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DETECTION AND EPIDEMIOLOGICAL TYPING OF LISTERIA STRAINS – DIAGNOSTIC METHODS FOR LISTERIA INFECTIONS

(A Review)

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Introduction

Different types of direct or indirect diagnostic methods can be applied for the detection of listeriae in human, animal, food and environmental samples or for the verification of listerial infection in human beings and animals. In this review the most important methods will be presented.

1. Cultivation of listeria strains

Culture media and methods have been summarized many times [1 – 12].

1.1. Growth in or on non-selective media

Cultivation of listeria cells from an otherwise uncontaminated sample is not a difficult task, even if they are injured, because they are nutritionally undemanding. They grow well in or on the usual bacteriological media Buffered Peptone Water (BPW), Nutrient Broth (NB) or Nutrient Agar (NA), Tryptose Broth (TB) or Tryptose Agar (TA), Brain Heart Infusion Broth (BHIB) or Brain Heart Infusion Agar (BHIA), Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA), Columbia Broth (CB) or Columbia Agar (CA) and Blood Agar (BA). Therefore, it is evident that non-selective enrichment broths (1.1.1.) and non-selective solid media (1.1.2.) provide satisfactory conditions for their growth.

1.1.1. Enrichment media

"Cold enrichment" (CE) at 4 °C for several weeks is the traditional enrichment method. It can be performed in different types of non-selective fluid media. Holman medium with blood [12] has been used successfully whereas Hayes et al. [13] have utilized MOPS buffered NB. Greenwood et al. [14] used BPW for the first step of enrichment at 30 °C. Lammerding and Doyle [15] applied a non-selective pre-enrichment broth (PE/FSIS; U.S. Food and Drug Administration [16] without selective agents) to recover low number of *Listeria monocytogenes*. Crawford et al. [17] studied recovery of *L. monocytogenes* from milk using various cultural methods including Trypticase Soy Yeast Extract Broth (TSYEB). Varabioff [18] also described a non-inhibitory pre-enrichment broth (Buffered Tryptic Soy Broth with Yeast Extract; BTSBYE) which allowed the recovery of listeria cells.

It was also observed that the cold enrichment method was not as effective as the U.S. Department of Agriculture Food Safety Inspection Service (USDA – FSIS) enrichment broths (University of Vermont Medium UVM_1 and UVM_2) described by McClain and Lee [19]. Hayes et al. [20], Ibrahim et al. [21], Crawford et al. [17] reported that any listeria cells surviving high-temperature, short-time pasteurization will be injured and unable to multiply either during cold-enrichment or in the FDA or the USDA – FSIS systems.

1.1.2. Plating agars

Fernandez-Garayzabal et al. [22] observed that the haemolysis of listeria strains on BHI BA was stronger than on CBA.

1.1.3. Differential media

Notermans et al. [23] examined 468 listeria strains for the presence of phosphatidylinositol-specific phospholipase C (PI-PLC) activity by using a simple assay that consisted of overlaying colonies formed on agar plates with L-alpha-phosphatidylinositol as substrate. PI-PLC-active colonies showed turbid halos around the colonies as a result of the release of insoluble diacylglycerol.

1.2. Media used for the isolation of uninjured listeria cells from contaminated samples and evaluation of methods

Successful isolation of listeria from contaminated samples sometimes requires the use of at least one selective enrichment broth (1.2.1.) and subculturing to a selective solid medium (1.2.2.).

1.2.1. Enrichment media

The selective enrichment broth of Ralovich et al. [24] containing trypaflavine, nalidixic acid and blood has been shown to be effective. Originally it was incubated at 37 °C for two days and at room temperature for further 5 days [24], but this has now been changed to 30 °C. Our medium and method were modified by Lovett et al. [16] when they published the FDA procedure which has been successfully used for isolation of listeriae from milk and dairy products. Dominguez-Rodriguez et al. [25] developed a two-step enrichment procedure in 1984. Donnelly and Baigent [26] modified their enrichment medium developing Listeria Enrichment Broth (LEB). McClain and Lee [19] published UVM_1 and UVM_2 broths which originated from LEB modifying the concentration of acriflavine. UVM_1 and UVM_2 have been proposed and widely used to isolate listeria strains from meats and poultry.

Since the present author's review of culture media for listeria in 1988 [4] the problems of selective enrichment have been discussed by several authors. Fraser Broth (FB) enable the presumptive detection of *Listeria* spp. within 48 h and was developed by modifying UVM_2 through the addition of lithium chloride and ferric ammonium citrate [27]. FB inoculated from UVM_1 was found to be more effective than FB inoculated from the FDA enrichment broth. On the basis of this observation they modified the original USDA-FSIS method. Usefulness of FB was assessed by Domján Kovács et al. [28] and also by Gasparik-Reichardt et al. [29]. The former studied 222 samples of meat products whilst the latter tested 191 swabs from surfaces sampled in different chambers of a meat plant. Incubation for 48 h was necessary for a reliable result because after 24 h not all FB tubes (which were listeria positive by

sub-cultivation) showed a black colouration. Blackening of the medium on its own without subculture to a solid medium was not precise enough for a positive diagnosis of the presence of listeria because other bacteria could also bring about such a colour change. This UVM_1 and FB combination was more effective than the UVM_1 and UVM_2 procedure, 82.4% of the samples being positive with both methods, 13.1% only with UVM_1 + FB + Modified Oxford Agar (MOX) [30] and 4.5% only by UVM_1 + UVM_2 + LiCl Phenylethanol Moxalactam Agar (LPM [31]). McClain and Lee [30] modified the original FB broth increasing the concentration of acriflavine. They proposed to use UVM_1 + modified FB in the modified USDA–FSIS procedure. Quist et al. [32] also observed that the UVM_1 + FB broth combination was better than the original USDA–FSIS method. Truscott and Mcnab [33] compared the sensitivity of the LEB of Donnelly and Baigent [26] to that of a Listeria Test Broth (LTB) containing horse serum and Tween 80. Their results suggested that LTB based protocols appeared to be more selective for isolation of *L. monocytogenes* than LEB. Swaminathan et al. [34] showed that quantitative recovery of *L. monocytogenes* from Brie cheese was significantly better in FDA broth than in USDA–FSIS broths. Lammerding and Doyle [15] compared six different enrichment media (U.S. Centers for Disease Control – CDC – thiocyanate + nalidixic acid – (TN)N – broth [13], selective enrichment procedure – SEP – [35], FDA, USDA–FSIS, modified USDA–FSIS procedure with PE/FSIS, CE). "Generally, the media and two stage enrichment protocol of USDA–FSIS with plating of samples after two consecutive 24 h incubation periods, yielded better recoveries than all other enrichment media incubated for 24 h." Fernandez-Garayzabal and Genigeorgis [36] compared three selective enrichment broths (FDA, USDA–FSIS and Dominguez-Rodriguez broth [25] and found that all listeria strains grew faster and yielded a higher number of cells in FDA enrichment broth. The use of a secondary broth (FDA) enrichment step improved the recovery of *Listeria* spp. from meat samples. Hitchins [37] compared the FDA and the USDA–FSIS enrichment methods and found both to be equally sensitive. Later Hitchins and Tran [38] observed that the FDA enrichment method performed better with LPM than with modified McBride agar (MMA) [39]. Bailey et al. [40] also compared the FDA and USDA–FSIS procedures and observed no significant differences between the two methods except that the level of mixed flora competitors was significantly higher in FDA broth than in USDA–FSIS media. The ratio of *L. monocytogenes* to mixed microflora was most favourable for recovery of *L. monocytogenes* after 2 d enrichment. Their observation and that of others [15, 41, 42] led the FDA to shorten the original incubation period of the enrichment broth to 2 d. Lewis and Corry [43] used a two-step CE technique. They compared this CE with the modified FDA enrichment method, and their procedure proved to be more effective than FDA method. Walker et al. [44] compared FDA broth, MOPS buffered FDA broth (MOPS), USDA–FSIS method

and UVM_1 + FB. MOPS and UVM_1 + FB media were the best. Cox et al. [45] used modified FDA and modified UVM_1 (MLEB) enrichment media for their studies. The latter contained trypaflavine in lower concentration and 15% of NaCl. They incubated their broths at 30 °C for 48 h. MLEB was considered as important for the detection of listeria. Yu and Fung [46] evaluated the usefulness of FB, FDA, UVM broth and UVM_1 with extra acriflavine for enumerating *L. monocytogenes* in ground beef. FDA broth combined with MOX was the most efficient. Noah et al. [47] used four enrichment procedures for the recovery of *Listeria* spp. from 211 samples of raw and processed seafoods. The enrichments used were FDA, MOPS, MOPS transferred to MOPS after 24 h, modified UVM_1 transferred after 24 h to UVM_1 containing additional acriflavine (UVM_2). All enrichments were incubated at 30 °C for a total of 48 h. Statistical analysis showed that recovery of *Listeria* spp. using FDA for 48 h without transfer did not differ significantly from that obtained with the revised FDA method. Lund et al. [48] compared three enrichments (FDA broth, UVM + FB and L-PALCAMY [49]) for the isolation of *Listeria* spp. from raw milk. Their results indicated that the enrichments were similar in their ability to enhance the growth of any *Listeria* spp. present in the sample. L-PALCAMY appeared to be the most effective enrichment with 16.7% positivity, UVM + FB resulted in 15.3% and FDA broth in 14.0%. Besides this L-PALCAMY may be more selective for *L. monocytogenes* in foods that have mixed bacterial populations. Hayes et al. [20] compared CE + CDC-TN and UVM enrichment broths for isolating *L. monocytogenes* by examining 402 food samples. The food samples were collected from refrigerators of patients. The USDA–FSIS method was significantly better than the two-step CE + CDC-TN method. Greenwood et al. [14] used a two-step enrichment with good results. First they incubated the samples in BPW at 30 °C for 24 h and that was followed with the 2nd enrichment in FDA broth for 2 d at 30 °C. In a Canadian comparative study Warburton et al. [41, 42] also observed that the use of modified FB [30], which contained an increased level of acriflavine, yielded better results. They modified the FDA method to a two-step procedure (FDA broth + modified FB) and used the modified USDA–FSIS method in which UVM_1 was followed by the modified FB. Approximately 92% of the positive samples were detected after 24 h of enrichment. They recommended that both FDA broth and UVM_1 should be subcultured into modified FB after 24 h and streaked onto the plating media after 24 and 48 h of incubation. Subculturing at 48 h improved the recovery of listeria.

1.2.2. Selective solid media

During the history of listeriosis research several selective plating media have been tested for the isolation of listeria strains. Most of these media were summarized in 1988 [4]. In the present paper those publications are mentioned which were not

cited earlier. Buchanan et al. [50] formulated Modified Vogel–Johnson Agar (MVJA) using moxalactam, nalidixic acid and bacitracin and observed that this medium proved highly effective for isolation of listeria. Swaminathan et al. [34] compared quantitative recovery of selected strains of *L. monocytogenes* in pure culture suspension from MMA, LPM, Gum Base–Nalidixic Acid–tryptone (GBNA) medium [51] and AC [52] agars. They recommended the use of at least two plating media (one with low selectivity GBNA, and one with high selectivity LPM) for isolation of *L. monocytogenes* from highly contaminated specimens. Curtis et al. [53] developed Oxford medium containing aesculin, ferric ammonium citrate, lithium chloride, cycloheximide, colistin sulphate, acriflavine, cefotetan and fosfomycin. Some 6 selective plating media were compared by us [54, 55]. Oxford agar proved to be the most convenient medium for the cultivation of listeriae. LPM agar, AC agar, PALCAM agar [49], Forray's medium [56] and TNSA medium [57, 58] were less suitable than Oxford agar, however, the number of positive samples enriched in UVM broths and subcultured on these selective media (done in parallel) was almost the same. In the same year Buchanan et al. [59] compared the effectiveness of MVJA and LPM for detection of *Listeria* spp. in foods by using the media to analyse retail-level meat, poultry and seafood both by direct plating and in conjunction with an enrichment. MVJA performed as well as did LPM and was considerably easier to use because of its ability to differentiate *Listeria* spp. from other microorganisms without special illumination techniques.

Bailey et al. [60] examined 90 broiler carcasses on LPM, MMA, GBNA and GBNA + media. The last medium contained also moxalactam and LiCl. After UVM enrichment, higher recoveries of both *L. monocytogenes* and *Listeria* spp. were made from LPM and GBNA + than the others. Lammerding and Doyle [15] examined five selective plating media McBride's agar (MA) [61], GBNA, CDC–MMA, FDA–MMA and LPM. LPM was the most effective plating agar for isolation of *L. monocytogenes* following enrichment of samples in any broth culture, and increased recoveries of *L. monocytogenes* by 19–40% compared with other selective agar media tested. Heisick et al. [62, 63] also found that recovery was higher with LPM than with MMA after FDA enrichment. Hitchins and Tran [38] came to similar conclusions comparing LPM and MMA. Fernandez-Garayzabal and Genigeorgis [36] evaluated LPM, MMA, Listeria Selective Agar (Dominguez-Rodriguez (LSA) that is a modification of the isolation medium III described [35]) and BHIA for their suitability to support the growth of six different species/strains of listeria. Any enrichment broth combined with plating on LPM or LSA gave 100% listeria recovery as compared to 50–67% for plating on MMA agar. Brackett et al. [8] wrote that media such as LPM routinely performed better than most others for direct plating, regardless of the food being analysed. Nevertheless, no one medium appeared to be suitable in all situations. GBNA was most convenient for milk and ice cream mix,

LPM for cabbage, Brie cheese, hams and Dominguez-Rodriguez agar for raw oysters. Tiwari and Aldenrath [64] studied four selective media (MMA, AC agar, LPM, Oxford agar). MMA was found to be substantially inferior in comparison with other media. AC agar was relatively more efficient. LPM and Oxford agars were approximately equally effective for isolation from all classes of foods. However, LPM performed poorly with *L. monocytogenes* serotype 3a. Oxford medium was consistently superior and gave higher recovery with all strains studied. Walker et al. [44] tested MMA, LPM and Oxford medium for the recovery of listeria strains. Oxford medium was superior to either MMA or LPM. Heizmann and Werner [65] compared PALCAM and Oxford agars. They concluded that both media supported growth of *L. monocytogenes*. Growth of most the remaining species investigated was suppressed, and PALCAM agar was more effective in suppression of staphylococci. Lachica [66] designed a new plating medium containing LiCl, glycine anhydride and ceftazidime (LCA). Comparison of LCA and LPM indicated that both were equally effective for enumeration of listeria in artificially and naturally contaminated foods. Al-Zoreky and Sandine [67] formulated a new selective medium (Al-Zoreky–Sandine listeria medium, ASLM) to recover *L. monocytogenes* from food samples. Their medium contains LSA base with cycloheximide, ceftazidime, acriflavine hydrochloride and moxalactam. That medium completely inhibited the common food microflora. Cox et al. [68] developed Enhanced Haemolysis Agar (EHA). It contains the selective agents of Oxford medium, sheep blood (5%), 4-methylumbelliferyl-beta-D-glucosidase (50 mg/l) and sphingomyelinase (10 units/l). Concentration of LiCl was reduced to 1%. They compared the usefulness of Oxford medium and EHA. Their results showed that EHA proved to be advantageous for both the isolation of *Listeria* spp. and the discrimination between haemolytic and non-haemolytic species. McClain and Lee [30] described MOX which is a slight modification of Oxford agar. It contains Columbia Blood Agar (CBA) base, aesculin, ferric ammonium citrate, lithium chloride and colistin. Later Lee [69] evaluated three commercial brands of Oxford agar base for the MOX agar. The three brands of MOX agar showing varying degrees of inhibition of the sensitive *L. monocytogenes* strains. Yu and Fung [46] compared MA, MMA, LPM and MOX. MOX agar proved to be the most effective to detect *L. monocytogenes* in ground beef. Lund et al. [48] compared four selective plating media – Oxford agar, MMA, LPM and L-PALCAM – to isolate listeria strains from raw milk samples. The most positive samples (27.3%) were observed on Oxford agar. It was followed by L-PALCAM (26.7%), LPM (25.3%) and finally MMA (21.3%). L-PALCAMY combined with Oxford agar or PALCAM and UVM broths combined with the same two agars resulted in similar responses – 15.7% and 15.0%, respectively. The combination of FDA broth and MMA was less selective and gave poor results.

Art and Andre [70] compared three selective isolation media for the detection of *L. monocytogenes* in foods. A total of 391 different foods were examined; 25 g of samples were enriched in UVM_1 and UVM_2 broths and after incubation the enrichment cultures were plated onto Oxford agar, PALCAM agar and BA with nalidixic acid, respectively. The percentage of positive samples was identical (15.8%) on each medium. However, PALCAM agar and Oxford agar inhibited to a greater extent the growth of the contaminating microflora. Huis in 't Veld and de Boer [71] published the results of 16 laboratories. They compared BA, PALCAMY agar as well as Oxford agar and did not find a significant difference between them. PALCAM agar resulted in poorer recovery of listeria strains. Hayes et al. [20] observed that after USDA–FSIS enrichment there were more isolations (96%) on LPM than after CE + CDC – TN procedure (59%) on LPM and AC agars. Eleven Canadian laboratories compared modified FDA and USDA–FSIS methods. The two methods were comparable in their ability to isolate listeria strains. Oxford agar proved to be marginally better than LPM and significantly better than MOX agar in isolating *L. monocytogenes*. Their opinion is that the modified USDA–FSIS method may be the method of choice for all food and environmental samples, and also that the use of two different plates (Oxford agar + LPM or MOX) results in more listeria positive samples [41, 42].

1.3. Media used for the isolation of injured listeria from mixed bacterial flora

Cultivation of injured listeria cells from samples is sometimes difficult. When such cells are present in contaminated samples resuscitation and effective enrichment are necessary for successful isolation. (Therefore, it is not surprising that several papers dealt with this problem.)

1.3.1. Enrichment media

Lammerding and Doyle [15] developed PE/FSIS medium and incorporated it in a modified USDA–FSIS procedure. With the help of this method they could significantly increase recoveries of low numbers of sublethally stressed *L. monocytogenes* Crawford et al. [17] studied recovery of *L. monocytogenes* from milk using different cultural methods (FDA, USDA–FSIS, non-selective broth enrichment NSB and CE) after heat treatment (71.7 °C). They observed the following D values: NSB, $D = 2.0 \pm 0.5$ s; FDA, $D = 1.4 \pm 0.3$ s; USDA–FSIS, $D = 0.6 \pm 0.2$ s; CE, $D \geq 1.2$ s. Their data suggested that any cells surviving high-temperature, short-time pasteurization will be injured and unable to multiply either during cold storage of milk or in the FDA, the USDA–FSIS or the CE systems. Thus, *L. monocytogenes* cells recovered in finished pasteurized milk products by these detection methods probably represent uninjured environmental contaminants. Varabioff [18] also described a non-inhibitory pre-enrichment broth which allowed

the recovery of injured cells. Bailey et al. [72] stated that when the number of viable cells was very low and listeria were exposed to substantial heat stress, UVM broths appeared to be better than FDA medium for recovery of the heat injured cells. The difference between the two media could not be attributed to the selective agents or the NaCl concentration but appeared to be related to the presence of the fermentable carbohydrate and the lack of an adequate buffering system in FDA broth. UVM_1 was recommended as a primary enrichment broth for recovery of heat-injured *L. monocytogenes*. McCarthy et al. [73] found that heat-stressed *L. monocytogenes* cells were recovered from cooked shrimp that had not been frozen; however, no cells were recovered from frozen cooked shrimp. These studies suggested that the effects of heating and freezing were synergistically lethal to *L. monocytogenes*. The FDA and USDA–FSIS procedures were not so effective than their new method. The essence of that method was that the thermally stressed cells were recovered following cold enrichment for 3 d without broth. Tiwari and Aldenrath [64] observed that incubation of the FDA enrichment broth culture for just one day was not sufficient and 2 d of incubation was necessary to achieve a satisfactory level of performance. Lovett et al. [74] showed that for isolating listeria from raw seafood and for recovering non-heat-stressed listeria, the USDA–FSIS procedure was preferable. In heat-treated products in which stressed cells and a relatively low aerobic plate count could be expected, the FDA 48 h and 7 d enrichment method would be advantageous. Busch and Donnelly [75] studied the ability of the divalent cations (Mg, Fe, Ca and Mn) yeast extract, pyruvate, catalase and the carbohydrates glucose, lactose, sucrose, aesculin, fructose, galactose, maltose and mannose to facilitate repair of heat-injured *L. monocytogenes* and *L. innocua*. On the basis of their result listeria repair broth (LRB) was formulated. When heat-injured listeria cells were incubated in LRB they became completely repaired in 5 h. After the repair, acriflavine, nalidixic acid and cycloheximide were added to LRB and it was used as a selective enrichment medium. The efficacy of the new procedure was compared with that of FDA broth, LEB and UVM broths and repair was not observed in them. McCarthy [76] stressed virulent *L. monocytogenes* cells by heating at 56 °C for 20 min and could resuscitate them in TSB at 25 °C for 2 to 14 days. She observed that the resuscitated heat-injured cells were as virulent as non-stressed cells and that the heat-stressed *L. monocytogenes* cells were probably less virulent than the non-stressed or resuscitated ones. Sokolovic and Goebel [77] claimed that listeriolysin was still very efficiently synthesized in one *L. monocytogenes* strain even intracellularly and induced under heat shock conditions in another *L. monocytogenes* strain. Listeriolysin appears to be the only extracellular protein synthesized under heat shock condition.

1.3.2. Selective plating agars

Cassiday et al. [78] evaluated LPM, MMA, MVJA and GBNA for recovering uninjured, heat-injured and freeze-injured cells of *L. monocytogenes* from pasteurized whole milk, ice cream mix, Brie cheese and cabbage and found that LPM was most suitable for analysing Brie cheese and cabbage. GBNA was most suitable for analysing milk and chocolate ice cream mix. Later they [79] studied the suitability of ten direct plating media for isolating and enumerating four strains of uninjured and injured *L. monocytogenes* from dry- and country-cured hams and raw oysters. LPM was selected as the most suitable medium for isolation and enumeration of *L. monocytogenes* from both types of ham, while Dominguez-Rodriguez agar was selected as most suitable for raw oysters. Lachica [66] compared LCA and LPM and observed that LCA was more effective than LPM in the recovery of sublethally heat-injured cells. Al-Zoreky and Sandine [67] published that their selective medium (ASLM) was able to recover heat injured cells with only 15% less count than the non-selective medium.

1.4. Chemically defined and minimal media

Properties of listeria strains and their nutritional demand have been studied either in fluid or on solid media since 1939 [80–98]. The first solid minimal medium for that purpose was published in 1977 [99]. Potassium phosphate buffer was chosen. The carbon and energy source was supplied by glucose. Sodium citrate without glucose was insufficient for growth. When glucose was combined with citrate the listeria strains grew better. It can be supposed that this was the result of chelating action of citrate. Ammonium sulphate did not provide the nitrogen requirements for the strains but when the three or four essential amino acids – leucine, iso-leucine, valine and cysteine – were supplied, the growth was supported by that salt. The presence of Fe^{++}, Mg^{++} and Ca^{++} ions as well as a low redox potential supplied by thioglycollate was necessary. DL-6,8-thioctic acid, pyridoxine, thiamine Ca-panthotenate and nicotinic acid were not essential. Using the minimal medium containing the four amino acids, glucose, citrate, sodium thioglycollate and inorganic salts the presence of both riboflavin and biotin was obligatory, but for a convenient colony size the use of thiamine was also necessary. Amino acid requirements of some 13 strains were not the same. The serotype, agglutinability, haemolytic effect, virulence and nutritional demand of these strains were not consistently linked. Two "ESM" mutants were also studied. Haemolytic effect, virulence and arginine, glutamine as well as methionine metabolism of one of them (025/4/4) was damaged.

Recently, it was observed that listeria strains could grow on that minimal medium without thioglycollate [100]. Siddiqi and Khan [101] published a complete chemically defined medium. In this medium riboflavin and Ca-pantothenate were essential for 4 *L. monocytogenes* and 2 *L. ivanovii* strains tested. Biotin, pyridoxal-

hydrochloride and p-aminobenzoic acid were either essential or stimulatory for those strains. Most strains did not require folic acid, thiamine, nicotinic acid and inositol but they were stimulatory for some strains. Adenine was essential for two *L. monocytogenes* strains. Later they observed [102] that most strains required cysteine, valine, isoleucine and leucine in a minimal medium. Phenylalanine was only stimulatory for them. Whilst tryptophan was essential for two *L. monocytogenes* and one *L. ivanovii* strains and stimulatory for one *L. monocytogenes* and one *L. ivanovii* strains, none of them required asparagine, glutamine, proline, histidine and/or tyrosine for their growth. Premaratne et al. [103] recently developed a chemically defined fluid minimal medium by modification of Welshimer's medium. *L. monocytogenes* Scott A strain required leucine, iso-leucine, arginine, methionine, valine, cysteine, riboflavin, biotin, thiamine and thioctic acid. Its growth was stimulated by ferric citrate. Glucose and glutamine were required as primary sources of carbon and nitrogen, respectively. Glucose could not be replaced by various organic acids or amino acids. Several sugars and amino sugars supported the growth in the absence of glucose. Evidence was found that chitin and cell walls of *Lactococcus lactis* supported survival of *L. monocytogenes*.

