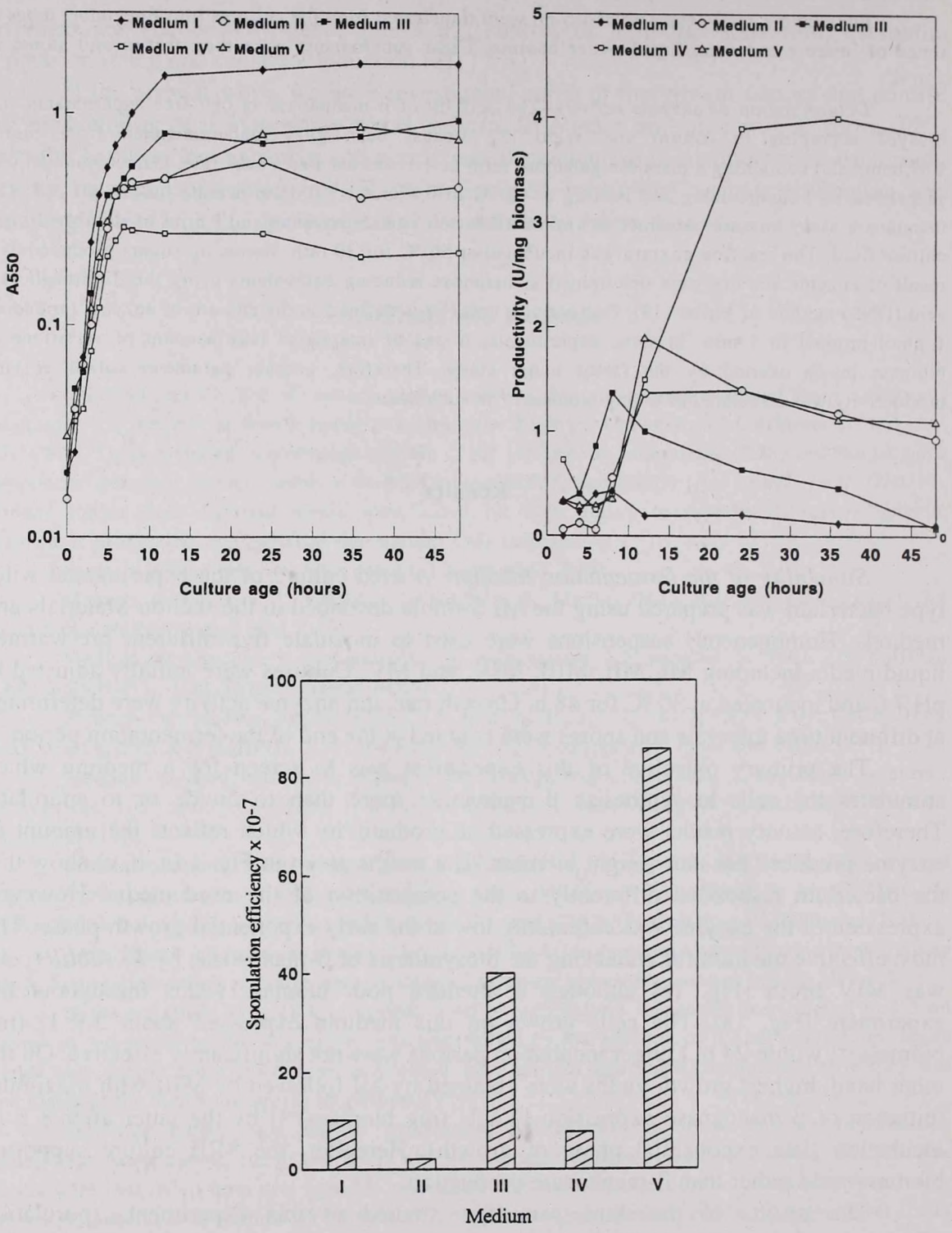

Fig. 1. Growth rates (a), β-mannanase productivity (b), and sporulation efficiency (c) of *B. subtilis* 168 grown at 30 °C in shaken flask cultures with different types of media. Pre-warmed fermentation media were inoculated with 2% (v/v) of OD (550 nm)≈0.4 seed culture. Sporulation efficiency was determined after 48 h growth and is expressed as number of spores (mg biomass)$^{-1}$

Optimum pH and temperature. The effect of 6 different initial pH values (ranging from 5 to 7.5) on β-mannanase synthesis by *B. subtilis* 168 was investigated. Adjustment of the pH was carried out either with 0.03 mol l^{-1} H_3PO_4 or 0.1 mol l^{-1} KOH before autoclaving. β-Mannanase activity was assayed after 24 h of fermentation. The enzyme was produced maximally at pH 7.0. Among 5 tested temperatures ranging from 30 to 42 °C, the condition of 37 °C was optimal (data not shown).

Regulation of β-mannanase expression by carbon sources. In this part of the work, the experimentally defined mineral medium was used as a fermentation medium to exclude the effects of natural additives as inducers.

Various carbohydrates including monosaccharides (glucose, galactose, mannose, and fructose), disaccharides (cellobiose and sucrose), and polysaccharides (galactomannan, pectin, starch, carboxy-methyl cellulose, chitin and xylan) were compared, each as a sole carbon source in the mineral medium. The biomass and activity of β-mannanase in each of the culture filtrates were determined after 24 h shaking. Enzyme assay results are presented as productivity in order to be informative about the direct effect of each carbon source on the amount of enzyme expressed by each cell. Carboxy-methyl cellulose, chitin and xylan failed to support considerable growth rates on the mineral medium. Cellobiose, glucose, and pectin cultures recorded the highest biomass yields accompanied by the most severe inhibitory effects on β-mannanase synthesis (Table I). Less inhibitory effects on enzyme synthesis were exerted by the monosaccharides other than glucose (galactose, fructose, and mannose). On the other hand, about a 217-fold increase in β-mannanase productivity was achieved as a result of induction by galactomannan when compared to the glucose culture.

Effect of inducer level. Different concentrations of the effective inducer, galactomannan, ranging from 0.5 to 14 g l^{-1} were used as glucose substituents in the basal enzyme production medium. Biomass yield and enzyme activity were determined after 24 h of growth. As shown in Fig. 2, increasing the concentration of galactomannan in the medium reduced the enzyme productivity parameter as a result of promoting cell replication (within 5.6-fold) markedly more than enzyme production (within 1.72-fold). Accordingly, high galactomannan concentrations in this medium seem to be not economic. The inducer concentration 4 g l^{-1} was chosen for the following experiments because it stimulated the cells to produce about 80% of the β-mannanase activity obtained by the highest examined galactomannan concentration.

Effect of substrate monomers as additional carbon sources. The objective of this experiment was to investigate the influence of the galactomannan hydrolysis products on the biosynthesis of its degrading enzyme by *B. subtilis*. The results shown in Table II prove that addition of 1 g l^{-1} mannose or galactose (monomers of galactomannan) to the inducing enzyme production medium attained deleterious effects on both β-mannanase production and biomass yield. They reduced the number of cells as well as the amount of enzyme synthesized by each cell.

Table I

Influence of carbon source on biomass yield and β-mannanase expression by B. subtilis 168 after 24 h shaking at 37 °C. Carbon sources were added in equal concentrations (1g l^{-1}) to the mineral medium

Carbon source	Biomass		β-mannanase	
	mg ml^{-1}	Fold increase	U (mg biomass)$^{-1}$	Fold increase
Glucose	0.324	1.00	0.03	1.00
Fructose	0.044	0.14	1.21	40.33
Galactose	0.083	0.26	2.02	67.33
Mannose	0.067	0.21	0.53	17.67
Cellobiose	0.350	1.08	0.11	3.67
Sucrose	0.148	0.46	1.13	37.67
Galactomannan	0.157	0.48	6.50	216.67
Pectin	0.210	0.65	0.07	2.33
Starch	0.110	0.34	0.56	18.67

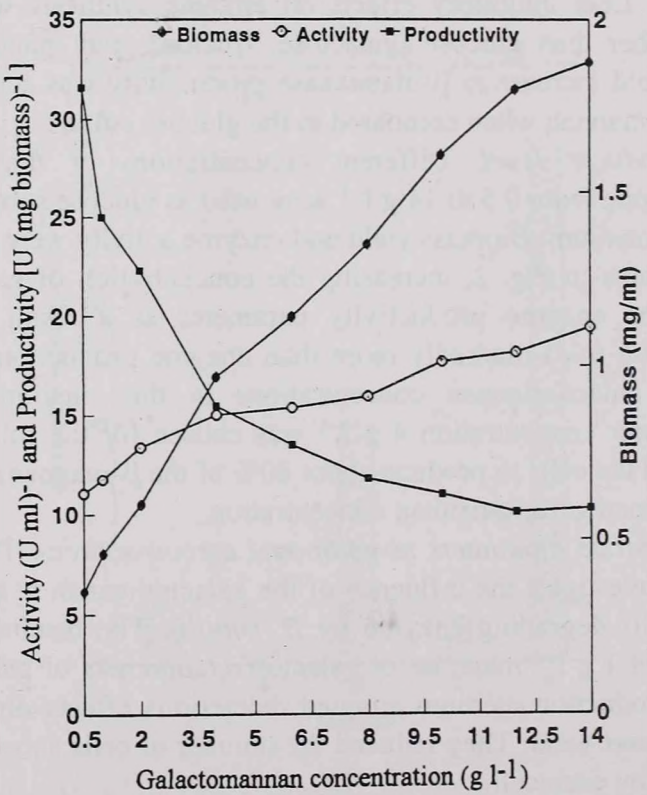

Fig. 2. Biomass yield and mannanase production by *B. subtilis* cultures as influenced by galactomannan concentration in the fermentation medium (MIV). Samples were collected for enzyme assay after growth for 24 h at 37 °C in shaken flask cultures

Table II

The effect of inducer monomers as additive carbon sources on biomass and β-mannanase production by B. subtilis 168. Additive carbon sources were added (1 g l^{-1}) to the enzyme production medium which further contained galactomannan (4 g l^{-1})

Additive carbon source	Biomass		β-mannanase	
	mg ml^{-1}	Fold increase	U ml^{-1}	Fold increase
Control	0.924	1.00	11.90	1.00
Galactose	0.368	0.40	0.75	0.06
Mannose	0.221	0.24	1.19	0.10

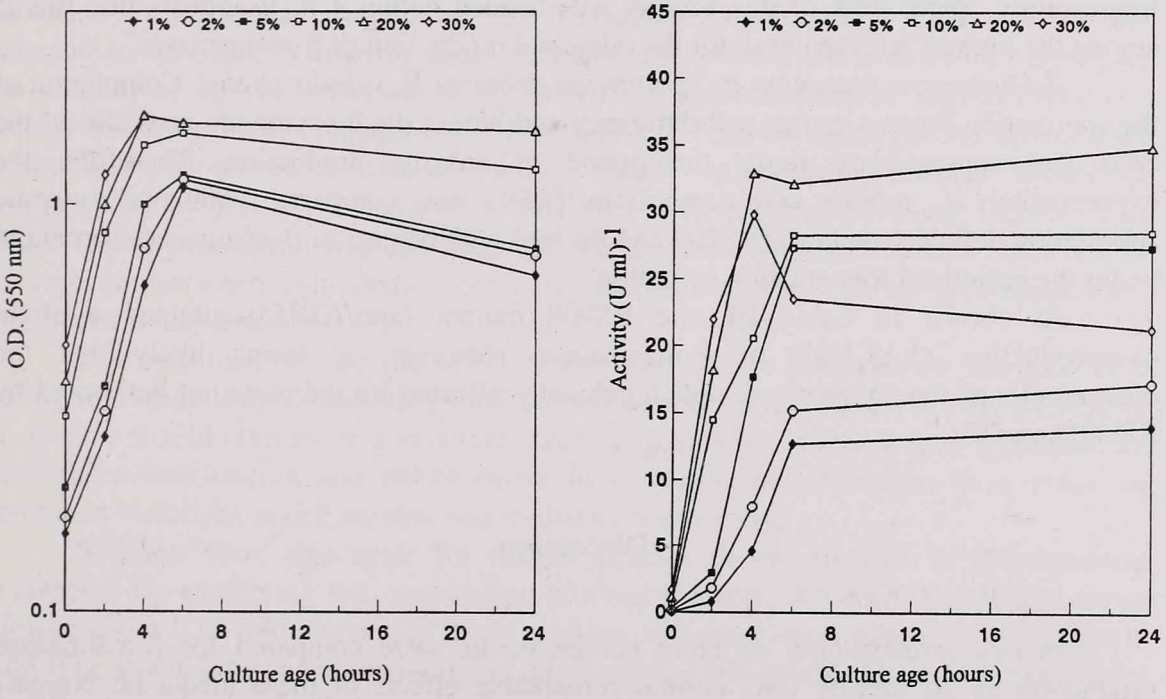

Fig. 3. Growth rates (a) and β-mannanase activity in the crude filtrates (b) of *B. subtilis* cultures as affected by the inoculum size-fermentation medium ratio (v/v). Cultures were shaken at 37 °C

Table III

Biomass and β-mannanase production by wild-type and sporulation defective B. subtilis strains grown for 4 h at optimal condition

Strain	Biomass		β-mannanase	
	mg ml^{-1}	Fold increase	U ml^{-1}	Fold increase
RS8 (wild-type)	0.951	1.00	34.21	1.00
RS908 (*spoIIGB*)	1.033	1.09	40.23	1.18
R8960 (*spoIIJ*)	1.060	1.11	33.92	0.99

Seed culture/fermentation medium ratio. Inocula with size ratios ranging from 1 to 30% of the total cultural volume were tried out with respect to β-mannanase formation after different periods of fermentation. The results shown in Fig. 3 (a, b) indicate that both growth and enzyme accumulation in the culture filtrate were positively affected by increasing the inoculum ratio up to the 20%. Maximum enzyme activity (34.7 U ml^{-1}) was obtained by the culture provided with 20% inoculum after 24 h shaking. Interestingly, about 95% of this activity was formed within 4 h. It appears that this is among the highest rates reported for the microbial production of β-mannanase.

β-Mannanase formation by sporulation deficient B. subtilis strains. Completion of the sporulation process causes cell dormancy and blocks the biosynthetic activities of the cells and consequently limits the period of enzyme production. Therefore, the experimental *B. subtilis* wild-type strain (RS8) was compared with two isogenic sporulation deficient mutants (RS908 and RS960) with respect to β-mannanase formation under the optimized fermentation condition.

As shown in Table III, the RS908 mutant (*spoIIGB55*) attained a slight overproduction (1.18-fold) of β-mannanase. However, it seams likely that the biosynthesis of the enzyme was not significantly affected by the mutation harboured by RS960 (*spoIIJ*).

Discussion

In this investigation, different culture media were compared for β-mannanase production by *B. subtilis* 168. Various remarkable effects of these media on biomass production, sporulation, and β-mannanase expression have been observed. However, a common late exponential activation of β-mannanase expression was demonstrated. A medium formula that maximizes β-mannanase productivity and minimizes sporulation efficiency was required. Such a medium would increase enzyme formation and elongate its production phase in a continuous culture. This objective was fulfilled by a relatively poor formula, MIV, that did not promote growth strongly. The high enzyme productivity observed in this case was possibly a result of sharp nutrient limitation which is known to

trigger the biosynthesis of numerous extracellular and intracellular degradative enzymes in *B. subtilis* [6, 14]. The biomass production deficiency of MIV cultures was mastered by choosing a seed culture medium that accelerated growth and initiation of β-mannanase synthesis.

The *B. subtilis* β-mannanase expression system appeared to be very sensitive to the nature of the carbon source in a mineral medium. It was maximally induced by the enzyme substrate, galactomannan. Similar results have been reported by Rättö and Poutanen [4] and Araujo and Ward [5]. However, its expression was most severely repressed by the carbon sources which supported highest biomass yields regardless to their molecular sizes. These include glucose, cellobiose, and pectin.

The severe repression exerted by glucose compared to the other monosaccharides such as galactose and fructose can be speculated through the regulation model proposed by Kraus et al. [15]. They demonstrated that normal CR of the *B. subtilis* XylA operon takes place through two different mechanisms that require two regulatory sequences, namely, *cis*-active element (CRE) and *xylR*. The response of the former is triggered by glucose, fructose, and glycerol while the latter is triggered by glucose only. Another possible route of glucose CR is indirect. That is, glucose represses the transcription of Spo0A, a key regulatory factor required for the initiation of sporulation and other stationary phenomena (Fisher [16]).

The substitution of glucose by galactomannan in the basal fermentation medium enhanced β-mannanase formation by *B. subtilis* significantly. The greater the galactomannan concentration the greater the biomass and enzyme yielded and the lesser the amount of enzyme synthesized by each cell. This observation suggests that cell density has a significant influence on the response of each bacterium to the surrounding environment. Moreover, the rate of releasing readily metabolizable substrate hydrolysis end products by a massive number of cells may cause inducer exclusion (Kraus et al. [15]). This interpretation was confirmed by the dramatic inhibitory effects observed on enzyme output when combinations of galactomannan and any of its monomers were introduced to the medium.

The investigation into the effect of the seed culture-fermentation medium ratio during this study proved that this parameter was so influential that it gave the possibility to obtain a 2-fold increase and maximize enzyme generation within 4 h of fermentation. This sharp acceleration and enhancement in β-mannanase production is a result of combined variations in cell number and medium composition.

Attempts were also made for further increase in the duration of β-mannanase expression by examining two sporulation blocked mutants. A *spoIIGB*-deficient strain showed about 1.18-fold increase in the expression of β-mannanase relative to the isogenic wild-type strain. It has been reported that the product of the *spoIIGB* gene is a sporulation specific sigma factor (SigE) essential for triggering the process beyond stage 0 (Rong et al. [17]). The other tested mutant (a *spoIIJ*-deficient strain) did not clearly alter enzyme production. The product of the *spoIIJ* gene, belongs to a class of environmentally responsive genes and regulates early developmental steps of sporulation in *B. subtilis* (Antoniewski et al. [18]). Strains with *spoIIJ* null genotypes showed extracellular alkaline protease overproduction and a sporulation delayed phenotype (Perego et al. [19]). Thus, it

can be concluded that deficiency in such *spoII* genes block, at least partially, a major branch of the *B. subtilis* developmental pathway, sporulation, saving energy which can be channelled to the biosynthesis of exoenzymes for extended expression phases especially in continuous cultures.

REFERENCES

1. McCleary,B.V.: β-D-Mannanases. Methods of Enzymology **160**, 596 (1988).
2. Kusakabe, I., Takahashi, R.: β-Mannanase of *Streptomyces.* Methods of Enzymology **160**, 611 (1988).
3. Torrie,I.P., Senior,D.J., Saddler,J.N.: Production of β-mannanases by *Trichoderma harzianum* E58. Applied Microbiology and Biotechnology **34**, 303 (1990).
4. Rättö,M., Poutanen,K.: Production of mannan-degrading enzymes. Biotechnology Letters **10**, 661 (1988).
5. Araujo,A., Ward,O.P.: Hemicellulases of *Bacillus* species: preliminary comparative studies on production and properties of mannanases and galactanases. Journal of Applied Bacteriology **68**, 253 (1990).
6. Schaeffer,P.: Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriological Reviews **33**, 48 (1969).
7. Sonenshein,A.L.: Metabolic regulation of sporulation and other stationary-phase phenomena. In Smith,I., Slepecky,R.A., Setlow,P. (eds): Regulation of Prokaryotic Development. Washington DC: American Society of Microbiology. 1989. pp. 109–130.
8. Wray,L.V., Pettengill,F.K., Fisher,S.H.: Catabolite repression of the *Bacillus subtilis* hut operon requires a cis-acting site located downstream of the transcription initiation site. Journal of Bacteriology **176**, 1894 (1994).
9. Yang,R.C.A., Mackenzie,C.R., Bilous,D., Narang,S.A.: Identification of two distinct *Bacillus circulans* xylanases by molecular cloning of the genes and expression in *Escherichia coli.* Applied and Environmental Microbiology **55**, 568 (1989).
10. Panbangred,W., Kando,T., Negoro,S., Shinmyo,A., Okada,H.: Molecular cloning of the genes for xylan degradation of *Bacillus pumilus* and their expression in *Escherichia coli.* Molecular and General Genetics **192**, 335 (1983).
11. Jones,G.H., Ballou,C.E.: Studies on the structure of yeast mannan. Journal of Biological Chemistry **244**, 1043 (1969).
12. Williams,A.G.: Staining reactions for the detection of hemicellulose degrading bacteria. FEMS Microbiology Letters **20**, 253 . (1983)
13. Miller,G.R.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry **31**, 426 (1959).
14. Stülke,J., Hanschke,R., Hecker,M.: Temporal activation of β-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. Journal of General Microbiology **139**, 2041 (1993).
15. Kraus,A., Hueck,C., Dormer,D. Hillen,W.: Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. Journal of Bacteriology **176**, 1738 (1994).
16. Fisher,S.H.: Catabolite repression in *Bacillus subtilis* and *Streptomyces.* Journal of Bacteriology **159**, 365 (1987).
17. Rong,S., Rosenkrantz,M.S., Sonenshein,A.L.: Transcriptional control of the *Bacillus subtilis spoIID* gene. Journal of Bacteriology **165**, 771 (1986).

18. Antoniewski,C., Savelli,B., Stragier,P.: The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. Journal of Bacteriology **172**, 86 (1990).
19. Perego,M., Cole,S.P., Burbulys,D., Trach,K., Hoch,J.A.: Characterisation of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. Journal of Bacteriology **171**, 6187 (1989).

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INCIDENCE OF WOUND INFECTION IN THREE DIFFERENT DEPARTMENTS AND THE ANTIBIOTIC SENSITIVITY PATTERN OF THE ISOLATES IN A SAUDI ARABIAN HOSPITAL

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The incidence of wound infection in three different departments: general surgery (GS), interdisciplinary intensive care (IIC) and pediatric intensive care {PIC) in King Khalid Hospital in Hail was studied during the hot months June–October. A total of 2331 wounds were examined. One hundred and ninety-three of them were infected (8%). The overall monthly infection rates in these departments were significantly different.

They were 18, 9 and 4% in the IIC, GS and PIC respectively. The monthly infection rates varied, the highest rates occurred in July. Two hundred and eighty three strains were isolated from the infected wounds. Seventy ones were isolated during June, and 69, 60, 42 and 41 strains were isolated during July, October, August and September, respectively- Thirty five per cent of these strains were identified as *Staphylococcus aureus*, 31% as *Escherichia coli*, 25% as *Pseudomonas aeruginosa*, and 10% as *Klebsiella*. *S. aureus* was the predominant organism during July; while *E. coli* predominated during August and October. *S. aureus* and *P. aeruginosa* strains were isolated in equal numbers during June.

The sensitivity of these bacteria to ampicillin, cephalotin, cefotaxime, cefoxitin, tetracycline, chloramphenicol and gentamicin was tested by the standard disk diffusion technique.

The most effective antibiotic against *S. aureus* was chloramphenicol (78%) followed by gentamicin (63%) and tetracycline (57%); against *E. coli* cefotaxime (82%) followed by cefoxitin (77%), cephalitin (62%) and chloramphenicol (53%); against *Klebsiella* cefotaxime (95%) followed by chloramphenicol (90%), tetracycline (70%), cephalotin and gentamicin (each 66%); and cefoxitin (60%); against *P. aeruginosa* cefotaxime (56%), gentamicin (33%) and chloramphenicol (29%).

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Wound infections are still one of the great unsolved problem in hospitals. They are caused mainly by *Staphylococcus aureus* and bacteria belonging to the Enterobacteriaceae family [1, 2]. However, the most significant microbial hazards vary among hospitals and from service to service within the hospital. The rate of wound infection also varies from hospital to hospital and from country to country. Values up to 20% have been reported in the literature [3–5]. Data on the incidence of wound infection have in the past been hard to obtain. With the general introduction of hospital infection-control programmes, such data are now being routinely collected. This study aimed to report on the rates of wound infection and sensitivity pattern of the predominant bacteria in three departments of this hospital which are mostly at high risk, during the hot months June–October.

Materials and methods

Wound swabs from patients in the general surgery (GS), interdisciplinary intensive care (IIC) and pediatric intensive care (PIC) departments were collected during the months June, July, August, September and October. Wound swabs were taken using a cotton-tipped swab moistened with sterile saline and sent to the microbiology laboratory where they were directly cultured on blood agar and MacConkey agar plates (Difco, USA) and incubated at 37 °C. Bacteria were identified on the basis of their morphological, cultural and biochemical characteristics [6]. To confirm the identification of some Gram-negative bacteria API 20 E system (from France) was used. For the determination of the antibiotic sensitivity of isolates the method of Bauer et al. [7] was used.

Results

A total of 2331 wounds were cultured during the study period. Out of these wounds 193 were clinically and microbiologically infected (8%). The infection rates are shown in Table I. The overall infection rate in the three departments were significantly different (F=10.24). They were 17.6% in the IIC, 8.6% in the GS and 3.7% in the PIC departments. The highest monthly infection rate occurred during July in all departments.

Table II shows the most common pathogens isolated from the infected wounds. Out of 283 strains *S. aureus* represented the highest frequency of isolation (35%) followed by *E. coli* (31%), *P. aeruginosa* (25%) and *Klebsiella* (10%). The highest frequency of *S. aureus* occurred during July (27.6%) while that of *P. aeruginosa*, *E. coli* and *Klebsiella* during June (34%), October (29%) and July (37%), respectively. On the other hand, the most frequently isolated organisms during June, July, August, September and October were *S. aureus* and *P. aeruginosa* (each 33.8%); *S. aureus* (39%); *E. coli* (33%); *S. aureus* (61%); and *E. coli* (41.7%) respectively.

Table III shows the sensitivity of the isolated pathogens to some antibiotics commonly used in this hospital. Chloramphenicol was the most effective antibiotic against *S. aureus* (78%) followed by gentamicin (63%) and tetracycline (57%). *E. coli* was the most frequently sensitive to cefotaxime (82%), followed by cefoxitin (77%), cephalotin (62%) and chloramphenicol (53%).

Against *Klebsiella* cefotaxime (95%), followed by chloramphenicol (90%), tetracycline (70%), gentamicin and cephalotin (each 66%) were the best choice of antibiotics. *P. aeruginosa* were the less frequently sensitive to the tested antibiotics.

Table I

Analysis of infection rates by hospital services and months

Months	Per cent of infected wounds in		
	GS	IIC	PIC
June	10.1	22.2	4
July	9.6	27.3	6.3
August	7.3	13.9	1.9
September	7.6	5.7	1.7
October	7.8	19.4	6
Overall	8.6	17.6	3.7

GS: general surgery; IIC: interdisciplinary intensive care; PIC: pediatric intensive care

Table II

Numbers of the most common bacteria isolated from the infected wounds during the study period

Months	Number of bacteria				
	S. aureus	*E. coli*	*P. aeruginosa*	*Klebsiella*	Total
June	24	18	24	5	71
July	27	22	10	10	69
August	11	14	12	5	42
September	25	8	7	1	41
October	11	25	18	6	60
Total	98	87	71	27	283

Discussion

Wound infection rate varies in literature from hospital to hospital and from department to department within the same hospital. Values between 2 and 20% are common [3–5, 8, 9]. Differences in the infection rate could be due to a number of factors including therapeutic factors, number and type of microorganisms present in wound, age and healthy condition of the patient, type and duration of operation as well as hygienic condition. The overall infection rate in this hospital is within the range reported in literature. The high infection rate in the IIC could be due to the fact that mostly burn cases are admitted in this unit. Infections in hospitals are caused by both Gram-positive and Gram-negative bacteria, the most frequent of them being *S. aureus* and *E. coli*, respectively [10–12]. Each environment has its own microflora and the three factors: environment, microorganism and host defense mechanism determine whether an infection develops or not.

Bacteria present in hospitals are generally more resistant to antibiotics and are infections caused by them difficult to treat, their resistance development is influenced by the frequency of antibiotic use. A study done by Rolinski et al. [10] showed a very high level of resistance of *E. coli* isolates (61–97%) to ampicillin and oxytetracycline. Also a high level of resistance characterized their isolates of *P. aeruginosa* to chloramphenicol. Among them *S. aureus* isolates 81% were resistant to erythromycin and 64% to chloramphenicol.

In this study *S. aureus* isolates were resistant to chloramphenicol at a lower rate. Among *E. coli* isolates in this study 88% were resistant to ampicillin and 57% to tetracycline. A high level of resistance characterized *P. aeruginosa* isolates to the tested antibiotics in this study. These results are in good line with others [11–13]. The frequency of resistance is particularly high among Gram-negative bacteria [14].

Table III

Antibiotic sensitivity of the isolates

Bacteria	Per cent of sensitive strains						
	AM	CR	CTX	FOX	TE	C	GM
S. aureus	16	11	11	11	57	78	63
E. coli	12	62	82	77	43	53	49
P. aeruginosa	8	25	56	25	22	29	33
Klebsiella	–	66	95	60	70	90	66

AM = ampicillin; CR = cephalotin; CTX = cefotaxime; FOX = cefoxicitin; TE = tetracycline; C = chloramphenicol; GM = gentamicin

The total incidence of drug-resistant bacteria may differ from study to study due to differences in local ecology, misuse of antibiotics and differences in the methodology employed [15]. Therefore sensitivity tests in hospitals are nowadays essential for successful treatment of infections with antibiotics and for the control of the spread of drug resistant pathogens.

REFERENCES

1. Altemeier,W.A.: Sepsis in surgery. Archives of Surgery **117**, 107 (1982).
2. Miller,W.A.: Postoperative wound infection in foot and ankle surgery. Foot Ankle **4**, 102 (1983).
3. Meers,P.D.: Infection in hospital. British Medical Journal **282**, 1246 (1981).
4. Abussaud,M.J.I., Meqdem,M.M.: A study of some factors associated with wound infection. J of Hospital Infection **8**, 300 (1986).
5. Abussaud,M.J.I.: Prevalence of nosocomial infections in a Saudi Arabian teaching hospital. J of Hospital Infection **17**, 235 (1991).
6. Cruickshank,R., Duguid,J.P., Marmion,B.A., Swan,R.H.A.: The practice of medical microbiology. Vol.2, 12th ed. Churchill Livingstone, Edinburgh. 1975.
7. Bauer,A.W., Kirby,W.M.M., Sherris,J.C., Turck,M.: Antibiotic susceptibility testing by a single Standardized disk method. American J Clin Pathology **45**, 493 (1966).
8. Osullivan,D.C., Murphy,D.M., Mclean,P., Donovan,M.G.: Transplant nephrectomy over 20 years: Factors involved in associated morbidity and mortality. Journal of Urology **151(4)**, 855 (1994).
9. Neidel,F.: Preoperative versus intraoperative one-shot prophylaxis with cefuroxime for the prevention of septic complications following biliary surgery. Infection **21(suppl.1)**, 549 (1993).
10. Rolinski,Z., Wiaz,P., Kowalski,C., Pacholczyk,S.: Computer analysis of antibiotic sensitivity pattern of bacteria isolated from polity. Medycyna Weterynaryjna **50(2)**, 65 (1994).
11. Samra,Z., Gadba,R.: Antibiotic susceptibility and phage typing of methicillin-resistant *Staphylococcus aureus* clinical isolates from blood cultures of 692 patients in 15 Israeli hospitals. Eur J of Epidemiology **9(5)**, 559 (1993).
12. Chattopadhyay,A.K., Nandal,A.K.: Microbes associated with urinary tract infection in Burdwan, West Bengal. Indian J of Physiology and Allied Sciences **47(2)**, 67 (1993).
13. Reali,D, Rosati,S.: Antibiotic susceptibility and serotyping of *Pseudomonas aeruginosa* strains isolated from surface water, thermomineral waters and clinical specimens. Zentralblatt für Hygiene und Umweltmedizin **196(1)**, 75 (1994).
14. Ahmad,I., Yadava,J.N.S., Ahmad,S.: High level transferable resistance among *Escherichia coli.* Indian J of Animal Sciences **64(5)**, 439 (1994).
15. Alsowaygh,I.A., Shibl,A.H.: Incidence of antimicrobial resistance among *Enterobacteriaceae.* Current Microbiology **5**, 143 (1981).

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IN VIVO STUDIES ON AUJESZKY'S DISEASE VIRUS MUTANTS

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We report the construction and in vivo analysis of three recombinant Aujeszky's disease virus (ADV) strains containing mutations at three different loci of the genome. Mutant **vE16lac** was generated by deleting of 2976 bp DNA fragment which covers 1851 bp of the right arm of UL component, the UL-US junction, the "*a*" element of the internal repeat (IR) region and a putative LAT promoter. Mutant **vRRlac** was generated by deletion of a 1805 bp fragment from the coding region of the large and small subunits of ribonucleotide reductase gene (rr). The third mutant, **vTKlac**, was constructed using insertional mutagenesis of the thymidine kinase gene (tk). In the constructed mutants a *lacZ* gene expression cassette was either inserted into the target gene (*vTKlac*) or replaced the deleted DNA segment (*vE16lac, vRRlac*). Constructed recombinant viruses were analyzed by infecting pigs and monitoring the virus excretion from nasal fluid and disease symptoms. Tissue specimens were collected for virus isolation and pathological examination. Strains *vTKlac* and *vRRlac* retained the ability to establish an infection, but showed reduced replication efficiency in the respiratory tract and were unable to attack the central nervous system (CNS) of pigs. Thus, both deletions induce significant attenuation of the virus measured by decrease of virulence in infected pigs. Strain *vE16lac* showed disease symptoms similar to that of wild type and could be detected in the CNS of pigs.

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Aujeszky's disease virus (ADV), also designated as pseudorabies virus (PrV), is a herpesvirus and belongs to the subfamily of *Alphaherpesvirinae*. ADV is economically important throughout the world resulting in severe losses in pig industry. Although pig is considered as the natural host of the virus, most mammals can be infected by ADV causing high mortality. Older pigs are usually resistant to the viral infection, but they show clinical signs of the disease, including fever, pneumonia and decrease in weight gain. Young pigs and other susceptible animals usually die of severe meningoencephalitis. ADV, like other herpesviruses, is composed of a complex-protein-DNA core surrounded by a lipid membrane that carries a set of glycoproteins. The genome of ADV is composed of a 135 kb linear double stranded DNA molecule which contains two components, UL (unique long) and US (unique short). The US region is bracketed by internal (IR) and terminal (TR) repeats [1]. US is present in two orientations relative to the UL component (class 2, or D type of genomes), and thus, viral DNA obtained either from virions or infected cells consists of two equimolar isomeric forms. In addition, under certain growth conditions, the UL component was found to be also inverted relative to the US component [2]. The physiological function of the inversions is yet unknown. In the genome of herpes simplex virus (HSV), both UL and US components are bracketed by inverted repeat sequences (class 3 or E type of genomes; [3]), and thus, in infected cells and in virions four isomeric forms of equal molarity can be found.

Upon entering the cell nucleus, end-to-end ligation of the ADV genome gives rise to a circular DNA [4], and the first round of DNA replication occurs by virtue of theta-type structures. Thereafter head-to-tail concatameric forms are generated by a rolling-circle mechanism [5]. Mature ADV genomes are cleaved from concatamers most likely by a site-specific mechanism [6], mediated by recognition signals homologous to pac-1 and pac-2 elements of HSV "*a*" sequences. In contrast to HSV, sequences at the termini of ADV genome are different. Sequences homologous to HSV pac-1 element can be found only at the right terminus and at the UL-IR junction of ADV genome. Sequences homologous to HSV pac-2 element are present only at the left terminus of the genome [7]. It has also been suggested that the "*a*" components are involved in genomic isomerization events [8]. Both homologous and site-specific recombination models have been invoked to explain the underlying mechanism [6, 9].

A characteristic feature of herpesviruses is that the inverted repeats flanking the unique regions of the viral genome are able to equalize themselves, which could be a result either of a copying mechanism [10], or alternatively, of an intermolecular recombination [11]. Rall et al. [12] analyzing intracellular DNA of an ADV mutant lacking internal pac-1 sequences found that, in contrast to the wild type (wt) virus, most of mutant genomes remained in concatameric forms. They also found, that inversion and equalization occurred in most of intracellular DNA of the mutant virus, while mature virions had an unequal repeat structure. This latter finding is reasonable as only those viruses are able to be cut out from concatamers and packaged which have intact cleavage-packaging signals at the termini of viral genome. The mechanism by which the wt TR is maintained has not been determined yet.

Alpha- and gamma-herpesviruses each encode a ribonucleotide reductase (RR) which comprises large (RR1) and small (RR2) subunits. RR converts ribonucleoside diphosphates to the corresponding deoxyribonucleotide diphosphates and is essential in

the de novo pathway of deoxyribonucleotide synthesis [13]. The entire DNA sequences specifying the two subunits of ADV ribonucleotide reductase were determined in our laboratory [14]. RR is translated from two transcripts which share coding sequences for RR2. It has been shown for HSV-1 that RR enzyme activity is non-essential for replication in tissue culture, although RR-deficient mutants replicate poorly in non-dividing cells [15]. RR mutants of HSV-1 proved to be avirulent in mice [16]. De Wind et al. [17] found that RR^- mutants of ADV are also avirulent for both mice and pigs.