1.4.1. Selective synthetic solid medium

Braveny and Grote [104] prepared a solid selective synthetic medium on the basis of the nutritional properties of listeria strains. It consisted of potassium and sodium phosphate buffer, Mg^{++} and Fe^{++}, glucose, leucine, iso-leucine, valine, cysteine, glutamine, arginine, methionine, histidine, tryptophan, riboflavin, biotin, thiamine, thioctic acid, potassium tellurite, nalidixic acid and nystatin. There have been no reports of the general use of this medium.

1.5. Selective agents

Selective agents used for listeria media have been reviewed [1, 2, 4, 9, 12]. Recently new data have been published. Curtis et al. [105] studied the minimum inhibitory concentrations of four antibiotics used in listeria selective agars at 30 and 37 °C. Differences of susceptibility to beta-lactam antibiotics and fosfomycin were observed in listeria strains. Incubation at 30 °C was recommended for listeria selective agars containing ceftazidime, cefotetam, latamocef and fosfomycin. Cox et al. [106] reexamined the effect of LiCl on the growth of listeria in enrichment broths. *L. monocytogenes* was able to grow in concentrations up to 2%, but above this level inhibition was noted. Trypaflavine was shown not to inhibit significantly the listeria strains studied in the presence of LiCl. The combination of LiCl, trypaflavine and nalidixic acid should be studied further in enrichment procedures for *L. monocytogenes*. A total of 74 isolates of *Listeria* (53 *L. monocytogenes*, 10 *L. inocua*, 3 *L. ivanovii*, 6 *L. welshimeri* and 2 *L. seeligeri*) were evaluated for their ability to grow

in the presence of 100 μg ml^{-1} sodium arsenite [107]. Six *L. monocytogenes* and one *L. inocua* strains were arsenite-resistant. Plasmid profiles of selected strains indicated that arsenite resistance is not a plasmid-linked trait in listeria. Fernandez-Garayzabal et al. [22] observed that potassium tellurite and BHIA can influence the haemolytic phenotype of *Listeria* spp. A bigger zone of haemolysis developed on BHI BA than on CBA. The addition of potassium tellurite increased the haemolysis of *L. monocytogenes* and decreased that of *L. ivanovii*.

1.6. Isolation of listeria colonies from plating media

This procedure requires practice. When a medium is transparent then Henry's oblique light illumination method can easily be applied. Under such conditions listeria colonies have characteristic colony morphology (glittering bluish surface) and suspicious colonies can be picked [12]. Application of Henry's method for both Oxford and PALCAM agars proved to be useful, too, because it was easier to differentiate among the black colonies of listeriae and that of the other bacteria grown [54, 55]. Lachica [108] modified the original Henry's technique. His simplified technique involved illuminating each colony directly with a high-intensity lamp while viewing it with a hand lens at a precise angle in place of a scanning light microscope. Dealler and Rotowa [109] developed a rapid screening method (Liststrip, Lab M) for the identification of aesculin positive colonies from Oxford agar. This strip method is based on the ability of listeriae to hydrolyse aesculin and to have rapid phosphatase activity but neither a pyroglutamic acid beta-naphthylamide amidase activity nor indole production. Some 194 listeria strains gave identical results in 10 min.

1.7. Identification and typing of listeria strains

Identification and typing of a suspicious strain is a very important task not only for the recognitation and verification of the identity of the bacterium but also for the determination of its antibiotic sensitivity and to perform epidemiological studies as well as animal experiments.

1.7.1. Methods for identification

1.7.1.1. Biochemical methods

Chemotaxonomic criteria have generally been used for the identification and classification of listeria strains until recently. Different methods have been published for that purpose. Robison et al. [110] proposed the use of Micro-ID Listeria identification system. The essence of this system is a self-contained unit with fifteen biochemical tests. It can be applied for rapid identification of the genus *Listeria* to species. Del Corral and Buchanan [111] evaluated the API–ZYM system for the same purpose. The system allowed listeria isolates to be rapidly analysed for 19 enzymatic activities. This kit could be used for rapid confirmation of listeria to the

subgenus level. Robison and Cunningham [112] identified a total of 63 cultures representing 7 species of *Listeria* and 10 cultures of other Gram-positive organisms with MICRO-ID^R Listeria and the conventional biochemical procedures. Both methods agreed on all 73 cultures. The test strips were easy to inoculate and read, and the results were obtained 24 h after inoculation, as compared to 7 days for the conventional procedures.

Kerr et al. [113] used the Rosco system for determining carbohydrate fermentation reactions of *Listeria* species. The system identified all species correctly. Results were obtained after 4 h if heavy inocula were used. The Rosco system can be used as a rapid, inexpensive method for the identification of listeriae.

Lachica [114] described a diagnostic scheme for the same-day identification of *L. monocytogenes* that emerged in large colonies on his medium after 40 h incubation at 30 °C. In this RAP-ID scheme he studied haemolytic activity, sugar acidification, catalase activity, KOH viscosity and motility by phase contrast microscope.

Kerr et al. [115] compared a multipoint inoculation technique (Mast ID) with the commercially available API 50CH system. Both methods successfully identified all 123 listeria strains tested. The Mast ID system was less expensive and time consuming than the API 50CH system.

Some 136 strains of the genus *Listeria* were characterized using 329 miniaturized tests by Kampfer et al. [116]. Overall similarities of the strains were determined by numerical taxonomic techniques. Strains of *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. murray* and *L. grayi* were found in separate clusters and formed homogeneous taxospecies. In contrast with this fact strains of *L. monocytogenes* and *L. ivanovii* could be found in five heterogeneous clusters and one single-member cluster. Members of these two species could be separated by hydrolysis of D-alanine-p-nitroanilide. The newest API identification system allows the determination of ten biochemical reactions using substrates aesculin, alpha-mannosidase, D-arabitol, D-xylose, rhamnose, alfa-methyl-D-glucosidase, ribose, glucose-1-phosphate, D-tagatose. After 24 h incubation the strains can be identified to species level [117].

1.7.1.2. Microcalorimetric investigation

During the 1960's microcalorimetry was observed as a useful technique in the characterization of microorganisms. Allerberger et al. [118] tested 61 listeria strains by microcalorimetry. *L. monocytogenes*, *L. welshimeri* and *L. inocua* isolates showed similar microcalorimetric curves which differed considerably from those of *L. ivanovii* and *L. seeligeri*. The latter displayed different and quite characteristic thermograms.

1.7.1.3. Pyrolysis-mass spectrometry (Py–MS)

This method was originally developed for the analysis of insoluble polymeric materials. Recently it has been used for the characterization of biological substances, too. With the help of Py–MS bacteria can be identified, classified, typed within species or chemically analysed. After cultivation the strains are smeared on pyrolysis foils and are pyrolysed at 530 °C for 4 s. The spectra are recorded and analysed. *L. monocytogenes* strains have spectra which are clearly distinct from those of corynebacteria [119]. Freeman et al. [120] examined 26 *L. monocytogenes* strains isolated from outbreaks and separate cases. The results correlated well with the epidemiological data. Py–MS clustered the epidemic strains and included in the same cluster some other strains, too. Four isolates originating from elsewhere had a similar phage type. The inclusion of three other strains suggests that the Py–MS analysis is to a degree independent of phage typing.

1.7.1.4. Genetical methods

1.7.1.4.1. DNA–DNA hybridization

During the last 20 years, on the basis of the results of DNA homology studies the former species of *L. monocytogenes* was divided into different genomic groups, thus emphasizing the importance of DNA–DNA hybridization technique in the identification of listeria strain [121–123].

1.7.1.4.2. Low molecular weight RNA profiles

Recently a new method for the identification of bacteria based on analysis of profiles of low molecular weight – LMW – RNA (5S rRNA and tRNAs) was described. Slade and Collins-Thompson [124] used this method to study LMW RNA profiles of listeria. The profiles of the five *Listeria* spp. were identical when cultured in three different media. This technique represents a simple, reproducible approach to the identification of species and possibly of relationships between species.

1.7.1.4.3. Pulsed-field gel electrophoresis (PFGE)

This is also an alternate method for differentiating microorganisms. Intact, high-molecular-weight genomic DNA was prepared from embedded listeria cells and digested by restriction endonuclease. Restriction fragments were separated with pulse-field system. First Vicente et al. [125] published the application of PFGE to the analysis of *L. monocytogenes*. Later, Howard et al. [126] observed that after ApaI, AscI, SmaI, or NotI digestion species- and serotype-specific differences in genomic fingerprints could be demonstrated in case of *L. monocytogenes*, *L. inocua*, *L. ivanovii* and *L. seeligeri* strains. Buchrieser et al. [127] characterized 35 *L. monocytogenes* strains belonging to serogroups 1/2 and 3 by whole cell DNA

restriction patterns using low-frequency cleavage enzymes and PFGE. They detected 17 restriction profiles. The strains could be divided into 24 distinct groups.

1.7.1.4.4. Sequencing of 16S rRNA with reverse transcriptase

This method being used increasingly as an alternative to oligonucleotide cataloging for elucidating the phylogenetic interrelationship of microorganisms. Collins et al. [128] studied the members of the genus *Listeria* with this method. Their data indicated that at intrageneric level the genus consisted of two closely related but distinct lines of descent. The *L. monocytogenes* group of species (including *L. inocua*, *L. ivanovii*, *L. seeliger* and *L. welshimeri*) and the species *L. grayi* and *L. murrayi*. At the intergeneric level a specific phylogenetic relationship between the genera *Listeria* and *Brochothrix* was evident.

1.7.1.4.5. Multilocus enzyme electrophoresis

This is not only a useful typing method for epidemiological investigations but is also able to make a contribution to taxonomic studies. Phenotypically similar *Listeria* species could be separated with the help of this method into six clusters at the species level. *L. monocytogenes*, *L. inocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii* each corresponded to one of these clusters without overlapping. *L. grayi* and *L. murrayi* formed a unique cluster and therefore these two species should be considered two biovars of a single species [129].

1.7.2. Methods for demonstration of virulent listeria

The genus *Listeria* consists of several species of which *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* have been implicated in human and animal infections. As only the pathogenic species have public health and veterinary importance it is a very important task to differentiate pathogenic listeria from non-pathogenic. The pathogenic property of listeria strains has been studied since 1924. The relevant publications were summarized during the last 20 years [12, 99, 130–134]. At present different methods can be applied to determine the virulence of listeria strains.

1.7.2.1. Cultural methods

Beta-type haemolysis on BA containing sheep or horse blood usually means that the given listeria strain is more or less virulent. The non-haemolytic listeria strains have never yet been proved to be virulent [130, 131]. McClain and Lee [19] proposed the use of a thin layer of horse BA (HL) to detect weak beta-haemolytic activity of listeria strains.

Dominguez et al. [135] developed an overlay technique for identification and counting of haemolytic listeria on selective plating media. The best results were obtained with LSA modified. Cassiday et al. [136] published a replica plating method. Bacterial colonies from LSAs were replica plated to sheep BA to screen for beta-

haemolysis. With the help of this method they could increase the number of listeria positive samples. van Netten et al. [137] used a special agar containing sheep erythrocytes and the supernatant fluid of a blood culture of a *Staphylococcus aureus* to overlay inoculated PALCAM agar plates. This method made possible the visual reading of haemolysis and increased recovery of *L. monocytogenes* from raw food samples.

It was observed that the colonies of a beta-haemolytic virulent listeria strain caused precipitation in LV agar but those of a non-haemolytic non-virulent strain failed to do so [12]. Notermans et al. [23] proposed the use of agar plates overlayed with L-alpha-phosphatidylinositol agar to determine pathogenic properties of listeria strains as only pathogenic *L. monocytogenes* strains caused a positive reaction in this medium. That activity was not present in any of the 167 strains of *L. seeligeri*, *L. welshimeri*, *L. inocua* and *L. greyi*.

The CAMP test may be of differential diagnostic value in distinguishing haemolytic listeria from non-haemolytic ones as well as from group B streptococci and *Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*) because sometimes it may be difficult to recognize weak haemolysis by nacked eye [12, 138, 139]. Ivanov [140] presented the so called Ivanov's test with *Rhodococcus equi* which bacterium intensively enhanced the haemolytic effect of *L. ivanovii* strains on BA but only weakly strengthened the haemolytic effect of other *Listeria* species [12, 138, 141]. In opinion of some authors this test gave only partly reproducible results [30, 142]. Brzin et al. [143] observed a functional similarity between *L. ivanovii* and *S. aureus* in the CAMP test. Vazquez-Boland et al. [144] revised the validity of both CAMP and Ivanov's tests. They thought that these tests were unreliable and proposed the use of a microplate technique [145].

Cowart and Foster [146] found that virulent *L. monocytogenes* strains exhibited faster rates of growth as a function of iron concentration than did the avirulent ones. So iron has a differential effect on the growth of virulent and avirulent *L. monocytogenes*.

1.7.2.2. Animal experiments

Different animal species – rabbits and guinea-pigs, white mice, monkeys, chick embryos, suckling mice, nude mice, rats, immunocompromised mice – have been used for the demonstration of the pathogenicity of listeria strains. The animals can be infected by intraperitoneal (ip), peroral (po), intragastric (ig), intraocular (io), intracerebral (ic) or respiratory routs. Virulent beta-haemolytic listeria strains can grow in the test animals and kill them or can cause keratoconjunctivitis in eyes of rabbits or guinea-pigs [99, 130, 131]. However, the level of haemolysin is not directly proportional to virulence [147], listeriolysin 0 is one of the virulence factors because it allows the intracellular growth and the extraintestinal dissemination of the

pathogens [148–152]. Results of Tabouret et al. [153] suggest that factor(s) other than listeriolysin could be involved in the virulence of *L. monocytogenes*. Non-haemolytic or R strains never kill the animals or never cause keratoconjunctivitis. Even these strains have also penetrated into the epithelial cells of eyes but they have not been able to multiply in that cells [12, 130, 131, 133]. It was observed that the loss of catalase activity did not reduce the level of virulence of these mutants in mice [154].

Notermans et al. [155] observed a good correlation between the result of chick embryo test and the mouse bio-assay, so both of them can be applied. Pine et al. [156] compared ig and ip approximate 50% lethal doses (ALD_{50}s) of *L. monocytogenes* in mice. They observed that although all strains gave ig ALD_{50}s comparable to or less than their ip ALD_{50}s, the ig feeding of most strains produced more deaths within the first 3 days of the 6-day test than did ip injection.

It seems that chicks of 2 days of age after oral infection are not susceptible to *L. monocytogenes*. Listeriae disappeared from the digestive tract and also from the body in most of the chicks within 9 days [157].

1.7.2.3. Infection of cells in vitro

Since the 1960's a number of cell types have been successfully infected by listeria *in vitro*. These are: macrophages, fibroblasts, epithelial cells, enterocyte-like Caco-2 cells and so on. Beta-haemolytic virulent listeriae not only invade these cells but also multiply in them and destroy the cells [158]. Although non-haemolytic mutants are still able to invade the cells, they are unable to survive within them. Haemolysin does not support the entry of listeria into the cells but it is necessary for the survival inside the cells mediating lysis of the phagocytic vacuoles [159]. The p60 extracellular protein seems to be necessary for the invasion [160]. Kathariou et al. [161] identified an (other?) extracellular protein with an apparent molecular weight of 32 000 which could induce opacity in egg yolk and was considered to be a phospholipase and involved in the ability of *L. monocytogenes* to enter mammalian cells. Later they purified and characterized this protein [162]. Once *L. monocytogenes* is free within the cytosol it acquires the capacity to spread intracellularly and intercellularly infecting adjacent cells by interacting with host cell microfilaments. The bacteria become encapsulated by short actin filaments and other actin-binding proteins. The actin-based structure is rearranged to form a long tail behind the bacterium, which appears to mediate movement of the bacterium through the cytoplasm to the cell periphery [163, 164].

1.7.2.4. Genetical methods

The gene coding for listeriolysin O (*hlyA*) has been cloned and sequenced [165]. The regulation of the expression of this essential virulence factor of *L.*

monocytogenes was also studied [166, 167]. The presence of two open reading frames (ORF) – D and U – was verified near the *hlyA* gene. Transposon insertions within the *hlyA* gene resulted in several non-haemolytic non-virulent mutants. This fact suggests that this region of the bacterial chromosome is important for virulence. An avirulent mutant transformed with a plasmid carrying only *hlyA* displayed a haemolytic phenotype identical to that of the wild strain and was virulent. The *prfA* gene also positively regulates the expression of a number of virulence factors. Köhler et al. [168] cloned the *iap* gene of *L. monocytogenes* which encodes the major extracellular protein (p60) and its complete nucleotide sequence was also determined. Mengaud et al. [169] observed that transposon inseration in ORF D could induce lecithinase-negative mutant of *L. monocytogenes*. Barry et al. [170] observed not only phenotypic defects (decreased haemolysin production, phospholipase C activity, intracellular growth, cell-to-cell spread in vitro and reduced virulence for BALB/c mice) of the transposon induced mutants of *L. monocytogenes* but also that two of these mutant strains were unable to escape phagolysosomes in infected J774 cells and could not transform these cells into targets of *L. monocytogenes*-immune cytotoxic cells. In DNA hybridization studies [171] with five DNA probes that encoded the listeriolysin O gene and surrounding sequences, highly homologous listeriolysin genes were found to be present in the species *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*. This study demonstrated the genetic homology between the various listeriolysins produced by *Listeria* species. Further analysis of the DNA fragments originating from two *L. monocytogenes* isolates harbouring the gene for listeriolysin, part of the gene for a phosphatidyl-inositol-specific phospholipases C, and part of the gene for a metalloprotease, revealed a significant number of nucleotide differences. These differences could be used to subdivide the species with the help of oligonucleotide probing of a larger number of *L. monocytogenes* strains [172]. When the *prfA* gene or an internal part of *iap* gene were used as a DNA probe they were found to be specific for demonstration of pathogenic *L. monocytogenes* [167, 168, 173].

1.7.3. Typing methods for listeria

Currently several methods can be used for typing or subtyping of listeria strains. These are biotyping, serotyping, phage typing, listeriocin typing, multilocus enzyme electrophoresis (MEE), restriction fragment length polymorphisms (RFLPs), restriction endonuclease analysis (REA), plasmid profile analysis and the use of type-specific oligonucleotides.

1.7.3.1. Biotyping

Emődy and Ralovich [174] studied the possibilities of biotyping. They presented a scheme on the basis of carbohydrate splitting character of *L.*

monocytogenes strains. The problem is that the fermentation property of *L. monocytogenes* strains for some carbohydrates is not always a stable character.

1.7.3.2. Serotyping

On the basis of O and H antigens listeria strains can be divided into more than 19 serogroups. Garcia Cabrera et al. [175] and Vazquez-Boland et al. [176] revised the original O antigenic scheme proposing some modifications. The antigenic markers alone are generally not enough for epidemiological studies because the greater part of listeriosis cases is caused by pathogenic listeria strains belonging to a small number of serovars: 1/2a, 1/2b, 1/2c, 3, 4a, 4b [177]. An interesting and useful exception was published by the English researchers. McLauchlin et al. [178] and McLauchlin et al. [179] observed an unusual variant of *L. monocytogenes* serogroup 4 during 1987–1989 in England, Wales and Northern Ireland as an epidemic strain. They designated this serovar 4b (x).

1.7.3.3. Phage typing

Up to now more than 300 listeria phages have been isolated from lysogenic strains, water and sewage with or without induction. Some phages, however, could only be isolated when the parent strain was induced. Loessner et al. [180] compared inducibility of bacteriophage in naturally lysogenic and lysogenized strains of *Listeria* spp. by UV light and Mitomycin C. Most strains were readily inducible. The yield of free phage could be increased. Mitomycin C was found to be a more powerful inducing agent than UV light. All listeria phages proved to be temperate except one (A511). Zink and Loessner [181] investigated 22 phages by electron microscopy and the proteins of these phages by electrophoresis for classification. Their classification corresponds well to the previous groupings based on host range. As to the research works on the field of listeria phages their main aim has been to develop a phage typing system for elucidating the epidemiology of listeriosis [182–185].

Although phage typing is more discriminating than serotyping, not all *L. monocytogenes* strains can be typed. Only 60 to 90% of the strains have been found typable. Loessner and Busse [186] developed a new bacteriophage typing scheme for differentiating listeria isolates using 16 selected phages. According to their lytic spectrum the isolates could be divided into 4 groups. When this set was used in typing 57 reference strains and, in addition, 454 listeria isolates, 41 distinct patterns of lysis were seen. Overall typability was 84.5%. Later Loessner [187] developed an improved method for phage typing of listeria strains with a reversed procedure. An extended set of 21 genus specific phages were employed. This method seems to be more reliable, efficient and convenient than the original one. The overall typability of listeria strains was 89.5%. Loessner et al. [188] studied the phagovar variability of listeria strains under the influence of virulent and temperate bacteriophages. Selected

strains were challenged against a variety of bacteriophages. The resistant mutants derived were characterized by the loss of sensitivity to defined groups of phages. When different phages were used in succession multiple mutants could be obtained. Their results indicated the possibility of shifting phagovars among listeria strains grown in mixed culture due to the potential action of free bacteriophages.

1.7.3.4. Listeriocin (monocin) typing

This method was discovered in 1961. The substances produced by listeria have an antibiotic-like effect against *L. monocytogenes* and other microorganisms such as staphylococci but they do not attack Gram-negative bacteria. Monocins could be induced by UV-irradiation from non-lysogenic strains of listeria. Listeriocins are somewhat trypsin-resistant proteins (defective phages with tail but without head) which are stable between pH 5.0 and 10.0. They can be classified into groups on the basis of their thermo-resistance and of their effect on the indicator strains [189]. Studies connected with listeriocins might be of practical epidemiological value [190].

1.7.3.5. Multilocus enzyme electrophoresis (MEE)

Ashton et al. [191] studied enzyme types of 46 Canadian listeria strains. Using nine enzyme loci seven electrophoretic types (ETs) were determined among 15 strains associated with sporadic cases which occurred across Canada. In contrast with this 27 out of 29 strains isolated during the Halifax outbreak had the same ETs. Bibb et al. [192] examined 310 strains by MEE and defined 56 ETs. Analysis revealed a sharp division among ETs which divided the species into two major clusters. In three separate epidemiologic investigations electrophoretic typing confirmed a common source as a cause of an outbreak and it was useful in documenting potential links between listeria-contaminated foods and persons. In another publication [193] they presented the results of MEE typing of 390 isolates, defining 82 ETs. Two distinct clusters of ETs (ETGA and ETGB) were identified. Strains of ETGB were associated with perinatal listeriosis. They started to establish a baseline for the study of the epidemiology of listeriosis by MEE.

Boerlin and Piffaretti [194] analysed 181 *L. monocytogenes* strains isolated from humans, animals, food, and the environment by MEE at 21 enzyme loci. The clone responsible for foodborne outbreaks in Switzerland and in North America was found frequently among strains isolated from animals. Thus, animals might represent a source of dissemination of this clone in the environment and in food. Two other unrelated clones were isolated from meat but not from animals. These findings might indicate that contamination of meat originated mainly from the environment in the meat industry rather than from the animals. Their results show that MEE is a tool for epidemiological studies.

1.7.3.6. Restriction fragment length polymorphisms

The basis of this typing method is the detection of interstrain RFLPs within the species *L. monocytogenes* using a cloned DNA probe. Saunders et al. [195] investigated the possibility of using RFLPs to distinguish different strains of *L. monocytogenes*. Cloned DNA fragments were selected from a bacteriophage lambda gene library of *L. monocytogenes*. DNAs from two lambda clones were labelled with biotinylated dUTP for use in these experiments. The criteria for probe selection were the ability both to hybridize all *L. monocytogenes* strains and to reveal RFLPs. Sixty-four strains of *L. monocytogenes* belonging to serogroup 1/2 which were not apparently epidemiologically associated were tested and 19 patterns were observed. Epidemiologically related strains gave identical patters of RFs. Carriere et al. [196] studied DNA polymorphism of 35 *L. monocytogenes* strains belonging to different serotypes. RFLPs varied among different serovars and were used for epidemiological studies.