Thymidine kinase catalyzes the phosphorylation of deoxythymidine to dTMP, which is further phosphorylated to dTTP. TK also facilitates the synthesis of dCTP, dATP and dGTP in an indirect way. The major role of the viral encoded TK is to support the viral replication in nerve cells. Therefore, TK plays an important role in the pathobiology of pig infection. Currently the most frequently used live ADV vaccine strains are deficient in TK.

In this work we generated three recombinant ADV strains containing mutations at three different loci of ADV genome. Mutant *vE16lac* was constructed by deleting a 2976 bp DNA fragment from the UL-IR junction region and by replacing the deleted fragment with the *ß-galactosidase* (*lacZ*) gene. Recombinant virus *vRRlac* was constructed by deleting DNA sequences coding for RR1 and RR2, and inserting *lacZ* gene cassette. Finally, the recombinant virus *vTKlac* was generated by introducing the *lacZ* gene into the *XhoI* site of TK gene.

Pigs were infected with each recombinant, marker-rescued (*vE16resc, vRRresc*) and wild type (strain KA) ADV strains, and the virulence was assayed by monitoring growth arrest, signs of disease, virus excretion from nasal fluid and tissue tropism of viruses in pigs. Serological response to viral infection was also examined.

Materials and methods

Cells and viruses. Strain KA [18] of ADV was used as a parental strain to produce recombinant viruses. Viruses were cultured in confluent monolayer of porcine kidney (PK15) and African green monkey kidney (Vero) cell lines. PK15 cell line was used for transfection and plaque purification of viruses. PK15 and MDBK (*Madin-Darby* bovine kidney) cell lines were used for titration. Cells were grown in Dulbecco's modified minimum essential medium supplemented with 5% foetal calf serum, 0.5 mg/l gentamicin and 0.25 mg/l amphotericin-B at 37 °C with 5% CO_2. Viruses were purified from the medium of infected cells showing total cytopathic effect by isopycnic centrifugation on a discontinuous gradient, as described previously [19].

Construction of recombinant plasmids and mutant viruses. Restriction enzymes and linkers used in plasmid constructions were obtained from "Fermentas" and "Amersham". Plasmids were prepared by alkaline lysis procedure [20]. *KpnI, BamHI* and *SalI* gene clonotheques and transfer vectors were constructed using bacterial positive-selection plasmid-vectors containing palindromes (pRL family) [14, 21].

Strain vE16lac. BamHI 8' restriction fragment of ADV was isolated from the agarose gel and then was cloned into *pRL425*, resulting in *pB8'*, which was used as a parent plasmid for constructing of the transfer vector. A 2976 bp fragment covering from −1185 to +1885 of UL-IR junction region and containing the "*a*" element of IR region and a putative LAT promoter [22] (Fig. 1.A) has been deleted

from *pB8'* by *SmaI* digestion, then *HindIII* linker was inserted at *SmaI* site resulting in the transfer plasmid *pEh2.*

The *lacZ* gene of *Escherichia coli* controlled by hCMV IE1 promoter/enhancer and containing a *polyA* signal of SV40 was ligated into the *HindIII* site of *pEh2* vector. The *lacZ* expression cassette was used for producing recombinant virus *vE16lac* (Fig. 1.B-C) in cotransfection experiments using purified *wt* viral DNA. Marker-rescued virus strain with reconstituted genome was obtained by cotransfecting of recombinant virus DNA with intact 5.1 kb *BamHI* 8' fragment of the *wt* virus and subsequent screening for white plaques.

Strain vRRlac. The largest (5 kbp) *SalI* restriction fragment of *KpnI 1* fragment was cloned into *pRL425* resulting in *p4R* and an 1805 bp DNA fragment was deleted by digesting *p4R* with *ScaI* and *MluI* restriction enzymes (Fig. 1.A), then the DNA was blunt-ended using Klenow enzyme and *HindIII* linker was attached to the plasmid resulting in *p4Rd.* The deleted DNA included 1789 bp sequence of the 3'-end of RR1, 8 bp of intergenic region and 7 bp of the 5' sequences of RR2 (Fig. 1.A). Viral DNA inserts of *p4Rd* served as flanking sequences for homologous recombination. The *lacZ* gene cassette described above was inserted into the *HindIII* site of *p4RRd* resulting in *pRRlac.* Recombinant virus *vRRlac* was generated by cotransfecting transfer vector *pRRlac* and wt viral DNA as described above.

Strain vTKlac. BamHI 11 fragment was cloned into vector *pRL425* resulting in *pTK,* which was digested with *XhoI* (Fig. 1.A), then blunt-ended using Klenow fragment of *E. coli* DNA polimerase. The *lacZ* expression casette was inserted into the plasmid using *HindIII* linker. The resultant plasmid *pTKlac* was used as a transfer vector for homologous recombination as described above.

Insertion of *lacZ* gene cassette into the coding regions of RR and TK led to termination of transcription and, in addition, recombinant strains acquired reporter *lacZ* gene which could be easily traced in colour reaction with X-Gal (see bellow).

Transfection of cells was carried out by electroporation of actively growing PK15 cells with 20 µg plasmid and 10 µg of viral DNA for 10^6 cells in a 4 mm electroporation cuvette, at 550 V (C=200mF, R=129W). Recombinant viruses carrying *lacZ* gene were screened as blue plaques by adding X-Gal, a chromogenic substrate of *ß-galactosidase*, to the agar overlay of infected cell culture followed by 6 to 8 plaque purification cycles.

Southern hybridization. For Southern analysis 2 µg of viral DNA was digested with *BamHI* restriction enzyme and DNA fragments were separated on 0.8% agarose gel, transferred to nitrocellulose, and hybridized using standard procedure [20]. Three ADV specific probes were used for hybridization: for vE16lac strain a 580 bp *DraI-NarI* fragment which included "*a*" sequences of internal repeat region of the viral genome; for *vRRlac* strain an 1115 bp *MluI-ScaI* fragment of plasmid *p4R;* and for *vTKlac* strain the 785 bp *BamHI-XhoI* fragment of *BamHI* 11 fragment (Fig. 1.A). Probes were labelled with ^{32}P-dCTP using random priming (USB).

Computer analysis. DNA sequences were analyzed with the sequence analysis software package GCG version 7.1 of the University of Wisconsin Genetics Computer Group [23].

Analysis of virulence and clinical observations in pigs. Pathogen-free 10-week-old KA-HYB pigs, 31 kg on average, purchased from a farm free of ADV were inoculated intranasally with 2 ml MEM media containing 10^5 p.f.u. of ADV. Seven experimental groups with 7 pigs in each were housed in a separate isolation room. Pigs mock-infected with MEM media or with marker-rescued virus strain served as controls. Pigs were weighed every second day. Rectal temperature was monitored daily. Nasal swabs were collected daily to determine the virus excretion. Virus titres were determined on PK15 cell using methyl-cellulose overlay. Animals were observed for signs of disease twice a day. Nasal discharge, coughing and forced respiration were observed as respiratory signs. The following neurological signs were observed: ataxia, itching, tremor, convulsion and paralysis. One pig from each group was exterminated at 24 h and the second one at 72 h, and then the survived pigs were sacrificed at day 16. Virus multiplication after infection was determined by titration of nasal fluid samples and organ suspensions on PK15 cell line.

Histochemical staining for detection of *ß-Gal* activity in infected pig tissues was used according to Sanes et al. [24].

Neutralization test. Blood samples were collected weekly. The standard technique (24 h SN test) in cell culture with constant virus and variable sera was applied to all infected and control pigs. Virus titres were expressed as $\log_{10}$ of the reciprocal of the end point serum dilution which inhibits the developing of cytopathic effect (c.p.e.) in 50% of the cells.

Results

Structure of recombinant viruses. Recombinant viruses carrying *lacZ* gene at the indicated loci (Fig. 1.C) were obtained as described in Materials and Methods. To ascertain that the deleted region of the ADV genome is solely responsible for the altered phenotype of the virus we obtained marker-rescued variants for two mutants (*vE16lac* and *vRRlac*). Rescuants were generated by the method described above using transfer plasmids containing intact genomic DNA which envolves the deleted sequences. These strains regained their initial structure of *wt* ADV and conferred white plaque phenotype.

To confirm the presence of deletion and/or insertion in the recombinant viruses DNAs were analyzed using Southern-blot hybridization (Figs 2, 3 and 4). Viral DNAs digested with *BamHI* were probed with the DNA sequences described in Materials and Methods. Using radioactive specific probe for **vE16lac**, both *wt* and marker-rescued strains showed two radioactive signals corresponding to *BamHI* 8' and 13 restriction fragments, respectively.

Recombinant virus propagated in tissue culture (PK15) showed only one signal (*BamHI* 13 fragment). However, a mixed population of the *vE16lac* strain was obtained from the nasal fluid of swine. Besides virus particles expressing *lacZ* and showing the same hybridization pattern as that of viruses obtained from the tissue culture, we also found mutants which formed white plaques showed two radioactive signals of *BamHI* 13 and the other one below the *BamHI* 8' fragment (Fig. 2), which indicates that in this variant the deleted UL sequences were missing, however the deleted IR sequences were restored.

Hibridization of DNA of wt and marker-rescued *vRRresc* strains with the specific probe for RR gene showed a radioactive signal of the *BamHI* 2 fragment, which indicated that strains were structurally identical. Mutant virus **vRRlac** lacked this DNA fragment (Fig. 3), which confirmed that the deletion occurred at the appropriate position of the viral genome.

The specific radioactive probe for the strain **vTKlac** gave a signal for the *BamHI* 11 fragment of *wt,* whereas a signal of lower molecular weight DNAs observed for the DNA of mutant virus. The fragment was formed due to additional *BamHI* restriction sites created by insertion of expression casette into the TK gene of the virus. Plaques of all studied mutant viruses in the presence of X-Gal developed blue colour, which indicated that viruses express the enzymatically active *ß-galactosidase* (data not shown).

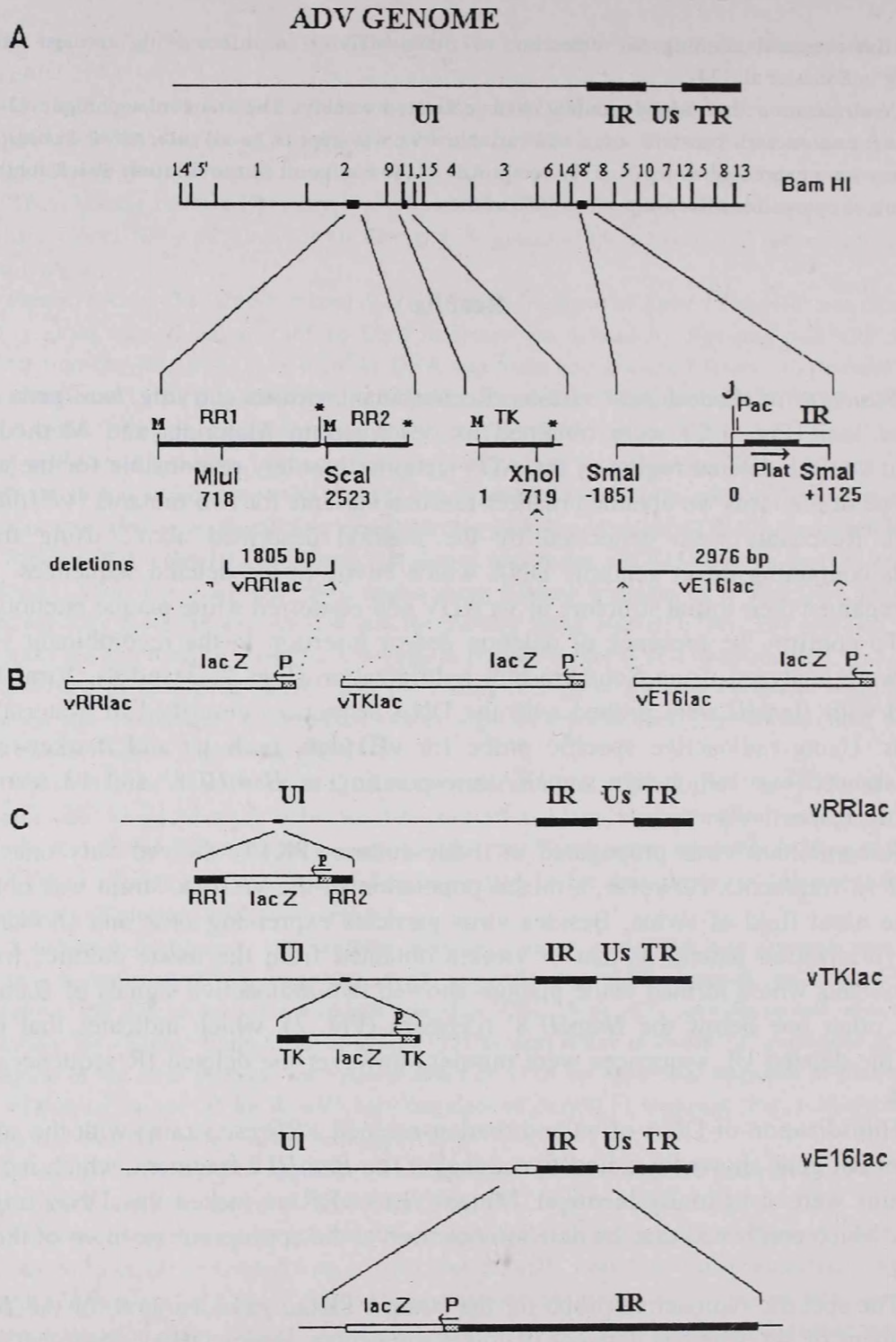

Fig. 1. A. Structural organization of ADV genome. ADV DNA is composed of a unique long (Ul) and a unique short (Us) regions. The Us component is bracketed by inverted internal repeat (IR) and terminal repeat (TR). The positions of BamHI restriction sites on ADV genome are indicated. Locations of genes coding for the two subunits of ribonucleotide reductase (RR1 and RR2), thymidine kinase (TK) and the region containing the putative LAT promoter and pac-1 signal are also indicated. Below the structure of deletions are shown. *B.* An expression casette containing *lacZ* gene was introduced into the viral genome as shown. *C.* Structural organization of constructed mutant ADV strains

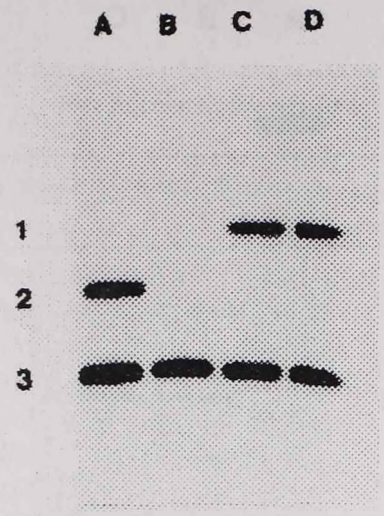

Fig. 2. Southern blot analysis of vE16lac strain. The 580 bp DraI-NarI fragment which included the "a" sequence of internal repeat region of the viral genome was used as a probe. Radioactive signals (3) indicate the BamHI 13 fragment which involves the pac-1 signal of TR. *Lane A.* White plaque forming strain recovered from brain of pig infected with vE16lac. Radioactive signal (2) indicates that this mutant restored its IR with intact pac-1 signal, while maintaining the deletion on Ul region. *Lane B.* Autoradiogram of vE16lac strains propagated in PK-15 cell line. This mutant possess only pac-1 signal at the TR (3). *Lanes C* and *D.* Autoradiograms of wt and rescuant strains, respectively. These strains have intact TR (3; BamHI 13 fragment) and IR (1; BamHI 8' fragment)

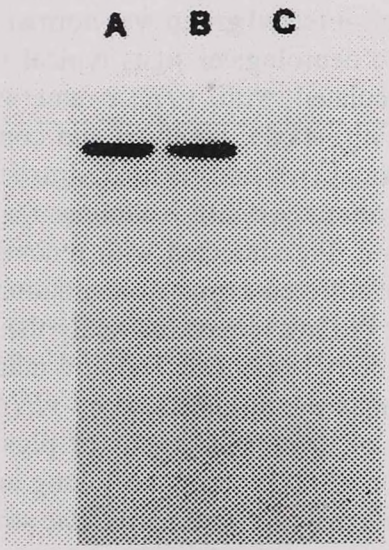

Fig. 3. Autoradiograms of wt (A), marker rescued (B; vRRresc) and vRRlàc (C) strains. The 1115 MluI-SacI fragment of BamHI 2 fragment was used as a probe for detection. Data indicate that deletion occurred at the appropriate site of RR gene (no radioactive signal was obtained; C), and that strain vRRresc has restored this deletion (B) as similarly to wt (A), it has an intact BamHI 2 fragment

Each recombinant ADV strains were assayed for virulence in pigs. A moderate increase of body temperature which had never exceeded 40.5 °C was observed at days 2–6 in pigs infected with mutants *vTKlac* and *vRRlac,* while pigs inoculated with *vE16lac,*

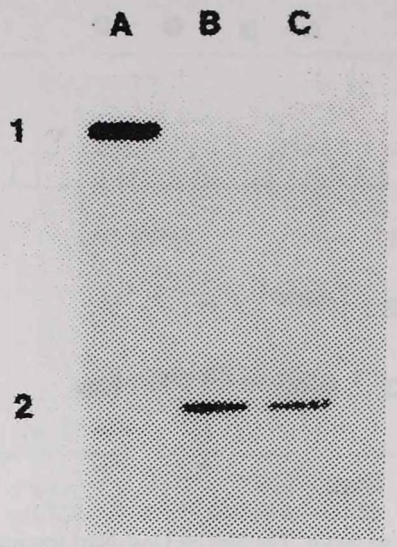

Fig. 4. Detection of TK gene of ADV. The 785 bp BamHI-XhoI fragment of BamHI 11 fragment was used as a probe for detection. Wt virus (A) has an intact BamHI 11 fragment (1), while smaller fragments could be detected in vTKlac propagated in PK-15 cell line (B) or recovered from OPF of swine (C). This latter finding was due to introducing additional BamHI restriction sites to the TK gene by inserting the lacZ gene

wt and rescued strains had a high temperature with peaks exceeding 41 °C (Table I). The body temperature of pigs in mock-infected group was normal. A mild nasal discharge and slightly reduced appetite, but no neurological signs typical for Aujeszky's disease were observed at days in pigs inoculated with *vTKlac* and *vRRlac.* In contrast, severe neurological signs (tremor, itching, ataxia, paralysis and convulsion) were developed in pigs infected with *vE16lac,* parental (*wt*) and rescued virus strains. These viruses evoked clinical signs of disease to various extent, including fever, loss of appetite, nasal discharge, and growth retardation. Pigs infected with recombinant *vE16lac* and rescued strains, similarly to the *wt* strain, suffered a growth retardation, while infection of animals with *vTKlac* and *vRRlac* strains did not reduce markedly the body weight gain. All virus strains induced neutralizing antibody responses. The titre induced by *vTKlac* and *vRRlac* was approximately 1 log lower than the titre induced by *vE16lac, wt* and rescued viruses. We recovered viruses from all of examined organs of pigs infected with *wt* and both rescued strains (see Table I). For *vE16lac* strain, only lung and lymph nodes were found to be free of viruses. In contrast, for *vTKlac* and *vRRlac* strains, the virus was detected only in the nasal mucosa, which means that mutant viruses were unable to spread in the body and to reach the CNS. Further studies are planned to examine tissue samples for the presence of viral antigens and viral DNA using immunocytochemical and PCR methods.

Table I

Virulence of ADV strains infecting 10-week-old pigs and the success of virus isolation from different tissues of swine

Group	Mortality	*Body weight gain	Fever	Respiratory signs	CNS signs	**Virus shedding	VN titre
vE16 lac	2/6	0.21	2–5	2–8	4–8	1.8×10^7	42
vTK lac	0/6	0.54	2–4	2–6	0	4.2×10^3	6.4
vRR lac	0/6	0.52	2–4	2–6	0	3.1×10^4	14.4
KA	5/6	–0.63	2–7	2–8	4–8	2.4×10^7	64
vE16 resc	6/6	–0.65	2–8	2–8	4–8	5.7×10^7	ND
vRR resc	6/6	–0.71	2–8	2–8	3–8	6.8×10^7	ND
Uninfected	0/6	0.61	0	0	0	0	0

Group	Nasal mucosa	Bulbus olfactorius	Gangl. Trigem.	Hypo-thalamus	Hypo-physis	Cortex	Cerebel-lum	Tonsilla	Lung	Lymph nodes
vE16 lac	+	+	+	+	+	+	+	+	–	–
vTK lac	+	–	–	–	–	–	–	–	–	–
vRR lac	+	–	–	–	–	–	–	–	–	–
KA	+	+	+	+	+	+	+	+	+	+
vE16 resc	+	+	+	+	+	+	+	+	+	+
vRR resc	+	+	+	+	+	+	+	+	+	+
Uninfected	–	–	–	–	–	–	–	–	–	–

* Mean body weight gain of groups between 0 and 7 days of post challenge (DPC) in kg/pig/day

** Total virus shedding of groups of pigs during the challenge in p.f.u./nasal discharge of group

Data suggest that inactivation of RR and TK gene of ADV produces avirulent virus strains, while extended deletion at the UI-IR junction including the internal "a" sequences and a putative LAT promoter does not affect the viability of the virus. This latter finding could be due to the ability of strain vE16lac to restore this deletion in vivo.

Discussion

We report data on construction and in vivo analysis of three recombinant ADV mutants. Mutant **vE16lac** contains a 2976 bp deletion at the viral genomic region comprising internal UL-IR junction and its flanking sequences. Our aim of this study was to alter the viral replication machinery by removing internal cleavage-packaging signal and a putative latency-associated transcript (LAT) promoter, and to explore the impact of this mutation on the viability of the virus. The structure of infectious virus during the propagation in PK15 cell line was stable. However, we observed that in infected pigs and mice due to equalization of repeat sequences, the recombinant virus *vE16lac* contained

variants with restored deletion at IR region. Similar mutation of ADV was constructed by Rall et al. [12], in which a 820 bp sequences were deleted from the UL-IR junction (–677 to +143) evolving regulatory element homologous to the HSV *pac-1* signal. The authors found that DNA of mature mutant virions had unequal repeat structure, while the majority of intracellular viral DNA molecules were equalized, which means that both ends of the inverted repeat bracketing the US region contained the mutation. The mutation prevented the maturation of intermediate concatameric viral DNA into monomer structure and thus, concatameric DNAs accumulated in the cell. The authors [12] also demonstrated that isomerization took place during the viral replication. The molecular mechanism of preserving intact TR region in viable virions is still uncertain.

Our result (obtaining no revertants in cell culture) might be explained by the fact that the recombinant virus (*vE16lac*) used for DNA analysis was propagated immediately following plaque purification steps. It cannot be excluded that additional passages of recombinant viruses in cell culture might produce revertants with low frequencies. This latter hypothesis, together with a detailed analysis of the restoration process will be examined in a later study. It is to be determined whether restoration occurs only in the cells of living organisms, or they only provide selective environment. In addition to *pac-1* sequences, deletion in *vE16lac* also involves a putative LAT promoter. Vlcek et al. [22] hypothesized that the expression of an antisense protein was driven from this promoter. However, we found [25–27] that the existence of long antisense ORFs found on the complementary strand of many herpesvirus genes, could be explained by mechanisms which did not require to suppose a coding function.

We also studied the virulence and tissue tropism of *vE16lac* by infecting pigs with the mutant virus. Studies showed that *vE16lac* was almost as virulent as the *wt* virus. Pigs infected with mutant strains developed clinical signs and virus excretion. VN titre and tissue tropism of this strain was similar to that of *wt* virus (Table I). This result may be explained by the ability of the mutant virus to restore the deleted sequences in IR region during replication in pig although, deletion has been retained in the UL segment. It is interesting that in the stained brain sections of infected pigs besides white plaques we also found blue plaques of the *vE16* strain, which indicates that the constructed mutant virus is somehow able to reach and infect the CNS. Additional experiments are needed to clarify whether the mutant virus itself infects the CNS, or for this process helper (revertant) virus is needed. Using mice, we found that brain suspension of some individuals almost exclusively contains the original mutant as well, which indicates that *vE16lac* is able to infect mice without the helper virus. Comparing *wt,* deletion-rescued (*vE16resc*) and mutant *vE16lac* strains we found some differences in pathogenicity between *vE16lac* and *wt,* but we could not find any difference between rescued and *wt* strains in multiplication and tissue tropism, which indicates that no other rearrangement in the genome of *vE16lac* had occurred. Meantime, we do not claim that altered growth characteristics of *vE16lac* in cell culture are caused exclusively by the deletion of "*a*" sequences of the IR, since the deletion contained the putative LAT promoter and 1851 bp of the UL component, as well.

The infectivity of the recombinant virus in pigs is only slightly decreased compared to the *wt* virus (Table I), which could be the result of restoration of the intact IR using TR as a template. This interesting finding might be an important clue for better understanding of molecular mechanism of viral replication.

As expected **vTKlac** proved to be highly avirulent for pigs. It only slightly affected the body weight gain, did not produce CNS signs, and did not cause death upon infection. VN titre induced by *vTKlac* was exactly one log lower than that of *wt* virus.

Data obtained in in vivo experiments in pigs with **vRRlac** strain were similar to that of **vTKlac** (Table I). The mutant proved avirulent for pigs and was unable to infect the CNS, which is in agreement with data of de Wind et al. [17]. Neutralizing antibody titre induced by infection of pigs with this mutant is considerably lower than that of *wt* strain (Table I), it may be advantageous for use as a vaccine. In addition, these strains have potentials for using as vectors for gene therapy.

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REFERENCES

1. Ben-Porat,T., Rixon,F.J., Blankenship,M.L.: Analysis of the structure of the genome of pseudorabies virus. Virology **95**, 285 (1979).
2. Lomniczi,B., Blankenship,M.L., Ben-Porat,T.: Deletions in the genome of pseudorabies vaccine strains and existence of four isomeric forms. J Virol **49**, 970 (1984).
3. Sheldrick,P., Berthelot,N.: Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp Quant Biol **39**, 667 (1977).
4. Ben-Porat,T., Veach,R.A., Ladin,B.F.: Replication of herpes virus DNA. VI. Virions containing either isomer of pseudorabies virus DNA are infectious. Virology **102**, 370 (1980).
5. Jean,J.H., Blankenship,M.L., Ben-Porat,T.: Replication of herpes virus DNA. I. Electron microscopic analysis of replicative structures. Virology **79**, 281 (1977).
6. Mocarski,E.S., Post,L.P., Roizman,B.: Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional inversions. Cell **22**, 243 (1980).
7. Hammerschmidt,W., Ludwig,H., Buhk,H.J.: Specificity of cleavage in replicative form DNA of bovine herpes virus 1. J Virol **62**, 1355 (1988).
8. Chou,J., Roizman,B.: Isomerization of herpes simplex virus type 1 genome. Identification of the cis-acting and recombinational sites within the domain of the a sequence. Cell **41**, 803 (1985).
9. Weber,P.C., Challberg,M.D., Nelson,N.J., Levine,M., Glorioso,J.C.: Inversion events in the HSV-1 genome are directly mediated by the viral replication machinery and lack sequence specificity. Cell **54**, 369 (1988).
10. Roizman,B.: The structure and isomerization of herpes simplex virus genome. Cell **16**, 481, (1979).
11. Ben-Porat,T., Deatly,A., Veach,R.A., Blankenship,M.L.: Equalization of the inverted repeat sequences of the pseudorabies virus genome by intermolecular recombination. Virology **132**, 303 (1984).
12. Rall,G.F., Kupershmidt,S., Sugg,N., Veach,R.A., Ben-porat,T.: Functions of the sequences at the ends of inverted repeats of pseudorabies virus. J Virol **66**, 1506 (1992).
13. Reichard,P.: Interactions between deoxyribonucleotide and DNA synthesis. Ann Rev Biochem **57**, 349 (1988).
14. Kaliman,A.V., Boldogkői,Z., Fodor,I.: Large and small subunits of the Aujeszky's disease virus ribonucleotide reductase: nucleotide sequence and putative structure. Biochimica et Biophysica Acta **1219**, 151 (1994).

15. Goldstein,D.J., Weller,S.K.: Herpes simplex virus type 1 induced ribonucleotide reductase activity is dispensible for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. J Virol **62**, 196 (1988).
16. Cameron,J.M., McDougall,I., Marsden,H.S., Preston,V.G., Ryan,D.M., Subak-Sharpe,J.H.: Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenecity of the virus in mice and a valid antiviral target. J Gen Virol **69**, 2607 (1988).
17. De Wind,N., Berns,A.J.M., Gielkens,A., Kimman,T.: Ribonucleotide-reductase-deficient mutants of pseudorabies virus are avirulent for pigs and induce partial protective immunity. J Gen Virol **74**, 351 (1993).
18. Kaplan,A.S., Watter,A.E.: A comparison of herpes simplex and pseudorabies virus. Virology **7**, 394 (1959).
19. Stevely,W.S., Katan,M., Sterling,V., Smith,G., Leader,D.P.: Protein kinase activities associated with the virions of pseudorabies and herpes simplex viruses. J Gen Virol **66**, 661 (1985).
20. Sambrook,J., Fritsch,E.F., Maniatis,T.: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, NY, 1989.
21. Elhai,J., Wolk,C.P.: A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene **68**, 119 (1988).
22. Vlcek,C., Kozmik,Z., Paces,V., Schirm,S., Schwyzer,M.: Pseudorabies virus immediate-early gene overlaps with an oppositely oriented open reading frame – characterization of their promoter and enhancer regions. Virology **179**, 365 (1993).
23. Devereux,J., Haeberli,P., Smithies,O.: A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Res **12**, 387 (1984).
24. Sanes,J.R., Rubenstein,J.L.R., Nicolas,J.F.: Use of a recombinant retrovirus to study post-inplantation cell lineage mouse embrios. EMBO J **5**, 3133 (1986).
25. Boldogkői,Z., Murvai,J.: A novel explanation for the existence of open reading frames on the Latency-associated transcripts of alphaherpesviruses. Virus genes. **9**, 47 (1994).
26. Boldogkői,Z., Kaliman,A., Murvai,J., Fodor,I.: Sense antisense DNA strand? Acta Veterinaria Hungarica **42**, 243 (1994).
27. Boldogkői,Z., Murvai,J., Fodor,I.: G and C accumulation at silent positions of codons produces additional ORFs. Trends in Genetics. **11**, 125 (1995).

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THE PROBLEMS OF FOOD-BORNE DISEASES IN HUNGARY*

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Data for food-borne diseases have been collected by the National Institute of Food Hygiene and Nutrition (NIFN) since 1954 [1]. The most important and most characteristic informations are presented in this paper. The aim of this publication is to show the epidemiological situation and problems connected with food-borne diseases.

1. Morbidity and mortality. Table I indicates the number of registered food-borne outbreaks, sick and hospitalized patients and that of the deaths of the last 35 years. For a comparison the number of salmonellosis patients supplied by the Department of Epidemiology, B. Johan National Institute of Hygiene (NIH) is also presented. The latter figures represent data reported independently from the source of salmonella infection. Since 1972 not only cases of salmonella enteritis and epidemic food-borne salmonellosis, but also sporadic food-borne salmonella patients have been reported. There is a marked difference between the number of registered patients with food-borne infection and with salmonellosis. It seems that the NIFN and NIH notification systems register somewhat different events.

Evaluating the figures for five year periods it can be stated that the number of food-borne outbreaks was the highest during the second half of the sixties. After this period the figures decreased till 1993.

During the last 35 years the number of patients with registered food-borne diseases showed several peaks. The greatest one was observed during the first half of the sixties. Two smaller ones occurred during the seventies. Since 1978 the number of registered patients increased till 1987 and afterwards there was a decrease.

The number of hospitalized patients had a similar trend to the occurrence of food-borne outbreaks.

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Table I

Characteristic data of registered food-borne outbreaks in Hungary between 1960 and 1994, and number of salmonellosis patients

Years	Number of				Number of deaths		
	food-borne outbreaks	registered patients	hospitalized patients	salmonellosis patients	mushroom poisoning	other causes	total
1960	420	4268	856	654	13	3	16
1961	575	4563	1142	972	1	3	4
1962	505	5841	1264	2468	6	6	12
1963	692	6311	1395	1471	13	4	17
1964	664	6522	1969	1825	7	7	14
1965	732	6079	2339	1637	7	4	11
1966	896	6005	2232	1791	18	1	19
1967	885	4264	2218	1747	5	1	6
1968	823	4792	2359	1625	7	1	8
1969	710	3604	1586	1514	8	2	10
1970	726	3952	1777	2869	13	1	14
1971	708	4578	1663	4871	2	4	6
1972	873	3932	1936	4880	8	1	9
1973	518	3503	1192	4859	1	1	2
1974	687	3709	1515	4539	7	4	11
1975	599	5156	1681	5661	18	1	19
1976	631	4683	1278	5319	11	1	12
1977	573	3523	1034	4817	5	0	5
1978	455	2947	897	4852	5	2	7
1979	637	4068	1361	5361	17	0	17
1980	549	4395	1177	7181	7	0	7
1981	502	4229	898	8379	9	2	11
1982	380	4678	731	8853	5	0	5
1983	326	3897	722	8584	6	0	6
1984	538	6054	1011	8353	1	1	2
1985	358	4964	967	9192	1	0	1
1986	295	3970	633	12012	5	1	6
1987	466	7856	903	15073	9	1	10
1988	561	4719	1032	14789	4	1	5
1989	316	5872	768	13524	2	1	3
1990	313	3679	473	11392	4	1	5
1991	322	5277	698	11198	14	3	17
1992	242	2928	466	12843	4	3	7
1993	351	5779	744	16452	5	5	10
1994	505	4377	892	19055	7	3	10
Total	19333	164974	43809	240012	255	69	324

Table II

Cases of food–borne diseases and number of outbreaks, patients and deaths in Hungary between 1985 and 1994

Causes	Outbreaks	Number of patients	Deaths
Salmonella	1453 (37.5)*	26119 (52.8)	8 (10.8)
S. aureus	156 (4.0)	2119 (4.3)	2 (2.7)
C. botulinum	106 (2.7)	181 (0.4)	4 (5.4)
Enterococcus	54 (1.4)	1268 (2.6)	
B. cereus	50 (1.3)	2067 (4.2)	
C. perfringens	43 (1.1)	2813 (5.7)	
Campylobacter	23 (0.6)	299 (0.6)	
Shigella	9 (0.2)	1917 (3.9)	
Aeromonas	8 (0.2)	583 (1.2)	
Y. enterocolitica	8 (0.2)	336 (0.7)	
Viruses	0	0	
Mushrooms	1152 (29.7)	2668 (5.3)	55 (74.3)
Chemicals	83 (2.1)	214 (0.4)	5 (6.7)
Other poisons	8 (0.2)	169 (0.3)	
Parasites	1 (0.03)	1 (0.002)	
Fungi	0	0	
Unknown	723 (18.6)	8665 (17.5)	
Total	3877 (99.8)	49428 (99.9)	74 (99.9)

* (In % of total)

As to the number of food-borne deaths during the years from 1960 till 1969, 127 persons died, between 1970 and 1979 102, 1980 and 1989 56 and finally between 1990 and 1994 49 deaths were registered. That is during the last 5 years the number of deaths increased again in consequence of bacterial infections, mushroom and chemical poisons.

2. Causes of food-borne diseases. Table II shows the causes of food-borne diseases diagnosed between 1985 and 1994. Different bacteria caused 49.1% of the outbreaks in which 76.4% of the patients were involved. Bacteria occupied the first place in spite of fact that regular examinations have been only partly done for pathogenic *Escherichia coli* and *Listeria monocytogenes.*

In four outbreaks conveyed by the meat of wild pigs trichinellosis affected a total of 87 persons.

Virological and mycological examinations have not been performed. Chemicals, poisonous plants and other foods caused 32.0% of the outbreaks which included in 6.1% of the ill persons. In 18.6% of the outbreaks the cause remained unknown. This means that in the case of 17.5% of the patients laboratory examinations were negative. Seventy-

four persons died during these 10 years. In 14 cases (18.9%) bacteria, in 55 cases (74.3%) mushrooms and in 5 cases (6.8%) poisons were the cause of the death.

Summarizing these data it can be concluded that bacteria, among them salmonellae play the most important role in food-borne diseases. Mushrooms are the second most important causative agent. The role of other poisonous materials (methanol, herbicides, pesticides and others) is less frequent.