Ribotyping, based on RFLPs in the chromosomal DNA containing rRNA genes, has been used as a means of detecting interspecies and interstrain differences. Baloga and Harlander [197] isolated total DNA from 28 *L. monocytogenes* isolated from food implicated in food-borne illness and from patients with listeriosis. The DNA was digested with three restriction endonucleases. Following agarose gel electrophoresis, the fragments were subjected to Southern blot hybridization with a digoxigenin-labelled cDNA probe transcribed from *Escherichia coli* 16S and 23S rRNA. The patterns of bands from genomic (DNA fingerprints) and rDNA fingerprints (ribotypes) were used for classification of *L. monocytogenes* strains. The most discriminating enzyme for ribotyping of strains was EcoRI, which divided the 28 *L. monocytogenes* into 6 ribotype groups. DNA fingerprinting and ribotyping differentiated *L. monocytogenes* from other *Listeria* spp. (*L. ivanovii*, *L. welshimeri* and *L. inocua*) as well as the lactic acid bacteria. *L. monocytogenes* strains isolated from four independent food-borne illness incidents were analysed by all typing methods. Patient and product isolates were not distinguishable by serotyping, ribotyping or multilocus enzyme electrophoresis. DNA fingerprinting was the only method capable of differentiating these strains or, conversely, of proving relatedness of patient-product pairs of isolates. Jacquet et al. [198] characterized 94 strains of *L. monocytogenes* of different serovars and phagovars as well as of varying origins by ribosomal RNA gene restriction polymorphism. After enzyme digestion chromosomal DNAs were hybridized with a cloned rDNA probe from *Bacillus subtilis* that included the 16S rRNA gene. The 94 strains were divided into 14 ribovars according to the different hybridization patterns generated by cleavage with EcoRI. EcoRI ribovar analysis allowed description of a new typing scheme which did not corroborate serotyping and phage typing.

1.7.3.7. Restriction endonuclease analysis

Nocera et al. [199] used REA of the chromosomal DNA to characterize *L. monocytogenes* collected from various sources during and after a Swiss outbreak. Ten different REA profiles were obtained. All 57 serotype 4b strains that were identified as Swiss epidemic strains by phage typing clustered into two closely related REA profiles. REA could characterize non-phage-typable strains and therefore, seemed to be a promising tool for *L. monocytogenes* subtyping. Wesley and Ashton [200] examined *L. monocytogenes* isolates from the Nova Scotia, Boston and Los Angeles outbreaks by REA with the endonuclease HhaI. Thirty-two human isolates were compared with a strain recovered from coleslaw; 29 of 32 strains exhibited the restriction enzyme pattern (REP) of the reference coleslaw isolate. The REP of the nine clinical strains from Boston were identical to each other but differed from those of raw milk isolates recovered from sources supplying the pasteurizer. REP of the 48 *L. monocytogenes* from human and from the suspect cheese samples were identical to those of four of five cheese factory environmental isolates. Isolates from each of these outbreaks exhibited a REP that was characteristic of that outbreak.

1.7.3.8. Plasmid profile analysis

Fistovici and Collins-Thompson [201] studied 48 listeria strains isolated from bulk raw milk. They determined plasmid and REA profiles of these strains. These characters can be used for monitoring *Listeria* spp. in the environment. Kolstad et al. [202] isolated extrachromosomal DNA from 107 (78%) out of 139 listeria strains. Only one of the isolates contained more than one plasmid. Plasmids from 51 of these strains were investigated. They covered a range of 8 different-sized molecules. Restriction analysis and hybridization experiments showed a high degree of homology between the different plasmids.

1.7.3.9. Use of type-specific oligonucleotides

Assays based on hybridization or PCR reaction with type-specific oligonucleotides may provide fast and easy alternative methods for strain typing [172].

2. Rapid detection methods

It is believed that the use of enrichment culture protocols coupled with rapid detection methods (immunoassays/2.1.) and nucleic acid probes/2.2.) may perhaps be the most efficient procedures to demonstrate listeria.

2.1. Immunoassays

Ziegler and Orlin [203] described several genus and species-specific monoclonal antibodies (MAbs). MAbs against listeria antigens were also produced by others [204–207]. With the help of MAbs different immunological methods have been developed.

2.1.1. ELISA procedures

Two ELISA kits (Organon and Noack) are commercially available. FB cultures were tested directly by some scientists [28, 44] by ELISA as described by Fraser and Sperberg [27]. UVM, LEB and MOPS cultures are also convenient for that purpose [28, 44, 208]. Sensitivity of the ELISA was studied by Mattingly et al. [209] who observed that for a positive reaction about 10^4 cells were necessary. Usefulness of Organon and Noack ELISA kits were compared by us [210, 211] and we found that presence of 2×10^4 colony forming units (c.f.u.) in the enrichment broth was the minimal requirement for a positive reaction with both kits. One step UVM enrichment was frequently insufficient. Sometimes, presumably when listeria cells were present in the food at low levels or in an injured condition, ELISA reaction was negative even after UVM_1 and UVM_2 enrichments. UVM broths were tested in parallel by conventional subculture. The latter was more sensitive than ELISA resulting in more positive results. Also when ELISA was positive it was still necessary to perform cultivation and isolation in order to obtain the species identification since the ELISA is genus specific. In contrast with our observation, others [44, 209] found that ELISA was more sensitive than conventional cultivation.

Beumer and Brinkman [212] stated that when the enrichment broth contained 10^6 cells/ml or more a positive ELISA was obtained. They detected some interference from product and/or competitive microorganisms. For the successful application of ELISA efficient enrichment procedures were necessary, especially when *Listeria* spp. were present at low levels.

Kerr et al. [213] examined 102 samples with culture and ELISA; 29 samples were positive with cultivation and only 24 with ELISA. Heisick et al. [62] compared four procedures to detect listeria strains in 309 food samples. They observed that culture procedures detected the most positive samples, ELISA was the next most sensitive and the GENE-TRAK DNA probe was the least sensitive. Durham et al. [214] found that Listeria-Tek had the sensitivity needed to conform to zero tolerance for listeria in food samples. Walker et al. [44] compared the abilities of conventional cultural procedures to detect listeria strains inoculated into foods and that of the Listeria-Tek ELISA kit. They cultivated the samples in MOPS-buffered enrichment broth and in UVM_1 + FB for the determination. The Listeria-Tek kit procedure produced results as good as the best cultural procedures used. Varabioff [18] stated that the cultural method gave greater recoveries of listeria from meat samples than

the TECRA ELISA kit. Vanderlinde and Grau [215] used an ELISA kit (TECRA™) for the detection of *Listeria* spp. in naturally contaminated meat and in environmental samples. Of the 170 samples examined, *L. monocytogenes* and *L. inocua* were detected in 74 by enrichment and selective plating. The ELISA kit detected 72 of these positive samples and gave 2 false-negative and 2 false-positive reactions. Norrung et al. [216] compared the ability to detect *Listeria* spp. of an ELISA and the FDA and USDA–FSIS culture procedures. One hundred samples of minced beef, artificially inoculated with *L. monocytogenes* and 149 natural samples were tested. The USDA–FSIS procedure proved to be the most sensitive method when samples contained less than 3 c.f.u. *L. monocytogenes* per g. When the count was higher than 3 c.f.u./g, then the ELISA and the USDA–FSIS method were almost equally sensitive, however, the detection limit for ELISA was 10^6 listeria cells and for the culture procedure 10^4 cells/g. Gavalchin et al. [217] isolated and characterized three MAbs that preferentially recognize the pathogenic *L. monocytogenes*. An indirect sandwich ELISA was developed and used to further confirm that these MAbs produced by Balb/c mouse reacted with a number of *L. monocytogenes* isolates and not with other common cross-reacting bacteria. Comi et al. [208] compared Listeria-Tek ELISA with the modified cultural procedure of the FIL/IDF (Federation International de Laiterie/International Dairy Federation). The ELISA assay was able to detect all *Listeria* species after 48 h enrichment. The ELISA test was positive when 10^4 c.f.u. listeria cells were present in each ml of the enrichment broths. For 313 food samples tested only 3 false positive results were observed. ELISA gave 0.9% more positive results than the cultural method.

2.1.2. Immunomagnetic separation

Skjerve et al. [218] reported separation of *L. monocytogenes* strains with immunomagnetic beads from both pure cultures and heterogeneous suspensions. The MAbs used recognized all six strains of serotype 4 but only one out of 3 strains of serotype 1. The sensitivity of the method was less than 10^2 cells/ml in pure cultures and less than 2×10^2 cells/ml in enriched foods. VICAM developed a commercially available procedure (Listertest™ Lift) for the detection of listeria in foods and environmental samples [219]. Fluid or liquefied samples after centrifugation can be mixed with magnetic immunobeads. The immunobeads capture listeria cells and so they can be separated magnetically from the sample and plated onto a solid medium containing little or no selective agents. Immediately after plating a membrane is overlaid on the medium to obtain an imprint of colonies for analysis. The number of viable listeria cells can be determined with this technique.

2.2. Nucleic acid probes

Recent progress in molecular biology has shown the possibility of detecting pathogens in samples without their isolation using target-specific gene probes.

2.2.1. Standard hybridization technique

Standard hybridization techniques detect genes coding for pathogenicity factors (beta-haemolysin, listeriolysin, CAMP-factor, msp, DTH factor, *p60*, *iap*, *prfA*) or specific 16S ribosomal RNA sequences [167, 168, 220–227]. Isotopic and non-isotopic (colorimetric or chemiluminescent) hybridization assays have been reported [220, 221, 228–232]. The colony hybridization can be performed on an agar plate or directly in enrichment medium by using a filtration technique. Some of the genes (*DTH–18*, those coding for alfa- and beta-listeriolysins, *p60*, *prfA*) seem to be specific for pathogenic listeria. These methods need relatively large numbers of target cells for a positive result. Heisick et al. [62] showed that the GENE-TRAK DNA probe was not so sensitive as cultivation or ELISA. Datta's opinion [233] was that the use of nucleic acid probes could substantially reduce the time required for identification and enumeration of listeria. However, the usefulness of radioactive techniques was limited. King et al. [231] developed a simple rapid non-isotopic assay. The comparison of the assay to the reference USDA–FSIS culture method indicated that the two procedures were essentially equivalent in sensitivity with both foods and environmental samples. The nucleic acid-based detection system proved itself to be highly accurate and sensitive. Peterkin et al. [234] isolated a DNA probe specific for *L. monocytogenes*. It was labelled with horseradish peroxidase and used in a direct colony hybridization method on hydrophobic grid-membrane filters for the detection of the organism. Following colour development of the chromogen, a commercial counter {HGMF Interpreter) was used to detect and count listeria electronically. Köhler et al. [168] cloned the gene of *L. monocytogenes* that encodes a major extracellular protein (p60) and designated it *iap* (invasion-associated protein). Hybridization with the *iap* gene revealed the presence of homologous sequences in most other *Listeria* species. In contrast, a 400-base-pair internal *iap*-probe hybridized only with genomic DNA of *L. monocytogenes*. Four oligonucleotides published by Datta et al. [235] proved to be a part of the *iap* gene. Kim et al. [236] prepared and used oligonucleotides to develop an *L. monocytogenes*-specific non-isotopic colony hybridization assay to confirm rapidly the presence of this organism on LPM agar plates. The probe reacted with all strains of *L. monocytogenes* tested and did not react with any other *Listeria* species or with other Gram-positive bacteria. Alden et al. [232] developed a chemiluminescent-labelled DNA probe which was complementary to ribosomal RNA of *L. monocytogenes*. Use of this probe in a hybrid protection assay format permits identification of *L. monocytogenes* from culture in under 40 min. This assay proved to be rapid and specific for *L.*

monocytogenes. Wang et al. [237] isolated crude rRNA from *L. monocytogenes*, *L. inocua* and *L. ivanovii* and sequenced by a reverse transcriptase method. Only two sequence regions were found to differ for *L. monocytogenes* versus *L. inocua* and *L. ivanovii*. Two oligonucleotide probes (RL-1 and RL-2) complementary to these two regions of rRNA of *L. monocytogenes* were synthesized. The RL-2 probe had two bases which differed for *L. monocytogenes* versus *L inocua* and *L. ivanovii*. Use of a dried gel hybridization in place of Northern hybridization or dot blot hybridization indicated that the RL-2 probe hybridized with all 36 *L. monocytogenes* strains tested but not with 6 other *Listeria* spp. and 11 other bacteria tested. So the RL-2 probe is specific for *L. monocytogenes* and is being used to develop a rapid method to detect it in contaminated foods. They [238] also published their new simple, sensitive, dried gel DNA hybridization method for detection of *L. monocytogenes* DNA fragments. DNA samples were fractionated on an agarose gel. The gel was then denatured in NaOH-NaCl and neutralized in Tris-NaCl. The resulting agarose gel was dried and hybridized with ^{32}P-labelled DNA probe. No transfer to nitrocellulose membrane was used.

2.2.2. Polymerase chain reaction (PCR)

The recently developed technique using thermostable DNA polymerase permits the rapid amplification of specific DNA sequences by a factor of up to 10^7. Border et al. [239] used five oligonucleotide sequences as primers in PCR. When all five primers were used in combination, three PCR products were possible; a listeria specific product that occurred with DNA from any *Listeria* spp., a *L. monocytogenes* specific product that occurred only in the presence of DNA from this organism and a universal product that was found using DNA from any bacterial source. Bessesen et al. [240] also developed a PCR method. They could amplify listeriolysin O gene and so their method was specific for *L. monocytogenes*. The sensitivity of the assay was 10^4 *L. monocytogenes*/ml and it must be improved before it can be used directly on clinical or food samples. Deneer and Boychuk [241] used PCR to detect and specifically identify *L. monocytogenes*. A 174-bp region of the listeriolysin O gene was shown to be specifically amplified in *L. monocytogenes* but not in other *Listeria* spp. or in a number of other Gram-positive and Gram-negative bacteria. Less than 50 organisms could routinely be detected by a procedure involving two rounds of 35 amplification cycles each and without the need for subsequent hybridization with labelled probes. Golsteyn Thomas et al. [242] also used five oligonucleotide primers complementary to the nucleotide sequence of the listeriolysin O gene. PCR products were generated with DNA from 72 *L. monocytogenes* strains with five different primer pairs. DNA from *L. ivanovii*, L. *inocua*, *L. seeligeri*, *L. welshimeri, L. grayi* and *L. murrayi* strains and a panel of 47 bacterial strains representing 17 genera did not generate PCR products with the primer pairs employed. The PCR assay on bacterial

DNA obtained from a two-step selective-enrichment protocol provides a sensitive and specific method for detection of *L. monocytogenes* in milk or ground-meat samples. The procedure takes less than 3 days to complete and can detect as little as 1 c.f.u. in the original food sample. Wernars et al. [243] used different sets of oligonucleotide primers. Parts of the *L. monocytogenes Dth18* gene could be amplified specifically when either a plasmid vector carrying the cloned gene or chromosomal DNA was used as a template. The detection limit for *L. monocytogenes* in dilutions of pure cultures was between 1 and 10 c.f.u. In extracts from soft cheeses containing *L. monocytogenes* DNA the amplification was strongly inhibited. Their opinion was that PCR was not yet convenient for direct detection of low numbers of *L. monocytogenes* in cheeses in routine examination. Wernars et al. [167] described a regulator gene, *prfA*, that positively regulates the expression of a number of virulence factors in *L. monocytogenes*. When the *prfA* gene was used as a DNA probe it was found to be extremely specific for the pathogenic species of *L. monocytogenes*. An oligonucleotide primer pair was developed that specifically amplifies the *prfA* gene in pathogenic listeria strains. They hoped that their method would be used successfully in clinical and food diagnostic laboratories. Rossen et al. [244] also developed a PCR protocol. Food samples were incubated in listeria enrichment broth at 37 °C for 40 h. The DNA sequence of *hlyA* gene specific for *L. monocytogenes* was used as a primer. Their method could detect 2×10^5 *L. monocytogenes* cells/ml of selective broth.

3. Methods other than cultivation to verify human listeria infection and to detect listeria in foods

3.1. Sero-immunological methods

Issekutz et al. [245] summarized our newest knowledge about the immune response to *L. monocytogenes* in infected humans. Serological diagnosis of listeria infection can be performed on the basis of a rise in specific antibody titre between two serum samples of a patient or with the help of enhanced blastogenic response of lymphocytes of that person. Serology is unreliable in newborns and immunocompromised patients and sometimes in other patients, too. Agglutination, complement fixation assay, microagglutination, ELISA and lymphocyte blastogenic assay can be used. Superiority of the complement fixation assay over agglutination, microagglutination and ELISA was observed. Evaluation of the results of serological tests and blastogenic assay is sometimes difficult because of antigenic cross-reactions existing among *Listeria*, *Staphylococcus* and *Streptococcus*.

Russell [246] used an immunofluorescent antibody test for the detection of IgG and IgM antibodies to *L. monocytogenes*. This test could diagnose listeriosis but it had some disadvantages (pre-absorption and subjective interpretation). He also stated that the ELISA system could be used to detect anti-listerial IgA, IgG and IgM

antibodies in sera of patients. In the case of IgG and IgM antibodies the test was not sufficiently specific but detection of IgA appeared to be sensitive, specific and easy to perform.

3.2. Detection of soluble listeria antigen

McLauchlin et al. [247] detected a soluble antigen of listeria in 399 samples of cerebrospinal fluid (CSF) using ELISA. Soluble antigen was demonstrated in 27% of CSF from patients infected with *L. monocytogenes* and 2% of patients where listeriosis was suspected.

3.3. Direct immunofluorescent detection of listeria

McLauchlin et al. [247] and McLauchlin and Pini [248] used MAbs in a direct immunofluorescence test for rapid demonstration and presumptive identification of *L. monocytogenes* in post mortem tissues and foods. High numbers of listeria had to be present in the samples for a reliable positive reaction.

3.4. Flow cytometric detection

To increase the specificity of fluorescent-antibody procedure a flow cytometry method has been used for the characterization of bacteria. A bacterial population can be characterized on the basis of its morphology, nucleic acid content and surface antigenicity with the help of this method. The cells can be treated with fluorescent-antibody and/or a suitable stain – for example propidium iodide – which labels cellular DNA. The treated suspension is exposed to a laser beam and the cells are analysed through their fluorescence. The use of immunofluorescence in combination with measures of DNA content by propidium iodine labelling and size by light scattering enable specific identification of *L. monocytogenes* among other bacteria in raw milk [26].

3.5. Microcolony technique combined with indirect immunofluorescence test

The microcolony epifluorescence technique was published during the 1980's [249]. This method was combined with a fluorescent antibody technique to detect salmonella in raw meats [250]. Sheridan et al. [251] combined the microcolony technique with a specific indirect immunofluorescence test to detect listeria. Listeria was detected with their method in artificially contaminated beef homogenates within 5 h. The detection level was 10^5 listeria/g meat.

3.6. Staining methods

A Gram staining method was used to detect colonies of bacteria in cryosections of food samples [252]. Dodd and Waites [253] used toluidine blue to detect sites of microbial growth. The method successfully detected colonies and single cells of both yeasts and bacteria at magnifications of $\times 400$ or below in the majority of foods.

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RESPONSE OF THE CYANOBACTERIUM *ANABAENA DOLIOLUM* TO ENDOSULFAN TOXICITY

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Anabaena doliolum Bhar. survived up to 3 mg/litre endosulfan on agar plates. Inhibition was visible from the beginning of growth at all tested concentrations; the LC_{50} was 2.15 ± 0.07 mg/l (p = 0.05). Though the growth and pigment inhibition at concentrations < 1.0 mg/l was not significant, these were severe at all other tested concentrations (≥ 1.0 mg/l). The pesticide was detoxified by *A. doliolum* at concentrations of 1.5, 2.5, and 3.0 mg/l. The growth rates were more than 90% of the control at 2.5 and 3.0 mg/l, while growth acceleration was observed at 1.5 mg/l after the third inoculation. The initial cell density was also found to be an influential factor in regulating the toxicity of the pesticide, the toxicity being inversely related to the cell density.

Cyanobacteria, notwithstanding their role in toxin production, have been implicated in the enrichment of surface water due to their ability to carry out both photosynthesis and nitrogen fixation. They also help in maintaining soil fertility through carbon fixation and synthesis of substances supporting plant growth. It has been reported that in the absence of chemical nitrogen fertilizer, application of cyanobacteria (heterocystous forms) can provide up to 30 kg of biologically fixed nitrogen per hectare, resulting in a 10 to 15% increase in the crop yield [1]. But the intense and indiscriminate use of pesticides like organochlorines, organophosphates and carbamate chemicals has hampered cyanobacterial growth and nitrogen fixation in wetlands [2, 3]. Though organochlorine pesticides have been banned in technologically advanced countries, these are still extensively used in India. This group of pesticides has a broad spectrum activity which extends to the non-target organisms besides the target ones. The present problem was undertaken in the context to study the effect of a widely used organochlorine pesticide endosulfan on

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Anabaena doliolum growing profusely under the local conditions. The detoxification capability of the cyanobacterium as well as its cell density effect on the pesticide toxicity were also evaluated.

Materials and methods

Cultivation. The cyanobacterium *A. doliolum* (strain Mohanty 1989/5) was grown in modified Chu No 10^+ medium [4] with A_6 micronutrients. The stock and experimental cultures were done in non-absorbent cotton-stoppered 250 ml and 100 ml borosilicate glass conical flasks and 18×150 mm tubes with 100 ml, 25 ml and 10 ml of culture, respectively. They were maintained at 27 ± 2 °C in a culture room illuminated with cool white fluorescent tube light/dark 12/12 h, PPFD 70 μmol/m² s (PAR: 400 – 700 nm), relative humidity 75%. Mid log stock cultures (7 days old) were used as initial inocula for the experiment. Stock solution (1 g/l) of acetone dissolved organochlorine pesticide endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6,9-methano-2,4,3-benzo – dioxanthiepin-3-oxide), obtained from Rallis India Ltd., Bombay was prepared with sterile culture medium under aseptic conditions for repeated use. The measurement for the survival test was done by culturing the alga on nutrient agar plates (12 filaments/cm²) with varying concentrations of the pesticide and counting the colonies after 10 days incubation. The rate of survivability was measured considering the survival in the control as 100%.

The toxic effect of the pesticide on the cyanobacterium was measured by inoculating the filaments at varying concentrations of the pesticide, incubating the cultures for 16 days and taking observations at every 4-day interval. The measurement of growth was made taking optical density (OD) of cultures at 660 nm, while that of the pigment content by extracting the pigments with 80% acetone and taking OD at 663 nm with the help of a SPEKOL spectrophotometer. For the detoxification study the organism was cultured in three higher concentrations (20, 30 and 40 mg/l) of pesticide for 21 days. Removal and reinoculation (1.40×10^6 cells/ml) of the organism was done at every 7 days under aseptic condition. ODs were measured at 7-day intervals to assess the rate of detoxification.

The toxicity of the pesticide at concentrations 0.1, 1.0 and 2.0 mg/l for different population levels was studied by taking three different sized initial inocula referred as low (0.35×10^6 cells/ml), medium (1.40×10^6 cells/ml) and high (2.45×10^6 cells/ml) in the text. The filaments were incubated for 8 days and observed every 2 day intervals [5]. The standard errors of the triplicates and confidence intervals (at $p = 0.05$) were statistically calculated for interpretation of the results [6].

Results and discussion

The survival of *A. doliolum* was reduced slowly from 0.1 mg/l up to 1 mg/l (92.8 ± 1.41%) of the pesticide, then decreased rapidly (Fig. 1). At most the cyanobacterium could tolerate 3 mg/l of the pesticide (showing 1.02 ± 0.0% survival) while the other higher concentrations were completely lethal. The LC_{50} and static dose were 2.15 ± 0.07 mg/l and 3.0 ± 0.01 mg/l ($p = 0.05$), respectively.

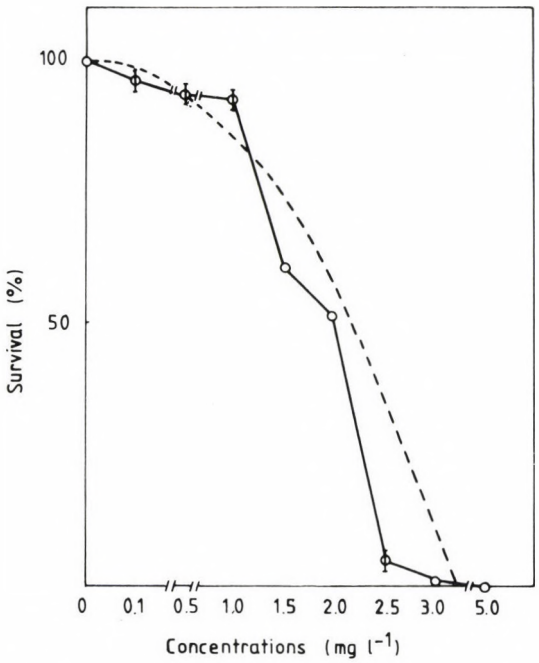

Fig. 1. Survival of *A. doliolum* at different concentrations of endosulfan; - - - - - survival curve; Φ standard error

The survival capability of *A. doliolum* was highly reduced at 2.0 mg/l concentrations of endosulfan indicating that the pesticide was highly toxic to the test organism. Reduction of the survival rate at a concentration as low as 0.1 mg/l might have been due to the death of certain colonies from the beginning of incubation as has been reported in case of other cyanobacteria [7, 8]. Similarly, the growth of *A. doliolum* was less affected at low concentrations ($\leq$ 1.0 mg/l) of the pesticide while significant growth reduction was observed at 1.0 mg/l and onwards (Fig. 2). However, the inhibitory effects at all the concentrations were visible right from the beginning. At 2.5 mg/l of the pesticide growth remained static till the 4th day while a slight increase in OD of the homogenized cultures was found at this concentration after 7 days though it did not increase thereafter. On the other hand, no change in OD at 3.0 mg/l, as compared to that of the initial till the 6th day indicated complete growth inhibition at this concentration. Since *A. doliolum* has been reported to be efficient in accumulating organochlorine pesticides [7, 9], the reason for the toxicity at higher doses corresponding to incubation time in the present study may be due to this.

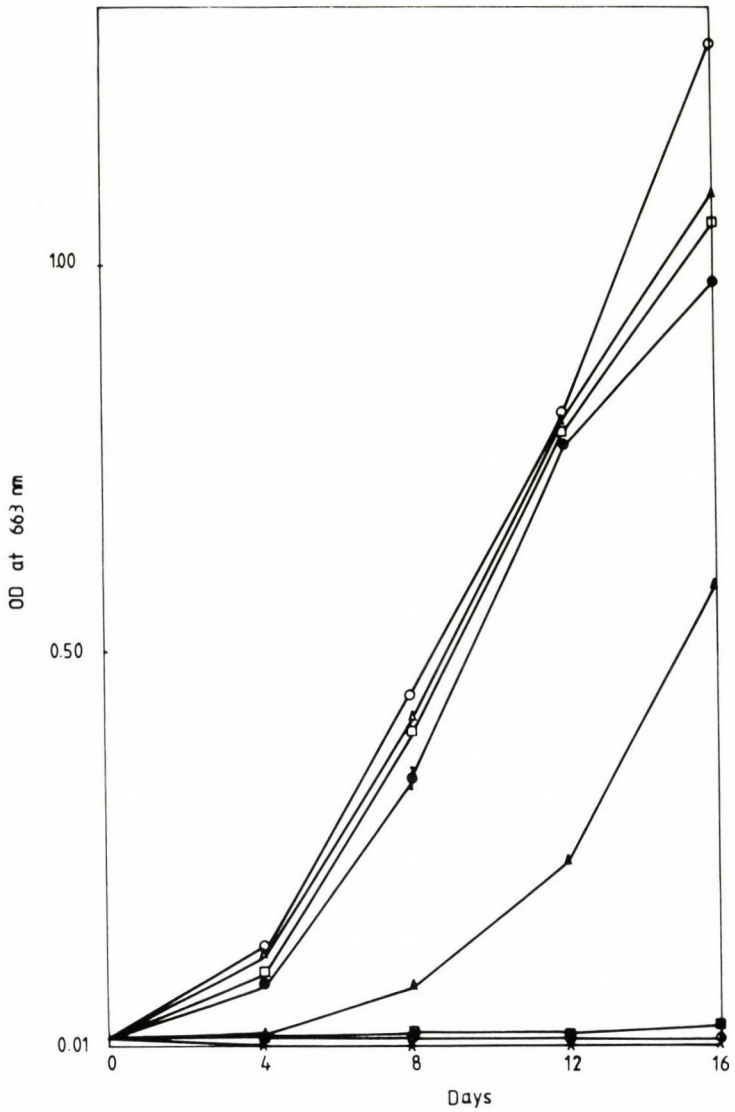

Fig. 2. Effect of endosulfan concentrations (mg/l) on growth of *A. doliolum*; O = 0; Δ = 0.1, □ = 0.5, • = 1.0, Δ = 2.0, ▪ = 2.5, O = 3.0, x = 3.5

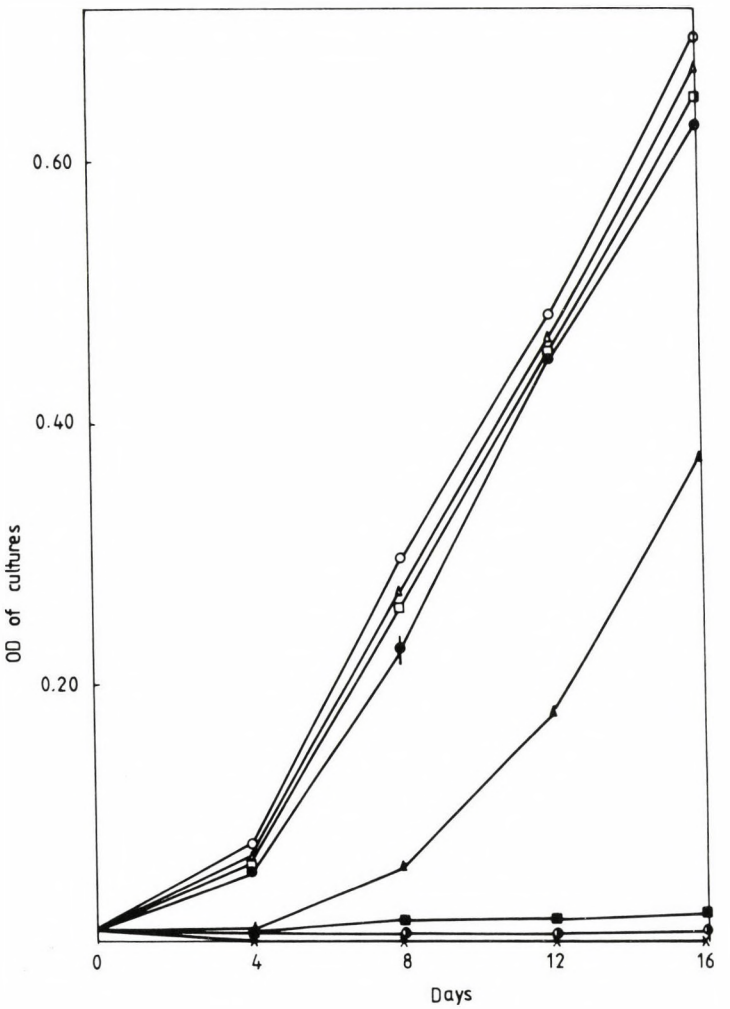

Fig. 3. Effect of endosulfan concentrations (mg/l) on chlorophyll content of *A. doliolum*. Point symbols: see Fig. 2

The pigment contents at concentrations $\leq$ 1 mg/l of the pesticide did not decrease significantly (Fig. 3). As in case of growth, there was direct relation of pigment inhibition and incubation at the higher doses. Complete chlorosis of the cells was observed at $\geq$ 3.0 mg/l. Organochlorines interact with the lipid bilayer of the chloroplast membrane and also affect lipid packing causing disordering effects [10]. This causes permanent physical and chemical changes in the chloroplast membrane resulting in chlorosis and imbalances in functioning of thylakoid membrane [11]. In

the present study the low rate of chlorophyll inhibition at low concentrations ($\leq$ 1.0 mg/l) of the pesticide may be due to inhibition of pigment synthesis; but, comparatively increased rates of inhibition at the high doses are probably further associated with acceleration of degradation rate.

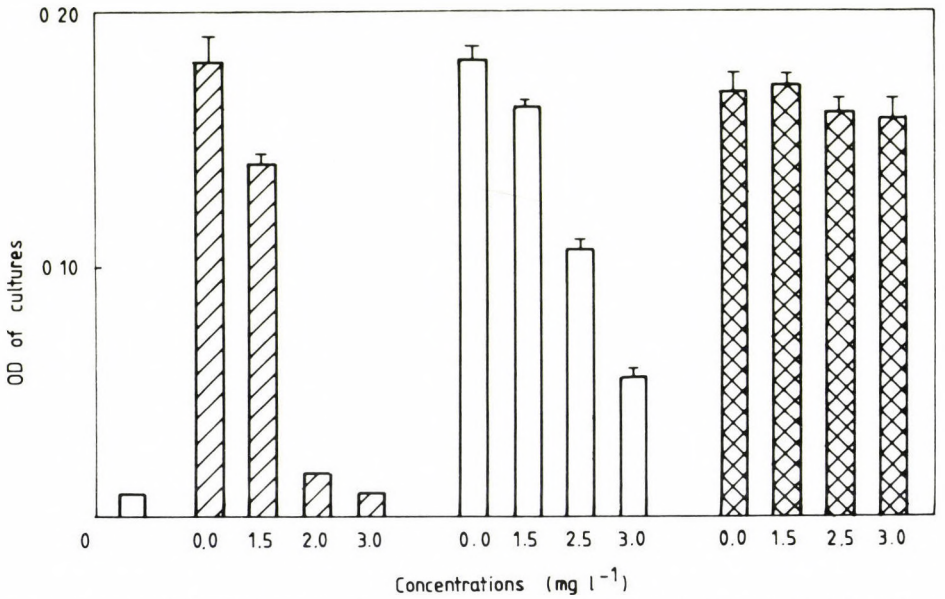

Fig. 4. Detoxification of endosulfan by *A. doliolum*. Solid columns: initial; shaded columns: first inoculation; open columns: second inoculation; hatched columns: third inoculation

From detoxification tests it was observed that endosulfan was detoxified, partly or completely, by *A. doliolum* at all the tested concentrations (Fig. 4). The cyanobacterium grew well at 1.5 mg/l but at 2.5 and 3.0 mg/l the growth rates were very slow, compared to control, after the first and second inoculation. However, the rates, after the third inoculation, increased to 101.49 ± 0.83%, 94.9 ± 2.81% and 94.02 ± 4.42%. Slight reduction of growth in the control after the third inoculation was most probably due to gradual lowering of nutrient status of the medium. *A. doliolum* has been reported very efficient in detoxicating another pesticide dimethoate lying between sublethal and static doses [5]. The present findings indicate that the cyanobacterium is capable to detoxicate endosulfan more successfully than dimethoate. The increase in growth of the cyanobacterium in the present study, with removal and reinoculation with fresh material may be due to accumulation of the pesticide by the filaments and decrease in the toxicity of the medium and/or degradation of the pesticide into simpler products with no toxic action by the organism [12, 13]. This also confirms the report [14] that cyanobacteria can be

successfully used against other organisms in eutrophic waters for removal of toxicity. Further, this finding puts forth the idea that frequent removal and reinoculation of cyanobacterium in the field may help in detoxifying polluted waters. However, such practice in natural habitats may bring in some secondary effects.

Table I

Effect of A. doliolum cell density on the toxicity of endosulfan (OD of cultures ± SE)

Cell density ($\times 10^5$/ml)	Days of exposure	Concentrations (mg/l) 0	0.1	1.0	2.0
1.48	2	0.012 ± 0.0013	0.009 ± 0.0007	0.00	0.00
	4	0.024 ± 0.0007	0.024 ± 0.0008	0.015 ± 0.00	0.00
	6	0.042 ± 0.0020	0.042 ± 0.0007	0.033 ± 0.0008	0.00
	8	0.078 ± 0.0007	0.077 ± 0.00	0.042 ± 0.0018	0.00
5.92	2	0.04 ± 0.0010	0.038 ± 0.00	0.028 ± 0.00	0.008 ± 0.00
	4	0.073 ± 0.0010	0.071 ± 0.0006	0.05 ± 0.00	0.008 ± 0.00
	6	0.128 ± 0.0036	0.111 ± 0.0030	0.098 ± 0.0010	0.010 ± 0.00
	8	0.238 ± 0.030	0.205 ± 0.0036	0.195 ± 0.0040	0.015 ± 0.00
10.40	2	0.050 ± 0.0029	0.048 ± 0.0010	0.030 ± 0.00	0.015 ± 0.00
	4	0.078 ± 0.0010	0.081 ± 0.0050	0.063 ± 0.00	0.035 ± 0.00
	6	0.157 ± 0.0040	0.162 ± 0.0030	0.145 ± 0.0023	0.097 ± 0.0039
	8	0.258 ± 0.0050	0.275 ± 0.0050	0.235 ± 0.0060	0.122 ± 0.0064

The growth measurement at every two-day interval showed that toxicity of the pesticide decreased with increase in initial cell density of the cultures. Even the same concentration of the pesticide showed differential toxicity effect to the test organism with change in the cell density (Tables I and II). The concentration of 0.1 mg/l which is the lowest tested dose of endosulfan, inhibited growth and pigment content of the cyanobacterium at low medium cell density accelerating the same at high cell density. This dose was found to be growth stimulating only at the level of high cell density. Similarly at the highest tested dose (2.0 mg/l) of the pesticide, though no growth and pigment content was observed at the low cell density level, the cyanobacterium could grow comparatively well at the high population. This may be due to high packed cell volume and/or decreased pesticide to organism ratio [5, 9, 15]. Since the pesticide is frequently used in crop protection practices, even its low concentration may become toxic to the microorganisms in the field at very low cell density underlining study of the existing population level while evaluating the toxicity of the chemicals.

Table II

Effect of A. Doliolum cell density on the toxicity of endosulfan (OD of acetone extracts ± SE)

Cell density ($\times 10^5$/ml)	Days of exposure	Concentrations (mg/l) 0	0.1	1.0	2.0
1.48	2	0.017 ± 0.00	0.010 ± 0.00	0.00	0.00
	4	0.040 ± 0.0018	0.040 ± 0.00	0.020 ± 0.00	0.00
	6	0.076 ± 0.0018	0.074 ± 0.0007	0.050 ± 0.00	0.00
	8	0.130 ± 0.0040	0.115 ± 0.0020	0.070 ± 0.00	0.00
5.92	2	0.068 ± 0.0010	0.063 ± 0.0020	0.035 ± 0.00	0.010 ± 0.00
	4	0.121 ± 0.0010	0.113 ± 0.0030	0.085 ± 0.0006	0.010 ± 0.00
	6	0.203 ± 0.0080	0.185 ± 0.0030	0.125 ± 0.0020	0.010 ± 0.00
	8	0.375 ± 0.0030	0.345 ± 0.0026	0.300 ± 0.0020	0.065 ± 0.0020
10.40	2	0.085 ± 0.0020	0.075 ± 0.0012	0.055 ± 0.0020	0.025 ± 0.0006
	4	0.120 ± 0.0050	0.130 ± 0.0030	0.098 ± 0.0030	0.065 ± 0.00
	6	0.238 ± 0.0050	0.293 ± 0.0030	0.205 ± 0.0020	0.090 ± 0.0036
	8	0.385 ± 0.0030	0.485 ± 0.0090	0.350 ± 0.0031	0.165 ± 0.0052

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WATER SOLUBLE COMPLEX OF PALMITIC ACID IN MEDIA FOR CULTIVATION OF LEPROSY-DERIVED PSYCHROPHILIC MYCOBACTERIA FROM *MYCOBACTERIUM LEPRAE* INFECTED TISSUES

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Palmitic acid and palmitates were transformed into water soluble complexes with crystalline heptakis-2,6-di-0-methyl-beta-cyclodextrin. This formulation was incorporated into liquid and solid chemically well-defined media. The fatty acid served as C and energy source, ammonium thioglycolate as the sole source of N with the SH group as further source of energy. Minute amount of dimethyl-sulfoxide added was used for its known effect on cell membrane permeability. The media were inoculated with host grown *Mycobacterium leprae* cells isolated from human, armadillo and Nu mice foot pad lepromata. No growth occurred in the liquid medium at 22 or 32 °C, but cultures and subcultures of acid fast rods were grown at 10 °C. Bacilli in the cultures were solid, strongly acid fast rods, growing in clumps like globi. Growth on the semisolid media was visible as smooth round colonies, of white to ivory in colour, slowly expanding flatly at the periphery of the colony on the agar surface. Colonies developed within 2–3 weeks and reached maximum size at 50–80 days depending on the size of inoculum. Subcultures grow faster and more abundantly with adaptation to the media. No growth was seen without the water soluble complexes of palmitic acid or palmitates in the media. The free fatty acid or its salts had an equal growth supporting effect. Identical psychrophilic cultures were obtained from 7 out of 9 armadillo, 12 out of 12 Nu mice and 1 out of 2 human lepromata. None of the cultures grow on Loewenstein, Dubos or 7H9 media at 10 °C, 20 °C or 32 °C, respectively. The tested 4th to 7th subcultures of the strains were strongly positive for phenolic glycolipid-1. Heat killed suspensions of up to 7th subcultures gave negative late skin reaction in all of 16 LL cases. In 19 I, B and T cases the late skin reactions were all similar to that obtained with authentic human lepromin.

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A unique marker of *Mycobacterium leprae* is that it cannot use substrates that other mycobacteria utilize for multiplication. This is just one reason why *M. leprae* dose not grow in any of the tested culture media. Yet it multiplies abundantly in tissues of susceptible hosts. For the above reasons, *M. leprae* is a most unusual and unique microbe. Cultivation trials under most unusual conditions have therefore been proposed repeatedly in our laboratories over four decades. Media were tried unsuccessfully with extreme pH and ionic strength, with rich and poor media, incorporating polysaccharides, steroids, hydrocarbons, a catalogue of prospective nutrients, even parabiotic cultures. The frequent proposal of new variations of media was motivated by consistent failure to achieve cultivation. The unusual conditions and compositions of the media were not some acrobatic exercise, but a follow-up of a research philosophy that a highly unusual microbe must grow under most unusual conditions. Why then should one persist with the same conventional media which do not work?

Following this leitmotif in cultivation trials, one of us (L.K.) obtained a flock of cultures. These strains were identical to the now well-known leprosy-derived cultivable mycobacteria (LDM). Some of them resembled *M. leprae* or were hard to identify, but only in the early subcultures. These were the mixed cultures, none of them the authentic *M. leprae*.

Recently the cultivation of several strains of a hitherto unknown cluster of cold loving mycobacteria from *M. leprae* infected tissues has been reported [1–3]. Tentatively these were named *M. psychrophilum* (L). Cautiously the successful cultivation of *M. leprae* was not claimed, although the cultures were regularly isolated from leprosy infected tissues. The cultures do not grow on media for cultivable mycobacteria, except on a multifactorial medium, optimally at 10 °C.

The recently discovered preparation of heat stable, water soluble cyclodextrin complexes of palmitates by Kátó, Szejtli and Szente permitted the development of chemically well defined new culture media for easily reproducible growth of the leprosy derived *M. psychrophilum* (L) [2, 3].

The purpose of the present investigations was to satisfy the authors' curiosity as to whether a hitherto unknown psychrophilic cluster of mycobacteria really existed, rather than to identify the cultures as being *M. leprae* or not.

Materials and methods

Chemicals. Water soluble inclusion complexes of palmitic acid, Na-palmitate and ascorbic palmitate were products of Cyclolab (Budapest, Hungary).

Multifactorial medium. Ammonium thioglycolate served as the sole source of N with an SH group as a prospective source of energy [4, 5]. Water soluble complexes of palmitic acid or its salts were used as potent energy sources [6–8].

Liquid medium. The chemically well defined medium was prepared as follows. In one liter distilled water are dissolved KH_2PO_4, 3.5 g; Na_2HPO_4, 2.0 g; $MgSO_4$, 0.01 g; ferric ammonium citrate, 0.05 g; ammonium thioglycolate (60% v/w), 10 ml; Na palmitate solution (10 mg/ml) (Cyclolab), 20 ml. Distributed into screw cap tubes, the culture media are autoclaved (120 °C, 15 lb pressure) for 25 min. The palmitate is precipitated in the hot media, but completely redissolved as cooled to room temperature or kept at 4 °C.

Dimethylsulfoxide (DMSO) 2 ml was mixed with 18 ml liquid medium and filter sterilized. To each of the 20 ml sterile media 0.4 ml of the dilute DMSO solution was pipetted aseptically.

Semisolid medium. Liquid medium was prepared without the addition of ammonium thioglycolate. To 1 litre liquid medium 30 g granulated agar was mixed by magnetic stirring. The agar suspension was dissolved by heating to 90 – 100 °C in a water bath or autoclave. The solution was cooled to 60 – 70 °C and with vigorous magnetic stirring 10 ml ammonium thioglycolate was slowly poured into the hot solution.

The medium thus prepared was distributed while hot into 20 ml/50 ml screw cap tubes, autoclaved at 120 °C (15 lb pressure) for 25 min and cooled in an inclined position to make agar slants. To each of the tubes, 0.5 ml liquid medium complete with DMSO was added aseptically.

Cultures. Mycobacterium leprae semipurified suspension was prepared as previously described [9] from an *M. leprae* infected armadillo nodule, from Nu mice foot pads or from human lepromata. The tissues were washed with sterile distilled water and cut aseptically with scissors into small pieces. Using a 4 blade Lourdes Model MMIB homogenizer, the tissues were homogenized 4 times for 5 s at maximal speed with cold distilled water, 10 times the weight of the tissue (v/w). A cca 20 × 20 cm nylon tissue (cut from nylon stockings) was fastened over a 250 ml beaker then autoclaved at 120 °C (15 lb pressure) for 30 min. The cell suspension was passed through the nylon filter and centrifuged at 6000 r.p.m. for 10 min. The sediment was washed twice with distilled water. Sterile distilled water was used rather than buffers or physiological saline t to promote lysis of blood cells and prevent swelling of tissue mucopolysaccharides. The semi purified suspension of *M. leprae* cells was adequately separated from undesirable impurities for cultivation trials, but not for metabolic studies. The sediment so washed was suspended for decontamination [10] in excess of 1% benzalkonium chloride (Zephiran) in 5% Na_3PO_4 solution in sterile distilled water, followed by constant slow magnetic stirring for 50 min at room temperature. The suspension was again centrifuged at 6000 r.p.m. for 10 min. The sediment was washed once with the sterile liquid medium. Cells were separated by centrifugation (8000 r.p.m. for 15 min) and the thick paste obtained was used as heavy inoculum to the semisolid media.

Following inoculation, the inoculated bacterial mass was gently homogenized on a ~ 2 cm^2 surface of the slope with a sterile platinum loop dipped in the condensed water. This was necessary in order to enable the cells to come into contact with the liquid media which contained DMSO and had previously been pipetted to the semisolid slants. Also twice a week the inoculated surface was gently wet with the condensed liquid, tilting the tubes carefully to avoid washing off the inoculum.

Liquid media were inoculated with a diluted suspension of *M. leprae* so as to obtain an average of 25 – 50 acid fast cells per microscopical field, as prepared on slides for estimation of growth.

Prevention of contamination. Commercially available antibacterial and antifungal preparation "Bacter-plus" was purchased from Becton Dickenson, USA. From the reconstructed solution 0.4 ml was pipetted aseptically to each of the tubes containing the liquid media. Similarly 0.1 ml of the reconstituted solution was added to each of the semisolid agar media. Media were also inoculated with heat killed autoclaved (30 min – 120 °C, 15 lb pressure) suspensions of *M. leprae*. These served as controls.

Cold temperature incubator. In a simple, regular used refrigerator, the thermoregulator switch was replaced by a thermoregulator to maintain a constant temperature of + 10 °C (± 1 °C) inside the 9 cubic foot space.

Estimation of growth in media. Since the palmitates and ammonium thioglycolate in the medium have a detergent effect, the cells are easily washed off from normal slides. Furthermore, the growth developed in huge clumps, impossible to disperse even with vigorous mechanical shaking. A special procedure was therefore necessary to visualize and quantify growth kinetics.

Following 10 s of shaking with a Vortex mixer, 1 ml of the culture was withdrawn by means of a sterile syringe and needle. This amount was transferred into a rubber stoppered glass tube (100×16 mm). Ten ml distilled water was pipetted into the tube, and thoroughly mixed. Cells were separated by 10 min centrifugation at 3000 r.p.m. The supernatant was withdrawn with a syringe attached to a 10 cm needle. The sediment was suspended in 1 ml distilled water and shaken for 10 s with a Vortex mixer. For declumpling during 10 s shaking with Vortex mixer, 1–2 drops of chloroform were added.

A 5 mm loopful of the so prepared declumped suspension or its dilutions was evenly spread over a 10 mm diameter surface of a slide. Slides were left at room temperature overnight for drying. Fixation over flame was followed by Ziehl-Nielsen staining and 15 s decolorization with 1% HCl in 75% ethanol. The number of cells was estimated by examining 20 microscopical fields at 1000× magnification, taking into account the dilutions of the cultures.