It is necessary to develop not only virological, parasitological and mycological diagnostics which practically do not exist in the laboratories dealing with foodstuffs but also bacteriological methods, too. The progress on the field of bacteriological diagnostics and epidemiological surveillance is also needed because the occurrence of epidemic listeriosis can be supposed [2] and illnesses caused by haemorrhagic *E. coli* were diagnosed [3] but the epidemiological background of these infections remained unknown. The Hungarian data are compared with the figures of other countries [4] in Table III.

The frequency of outbreaks caused by different food-borne bacterial pathogens is similar in the countries listed. Most frequently *Salmonella* species caused diseases. They are followed by *Campylobacter*, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus* and *E. coli* species. Data for *L. monocytogenes* are missing from Table III.

Data for foods causing food-borne diseases as well as that of producers have also been collected and studied to assess the epidemiological role of different foodstuffs as well as the ways of handling, preparation and cooking technics (Table IV).

Table III

Epidemiological importance of food-borne bacterial pathogens

Country	Period	Number or mean annual number of food-borne outbreaks					
		Salmonella spp.	*S. aureus*	*C. perfringens*	*Bacillus* spp.	*Campylobacter* spp.	*E. coli*
Canada	1985–86	50.5	20.5	16.0	14.5	9.5	5.0
England and Wales	1986–88	438.3	10.0	53.0	19.3	12.0	0.7
France	1991	477.0	48.0	25.0	1.0	0	0
Hungary	1985–94	145.3	15.6	4.3	5.0	0.9	0
Spain	1985	213.0	25.0	7.0	2.0	1.0	0
The Netherlands	1981–82	15.5	14.0	14.0	16.0	12.0	0
United States	1983–87	68.4	9.4	4.8	3.2	5.6	1.4

Table IV

Place of preparation of foods causing outbreaks in Hungary between 1985 and 1994

Place of preparation	Relative frequency in % of foodstuffs
Home	69.8–85.3
Restaurant	10.2–25.6
Industry	2.5– 9.8

Table V

Relative frequency of different kinds of foods involved in salmonellosis between 1981–1994 in Hungary

Foods	Years/Relative frequency in %		
	1981**	1984**	1994***
Eggs	5.2	27.5	*
Ice creams	2.6	1.5	
Creamy pastry	3.9	9.9	
Cold dishes	0.0	4.6	
Raw meats	10.4	9.9	
Sausages, brawn	52.0	22.1	
Boiled pastes	5.2	1.5	
Others	15.6	19.6	17.5
Pork		6.1	
Poultry and eggs		76.4	

* no data

** These data were presented by I. Straub

*** These data were presented by Gy. Bíró

It is characteristic in Hungary that in each years 69.8–85.3% of the foods involved in outbreaks were prepared at home, 10.2–25.6% of them in restaurants or other food serving establishments and only 2.5–9.8% of the foods were produced on industrial scale. It is quite clear that non-adequate kitchen technics, special habits and insufficient educational level of the population contribute to the occurrence of food-borne diseases.

Table VI

Results of microbiological control examinations of industrial food products in Hungary between 1986 and 1994

Year	Samples	Suitable samples	Acceptable samples under MSL*	Over MSL	Number of untolerated samples		
					Presence of facultative pathogenic germs**	Presence of salmonellae	In % of all samples
1986	190412	165268 (86.7)***	7182	12528	2266	3168 (1.6)	9.4
1987	219359	189800 (86.5)	9652	13257	1897	4688 (2.1)	9.0
1988	235307	202799 (86.1)	10714	15926	2363	3505 (1.4)	9.3
1989	226688	192588 (84.9)	12152	13736	3195	3727 (1.6)	9.1
1990	221506	192818 (87.0)	10550	11628	2421	4089 (1.8)	8.2
1991	197647	174216 (88.1)	9045	9215	2728	2443 (1.2)	7.2
1992	191239	172195 (90.0)	7808	7790	1313	2053 (1.0)	5.8
1993	193017	169218 (87.6)	6976	7037	1590	2070 (1.0)	5.5
1994	196284	178388 (90.8)	6889	7318	1456	2239 (1.1)	5.6
Total	1871459	1637290 (87.4)	80968	98435	19229	27982 (1.4)	7.7

* Maximum Spoilage Level

** *S. aureus, B. cereus, C. perfringens, Enterococcus faecalis, Pseudomonas aeruginosa*

*** in % of all samples

For example, mushrooms cause problems in each year because many persons gathering them cannot distinguish the poisonous species and fail to show their collection to an expert. They may offer their mushrooms collections as a present or for sale, at relatively cheap prices. On the other hand after slaughtering of a pig at home great part of its meat and meat products are stored for long periods under inadequate conditions. In consequence of this practice the number of botulismus cases increased. It is a tradition to organize big marriage parties mainly in villages. The number of invited guests may be one hundred or even more. However, kitchen conditions are frequently poor, preparation processes may last for days and storing conditions are inadequate. Proper hygienic handling, cooking and storing rules are frequently not known by the inhabitants.

The role of foods prepared in restaurants, other catering services and mainly in food industry is less. Perhaps professional persons keep the rules better. This fact may partly be the result of efforts of the Veterinary and Food Control Service and that of the Public Health Service and partly the result of economic and legal consequences of a food-borne outbreak.

It is interesting, how did the kinds of food influence salmonellosis during the last years. It can be seen in Table V that the importance of eggs and foodstuffs containing egg and chicken has increased. Besides these, new kinds of food – for example ready to eat

cold dishes – became also involved. The transmitter role of ice creams, different kinds of sausages and brawn decreased. That fact can be explained by the better microbiological quality of raw materials of these foods and with the more efficient heat treatment.

3. Quality of industrial food products. Tables VI, VII, VIII, IX and X present the results of bacteriological control examinations of industrial food products. The examinations were performed during 1988–1994 in the laboratories of the Veterinary and Food Control Service and in the National Food Investigation Institute. The data are presented in the Tables. In each year around 200 thousand samples were tested. The rate of the products suitable for human consumption was between 84.9% and 90.8%, 87.4% in average. The rate of the suitable products has slowly increased. Naturally, in the same time the rate of tolerated samples and that of different unaccepted ones have decreased. This is a favourable process. The rate of pathogenic agents, which practically represents that of salmonellae as these samples have rarely been tested for other pathogenic microorganisms was between 2.1% and 1.0%.

Table VII shows those kinds of food industry in which rate of untolerated products was higher during the control period. The highest rate of unacceptable samples because of the presence of spoilage germs was observed with products of the refrigerating industry. They were followed by dairy products, products of poultry industry, dried pastes of cereal industry and meat industry products.

Table VII

Rate of products unsuitable for human consumption because of spoilage microorganisms and salmonellae in % in Hungary between 1986 and 1994

Year	Industry				
	Poultry	Meat	Dairy	Refrigerating	Cereal
1986	19.5* (13.5)**	7.1 (1.3)	17.4 (0.02)	8.8 (0.8)	10.7 (0.0)
1987	16.0 (10.1)	7.0 (1.9)	18.0 (0.01)	7.8 (0.5)	10.4 (0.0)
1988	15.2 (7.6)	7.2 (1.3)	17.0 (0.1)	38.3 (0.3)	5.9 (0.7)
1989	17.5 (9.8)	7.1 (1.5)	16.4 (0.02)	23.5 (0.2)	12.9 (0.4)
1990	17.2 (6.8)	6.2 (1.8)	17.5 (0.02)	17.9 (1.3)	11.5 (1.1)
1991	9.9 (5.7)	5.8 (1.3)	14.6 (0.01)	12.4 (0.1)	7.9 (0.0)
1992	6.7 (5.0)	4.3 (0.9)	11.7 (0.0)	13.8 (0.6)	8.6 (0.0)
1993	7.0 (3.9)	4.2 (1.0)	10.7 (0.3)	10.7 (2.4)	7.4 (1.5)
1994	6.0 (3.9)	4.1 (0.8)	10.1 (0.5)	14.8 (0.5)	15.3 (0.8)

* Total untolerated

** Untolerated because of samonellae

Table VIII

Rate of untolerated samples of poultry industrial products in % in Hungary between 1986–1994

Year	Products			
	Poultry	Liquid egg	Egg powder	Meat products
1986	12.6* (12.5)**	6.4 (6.1)	29.1 (20.6)	0.0 (0.0)
1987	20.5 (19.9)	8.4 (7.7)	25.8 (18.7)	12.4 (0.0)
1988	24.4 (23.8)	47.4 (4.0)	21.9 (15.8)	7.5 (0.06)
1989	23.5 (22.7)	34.2 (2.2)	25.3 (21.2)	12.4 (0.6)
1990	21.7 (19.2)	54.6 (4.4)	28.6 (20.0)	1.1 (0.1)
1991	7.7 (6.3)	25.5 (1.7)	20.3 (16.4)	2.1 (0.0)
1992	9.7 (9.3)	15.1 (1.6)	16.8 (15.5)	2.0 (0.1)
1993	11.7 (9.7)	10.0 (4.5)	14.4 (7.1)	4.0 (0.1)
1994	12.3 (10.1)	23.1 (7.4)	27.2 (27.1)	5.4 (0.5)

* Total untolerated

** Untolerated because of salmonellae

On the basis of the presence of salmonellae or *S. aureus* the products of poultry industry proved to be the most contaminated. They were followed by dried pastes, meat industry products, refrigerating industry products, foodstuffs of cereal industry and finally by dairy products. It is interesting to mention that salmonellae could be isolated from flour, too. It may also be stated that the microbiological quality of different food industrial products has improved.

As mentioned before, practically only the presence of salmonellae has been officially controlled but it is known from other studies that *Listeria* and *S. aureus* species may also be in or on raw materials and food products of dairy, meat and confectionery industries [2, 5].

It is worthwhile to analyse the experience collected during examination of foodstuffs produced by both poultry and meat industries (Table VIII). Egg powders proved to be the most contaminated poultry products. As their selling is only possible when their salmonella-free quality is officially proved, they are of a very limited danger. The quality of liquid egg is also not convenient, but that product is used only for preparation of egg powder which is under strict control. Raw poultry meats mean the most serious problem because their surface may frequently be contaminated with salmonellae. So raw chickens can transmit and spread these bacteria. Nowadays raw hen eggs may have a similar role. Figures of the last column show that heat treatment of poultry meat products is not always efficient.

Table IX

Rate of untolerated samples of meat industrial products in % in Hungary between 1986–1994

Year	Products				
	Raw meat organs	Raw sausage	Dried smoked sausage	Heat treated meat products	Semipreserved products
1986	1.7* (1.3**	24.5 (5.3)	5.8 (1.1)	4.9 (0.01)	13.0 (0.2)
1987	1.8 (1.4)	12.8 (8.9)	10.5 (2.5)	5.1 (0.03)	4.5 (4.5)
1988	1.6 (1.5)	5.5 (0.3)	7.9 (1.1)	7.2 (0.004)	3.8 (0.01)
1989	1.9 (1.6)	7.5 (4.8)	9.5 (2.3)	7.1 (0.0)	4.4 (0.02)
1990	3.1 (2.0)	5.2 (4.7)	5.7 (1.0)	4.9 (0.02)	4.4 (0.1)
1991	1.3 (1.2)	2.5 (1.5)	6.5 (0.9)	6.1 (0.0)	4.4 (0.3)
1992	0.5 (0.5)	5.6 (3.9)	6.1 (1.1)	8.7 (0.03)	2.2 (0.06)
1993	0.7 (0.7)	6.1 (5.0)	7.8 (0.9)	4.9 (0.04)	2.5 (0.01)
1994	0.8 (0.5)	3.9 (3.3)	7.9 (0.8)	5.3 (0.03)	2.5 (0.1)

* Total untolerated

** Untolerated because of samonellae

The situation is somewhat different in cases of meat industrial products (Table IX). The quality of raw meats and organs is better than that of raw poultry. As microbial quality of samples of raw sausages is more often worse than that of the raw meats this fact emphasizes importance of ensuring of hygienic conditions during the preparation process. The effect of ripening can be seen in the 3rd column. Bacteriological control of sausages and salamies before selling is obligatory and only salmonella-free products can be sold. Figures of the last two columns direct the attention to the importance of keeping HACCP principles and of control of sufficient heat treatment in the factories.

4. Hygienic conditions in food industry. Hygienic situation in the plants is also regularly controlled. The results can be seen in the Table X.

In meat industrial factories there is no observable change as to the % of untolerated surface samples. In contrast with this it seems that the hygienic situation slowly improved in poultry industry. Different kinds of surveys verify that not only salmonellae but *L. monocytogenes* and *S. aureus* can be found in environmental samples from factories of meat, dairy and confectionery industries [2, 5].

It may be concluded that the general hygienic situation is not too bad in our food industry. Results of progress already appear, however further development is needed. Rules of free market and control efforts of veterinary and food control as well as public health authorities force producers to develop the quality of their products, to apply

principles of HACCP in processes of production and to strengthen own internal quality control. Further progress may be expected when the quality of food industrial raw materials – poultry, pigs and milk – will be better.

5. Tasks of future. In the light of the above-mentioned problems it seems obvious that the conditions of keeping and breeding of animals have to be improved. For example, it is necessary to establish salmonella-free breeding farms. Such efforts have been performed since the eighties [6], realizing the proposals of WHO [7–10].

Another important task is to perform more effective educational programmes in general for citizens and in special for industrial and catering employees in connection with proper handling, cooking and storing practices of foods as well as for farmers in relations to the rulers of hygienic husbandry. So far these efforts have not been effective enough.

Table X

Rate of unaccepted surface samples taken in factories of poultry and meat industries in Hungary between 1986 and 1994

Year	Meat	Poultry
1986	16.3* (1.3)**	11.6 (5.4)
1987	18.7 (1.4)	12.0 (6.5)
1988	18.4 (1.1)	11.6 (5.8)
1989	12.0 (1.6)	8.0 (2.7)
1990	9.4 (1.0)	10.3 (2.8)
1991	17.2 (1.0)	7.1 (1.9)
1992	10.5 (1.1)	5.1 (3.4)
1993	17.1 (0.8)	3.4 (2.2)
1994	13.8 (1.4)	4.7 (1.7)

* Total unaccepted in %

** Unaccepted because of salmonellae in %

6. Problems of notification and surveillance. It is well known that neither the total number of food-borne diseases nor that of enteric infections or, more general of infectious diseases can exactly be registered. Even in developed countries the notified cases represent only the peak of the iceberg.

Table XI contains the data for those infections which can be conveyed by foods and other ways, too. Great differences can be observed among the figures. The following explanations can be offered:

Table XI

Distribution of patients with food-borne and infectious diseases according to pathogenic microorganisms in Hungary between 1985 and 1994

Microorganisms	Number of patients with notified	
	food-borne diseases	infectious diseases
Salmonella	26119	135530
S. aureus	2112	*
C. botulinum	181	
Enterococcus	1268	
B. cereus	2067	
C. perfringens	2813	
Campylobacter	299	61309
Shigella	1917	25875
Aeromonas	583	
Y. enterocolica	9	3571
EPEC		3348
EIEC		3652
EHEC		4
F. tularensis		642
Brucella		36**
L. monocytogenes		145
C. burnetii		57
Entamoeba		905
Toxoplasma		1990
Trichinella	1	97
Taenia		202**
Hepatitis viruses		24356***
Total	37369	261719

* No data

** Imported cases are included

*** About 60–70% of the patients were infected by hepatitis A virus

Part of inhabitants, mainly those who are under-educated, even when having health (enteric) problems, are quite tolerant and do not contact with their G.P. The situation is the same in cases of patients with mild infection. Part of the affected persons remain symptom-free after the exposition. Without exact surveillance these cases are missing from the statistics.

When (diarrhoeal) patients go to their G.P. for treatment, a well-trained doctor wants to receive aetiological diagnosis and sends a (faecal) sample with the filled notification card to the local Public Health Service (P.H.S.). In such case the (diarrhoeal)

patient is notified on the list of infectious diseases but there will not be any note among patients of food-borne disease even the agent had been transmitted by foodstuff. In a sporadic "simple diarrhoea" an active epidemiological surveillance is only performed when a G.P. supposes salmonellosis, shigellosis, campylobacteriosis or *E. coli* O124 infection P.H.L. findings show the presence of any of these agents. In Hungary, an epidemiological surveillance has started in 1972 for salmonella, shigella and *E. coli* O124, and in 1990 for campylobacter. The importance of this work is verified by the next figures. In 1994, 19055 salmonellosis cases were reported and notified. During the survey of these patients and their environment 6751 further bacteriologically positive persons could be detected and on the basis of epidemiological findings the involvement of further 1396 bacteriologically negative persons could be verified.

When the patient's history indicates a food-borne disease and the G.P. notifies the P.H.S., the case will be surveyed and reported to the NIFN. That case will be listed among food-borne diseases. The role of the G.P. is very important in respect of both diagnosis and notification. The sporadic food-borne diseases are poorly reported and registered.

The other problem which has been mentioned before is that our food control laboratories in P.H.S. do not deal with all agents which may cause food-borne diseases. To get more exact picture about the agent-transmitter role of foods in infectious (diarrhoeal) diseases, the knowledge of doctors and inhabitants as well as the spectrum of diagnostic and surveillance activities have to be further developed.

There are more possibilities to determine the estimated country-wide number of food-borne diarrhoeal patients [11–13].

Table XII

Estimated number of food-borne diarrhoeal cases in Hungary

Mean annual number of registered patients	Way of calculation	Supposed number of patients (in mill.)
	With the help of factors	
4713	American: 5000 [11]	23.5
	Canadian: 350 [14]	1.6
	On the basis of the rate of inhabitants	
	American 1 [12]	0.9–3.2
	American 2 [12]	2.7–11.0
	Canadian 1 [12]	0.05–0.2
	Canadian 2 [12]	1.0–1.9

The ways and the results of estimations are summarized in Table XII. These estimations represent enormous numbers in comparison with the registered figures. An exact importance of food-borne diseases may only be estimated if the most realistic number of diarrhoeal patients will be known and the source of their infection will be cleared up. Some of our experts believe that to get a more realistic picture, the registered number of patients should be multiplied by 10. It would be necessary to perform a more effective surveillance programme which deals with both the sporadic enteric and food-borne cases.

7. Costs of food-borne diseases. It is well known that food-borne diseases have economic, social and legal consequences [13]. As to the economic question the value of total losses due to food-borne diseases can be calculated. For a real calculation it is necessary to know the number and data of patients, length and way of treatment of their disease, costs of hospitalization and that of medical services in case of outpatients, number and value of lost working hours, value of losses in cases of premature deaths, economic losses of food producers, value of penalties, that of compensations and so on.

In the USA the estimated losses of food-borne diseases are estimated as 5–17 billion USDs per year [12]. In Canada the same value was supposed to be about 1.33 billion USDs in 1985 [14].

At present only one but not total calculation is known in Hungary. It was published by Bíró [15]. On the basis of his calculation the estimated losses in consequence of only salmonellosis in 1989 were more than 200 million Fts which were roughly equal with 30 million USDs. The value of the real total losses, may be much higher. Different experts state on the basis of cost-benefit calculations that it is worthwhile to spend some more money to prevent food-borne illnesses, since the total costs of manifest diseases are higher than that of prevention [14, 16].

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REFERENCES

1. Nikodémusz,I., Novotny,T., Bouquet,D., Tarján,R.: Über die Ätiologie der Lebensmittelvergiftungen in Ungarn. Zbl Bakt I Abt Orig **203**, 137 (1967).
2. Ralovich,B., Kovács Domján,H.: Experience of Listeriosis in Hungary. Proceedings of the XIIth International Symposium on Problems of Listeriosis. Perth, Western Australia, 2–6 October 1995. Promaco Conventions Pty. Ltd. Perth, Western Australia, p. 483. Accepted for publication in Acta Veterinaria Hungarica.
3. Czirók,É.: Personal communication
4. D Aoust,J.Y.: Salmonella and the international food trade. Int J Food Microbiol **24**, 11 (1994).
5. Németh,A., Gasparik Reichardt,J., Ralovich,B.: Presence of pathogenic bacteria in slaughterhouses and meat processing plants (in Hungarian). A Hús **2**(2), 96 (1992).

6. Nagy,B., Kostyák,Z., Bitay,G., Szmollény,G., Kovács,S.: Most important serotype of Salmonella and their prevention in Hungarian poultry products. 9th World Congress of Food Science and Technology, Budapest, Hungary, July 3–August 4 1995. Abstracts, Vol. II. p. 161. P466
7. WHO, VPH Unit.: Report of WHO Consultation on Epidemiology and Control of Campylobacteriosis in Animals and Humans. Bilthoven, The Netherlands. 25–27 April 1994. WHO/CDS/VPH/94. 135. pp. 1–184.
8. WHO, VPH Unit.: Guidelines on Cleaning, Disinfection and Vector Control in Salmonella Infected Poultry Flocks – With particular reference to *S. enteritidis*, Bakum/Vechta, Germany, 7–11 June 1993. WHO/Zoon./94. 172. pp. 1–33.
9. WHO, VPH Unit.: Guidelines on Detection and Monitoring of Salmonella Infected Poultry Flocks with Particular Reference to *S. enteritidis*. In Wray,C., Davies,R.H. (eds): Graz, Austria, 11–15 April 1994. WHO/Zoon./94. 173. pp. 1–48.
10. WHO-FEDESTA-FEP Workshop: On Competitive Exclusion, Vaccination and Antimicrobials in Salmonella Control in Poultry. Obernkirchen, Germany, 29 August–1 September 1994. WHO/CDS/VPH/94. 134. pp. 1–20.
11. Kvenberg,J.E., Archer,D.L.: Economic impact of colonization control on food-borne disease. Food Technol 41(7), 77, 80, 81, 98 (1987).
12. Institute of Food Technologists Expert Panel of Food Safety and Nutrition: Bacteria associated with foodborne diseases. Food Technol **42**, 181 (1988).
13. Todd,E.C.D.: Costs of acute bacterial foodborne diseases in Canada and the United States. Int J Food Microbiol **9**, 313 (1989a).
14. Todd,E.C.D.: Preliminary estimates of costs of foodborne diseases in Canada and costs to reduce salmonellosis. J Food Prot **52**, 586 (1989b).
15. Bíró,Gy.: Economic consequences of food-borne diseases caused by microbiologically unsuitable foods (in Hungarian). Élelmezési Ipar **45**, 85 (1991).
16. Notermans,S.: Emerging foodborne diseases with special reference to *S. enteritidis*. 9th World Congress of Food Science and Technology, Budapest, Hungary, July 30–August 4 1995. Abstracts, Vol. I. p. 102. L247

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THE SPECIES COMPOSITION OF OLIGOTROPHIC BACTERIAL COMMUNITIES OF LAKE BALATON – A NUMERICAL ANALYSIS

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From among the 565 aerobic and facultatively anaerobic, oligotrophic bacterial isolates obtained from the open water region of the Balatonfüred-bay, 58 representative strains were selected for detailed taxonomic-physiological studies and numerical analyses as well as simultaneous comparisons with authentic bacterial strains isolated earlier by Langó (1987) from different water habitats of the Lake Balaton. On the basis of 186 ceded differential diagnostic characteristics involved in these analyses and comparisons, the representative strains were classified into 6 larger similarity groups (I. *Micrococcus* spp.; II. *Arthrobacter* "*globiformis*"; III. *Acinetobacter calcoaceticus*; IV. *Pseudomonas* spp. I.; V. *Pseudomonas* spp. II.; VI. *Aeromonas* spp.) which were partly further divided into smaller subgroups of species level (IV.a. *Pseudomonas palleronii*; V.a. *Pseudomonas pseudoalcaligenes*; V.b. *Pseudomonas acidovorans*; V.c. *Pseudomonas fluorescens*; VI.a. *Aeromonas caviae*; VI.b. *Aeromonas hydrophila*).

The Lake Balaton in Hungary belongs to the most intensively researched large water bodies of the European Continent. The results of many studies clearly show that in its open water the total number and the produced biomass of bacteria as well as algae have, during the last decades, increasing tendencies. While the waters of the Western basin, especially those of the Keszthely-bay are already strongly contaminated and rich in bacteria, the quality of the waters of the Eastern basin changed only slowly and showed still at the beginning of the 80th years only mezotrophic values [1]. The composition of bacterial communities in different water and mud regions of the Lake Balaton were investigated also qualitatively by several workers, first by Haranghy in 1941 [2]. Szabó (1978–79) detected a predominance of the genus *Micromonospora* in the bottom mud [3].Other *Actinomycetes* (mostly *Streptomycetes*) occurring in the water and the mud of the Lake Balaton and the River Zala were examined by Suman and Szabó [4], furthermore by Farkas [5]. Analysing the bacterial populations of the open water Kotsis et

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al. [6] found that the most common members of these belonged to the genus *Micrococcus*. The complex *Bacillus* populations of the terrestric soils and the littoral detritus in the northern littoral region were thoroughly studied by Langó [7]. In contrast to many published data, however, our knowledge on the composition and biochemical activities of the very complex microbial biotas of the Lake Balaton, which can be considered as a natural and selected model by many workers for the large shallow waters of our Continent, is still very meagre.

In this work I am trying to give a relatively deeper insight into the species composition of the up till now scarcely studied but widely distributed oligotrophic bacterial communities of the Balaton's open water region.

Materials and methods

Water samples for isolating bacteria were collected from the open water of the Balatonfüred-bay two times at 3–3 sampling sites, first on 6th of April in 1988 (water temperature: 10.5 °C), second time on 5th of July in 1988 (water temperature 23 °C). Water samples taken aseptically from under the water surface with sterile pipettes were cooled and transported within 24 h to the laboratory. Plating was carried out on simple water agar plates prepared with Balaton water. After incubation for 7 or 10 days at 28 °C we nonselectively isolated a large number of colonies from the plates. The obtained isolates were separated into distinct similarity groups partly according to the site and time of sampling, partly on the basis of certain selected macromorphological and cultural diagnostic properties. From every individual similarity group, proportionally to the number of isolates, representative strains were selected for further detailed investigations, all together 58. In order to obtain pure isolates, representative strains were then subjected to repeated distributions and reisolations on nutrient agar plates. Into the comparative taxonomic studies we involved also 5 authentic strains isolated earlier from the Lake Balaton and identified by Langó [7]: *Aeromonas hydrophila* (T5KV/72), *Pseudomonas alcaligenes* (T2-V/1), *Bacillus subtilis* (TKÖ/4) *Micrococcus luteus* (MIII-2/2) and *Micrococcus roseus* (xl/vp). All of our representative and also the authentic strains were simultaneously tested for the following morphological-cultural properties: size, colour, shape, consistency, surface and elevation of the colonies, the cells' size and shape from 24 and 48 h cultures, their Gram staining and ability to produce endospores. Other characteristics determined were as follows. Catalase activity: 48 h slant agar cultures treated with 10% H_2O_2; the oxidase test was performed with 1% solution of p-aminodimethylanilin-monohydrochloride; NaCl tolerancy was detected in peptone water with 5, 7, 9 and 12% NaCl and pH tolerancy in peptone water containing 5 g peptone and adjusted to pH 4, 5, 7 and 9; temperature range of growth (4, 28, 37, 45 and 50 °C) was tested on nutrient agar slants containing 5 g peptone and 5 g meat extract; oxidative and fermentative utilization of glucose, fructose, lactose, xylose and salicin was detected according to Cowan and Steel [8]. The hydrolysis of starch, production of indole, phosphatase activity, reduction of nitrate, lipolytic activities (Tween-20, -40, -60 and -80), urease activity, decomposition of tyrosine, and deamination of phenylalanine were performed using the methods of Cowan and Steel [8]. Utilization of citrate as sole source of carbon was tested on Simmons' citrate medium. For studying the susceptibility to antibacterial substances on nutrient plates Resistest discs (Human Ltd., Budapest)were used. The below listed antibiotics were tested: ampicillin (20 µg), carbenicillin (100 µg), chloramphenicol (30 µg), chlortetracycline (30 µg) clindamycin (10 µg), erythromycin (10 µg), gentamicin (20 µg), kanamycin (30 µg), lincomycin (10 µg), neomycin (30 µg), nalidix acid (30 µg), nitrofurantoin (300 µg), nystatin (100 µg), oleandomycin (30 µg), oxacillin (10 µg) oxytetracycline (30 µg), penicillin (30 NE), spiramycin (30 µg), co-trimoxazole (25 µg), sulphadimidine

(400 μg), streptomycin (30 μg), tetracycline (20 μg), vancomycin (50 μg). Utilization and acid production from carbohydrates were studied using the following substrates: xylose, arabinose, mannitol, sorbose, inositol, inulin, mannose, glycerol, trehalose, raffinose, saccharose, sorbitol, levulose, galactose, melibiose and lactose. Hydrolysis of arginine, aesculin and gelatin, furthermore the decomposition of RNA and DNA were examined by the method of Cowan and Steel [8]. β-Galactosidase activity was tested after Lowe [9]. Lysosyme-resistence was tested in nutrient broth containing 0.001% lysosyme [10]. Production of H_2S was detected in peptone water. The capability of using methylene blue as terminal electron acceptor was tested in nutrient broth containing tryptone and 0.1% methylene blue. The ability to utilize salts of organic acids (acetate, pyruvate, gluconate, salicylate, benzoate and lactate) as sole sources of carbon was detected by observing the intensity of growth and the pH shifting into alcalic direction. Utilization of different amino acids (cysteine, asparagine, glycine, arginine, alanine and methionine) as C- and N-sources as well as KNO_3 as sole source of nitrogen were tested on slant agar media containing a total of 280 mg/l [11] nitrogen. To study the growth on a synthetic medium, glucose as carbon and $(NH_4)_2HPO_4$ as nitrogen source were added. To detect the utilization of $Ca_3(PO_4)_2$ plates containing an inorganic nitrogen source, 1.8 g/l each of $Ca_3(PO_4)_2$, glycerine and glucose were employed. The resistance to wet heat treatment was studied with cultures of bacteria cultivated in peptone and Balaton water. Antibiotic activity against strains of *Escherichia coli* and *Bacillus subtilis* was studied by the hole-test method using cell-free filtrates of 5 days old broth cultures of our strains. Mobility was checked by observation of the living cells in phase-contrast illumination and in their preparations for presence of cilia by electron microscope.

Numerical analysis based on 186 coded characteristics was performed with our 58 representative (2 strains died out during the investigation) and the 5 authentic bacterial strains obtained from Langó, according to the principles of Adansonian taxonomy. The similarity index of Sokal and Michener [18] was calculated between all of the compared pairs of strains. After comparisons the computer clustered the strains into similarity groups.

Results and discussion

The phenetic relationships of the 58 representative bacterial strains isolated and selected by us and of the 5 authentic strains used for comparisons are demonstrated on Fig. 1. In the last analysis the computer clustered the 63 bacterial strains together on 75.4% level of similarity (similarity index: 0.75) into one single and large group.

With 48 strains the computer created 6 separate (I–VI) similarity groups on the dendrogram, while the others were placed in intergroup positions. As can be seen the numbers of strains which were placed into the individual groups are very different. But the size of a group can give information about the relative frequency of occurrence of its members at their sampling habitats, because the selection of the 58 representative strains from among the similar isolates happened proportionally. For the taxonomic identification of our bacterial strains at genus and species levels we used partly the key characteristics of Bergey's Manual (1984–86), partly the numerical values of similarities among our and the authentic strains.

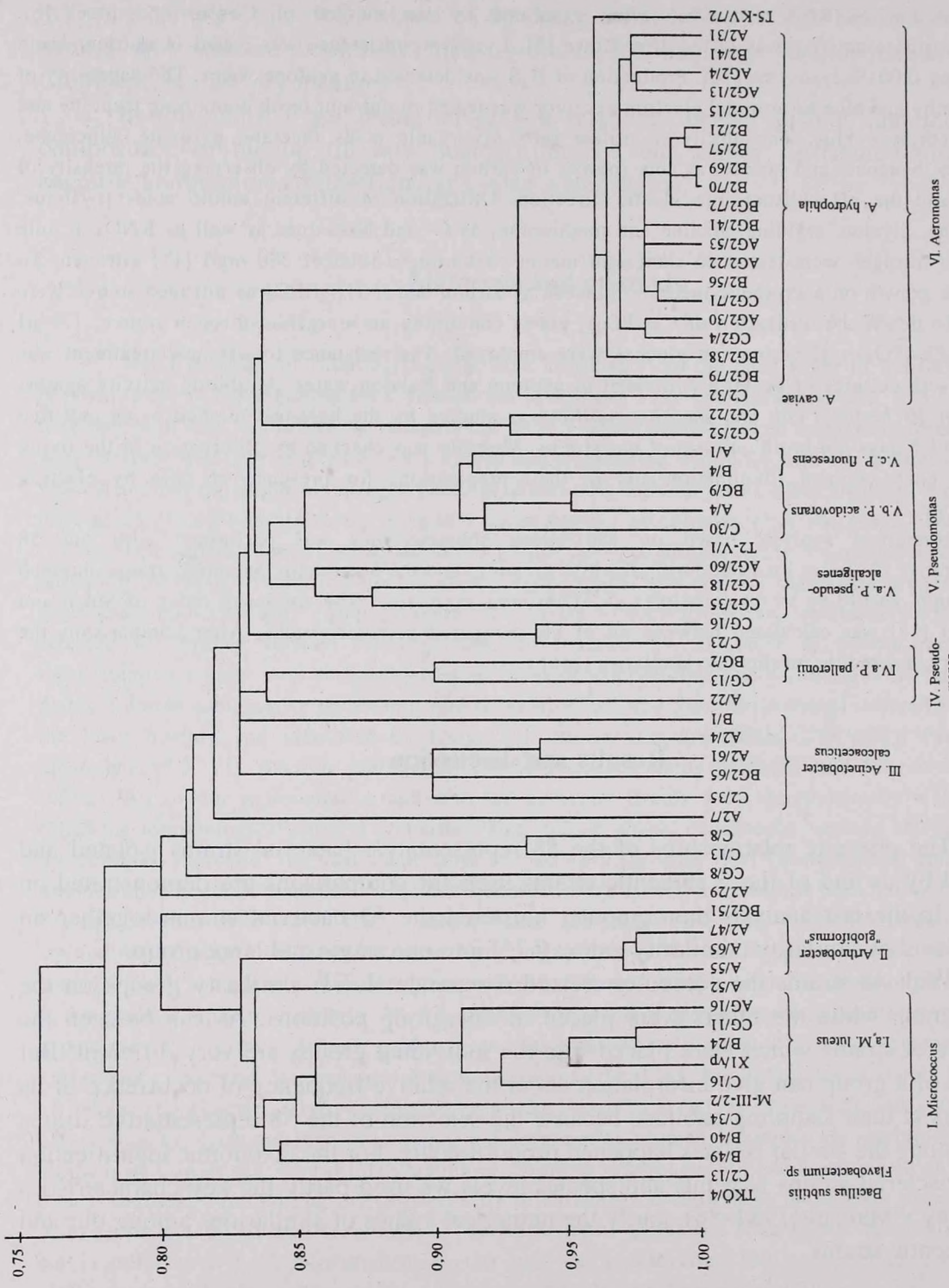

Fig. 1. Dendrogram showing the phenetic relationships (similarity index: 0–1) between 5 authentic and 58 representative bacterial strains isolated from the Lake Balaton

Into group I the computer placed the typical representatives of the genus *Micrococcus*. Within this group, at 91.2% level of similarity, a subgroup of *M. luteus* has been separated. This subgroup contains also the authentic strain of *M. luteus* marked x1/vp. The members of this subgroup formed light yellow colonies. They grew well in the presence of 5 and 7% NaCl but showed only slow growth with 10% NaCl. They produced no indole. Their citrate utilization proved to be negative and they reduced nitrate weakly. The computer put the authentic strain of *M. roseus* marked M-III-2/2 into group I as well. It is to be noted that in contrast to the data in the literature, our *Micrococcus* strains isolated from Lake Balaton, exerted positive phosphatase activity. This ability can be important for their survival in the Balaton water, as it helps to utilize biologically hardly mobilizable $Ca_3(PO_4)_2$ pools.

Into group II at 97.3% similarity level 3 Gram-positive or -variable, catalase positive and oxidase negative strains were placed. These 3 strains and a further strain marked A/52 and placed near to the former show the key diagnostic properties of the genus *Arthrobacter* [13]. During their life-cycle they revealed typical cell morphological changes: in their 24 h cultures they can develop as regular rods, but after 48 h they become spherical or transform into coccoid cells similarly to the members of the *Arthrobacter "globiformis"* group.

Four representative strains identified as typical members of *Acinetobacter calcoaceticus* [14] were placed into group III at 90.1% similarity level.

Into groups IV and V the computer placed 14 strains belonging to diverse species within the genus *Pseudomonas* [15]. In group IV one and inside group V three little subgroups were separated. Into group V in intergroup position also the authentic strain T2-V/1 of *Pseudomonas alcaligenes* has been incorporated.

Two non-pigmented strains of *Pseudomonas palleronii* were placed into subgroup IVa containing only one typical *P. palleronii* strain. The correctness of their identification was confirmed also by the fact that we could show the presence of subpolar flagellas on the cells of both non-pigmented strains. In subgroup Va Balaton strains of the species *Pseudomonas pseudoalcaligenes* were grouped together. Subgroup Vb contains 3 typical strains of *Pseudomonas acidovorans* gathered at 91.7% level of similarity. The validity of this grouping has been corroborated by their multitrichous flagellation, their negative starch hydrolysis, PHB-accumulation, positive fructose utilization and their positive lipolytic activity detected at using Tween 80 as substrate. In contrast to data in the literature, our Balaton-isolates proved to be able to utilize both glucose and arabinose. In subgroup Vc two strains of *Pseudomonas fluorescens* were clustered at 91.1% level of similarity together. Typically of this species, they produced fluorescent pigments on King's agar medium, their arginine hydrolysis and oxidase reaction were positive but starch was not decomposed by them.

Those 20 strains which were grouped together on the right side of the dendrogram at 95.6% level of similarity, we identified as members of *Aeromonas hydrophila* [16]. An authentic strain of this species, strain No. T5-KV/72 was also placed into this group. It is worthy of note, that *A. hydrophila* strains could be isolated only from the summer samples. The yearly population dynamics of *A. hydrophila* in the Lake Balaton exhibit a characteristic seasonal cycle: from the end of autumn their number strongly decreases, but

in spring ,with increasing water temperature they appear again and can reproduce quickly. Our findings agree with the data of Kaper et al. [17].

The computer incorporated into this big group at 92.9% level of similarity and in a border position also the strain C2/32, which we identified as a member of the species *Aeromonas caviae.*

REFERENCES

1. Tóth,G.,L., Padisák,J.: A Balaton tihanyi térségében végzett algológiai és bakteriológiai tanulmányok eredményeinek értékelése a zooplankton táplákozása szempontjából. A Balatonkutatás újabb eredményei **II**, 105 (1982).
2. Haranghy,L.: Adatok a Balaton bakteriológiájához. Magy Biol Kut Munk **13**, 57 (1941).
3. Szabó,Zs.: Possibilities of taxonomic differentiation between *Micromonospora chalcea* and gentamycin-producing *Micromonospora* species. Annual Univ Sci Budapest de R Eötvös Sect Biol **20–21**, 163 (1978-79)
4. Suman,Y., Szabó,I.M.: A Zala folyó alsó szakasza fenéküledékének baktériumnépessége. MTA Biol Oszt Közl **25**, 179 (1982).
5. Farkas,I.: Comparative investigations on reed-surface epiphytic bacterial populations in different regions of Lake Balaton. Annales Universitatis Scienfiarium Budapestinensis. Sectio Biologica. Tomus XXIV–XXVI. 1984.
6. Kotsis,I., Fehér,E., Cziráki,R.: A Balaton nyílt vize baktériumnépességének összetételéről. MTA Biol Oszt Közl 25, 245 (1982).
7. Langó,Zs.: Studies on the *Bacillus*-population of the NW-littoral zone of the Lake Balaton (Hungary). Arch Hydrobiol **108**, 449 (1987).
8. Cowan,S.T., Steel,K.J.: Manual for identification of medical bacteria. Cambridge, Univ. Press. 1974.
9. Lowe,G.H.: The rapid detection of lactose fermentation in Paracolon organisms by the demonstration of β-D galactosidase. J Med Lab Technol **19**, 21 (1962).
10. Gordon,R.E., Hayness,W.C., Pang,C.H.-N.: The Genus *Bacillus.* Agricultural handbook, US Dept. of Agriculture, Washington. 1973. p.427.
11. Szabó,I.M.: Microbial Communities in a Forest Rendzina Ecosystem. The pattern of microbial communities. Akadémiai Kiadó, Budapest, 1974.
12. Kocur,M.: The Genus *Micrococcus.* In Buchanan,R.E., Gibbons,N.E. (eds): Bergey's Manual of Determinative Bacteriology. 9th Edition, Williams and Wilkins Co., Baltimore, 1986.
13. Keddie,MR., Collins,M.D., Jones,D.: The Genus *Arthrobacter.* In Buchanan,R.E., Gibbons,N.E. (eds): Bergey's Manual of Determinative Bacteriology. 9th Edition, Williams and Wilkins Co., Baltimore, 1986.
14. Juni,E.: The Genus *Acinetobacter.* In Buchanan,R.E., Gibbons,N.E. (eds): Bergey's Manual of Determinative Bacteriology. 9th Edition, Williams and Wilkins Co., Baltimore, 1986.
15. Palleronii,J.N.: The Genus *Pseudomonas.* In Buchanan,R.E., Gibbons,N.E. (eds): Bergey's Manual of Determinative Bacteriology. 9th Edition, Williams and Wilkins Co., Baltimore, 1986.
16. Popoff,M.: The Genus *Aeromonas.* In Buchanan,R.E., Gibbons,N.E. (eds): Bergey's Manual of Determinative Bacteriology. 9th Edition, Williams and Wilkins Co., Baltimore, 1986.
17. Kaper,J.B., Lockman,H., Colwell,L.L.: *Aeromonas hydrophila*: ecology and toxigenicity of isolates from an estuary. J Appl Bact **50**, 359 (1981).

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DETECTION OF BULLOUS PEMPHIGOID ANTIBODIES BY MEANS OF SYNTHETIC PEPTIDES AS ANTIGENIC EPITOPES

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In an earlier study, the circulating autoantibodies in patients with bullous pemphigoid (BP) were demonstrated in higher frequencies by means of an immunoblot technique then via indirect immunofluorescence. In the present work with the help of Peptide Structure software, two matched antigenic epitopes were chosen and synthesized. The sera of 34 patients with BP were investigated in parallel by an immunoblot technique using a human epidermal extract, and by an ELISA technique with synthetic peptides. The sera of 16 healthy persons and 10 patients with other bullous diseases served as controls. Among the 34 patients with BP, 23 sera proved positive for at least one synthetic peptide. Positive reactions with the major BP antigen (230 kD) were found in 22 patients by immunoblot technique and in 16 patients by the ELISA technique with the synthetic peptide, whereas with the minor BP antigen (180 kD), positive reactions were found in 9 patients by the immunoblot technique and 18 patients by the ELISA technique using the synthetic antigenic epitope of the minor BP antigen. All control sera were negative for both antigens in ELISA investigations. In 3 patients with pemphigus vulgaris, characteristic bands against pemphigus vulgaris antigen were identified by means of the immunoblot technique, but all other cases were negative. It is suggested that the development of this technique may lead to an opportunity for the rapid and simple diagnosis of BP.

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Bullous pemphigoid (BP) is a subepidermal blistering disease of elderly people, but the condition can occur in the under-forties and occasionally in children, too. Direct immunofluorescence (DIF) studies revealed preformed Ig and C3 linear deposition along the basement membrane zone (BMZ). About three-quarters of these patients have a circulating antibody to the BMZ. The BP antigen appears to exhibit molecular heterogeneity. There is a major 230 kD and a minor 180 kD peptide antigen. Through a use of patients' autoantibodies, complementary DNAs (cDNAs) of both BP antigens have been isolated, and the amino acid sequences for the human 230 kD and 180 kD autoantigens were deduced [1, 2].

We have now addressed the question of whether the demonstration of circulating antibodies against the minor (180 kD) and major (230 kD) BP antigens is possible by means of an ELISA technique with antigenic synthetic peptides.

Material and methods

With the help of Peptide Structure software, several antigenic epitopes were chosen and synthesized. The predicted epitopic peptides were synthesized by solid-phase technique utilizing 'Boc chemistry [3]. The peptide chain was elongated on an MBHA (p-methyl-benzhydril-amine) resin and the syntheses were carried out using an ABI 430A automatic machine with certain minor modifications on the standard protocol. The completed peptide resins were treated with liquid HF and the resulted free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized. The crude peptides were purified by semipreparative reverse phase HPLC. The purified peptides were characterized by am[no acid composition and mass spectrometry. Peptide purifies were above 95% (HPLC and capillary electrophoresis). According to previous investigations, two of the peptides gave positive reactions with BP patients' sera by means of the ELISA technique. We therefore further investigated these two synthetic peptides. One of them represented the 230 kD, and the other the 180 kD antigen. Thirty-four patients with proved BP were investigated (17 females and 17 males, aged 43–89 years, mean 72 years). The diagnosis was established on the basis of the clinical picture and by histology, as well as by DIF investigations. In all patients, the sera were examined by indirect immunofluorescence (IIF) and by immunoblotting too, as described earlier [4].

The patients' sera were investigated by ELISA technique to determine antibodies against the antigenic epitopes representing either the 230 kD or the 180 kD antigen. For this technique, 0.1 µg/100 µl concentrations of antigen and 1:100 dilutions of sera were used. Our earlier studies indicated a positive result when OD>X+2SD (OD = optical density, X = mean OD of control subjects, and SD = standard deviation of control OD).

Sera from 16 blood bank donors (8 females and 8 males, aged 25–53 years, mean 33 years), and 10 patients (7 females and 3 males, aged 39–83 years, mean 49 years) with other bullous diseases (drug allergy, porphyria cutanea tarda and 3 patients with pemphigus vulgaris) served as controls.

Results

All control sera were negative for both antigens in the ELISA investigations. In 3 patients with pemphigus vulgaris, characteristic bands against pemphigus vulgaris antigen (130 kD and 80 kD) were identified by means of the immunoblot technique, but all the other cases were negative.

Among the 34 patients with BP, 23 sera proved positive for at least one synthetic peptide. Positive reactions with the major BP antigen were found in 22 patients by the immunoblot technique and in 16 patients by the ELISA technique with synthetic peptide, with the minor BP antigen, positive reactions were found in 9 patients by the immunoblot technique and in 18 patients by the ELISA technique when the synthetic antigenic epitope of the minor antigen was used.

The summarized results are to be seen in Tables I, II, and III. Statistical differences between the various positive and negative results were not found by the Fischer exact test.

Table I

Correlation between indirect immunofluorescence (IIF), immunoblotting (IB) and ELISA investigations of sera of patients with bullous pemphigoid (positive by direct immunofluorescence technique)

Bullous pemphigoid (n=34)	ELISA only 230 kD positive	ELISA only 180 kD positive	ELISA both 230 and 180 kD positive	ELISA both 230 and 180 kD negative
*IB+, IIF+ (n=8)	0	1	3	4
IB+, IIF– (n=13)	4	1	6	2
IB–, IIF+ (n=1)	0	0	0	1
IB–, IIF– (n=12)	1	5	2	4
DIF-positive (n=34)	5	7	11	11

*IB+ = immunoblot : positive result
IIF– = indirect immunofluorescence : negative result

Discussion

About three quarters of patients with BP have circulating antibodies to the basement membrane demonstrable by IIF. In our study, immunoblotting proved to be more sensitive than IIF for the identification of different antibodies. Eight out of 34 (22%) sera of patients with BP gave positivity by means of IIF and 21 of 34 (61%) sera by means of immunoblotting assay. Our results are in accordance with the findings of Bernard et al. [5]. Earlier investigations [6, 7] demonstrated either that 80% of patients with BP have circulating IgG that binds to fusion proteins encoding for both the amino and carboxyl terminal regions of the putative alpha helical coiled-coil rod of the 230 kD cDNA or that 53% of patients with BP have circulating IgG by means of an ELISA technique with a segment of a human cDNA encoding a 42-amino-acid stretch of the BP 180 kD antigen (GST-S 1).

Table II

Summarized results of correlation between ELISA and immunoblotting investigations

	ELISA positive	ELISA negative
Blot positive	15	6
Blot negative	8	5
DIF positive	23	11

Table III

Correlation of the results of ELISA and immunoblotting investigations according to different antigens

Bullous pemphigoid (n=34)	230 kD BP antigen	180 kD BP antigen
ELISA positive	17	19
Blot positive	22	9

In our investigations with synthetic antigenic epitopes, the ELISA technique revealed serum positivity in 67.6% of the patients with BP. Our results suggest that the epitope of the 180 kD antigen gave much higher positivity than that of the 230 kD protein

by ELISA technique respective to the immunoblotting method. Therefore, to increase the usefulness of the 230 kD antigen as a diagnostic tool, other epitopes within the protein should be learned.

We consider that this kind of investigation offers a new possibility to determine antibodies in patients with BP. By using a mixture of these synthetic antigenic epitopes it is possible to diagnose patients with BP in a relative simple way by means of an ELISA technique, which is much cheaper, and a highly equipped laboratory is not necessary.

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REFERENCES

1. Sawamura,D., Li,K., Chu,M-L., Uitto,J.: Human bullous pemphigoid antigen (BPAG1). J Biol Chem **266**, 17784 (1991).
2. Giudice,G.J., Emery,D.J., Diaz,L.A.: Cloning and primary structural analysis of the bullous pemphigoid autoantigen, BP180. J Invest Dermatol **99**, 243 (1992).
3. Merrifield,R.B.J.: The synthesis of a tetrapeptide. Solid phase peptide synthesis. J Amer Chem Soc **85**, 2149 (1963).
4. Kiss,M., Husz,S., Dobozy,A.: Identification of different circulating autoantibodies in patients with bullous pemphigoid and pemphigus vulgaris by means of immunoblotting. Acta Microbiol Immunol Hung (accepted for publication)
5. Bernard,P., Didierjean,L., Denis,F., Saurat,J-H., Bonnetblanc,J-M.: Heterogeneous bullous pemphigoid antibodies: Detection and characterization by immunoblotting when absent by indirect immunofluorescence. J Invest Dermatol **92**, 171 (1989).
6. Rico,M.J., Streilein,R.D., Hall,R.P.: Peripheral blood mononuclear cells from patients with bullous pemphigoid have an increased frequency of response to synthetic peptides encoded by BPAG1. J Invest Dermatol **103**, 73 (1994).
7. Giudice,G.J., Wilske,K.C., Anhalt,G.J., Fairley,J.A., Taylor,A.F., Emery,D.J., Hoffman,R.G., Diaz,L.A.: Development of an ELISA to detect anti-BP180 autoantibodies in bullous pemphigoid and herpes gestationis. J Invest Dermatol **102**, 878 (1994).

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LARGE SCALE CULTIVATION OF AN ANTI-D (IgM) ANTIBODY PRODUCING HUMAN/MOUSE HETEROHYBRIDOMA

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A human/mouse heterohybridoma cell line secreting anti-Rh(D) antibody was established by the fusion of Epstein–Barr Virus (EBV) transformed human B lymphocytes and human/mouse heteromyelomas. During scaling-up the cell line was adapted to suspension culture, then medium composition was gradually altered into a more economical one. The adapted cell line has kept its multiplication and antibody secreting characteristics in the new medium. Parameters of large scale batch cultivation in bioreactors were optimized in working volume of 3.6 litre and production was carried out under these parameters in working volume of 24 litre. Optimized parameters were the follows: (i) the cell line is highly sensitive to dissolved oxygen (DO) level, its optimal DO is 10%; (ii) optimal inoculum cell count is 3×10^5 cell/ml; (iii) Marine-blade impeller was chosen for supporting the requested oxygen transfer. Using the optimized parameters HUMAN Co. Ltd. produces the Monoclonal Anti-D (IgM) reagent, which has been registered in 1995 in Hungary and now it is on the market.

Identification of the Rh(D) antigen has a particular importance in blood group serology. Traditional reagents routinely used for this purpose are made of human serum which is getting less and less available. Reagents based on the appropriate monoclonal antibodies are able to substitute the human serum based ones. For this reason consumption of monoclonal antibodies which can be produced in vitro in large scale is increasing all over the world.

In biotechnological industry as well as in research laboratories monoclonal antibodies are produced by "immortalized" immune cells (lymphocytes) of different mammalians including humans. Immortalization is usually achieved by in vitro infection of lymphocytes by certain viruses (e.g. Epstein–Barr Virus, EBV) or transfection by oncogens and/or by fusion with different myeloma cell lines [1, 2].

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Production of large amounts of monoclonal antibodies means that the volume of cultivation of these mammalian cells grown in laboratory scale have to be enlarged up to several hundred times (up to 5–500 litres). This huge augmentation of volume can be realized only step by step, applying special techniques and adaptation strategies. It is necessary because mammalian cells are extremely sensitive to environmental changes like variation of oxygenation, medium composition, altered diffusion of nutrients and metabolites, decreased concentration of some self-secreted factors and the hydrodynamic shear rate caused by the agitation of the whole culture [3]. During upscaling lucrativity of the process also should be considered. Scaling up and optimization of cultivation parameters have to be carried out individually with each individual cell line [4, 5].

The most common techniques are based on cultivation in stirred tank bioreactors. These bioreactors were originally developed from fermentors used for conventional bacteria or yeast fermentation considering the special requirements of mammalian cells [6]. The biggest difference between bacterial and mammalian bioreactors is usually in their aeration–agitation system. While bacteria and yeast cultures need very high (several hundred rpm) stirring speed for the maximal oxygenation at very high cell densities (above the range of 10^8 cells/ml), mammalian cells do not survive such a high dissolved oxygen (DO) level and agitation speed. Suspended mammalian cell cultures require precisely controlled DO level between 0–50% and gentle agitation system which keeps the cells in suspension without damaging their sensitive membrane. These bioreactors can be operated in batch, fed-batch, perfusion or diafiltration mode [7].

Materials and methods

Cell line. We have established a human/mouse heterohybridoma cell line by the fusion of EBV-transformed peripheral human B lymphocytes with C73H11 human/mouse heteromyelomas [2]. The obtained cell line marked as 1C2 secretes monoclonal antibody of IgM isotype which is a suitable base of an anti-D reagent. The mother cell line was recloned several times by limiting dilution, subcultured in static culture (in 96 well microplates and T-flasks, Greiner, Germany) and now it is stored in liquid nitrogen.

Static cultivation in tissue culture dishes. Static cultivation of 1C2 was continued in T-flasks (Greiner, Germany), using Iscove Modified Dulbecco's Medium (Sigma Co., USA) supplemented with 15% Foetal Calf Serum (FCS, Gibco, USA) and with metabolic additive (FM) which contained 132 µg/ml cis-oxalacetic acid (Sigma Co., USA), 1 µg/ml bovine insulin (Sigma Co., USA), and 45 µg/ml sodium pyruvate (Gibco, USA). Cultures were incubated in CO_2 incubator (Biocenter 2000; Salvis, Switzerland) at 37 °C, 5% CO_2, and 97% humidity. Medium was changed every second day. Cell density was maintained below 10^6 cells/ml.

Bioreactors. Enlargement of cultivation was carried out in a Celligen bioreactor (New Brunswick Sci., USA) in 3.6 litre and in Microlift bioreactor (New Brunswick Sci., USA) in 24 l working volume. These bioreactors are microprocessor-controlled, pH, DO, agitation speed, temperature setpoints are maintained by PID controllers. DO and pH are maintained by a 4-gas control system, which automatically regulates the composition of the air, oxygen, CO_2, and nitrogen mixture. Base-solution pump is also driven by the control system to increase pH, if it is necessary.

Celligen and Microlift bioreactors can be set up with Cell-Lift or Marine-Blade impeller as well. The Cell-Lift impeller is a specially designed system which provides a gentle agitation of the

culture vertically and horizontally at the same time. Cells are separated from the bubbles of aeration part by a mesh screen, therefore no foaming and no damaging of cells caused by bubbles occurs. The Marine Blade impeller system consists of two propeller-shaped stirrer blades fixed on one vertical shaft and an air-sparger ring positioned below the lower blade. Marine Blade impeller provides higher oxygen transfer, but foaming occurs and so air bubbles may damage cells [7].

Fed-batch cultivation in Roller bottles. Roller bottles (maximal working volume: 500 ml) were inoculated with 100 ml of cell suspension ($1–4\times10^5$ cells/ml) made with fresh IMDM medium supplemented with 15% FCS and FM additive. Bottles were placed on the roller apparatus (Wheaton, USA) in a 37 °C incubator. Samples were taken every day. Cell count and viability was measured. Fresh medium was added to maintain cell density at $3–5\times10^5$ cells/ml. When the bottles had been filled, the cultures were divided into two bottles or the excess was discarded.

Batch cultivation in Celligen and Microlift bioreactors. In bioreactors batch cultivation was performed. For the best productivity the following parameters had to be optimized: (i) initial (inoculum) cell density; (ii) agitation/aeration system; (iii) DO. Other setpoints were set on the usual values: pH: 7.2; temperature: 37 °C; agitation speed: 40 rpm; gas flow: 1 l/min (Celligen) or 4 l/min (Microlift). Agitation aeration system: Cell-Lift impeller.

Batch cultivation was performed as follows. The reactor vessel was sterilized in autoclave (Celligen) or in situ (Microlift), filled with phosphate buffered saline (PBS) pH 7.2 solution at 121 °C for 40 min, then pH and DO electrodes were calibrated. After reaching the operation temperature, PBS was withdrawn, the vessel was filled with medium containing hybridoma cells at the appropriate initial cell density and setpoints were entered in the control unit. Samples were taken every day and cell density, viability and antibody titre were determined. When viability decreased below 60%, the culture was harvested.

Cell count. Cell concentration and cell viability were determined by light microscopy and the trypan blue dye exclusion method [8]. Calculation of viability:

$$v = \frac{\text{viable}}{\text{total}} \cdot 100\%$$

Specific growth rate (μ). Specific growth rate was calculated from the definition of it using the cell count results [9, 10], as follows:

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt}$$

where: μ = specific growth rate (1/h); x = viable cell concentration (cells/ml); t = duration of cultivation (h).

Determination of antibody titre. Antibody titres of cell-free culture supernatant samples were determined using pooled Rh(D) positive red blood cells in saline by the slide method. Rh(D) negative cells were used as control. Samples were serially diluted by saline.

Antibody titre was considered as the highest dilution of the sample at which test cells showed agglutination of at least ++ strength.

Results and discussion

Adaptation to suspension culture. During scaling-up the 1C2 cell line was first adapted to suspension culture by fed-batch cultivation in Roller bottles (Fig. 1). FCS and FM additive content of the feeding IMDM medium was gradually decreased. FCS content was decreased from 15% to 7.5% and FM additive was completely withdrawn by the end of the adaptation procedure. In the last third period of the process IMDM was gradually changed for the cheaper DMEM medium.

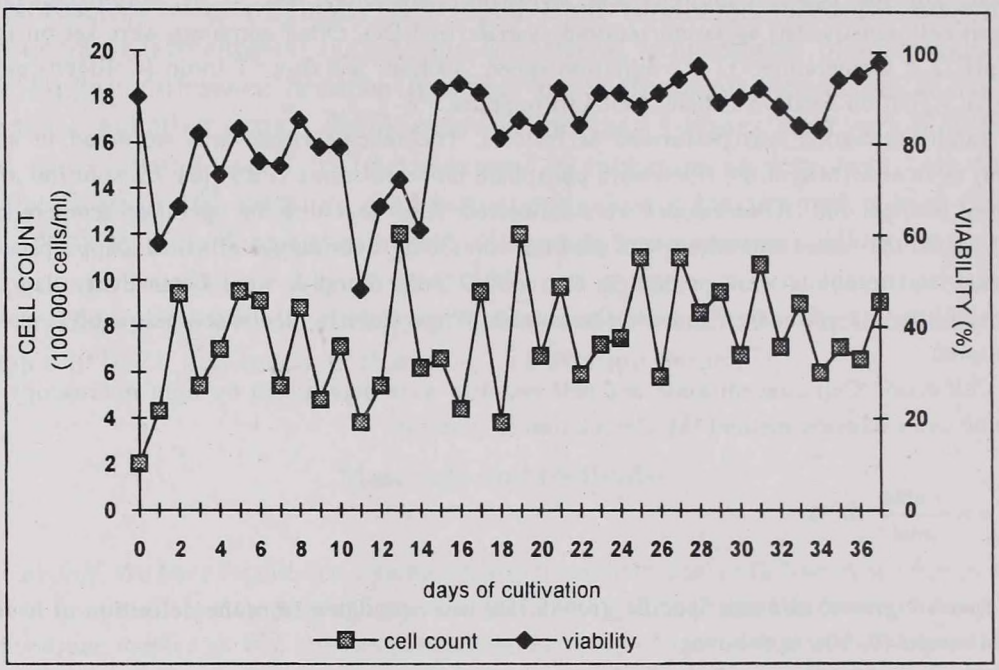

Fig. 1. Development of cell density and viability during 37 days adaptation of suspended 1C2 heterohybridoma cell line. Propagation was carried out in Roller bottles in 500 ml at 37 °C

During the adaptation procedure feeding strategy was mainly directed by viability (v) and specific growth rate (μ) data [9, 10] (Figs 1 and 2).

Days 0–4. Inoculation of roller bottle – initial cell density: 2×10^5 cells/ml. Medium: IMDM + 15% FCS + FM. At the beginning of the propagation in suspension culture viability (v) and specific growth rate (μ) dropped, then reached the normal values (v: cca. 90%, μ: cca. 0.02 per hour).

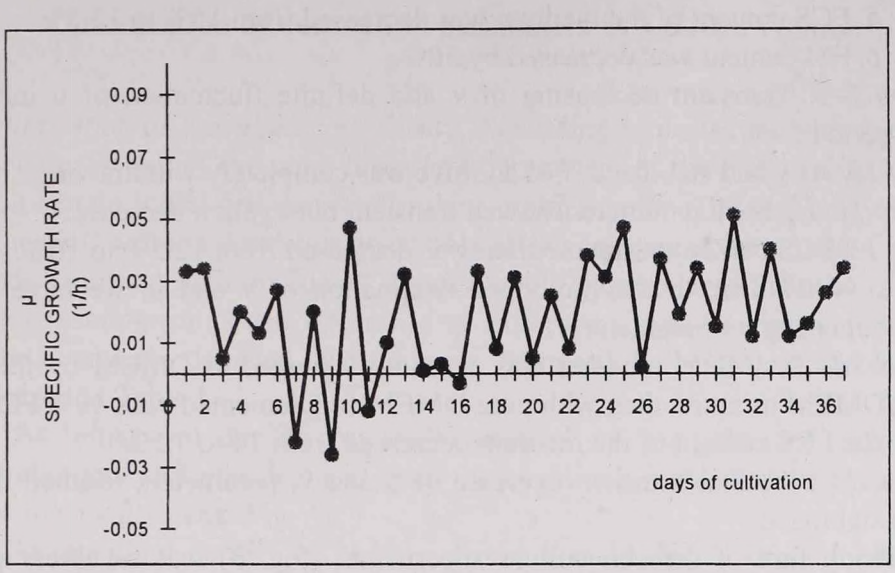

Fig. 2. Development of specific growth rate during 37 days adaptation of suspended 1C2 heterohybridoma cell line. Propagation was carried out in Roller bottles in 500 ml at 37 °C

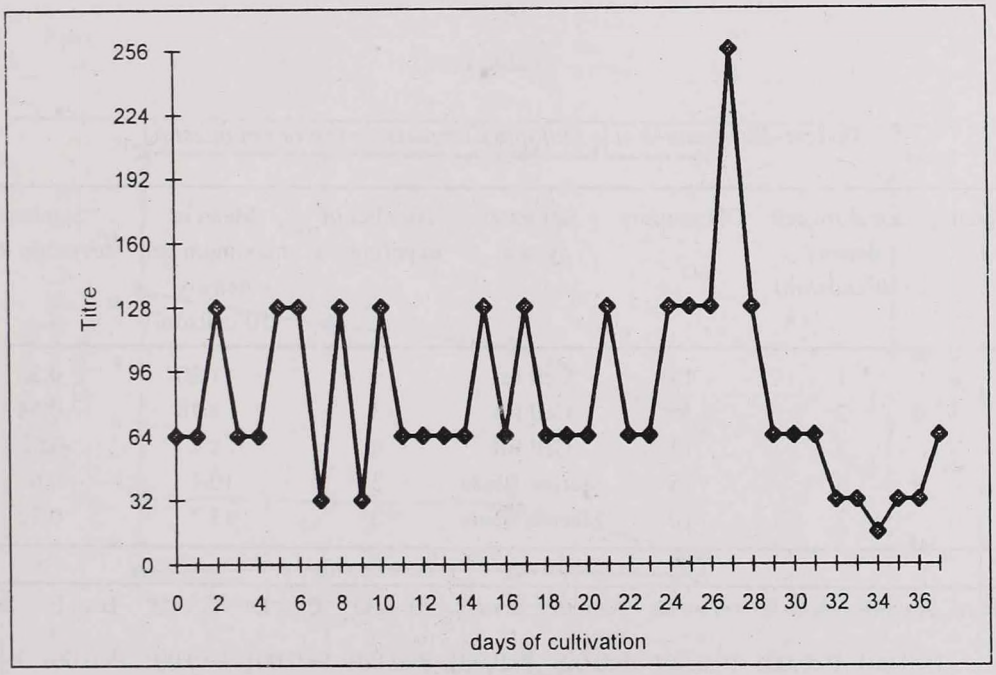

Fig. 3. Development of antibody titre during 37 days adaptation of suspended 1C2 heterohybridoma cell line. Propagation was carried out in Roller bottles in 500 ml at 37 °C

Day 5. FCS content of the medium was decreased from 15% to 12.5%

Day 6. FM content was decreased by 50%.

Days 7–9. Transient decreasing of v and definite fluctuation of μ indicates the adaptation period.

Day 10. As v had stabilized, FM additive was completely withdrawn.

Days 11–12. Each parameter showed transient but explicit decrease.

Day 13. FCS content of the medium was decreased from 12.5% to 10%.

Days 14–23. Due to the prolonged declination of v and μ, we have let longer period without changing parameters.

Days 24–25. IMDM medium was gradually changed to DMEM by feeding with 1:2 IMDM/DMEM mixture, then with pure DMEM supplemented with 10% FCS.

Day 26. FCS content of the medium decreased from 10 to 7.5%.

Days 27–37. After transient decrease of μ and v, parameters reached the normal values, and stabilized.

Antibody titre of the cell culture supernatant (Fig. 3) was relatively permanent (64–128) during the adaptation procedure. Transient decrease was found on days 7 and 9 when FCS and FM content decreased. After day 28 the titre gradually declined probably because of gene loss and growing up of non-producer clones [11]. The adapted 1C2 clone was recloned and now the subclones are stored frozen in liquid nitrogen as master-seed of forthcoming antibody productions.

Table I

Student–Newmann–Keuls Multiple Comparisons test of optimization

Experiment	Inoculum cell density (10^5cells/ml)	DO setpoint	Agitation system	Number of experiments	Mean of maximum cell density (10^5cells/ml)	Standard deviation (SD)
A	1	15	Cell lift	3	1.20	0.20
B	2	15	Cell lift	3	3.78	0.58
C	3	15	Cell lift	3	8.4	0.62
D	3	15	Marine-Blade	3	10.7	0.67
E	3	10	Marine-Blade	3	13.7	0.72

Comparison	A vs B	A vs C	A vs D	A vs E	B vs C	B vs D	C vs D	C vs E	B vs E	D vs E
P value	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Optimization of cultivation parameters in 3.6 litre volume

Optimization of inoculum cell density. According to our experience the cell count of the inoculum is one of the most important parameters of the cultivation. Mammalian cells need a certain initial cell density for their multiplication. If cell density is less than required, the cells will not divide, or do so only after a long incubation period (lag phase). On the other hand, too high initial cell density would require large quantity of inoculum, which is not economical. Each optimization experiment was carried out as triplicate. Mean of the results are depicted in the figures. Standard deviation and significance data are summarized in Table I.

(a) At initial cell density of 1×10^5 cells/ml no multiplication was observed. Viability, cell count and titre declined from the beginning. After 3 days propagation the culture was practically dead (Fig. 4).

(b) At inoculum density of 2×10^5 cells/ml a small degree of multiplication was observed, but maximal cell counts (3.78×10^5 cells/ml) and antibody titre (32) were far from the satisfactory. The yielded supernatant was not suitable for economical reagent production (Fig. 5).

(c) At inoculum density of 3×10^5 cells/ml multiplication and antibody production were sufficient (maximum cell density: 8.4×10^5 cells/ml). The yielded cell culture supernatant having a titre of 256 was suitable for reagent formulation (Fig. 6).