Growth on the semisolid agar media was easily detected by increased bacterial mass and colony formation on the surface, as compared to the control tubes inoculated with heat killed *M. leprae* cells.

Percentage of solidly stained acid fast rods was counted microscopically on siliconized slides following Ziehl–Nielsen staining of thin smears of cell suspensions after declumping with chloroform.

Results

Table I

Growth of M. psychrophilum (L) at different incubation temperatures in sodium palmitate liquid media. Cultures were isolated from an M. leprae infected armadillo (A7) and foot pads of Nu mice (M3)

Culture	°C	Incubation time in weeks					
		0	1	2	4	8	10
A7	10	+	+	+++	++++	>++++	++++
	22	+	+	±	±	±	±
	32	+	±	±	–	–	–
M3	10	+	+++	++++	>++++	>++++	>++++
	22	+	+	±	±	±	±
	32	+	+	±	–	–	–

Acid fast bacilli per microscopical field; +, 20–40; ++, 50–75; +++, 100–200; ++++, >200; >++++, heavy growth. ±, deteriorating morphology

Results presented in Tables I and II are only relative values, but express fairly the multiplication of inoculated acid fast rods in the liquid media. Counting of bacilli in stained preparations was difficult because the number of cells washed off from the slides during staining could not be estimated. Furthermore, growth occurred as huge

clumps, that did not disintegrate following declumping with chloroform. The growth, however, was so obvious for the trained eyes of students of bacteriology, that the microscopical readings offered semiquantitative, but acceptable information concerning growth of the cultures.

Results in Table I show multiplication of inoculated acid fast rods at 10 °C during 10 weeks of incubation in the liquid media. No growth was observed at 22 and at 32 °C during the observation period. At 10 °C incubation the morphology of cells was not only well preserved, but improved with multiplication in progress. By contrast, at 22 and 32 °C incubation, cellular morphology deteriorated after 2–4 weeks of incubation. Table I shows results obtained on the primary cultures in media inoculated from subcutaneous lepromata of an armadillo (A7) and cells collected from foot pads of *M. leprae* infected Nu mice (M3). The table also shows that multiplication at 10 °C started before the 2nd week after inoculation of the armadillo derived bacilli, but about a week earlier in media inoculated with cells from foot pads of Nu mice.

Heat killed A7 and M3 cell suspensions did not grow in the sodium palmitate liquid media. Acid fast cell suspensions from A7 and M3 lepromata did not grow on Loewenstein or in 7H9 or Dubos liquid media at 10, 22 and 32 °C.

Results presented in Table I indicate that maximal growth was probably achieved at 8 weeks of incubation. Cultures were therefore transferred into fresh homologous media at that time as a 1:10 dilution.

Table II shows schematic representation of growth of *M. psychrophilum* (L) in the primary culture as well as in several subcultures. Inoculated cells of acid fast bacilli were separated from an armadillo (A6) and a human (H4) leproma, grown in sodium palmitate liquid media at 10 °C. It took 2 to 4 weeks of incubation before multiplication of cells occurred in the primary cultures. This latency period of growth was considerably shortened in the subcultures. These results are a clear indication of the adaptation of host grown cells to the chemical and physical conditions for in vitro growth.

Armadillo (A6, A7), human (H4) and mouse (M3) derived authentic host grown *M. leprae* cell suspensions from the corresponding lepromata were also inoculated into liquid media prepared with the same concentrations of water soluble complexes of palmitic acid, Na palmitate and ascorbic palmitate. Results not presented in the Tables left no doubt that multiplication of acid fast rods was practically the same with water soluble palmitic acid or any of its salts.

Table II

Growth of M. psychrophilum (L) in sodium palmitate liquid media primary cultures and subcultures at 10 °C incubation temperature. Cultures were isolated from an armadillo (A6) and human (H4) subcutaneous lepromata

Strain	Cultures	Transfer time in weeks	Incubation (10 °C) weeks 0	1	2	4	8
A6	Primary	0	+	+	++	++++	>++++
	1st sub.	8	+	+	+++	>++++	
	4th sub.	8	+	++	++++	>++++	
	7th sub.	8	+	+++	++++	>++++	
H4	Primary	0	+	+	+	+++	++++
	1st sub.	12	+	++	++	++++	
	2nd sub.	8	+	++	+++	++++	
	3rd sub.	8	+	++	+++	++++	

Acid fast bacilli per microscopical field; +, 20–40; ++, 50–75; +++, 100–200; ++++, >200; >++++, heavy growth. ±, deteriorating morphology

The inoculum from authentic *M. leprae* suspensions isolated from human (H), nude mice (M) or armadillo (A) lepromata, as well as the subcultures of *M. psychrophilum* (L) grow slowly but abundantly on the semisolid agar media containing the water soluble complexes of palmitic acid or palmitates at 10 °C, but not at 22 °C nor at 32 °C. Growth was visible as a smooth, off white opaque growth on the agar surface. The bacterial mass increased slowly in size during 10 to 90 days of incubation. The colonies turned light yellow with aging of the cultures. Bacilli were strongly acid fast, arranged in small to large clumps. At 32 °C most of the cells became granulated or beaded after 20 to 30 days of incubation. The growth is seemingly maximal at 50 to 100 days of incubation. At that time cultures were regularly transferred into fresh homologous agar media. Heavy inoculum was used for transfer to fresh media. No changes in growth pattern were observed when cultures were transferred to the fresh semisolid media. None of the subcultures grew in 7H9, Loewenstein or Dubos media at 32 °C.

Counting of bacilli was not necessary to register growth nor was it feasible in semisolid media because of difficulties of declumping with chloroform. Percentage of solidly stained cells in the primary cultures and subcultures was therefore registered as an indication of the quality of cells during growth visible to the naked eye on the surface of the media. Results are shown in Table III. The percentage of solidly stained acid fast rods is extremely low – about 14% – in suspensions of leprosy bacilli freshly isolated from a human leproma (H4), considerably higher in the armadillo derived suspensions (A6 and A7), and even higher in the inoculum

obtained from Nu mice foot pads (M3). As cells multiplied in the liquid and agar primary culture, the percentage of solidly stained cells reached relatively high levels – up to 80–91% in the Nu mice and armadillo derived cultures, most likely because of cell divisions of the solidly stained live bacilli.

Table III

Solidly stained cells (%) of M. psychrophilum (L) isolated from human (H4), mouse foot pad (M3) and armadillo (A6, A7) lepromata. The primary cultures and subcultures were grown at 10 °C in liquid and solid media

Culture	Medium	% Solidly stained cells, percent Incubation at 10 °C, time in weeks					
		primary culture			subculture		
		0	4	8	0	4	8
H4	Liquid	14	28	69	75	82	86
	Solid	14	37	42	79	88	92
M3	Liquid	36	52	80	86	89	86
	Solid	36	59	84	80	92	88
A6	Liquid	24	47	69	67	88	80
	Solid	24	52	77	74	90	94
A7	Liquid	42	62	82	79	86	90
	Solid	42	70	91	88	89	89

A high percentage of solidly stained cells were counted in all the subcultures, whether of human, mice or armadillo origin. These results are in agreement with the well-known observations that most of the host grown *M. leprae* cells are fragmented, nonsolid, probably dead.

Authentic *M. leprae* freshly isolated from a human leproma, armadillos or foot pads of Nu mice did not grow on 7H9 media at 10 or 32 °C. Similarly the leprosy derived *M. psychrophilum* (L) did not grow in 7H9 media at any of the incubation temperatures. Growth was not estimated in media without the palmitates added, but experience left no doubt that there was no growth in the primary cultures or in the subcultures in the absence of palmitates.

Table IV

Late skin reactions (Mitsuda) in the skin of human volunteers with indeterminate (I), borderline (B), tuberculoid (T) or lepromatous leprosy (LL) injected intradermally with authentic human lepromin (H.-LEPR) and heat killed suspension of M. psychrophilum (L), (MPL-H). Both antigens contained 10^7 acid fast cells in 0.1 ml physiological saline solution. MPL-H antigen was prepared from the 6th subculture of M. psychrophilum (L) (H-3) isolated from a human leproma

Patient	Case	Skin reaction to H-LEPR	Skin reaction to MPL-H	Patient	Case	Skin reaction to H-LEPR	Skin reaction to MPL-H
N.S.	I	4×4	6×6	M.M.B.	LL	-	-
D.C.M.	I	-	-	R.T.	LL	-	-
O.L.F.	I	-	-	A.J.	LL	-	-
V.A.T.	B	-	-	S.O. de S.	LL	-	-
I.R.S.	T	15×15	13×15	P.C.G.	LL	-	-
M.I.G.S.	T	12×12	10×9	M.A.G.	LL	-	-
C.R.R.	T	6×11	4×6	D.B.	LL	-	-
A.M.G.F.	T	8×8	7×8	J.M.T.	LL	-	-
A.F.S.	T	5×6	10×9				

Table V

Late skin reactions (Mitsuda) in the skin of human volunteers with indeterminate (I), borderline (B), tuberculoid (T) or lepromatous leprosy (LL) injected intradermally with authentic human lepromin (H.-LEPR) and heat killed suspension of M. psychrophilum (L), (MPL-A). Both antigens contained 10^7 acid fast cells in 0.1 ml physiological saline solution. MPL-A antigen was prepared from the 7th subculture of M. psychrophilum (L) (A-5) isolated from an armadillo

Patient	Case	Skin reaction to H-LEPR	Skin reaction to MPL-A	Patient	Case	Skin reaction to H-LEPR	Skin reaction to MPL-A
E. de O.	I	6×8	5×8	H.A.	LL		-
W.P.	I	6×5	8×8	J.M.T.	LL	-	-
J.C.S.O.	I	5×6	5×6	J.O.B.	LL	-	-
J.A.M.	I	8×7	6×6	D.R.B.	LL		-
D.F.T.	B		-	A.R.G.	LL		-
A.C.S.	BT	Eryth.3	Eryth.6	R.A.S.	LL		-
M.Ap.V.	T		2×2	A.W.	LL		-
M.S.	T	4×6	6×6	J.A.M.	LL	-	-
I.B. de S.F.	T		8×5				
C.A.S.	T		8×5				

Dr. Enzo Melchior, Jr., University of Sao Paulo, Department of Dermatology, Ribierao Preto, Sao Paulo, Brazil, kindly tested M3 (6th subculture) and A5 (7th subculture) for Mitsuda type skin reaction in volunteer patients. Dr. Enzo Melchior found that the heat killed suspensions (10^7/0.1 ml) gave negative late reactions in all LL cases (as in the case of the human lepromin). In I, B, and T cases the late

reaction was similar to that obtained with human lepromin. Results are presented with Dr. Enzo Melchior's kind permission in Tables IV and V.

Table VI shows some characteristics of *M. leprae* compared to *M. psychrophilum* (L) and leprosy derived cultivable mycobacteria (MAIS).

Table VI

Characteristics of M. leprae, compared to M. Psychrophilum (L) and leprosy derived mycobacteria (MAIS)

Characteristics	Leprosy derived mycobacteria (MAIS)	Authentic *M. leprae*	*M. psychrophilum* (L)
1. Regularly isolated from *M. leprae* infected tissues	–	+	+
2. Cultures and subcultures only in special media and conditions	–	not cultivable in vitro	+
3. Growth in 7H9, Dubos Loewenstein or Ogawa media	+	–	–
4. Acid fastness	+	+	+
5. Phenolic glycolipid-1	–	+	+
6. Polymerase-chain reaction	–	+	result not yet available
7. Late skin reaction in lepromatous leprosy patients	+	–	–
8. Late skin reaction in tuberculoid leprosy patients	+	+	+
9. Multiplication in mouse foot pad	–	+	result not yet available
10. Leprosy in the armadillo	–	+	result not yet available

Discussion

The presented experiments were initiated and conducted according to a previously formulated working theory as follows: all components of the medium – palmitic acid and palmitates included – must be biologically available as water soluble complexes.

Special attention was given to the observations of Binford [11] that *M. leprae* grow best in the cooler tissues of humans. A similar association between damage from leprosy and temperature was reported by Brand [12]. These findings raised the question as to whether temperature cooler than the coolest parts of a human or an animal body is optimal for growth of *M. leprae*. How cool is cool enough for *M. leprae* to grow faster (or at all) in vitro as the well documented slow growth in vivo? Does

M. leprae not spare the skin fossae, intergluteal regions and other warmer areas, as shown by Brand [12]? In cool parts of the body the lesions are heavily parasitized by *M. leprae*, but the proliferation of leprosy bacilli is limited to microscopic lesions in warmer parts of the body [11, 12]. The thermal difference between normal body temperature and the cool sites is only a few degrees – not more than 4 °C. However, this small thermal difference is sufficient to result in heavy growth at the cool site to limited growth at the warm site of the narrow thermal spectrum. These facts suggest that the growth-temperature relation is optimal for multiplication at a temperature much lower than ever recorded in the living body of any of the hosts susceptible to leprosy. Exploration of possible in vitro psychrophilic characteristics of *M. leprae* seemed imperative.

A special, chemically well defined cultural medium was necessary to grow the leprosy derived *M. psychrophilum* (L).

(i) It was necessary to supplement the medium with an SH group compound, ammonium thioglycolate. It was previously shown that SH compounds are the rare substrates which *M. leprae* can oxidize [4].

(ii) The water soluble complexes of palmitic acid and its salts are the key components of the culture media. These compounds merit special attention as carbon and energy sources. Experimental evidence is accumulating which suggests that palmitic acid might play a major role in the energy-metabolism and probably in the energy-dependent biological processes of *M. leprae*.

Franzblau [6] reported oxidation of palmitic acid by *M. leprae* in an axenic environment. Oxidation of palmitic acid resulted in increased synthesis of energy rich phosphate (ATP) and of PGL-1. Using manometric technics Ishaque [7] provided direct evidence that *M. leprae* oxidize palmitic acid. This was a beta-oxidation process via the tricarboxylic acid cycle and the electron-transport chain with O_2 as the terminal electron acceptor. Cytochromes of *M. leprae* were reduced by palmitic acid and fully oxidized by O_2. Palmitic acid was more actively oxidized than any other substrate known to be oxidized by *M. leprae*. Since the 16C fatty acid provides close to three times more energy than glucose, palmitic acid should be considered as a candidate energy source in axenic media for *M. leprae*.

Wheeler et al. [13] expressed the view that fatty acid synthetase activity of host grown *M. leprae* being very low, it was insufficient to provide enough fatty acids for growth. "Hence *M. leprae* requires an exogenous source of fatty acid." The authors propose that since in vivo grown mycobacteria scavenge lipids from host cells, a source of lipid might promote in vitro growth of *M. leprae*. As shown by Barclay and Wheeler [14], *M. leprae* can indeed hydrolyze lipids and release fatty acid. Fatty acid acquisition from media is therefore an important step in the synthesis of lipids, necessary for multiplication. At least one of the fatty acids might have another role of primary importance: a powerful source of energy. This became evident in the

experiments of Franzblau [6] and Ishaque [7], also supported by the results of Wheeler and Ratledge [15], concerning CO_2 evolution when incubating *M. leprae* with palmitic acid.

The above results were obtained with host grown *M. leprae*, thus adapted to utilize chemistry of the host cells. When proposing palmitic acid as an energy and carbon source in vitro, one must also consider that free fatty acids are scarcely available in the host [15]. The slow growth of *M. leprae* in vivo is probably due (among other reasons) to the dependence on host constituents, where massive scavenging of host molecules precedes utilization of substrates, whether as an energy source or as building blocks for further biosynthetic processes.

Palmitic acid and its salts are insoluble in water. It was used in biological systems as a suspension, or in liposomes. In none of these conditions is palmitic acid biologically easily available in a solution for mycobacteria, although most microbes can scavenge any substance from a solid state.

Water soluble inclusion complex of palmitic acid, Na palmitate or ascorbic palmitate, respectively, was incorporated into the above described semisolid and liquid media. This method is based on results reviewed by Szejtli [16]. The solubilization of the fatty acids, their salts and esters was carried out by formation of their water soluble inclusion complexes with heptakis-2,6-di-0-methyl-beta-cyclodextrin. This alkylated cyclodextrin has been known to enhance the water solubility of lipophilic potential guest molecules via molecular encapsulation. Among cyclodextrins and their derivatives heptakis-2,6-di-0-methyl-beta-cyclodextrin is the most potent solvent for most of the guest substances of biological importance [16]. Since the preparation of the water soluble inclusion complexes of lipophiles is a rather simple technology and the solvent itself does not possess microbiological effects [8], the present solubilization technique seems to be of practical significance.

Incorporation of lipophilic substrates into culture media with the help of hydrophilic molecules was first described by Bar [17]. His method permitted the preparation of media containing Na palmitate accessible for the inoculum as a fine dispersion. We are now able to report that palmitic acid or palmitates perfectly soluble in water [3] have been incorporated into culture media.

In the presence of excess water or of a third component the dissociation of the complex takes place and the continuous release of the molecularly dispersed guest substance is ensured. This unique method of release of an active substance moreover results in an improved bioavailability [16].

Recently Ishaque [7] reported that the water soluble complexes of palmitic acid salts are oxidized by *M. leprae* at the same rate as the palmitates insoluble in water. The methylated cyclodextrin part of the complexes was not oxidized. Latency period of oxidation was considerably shorter with the soluble complexes as compared to the palmitates insoluble in water. Ishaque also described many advantages of the soluble

palmitates versus the hydrophobic forms when proposed for cultivation trials or metabolic studies with *M. leprae*.

One of us (L.K. [1]) reported for the first time that a mycobacterium had been cultivated optimally at an incubation temperature as low as 10 °C compared to practically no growth at 22 °C and 32 °C. Results presented in this communication brought further experimental evidence that there exists a leprosy derived species of psychrophilic mycobacteria. It did not become evident whether these cultures were identical to *M. leprae*. They are certainly related to the disease, since identical psychrophilic cultures have been obtained so far from 7 out of 9 leprosy infected armadillos, 12 out of 12 *M. leprae* infected Nu mice foot pads and 1 out of 2 human lepromata.

The presence of PGL–1 in the cultures as well as the Mitsuda type skin reactions provoked by heat killed suspensions of the cultures, as found by Enzo and Bechelli, merit special attention.

Acknowledgements. This study was supported by grants from the Cardinal Léger Institute against leprosy. Dr. Eleanor Storrs (Florida Institute of Technology) deserves gratitude for tirelessly supplying *M. leprae* infected armadillo tissues. Nu mice foot pads were donated by Dr. M. Ishaque, (Montreal) and Dr. K. Kohsaka, Osaka, Japan. Human lepromata were kindly supplied by Dr. R. N. Miranda, CELSA, Curitibo, Brazil. With gratitude we acknowledge the expert cooperation of Dr. Enzo Melchior, Jr. and Professor L. M. Bechelli for performing and evaluating the late skin reaction to *M. psychrophilum* (L) antigens.

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SENSITIVITY OF DETECTION OF ENTEROTOXIGENIC *ESCHERICHIA COLI* FROM STOOL SAMPLE BY DNA PROBE

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An attempt has been made to detect the minimum counts of enterotoxigenic *Escherichia coli* (ETEC) in stool sample under simulated clinical condition. Thermostable (ST-la) enterotoxin-producing ETEC culture was mixed with stool sample and normal saline, centrifuged, then the supernatant was further diluted with saline and different volumes were spotted on nitrocellulose paper. Hybridization with ^{32}P labelled pDAS–101 DNA and viable count of original culture on MacConkey agar plates with ampicillin revealed that minimum 8 cells of ETEC (ST) could be detected. The method of labelling used was sequential harnessing of the catalytic and synthetic activity of the large Klenow fragment of DNA polymerase–I. Linearizing of the DNA was dispensed with as the nicked circular DNA was excised with the gel and used for labelling directly.

Infectious diarrhoea is the third leading cause of morbidity and a major cause of infant mortality in the developing world [1]. Enterotoxigenic *Escherichia coli* (ETEC) is one of the common causes of diarrhoea in India and other developing countries. The conventional methods of detection of thermolabile (LT) and thermostable (ST) toxin include tissue culture [2] immunologic [3], in vivo testing in the rabbit ileo-caecal loops [4] and by infant mouse assay [5]. Almost all these techniques require culture in the selective medium, purification of isolates and their identification thus takes long time and even then are not free from giving false positive and false negative results because of various reasons like cross-reactivity [6], interference growth, lack of sensitivity, poor viability, presence of debilitated organisms and absence of preformed toxin [7]. Detection by DNA–DNA or DNA–RNA hybridization is free from all these problems and provides a rapid sensitive tool that enables large number of samples that can be screened simultaneously.

In this experiment we have attempted to detect the lowest count of ETEC (ST) by DNA probe directly from stool sample in a simulated clinical condition.

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Materials and methods

Strain. The *E. coli* with the plasmid DAS-101 containing a 157 bp ECO-R-I/Bam H-I fragment of toxin gene and an amp. marker was obtained from Professor Y. Takeda of Kyotot University, Japan.

Chemicals. The enzyme DNA polymerase-I (Klenow fragment) was obtained from M/s. Bangalore Genei Pvt. Ltd., India. The ^{32}P deoxycytidine triphosphate was from the Board of Radiation and Isotope Technology, Bombay and the deoxyribonucleotide triphosphates were purchased from M/s. Sigma Chemicals, USA.

Sample preparation. E. coli culture (20 µl) containing with the plasmid DAS-101 was mixed thoroughly with 1 g of stool sample and 3 ml of sterile normal saline (SNS). The mixture was centrifuged at 2500 *g* for 10 min and 20 µl of supernatant was pipetted out and mixed with 10 ml of SNS.

Filter preparation. The diluted culture thus obtained was spotted on nitrocellulose filter at volumes ranging from 1 to 10 µl at 1 µl intervals and from 20 to 390 µl at 10 µl intervals with the help of a Dot-blot apparatus (Bioteck, India). Cells were lysed and DNA denatured by placing the filter on a Whatman 3 MM filter presoaked with 0.5 N sodium hydroxide. Neutralization was effected by similar routing through 0.5 M Tris-HCl (pH 7.0) and 1.5 M sodium chloride. The infra-red dried filter was baked at 80 °C under vacuum for 2 h.

Probe generation. Probe was generated by sequentially harnessing the 3'-5' exonuclease and 5'-3' polymerase activity of the large Klenow fragment of DNA polymerase-I [8]. The DNA used for labelling was the 2.8 kb pDAS-101 which contains the 157 bp ECO-R-I/Bam H-I ST toxin gene [9]. The catalytic activity of the enzyme was continued for 1.5 h at 37 °C followed by the synthetic activity, initiated by the addition of the deoxyribonucleotide triphosphates (both labelled and unlabelled) and continued overnight at room temperature.

Plasmid DNA, isolated by the alkaline lysis method [8] was electrophoresed on 1% low melting point agarose and the nicked circular DNA that bands ahead of the covalently closed circular DNA was chopped out along with the gel. The 130.38 mg gel containing the DNA was dissolved in 391 µl of sterile distilled water by mild heating and 1 µl of the DNA in water was used for exonuclease action (1 µl containing 5 units of enzyme) in 10 µl reaction volume.

Hybridization. The DNA bound nitrocellulose filter was prehybridized in 6× SSC (1× is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), 5× Denhardts solution (1× is 0.02% each of Ficoll, polyvinyle pyrrolidone and bovine serum albumin), 0.1% sodium dodecyl sulphate (SDS) and 100 µg/ml denatured sonicated salmon sperm DNA in a hybridization bag for 1 h at 68 °C. Hybridization was initiated by the addition of heat denatured probe and continued overnight at the same temperature.

The filter was washed thrice in 2× SSC and 0.1% SDS for 10 min each at room temperature followed by twice in 1× SSC and 0.1% SDS at 68 °C for 1 h each. The infra-red dried filter was put for autoradiography.

Viable count. Viable count of original culture was made by serial dilutions of 0.1 ml of culture in 0.9 ml of SNS. Dilutions ranging from 10^{-4} to 10^{-7} were plated on MacConkey agar plates containing 20 µg/ml of ampicillin. Similarly, the diluted supernatant of centrifugation was plated at 10^{-1} to 10^{-3} dilutions. The colony forming units (c.f.u.) were counted after 48 h incubation at 37 °C.

Results and discussion

Hybridization was observed (Fig. 1) at all the spots above 100 µl application volume. No hybridization was observed below 100 µl application spot.