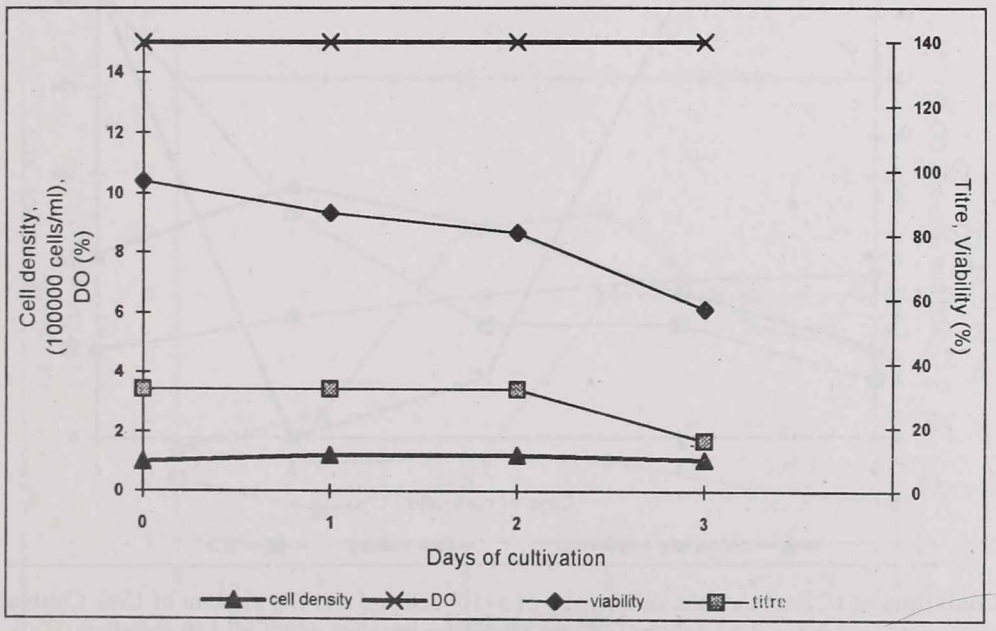

Fig. 4. Cultivation of 1C2 at inoculum cell density of 10^5 cells/ml and DO setpoint of 15%. Cultivation was carried out in 3.6 l volume Celligen bioreactor installed with Cell-Lift impeller

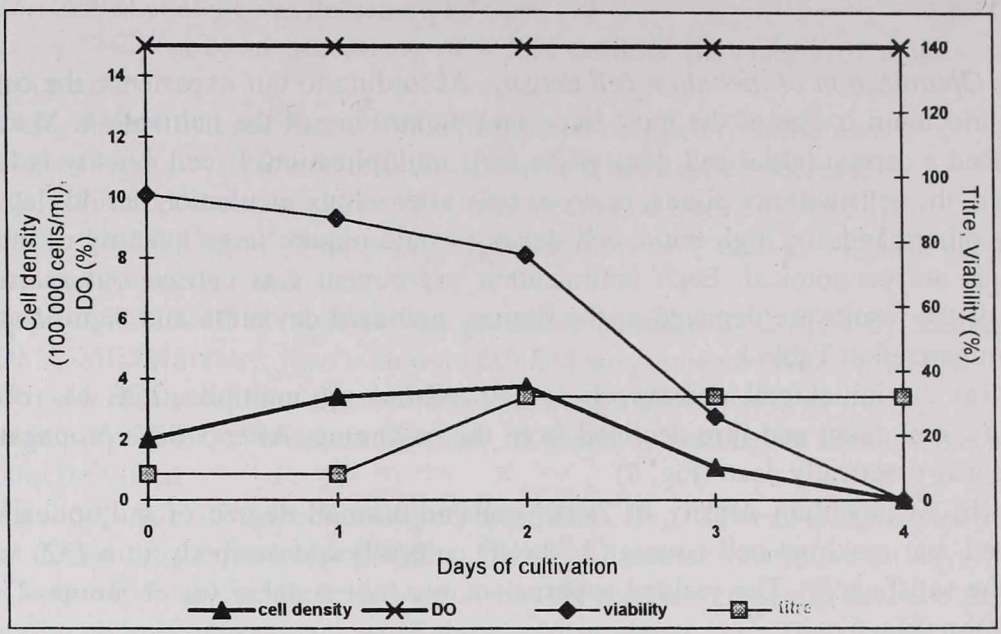

Fig. 5. Cultivation of 1C2 at inoculum cell density of 2×10^5 cells/ml and DO setpoint of 15%. Cultivation was carried out in 3.6 l volume Celligen bioreactor installed with Cell-Lift impeller

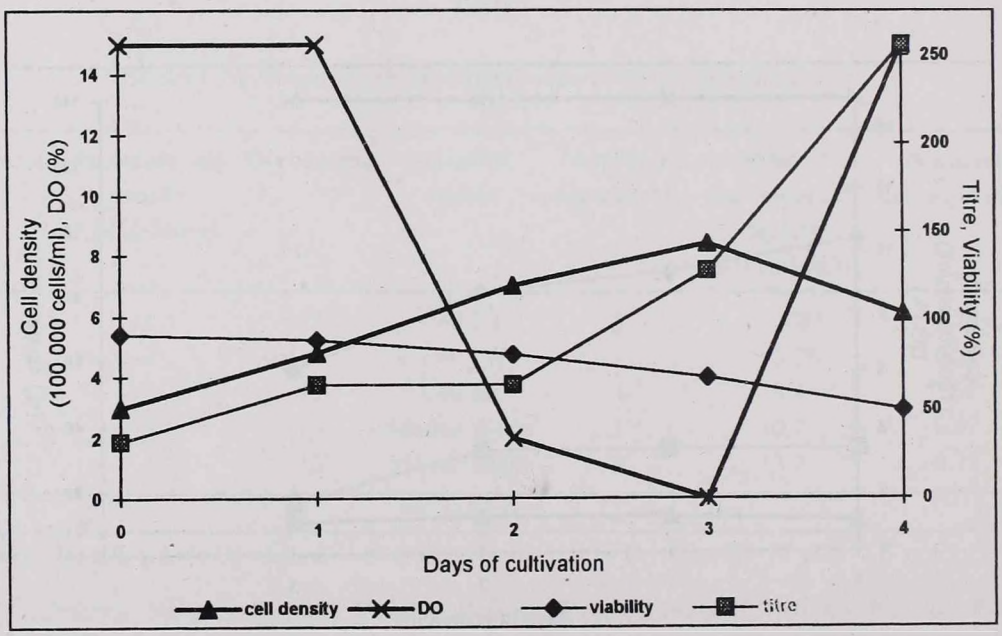

Fig. 6. Cultivation of 1C2 at inoculum cell density of 3×10^5 cells/ml and DO setpoint of 15%. Cultivation was carried out in 3.6 l volume Celligen bioreactor installed with Cell-Lift impeller

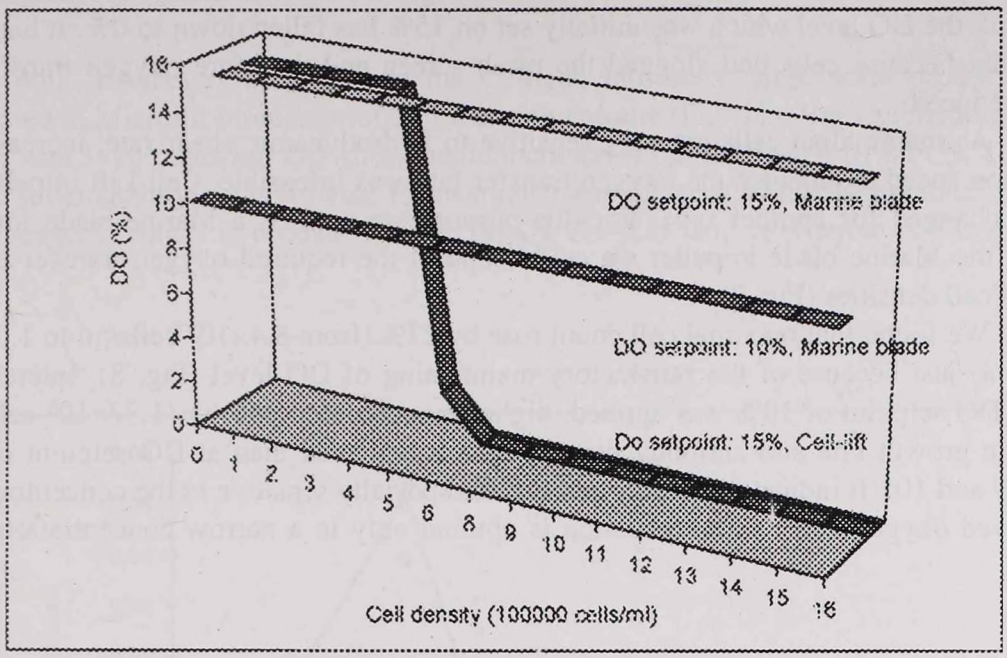

Fig. 7. Maintenance of DO level at 3.6 l volume batch cultivation with different agitation systems. Agitation speed: 40 rpm

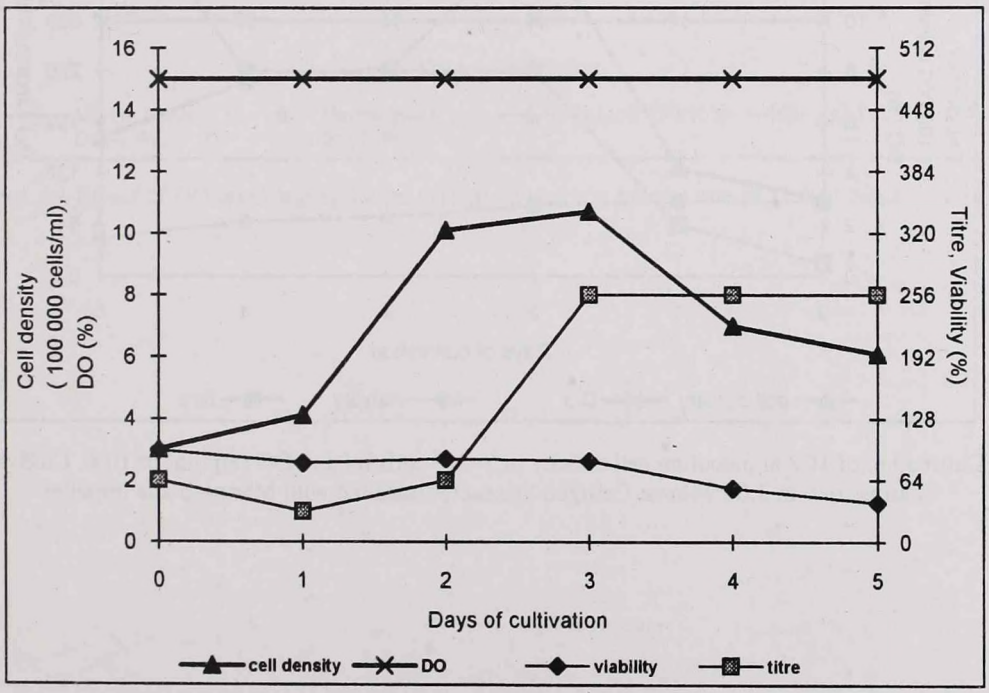

Fig. 8. Cultivation of 1C2 at inoculum cell density of 3×10^5 cells/ml and DO setpoint of 15%. Cultivation was carried out in 3.6 l volume Celligen bioreactor installed with Marine-blade impeller

Optimization of DO level. As shown in Fig. 5, when cell density reached 4.8×10^5 cells/ml, the DO level which was initially set on 15% has fallen down to 0%. It happened probably because cells had clogged the mesh screen and therefore oxygen transfer had been reduced.

As mammalian cells are very sensitive to hydrodynamic shear rate, increasing of agitation speed to enhance the oxygen transfer rate was infeasible. Cell-Lift impeller had to be changed for another type. For this purpose we applied a Marine-blade impeller. Using the Marine-blade impeller we could support the required oxygen transfer even at higher cell densities (Fig. 7).

We found that maximal cell count rose by 27% (from 8.4×10^5 cells/ml to 1.07×10^6 cells/ml) just because of the satisfactory maintaining of DO level (Fig. 8). Interestingly when DO setpoint of 10% was applied, higher maximal cell density (1.37×10^6 cells/ml), specific growth rate and antibody titre (512) was achieved than at DO setpoint of 15% (Figs 9 and 10). It indicates that this cell line is especially sensitive to the concentration of dissolved oxygen and its multiplication is optimal only in a narrow concentration range [7].

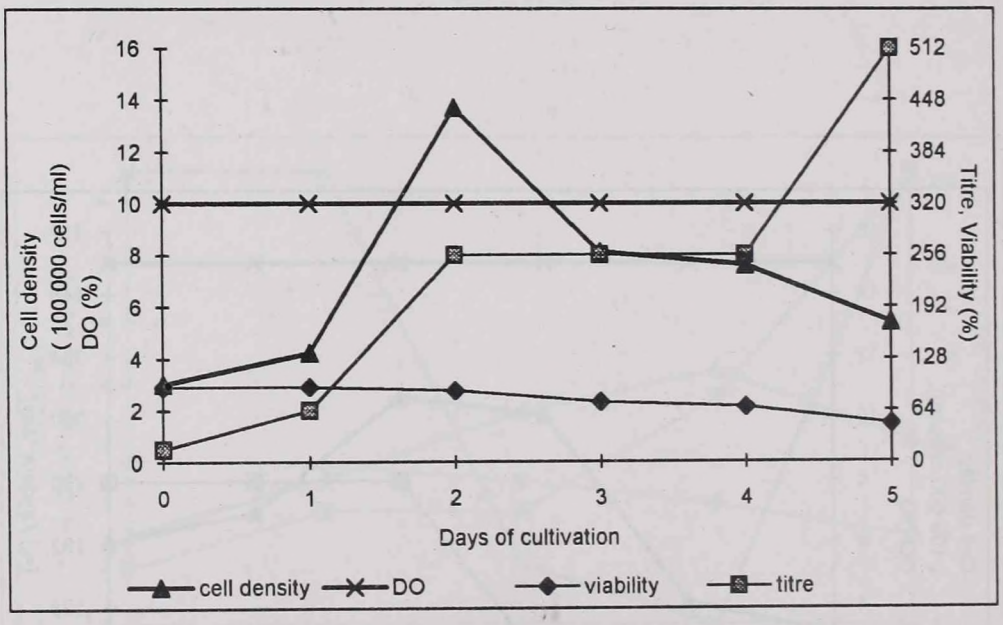

Fig. 9. Cultivation of 1C2 at inoculum cell density of 3×10^5 cells/ml and DO setpoint of 10%. Cultivation was carried out in 3.6 l volume Celligen bioreactor installed with Marine-blade impeller

Large scale production in 24 litre volume in Microlift bioreactor

With parameters optimized in the Celligen bioreactor large scale production is performed in Microlift bioreactor of 24 l working volume (Fig. 11). Briefly: inoculum cell density was 3×10^5 cells/ml, DO 10%, medium composition: DMEM + 10% FCS. The cell culture supernatant yield is suitable for using as basic material of the Monoclonal Anti-D (IgM) reagent, which is produced by HUMAN Co. Ltd. and registered in Hungary in 1995.

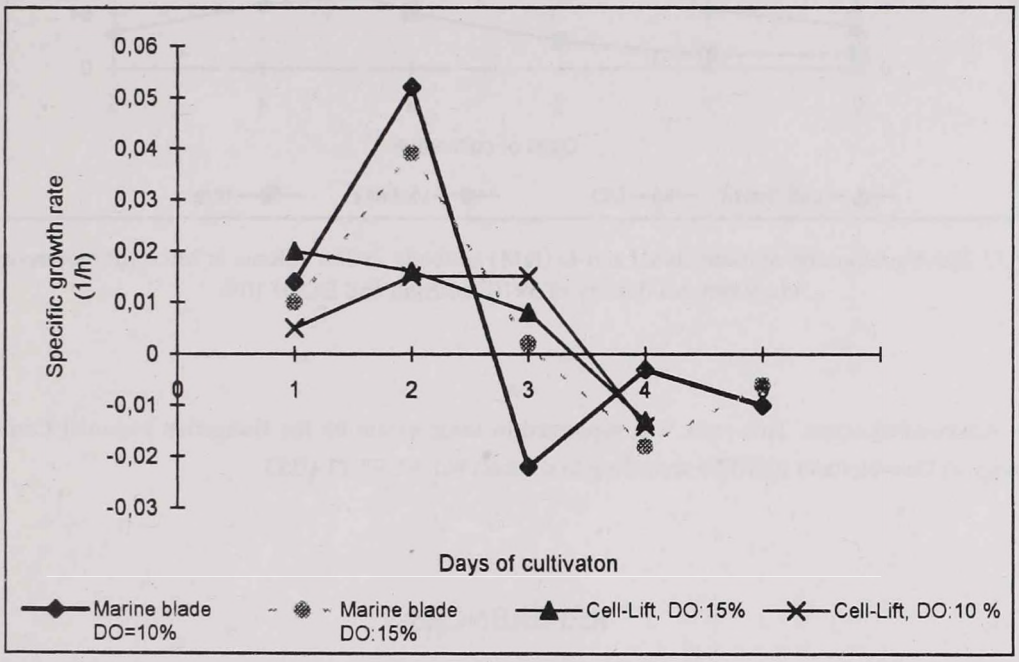

Fig. 10. Effect of DO level and agitation system on specific growth rate of 1C2 in batch culture

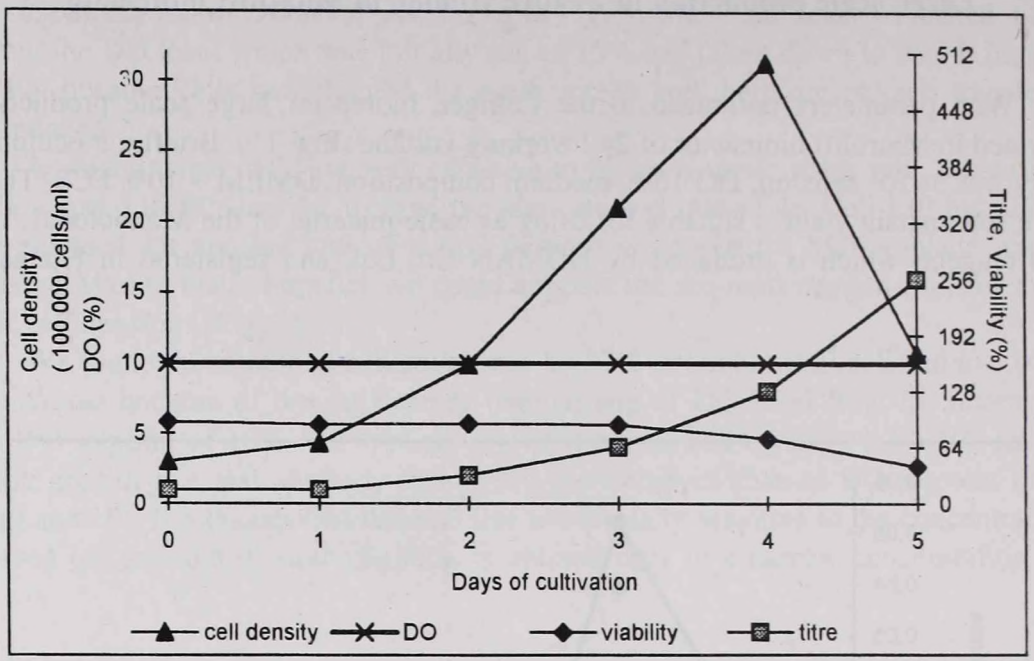

Fig 11. Batch production of monoclonal anti-D (IgM) antibody in 24 l volume in Microlift bioreactor at inoculum cell density of 3×10^5 cells/ml and DO of 10%

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REFERENCES

1. Thompson,K.M., Hough,D.W., Maddison,P.J., Melamed,M.D., Hugh-Jones,N.C.: Production of human monoclonal IgG and IgM antibodies with anti-D (Rhesus) specificity using heterohybridomas. Immunology, **58**, 157 (1986).
2. Goossens,D., Champomier,F., Rouger,P., Salmon,C.: Human monoclonal antibodies against blood group antigens. Preparation of a series of stable EBV immortalized B clones producing high levels of antibody of different isotypes and specificities. J Immun Meth **101**, 193 (1987).
3. Graf,H., Schügerl,K.: Some aspects of hybridoma cell cultivation. Appl Microbiol Biotechnol **13**, 165 (1991).
4. Long,W.J., Palombo,A., Schotfield,T.L., Emini,E.A.: Effect of culture media on murine hybridomas; Definition of optimal conditions for hybridoma viability, cellular proliferation and antibody production. Hybridoma **7**, 69 (1988).
5. Backer,M.P., Metzger,L.S., Slaber,P.L., Naritt,K.L., Bodre,G.B.: Large-scale production of monoclonal antibodies in suspesion culture. Biotechnol Bioeng **32**, 993 (1988).
6. de St.Groth,F.S.: Automated production of monoclonal antibodies in a cytostat. J Immunol Methods **57**, 121 (1983).
7. Zhang,S., Handa-Corrigan,A., Spier,R.E.: Oxygen transfer properties of bubbles in animal cell culture media. Biotechnol Bioeng **40**, 252 (1992).

8. Patterson,M.K.jr.: Measurement of growth and viability of cells in culture. Methods in Enzymology **58**, 141 (1979).
9. Linardos,T.I., Kalogerakis,N., Behie,L.A.: The effect of specific growth rate and death rate on monoclonal antibody production in chemostat cultures. Can J Chem Eng **69**, 429 (1991).
10. Leno,M., Merten,O-W., Hache,J.: Kinetic analysis of hybridoma growth and Monoclonal antibody production in semicontinuous culture. Biotechnol and Bioeng **39**, 596 (1992).
11. Couture,M.L., Heath,C.A.: Relationship between loss of heavy chains and the appearance of nonproducing hybridomas. Biotechnol and Bioeng **47**, 270 (1995).

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EFFECT OF MEDIUM COMPOSITION ON HYBRIDOMA GROWTH AND ANTIBODY PRODUCTION

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Two murine hybridoma cell lines named 1D12 and 3B1 secreting antibody against human blood group B were developed. Effect of medium composition and hyperosmotic stress on cell growth, viability and monoclonal antibody (MAb) production was examined during down-stream process in suspension culture. Both of the cell lines secreted MAb of higher titre if the basal medium was supplemented with 2.5 g/l L-glucose and 419 mg/l L-glutamine. Additional nutrients like transferrine, amino acids, ethanolamine, selenium were added to promote cell growth and viability. In case of cell line 1D12 enhancement of MAb production was observed using hyper-osmotic medium as well. Having data of these preliminary experiments we optimized the large scale production of MAbs in bioreactor. These MAbs serve as a base of the anti-B blood group reagent registered in Hungary.

A wide range of biological scientists are using monoclonal antibodies (MAbs) including basic researchers, clinical laboratories, commercial diagnostic reagent producers, and even medicals for therapeutic purposes.

The first monoclonal antibody (anti-A) used for blood group serology was produced by Voak et al. in 1980 [1]. As sources of basic materials of human origin are being exhausted, human serum is being substituted by monoclonal antibodies in large scale reagent production.

Due to the increasing consumption of monoclonal blood group reagents, we aimed at the large scale production of antibodies applicable for that purpose. Production of large quantity, high quality monoclonal antibodies needs the ability of large scale cultivation of mammalian cells. This requires among many others the following conditions: (i) well defined, genetically stable cell lines which are sufficiently growing and producing antibodies in cell culture medium [2]; (ii) specially formulated cell culture medium which supports the highest growth and antibody production rates in the most economical way [3, 4]; (iii) cell culture systems (bioreactors) specially designed for cultivation of mammalian

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cells – such systems were first described in 1983 [5]. The effect of medium composition and additives on cell multiplication and antibody production was studied in detail.

A number of studies have shown that MAb concentration increases not only during the exponential growth phase but also during stationary phase. It appears that the formation of MAb occurs as long as the cells are in a viable state and the precursors needed for synthesis of MAb are available in the medium. Therefore, if the cells could be maintained in a viable state for longer time periods, additional MAb could be produced.

Many researchers have examined the effects of the primary energy substrates, glucose and glutamine, on mammalian cell metabolism. Luan et al. [6] examined the effect of varying glucose/glutamine ratios (r) on both cell growth and viability as well as MAb formation in plastic cell culture flasks.Various models describing hybridoma cell growth and antibody production have been published recently. Dalili et al. [7] modelled specific growth rate and specific antibody secretion rate as function of initial serum concentration and glutamine concentration. Flickinger et al. [8] reported stimulation of antibody synthesis and secretion after addition of 5–40 mM L-glutamine during slow hybridoma growth. Jeong et al. [9] found that glutamine enhanced MAb production not only by stimulation of cell growth but also by increasing the specific MAb production.

In an earlier study [10] we examined the alteration of growth rate and antibody production by changing the rate of glucose and glutamine in the culture medium. At a rate of 5.56 we found significant enhancement of both parameters. At the present study we made attempts to examine how growth rate, viability and MAb production of hybridoma cells changes in suspension culture under different conditions.

Materials and methods

Cell lines. Two murine hybridoma cell lines (named 1D12 and 3B1) were established in our monoclonal laboratory by fusion of Sp2/0-Ag-14 myeloma cell line with activated B-lymphocytes of an immunized mouse. Murine hybridoma cells obtained by this method produce MAb anti-B of isotype IgM. Isotype was determined with Mouse monoclonal antibody isotyping kit (Gibco). The MAbs did not react with red cells belonging to groups A and O. Hybridoma cell lines were tested for the absence of mycoplasma according to the methods described at European Pharmacopeia (1991.V.2.1.3.6.-1-3.) and Sterility test for Mycoplasmas (WHO Expert Committee on Biological Standardization 25th Report, 1973. p. 52, 56–57; WHO Organization Report Series 530).

Culture media. The basal medium used was RPMI 1640 medium (Gibco) containing 2 g/l glucose and 328.4 mg/l L-glutamine, supplemented with 7.5% Foetal Calf Serum (FCS, Protein GMK, Hungary), 2 g/l sodium bicarbonate (Gibco), 110 mg/l sodium pyruvate (Gibco), 10^5 IU/l sodium penicillin G (Biogal, Hungary), 100 mg/l streptomycin sulphate (EGIS, Hungary) and 0.05 mM mercaptoethanole (Sigma, USA). Composition of cell culture media used in the experiments is shown in Table I. Medium composition and osmolarity applied in osmotic stress experiments were as set out in Table II.

Medium osmolarity was measured by Osmomat 030 (Gonotec, Germany) osmometer. NutriBoost (J. Brooks lab. Inc., USA) is a supplement sold for experimental laboratories for boosting of immunglobulin production of hybridomas.

Table I

Supplements used in cell culture media

Supplements	RPMI (basal, r*=6.66)	RPMI R556 (r=5.56)	RPMI R556F (r=5.56+amino acids+factors)
Sodium bicarbonate (g/l)	2	2	2
Sodium pyruvate (mg/l)	110	110	110
Penicilline (IU/l)	100 000	100 000	100 000
Streptomycin (mg/l)	100	100	100
Mercaptoethanol (mM/l)	0.05	0.05	0.05
L-Glucose (g/l)	–	2.5	2.5
L-Glutamine (mg/l)	–	419	419
L-Isoleucine (mg/l)	–	–	50
L-Leucine (mg/l)	–	–	50
L-Valine (mg/l)	–	–	20
L-Tryptophan (mg/l)	–	–	5
Transferrine (mg/l)	–	–	10
Ethanolamine (µg/l)	–	–	10
Selenite (µg/l)	–	–	5
Foetal calf serum (%)	7.5	7.5	7.5

* r= glucose/glutamine ratio

Table II

Osmolarity of media

Medium composition	Osmolarity (Osmol/kg)
RPMI R556	0.330
RPMI R556F	0.328
RPMI R556 + 10µl/ml NutriBoost	0.327
RPMI R556 + 5 mg/ml NaCl	0.380
RPMI R556 +14.2 mg/ml fructose	0.395

Cell count. Cell concentration and cell viability were determined with light microscopy and the Trypan blue dye exclusion method [18].

Propagation in cell culture flasks. Cells were routinely grown in 75 cm^2 static flasks (Greiner) at 37 °C and 5% CO_2 in humidified incubator (Biocenter 2001, Salvis AG, Switzerland).

Batch cultivation in Roller bottles and bioreactors. Cultivation in Roller bottles (Wheaton, USA) was performed in 100–500 ml volume in thermostat at 37 °C. Cultivation in Celligen bioreactor (New Brunswick Scientific Inc., Edison, USA) was carried out in 3600 ml working volume, at 37 °C, agitation: 40 rpm, pH: 7.2, dissolved oxygen (DO): 15%. Cultivation in Microlift bioreactor (New Brunswick Scientific Inc., Edison, USA) was performed in 22 litre working volume, at 37 °C, agitation: 30 rpm, pH: 7.2, DO: 10%.

Serological activity by direct haemagglutination. Red cells of blood group 'B' not older than 48 hours were used as test cells. Cells were washed three times with saline and suspended in saline at 2% concentration.

Haemagglutinating activity of supernatants was determined by U well microplate (Greiner) technique. Thirty µl cell-free cell culture supernatant was mixed with 30 µl 2% suspension of test red cells. Agglutination was examined after incubation at room temperature for 1 h. Results were read visually by tilting the microplate.

Haemagglutinating activity was expressed in titre score. Calculation of titre score was made by the method of Marsh [19]. According to Marsh at least a 10 point difference between scores should be considered as significant.

Cell culture supernatants showing titre score at least 66 are considered as suitable for reagent formulation.

Results and discussion

A number of studies show that mammalian cells demand various low-molecular nutritional factors and high-molecular weight growth factors for their growth and expression of differentiated functions.

Transferrin stimulates the growth of various type of cells. Iron – which binds to transferrin in vivo and incorporated into cells through receptor-mediated endocytosis – is an essential element for cell growth, because it is important for the expression of such enzyme activities as oxidoreductases. These enzymes are necessary not only for cell growth but also for the maintenance of viability [11]. Increased viability of cells grown in medium supplemented with transferrin, ethanolamine and selenium was found.

The effect of amino acids on the growth of mammalian cells in culture has also been studied by number of groups. Significant increases in viable cell concentration and final antibody concentration were achieved through the supplementation of cell culture medium with selected amino acids and vitamins. The addition of tryptophan and of isoleucine and valine had the largest positive effects on the viable cell number [3, 12].

Growth rate, antibody production and viability of two hybridoma cell lines – one is marked 1D12 other is 3B1 – producing anti-B antibody were examined in suspension culture, in three cultivation systems. Results obtained from Roller bottle apparatus, Celligen and Microlift bioreactors are depicted on distinct figures.

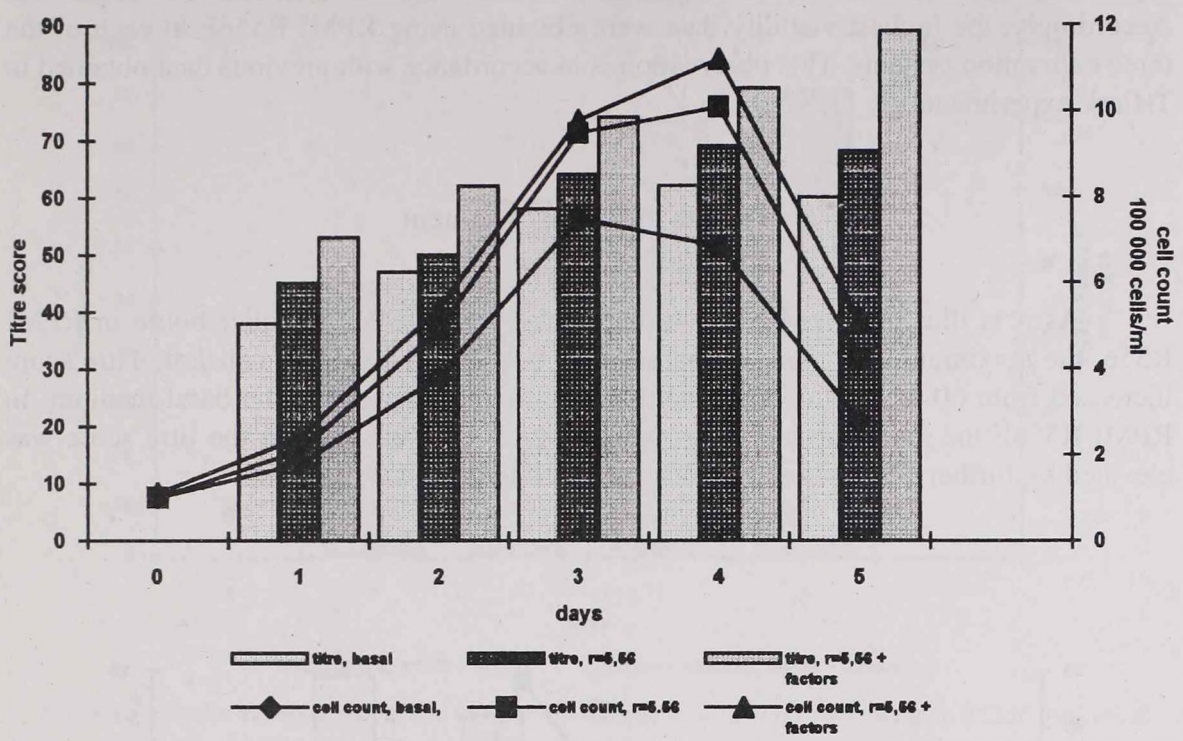

Fig. 1. Development of cell count and titre during cultivation of 1D12 in Roller bottles in different compositions of RPMI medium at initial cell density of 10^5 cells/ml (results are average of 8 experiments)

Results of 1D12 experiments

As it is shown in Fig. 1, during cultivation 1D12 in Roller bottle in RPMI R556 maximum cell count increased by 35% from 7.5×10^5 to 1.01×10^6 cells/ml and the titre score increased from 62 to 69 points (the latter being non significant) compared to basal medium. In RPMI R556F the highest cell count increased by further 11% to 1.12×10^6 cells/ml (49% compared to the basal medium) and the titre reached 89 points which is a significant difference.

Multiplication and antibody production in RPMI R556 and in RPMI R556F were compared in Celligen bioreactor, too. As it is illustrated in Fig. 2 the same significant increase of maximal cell count and titre score was observed as in Roller bottles.

Large scale antibody production in Microlift bioreactor with 1D12 hybridoma. Since the best production and multiplication data were attained with RPMI R556F either in the Roller bottles or in the Celligen bioreactor, large scale production in Microlift bioreactor was performed using this medium composition. The results achieved in large scale propagation are comparable to those in Celligen bioreactor (Fig. 3). Cell culture supernatant batches yielded from the Microlift bioreactor are suitable for economical production of high quality blood group reagents.

Viability data of 1D12 experiments. Data are summarized in Table III. Accordingly, the highest viability data were obtained using RPMI R556F in each of the three cultivation systems. This observation is in accordance with previous data obtained in T-flask experiments [3, 11, 12].

Results of 3B1 experiment

As it is illustrated in Fig. 4, during cultivation of 3B1 in Roller bottle in RPMI R556, the maximum cell count increased from 6.9×10^5 to 1.02×10^6 cells/ml. Titre score increased from 60 to 72 which is a significant change compared to the basal medium. In RPMI R556F the maximal cell count reached 1.21×10^6 cells/ml and the titre score was elevated by further 12 points (24 points compared to the basal medium).

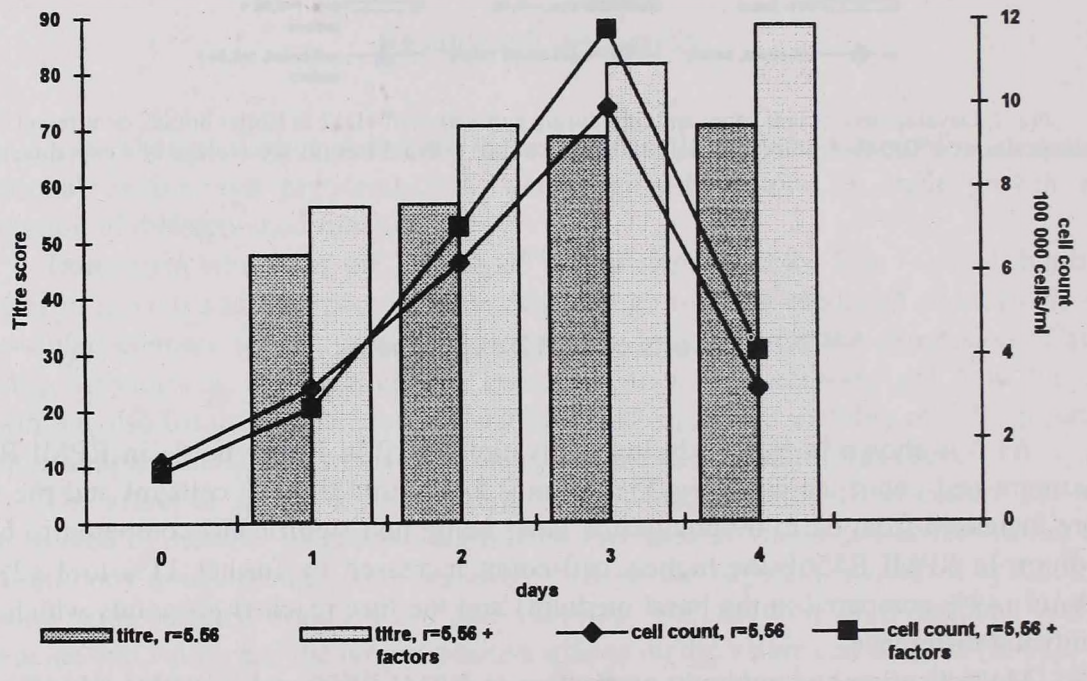

Fig. 2. Development of cell count and titre during cultivation of 1D12 in Celligen bioreactor in RPMI R556 and R556F at initial cell density of $1.2–1.3\times10^5$ cells/ml (results are average of 8 experiments)

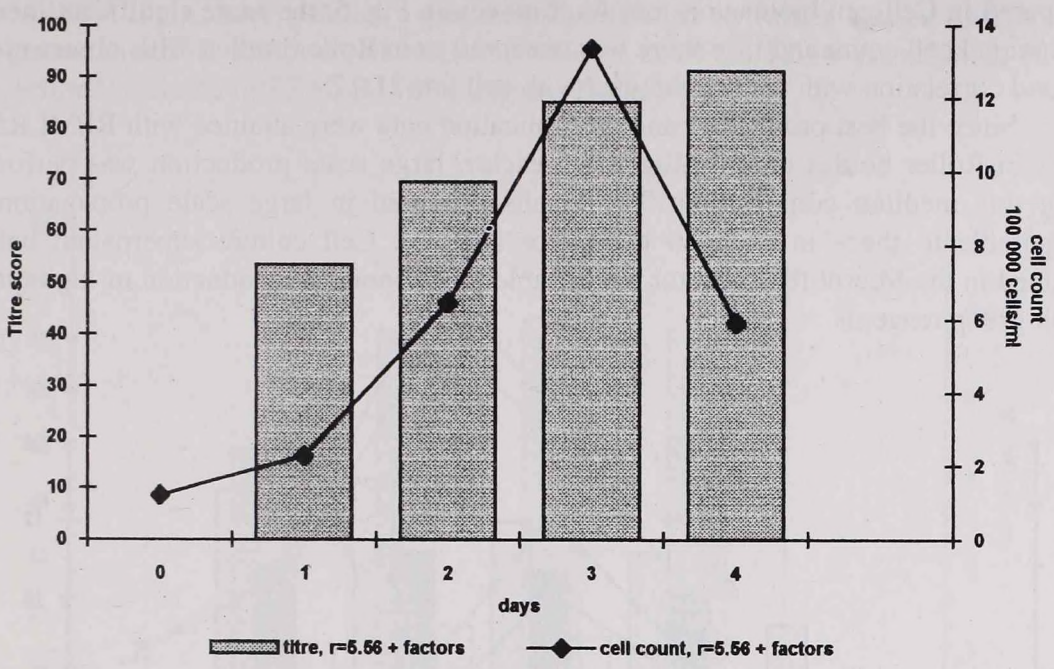

Fig. 3. Large scale antibody production in Microlift bioreactor with 1D12 hybridoma in R556F medium at initial cell density of 1.2×10^5 cells/ml (results are average of 8 batches)

Table III

Summarized viability data of 1D12

Glucose/glutamine (r)	Viability (%)				
	day 1	day 2	day 3	day 4	day 5
Roller bottles					
6.66	78	83	81	71	30
5.56	85	89	87	82	28
5.56 +factors	91	94	92	88	36
Celligen bioreactor					
5.56	86	88	78	45	
5.56+factors	90	93	87	38	
Microlift bioreactor					
5.56+factors	91	95	88	41	

Multiplication and antibody production in RPMI R556 and in RPMI R556F were compared in Celligen bioreactor, too. As it is seen in Fig. 5, the same significant increase of maximal cell count and titre score was observed as in Roller bottles. This observation is in good correlation with results obtained with cell line 1D12.