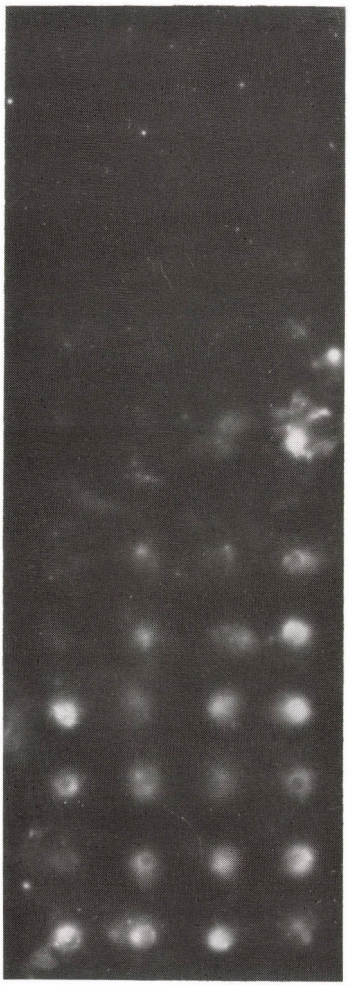

Fig. 1. Hybridization seen at all points above 100 μl application spot

It was also observed (Fig. 2) that 60 c.f.u. were present in 10^{-4} dilution and 6 colonies in 10^{-5} dilution MacConkey plates. There was no growth in 10^{-6} and 10^{-7} dilution plates. No c.f.u. was detected in the stool processed and diluted culture.

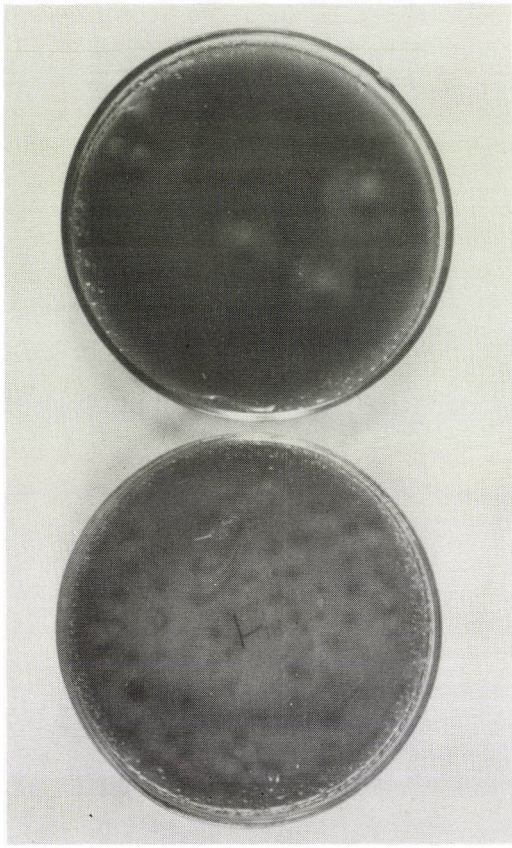

Fig. 2. Amp – MacConkey agar sustains 60 c.f.u. in 10^{-4} dilution and 6 c.f.u. in 10^{-5} dilution plates

Calculating from the viable count figures and the dilution factors used in sample preparation, the minimum number of ETEC (ST) cells that can be detected by hybridization (contained in 100 μl application volume) was 8. That is, a load of 8 cells present in the stool sample can be detected by the above method. We have in a separate experiment (not reported here) observed that 25% of ETEC gets trapped in the stool sample and 75% are recovered in the supernatant. Therefore it can be interpretated that a minimum of 6 ETEC was actually detected.

Detection of 10 ETEC by directly spotting stool sample on nitrocellulose membrane has been reported [10]. Similarly, detection of 100 *Salmonella typhi* from stool by using a 8.4 Kb DNA probe has been described [11].

In this experiment we have utilized both the catalytic (3' – 5' exonuclease) and synthetic (5' – 3' polymerase) activity of the Klenow fragment of DNA polymerase-I.

This dispensed the need for use of random hexanucleotide primers which besides been expensive involves sensitive oligoannealing to denatured DNA strand at 0 °C.

We have in this experiment also dispensed the need for linearizing the plasmid DNA as required by the enzyme. This was possible by collecting the nicked circular DNA that bands ahead of the closed circular DNA and using it for labelling directly.

Accordingly, this is a sensitive and economic method of detection that can be practised for molecular epidemiological studies.

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MUTAGENIC EFFECT OF CADMIUM ON *TRICHODERMA VIRIDE*

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The effect of cadmium ion on growth and differentiation of the filamentous fungus *Trichoderma viride* was studied. Cd^{++} at a concentration of 10 mM strongly retarded growth and caused morphological changes. From cultures treated with cadmium, two white mutants and one yellow mutant were isolated. The white mutants formed no conidiophores and one of them produced colourless crystals in the cultivation medium. The yellow mutant differed from the parental strain mainly by the production of differently coloured conidia and by a slower growth rate.

Heavy metals are among the most dangerous pollutants. They have a tendency to accumulate in nature. Cadmium is one of the most frequent metals that enters the environment through a variety of human activities and, to a lesser extent, by natural weathering. The cadmium ion is a well-known toxicant. Many authors have demonstrated and confirmed its harmful effects on various organisms. However, the majority of these studies have dealt with the toxicity of cadmium in humans and animals and only to a lesser degree with its effect on plants and microorganisms [1–6]. Relatively little is known about the effect of cadmium on filamentous fungi. Their significant role in the environment is obvious. In our laboratory the effect of cadmium on the growth and differentiation of the filamentous fungus *Trichoderma viride* was studied.

Materials and methods

Cultivation media. The following media were used: A, basic growth medium containing Czapek-Dox agar + 0.5% yeast autolyzate; B, medium with cadmium ion: basic growth medium A to which 10 mM Cd^{++} (as $CdCl_2 \cdot 2.5\ H_2O$) were added; C, selective medium: basic growth medium A supplemented with sodium deoxycholate at a concentration of 1 g l^{-1}. On this medium only small and slow growing colonies developed [7].

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Strain. The wild-type *T. viride* 8–7 Pers. ex S. F. Gray with smooth conidia was used. This strain is maintained in The Czech Collection of Microorganisms, Brno, as CCM F–534. In our study this strain was maintained on slants of basic medium A.

Cultivation. All of the culture studies were carried out on solid cultivation medium in Petri dishes. *T. viride* was propagated from medium A agar slants. Following satisfactory conidiation the conidia were used to inoculate Petri dishes containing agar A. After 5 days, sterile filter paper discs (d = 4 mm) were placed on the agar medium. After further two days of incubation, when the discs were overgrown with mycelium, the discs were transferred to the centre of plates with agar medium B. Since the fungus grew very slowly on this medium, the Petri dishes were kept in a moist glass chamber to avoid drying of the agar. All cultures were grown under daylight conditions at room temperature. After several weeks, when the colonies growing on medium B reached a diameter of 2–3 cm, inocula were taken from morphologically changed areas at several places of every colony. A suspension of conidia and hyphae in sterile water was then used to inoculate Petri dishes of medium A. For all subsequent cultivations medium without cadmium was used.

Observation of growth and morphology. The growth and morphology of the cultures were observed on cadmium-free medium. These observations were done both macroscopically and microscopically. In the macroscopic observations several criteria were evaluated: growth, shape, colour and morphology of colonies, the manner and intensity of conidiation, etc. Microscopic observation: branching and structure of mycelium, morphology of conidiophores, character of conidia.

Isolation of mutants. Mutants were isolated from the first or successive cultivations after cadmium had been applied. Isolation was carried out in two ways: (a) direct transfer of inoculum to basal medium A; (b) inoculation of suspended inocula on plates with selective medium C. Inocula taken from individual small colonies were then again subcultured on medium A.

Results and discussion

The direct effects of cadmium on medium B were manifested by evident changes in the growth and morphology of *T. viride*. In the first place, the growth rate of the colonies was severely depressed (Fig. 1). While the control cultures, growing on basal growth medium A, reached a diameter of 7 cm within 3 days, the colonies growing on medium B reached this size only after more than 3 weeks of incubation. The morphological changes also were very pronounced. Control colonies had an approximately circular form with a plain white edge. Sporulating zones created typical concentric green rings. Cultures growing in the presence of cadmium formed irregularly lobed colonies and on their white surface irregular yellow or pale green zones appeared. Conidia and pieces of hyphae taken from the white and coloured parts of the colonies were then inoculated on plates of the growth medium A. In subcultures on this medium, these morphologic, growth and colour changes persisted. Mutants isolated from these cultures differed from the wild strain of *T. viride* mainly by retarded growth, reduced conidiation, changed pigmentation, different morphology of colonies, unusual form and branching of hyphae, etc. Also intensive vacuolization and hyphae crumbling often occurred. Up to now three mutants have been isolated: *MCd* 1: milky white, heavily retarded growth, no

conidiation, appearance of crystals in cultivation medium, straight and scarely branched hyphae; *MCd* 2: milky white, heavily retarded growth, no conidiation, curly and vacuolated hyphae, many hypae crumble intensively to small pieces; *MCd* 3: yellow coloured, slightly retarded growth, reduced conidiation, normal hyphae and conidiation.

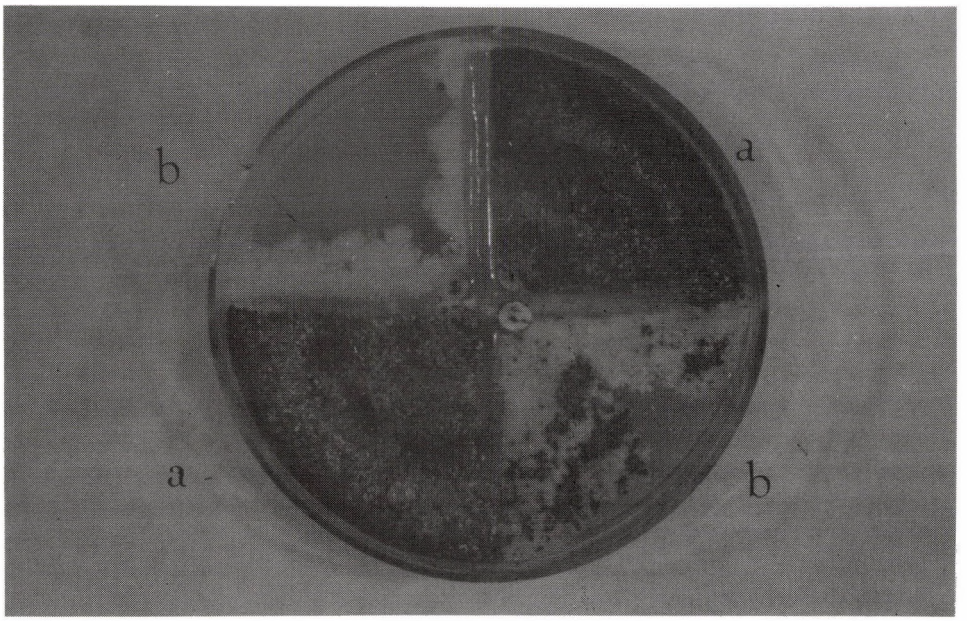

Fig. 1. Growth of *T. viride* on normal medium (a) and on medium with cadmium (b)

Both white mutants, *MCd* 1 and *MCd* 2, showed substantially lowered growth rate. The yellow *MCd* 3 mutant grew at a rate very similar to that of the wild strain (Fig. 2). Both white mutants failed to form conidia and conidiophores. Only rarely were some single phialides with 1–2 conidia found. The mycelium of both of the white mutants was very thin and sparse near the centre of the colonies. The periphery of these colonies was irregular. Old *MCd* 1 mutant colonies became farinaceous (Fig. 3). The surface of old *MCd* 2 colonies was wooly.

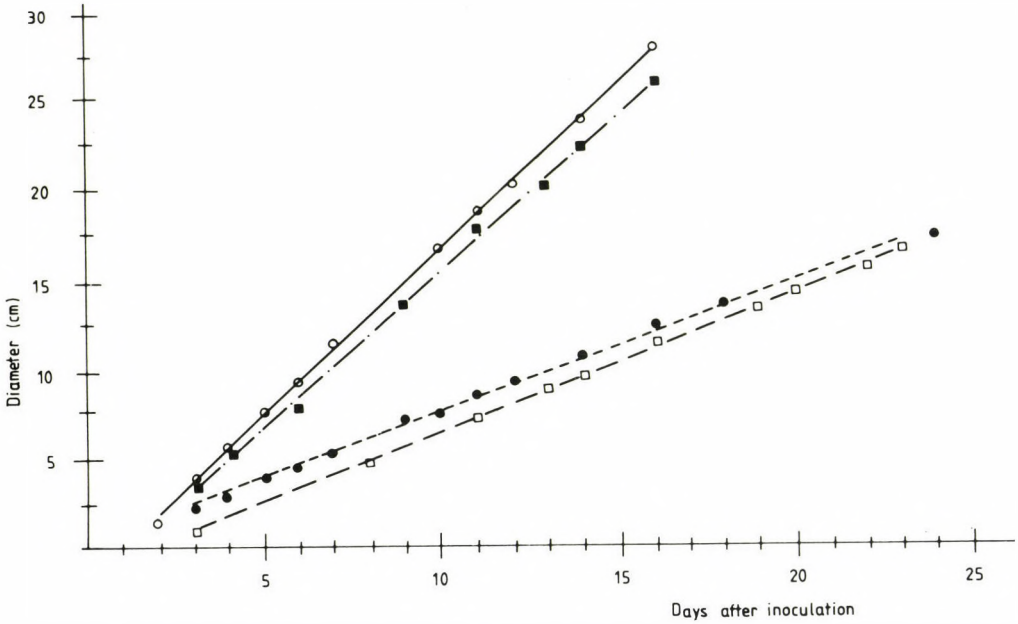

Fig. 2. Growth rates of *T. viride* mutants; O - - - O *MCd* 1; • - - - • *MCd* 2; ■ - - - ■ *MCd* 3; O——O control

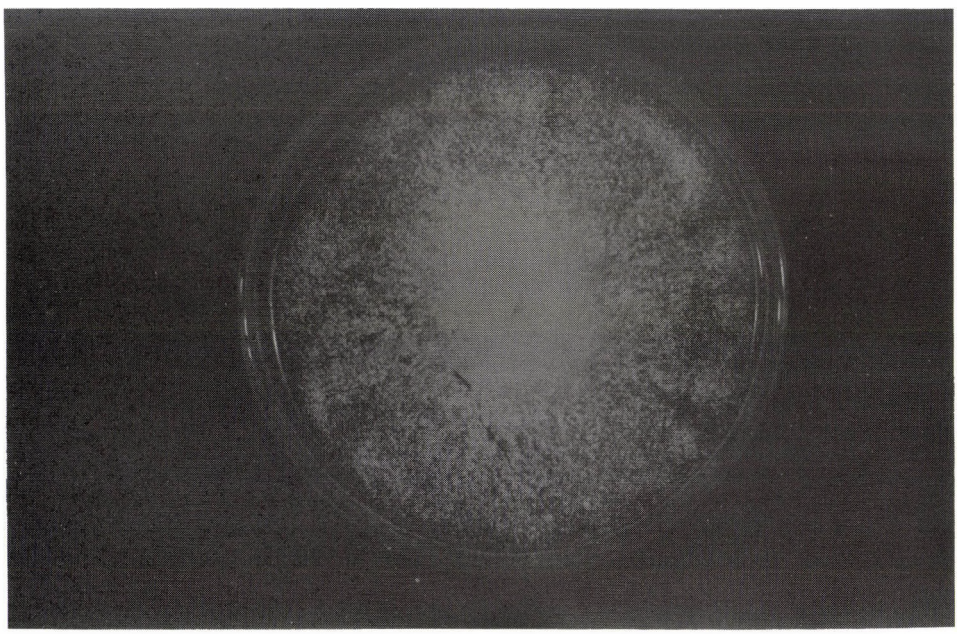

Fig. 3. White mutant *MCd* 1 after three weeks cultivation

Production of crystals by *MCd* 1 was noteworthy and surprising. These crystals were regular, colourless octahedrons of various sizes and their quantity rose during cultivation. Both of the white mutants tended to form many chlamydoconidia. Their hyphae were brittle and, mainly on the *MCd* 2 mutant, they often crumbled into small fragments. The yellow *MCd* 3 mutant did not exhibit such striking morphologic or physiologic changes. Beside its differing pigmentation, only less intensive conidiation with a lower number of phialoconidia was observed. The thickness, density and branching of the hyphae, as well as the morphology of the conidiophores were not apparently changed. Pale green pigmentation sometimes appeared, where the colonies were in contact with the Petri dish wall or with any other object placed on the surface of the cultivation medium. All three mutants maintained their properties after multiple passages on cadmium-free media.

The mutants isolated after exposure to cadmium strikingly resembled mutants obtained after UV-radiation. Besides white and yellow mutants also brown colonies were isolated [8]. After cadmium treatment, we also observed some brown mutants, but so far their properties have not been sufficiently stabilized.

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ISOELECTRIC FOCUSING ISOZYME PROFILES AND TAXONOMIC DISTANCES AMONG *FUSARIUM* SPECIES OF THE SECTIONS ARTHROSPORIELLA AND SPOROTRICHIELLA

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Isozymes from 18 isolates representing seven species of the *Fusarium* sections Arthrosporiella and Sporotrichiella were compared by isoelectric focusing in polyacrylamide gels. Of the six enzyme systems tested esterase and malate dehydrogenase showed the largest variation. A numerical analysis of the *p*I values determined for acid phosphatase, esterase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase resulted in a dendrogam demonstrating the taxonomical relationships of the seven species. *Fusarium avenaceum* and *Fusarium pallidoroseum* were the two most closely related species. The high degree of isoenzyme dissimilarity among *Fusarium chlamydosporum, Fusarium poae, Fusarium tricinctum*, the fungi that produce pyriform or citriform microconidia, suggests that they are distinct species and their reduction to a variety level is not reasonable. The taxonomical distinctness of *Fusarium camptoceras*, a lesser known and rarely occurring fungus was also proven.

Arthrosporiella and Sporotrichiella sections of the genus *Fusarium* Link contain several species known as secondary parasites of small-grain cereals. Other members of these groups, e.g. *Fusarium avanaceum* (Fr.) Sacc. cause root rots and damping-off of many herbaceous plants. The majority of these species are widely distributed throughout the world and some of them are of particular importance as producers of trichothecene mycotoxins.

The taxonomy of these fungi, which in most cases lack the sexual form, is exclusively based on morphological characteristics and this fact leads to

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controversies. There are considerable disagreements over the status of several taxa and confusions in placing certain species in the correct section. According to Booth [1] seven species are included in the two groups: *Fusarium poae* (Peck.) Wollenw. and *Fusarium tricinctum* (Corda) Sacc. belong to Sporotrichiella, while *Fusarium avenaceum*, *Fusarium camptoceras* Wollenw. et Reinking, *Fusarium fusarioides* (Frag. et Cif.) C. Booth, *Fusarium semitectum* Berk. et Rav., and *Fusarium sporotrichioides* Sherb. are put into Arthrosporiella. This sectional arrangement is based on conidium ontogeny, i.e. members of Arthrosporielly produce – at least at a certain stage in their development – polyphialides or polyblastic conidiogenous cells, whereas Sporotrichiella species are exclusively monophialidic. Other taxonomists – Billay [2], Gerlach and Nirenberg [3], Nelson et al. [4], Joffe [5] – regarded the presence of ovoid, pyriform or citriform microconidia as a guiding principle and assembled the species *F. fusarioides*, *F. poae*, *F. sporotrichioides* and *F. tricinctum* that produce such conidia into Sporotrichiella irrespective of the conidiogenous cells; they also preferred the original name, *Fusarium chlamydosporum* Wollenw. et Reinking to *F. fusarioides*. The other major difference between the Booth-system and the latter is the place of *F. avenaceum*. All *Fusarium* specialists subsequent to Booth disclaimed the affinity of *F. avenaceum* to Arthrosporiella and redirected this species to Roseum. A minor modification was proposed by Billay [2] and Joffe [5] who reduced both *F. chlamydosporum* and *F. tricinctum* to varieties of *F. sporotrichioides*. Worthy of note is, that only two of the species considered here, *F. avenaceum* and *F. tricinctum* have teleomorphs named as *Gibberella avenacea* R. J. Cook and *Giberella tricincta* El-Gholl, McRitchie, Schoulties et Ridings, respectively.

For the sake of a firm starting-point, we decided to follow the earliest modern system of *Fusarium* constructed by Booth [1]. We accept his nomenclature for five species (*F. avenaceum*, *F. camptoceras*, *F. poae*, *F. sporotrichioides*, and *F. tricinctum*) but use the widespread name *F. chlamydosporum* instead of *F. fusarioides* and replace *F. semitectum* by the more reasonable name *Fusarium pallidoroseum* (Cooke) Sacc. [6]. *Fusarium semitectum* Berk. et Rav. var. *majus* Wollenw. was omitted due to the general skepticism that concerns the existence of this taxon as well as the difficulties in obtaining authentic type materials.

In the last two decades, and especially in recent years, gel electrophoresis of isozymes has proved to be a powerful technique for the investigation of taxonomical relationships among species in fungus genera such as *Basidiobolus* [7], *Candida* [8], *Cronartium* [9], *Endothia* [10], *Fusarium* [11–13], *Kluyveromyces* [14], *Mucor* [15, 16], *Peronosclerospora* [17], *Phyllosticta* [18], *Phytophthora* [19], *Pleurotus* [20], *Puccinia* [21], *Saprolegnia* [22] and *Trichoderma* [23]. Enzyme polymorphisms of taxonomical and diagnostic value have also been detected within species, among genotypes, strains, races or formae speciales in *Atkinsonella hypoxylon* [24], *Erysiphe graminis* [25, 26], *Fusarium graminearum* [27], *Fusarium oxysporum* [28] and *Trichoderma*

harzianum [29]. Because isozymes are controlled by different alleles they were successfully traced as molecular markers in the analysis of hybrids obtained from matings [30] or after protoplast fusion of genetically unlike organisms [31, 32].

Since *Fusarium* species of the sections Arthrosporiella and Sporotrichiella have not been thoroughly investigated by molecular taxonomical approaches we decided to compare isozyme of 18 isolates of seven species from these groups to indicate some resolution of the afore-mentioned taxonomical disagreements. Polyacrylamide gel electrophoresis isoelectric focusing was applied to reveal minor differences between the enzyme patterns. Data were subjected to cluster analysis purporting a more objective grouping of the species. Six enzyme systems were examined in order to find the most discriminating ones suitable for use as molecular markers in these fusaria.

Materials and methods

Isolates and culture methods. The origins of the 18 *Fusarium* isolates as well as their culture collection numbers are given in Table I. The fungi were maintained on potato sucrose agar (commercial potato flakes, 20 g; sucrose, 20 g; agar, 20 g; made up to 1 litre with distilled water) at 10 °C in the dark. For mycelium production 50 ml of a complete medium (salts of the standard Czapek solution; glucose, 30 g; yeast extract, 3 g; distilled water, 1 l) in 250 ml Erlenmeyer flasks were inoculated with 10^6 conidia and incubated on a rotary shaker for 48 h at 180 r.p.m. at 25 °C.

Preparation of samples. Mycelium was harvested by filtration through a nylon mesh, washed several times with cold water, blotted dry between paper towels and frozen at −70 °C. Frozen mycelium (4 g), 10 ml of glass beads, 0.1 mm diameter (Biospec, Bartlesville, Oklahoma, USA), and 10 ml extraction buffer (0.05 M Tris–HCl, pH 7.0) were agitated in a Bead-Beater homogenizer (Biospec) at full speed for 60 s in an ice-water jacket. Slurries obtained were centrifuged first for 10 min at 4000 r.p.m., then again for 10 min at 15 000 r.p.m. at 4 °C. The protein concentrations of the supernatants as determined by the method of Bradford [33] were about 4–5 mg ml^{-1}.

Isoelectric focusing. A 5% (w/v) polyacrylamide gel, 0.4 mm thick containing 5% (v/v) ampholyte (Servalyte, pH 3–10; Serva, Heidelberg, Germany) was casted on 125 × 65 mm gel support film (Bio-Rad Laboratories, Richmond, California, USA). After photopolymerization 15–30 µl of mycelium extracts were dropped onto filter paper application strips. Isoelectric focussing was performed at 4 °C in a Model 111 IEF Cell (Bio-Rad) for 90 min with stepwise increases of voltage: 100, 200, 450 V for 15, 15, and 60 min, respectively. Gels were stained for enzyme activities according to the methods published by Harris and Hopkinson [34]. The enzyme names with the Enzyme Commission numbers (Internation Union of Biochemistry, Nomenclature Committee, 1984), abbreviations, numbers of electromorphs detected, and isoelectric points (*p*I) are given in Table II; *p*I values were estimated to point nought five precision by comparing fungal patterns to those of Protein Test Mix 9 (Serva) run in parallel with the samples.

Data analysis. The presence (1) or absence (0) of each of the 25 isoenzymes summarized in Table II served to construct a dendrogram by cluster analysis [35] using the "mean" method of the PHYLO program described by Baertlein et al. [36]. The analysis was performed on a Hewlett-Packard 9845B computer.

Results

Mycelial extracts of the fungi investigated in this experiment were independently isoelectric focused on separate gels for each staining procedure, at least in duplicate. Extracts were prepared from different culture batches. Only invariant bands of the zymograms were taken into account, those appearing at the same position with similar intensity in repeated runs.

Six different enzymes were examined from 18 strains of seven *Fusarium* species. All these strains expressed an appropriate enzyme activity and the bands were clearly resolved in the selected enzyme systems. As can be seen in Table II only two enzyme (G-6PDH and PG1) proved to be monomorphic, the others comprised 3-10 isoenzymes.

Table I

List of Fusarium species examined in this study

Reference no.	Species	Strain number	Origin	Source
(1)	*F. avenaceum*	22-2	Hungary, alfalfa, 1972	own culture
(2)	*F. avenaceum*	22-170	Hungary, maize, 1972	own culture
(3)	*F. avenaceum*	A-20	Hungary, wheat, 1990	own culture
(4)	*F. camptoceras*	IMI 162622	Australia, wheat, 1971	S. Chambers
(5)	*F. chlamydosporum*	IMI 153430	Australia, wheat, 1971	S. Chambers
(6)	*F. chlamydosporum*	IMI 128101	Pakistan, soil, 1971	C. Booth
(7)	*F. pallidoroseum*	22-22	Hungary, alfalfa, 1972	own culture
(8)	*F. pallidoroseum*	R-2813	Australia, ?	P. E. Nelson
(9)	*F. pallidoroseum*	A-1	Hungary, maize, 1990	own culture
(10)	*F. poae*	22-189	Hungary, wheat, 1970	own culture
(11)	*F. poae*	72-178	Finland, barley, 1972	A. Ylimäki
(12)	*F. poae*	A-11	Hungary, wheat, 1990	own culture
(13)	*F. sporotrichioides*	22-26	Hungary, alfalfa, 1972	own culture
(14)	*F. sporotrichioides*	22-205	Hungary, fodder, 1972	own culture
(15)	*F. sporotrichioides*	A-2	Hungary, maize, 1990	own culture
(16)	*F. tricinctum*	22-144	Hungary, onion, 1972	own culture
(17)	*F. tricinctum*	72-297	Finland, barley, 1972	A. Ylimäki
(18)	*F. tricinctum*	A-12	Hungary, wheat, 1990	own culture

Table II

List of enzymes with Enzyme Commission (EC) number, abbreviations, number of electromorphs, isoelectric points (pI) and presence (+) or absence (0) of isozymes in the 18 Fusarium strains tested

Enzyme (EC no.)	Abbreviation	Number of electromorphs determined	Band pattern (*pI*)	Strains (ref. nos. in Table I) (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)
Acid phosphatase (3.1.3.2)	ACP	4	4.70	+	+	+	+	0	0	+	+	+	+	+	0	+	+	+	+	+	+
			5.00	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0
			5.20	0	0	0	0	+	0	0	0	0	0	0	+	0	0	0	0	0	0
			5.85	0	0	0	+	0	0	0	0	0	0	0	0	+	+	+	0	0	0
Esterase (3.1.1.1)	EST	13	4.30	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	0	0	0
			4.50	0	0	0	0	+	0	0	0	0	0	0	0	+	+	+	0	0	0
			4.65	+	+	0	0	0	0	0	+	0	+	0	+	0	0	0	0	0	0
			4.95	0	0	0	+	0	0	0	0	+	+	+	0	+	+	+	+	+	+
			5.20	+	+	+	+	+	+	+	+	+	+	0	+	0	+	+	+	+	+
			5.40	+	+	0	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0
			5.55	+	+	+	+	+	0	0	+	0	+	+	+	0	0	0	0	0	0
			5.70	0	0	0	0	0	0	0	0	0	+	+	+	0	0	0	0	0	0
			6.40	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			6.50	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G-6 PDH	1	4.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malate dehydrogenase (1.1.1.37)	MDH	7	5.70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+
			5.95	+	+	+	+	+	0	+	+	+	0	0	0	+	+	+	0	0	0
			6.20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
			6.40	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0
			6.60	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	0	0	0
			7.40	0	0	0	+	+	+	0	0	0	0	0	0	+	+	+	+	+	+
Phosphoglucose Isomerase (5.3.1.9)	PGI	1	4.80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphoglucomutase (2.7.5.1)	PGM	4	5.20	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+
			5.40	0	0	0	0	+	0	0	0	0	0	0	0	+	+	+	0	0	0
			5.55	+	+	+	0	+	0	+	+	+	0	0	0	0	0	0	+	+	+

Zymograms and *p*I ranges widely varied depending on enzymes. Four acid phosphatase (ACP) bands with *p*I values between 4.70 and 5.85 were detected. Except for *F. camptoceras* and isolates of *F. sporotrichioides* in which two bands were resolved, all the other fungi showed only one isoenzyme. Nearly all strains – with the exception of those of *F. chlamydosporum* – express – an ACP activity at *p*I 4.70. The highest polymorphism was found for esterase (EST), which seems to be the most powerful molecular marker to differentiate either between or within species. Ten distinct bands with *p*I values between 4.30 and 6.50 were visualized and, as almost all strains proved to be unique, 13 electromorphs were identified. While EST is suitable for the characterization of species, significant interspecific variations were also revealed by these isozymes. Although no more than three isolates of each species were run, *F. tricinctum* was the only one in which no interstrain differences occurred after staining for EST. Glucose-6-phosphate dehydrogenase (G-6PDH) showed no variation among species of the two *Fusarium* sections, a single band appeared in all cases at *p*I 4.45. A moderate diversity was observed for malate dehydrogenase (MDH) patterns with a total of six bands detected in a *p*I range of 5.70 and 7.40. Seven electromorphs were identified for this enzyme activity. Several species were indistinguishable: all strains of *F. avenaceum* and *F. pallidoroseum* produced identical patterns as did isolates of *F. camptoceras* and *F. sporotrichioides*. Phosphoglucose isomerase (PG1) proved to be as invariable as G-6PDH. All species of the sections Arthrosporiella and Sporotrichiella showed one band at *p*I 4.80. On the other hand, three phosphoglucomutase (PGM) isoenzymes were detected between *p*I 5.20-5.55; the isolates gave one or two bands, and four electromorphs were identified. The occurrence of a PGM band of *p*I 5.20 was universal, the difference among species derived from the presence or absence of a second isoenzyme, or from the position of the latter. A band with a *p*I = 5.40 characterized all *F. sporotrichioides* isolates, while *F. poae* strains were distinguished by the absence of the second PGM band.

When electromorphs found for the different enzyme systems were combined, 15 distinct isozyme phenotypes could be identified in the seven *Fusarium* species. Two strains of *F. sporotrichioides* (22-205 and A-2) and *F. tricinctum* (22-144 and 72 297), as well as one of *F. avenaceum* (22-170) and *F. pallidoroseum* (R-2813) exhibited identical phenotypes. These identities – over and above the unquestionable phylogenetic affinity – can be explained by the limitations of the isoelectric focusing technique used in this study. The clonal origin of these three pairs of strains can be excluded because of the geographical and chronological differences in their collection data (Table I). Moreover, isolates nos. 22-170 and R-2813 retained their morphological characteristics typical of *F. avenaceum* and *F. pallidoroseum*, respectively.

From the data matrix given in Table II taxonomical distance (D) and similarity (S = 1-D) were computed and a dendrogram was constructed by means of cluster analysis (Fig. 1). In this dendrogram a taxonomical distance of 33% (D = 0.33) was the dividing line above which species could be separated.

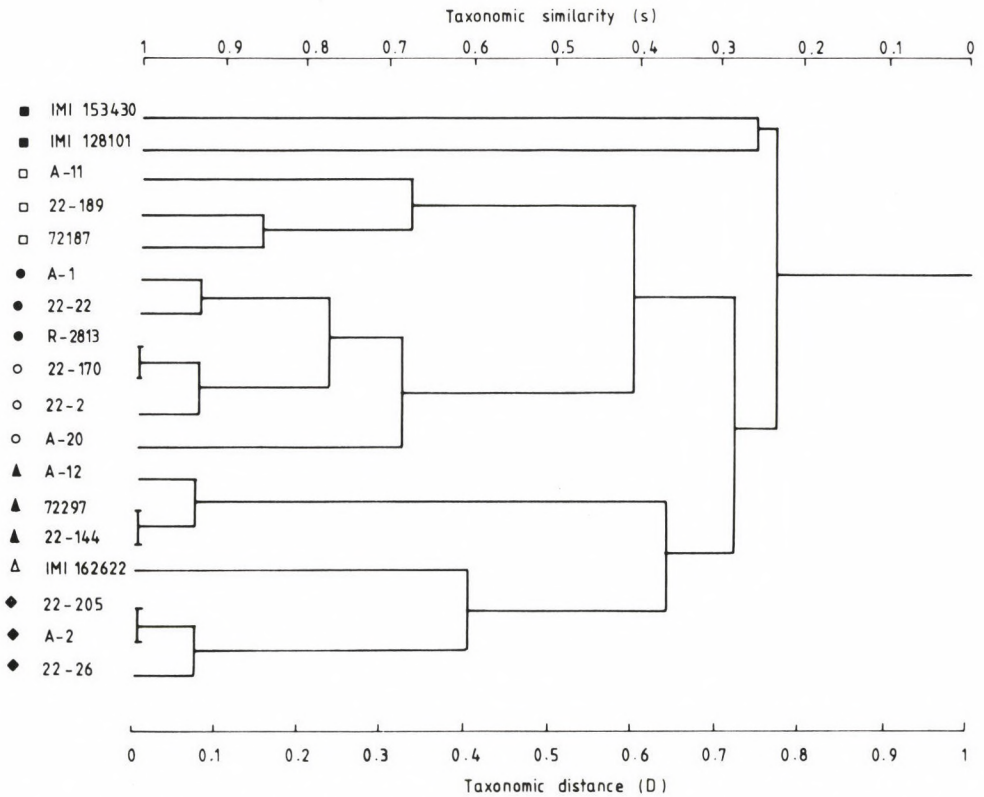

Fig. 1. Dendrogram showing taxonomic relationships of the 18 *Fusarium* isolates examined (■, *F. chlamydosporum*; □, *F. poae*; •, *F. pallidoroseum*; O, *F. avenaceum*; ▲, *F. tricinctum*; Δ, *F. camptoceras*; ◆, *F. sporotrichioides*)

Strains that belong to *F. sporotrichioides* and *F. tricinctum* revealed close intraspecific relationships at similarity levels of 92% (S = 0.92). Isolates of *F. pallidoroseum* were more diverse (D > 0.24) and even greater electrophoretic variations occurred in *F. avenaceum* (D = 0.32) and *F. poae* (D = 0.33). However, the interstrain diversity within these three species do not exceed the interspecific differences found in Arthrosporiella and Sporotrichiella. Very high within-species variation was observed in *F. chlamydosporum* represented by two isolates. This might be due in part to the wide geographical separation of these isolates, but the considerable isoenzyme diversity would rather suggest that they belong to different taxa. The single isolate of *F. camptoceras* had no particular isoenzymic relationship with the other species of Arthrosporiella and Sporotrichiella. A moderate affinity expressed toward *F. sporotrichioides* does not disprove the taxonomical distinctness of this rarely occurring species.

Discussion

In the experiments presented here six isozyme activities were examined by names of isoelectric focusing in order to provide a molecular approach supplementary to morphological characteristics in the classification of *Fusarium* species that belong to the sections Arthrosporiella and Sporotrichiella. The isozyme analysis demonstrated noteworthy differences among the seven species tested. Two of the enzymes revealed considerable diversity between species, the greatest inter- and intraspecific variation was recorded for EST. Less complex, but still informative zymograms were found for MDH. APC and PGM brought to light little polymorphisms, while G-6PDH and PGI did not vary at all.

The main taxonomical conclusions from these investigations were as follows. (i) Cluster analysis of the isozyme data confirmed the taxonomical distinctness of all species of the two sections. (ii) *F. avenaceum* and *F. pallidoroseum* were found to be the most closely related species indicating the affinity of the former towards members of Arthrosporiella and Sporotrichiella. (iii) The relatively high degree of isozyme dissimilarity among *F. chlamydosporum*, *F. poae*, *F. sporotrichioides* and *F. tricinctum* suggests that these are real species and their reduction to a variety level is not reasonable. (iv) The position of the afore-mentioned four species in the dendogram indicates that they are not more closely related to one another than to any other species of the sections examined. Hence, their arrangement into two different sections, as proposed by Booth [1] seems to be unjustified. (v) The magnitude of variation with the exception of *F. chlamydosporum* was always smaller within species than that observed among species. (vi) As far as *F. camptoceras* is concerned, this fungus should be considered as a distinct species.

The only molecular approach to understanding the phylogenetic relationships of some of these fungi has recently been published [37]. Three regions of small and large subunit rRNA were partially sequenced in single isolates of *F. chlamydosporum*, *F. poae*, *F. sporotrichioides* and *F. tricinctum*. The numbers of base substitutions in these fungi indicated, that among the four species *F. poae* and *F. sporotrichioides* are the most closely related ones.

The results presented here demonstrate again that isozyme analysis may help in resolving taxonomical problems in morphologically highly variable fungi like *Fusarium*. Isozymes, especially those of EST are valuable molecular markers to reveal either intra- or interspecific taxonomic differences.

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IN MEMORIAM ZOLTÁN ALFÖLDY
1904–1992

Professor Zoltán Alföldy graduated as M. D. from the University of Debrecen, Hungary, director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest has died. His pupils and admirers would like to express their deep respects by papers published in this volume.

Professor Alföldy was an outstanding personality of medical microbiology in Hungary. He was a member of the generation of physicians who, despite the inevitable specialization characteristic of his age, retained his interest in the whole of medicine till his death. He started his career in 1928 in the Department of Medicine of the University of Debrecen, where he based his humane medical attitude, so characteristic of him, on active therapeutical practice. He was the head of both the infectious disease and pulmonological units of the Department and, attracted by the modern medical technology, he soon became deputy-leader of the radiological and EKG laboratories.

Akadémiai Kiadó, Budapest

The severe typhoid epidemics of the 'thirties directed his attention and interest to the bacteriological laboratory. In 1937 he became a member of the National Institute of Hygiene in Budapest, where he soon joined the national anti-typhoid programme (1938). From 1946 to 1951 he was the head of the Department of Bacteriology of the same institute. In 1948 he qualified as lecturer at university. In 1952 he won the postgraduate (academic) degree "candidate of medical science".

In 1950 he was appointed to the chair of microbiology at the University Medical School (now Semmelweis University) and he was active as director of the Institute of Microbiology of the same university till 1974, when he retired. He was the "host of microbiology" at the university over a quarter of century; his lectures can never be forgotten. He was the master of a great part of the physicians being active in Hungary nowadays; they learned microbiology from his books.

Professor Alföldy was the author of 70 papers, 10 books and many chapters in several books. His scientific research in the field of infectious diseases was the most successful on leptospirosis.

He was the first in Hungary who isolated leptospires from patients. Subsequently, he revealed the regularities of the incidence of leptospiroses and proved the leptospiral origin of "aseptic" meningitis epidemics in this country. Examination of thousands on animals proved the main role of domestic animals in the spreading of leptospiroses, further, the sources of infections conveyed by rodents were cleared up also by him.

His life, being most successful in education and research, was continually intervowen by scientific activities in public life. He was active especially as secretary (later vice-president) of the Scientific Council of the Ministry of Health, where he was the head of the Scientific Research Department. He was member of a number of scientific societies and editorial boards and considerably contributed to the activities of these organizations. He was in close contact with prominent microbiologists both in this country and abroad.

Professor Alföldy was a pioneer and organizer of specialization of physicians. As a member of the National Council of Medical Specialization he examined the specialist candidates over 40 years.

He received many decorations and awards, among them, Kossuth-prize for his fundamental leptospirosis research; the title Eminent Physician and Eminent Paedagogue; the gold medal of the Order of Labour (three times); the Endre Hőgyes medal, the Rezső Manninger medal; for high-level merits in the history of medicine he received the Weszprémi medal. His handbook of microbiology, written for medical students (Medicina, Budapest), reached many editions.

In addition to being highly active in research and education, Professor Alföldy devoted much time and energy to education of successors both in his private family and the large family of his co-workers.

All these characterize a man who lived an intensive full life, who did not bate a jot of completeness, retained his wide interest and critical sense and fructified his activity even when he was severely ill, till the last moment of his life.

Alföldy's path of life, deep humanity and unselfish modesty are exemplary for all of us – pupils and co-workers – either living in this country or abroad.

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Acta Microbiologica Hungarica, 40 (2), pp. 85–90 (1993)

LATEX AGGLUTINATION AND ADENOVIRUSES* II. DETECTION OF ANTIGENS

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(Received November 5, 1992)

Latex particles were coated with different monoclonal antibodies (MAbs) reacting with different epitopes of the hexons of adenovirus (AV) type h1 and h35. The coated particles were tested with the purified hexon of 22 different mammalian AV types and were agglutinated by the respective hexon antigen(s) with high specificity. The sensitivity of the reaction was influenced by the amount of MAb adsorbed to latex particles. The latex, coated with a MAb of genus specific reactivity can be a valuable tool in the rapid immunodiagnosis of adenovirus infections.

Latex agglutination (LA) is used since several years for different purposes in immunology [1]. It proved to be very useful among the rapid methods for immunodiagnosis in microbiology [2, 3]. In the last decade the introduction of monoclonal antibodies (MAbs) provided significant improvement of the LA tests, for detection of different bacterial [4–9], fungal [10], parasitic [11] and viral [12–16] infections.

Concerning adenoviruses (AV-es), data are few about LA, dealing only with the detection of enteric AV-es h40 and h41, mostly by a commercial reagent with polyclonal rabbit serum [17–20]. A single paper [21] reported on the preparation of a reagent for LA test, also for AV h41, using a polyclonal guinea-pig serum. Recently we developed a LA test for detection and measurement of AV antibodies (Ab-s) [22]. To find further possibilities to introduce LA test in the AV research, experiments were made to detect AV antigens by means of LA, using MAbs. The present paper reports on the first results of these studies.

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* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

Akadémiai Kiadó, Budapest

Materials and methods

Viruses, antigens. Human AV types 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 18, 19, 26, 27, simian AV sim16 and canine AV can1 were prepared in HEp-2 h35, h40 and h41 in "293" cells [23, 24]. The bovine strain bos3 was kindly supplied by A. Bartha. Purified hexon antigens were prepared by repeated anion exchange and hydrophobic interaction chromatography, and by gel filtration, as described [24, 25].

For the examination of the relationship between the appearance of latex-agglutinating antigens and cytopathic changes, "293" cultures were infected with AV types h8 and h41.

Antibodies (Ab-s). Polyclonal anti-hexon sera to AV types h1 and h5 were produced in rabbits [24]. MAbs were prepared by hybridoma technique, ascitic fluids were produced in Balb/c mice as described earlier [26, 27]. The MAbs included in this study originated from two panels of MAbs directed against the purified hexons of AV h1 and h35, respectively [27, 28]. The reactivity of MAbs 1A3 and 2C2 has been found genus specific, that of MAb 5D2 type specific for AV h1 hexon, as determined with ELISA and passive haemagglutination (HA) assays [27]. MAbs 35H58 and 35H62 reacted with types h7 and h35 in ELISA, but not in HA [28]. The antibodies were purified by ammonium sulphate precipitation, as reported [27].

Protein content of reagents was determined by Lowry's method [29].

Faecal specimens. Six samples, previously proved to contain different types of AV-es, were kindly supplied by Gy. Szücs. A tenfold extract of faeces was prepared in PBS, purified with Arcton, and stored at −20 °C before testing.

Latex reagent. Suspension of sulphate latex particles, diameter 137 nm, was kindly supplied by L. Bánkuti (Human Institute for Serobacteriological Production and Research, Budapest). A 1% suspension of latex in PBS was mixed with equal volume of MAb, diluted in the same solution. The coating procedure was the same as used previously with antigens [22], as well as the control reagent, coated by bovine serum albumin (BSA), without MAb. The reagents were stored at 4 °C and the reactivity remained stable for at least 5 years.

Inhibition of LA. Equal volume of appropriate dilutions of monoclonal and polyclonal Ab-s were mixed with 5 μg/ml purified hexon and after 30 min incubation at room temperature LA test was performed with the mixtures.

Results

Specificity of LA test. Four MAbs were used for coating of latex particles. The reactivity of them, as tested with 19 human and 3 non-human mammalian AV types are summarized in Table I. Latex suspension coated with MAb 1A3 (L-1A3) reacted with all hexons tested. Latex coated with MAb 5D2 (L-5D2) reacted only with type 1 hexon, while two MAbs (35H58 and 35H62) directed against h35 hexon were agglutinated only by types h7 and h35 hexons. BSA-coated control reagent was not agglutinated by any antigen preparation.

The specificity of the reaction was tested in inhibition experiments, too. As Table II shows, agglutination of the genus specific L-1A3 was inhibited by both genus specific MAbs, i.e. the homologous 1A3 and the MAb 2C2 and by both polyclonal anti-hexon sera, but not by the two MAbs of narrow reactivity. The type

specific L–5D2 was inhibited only by the homologous type specific MAb, 5D2 and by the two polyclonal sera.

Table I

Reactivity of MAb-coated latex reagents

MAb adsorbed to latex	Agglutination of reagent by purified hexon of AV type	Reactivity of latex reagent
1A3	h1 to 10, 12, 13, 18, 19, 26, 27, 35, 40 and 41 bos3, sim16 and can1	genus specific for Mastadenovirus
5D2	h1	type specific for h1
35H58 35H62	h7 and h35	intertype specific within subgenus B

Table II

Inhibition of agglutination of MAb-coated latex reagents by different monoclonal and polyclonal antibodies

Type of agglutinating hexon	Coating MAb on latex reagent	Inhibitor antibody: MAb 1A3	MAb 2C2	MAb 5D2	MAb 35H58	anti-h1[1]	anti-h5[1]
h1	1A3	+[2]	+	–[3]	–	+	+
	5D2	–	–	+	–	+	+
h3	1A3	+	+	–	–	+	+
	5D2	[4]					
h35	1A3	+	+	–	–	+	+
	5D2						
h41	1A3	+	+	–	–	+	+
	5D2						

[1] Polyclonal rabbit anti-hexon serum

[2] Agglutination was inhibited

[3] No inhibition

[4] Blank: this reagent was not agglutinated by the respective hexon

Sensitivity of LA test. To determine the sensitivity of the reaction, latex suspensions were coated with different dilutions of the MAbs. Figure 1 shows the relation between the concentrations of the MAb and that of the antigen in the case of MAbs 1A3 and 5D2. Accordingly, the optimal concentration for coating is not the highest, and for the different MAbs it is individual.

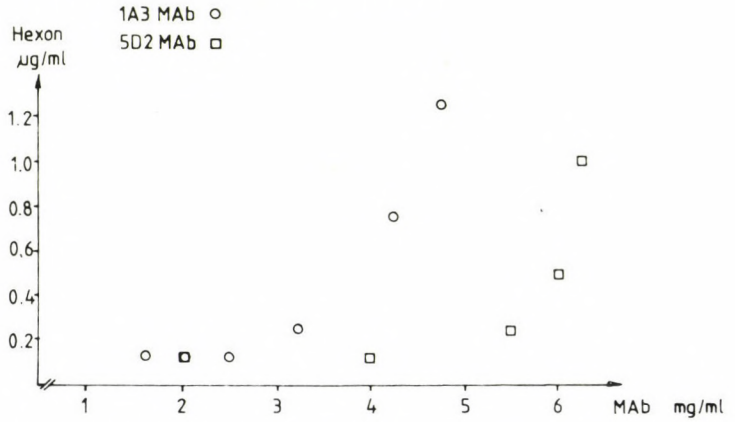

Fig. 1. Sensitivity of LA with MAb 1A3 and 5D2 coated latex reagents, tested with purified hexon of AV h1

The lowest concentrations of different hexons, detectable by LA were 0.1 to 0.12 μg/ml with the homologous type and between 0.25 to 5 μg/ml with others.

The use of LA test. Model experiments were made with L–1A3 latex reagent, coated with a genus specific MAb. Cultures of "293" cells were infected with 50 $TCID_{50}$ of AV h8 and h41, and the supernate of the cultures were tested daily with L–1A3 reagent. The samples agglutinated L–1A3 24 to 48 h after inoculation, while cytopathic effect could have been observed only after 4–5 days. The supernate of uninfected cells did not react.