Since the best production and multiplication data were attained with RPMI R556F either in Roller bottles or in Celligen bioreactor, large scale production was performed using this medium composition. The results achieved in large scale propagation are comparable to those in Celligen bioreactor (Fig. 6). Cell culture supernatant batches obtained in the Microlift bioreactor are suitable for economical production of high quality blood group reagents.

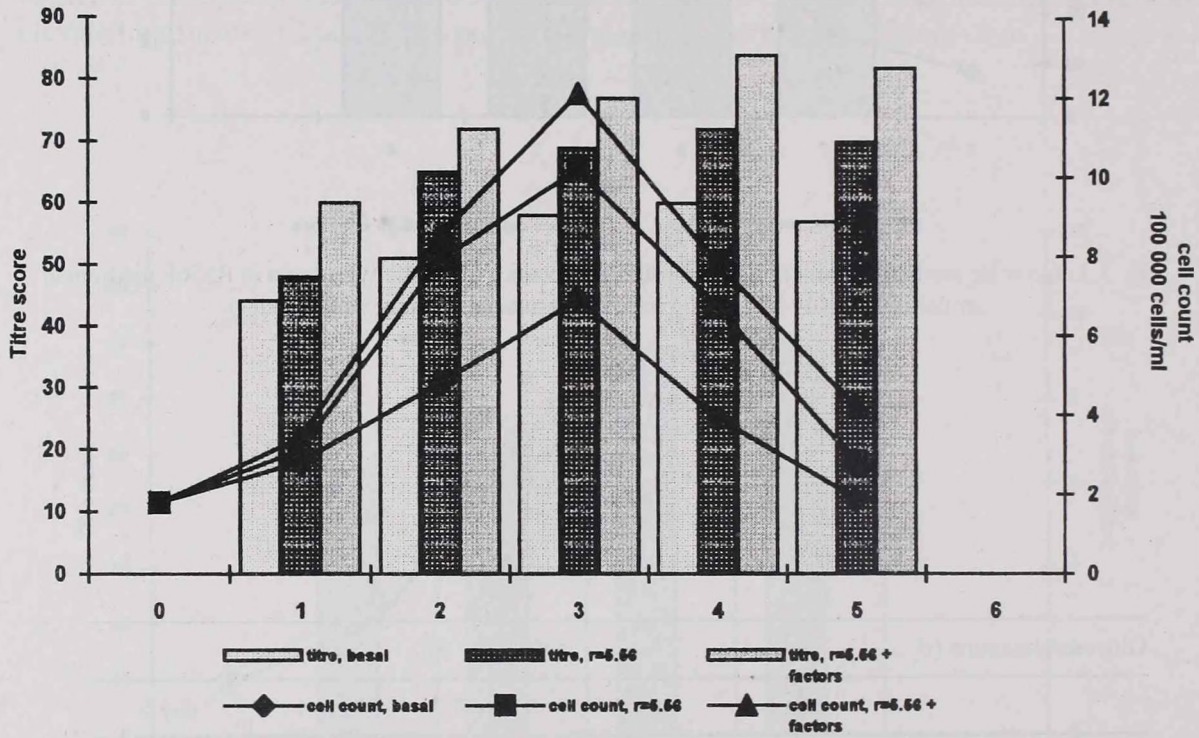

Fig. 4. Development of cell count and titre during cultivation of 3B1 in Roller bottles in different compositions of RPMI medium at initial cell density of 1.8×10^5 cells/ml (results are average of 8 experiments)

Viability data of 3B1 experiments. Data are summarized in Table IV, the highest viability data were obtained using RPMI R556F in each of the three cultivation systems just like in the case of 1D12.

Effect of hyper-osmotic shock on antibody secretion. Osmolarity is an important process variable during cultivation of mammalian cells in vitro. Cell culture medium is designed to have osmolarity in the range of 260 and 320 mOsmol/kg, basically to mimic the osmolarity of serum at 290 mOsmol/kg. There are a few publications on the effect of osmolarity on hybridoma growth and immunoglobulin production [13, 14]. Some researchers published significant enhancement of immunoglobulin production by hyper-

osmotic shock. Others reported no significant increase in antibody titres in hyper-osmotic cultures [15–17]. In three parallel experiments performed in Roller bottles hybridomas were grown in RPMI R556, in RPMI R556 +NaCl, in RPMI R556+fructose, in RPMI R556 +NutriBoost and in RPMI R556F medium (Table V).

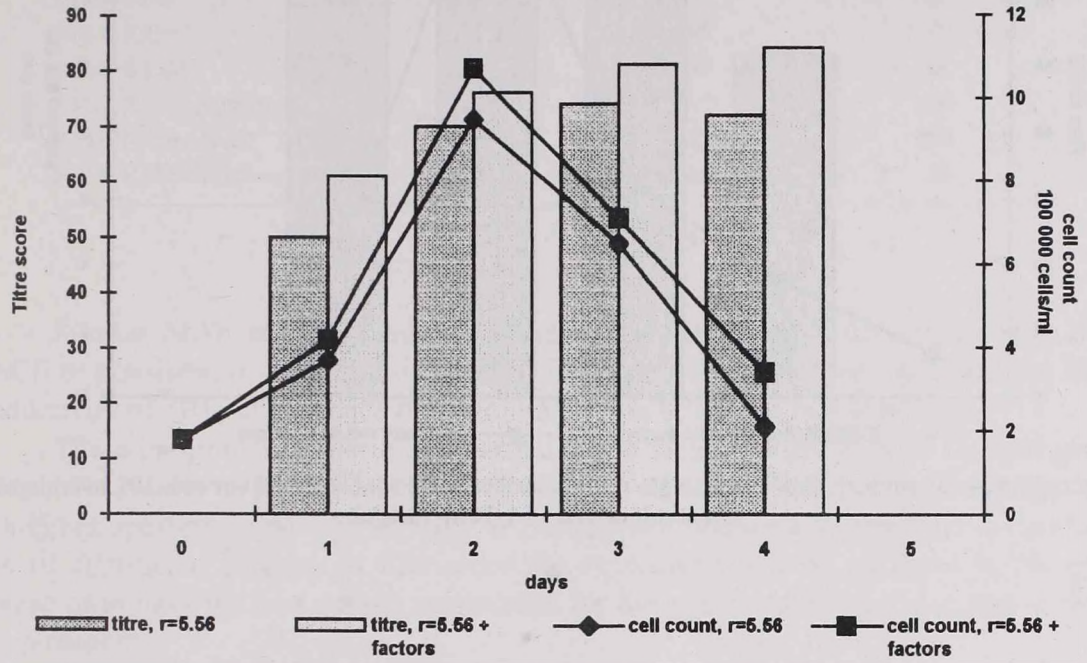

Fig. 5. Development of cell count and titre during cultivation of 3B1 in Celligen bioreactor in RPMI R556 and R556F at initial cell density of 1.8×10^5 cells/ml (results are average of 6 experiments)

As shown in Table V, increasing the osmolarity of R556 medium did not induce a significant alteration in the titre of 3B1, but a significant increase was observed in the titre of cell line 1D12. Rising of titre using NutriBoost was significant in the case of 1D12 in comparison with R556 medium, but 3B1 showed significancy only in comparison with basal RPMI. It is worth noting that commercially available supplement NutriBoost had no effect on medium osmolarity but it enhanced MAb production of 1D12. In accordance with previous observations [3, 11, 12] amino acids and nutrient factors (F) used in culture media induced significant increase in MAb production of both cell lines compared to RPMI R556 medium. Titre score was elevated by 20 and 12 points in case of 1D12 and 3B1, respectively.

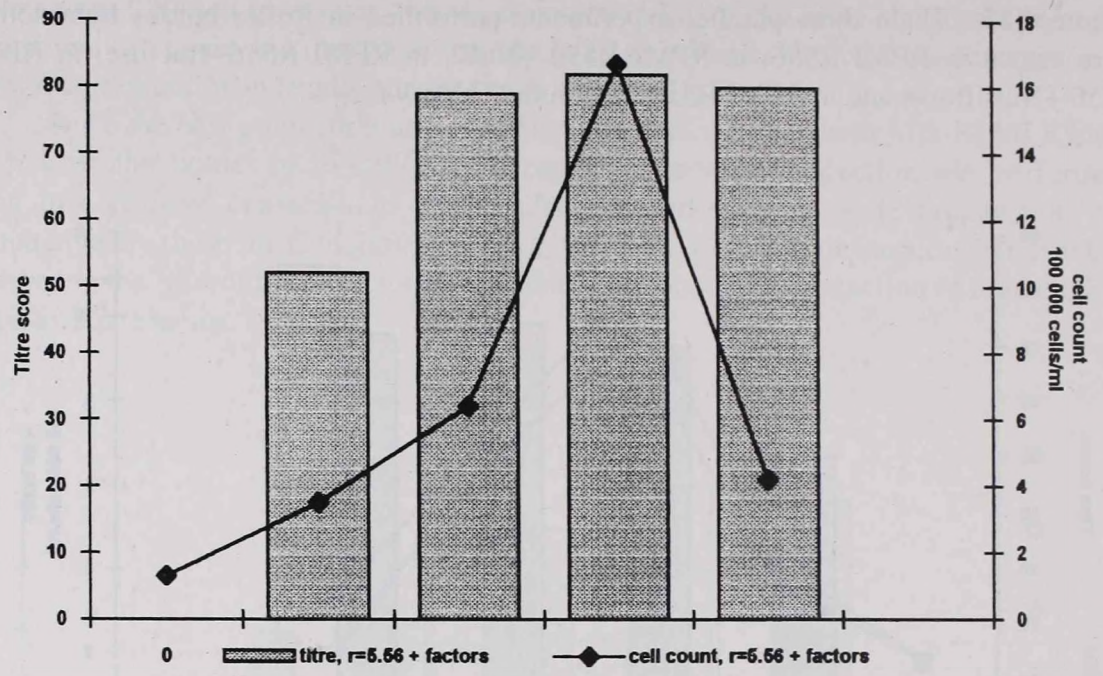

Fig. 6. Large scale antibody production in Microlift bioreactor in RPMI 556F medium with 3B1 hybridoma at initial cell density of 1.3×10^5 cells/ml

Table IV

Summarized viability data of 3B1

Glucose/glutamine (r)	Viability (%)				
	day 1	day 2	day 3	day 4	day 5
Roller bottles					
6.66	78	81	85	65	27
5.56	84	90	89	75	44
5.56 +factors	87	92	95	87	32
Celligen bioreactor					
5.56	78	90	85	27	
5.56+factors	88	93	81	31	
Microlift bioreactor					
5.56+factors	84	92	86	38	

Table V

Titre scores attained in media of different osmolarity

Medium	Osmolarity (mOsmol/kg)	Titre score Cell line 1D12	Titre score Cell line 3B1
RPMI basal	315	62	60
RPMI R556	330	69	72
RPMI R556F	328	89	84
RPMI R556+NutriBoost	327	87.5	80
RPMI R556+ NaCl	380	88.5	79
RPMI R556+ fructose	395	88	79

Similar MAb enhancement was observed via hyper-osmotic stress using ionic (NaCl) or non-ionic (L-fructose) substances. Increased osmolarity did not effect the MAb productivity of 3B1, so influence of osmolarity seems to be cell line dependent.

These preliminary experiments carried out in Roller bottles have to be widened to get reliable results of MAb production of 1D12 in RPMI R556F medium supplemented with either appropriate amount of NaCl or L-fructose to induce a hyper-osmotic shock. In case of significant increase in titre score the experiment will be enlarged in Microlift bioreactor to have the best culture supernatant for the registered MAb-based anti-B blood group reagent.

Since MAb secretion of 3B1 is independent of hyper-osmotic shock its optimized large scale production is already solved.

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REFERENCES

1. Voak,D., Sacks,S., Milstein,C. et al.: Monoclonal anti-A from hybrid-myeloma: Evaluation as a blood grouping reagent. Vox Sang **39,** 134 (1980).
2. Seaver,S.: Enchancing monoclonal antibodies and hybridoma cell lines. Cytotechnology **9**, 131 (1992).
3. Jo,E.-C., Park,H.-J., Park,J.-M., Kim,K.-H.: Balanced nutrient fortification enables high density hybridoma cell culture in batch culture. Biotechnol Bioeng **36**, 717 (1990).
4. Jayme,D.W., Greenwold,D.J.: Media selection and design: wise choices and common mistakes. Bio/Technology **9**, 716 (1991).
5. de St.Groth,F.S.: Automated production of monoclonal antibodies in a cytostat. J Immunol Methods **57**, 121 (1983).
6. Luan,Y.T., Mutharasan,R., Magee,W.E.: Effect of various glucose/glutamine ration on hybridoma growth, viability and monoclonal antibody formation. Biotechnol Lett **9**, 535 (1987).

7. Dalili,M., Sayles,G.D., Ollis,D.F.: Glutamine-limited batch hybridoma growth and antibody production: experiment and model. Biotechnol Bioeng **36**, 74 (1990).
8. Flickinger,M.C., Goebel,N.K., Bibila,T., Boyce-Jacino,S.: Evidence for post-transcriptional stimulation of monoclonal antibody secretion by L-glutamine during slow hybridoma growth. J Biotechnol **22**, 201 (1992).
9. Jeong,Y-H., Wang,S.S.: Role of glutamine in hybridoma cell culture: Effects on cell growth, antibody production, and cell metabolism. Enzyme and Microbial Technology **17**, 47 (1995).
10. Hencsey,Zs., Inzelt-Kovács,M., Szabó,Gy., Bánkúti,L.: B sejtes hibridoma szaporodásának és ellenanyagtermelésének vizsgálata szuszpenziós kultúrában. Acta Biologica Debrecina Supplementum 241 (1992).
11. Patterson,M.K.jr.: Measurement of growth and viability of cells in culture. Methods in Enzymology. Academic Press Inc., New York **58**, 141 (1979).
12. Marsh,W.L.: Scoring of haemagglutination reactions. Transfusion **12**, 352 (1972).
13. Eto,N., Yamada,K., Shito,T., Shirahata,S., Murakami,H.: Development of a protein-free medium with ferric citrate substituting transferrin for the cultivation of mouse-mouse hybridomas. Agric Biol Chem 55, 863 (1991).
14. Hiller,G.W., Clark,D.S., Blanch,H.W.: Transient responses of hybridoma cells in continuous culture to step shanges in amino acid and vitamin concentrations. Biotechnol Bioeng **44**, 303 (1994).
15. Oh,S.K.W., Vig,P., Chua,F., Teo,W.K., Yap,M.G.S.: Substantial overproduction of antibodies by applying osmotic pressure and sodium butyrate. Biotechnol Bioeng **42**, 601 (1993).
16. Chua,F.K.F., Yap,M.G.S., Oh,S.K.W.: Hyper-stimulation of monoclonal antibody production by high osmolarity stress in eRDF medium. J Biotechnol **37**, 265 (1994).
17. Marshall,C.L., Boraston,R., Brown,M.E.: The effect of osmolarity on hybridoma cell growth and antibody production in serum-free media. In Spier,R.E., Griffiths,J.B., Meignier,B. (eds): Production of Biologicals from Animal Cells in Culture. Butterworth, London 1991. p. 259.
18. Ozturk,S.S., Palsson,B.O.: Effect of medium osmolarity on hybridoma growth, metabolism and antibody production. Biotechnol Bioeng **37**, 989 (1991).
19. Reddy,S., Bauer,K.D., Miller,W.M.: Determination of antibody content in live versus dead hybridoma cells: analysis of antibody production in osmotically stressed cultures. Biotechnol Bioeng **40**, 947 (1992).

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EFFECT OF MICROWAVES ON SURVIVAL OF SOME BACTERIAL STRAINS

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While the inhibitory effect of microwave radiation on microorganisms is being researched intensively, how microwave radiation brings about this effect has been a matter of discussion. Some researchers support that this effect is of a thermal character, whereas some others maintain a non-thermal effect. In this work, 1 ml suspensions of *Pseudomonas aeruginosa*, *Pseudomonas acidovorans*, *Staphylococcus aureus* and *Staphylococcus epidermidis* bacteria were subjected to microwave radiation at 2450 MHz and 550 Watts for periods of 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 25 and 30 seconds. When each result was compared with the CFU/ml results obtained from unradiated control group bacterial suspensions derived from stock cultures, significant conclusions were attained ($P<0.001$). The same experiments were repeated with the application of conventional heating. The difference between the CFU/ml values of similar bacterial suspensions subjected to microwave radiation and conventional heating was significant ($P<0.001$). Concurrently, the fact that the effect was exacerbated upon increasing of liquid volume during the application of microwave radiation was established via the results obtained through the application of microwave radiation to 1 ml and 5 ml bacterial suspensions ($P<0.001$).

The spread of the use of microwaves in parallel with the development of science and technology has directed researchers towards work on the biological effects of microwaves [1–5]. Microorganisms were widely used in experiment conducted on the biological effects of microwaves in connection with reproduction because of the ease of observation of their enzymatic activities and reproductive characteristics. Microwaves have also been used in the field of sterilization as a result of its inhibitory effect on microorganisms [6–8]. While some researchers support that inhibition of microorganisms is a result of the heat rise stemming from the transfer of thermal effect due to microwave radiation, other researchers deny the direct effect of thermal heat on the reproductive

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characteristics of microorganisms, and suggest some theories claiming that microwaves affect the nucleic acid, protein, and the protein-lipopolysaccharide compound of cell membranes [9–12]. However, it has been reported that microwaves inhibit microorganisms only in the presence of water, and that dry or lyophilized organisms are unaffected by even extended exposures [13, 14]. This work deals with the effects of microwave radiation at 2450 MHz frequency and 550 W power applied for various lengths of time on the reproductive characteristics of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Pseudomonas acidovorans*, and with a comparative analysis incorporating the effects of the application of conventional heat on the reproduction of these microorganisms.

Materials and methods

Bacterial strains. All throughout the work, 4 bacterial strains were used: *S. aureus* (ATCC 25923) was obtained from the Ankara Institution of Protection of Health (Hifizisihha), *S. epidermidis* (F11, powerful slime positive) from Dr. Roger Bayston of Nottingham City Hospital, *P. aeruginosa* and *P. acidovorans* from the Microbiology Laboratory of the Faculty of Medicine of Nottingham City Hospital (England).

Source of irradiation. In this work, a touchmatic microwave oven operating at a frequency of 2450 MHz and a power range of 55 W to 550 W (Imperial V8505 T) was used.

Application of conventional heat. A Benmari apparatus of no. 68/385 produced by Baird and Tatlock Ltd. (London) was used.

Thermometer. A Jenway 2103 digital thermometer was used. Throughout the work, the intratube temperatures of 1 ml bacterial solutions were measured pre- and post-experiment with this instrument.

Growth media. Blood agar plates were used for *S. aureus* and *S. epidermidis* whereas for *P. aeruginosa* and *P. acidovorans*, EMB (Eosin Methylene Blue, Oxoid CM69) agar plates were employed.

Experimental design. 1. Exposure of bacterial suspensions to microwaves. One hundred ml stock cultures of each bacteria were prepared. After the colony count was effected via the cast plate method, and the number of live bacteria (CFU/ml) per each ml of these stock cultures was determined [15] the number of live bacteria in each ml was estimated according to the following formula:

$$\text{CFU / ml} = \frac{\text{No. of colonies per 0.1 ml} \times \text{volume of solution}}{\text{degree of dilution} \times 0.1}$$

Subsequently, 1 ml aliquots of bacterial suspensions taken from these stock solutions were exposed to microwave radiation for 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 25, and 30 s at 550 Watts. Following the exposures, each 1 ml bacterial suspension was duly diluted and placed for culture in appropriate plates at 37 °C for 24 h. Following the determination of colony counts on disks on which reproduction was observed, the number of live bacteria (CFU/ml) per milliliter was assessed using the above formula. Throughout the study, prior to and following each microwave application, the intra-tube temperatures of bacterial suspensions were measured. The experiments were repeated 5 times for each bacteria and each duration of exposure. The average live bacterial count obtained in the 5 experiments was accepted as the result. The results were compared with the live bacterial count obtained from unexposed control group bacterial suspensions derived from stock cultures.

2. Conventional heat application. This experiment was conducted for the purpose of comparing the effects of exposure to microwave radiation and conventional heat. The experiments were effected for durations of 16, 20, 25 and 30 s and without varying the volumes of bacterial suspensions. The reason for the choice of these time periods was that the live bacterial count per ml had been observed to exhibit a significant drop during the application of conventional heat set to match the intra-tube temperature measured during each period of exposure to microwave radiation (16 s 85.1 °C, 20 s 91 °C, 25 s 91.3 °C, 30 s 92 °C). The recovery of live bacteria after application of 30 s of conventional heat indicated an addition of 1 and 2 min application to this set of experiments. For this 2-min period, the interior heat of 92 °C was measured at 30 s of exposure to microwave radiation.

Results obtained were compared with the live bacterial count per ml of suspensions exposed to microwave radiation for the same duration.

3. Increase of volume. Throughout the experiment, while the 4 bacterial suspensions were exposed to microwave radiation at 1 ml volume, the experiments were repeated for 14, 16, 18, 20, 25 and 30 s periods with 5 ml bacterial suspensions. The live bacterial counts obtained were compared with the results obtained with 1 ml samples.

Results

The decrease in the live bacterial count of *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *P. acidovorans* exposed to microwave radiation at 2450 MHz for 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 25 and 30 s in relation to control samples (0 s) was found to be statistically significant through the use of variance analysis test ($P<0.001$). The graph for live bacterial count per ml plotted against the duration of exposure to microwave radiation is exhibited in Fig. 1.

The decrease in the live bacterial count of the above-mentioned bacteria exposed to conventional heat against time and temperature, was found to be statistically significant when compared to the live bacterial count obtained during a series of experiments involving exposure to microwave radiation (Students' *t* test, which effects the comparison between pairs, $P<0.001$). Contrary to the findings of exposure to microwave radiation, as reproduction was observed at 30 s, this series of experiments were extended to periods of 1 and 2 min. The decline in the live bacterial count against time of bacterial suspensions exposed to conventional (thermal) heat is exhibited in Fig. 2.

In order to assess how the increase of liquid volume affects the live bacterial count per ml, the results of exposure to microwave radiation of 5 ml samples of *S. aureus* and *P. aeruginosa* for durations of 14, 16, 18, 20 and 30 s were compared with results obtained for 1 ml samples. The increase of liquid volume exacerbated the destructive effects of microwave radiation on live bacteria. In the statistical comparison effected through variance analysis test, the results were found to be significant ($P<0.001$) (Fig. 3).

During all microwave exposures, temperatures of bacterial suspensions were measured pre- and post-experiment, and the rise in temperature observed subsequent to exposure to microwave radiation plotted against time is displayed in Fig. 4. In Fig. 4, the arithmetic mean of the temperatures of the bacterial suspensions prior to exposure were taken as the starting point.

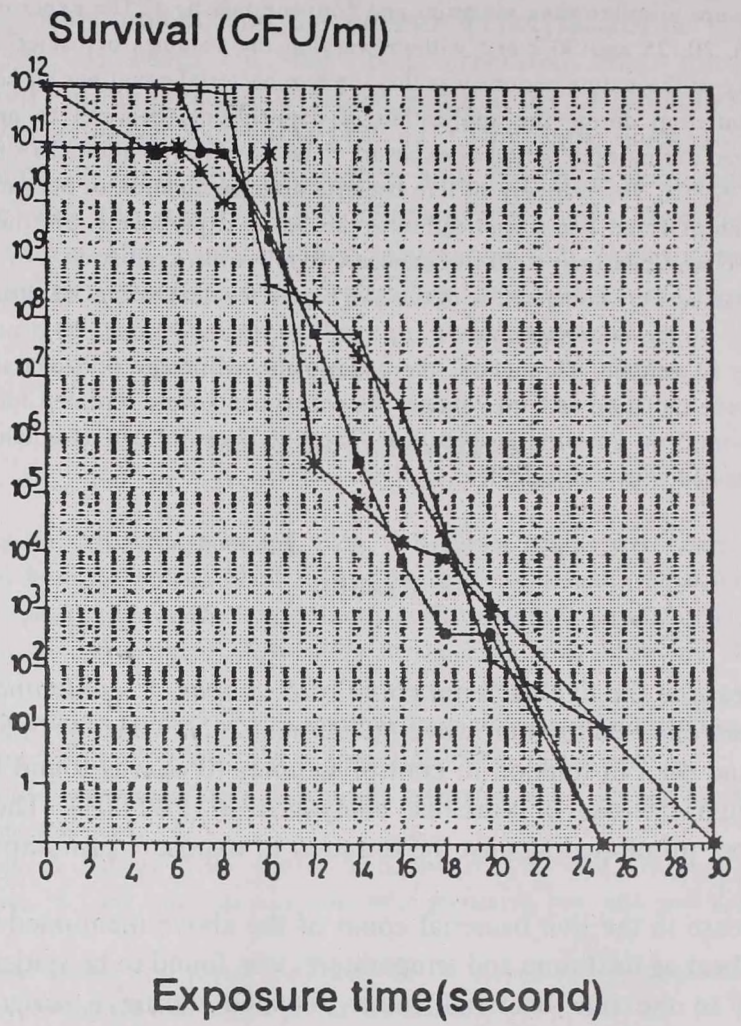

Fig. 1. The graph of 4 bacteria with live bacteria count per ml plotted against the duration of exposure to microwave radiation. • *S. aureus*, + *S. epidermidis*, * *P. aeruginosa*, ■ *P. acidovorans*

Discussion

Some researchers, assumed that microwave radiation is a form of energy different from heat [9]. The studies concentrated on the mechanism of inhibition of bacteria by microwave radiation. Besides researchers who uphold that the effect of microwave radiation on bacteria is directly of thermal character, others refuse the direct effect and claim that microwave radiation affects the nucleic acid, protein and the protein-lipopolysaccharide compound of cell membrane of microorganisms, thus stopping the vital activities of bacteria [12]. Along with these, there are studies that present data on the fact that the volume of liquid present in the medium modifies the intensity of the effect of microwave radiation on bacteria [13, 14].

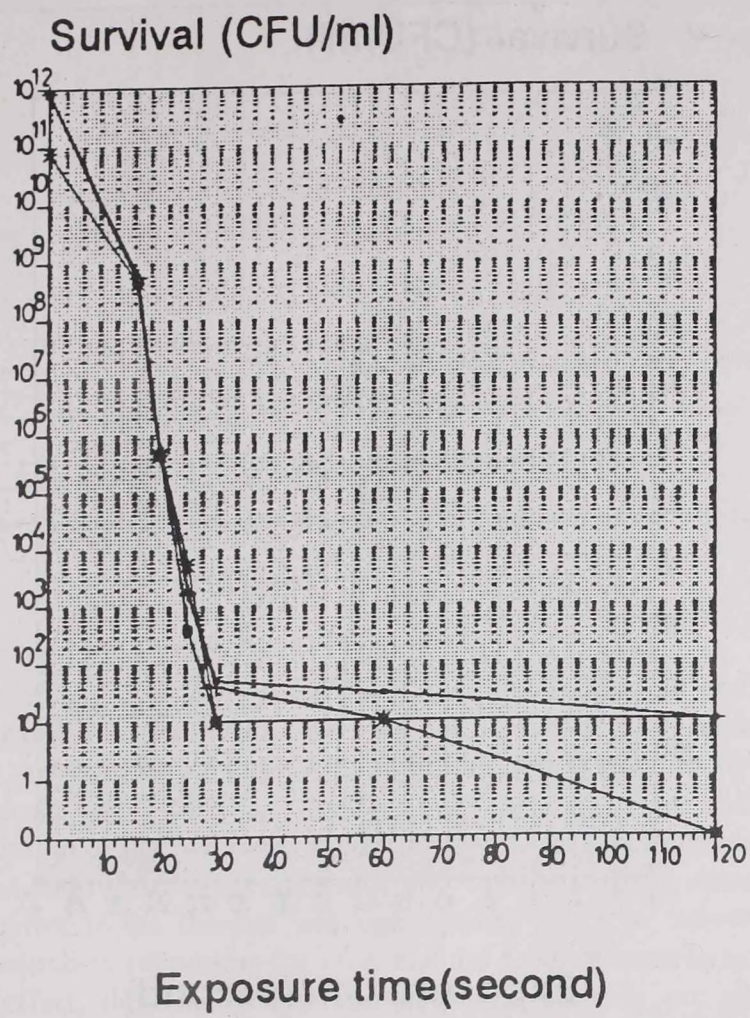

Fig. 2. The decline in the live bacterial count against time of bacterial suspensions exposed to conventional heating. • *S. aureus*, + *S. epidermidis*, * *P. aeruginosa*, ■ *P. acidovorans*

In our work, we have reached the conclusion that increasing the volume of liquid definitely increases the inhibitory effect of microwave radiation on bacteria. Another study, with findings supporting our work, was conducted by Vela and Wu [13]. They have reported, while studying the lethal effects of 2450 MHz microwave radiation on microorganisms, that in liquid media and in cases when the amount of liquid is increased, this effect is exacerbated. These workers have also pointed out, on the other hand, that there is little effect on dry or lyophilized microorganisms, indicating that the liquid in the media plays a major role in absorbing the energy from microwave radiation [13].

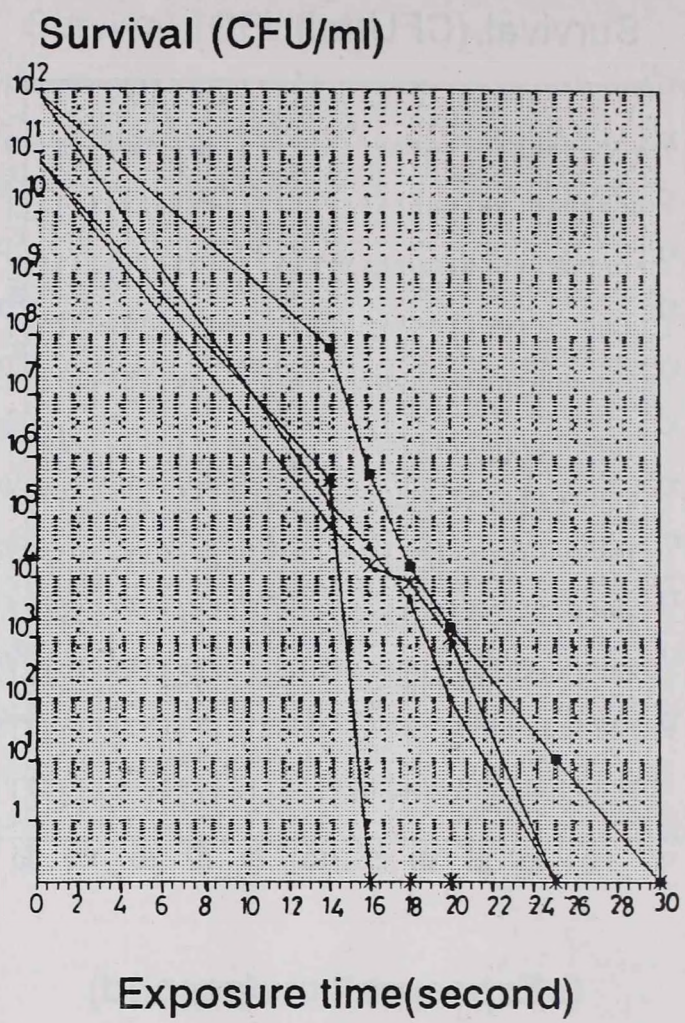

Fig. 3. Comparison of exposures of 5 ml and 1 ml volumes of *S. aureus* and *P. aeruginosa* for 14, 16, 18, 20 and 30 s. • *S. aureus*, + *S. epidermidis*, * *P. aeruginosa*, ■ *P. acidovorans*

Lin and Sawyer [16], covering the meat that they had infected with *S. aureus* (ATCC6538) and *E. coli* (O157:H7) bacteria with polyvinylidine chloride (PVDC), studied the lethal effect of microwave radiation at 2450 MHz, 713 and 356 W on these bacteria. The above-mentioned researchers conducted the same experiment on meat not covered with PVDC, and upon comparison of results, observed that the rate of inhibition of the bacteria in meat covered with PVDC was higher than that in meat which was not covered with PVDC. Lin and Sawyer [16] explaining the findings, pointed out that PVDC played an important role in the absorption, by meat, of the heat created by microwave radiation. Blevins et al. [12], studying the effects of microwave radiation on *Salmonella typhimurium* LT2 against time, found that it was completely inhibited at 2450 MHz in 23 seconds. They worked with suspensions of 2 ml all throughout his work. Although they

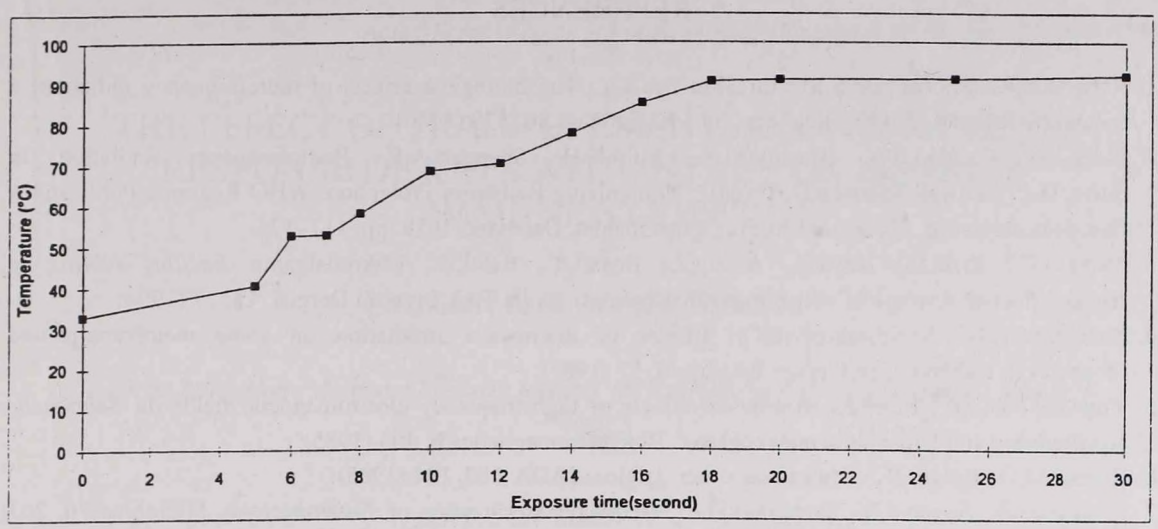

Fig. 4. The graph of temperatures measured in bacterial solutions plotted against time during exposure to microwave radiation

worked with a bacteria different from ours, we have noticed that the period required for inhibition in his work was less than the ones we needed for the bacteria in our study. We have determined that complete inhibition of *S. aureus* and *S. epidermidis* took 30 s and that *of P. aeruginosa* and *P. acidovorans* 25 s. We can explain the difference in time required for inhibition in the two experiments through both the employment of different strains of bacteria and our using samples of a low volume of 1 ml. Another area where discussion intensifies is the thermal and non-thermal effect of microwave radiation. Besides those researchers supporting the view that the death of bacteria occurs as a result of the thermal effect, there are those who hold that bacteria are inhibited because microwave radiation affects the dark repair zones and DNA SOS repair zones of bacteria, and that these are the zones responsible for the extension of the life spans of bacteria and thus the bacteria are inhibited [13, 17]. Dreyfus and Chipley [18], studying the enzymatic activities of *S. aureus* upon exposure to microwave radiation and thermal heat, have found that bacteria exposed to microwave radiation exhibit different enzymatic activity from *S. aureus* subjected to thermal heat.

Our work has lead us to the conviction that microwave radiation portrays a different effect in liquid media than thermal heat. As a conclusion, we can say that microwave radiation inhibits bacteria with an effect which is different from thermal heat, and that the amount of liquid in the medium plays an important role in the absorption of the energy created by microwave radiation.

REFERENCES

1. Roberts,N.R., Michaelson,S.M., Lu Shin-Tsu Lu.: The biological effects of radiofrequency radiation: a critical review and recommendations. Int J Radiat Biol **50**, 379 (1986).
2. Elder,J.A., Czerski,P.A., Stuchly,M.A., Mild,K.H., Sheppart,A.R.: Radiofrequency Radiation. In Suess,M.J., Benwell-Morison,D.A. (eds): Nonionizing Radiation Protection. WHO Regional Publications European series No. 25, second edition. Copenhagen, Denmark, 1989. pp. 117–174.
3. Aytekin,C., Kizil,M., Kaya,Z., Akdag,Z., Ensari,Y., Balci,K.: Mikrodalganin *Bacillus subtilis* ve *Escherichia coli* üzerindeki etkisinin karsilastirilmasi. Doga Türk Biyoloji Dergisi. **18**, 77 (1994).
4. Kazbekow,E.N., Vyacheslayev,L.G.: Effects of microwave irradiation on some membrane-related processes in bacteria. Gen Physiol Biophys **6**, 57 (1987).
5. Yngve,H., Asa,R., Bertil,R.: Biological effects of high-frequency electromagnetic fields on *Salmonella typhimurium* and Drosophila melanogaster. Bioelectromagnetics **6**, 405 (1985).
6. Rohrer,M.D., Bulard,R.A.: Microwave sterilization. JADA **110**, 194 (1985).
7. Rosaspina,S., Anzanel,D., Salvatorelli,G.: Microwave sterilization of *Enterobacteria*. Microbios **76**, 263 (1993).
8. Akdag,Z., Turfan,M., Aksen,F., Celik,S., Arikan,E., Dasdag,S.: Microwave sterilization of disposable plastic micropipet tips. Proceedings of the ISRAMT'95 (5th International Symposium on Recent Advances in Microwave Technology). Kiev, Ukraine, 525 (1995).
9. Chipley,J.R.: Effects of microwave irradiation on microorganisms. Advanc Appl Microbiol **26**, 129 (1980).
10. Culkin,K.A., Fung,D.Y.C.: Destruction of *E. coli* and *Salmonella typhimurium* in microwave-cooked soups. J Milk Food Technol **38**, 8 (1975).
11. Dardalhon,M., Averbeck,D., Bertaud,A.J.: Studies on possible genetic effects of microwaves in procariotic and eucariotic cell. Radiat Environ Biophys **20**, 37 (1981).
12. Blevins,R.D., Crenshaw,R.C., Hougland,A.E., Clark,C.E.: The effect of microwave radiation and heat on specific mutants of *Salmonella typhimurium* LT2. Radiation Research **82**, 511 (1980).
13. Vela,G.R., Wu,J.F.: Mechanism of lethal Action of 2450 MHz radiation on microorganisms. Appl Environ Microbiol **37**, 550 (1979).
14. Najdovski,L., Dragos,A.Z., Kotnik,V.: The killing activity of microwaves on some non sporogenic medically important bacterial strains. J Hosp Infect **19**, 239 (1991).
15. Bilgehan,H.: Bakteri sayma yöntemleri. Klinik Mikrobiyolojik Tani. I .Baski, Baris yayinlari. Ankara. 1992. pp. 121–134.
16. Lin,W., Sawyer,C.: Bacterial survival and thermal responses of beef after microwave processing. J Microwave Power and Electromagnetic Energy **23**, 183 (1988).
17. Rojavin,M.A., Ziskin,M.C.: Effect of milimeterwaves on survival of UVC-Exposed *E. coli*. Bioelectromagnetics **16**, 188 (1995).
18. Dreyfus,M.S., Chipley,J.R.: Comparison of effects of sublethal microwave radiation and conventional heating on metabolic activity of *Staphylococcus aureus*. Appl Environ Microbiol **39**, 13 (1980).

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THE EFFECT OF TRACE ELEMENTS ON THE IMMUNE RESPONSE OF RATS WITH INTACT OR INJURED IMMUNE SYSTEM

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We studied the effect of a trace element combination (Béres Drop Plus; BDP) on the immune response of rats, with intact and with injured immune system. Rats were treated with different doses of BDP for 26 days. The immune system was injured by 7.0 Gy whole body gamma irradiation on the first day of BDP treatment. Rats were immunized on the 21st day of the BDP treatment with sheep red blood cells. In the spleen the cell count and the number of antibody producing cells were determined. In the serum the titre of hemolysin was tested.

In rats with intact immune system all used doses of BDP induced elevation of the immune response. The moderate high doses were especially effective. In rats with injured immune system the effect was less. Only the 250 µl/kg body weight dose could elevate the immune response, and the highest (500 µl/kg) dose reduced the response.

The function of immune system (human and animal) can be influenced not only by generalized malnutrition, but also by select nutrient deficiencies [1–5]. The deficiency in zinc results in lymphoid atrophy and decreased polyclonal T cell activation, reduces the activity of mononuclear cells [1, 6]. Zinc supplementation normalizes the CD_4/CD_8 ratio and induces significant elevation of other lymphocyte subpopulation in humans, inducing immunomodulating effects [7]. Copper deficiency results in decreased antibody producing cell response and increased susceptibility to microorganisms [1, 5], furthermore, induced anaemia and splenomegaly altered the protein and lipid composition of lymphocyte plasma membranes of mice [1, 5, 8, 9]. Addition of zinc and copper to human peripheral mononuclear cells in the presence of endotoxin influenced the cytokine secretion, i.e. stimulated the TNF secretion. At the same culture condition copper inhibited the Il-1ß and Il-6 secretion, while zinc stimulated the Il-1 secretion and did not influenced the Il-6 production [10]. Experimental animals fed manganese deficient diets showed decreased antibody production [5].

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Ionizing radiation destroys the immune system and hematopoiesis [11, 12]. The destruction appears in the spleen, manifesting among others in the reduction of spleen weight [11, 13] and also in the decrease of antibody production [12, 14]. To restore the decrease of the immune response induced by sublethal irradiation, seems to be very difficult [12, 14]. The antibody production can be enhanced by bacterial lipopolysaccharide only following a longer period after the irradiation. Because of the difficulties in enhancing of the immune response following irradiation, all possibilities of enhancement must be tested. Such a possibility suggests the use of trace element or much rather of combination of trace elements. The supplementation of trace elements in form of a mixture seems to be useful, because combined deficiency of trace elements often appears. The Béres Drop Plus enhances the reticulo-endothelial function in rats [15] and the Il-6 production in human monocytes [16].

The aim of the present work was to test the effect of different doses of trace element mixture on the immune response of rats, with intact and injured immune system.

Materials and methods

Animals. In the experiments Wistar Rats (LATI, Gödöllő) of both sexes were used. Animals (5 rats per each boxes) were kept in plastic boxes of 30×40 cm size, on softwood shavings. Animals were fed with pelleted rat-chow (LATI, Gödöllő) and water ad libitum. Rats used in this study were maintained and used in accordance with the Guidelines for Care and Use of Animals in Scientific Research of the Hungarian Academy of Sciences, Budapest, Hungary.

Trace element treatment. The trace element preparation Béres Drop Plus (BDP, Béres Inc., Budapest, Hungary) contains the following inorganic components in coordinative binding to organic molecules in mg/60 drops (18 drops=1 ml): Fe (6.70), Zn 3.79), Na (2.14), Mg (1.35), Mn (1.02), K (0.924), Cu (0.848), Mo (0.634), V (0.406), Ni (0.362), B (0.35), F (0.301), Cl (0.099), Co (0.083). Organic components (mg/60 drops): glycerol (20.0), L-(+)ascorbic acid (10.0), EDTA (7.85), glycin (7.67), L-(+)tartaric acid (5.21), succinic acid (1.67), 2,4,5,7-tetraiodofluorescein (0.0159), dissolved in water.

Animals were treated with different dilutions of BDP for 26 days. The dilutions calculated pro kg were the followings: 500.0 µl/kg, 250.0 µl/kg, 125.0 µl/kg or 62.5 µl/kg. The used BDP doses/dilutions were prepared in distilled water. Rats were fed of the appropriate dilution 1 ml/100 g body weight. The calculated dose was given daily once through gavage, on the basis of the individual weight, corrected by the body weight weekly.

Other treatments. At the beginning of the BDP treatment rats were treated with 7.0 Gy ^{60}Co gamma whole body irradiation in Gammatron-3 source (air-dose-rate:0.5202 Gy/min). On the 21th day of the treatment animals were immunized with 4×10^8 sheep red blood cells (SRBC) intraperitoneally.

Determination of antibody production. On the fifth day of the immune response, i.e. on the 26th day following the beginning of BDP treatment and irradiation, the animals were sacrificed. Spleen cell suspensions were prepared from each animal in TC 199 tissue culture medium. The number of antibody producing cells (PFC) was counted according to the method of Jerne et al. [17]. Briefly, 0.1 ml of spleen cell suspension and 0.1 ml of 20% suspension of SRBC was mixed with agarose suspension in TC 199 tissue culture medium at 49 °C, then placed on a preformed agar layer. Plates were incubated at 37 °C for 1 h without complement, and for an other hour with complement. This incubation was followed for 1 h at room temperature then to the next morning at 4 °C, when the number

of PFCs on the plates were determined. Results were calculated on the basis of number of PFCs per million spleen cells and of nucleated cell count in the whole spleen.

Haemagglutinin and haemolysin titres of the serum samples were determined by micromethod of Takátsy [18]. Briefly, twofold serial dilutions were prepared from the serum samples in microtiter plates and equal volumes of SRBC suspension and with or without complement were added and incubated at 37 °C for 1 h.

Statistical analysis. Mean value ± SE and spleen index (spleen weight mg/body weight g) were calculated. Significance was calculated by Student's t test.

Results

Body weight, spleen weight and the spleen index of mice treated with Béres Drop Plus. The body weight was unchanged or moderately altered in both groups of rats, i.e. in the normal and in the irradiated ones. The spleen weight does not show parallelism with the body weight (Table I). In rats with intact immune system the 250–62.5 µl/kg BDP doses induced moderate elevation of the spleen weight. The irradiation and the highest, 500 µl/kg dose of BDP together induced a pronounced (34%) decrease, the 125 and 62.5 µl/kg doses induced only a moderate decrease of the spleen weight (Table I). In normal animals the elevation of spleen weight manifests itself in the elevation of spleen index. In the irradiated rats the spleen weight and the spleen index showed an approximately parallelism (Table II).

The alteration of the number of cells in the spleen of BDP treated rats with intact or injured immune system. In rats with intact immune system, the BDP treatment altered the number of spleen cells moderately, by less than 10% (Table III). The relative density, the number of cells in one gram of spleen, was reduced by all doses of BDP, but the 250 µl/kg dose induced a 23%, 125 µl/kg dose a 19% reduction.

In the number of spleen cells the irradiation and BDP treatment resulted in 14% reduction following treatment with 125 µl/kg, and 31% following treatment with 62.5 µl/kg. The relative density of cells in the spleen was markedly reduced only by the 62.5 µl/kg dose (Table III).

The number of antibody producing cells, following treatment with BDP in intact and irradiated rats. The chronic treatment with BDP influenced the ratio of the number of antibody producing cells per million spleen cells, and their absolute number in the spleen.

In rats with intact immune system all doses of BDP induced elevation of antibody producing cells. The relative number was elevated by 250 µl/kg dose significantly ($P<0.05$), and by the dose of 125 µl/kg almost significantly ($0.1>P>0.05$; Fig. 1). Also, all doses induced elevation of antibody producing cells in the whole spleen, but this elevation was a little less marked than the elevation of the relative ones. In this parameter 250 µl/kg dose induced almost significant elevation ($0.1>P>0.05$; Fig. 2).

In the irradiated rats the effect of BDP treatment was moderate. The relative number of antibody producing cells was elevated by the dose of 250 (+13%) and 62.5 µl/kg doses (+30%). The used highest does, 500 µl/kg dose induced 24% decrease (Fig. 3). In the whole spleen only the 250 µl/kg dose induced 54% elevation of antibody

producing cells. The highest 500 µl/kg dose induced 35% depression of antibody producing cells (Fig. 4).

The effect of BDP treatment on the serum antibody titre in rats with intact or injured immune system. In rats with intact immune system the 500 and 250 µl/kg doses, in irradiated rats only the 250 µl/kg dose could enhance the haemolysin titre in blood serum. The alterations observed in the haemolysin titre did not reflect in the haemagglutinin titre (Table IV). The IgG level was markedly elevated (23%) only in irradiated rats treated with 250 µl/kg BDP treated rats (data not seen).

Table I

The effect of Béres Drop Plus on the body weight and spleen weight of rats with normal or injured immune system

Group	n	Body wt. (g)	%	Spleen wt. (mg)	%
Normal immune system					
control	6	203.12±4.81	100	463.94±21.43	100
BDP 500 µl/kg	6	204.00±18.86	100.43	495.24±37.02	106.75
250 µl/kg	6	202.50±11.53	99.69	564.72±46.86	121.72
125 µl/kg	6	190.83±9.61	93.95	517.32±47.01	111.51
62.5 µl/kg	6	199.17±6.76	98.05	557.97±57.97	120.27
Injured immune system					
control	17	247.06±10.53	100	864.28±79.72	100
BDP 500 µl/kg	14	264.29±11.71	106.97	746.79±48.50	66.41
250 µl/kg	13	253.08±13.10	102.44	895.05±116.00	103.56
125 µl/kg	15	150.33±11.50	101.33	786.87±76.02	91.04
62.5 µl/kg	12	220.83±15.40	89.34	749.94±53.15	86.77

Rats were treated with different doses of Béres Drop Plus (BDP) for 26 days. The immune system was injured with 7.0 Gy whole body gamma irradiation at the beginning of the BDP treatment and immunized with SRBC before the end of the experiment

Discussion

From the literature it is well known, that the trace element deprivation depress the function of immune system [1–5] and the supplementation can correct the negative consequence in human and animal [19, 20]. The trace element supplementation can further influence the toxicity of heavy metals [21, 22].

Table II

The effect of Béres Drop Plus on the spleen index of normal and irradiated rats

Group	n	Spleen index*	%
Normal immune system			
control	6	2.28±0.08	100
BDP 500 µl/kg	6	2.43±0.08	106.57
250 µl/kg	6	2.79±0.15	122.26
125 µl/kg	6	2.69±0.15	118.05
62.5 µl/kg	6	2.81±0.23	123.18
Injured immune system			
control	17	3.64±0.39	100
BDP 500 µl/kg	14	2.91±0.24	79.89
250 µl/kg	13	3.68±0.51	101.10
125 µl/kg	15	3.16±0.26	86.79
62.5 µl/kg	11	3.63±0.40	99.70

*Spleen index = spleen weight (mg)/body weight (g). Other details, see Table I

Table III

The effect of Béres Drop Plus on the spleen cell number of normal and irradiated rats

Group	n	$\times 10^7$ spl.c/spl*	%	$\times 10^7$spl.c./g**	%
Normal immune system					
control	6	18.23±1.56	100	39.20±2.71	100
BDP 500 µl/kg	6	17.32±1.87	94.50	35.61±1.87	90.85
250 µl/kg	6	16.68±1.11	91.48	30.15±2.28	76.93
125 µl/kg	6	16.82±1.76	92.23	31.87±1.61	81.32
62.5 µl/kg	6	19.62±1.05	107.76	35.55±2.37	90.71
Injured immune system					
control	17	17.12±2.01	100	20.85±2.14	100
BDP 500 µl/kg	14	16.09±1.53	93.98	22.45±2.39	107.66
250 µl/kg	13	18.17±2.89	106.13	20.04±2.35	96.1
125 µl/kg	15	14.82±1.62	86.57	20.39±2.48	97.77
62.5 µl/kg	11	11.87±1.89	69.33	15.80±2.02	75.8

* spl.c./spl. = spleen cell/spleen

** spl.c./g = spleen cell/gram; other details see Table I

Table IV

The effect of Béres Drop Plus on the haemolysin and haemagglutinin titre in blood serum of rats with intact or injured immune system

Group	n	Haemolysin (2^{-n})	Haemagglutinin (2^{-n})
Normal immune system			
control	6	9.72±0.31	6.00±0.18
BDP 500 µl/kg	6	10.89±0.64	6.18±0.28
250 µl/kg	6	10.34±0.42	6.44±0.25
125 µl/kg	6	9.87±0.51	6.19±0.46
62.5 µl/kg	6	9.94±0.68	5.72±0.39
Injured immune system			
control	17	4.85±0.37	3.61±0.17
BDP 500 µl/kg	14	4.69±0.38	3.73±0.16
250 µl/kg	13	5.23±0.32	3.67±0.24
125 µl/kg	15	4.83±0.20	3.60±0.18
62.5 µl/kg	12	4.40±0.51	3.69±0.29

Details, see Table I

In the experiments we wanted to test the effect of different doses of trace element mixture on the immune response in normal animals. It was also interesting, whether or not the damaging effect of sublethal ionizing radiation could have been repaired by BDP.

The BDP treatment did not influence the body weight (Table I). The spleen weight and the spleen index (Tables I and II) were moderately elevated. In the number of spleen cells (Table III) exhibited a moderate decrease. The density of cells (Table III) was also by all BDP doses reduced. The absolute number of spleen cells and their density decreased differently. From this seems that the BDP treatment had general effect on the spleen. The decrease of the number of cells in the spleen may be a consequence of a cell efflux.

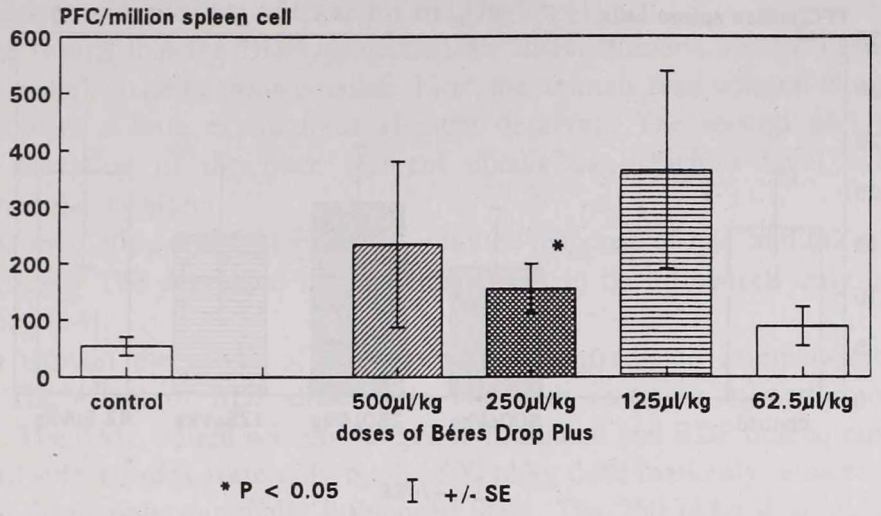

Fig. 1. The relative number of antibody producing cells (PFC/million cells) in the spleen of rats treated with different doses of Béres Drop Plus (BDP) chronically. Animals were treated with BDP for 26 days and immunized with sheep red blood cells (SRBC) on the 21st day of the treatment. The number of PFC was determined by the plaque method according to Jerne et al. [17]

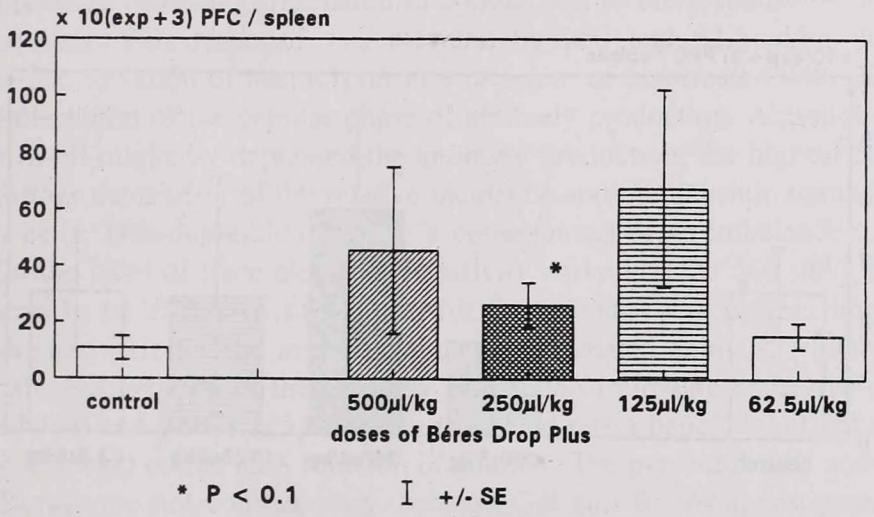

Fig. 2. The whole number of PFC in the spleen of rats treated with different doses of BDP chronically. Details, see Fig. 1

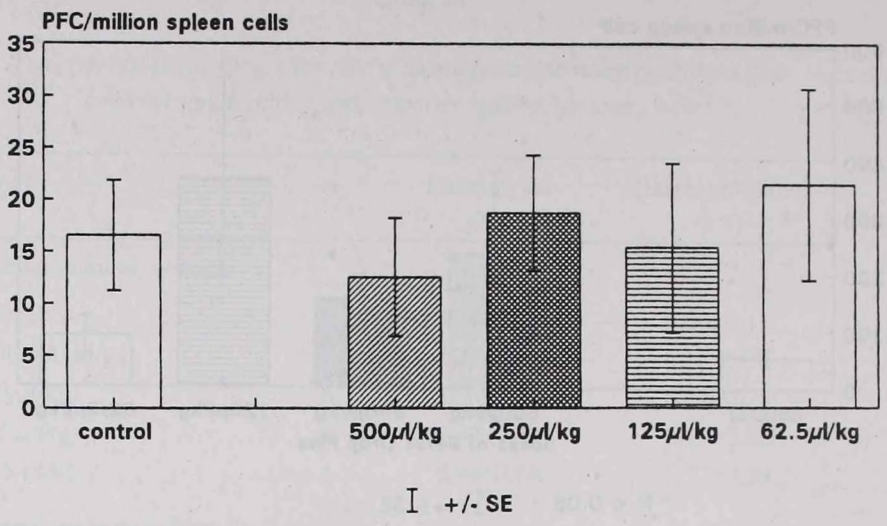

Fig. 3. The relative number of antibody producing cells (PFC/million cells) in the spleen of irradiated rats treated with different doses of Béres Drop Plus (BDP) chronically. Animals were treated with BDP for 26 days and treated with 7.0 Gy ^{60}Co gamma irradiation on the 1st day of the treatment. They were furthermore immunized with sheep red blood cells (SRBC) on the 21st day of the treatment. The number of PFC was determined by the plaque method according to Jerne et al. [17]

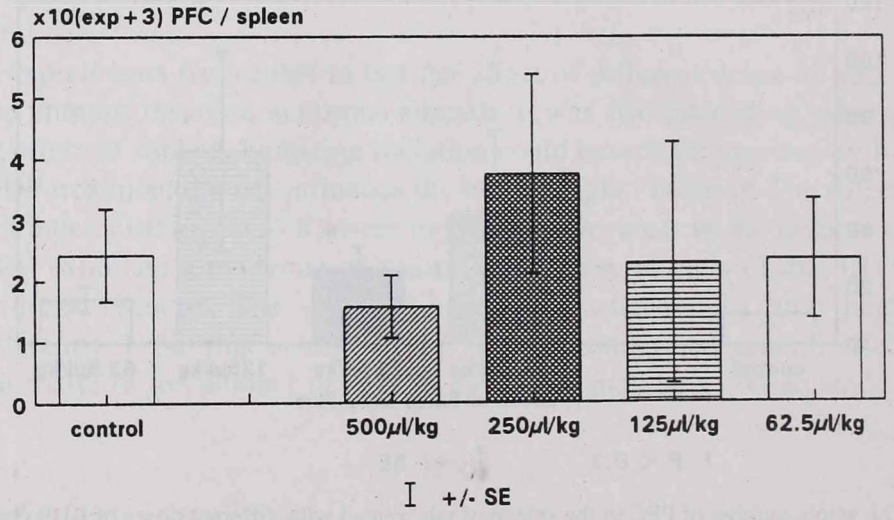

Fig. 4. The whole number of PFC in the spleen of irradiated rats treated with different doses of BDP chronically. Details, see Fig. 1

From the used doses the 250 µl/kg dose elevated the spleen weight, most markedly influenced the cell number and environment and the antibody production, although all doses elevated the antibody production (Fig. 2). This makes probable that this dose was

optimal in our experimental conditions, and the other doses were suboptimal. This result is in agreement with the data of Chandra and Dayton [1].

The results that the BDP influenced the intact immune system (Tables I, II, III, Figs 1, 2) make two deductions possible. First, the animals feed with rat chow from LATI (Gödöllő) were a little extent trace element deprived. The second possibility, that a moderate elevation of the trace element uptake can reach a level, which has an immunomodulatory effect.

The irradiation deeply depress the immune response of rats, and the regeneration is a slow process. The depressed immune response can be influenced only on a stage of regeneration [14].

To explain the effect of BDP in rats with irradiated immune system is very difficult. The effect of BDP differed in rats with intact or injured immune system markedly. The body weight was unchanged in irradiated and BDP treated rats. The spleen weight and spleen index were only by the 500 µl/kg dose markedly reduced, the 125 and 62.5 µl/kg doses only minimally influenced them. The 250 µl/kg dose did not alter the spleen weight and spleen index (Tables I and II). The number of spleen cells was reduced by all doses except 250 µl/kg (Table III). The irradiation in itself markedly reduced the ratio of cells and connective tissue in the spleen. From the used BDP doses the 62.5 µl/kg dose induced further reduction of cell density. The alteration of cell density may be partly consequence of cell efflux, as it was supposed in the case of intact immune system, or partly consequence of the fibrosis of spleen as a biological effect of irradiation.

The relative and also the absolute number of antibody producing cells were markedly reduced following irradiation in comparison to the response of rats with intact immune system. This response was elevated by 250 and 62.5 µl/kg doses of BDP treatment. The elevation of haemolysin titre in serum of rats treated with 250 µl/kg BDP was a manifestation of the cellular phase of antibody production. Although the sublethal irradiation itself markedly depressed the antibody production, the highest 500 µl/kg dose induced further depression of the relative incidence and the absolute number of antibody producing cells. This depression may be a consequence of an imbalance in the immune system. On the basis of trace elements effectivity curve [1], the 500 µl/kg dose of BDP, which seems to be excessive suboptimal for the animals with normal immune system, may be already toxic for the immune system in irradiated animals. Mulhern et al. [23] observed also suppression of the antibody production following excessive dietary intake of zinc. But Gál and Bertók [15] found in irradiated rats a beneficial effect of BDP (2 ml per rat for 4 weeks) on the RES function of animals. The present results and the results of Gál and Bertók are not contradictory, because Gál and Bertók investigated the in vivo phagocytic function of macrophages, whereas in the present work the number of antibody producing cells and the produced antibody in the circulation were tested. In the process of antibody production the macrophages are involved, but their function is only part of the whole process. The alteration of the phagocytic function and of the antibody production should not be parallel.

REFERENCES

1. Chandra,R.K , Dayton,D.H.: Trace element regulation of immunity and infection. Nutr Res **2**, 721 (1982).
2. Chandra,R.K.: Effect of macro- and micronutrient deficiencies and excesses on immune response. Food Technology **39**, 91 (1985).
3. Keen,C.L., Gershwin,M.E.: Zinc deficiency and immune function. Ann Rev Nutr **10**, 415 (1990).
4. Hamdard,M.E., Vohora,S.B.: Medical elementology: Practical applications in Third International Congress on Trace Elements in Health and Disease. Cukurova Univ Adana, Turkey, 1989. pp. 1–32.
5. Gershwin,M.E., Hurley, L.: Trace metals and immune function in elderly. Comprehensive Therapy **13**, 18 (1987).
6. Kruse-Jarres,J.D.: The significance of zinc for humoral and cellular immunity. J Trace Elem Electrolytes Health Dis **3**, 1 (1989).
7. Lutz,G., Kreysel,H.W.: Selective changes in lymphocytic differentiation antigens in the peripheral blood of patients with alopecia areata treated with oral zinc. Z Hautkr **65**, 132 (1990).
8. Korte,J.J., Prohaska,J.R.: Dietary copper deficiency alters protein and lipid composition of murine lymphocyte plasma membranes. J Nutr **117**, 1076 (1987).
9. Lukasewycz,O.A., Prohaska,J.R.: The immune response in copper deficiency. Micronutrienta Immune Functions **587**, 147 (1990).
10. Scuderi Ph.: Differential effects of copper and zinc on human peripheral blood monocyte cytokine secretion. Cell Immunol **126**, 391 (1990).
11. Fritz-Niggli,H.: Strahlenbiologie, Grundlagen und Ergebnisse. Georg Thieme Verlag, Stuttgart 1959. pp. 290–301.
12. Simic,M.M., Slivic,V.S., Petrovic,M.Z., Cirkovic,D.M.: Antibody formation in irradiated rats. 1965 Boris Kidric Institute of Nuclear Sciences, Beograd. 16. Suppl. 1.
13. Maruyama,Y., Grider,C., Williams,A.: Combined chemotherapy-radiotherapy on spleen hypertrophy after C. parvum: Combined treatment with 5-FU, cyclophosphamide and radiation. Int J Radiat Biol **40**, 475 (1981).
14. Elekes,E., Merétey,K., Várterész,V.: Adjuvant effect of endotoxin on primary antibody formation in irradiated rats. Path Microbiol **37**, 302 (1971).
15. Gál,K., Bertók,L.: The effect of X-radiation on reticulo-endothelial system and its treatment with radiodetoxified-endotoxin and trace elements in rats. Acta Microbiol et Immunol Hung **41**, 457 (1994).
16. Falus,A.: Stimulatory effect of mixture of trace elements ("Béres Drops Plus") on the production of interleukin-6 in human monocytes and glia cells. In I. Pais (ed.): Proc 6th Int Symp on New Results in Res of Hardly Know ı Trace Elements and Element-interaction. University of Horticulture and Food Industry, Budapest 1994. p. 289.
17. Jerne,N.K., Nordin,A., Henry,C.: The agar plaque technique for recognizing antibody-producing cells. In Amos,B., Koprowski,H. (eds): Cell bound antibodies. Wistar Institute Press, Philadelphia 1963. pp. 109–125.
18. Takátsy,G.: The use of spiral loops in serological and virological micromethods. Acta Microbiol Acad Sci Hung **3**, 191 (1956).
19. Tanaka,Y.: A study on the role of zinc on the immune response and body metabolism – a contribution of trace elements. Kobe J Med Sci **35**, 299 (1989).
20. Tanaka,Y., Shiozawa,S., Morimoto,I., Fujita,T.: Zinc inhibits pokeweed mitogen-induced development of immunoglobulin-secreting cells through augmentation of both CD_4 and CD_8 cells. Int J Immunopharmacol **11**, 673 (1989).
21. Waalkes,M.P., Kovatch,R., Rehm,S.: Effect of dietary zinc on cadmium toxicity and carcinogenesis in the male Wistar [Hsd:9WI)BR] rat. Toxicol Appl Pharmacol **108**, 448 (1991).

22. Jackl,G., Reidel, G.: Bindungsverhalten von Cd und Zn an Darminhaltstoffen der Ratte bei Zn-armer Ernährung. In Mengen und Supraelementen. 10. Arbeitsstagung Herausgeber Fridrich-Schiller-Universität, Jena. 1990. pp. 16–23.
23. Mulhern,S.A., Vessey,R.A., Taylor,G.L., Magruder,L.E.: Suppression of the antibody response by excess dietary zinc exposure during certain stages of ontogeny. Proc Soc Exp Biol Med **180**, 453 (1985).

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HUMAN HERPESVIRUS 8 IN CLASSIC KAPOSI SARCOMA

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Recent studies suggest the role of a new human herpesvirus (HHV8) in the pathogenesis of different forms of Kaposi sarcoma (KS). In the present work we investigated the presence of HHV8 sequences in KS tumour tissues from patient with classic KS. Since clear evidences point to the role of immune suppression in the development of AIDS-associated KS or patients receiving immunosuppressive therapy, immunological investigations were also performed. We could show a highly consequent association of HHV8 sequences with classic KS in the large series of patients supporting our previous findings that this virus might be in some way involved in the pathogenesis of this tumour. In addition, immunological examination of the patients revealed a mild decrease in the CD4 positive cell number, a significantly reduced CD4/CD8 ratio, a diminished PHA reactivity and leukocyte migration factor production of lymphocytes. The changes observed in the present study are similar, but much less pronounced than those may be observed in HIV infection.

Kaposi sarcoma (KS) is a malignant, multicentral proliferation of capillaries and perivascular connective tissue cells of the skin and internal organs. Epidemiologic evidences indicate an infectious aetiology of the disease. Recently, herpesvirus-like sequences have been identified in skin tumours from patients with AIDS-associated KS using representational difference analysis [1]. This new herpesvirus was first descriptively named KS-associated herpesvirus and most recently designated as human herpesvirus 8 (HHVS) [2]. These HHV8 DNA sequences are homologous to the lymphotropic γ-herpesviruses Epstein–Barr virus and herpesvirus saimiri. We and others have shown that HHV8 DNA is also found in most cases of HIV-negative classic KS, as well as in KS occurring in patients receiving immunosuppressive therapy [3–5]. Although clear

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evidences point to the role of immune-suppression in the development of AIDS-associated KS or patients receiving immunosuppressive treatment, the data concerning the immune functions in classic KS are contradictory [6, 7]. In the present study we investigated paralelly the immune functions and the presence of HHV8 sequences in KS tumour tissues.

Materials and methods

Analysis of HHV8 sequences. DNA was obtained from the tissue blocks by proteinase-K digestion, phenol–chloroform extraction, and ethanol precipitation from formalin-fixed, paraffin-embedded tissue specimens of 14 patients with classic KS (11 non-homosexual men, 55–93 years; 3 females 51–79 years; all patients were HIV-negative and had not received any immunosuppressive therapy). The diagnosis was based on clinical and histopathological examination and was confirmed immunohistochemically where it was necessary. Biopsy of normal skin from a healthy person undergoing plastic surgery served as negative control. The quality of the extracted DNA was tested by amplifying the human β-globin gene as described [8]. DNA was analysed for the presence of HHV8 by PCR, using primers specific for this herpesvirus, which amplifies a 233 bp sequence designated the $KS330_{233}$ fragment as described by Chang et al. [1]. The PCR products were visualized with UV on 2% agarose gel stained with ethidium bromide. The amplification products were then transferred to a Hybond N+ membrane filter prehybridized in Rapid Hybridization buffer and subjected to Southern blot hybridization with a previously sequenced and P^{32}-labelled 233-bp KSHV probe obtained from KS tissue.

Immunological investigations. The number of CD3, CD4, CD8 positive T-cells was determined by flow cytometry using monoclonal antibodies. The functional activity of T-cells was investigated by measuring the H^3-thymidine incorporation of phytohaemagglutinin (PHA) stimulated lymphocytes and by the measurement of the leukocyte migration factor production of purified protein derivate (PPD)-stimulated mononuclear cells as described earlier [9, 10].

Results

The PCR reaction with a primer pair specific for a 268-bp region of the human β-globin gene was positive in all samples, indicating the absence of possible PCR inhibitors (not shown). With the primer pair specific for the $KS330_{233}$ sequence and subsequent Southern hybridization, we could detect the predicted 233-bp bands in each of the 14 KS lesions; Fig. 1 shows a representative Southern blot analysis. HHV8 sequences were not found in the normal skin used as negative control.

Table I shows the results of the immunological examinations. There was a slight, but not significant decrease in the number of CD3 positive T-lymphocytes. The ratio of the CD4/CD8 positive T-cells was significantly lower as compared to the normal range obtained from 30 healthy individuals. The functional damage of the lymphocytes was suggested by the significantly decreased PHA stimulation and PPD-induced migration factor production.

Table I

Immunological investigations in patients with classic Kaposi sarcoma

	Kaposi sarcoma (n=14)	Healthy controls (n=20)
CD3+ cells (%)	65.4 ± 5.1	78.3 ± 7.6
CD4/CD8 ratio	1.1 ± 0.3*	1.8 ± 0.3
PHA stimulation (cpm/10^6 cells)	10600 ± 4900*	20610 ± 5710
Mi (PPD)	0.92 ± 0.0S*	0.79 ± 0.12

PHA: phytohaemagglutinin, Mi (PPD): migration index obtained from purified protein derivate (PPD) stimulated leukocytes.
Data are expressed in mean ± SD, * $p<0.05$, Student *t*-test as compared to the controls

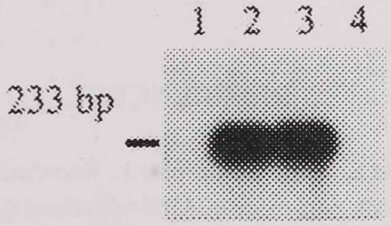

Fig. 1. Southern blot hybridization of the $KS330_{233}$ PCR products from tissue specimens from Kaposi sarcoma (KS) with P^{32}-labelled HHVS-probe. The $KS330_{233}$ PCR products were transferred to nitrocellulose filters and hybridization was performed with the previously cloned and sequenced HHV8 probe [5]. Line 1: negative control from normal skin sample, lines 2 and 3: specimens from KS tumours, line 4: water control

Discussion

The highly consequent association of HHV8 sequences with classic KS in the large series of patients reported here also supports our previous findings that this virus might be in some way involved in the pathogenesis of this tumour. Recent studies have shown that HHV8 DNA sequences are not specifically associated with KS, but these virus sequences can be found in an other malignant tumour of endothelial cells such as angiosarcoma of the face [11], and in both benign and malignant lymphoproliferative disorders such as multicentric Castleman disease and body-cavity-based B-cell lymphomas [12, 13]. The wide spectrum of disorders that has been shown to contain the HHV8 sequences suggests that the virus alone is not sufficient to produce a specific lesion, rather the virus might act

as a cofactor leading to immune disregulation and vascular proliferation. The immune status of the individual or infection with other herpesviruses might be another important factor in the development of a specific lesion. Although previously we detected immune deficiencies in patients with classic KS [6], the data concerning the immune functions in classic KS are contradictory. In the present study we have found a mild decrease in the CD4 positive cell number, a significantly reduced CD4/CD8 ratio, a diminished PHA reactivity and leukocyte migration factor production of lymphocytes. The changes observed in the present study are similar, but much less pronounced than those may be observed in HIV infection. The different forms of KS might, therefore be due to differences in the host immune protection. Solid tumours affecting only the skin are found in classic type of KS with mild immune deficiency, while more disseminated mucocutaneous tumours with internal organ involvement are associated with severe immune dysfunctions in AIDS. The importance of the immune system in HHV8 infection was further strengthened recently by Rady et al. [14], who detected HHV8 DNA sequences in different non-KS skin tumours of patients receiving immunosuppressive therapy, suggesting that HHV8 is a widespread latent virus activated by immunosuppressive conditions. Further investigations are necessary to establish the exact role of HHV8 and the immune functions in the pathogenesis of KS.

REFERENCES

1. Chang,Y., Cesarman,F., Pessin,M.S., Lee,F., Culpepper,J., Knowles,D.M., Moore,P.S.: Identification of a new human herpes virus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266**, 1865 (1994).
2. Levy,J.A.: A new human herpesvirus: KSHV or HHV8? Lancet **34**, 786 (1995).
3. Huang,Y.Q., Li,J.J., Kaplan,M.H., Poiesz,B., Katabira,E., Zhang,W.C., Feiner,D., Friedmann-Kien,A.E.: Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. Lancet **345**, 759 (1995).
4. Moore,P.S., Chang,Y.: Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. New Engl J Med **332**, 1181 (1995).
5. Kemény,L., Kiss,M., Gyulai,R., Dobozy,A., Ádám,É., Nagy,F.: Herpesvirus-like nucleic acid sequences in patients with Eastern European sporadic Kaposi's sarcoma. J Invest Dermatol **106**, 381 (1996).
6. Dobozy,A., Husz,S., Hunyadi,J., Simon,N.: Immune deficiencies and Kaposi's sarcoma. Lancet **ii**, 625 (1973).
7. Peterman,T.A., Jaffe,H.W., Friedmann-Kien,A.E., Weiss,R.A.: The etiology of Kaposi's sarcoma. Cancer Surv **10**, 23 (1991).
8. Resnick,R.M., Cornelissen,M.T.E., Wright,D.K. et al.: Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. J Natl Cancer Inst **82**, 1477 (1990).
9. Dobozy,A., Hunyadi,J., Simon,N.: Lymphocyte-transformation test in detection of drug hypersensitivity. Lancet **ii**, 1319 (1972).
10. Dobozy,A., Csató,M., Kenderessy,Sz.A., Hunyadi,J., Kemény,L., Simon,N.: Regulation of lymphokine production in peripheral blood mononuclear cell cultures. Experientia **38**, 505 (1982).
11. Gyulai,R., Kemény,L., Kiss,M.. Ádám,É.., Nagy,F., Dobozy,A.: Herpesvirus-like nucleic acid sequence in angiosarcoma in a patient without HIV infection. N Engl J Med **334**, 540 (1996)

12. Soulier,J.S., Grollet,L., Oksenhendler,E., Cacoub,P., Cazals-Hatem,D., Babinet,P., D'Agay,M.F., Clauvel,J.-P., Raphael,M., Degos,L., Sigaux,F.: Kaposi sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood **86**, 1276 (1995).
13. Cesarman,E., Chang,Y., Moore,P.S., Said,J.W., Knowles,D.M.: Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med **332**, 1186 (1995).
14. Rady,P.L., Yen,A., Rollefson,J.L., Orengo,I., Bruce,S., Hughes,T.K., Tyring,S.K.: Herpesvirus-like DNA sequences in non-Kaposi's sarcoma skin lesions of transplant patients. Lancet **345**, 1339 (1995).

Acta Microbiologica et Immunologica Hungarica, 43 (4), p. 397 (1996)

BOOK RECEIVED

J. Heritage, E. G. V. Evans, R. A. Killington: Introductory Microbiology. Studies in Biology, Cambridge University Press. 1996. 234 pp.
Price: £9.95 (paperback, ISBN 0 521 44977 4), £27.95 (hardback, ISBN 0 521 44516 7)

"Introductory Microbiology" by J. Heritage et al. from Department of Microbiology, University of Leeds gives a really good practical overview of bacteriology, mycology and virology.

I suggest and highly recommend this book for both students – who participate in practical courses of Microbiology – and their teachers. The book provides a perfect theoretical background for most of the material to do, study, investigate and manipulate during a laboratory practical of a Microbiology course. The basic things like safety rules, sterilization, disinfecting, microscopic morphology, making a smear, staining, cultivation, making single colonies, counting of bacteria etc. are just the same, no matter if medical, veterinary or food industry etc. microbiology practical course one is attending.

Hereby I would like to grab the chance to express my special thanks towards the authors for my favorite figure: Fig. 2.1 on page 71 – solving my forever problem with the student of two left hands...

I found the black and white drawings very good – giving a chance for the readers to colour by themselves, e.g. Fig. 1.13 or Fig. 1.17.

I found quite uncomfortable to turn pages on and back to see the figures and read the text. (e.g. Fig. 1.15 - page 40; Fig. 1.20 - page 47; Fig. 1.21 - page 49–50 etc.)

I guess this was more an editorial or make-up-in-slips problem and page number limitation than the authors intention.

I think it was an excellent idea to explain etymologically the microbiological terms, how they come from Greek and Latin words.

I wonder how this book can fit into the teaching programme and system of high/secondary school students in foreign countries. As for Hungary I know it can not be applied in the present system of general education, maybe only special fields, e.g. school for laboratory technicians. Therefore I recommend and suggest this book for sixth form (17–18 years old) students only, who participate in a high/secondary school system of Anglo-Saxon tradition.

The authors address this book to "non-specialist audience" as well. Reading through the book as a medical doctor, and specialist and teacher of medical microbiology, I have to say, that the "non-specialist audience" must have a basic knowledge in (bio)chemistry, immunology and biology (cell and molecular) before reading this book. E.g. I hardly believe that a "non-specialist audience" will understand the explanation of Salk and Sabin vaccination at page 150 without immunological and infectological background despite the best "Glossary" of the book I ever met.

Zsuzsa Berek

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SYMPOSIUM

ON THE

PATHOMECHANISMS AND PREVENTION OF VIRAL AND BACTERIAL DISEASES

SZEGED, MAY 29–JUNE 2, 1996

ABSTRACTS OF PAPERS AND POSTERS

Acta Microbiologica et Immunologica Hungarica, 43 (4), pp. 401–414 (1996)

E. DUDA[1], ZS. GYŐRFY[1], T. NAGY[1], L. VIGH[1], and B. MARESCA[2]

Use of cellular genes to enhance effectiveness of viral vaccines

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More and more data support the hypothesis that the existence of latent or chronic viral infections and the development of malignant tumours are not the results of insufficient immune responses, but rather the consequence of an inadequate immune response. Cells infected by certain DNA and RNA viruses and many tumour types produce immunomodulatory molecules (growth factors, cytokines, receptors of these and complement proteins, protease inhibitors, etc.) which inhibit the development of full scale, protective immune responses.

We made efforts to produce tumour cell lines expressing the genes of inflammatory cytokines (interferon gamma, interleukines, TNF, etc.) under the control of strong constitutive promoters. These cell lines exhibited unaltered growth characteristics in vitro, however their oncogenic potential was decreased or completely diminished when injected into experimental animals. Their presence elicited an effective cell mediated toxicity against both the modified and the parental tumour cells. Tumour cells producing the membrane form of TNF were capable to activate macrophages and monocytes even in cell cultures.

Previous studies on TNF sensitivity of target cells indicated that resistance of tumour cells to the cytotoxic action of TNF is determined by membrane fluidity. It is well known that the physical condition of membrane lipids is largely a property of their level of unsaturation and introduction of the "first" unsaturation bond has by far the greatest effect.

In order to elucidate the molecular basis of TNF sensitivity (and other fluidity-related phenomena), we established L929 mouse fibroblastoid cell clones which express the *OLE1* gene of *S. cerevisiae*, coding the Δ-9-desaturase. In these cells the yeast desaturase coding sequence is under the control of promoters derived either from wild type or Downs strains of the dimorphic fungus *Histoplasma capsulatum*.

Testing both the fatty acid composition (total lipids, phospholipids) and membrane microviscosity of control vs. transfected cells, the results confirmed that the manipulation was highly effective. Preliminary studies with the transfected clones fully supports the notion that membrane physical state controls the resistance of target cells to these cytokines. D/5, one of the L292 clones proved to be 100 times more sensitive to TNF as the parental cells.

We started experiments to construct virus strains which carry TNF and desaturase genes. We expect that the expression of these cellular genes would render the cells

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infected with these recombinant viruses highly immunogenic and very sensitive. These virus strains might serve as valuable starting material for the development of effective virus vaccines.

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Detection of human herpesvirus 8 DNA sequences in skin tumours

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Recent studies suggest the role of a new human herpesvirus (HHV8) in the pathogenesis of different forms of Kaposi's sarcoma (KS) and in both benign and malignant lymphoproliferative disorders such as multicentric Castleman disease and body-cavity-based B-cell lymphomas. In the present work we investigated the presence of HHV8 sequences in different skin turnout tissues from patient without HIV-infection. Polymerase chain reaction (PCR) followed by Southern blot analysis from paraffin embedded tissue specimens were performed from patients with different skin tumours. We could show that HHV8 DNA sequences are also present in endothelial cell-derived malignant tumours such as angiosarcoma of the face, and in a rare benign tumour of atypical endothelial cells in patients with angiolymphoid hyperplasia with eosinophilia. HHV8 sequences could not be detected in specimens with basalioma, squamous cell carcinoma, pyogenic granuloma or haemangioma. The broad spectrum of disorders that has been demonstrated to contain the HHV8 sequences suggests that the virus alone is not sufficient to produce a specific lesion; it might rather be the trigger of a complex reaction. The role of HHV8 in inducing proliferative diseases demand further elucidation.

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Relevance of ICAM-1 to inflammatory diseases

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Intercellular adhesion molecule-1 (ICAM-1) is a cytokine inducible adhesion molecule expressed on cells of multiple lineages at the site of inflammation. Shedding of ICAM-1 from these cells is enhanced by proinflammatory cytokines such as interferon, TNF or IL-1. The elevated levels of these soluble adhesins in serum of patients with inflammatory diseases or with tissue injury may therefore provide some diagnostic or prognostic information. The aim of the study was therefore to investigate the serum sICAM-1 level of septic patients, and of patients with alcoholic liver cirrhosis.

In septic patients a positive correlation was observed between serum IL-6 level and the soluble ICAM-1 level ($r=0.6837$, $p<0.001$) The circulating sICAM-1 level was significantly higher (1000±200 ng/ml) than in normal controls (250±125 ng/ml) The administration of pentoxifylline resulted in a rapid decrease in sICAM-1 level, and it decreased parallel with a decrease of APACHE score of patients. Although the vascular endothelium could be the main source of ICAM-1, flow cytometric (FACS) analysis revealed that the expression of ICAM-1 on monocytes of septic patients was higher than that of normal blood donors.

Elevated serum concentration of sICAM-1 was observed in 25 patients with alcoholic liver cirrhosis and these were strongly related to the actual activity of liver disease and antecedent alcohol consumption In alcoholic liver cirrhosis an increased TNF and IL-6 producing capacities of the peripheral leukocytes was observed. This overresponsiveness in cytokine secretion may be involved in the elevation of the sICAM-1 in the serum and in the pathogenesis and perpetuation of alcoholic liver damage. Proliferating bile ducts and parenchymal cells of cirrhotic livers showed positive ICAM-1 immunoreaction in 3 autopsy cases.

It is concluded that monitoring of the serum ICAM-1 level might be informative not only in sepsis but also in liver diseases.

K. MEGYERI, I. ROSZTÓCZY, Y. MÁNDI, T. LÉVAI and D. VIRÓK

Induction of interferon and Interleukin 6 by different *Staphylococcus* strains in human leukocytes

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The aim of our studies was to compare the interferon (IFN) and interleukin 6 (IL-6) inducing activities of different *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* strains.

Heat killed bacterial suspensions were added to cultures of human mononuclear cells (MNC) isolated by density gradient centrifugation. MNC cultures were incubated with the inducer, then centrifuged, and supernatants were assayed for their IFN and IL-6 activities in cytopathogenic effect inhibition assays and on the IL-6 dependent B9 cell line, respectively. The levels of IFNA-specific mRNA were assessed by RT-PCR method. Total cellular RNA was prepared by the guanidine thiocyanate method. RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Amersham). cDNA was amplified using Taq DNA polymerase (Amersham). PCR was conducted in a PDR-91 (BLS) thermocycler. The PCR product was analyzed on a 1.5% agarose gel. The amplified DNA was characterized by restriction analysis, as well.

S. aureus strains induced a transient IFN production, with the highest yield between 12 and 20 h after induction. The IFN activity ranged from several hundred to one thousand IU/ml. Serological characterization of *S. aureus*-induced IFN indicated that IFN-α was the predominant type in these preparations. The presence of IFN-gamma was also shown. *S. epidermidis* strains induced low-titre (10–20 IU/ml) IFN production. No IFN

was detected in supernatants of MNC stimulated with *S. saprophyticus*. Our RT-PCR assay demonstrated S. *aureus*-specific increase in IFNA mRNA. Transcription of IFNA gene was transient, reaching a maximum between 4 and 8 h after induction and decreasing to baseline level at 24 h. A very weak expression was detectable in MNC stimulated with *S. epidermidis* at 4 h postinduction. We were unable to identify *S. saprophyticus*-specific increase in IFNA mRNA. Restriction analysis with Hinc II. endonuclease which is specific for IFNA2, suggests the presence of this subtype in *S. aureus*-induced MNCs. The secreted IL-6 was also measured. The *S. aureus*, *S. epidermidis* and *S. saprophyticus* species stimulated high-titre IL-6 production. Only slight differences were observed in the IL-6 inducing activities of the *Staphylococcus* strains tested.

Conclusions: i) The higher IFN-inducing activity of *S. aureus* may be characteristic of this species. ii) All of the tested *Staphylococcus* strains proved to be very efficient inducers of IL-6 synthesis.

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Reversal of drug resistance in bacteria and tumour cells by some substituted phenothiazines

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Efflux pump may increase the level of resistance to antibiotics in bacteria and to cytostatics in tumour cells due to a decreased drug accumulation. Some Ca-channel blockers are able to enhance drug retention in the cells by inhibiting efflux pump mechanism of *mdr* tumour cells and bacteria. The effects of substituted chlorpromazines were studied in *Escherichia coli* and mouse tumour cells simultaneously.

Haemolysin transporter plasmids were eliminated from *E. coli* by some phenothiazines. Minimal inhibitory concentrations of tetracycline and phenothiazine for plasmidless bacteria decreased comparing to the parent, plasmid carrying strains.

The effect of the same phenothiazines on p-glycoprotein responsible for *mdr* resistance in tumour cells was also studied. Murine lymphoma cell line and its human *mdr*1 gene transfected subline were treated and the accumulation of the fluorescent rhodamine 123 was measured by flow cytometry. The 3,7,8-trihydroxy- and 7,8-dihydroxy derivatives of chlorpromazine were effective p-glycoprotein blockers, however, 7,8-diacetoxy-, 7,8-dimetoxy-, 7-semicarbazone-, and 5-oxo-chlorpromazine derivatives had moderate effects on the function of p-glycoprotein. The surface of treated

tumour cells was found to be less corrugated in atomic force microscopy that the untreated cells.

A possible correlation between the biological activity and electrostatic potential of the studied molecules was analysed by MM-II, CNDO programmes. Our results suggest that the inhibition of p-glycoprotein function in murine tumour cells and haemolysin transporter in *E. coli* bacteria may depend on π-electron superdelocalizibility and electrophil binding of the compounds to the transporter proteins.

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Expression of monoclonal antibodies in transgenic plants: a novel way to produce antibodies and to introduce virus resistance

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Immune protection by synthesis of specific antibodies against invading foreign antigens is restricted to higher animals in nature. However, the development of new techniques during the last few years has made it possible to express both assembled full-size antibodies as well as various active antibody fragments in transgenic plants [1, 2]. To obtain high antibody concentrations of up to 1–3% of the extractable protein, it is necessary to express antibody chains that contain the native or a heterologous signal peptide. In plants which simultaneously synthesize complete heavy and light antibody chains coupled to a signal sequence, correctly folded and assembled tetrameric H2L2 IgG molecules are formed in the endoplasmic reticulum. These antibodies are subsequently secreted into the apoplast from where they can be easily extracted. The results show that transgenic plants offer a powerful alternative to other methods of antibody production for diagnostic as well as for therapeutic purposes.

A detailed analysis of antibody expression in transgenic tobacco has been performed. The plants were transformed with different constructions of the double-stranded RNA (dsRNA) specific antibody J2 under the control of the strong constitutive 35S-promoter from cauliflower mosaic virus. The J2 antibody recognizes the A-helix structure of dsRNA independent of the sequence and nucleotide composition of the antigen, provided that a helix of >40 bp is present. The antibody can therefore be used to detect dsRNA of any RNA viruses. At present we investigate whether the antibody can counteract viral helicase activity by stabilizing double-stranded replication intermediates and thereby inhibit the replication of single-stranded (+)-RNA viruses in vivo in transgenic plants.

[1] Hiatt,A., Cafferkey,R., Bowdish,K.: Nature **342**, 76 (1989); [2] Conrad,U., Fiedler,U.: Plant Molecular Biology **26**, 1023 (1994).

B. TARÓDI

BCL2 and homologues: a family of multifunctional antiapoptotic gene products

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Adenovirus E1B 19 kD protein cooperates efficiently with E1A in the transformation of primary rhodent cells. Adenovirus mutants defective in 19 kD protein induced cell death similar to that observed with known apoptosis inducing agents. Mutational dissection of the 19 kD gene indicates that its transforming activity and its ability to protect cells against apoptosis are linked. We have carried out mutant complementation analysis with 19 kD and Bc12, the known anti-apoptotic gene product. They proved to be similar in suppression of cell death. To understand the mechanism by which 19 kD cooperates with E1A in transformation and protects against cell death we undertook a study to identify the cellular proteins with which 19 kD interacts using two hybrid genetic screen. These experiments suggest correlation between the biological functions of 19 kD and its association with cellular proteins. Moreover it seems that 19 kD and Bc12 may suppress cell death by interacting with common cellular proteins.

BHRF-1 an EBV gene product sharing some sequence homology was also included in this study and the results suggest that BHRF-1 is indeed functionally similar to Bc12 and 19 kD. Possible physiological role of viral anti-apoptotic genes and also implication for immune response has been discussed.

K. BERENCSI

Human CTL responses to cytomegalovirus

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The major histocompatibility complex (MHC) class I restricted cytotoxic T lymphocyte (CTL) response to human cytomegalovirus CHCMV) assists in preventing progression of HCMV infection to severe or life-threatening disease. The vital antigens recognized by CTL, however, are not well characterized and CTL specificity of individuals with low and high virus neutralizing antibody titres (NA) have not been compared. We examined CTL responses of 12 healthy blood donors with NA titres < 4–1:384. Peripheral blood mononuclear cells were restimulated in vitro with canarypox virus recombinants expressing I tCMV immediate early (IE) protein and structural proteins: phosphoprotein 65 (pp65) and phosphoprotein 150 (pp150), CTL activity was tested on autologous and heterologous target cells infected with vaccinia recombinants expressing the corresponding HCMV antigen. Donors with low NA and seronegative donors did not show HCMV specific CTL. PBMC of 7 donors with NA of 1:128–1:384 lysed target cells expressing pp65, IE and pp150, the spectrum of CTL specificity, however varied: 6 donors showed pp65-, 4 IE-, and 2 pp150-specific lysis. Presence of

pp65-specific antibody correlated with pp65-specific CTL activity. One of 2 donors with moderate NA (1:64) and pp65-specific antibody showed pp65- specific lysis. Seven month follow up of 2 individuals did not show changes in CTL specificity.

The majority of antigens gain access to MI IC class I molecules via the endogenous antigen pathway. Earlier reports have shown that HCMV-pp65 specific CTL recognize target cells in vitro without endogenous viral gene expression. We examined whether exogenous pp65 is also able to restimulate memory CTL in vitro and generate CTL responses in vivo. PBMC of CTL responder donors showed pp65 specific lysis not only after pp65-expressing recombinant restimulation, but also after HCMV restimulation. Spleen cells of mice immunized with HCMV or UV inactivated HCMV exerted pp65 specific lysis. These results demonstrate that pp65 is able to restimulate memory CTL in vitro and generate CTL responses in vivo without de novo protein synthesis.

POSTER PRESENTATIONS

G. GÁSPÁR

Quantitative analysis of the histopathological parameters of HCV induced chronic hepatitis

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Hepatitis C virus has recently been recognized as agent of the parenterally transmitted form of non-A, non-B hepatitis.

The most common histopathological picture of chronic hepatitis C virus infection is that of a mild chronic hepatitis with dense portal inflammation composed mainly of lymphocytes. Bile duct damage is often present along with bile ductular proliferation of various degrees and lobular activity tends to be mild to moderate with spotty necrosis. In the majority, mild activation of the sinusoidal lining cells, steatosis and focal piecemeal necrosis can be seen as well.

In our non-controlled randomized study we selected 84 patients suffering from chronic hepatitis C which was confirmed by both second generation ELISA test and PCR technique. (The ALT levels of the patients exceeded the upper limit of the normal enzyme value by more than 50 % for at least six months.)

Using the numerical score system of the histological activity index, introduced by Knodell et al. (Hepatology, Vol 1, No 5, 1981), in evaluating the liver biopsies of these patients we obtained the following results:

periportal necrosis: 1.39±0.09
intralobular necrosis: 1.21±0.06
portal intimation: 2.64±0.08
fibrosis: 1.32±0.13

A. HEVÉR[1], I. OCSOVSZKI[2], J. O. MOLNÁR[1] and J. BARBE[3]

Acridine derivatives reversing multidrug resistance in bacteria and tumour cells

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A wide variety of drugs have been shown to reverse the antibiotic resistance in bacteria and multidrug resistance (Mdr) in tumour cells as well.

In our experiments the plasmid curing effect of several acridines were studied on *Escherichia coli* Flac plasmid. In addition, the inhibition of antibiotic resistance transfer was also studied. For studying the Mdr function, the mouse lymphoma cell line L5178Y and its Mdr subline L5178A was used (transfected with human Mdr 1 gene). The fluorescent substrate rhodamine 123 was the test compound, which shows the highest differential fluorescence between drug sensitive and resistant cells.

The majority of the acridines exhibited weak antibacterial effect, with MIC value being above 200 μg/ml. Some compounds had Mdr inhibitory effect and antiplasmid effect also, however some resistance modifiers were without antiplasmid effect. We conclude that although the genetic basis of multidrug resistance in bacteria and tumour cells may be similar, the operating mechanisms should be different.

A. KÁTAI, K. KIRÁLY, M. BENKŐ and GY. BÁLINT

National programme for the prevention of Hepatitis B virus vertical transmission. Local experiences in Csongrád

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It is well known that Hepatitis B virus (HBV) infection is commonly transmitted from HBsAg positive mothers to their infants. Almost all infants who are infected neo- or perinatally become carriers. These cases develop at a rate of about 90% into the chronic form of hepatitis and some decades later a quarter of them may progress to cirrhosis, hepatocellular carcinoma and death.

The applied specific immunoglobulin (HBIG) within 12 hours of birth and the concomitant initiation of a tree-dose Engerix B vaccine series prevents the vertically transmitted infection with more than 90% effectiveness.

In a four and half year period (Sept. 1991.–Apr. 1996.) 47 HBsAg positive women (0.3%) were discovered among the nearly 15 000 pregnants screened by the virological laboratory of the Institute. The effectiveness of the immunization among the vaccinated infants was controlled by HBsAg and anti-HBs microelisa tests at the age of 15 months. The results showed no HBsAg positivity in the children checked. The antibody titres were

also mostly satisfactory with the exception of three cases when a fourth dose of vaccine administration was suggested.

Considering that before the mentioned period no screenings were carded out, HBsAg positivity was not always revealed during the first pregnancy, so the investigation was focused on children born earlier in these families. In the case of four families former HBV infection with maternal origin could be detected. These data have also been discussed.

On the basis of the results it is clear that for the lack of a general HBV vaccination programme the screening of pregnant women for HBsAg is beneficial. With the help of immunization the health of the affected offspring could be protected and the formerly infected, asymptomatic children revealed as I-IBV carriers could get into infectological care. Furthermore interferon therapy could be applied when it was necessary.

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Development of an HBxAG and anti-HBx. Dg diagnostic system

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The hepatitis B virus ([H]3V) is one of the causative agents of acute and chronic liver diseases in humans. Epidemiological and molecular data indicate that the chronic infection by HBV is associated with an increased incidence of human hepatocellular carcinoma (HHC) [1–4]. The X gene product of the HBV genome ([H]3xAg) transactivates both viral and cellular genes [5] and it is considered as an essential factor for establishing chronic infection as well as mediating hepatocarcinogenesis [6–8]. Therefore, for the chronic state of elisense and its progression to malignant transformation, monitoring of the expression of HBxAg may have prognostic value.

The aim of the experiment was the production of HBxAg based on recombinant techniques and development of anti-HBx antibodies and of a diagnostic system for detection of HBxAg by immunoserological and immunohistochemical methods as well as quantitative detection of anti HBx antibodies.

A truncated form of HBV X gene (HBx) was cloned into the fusion expression vector of pGEX-3X (Pharmacia) resulting in a GST-HBx fusion gene construction (pGEX-3XXBF). This plasmid was transformed into and expressed by the *Escherichia coli* strain DH5 α. More than 80% of the expressed fusion protein was found in the insoluble fraction (inclusion body) of the cell lysate. The fusion protein was selectively extracted by 8M urea with high purity and recovery (10–15 mg/100 ml cell culture) from the inclusion bodies. Rapid refolding by dilution method at 2–3 mg/ml protein concentration was performed. Removing of the affinity handle by X_a factor was efficient. The vital portion of the cleaved fusion protein was separated by gel filtration (FPLC, Superdex 75). Milligram quantities of HBx protein were yielded by the applied

purification/cleavage procedure. Polyclonal and monoclonal antibodies against HBxAg were developed. Here we demonstrate the results of serological and immunohistochemical examinations with some representative data using our diagnostic system.

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[1] Beasly: Cancer **61**, 1942 (1988); [2] Ganem, Vermus: Annu Rev Biochem **56**, 651 (1987); [3] London et al.: Intervirol **38**, 155 (1995); [4] Tiollais et al.: Nature **317**, 489 (1985); [5] Colgrove et al.: J Virol **63**, 4019 (1989); [6] Feitelson et al.: Oncogene **8**, 1109 (1993); [7] Koike: Intervirol **38**, 134 (1995); [8] Zahm et al.: Oncogene **3**, 169 (1988).

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Natural cytotoxicity of human NK cells and granulocytes

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The natural or nonadaptive immune system consists of a variety of leukocytes that exhibit natural cytotoxicity. NK cells are a discrete subset of lymphocytes with characteristic morphological, immunophenotypic and functional attributes. There is increasing evidence that neurohormones affect various immune responses, therefore we investigated the effect of serotonin on human NK cell activity. A marked decrease in NK activity was observed in the presence of 5 HT (serotonin) , especially applying whole blood. The use of whole blood instead of purified lymphocytes offers a model which can be regarded as intermediate between in vivo and in vitro studies. Depletion of CD 56+ cells abrogated the cytotoxicity, which verifies that the reaction reflect the behaviour of the NK cells in the whole blood. The inhibitory effect of serotonin was partially reversed by interferon alpha. Membrane depolarization was observed in the presence of serotonin, which was reversed by interferon.

The natural cytotoxicity of granulocytes is rather neglected. However, these cells are able to kill TNF sensitive WEHI 164 cells in a 18 h ^{51}Cr release reaction. In addition, the ADCC activity of granulocytes is an effective mechanism of host defense as well. Investigation of the cytotoxicity of granulocytes against WEHI cells and against sensitized red blood cells revealed that both functions were impaired in patients with malignant diseases. So we conclude that not only NK cells but granulocytes themselves can participate in natural defense against cancer.

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Inhibition of reverse transcriptase activity by metal complexes of TIFF cyclic compounds

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Reverse transcriptase (RT) plays an essential role in the early steps of the replication cycle of retroviruses. While nucleoside analogs such as AZT and ddI have exhibited usefulness in slowing the rate at which AIDS progress, the development of the in vivo resistance by HIV-1 RT in response to these inhibitors has been reported. Some phenothiazines which differ in mechanism of action from the chain terminators AZT and ddI can inhibit the activity of the reverse transcriptase. To improve the biological effect we prepared various metal coordination complexes with Chlorpromazine and Trifluoperazine (CPZ-Pt, CPZ-Sn, TFP-Sn, TFP-Cu, TFP-Pd, TFP-Ni, TFP-V). These compounds were tested in Murine Leukemia Virus reverse transcriptase assay. AZT was used as a control compound. The inhibitor activity of some metal complexes, TFP-Pd, TFP-V and CPZ-Pt were definitely higher compared to the basic tricyclic chemicals. Therefore we conclude that the conformation and the improving biological effect depend on the formation of coordination complexes. Testing the acute toxicity on mice the LD_{50} values did not exceed that of the basic phenothiazines. So these metal coordination complexes may become a new group of effective compounds inhibiting the HIV replication.

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The complex pattern of cytokines in sepsis

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The activation of cytokine network has a pivotal role in the pathogenesis of sepsis. During this process the stimulation of white blood cells and increase of the expression of adhesion molecules are essential in the development of metabolic and cardiovascular symptoms of sepsis. We investigated the connection between plasma cytokine levels and the process and prognosis of illness. We also studied, whether the administration of pentoxifylline (PTX) influences the serum cytokine and inflammatory mediator values.

Fourteen surgical patients with clinical sepsis were studied. We measured circulating TNF, IL-6, IL-8, IL-12 and neopterin levels every day during the septic condition. The in vitro TNF- and IL-6-producing capacity of leukocytes was checked as well. Determination of serum cytokine and neopterin levels were performed by ELISA and in vitro TNF and IL-6 production was measured by bioassay.

Increasing TNF serum levels (>20 pg/ml) were found in 36% of the septic patients, while the IL-6 levels were elevated (>40 pg/ml) in 93% of them. The values of serum TNF level were decreased following the first days of illness, while the IL-6 serum levels remained elevated until the end of the septic period. The plasma IL-8 concentration was also elevated (>40 pg/ml) in 86% of the patients. Neopterin is the specific marker of monocyte activation, its serum level was increased (>20 nmol/1) in 86%, and it showed a inverse correlation with survival. There was no correlation between the levels of IL-12 and other cytokines. We observed that the change of plasma cytokine levels are in connection with the clinical condition. The in vitro TNF and IL-6 producing capacities in septic patients were higher than in healthy blood donors.

According to previous data pentoxifylline (PTX) inhibits the TNF production of human white blood cells. Administration of PTX to septic patients resulted in the normalization of TNF and IL-6 synthesis.

Investigation of the complex pattern of cytokines and the inflammatory mediators is beneficial from the point of view of the course, the prognosis and the therapy of sepsis. The multiple effects of PTX concerning the inhibition of the activity of the leukocytes responsible for the production of cytokine and inflammatory mediators suggest that it may improve therapeutic strategies in the treatment of septic syndrome.

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Antiherpetic activity, antimutagenicity and mutagenicity of benzo[α]phenothiazines

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Antipsychotic phenothiazines have been found to exert a broad spectrum of antimicrobial activities. Antiherpetic effect of seven benzo[α]phenothiazines were studied on the cytopathic effect of herpes simplex virus type 2 (HSV-2) in Vero cells and their mutagenic effect was screened on *Salmonella typhimurium* TA98 strain. Benzo[α]phenothiazines were variously substituted by methyl-, oxo- and/or hydroxyl-substituent(s) at 5, 6, 9 and/or 10 position(s). 5-Oxo-5*H*-benzo[α]phenothiazine and 6-methoxyl-5-oxo-benzo[α]phenothiazine were observed to have the most potent virucidal effect against HSV-2 with EC50 of 7.1 BM and 5.0 BM, respectively. Antimutagenicity of benzo[a]phenothiazines were screened on *S. typhimurium* strain TA98 treated with 4-nitro-*o*-phenylenediamine (4-NPD) which is a specific mutagen to the strain. 9-Methyl-12*H*-benzo[α]phenothiazine [3] reduced the 4-NPD-induced mutation by 30%. None of the test chemicals showed mutagenic activity. The antiherpetic activity of benzo[α]phenothiazines presumably depends on the substitution of the tricyclic skeleton.

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Construction of plasmids for genetic immunization against human cytomegalovirus diseases

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A novel approach for induction of primary immune responses based on plasmid vectors carrying vital genes has been developed recently.

Here we show construction of plasmid vectors expressing HCMV proteins with the aim – to test whether HCMV-specific immunity can be induced by naked DNA immunization; and – to study how a regulatable plasmid works for immunization in vivo.

An expression plasmid: pΔRC was derived from the commercially available (Invitrogen) pRC/CMV plasmid by deleting the PvuII 1290 - PvuII 3557 fragment to obtain more unique restriction sites. This vector was used to construct:

- pΔRC expressing the full glycoprotein B (gB) of HCMV
- pΔRC-gB680 coding for the N-terminal 680 amino acids of tile gB protein
- pΔRC-pp65. The tegument protein of HCMV is expressed
- pΔRC-pp150. The pp150 tegument protein is expressed
- pΔRC-Exon-4. The IE-Exon-4 (truncated UL123) is expressed

The pTeto-gBtTA expresses the full length gB under the control of a tetracycline regulatable promoter. The structure of the plasmid is, in addition to *Escherichia coli*-part, tetracycline-regulatable promoter (7 teto + minimal hCMV promoter) followed by the hCMV-gB gene, followed by the SV40 polyA signal, followed by the hCMV-IE promoter-enhancer, followed by the tTA gene and ending with the SV40 polyA signal.

All plasmids we constructed express the insert in transfected human (293 or A-549) cells. Expression of gB, pp65 and pp150 is strong by using any vectors, the expression of Exon 4 is weak.

The expression of gB after transfection with pTeto-gBtTA can be manipulated by the presence or absence of tetracycline; it is expressed in the absence, but not expressed in the presence of tetracycline, as tested by an immunofluorescence assay using a monoclonal antibody to gB protein.

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Genetic immunization against human Cytomegalovirus diseases

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Plasmid DNA encoding proteins represent a convenient novel approach to vaccination. We have investigated this genetic immunization approach as a means to induce human cytomegalovirus (HCMV) specific immune responses in mice. BALB/c mice were first injected with 0.1 ml Bupivacaine i.m., 1 day later 80 μg plasmid was

administered into the same site. Booster immunizations were given without Bupivacaine pretreatment.

After immunization with plasmid DNA encoding glycoprotein B (pΔRC-gB), all of 6 mice responded with both ELISA antibody and NA (HCMV neutralizing antibody titer) ranging 1:16–1:128. The HCMV phosphoprotein 65 expressing plasmid (pARC-pp65) induces pp65 specific antibody and CTL responses.

The pTeto-gBtTA construct expressing gB under the control of a tetracycline regulatable promoter induced ELISA and NA responses. Four of 10 mice developed HCMV NA>1:8, while 9 of 10 were positive for ELISA antibodies.

Combined immunization with 2 kinds of plasmids expressing different HCMV antigens (pTeto-gBtTA and pΔRC-pp65) given mixed at one site or separately at two sites resulted antibody responses to both proteins, indicating that there is no significant antigenic competition between gB and pp65.

Immunization with the pTeto-gBtTA primed NA responses to a booster, i.e. combination of a pTeto-gBtTA priming and purified gB protein (in Alum adjuvant) booster gave higher number of responder mice and slightly higher NA titres, than pTeto-gBtTA given 2 times.

The protective effect of DNA immunization was tested in mice challenged with Vaccinia recombinants expressing HCMV gB or pp65. Mice were immunized either with pΔRC-gB or pΔRC-pp65, than challenged with Vac-gB or Vac-pp65. Virus titres of lungs showed that immunization with either plasmid reduced the titre of the corresponding challenge virus by 0.5–1.4 log on days 3, 4, 5 and 7 after the challenge.

The above results demonstrate that our constructs are potent immunogens, induce both antibody and CTL responses and protect against a challenge virus.

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