Six faecal extracts, known to contain AV (unpublished data), were tested with L–1A3. Four of them agglutinated the reagent. In a fourfold dilution all six samples agglutinated L–1A3, while control with PBS and with BSA-coated control reagent gave negative results.

Discussion

The reaction of MAb-coated latex reagent proved to be highly specific. Similar results were reported with other viruses, both with polyclonal and monoclonal Ab-s [12, 14, 21, 30], but with polyclonal Ab-s the reaction is not always type specific [17]. In comparison with other methods, the specificity was more advantageous with type

h41 as electron microscopy [21], or even as enzyme immunoassay [30]. Results shown in Tables I and II are in good accordance with earlier results [27, 28], the results with inhibition experiments tally with those obtained by reciprocal competitive binding ELISA. They attest the evidence found for the existence of distinct epitopes on different antigenic sites of AV hexon [27].

The sensitivity of LA was found not so favourable in different systems [12, 13, 30], the smallest antigen concentration detectable was less in other immunoassays than in our experiments with LA, shown in Fig. 1 [6, 13]. In previous experiments we succeeded in detecting 6 ng/ml hexon with double MAb sandwich ELISA [31], while for LA at least 100 ng/ml has been necessary for the reaction. Similar results were found with bacterial antigens [32].

The sensitivity of the reaction has been influenced by the amount of MAb adsorbed to latex particles. The optimal concentration has to be determined individually with the different MAbs.

As to the experiments with cell cultures, we could detect viral antigens 24 h after infection. With cytomegalovirus viral antigen was detected within a few hours after inoculation of cell cultures, too [16]. Considering these results together with those with faecal specimens, as well as the simple and rapid accomplishment of the LA test, the method can become a valuable tool in the immunodiagnosis of AV infections.

Acknowledgements. We are indebted to J. ERDEI for help in the production of MAbs, and to L. BÁNKUTI providing the latex preparation. The skillful technical assistance of Miss Z. BAKONYI and Mrs M. SÓSKUTI is highly appreciated.

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MULTIPLE EFFECTS OF PROMETHAZINE IN *STAPHYLOCOCCUS AUREUS**

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The antibiotic resistance of 6 *Staphylococcus aureus* strains was eliminated with a frequency from 1.2 to 10% in the presence of subinhibitory concentrations of promethazine. The pigment production of the cells was also eliminated by the promethazine to an extent of 0 to 5%. The cell size was increased and the protein A production was markedly decreased in *S. aureus* cells cultured in the presence of promethazine. Complex formation between protein A and promethazine was detected by differential spectrophotometry. The biological activity of staphylococcus protein A was abolished by promethazine in the passive haemagglutination of rabbit antiserum treated sheep red blood cells. Evidence has been found that plasmid-encoded functions of *S. aureus* cells can be altered in the presence of promethazine, and the chromosomally controlled synthesis of protein A, one of the weakest virulence factor of *S. aureus* is also lowered by promethazine.

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* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904 – 1992) director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

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The effects of tricyclic psychopharmacons binding to bacteria and their extrachromosomal genetic elements were studied earlier in relation to the electronic structure of the compounds [1].

It was found that binding of such drugs to the plasmid DNA leads to inactivation of the plasmid replication system in the cells. Some of the phenothiazines, including promethazine were bound to the bacterial cell wall and membrane, inducing birefringence [1], disorganization of the cell wall [2], inhibition of the potassium efflux [3] and change in the chemical composition of the cell envelope proteins [4].

Besides antibiotic resistance, pigment production is also an unstable plasmid-encoded property of *S. aureus* [5]. In this report, the effects of promethazine, a phenothiazine drug, were tested on the plasmid-encoded antibiotic resistance of *S. aureus*, and some of its morphological characteristics, such as cell size and surface properties. The experiments also focused on the virulence factor protein A [6, 7]. Protein A has a special role in the pathogenicity of *S. aureus*, being a surface located protein, which binds immunoglobulin molecules at their Fc region [8].

Materials and methods

Bacterial strains. S. aureus Brandy (Km^r, Ap^r); *S. aureus* 4972 ($Na1^r$, a strong protein A producer); *S. aureus* P. 19237/78 (Str^r); *S. aureus* J. 63027 (sensitive to all antibiotics); *S. aureus* Hoffmann (Km^r) and *S. aureus* Mo Gm^r.

Culture media. MTY (minimal trypton-yeast extract) broth prepared according to Alföldy et al. [9] was used for culturing the bacteria. Mueller-Hinton agar was employed for testing antibiotic susceptibility.

Chemicals. Promethazine (Pipolphen) was a product of EGIS (Budapest). Anti-gammaglobulin rabbit serum, FITC-labelled, was produced by the Institute of Serobacterial Production and Research Human (Gödöllő-Budapest, Hungary); specificity: fluorescein conjugated protein 6.9×10^{-3} g/g = 2.8 mol. The serum was used for labelling the protein A content on the surface of the staphylococcus cells. Formaldehyde 10% (Reanal, Budapest) was used to fix protein A on the surface and kill bacterial cells. Ethidium bromide (Reanal, Budapest) was used to distinguish bacterial cells from strong background caused by extreme amplifier gain. Protein A was prepared by Sigma.

Elimination of antibiotic resistance and pigment production of S. aureus. An overnight preculture of the strains ($1-5 \times 10^3$ cells) was inoculated into MTY broth, which was distributed into 5.0 ml aliquots in test-tubes [10]. Promethazine, from 0 to 120 μg/ml was added to the samples. Incubation lasted for 24–48 h at 37 °C.

Dilutions were prepared of the samples grown in the presence of subinhibitory concentrations of the drugs, and 0.1 ml aliquots of the dilutions were then plated on the surface of Mueller-Hinton agar. The plates were incubated at 37 °C for 24 h, then the colonies were transferred to antibiotic-containing Mueller-Hinton agar by the replica plating method of Lederberg [11]. The colonies on antibiotic-containing plates were compared with the master plates, and the antibiotic-sensitive colonies were checked for biochemical activity.

Testing of protein A expression in S. aureus cultures. S. aureus strain Brandy and strain 4972 were cultured in the presence of different concentrations of promethazine in MTY broth, without glucose and magnesium addition, at 37 °C for 48 h. The cultures showing growth (at subinhibitory concentrations) were treated with 1/10 vol formaldehyde for 60 min at 4 °C. The samples were then washed in PBS buffer 3 times and the sediment was resuspended in 300 μl of PBS; 25 μl of the cell suspension was

added to 200 µl of FITC-labelled anti-gammaglobulin to label the protein A-containing cells. The serum was diluted 1:30 in PBS. Samples were mixed and incubated at 4 °C for 45 min, then washed twice in 300 µl of PBS. After the last centrifugation, at 3000 r.p.m. for 5 min in an Eppendorf table centrifuge, the sediment was resuspended in 300 µl of PBS. To label all the bacterial cells in samples, 250 µl of ethidium bromide (10 µg/ml) were added to each FITC-labelled samples. The samples were then analyzed in a Beckton Dickinson Cell Sorter. In each sample 5000 individual bacteria were analyzed for fluorescence intensity, cell size and surface homogeneity.

Analysis of promethazine-protein A complex by differential spectrophotometry. The complex formation between promethazine and protein A was studied with a Unicam SP-800 automatic spectrophotometer. Protein A (50-200 µg/ml) and promethazine (1000 µg/ml) were dissolved in 1% NaCl, and the pH was adjusted to 6.8-7. In the sample cell, 500 µl of protein A was mixed with 50 µl of promethazine, while in the reference 500 µl of saline was mixed with 50 µl of promethazine. The spectrum of the protein A-promethazine system was measured from 200 nm to 350 nm. For comparison, the individual spectrum of each compounds was also registered in saline.

Haemagglutination activity of promethazine-protein A complex. Sheep red blood cells sensitized by rabbit antibody were used (as for the complement fixation test). The cells were distributed into 150 µl aliquots in U-shaped microtiter plates. Protein A (150 µl/ml) solution was added to the sensitized red blood cells (0.7-30 µg/ml). The protein A-promethazine complex was tested for haemagglutinating activity. Plates were incubated at 37 °C for 15 min and 4 °C for 16 h, and then haemagglutination was checked [12].

Results

The antibiotic resistance of the 6 strains of *S. aureus* could be eliminated at very low frequency in the presence of promethazine. The Km^r and Ap^r of *S. aureus* strain Brandy were eliminated in 0.6-4%, while Str^r of strain P. 19237 was cured in 0.8-10%. Strain Hoffmann lost the Km^r in 0.2-1.3%, and strain 4972 lost resistance to nalidix acid at 0.3-1.5% frequency. Isolate J. 63027 was susceptible to all antibiotics tested; 0-4% of the cells lost the yellow pigment production in the presence of a subinhibitory concentration of promethazine (Table I). Strains Brandy 19.238/78 and Mo Gm^r seemed to be more sensitive than the others to the action of promethazine. Nevertheless, the change in the population was rather low. There was no spontaneous loss of antibiotic resistance or pigment production except in the case of strain Mo Gm^r that lost the yellow pigment production at a ratio of 1-5/1000 colonies. The number of tested colonies of each sample varied between 250 and 1000. Besides the antibiotic resistance, the pigment production of *S. aureus* cells could be destabilized by the plasmid-curing promethazine.

Table I

Instability of antibiotic resistance of S. aureus cells growing in the presence of promethazine

S. aureus strains	Promethazine μg/ml		Plasmid elimination in %	
	Curing	MIC	Antibiotic resistance	Pigment production
Brandy (Km^r, Ap^r)	80 – 90	100 – 105	0.6 – 4.0	0.5 – 2.0
4972 (Na1^r)	80 – 90	100 – 105	0.3 – 1.5	0.1 – 3.0
P. 19237/78 (Str^r)	80 – 90	100 – 105	0.8 – 10.0	2.0 – 10.0
J. 63027	100 – 120	120 – 135	No res. markers known	0 – 4.0
Hoffmann (Km^r)	90 – 120	125 – 140	0.2 – 1.3	0.3 – 2.6
Mo 99 (Gm^r)	60 – 80	90 – 150	0.5 – 5.0	1.0 – 4.0

The bacterial strains were inoculated into MTY broth and the cultures were incubated at 37 °C for 48 h. The number of tested colonies were between 250 and 1000 in each case

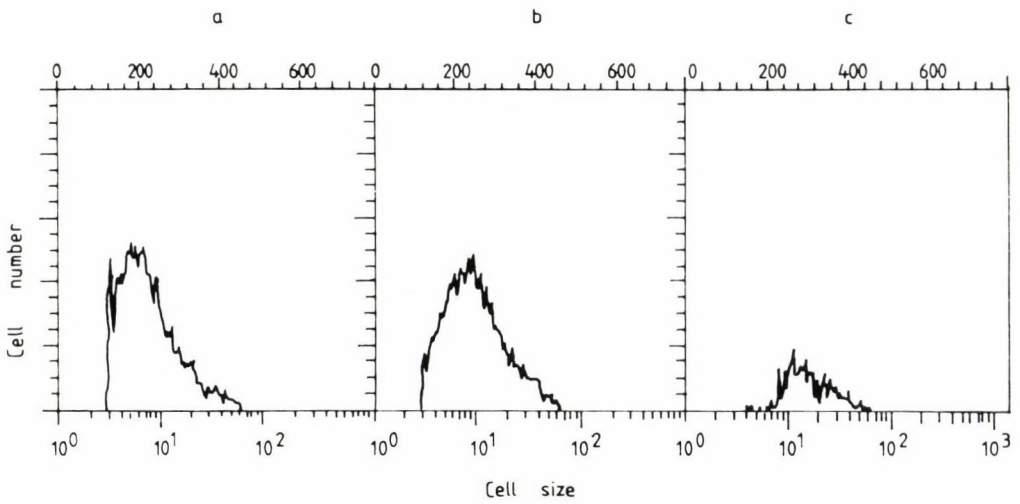

Fig. 1. The size distribution of cells in *S. aureus* 4972 culture growing in the presence of 60 μg/ml promethazine. a, control; b, sample; c, difference between control and sample

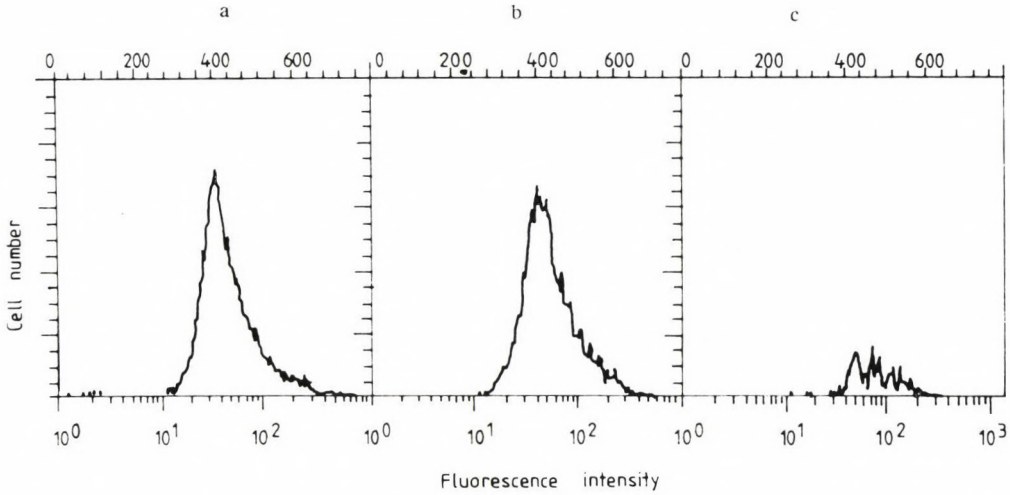

Fig. 2. The distribution of protein A intensity on the cells *S. aureus* growing in the presence of 60 μg/ml promethazine. a, control; b, sample; c, difference between control and sample

The changes caused by promethazine in the *S. aureus* population were further analyzed in a cell sorter. When *S. aureus* 4972 was cultured in the presence of 40 μg/ml promethazine, 14% of the cell population became larger than in the control sample. The frequency of larger cells in the population increased to 23% in the presence of 60 μg/ml. The increase in cell size from 0.8 to 2 μm can be seen in Fig. 1. The fluorescence intensity of the individual cells was increased insignificantly (19%), as the consequence of larger cell surface it can be considered as decreased protein A production (Fig. 2). The promethazine sample curve was shifted slightly, and was not quite overlapped by the control.

Investigation of the *S. aureus* strain Brandy revealed that 17% of the population became enlarged in the presence of 60 μg/ml promethazine; this value increased up to 25% in the presence of 80 μg/ml promethazine (Fig. 3). The protein A-dependent fluorescence of the cells decreased more efficiently than in *S. aureus* 4972 in the presence of promethazine, which means that the weakly or hardly labelled protein A-less cells could overgrowth in the population. At the same time, the number of cells with original fluorescence due to the strong protein A expression was decreased in the population. Indeed, the difference in the fluorescence of the control sample and cells grown in the presence of 80 μg/ml promethazine was found to be 68%. The number of cells with low fluorescence increased and the original population was markedly reduced (Fig. 4). When the two (the weakly and the strongly fluorescing) populations were further analyzed, the size and the surface homogeneity of cells (SSC) were found to be in the same range. There were also some non-labelled cells in the control, but the number was subtracted by the computer. This is why the difference in the two determination is not the same.

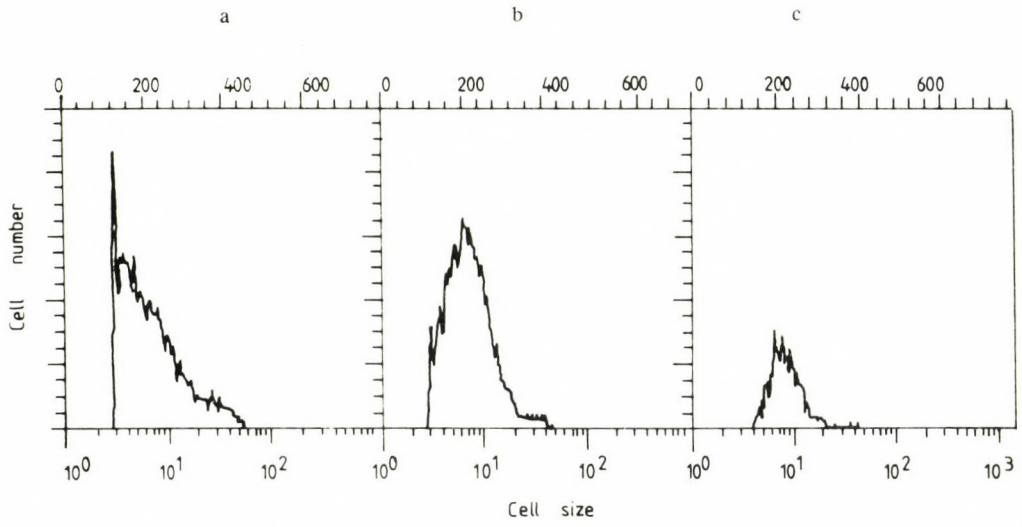

Fig. 3. The size distribution of cells in *S. aureus* Brandy culture growing in the presence of 80 μg/ml promethazine. a, control; b, sample; c, difference between control and sample

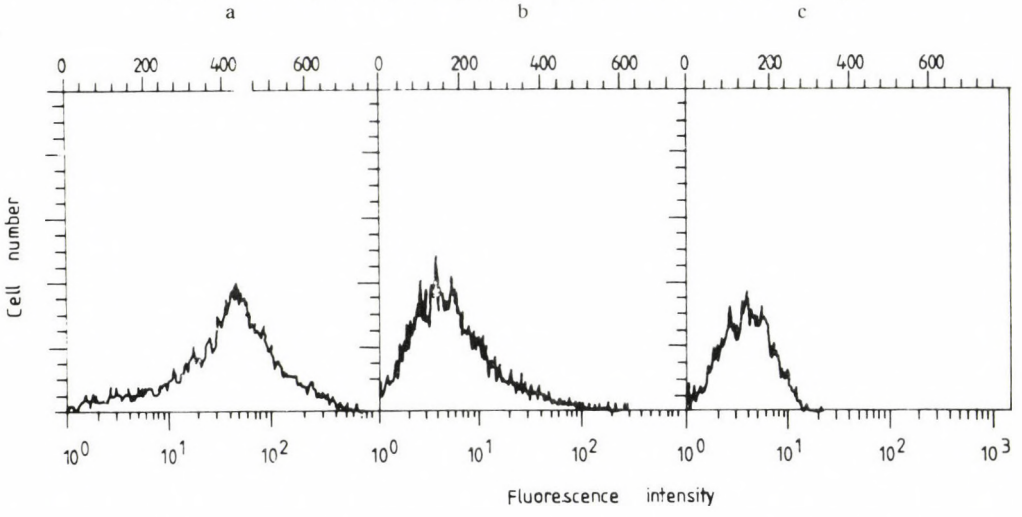

Fig. 4. The distribution of protein A intensity on *S. aureus* Brandy cells growing in the presence of 80 μg/ml promethazine. a, control; b, sample; c, difference between control and sample

The great change in the fluorescence intensity could be due to a direct interaction between promethazine and protein A. Such a direct interaction between promethazine and protein A was found on differential spectrophotometry. As Fig. 5 shows, promethazine induced a strong hypochromicity in the spectrum of protein A. In the absence of an interaction between the drug and protein A, one would expect

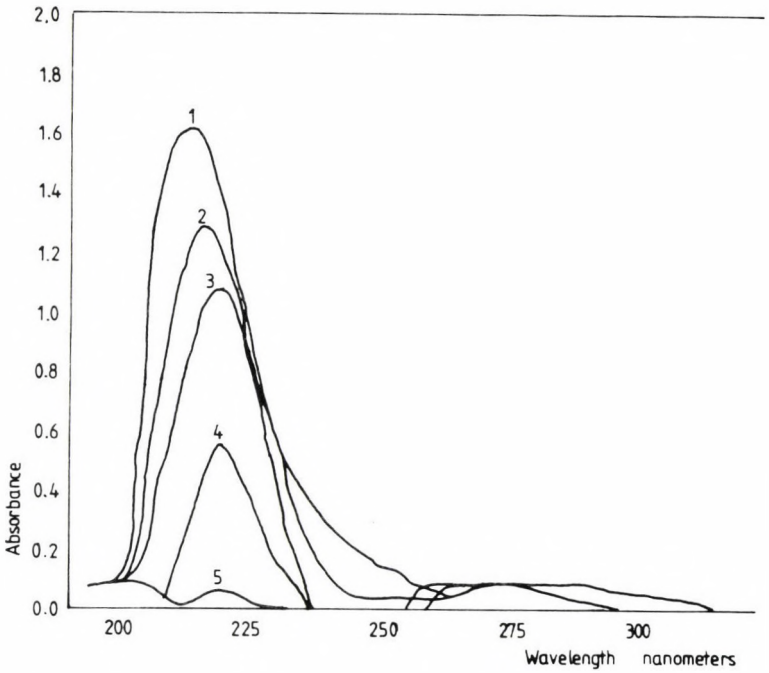

Fig. 5. Complex formation between *S. aureus* protein A and promethazine in differential spectrophotometry. In all the sample protein A was applied at 150 μg/ml and promethazine at 0, 10, 20, 50 and 100 μg/ml for samples 1, 2, 3, 4 and 5, respectively. The same concentration of promethazine was applied in the reference cuvette as in the sample

Table II

The effect of promethazine on the haemagglutinating activity of staphylococcus protein

Samples	Haemagglutination at various protein A concentrations (μg/sample ml)								
	0.0	0.7	1.0	3.0	4.5	6.0	15.0	20.0	30.0
Protein A (control)	–	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Protein A + Promethazine 3.0 μg/sample	–	–	–	–	+	+	+ +	+ +	+ + +

Promethazine 3.0 μg/sample ml and protein A 0.7–30 μg/sample ml were mixed and the complex was then added to sensitized red blood cells

In some experiments the biological activity of the protein A-promethazine complex was tested on the haemagglutination of antibody sensitized red blood cells. The haemagglutinating activity of the complex decreased, however, 3.0 μg/ml promethazine was able to prevent haemagglutionation by 0.7–3.0 μg/ml protein A (Table II). The pretreatment of sheep red blood cells with promethazine did not prevent the haemagglutination induced by protein A (data not shown). The altered structure of protein A in the promethazine complex was unable to agglutinate antibody-coated red blood cells.

Discussion

The plasmid elimination of promethazine is relatively high in some Gram negative bacteria, e.g. *Escherichia coli* [13, 14], *Yersinia enterocolitica* [15] and also effective in a few *S. aureus* strains [16]. It was surprising that in the protein A-producing *S. aureus* the curing effect on antibiotic resistance markers and pigment production was weaker than was previously described by Lantos et al. [16]. The basis of the difference can be the different sensitivity of bacteria used. The asymmetrical cell division in staphylococci in the presence of psychopharmacons [17], might affect the partition of plasmids in the daughter cells before these are separated completely during the cell division.

On the other hand, some chromosomally encoded properties of *S. aureus* cells and other bacterial species are changed considerably in the presence of promethazine, e.g. the cell size, adhesion properties [18, 19]. Similar results were obtained earlier [4, 14, 20] in the presence of other phenothiazines. The high number of enlarged cells in the population suggests that the DNA synthesis could be blocked although the protein synthesis in the cells might not be affected. Since promethazine has relatively high MIC values for the tested *S. aureus* strains as compared to previous reports for Gram negative bacteria [10], it has been assumed that the protein A on the surface of the cells can reduce the penetration or the biological activity of the phenothiazines in some way. The surface homogeneity was not changed significantly, as shown in ethidium bromide-stained cells, but the protein A expression was sharply decreased in the majority of cells cultured in the presence of promethazine. There are several possible explanations for these findings. The bacteria simultaneously lose the phenotypic expression of protein A in some way. The second explanation is a complex formation between promethazine and protein A, resulting in a decreased binding of the FITC-labelled antibody. Indeed, spectrophotometric experiments demonstrate a strong interaction between the drug and protein A. The third possibility is that the drug inhibit synthesis of protein A. The interaction observed between protein A and promethazine might support the second possibility. The biological activity of the protein A and promethazine complex

in indirect haemagglutination supports complex formation. Complex formation of phenothiazines and xanthenes [21] or structural elements of bacterial cells was shown by Molnár et al. [1, 22]. Whether this complex formation can affect the pathogenicity of *S. aureus* in vivo needs further investigation.

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THE ROLE OF RECEPTORS OF THE PLASMA MEMBRANE AND NUCLEAR ENVELOPE IN THE FAILURE OF INSULIN IMPRINTING IN STARVING *TETRAHYMENA**

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Starvation for 2 h does not disturb the insulin binding of *Tetrahymena*. At the same time imprinting does not develop at the first encounter with insulin and a down regulation is observed after 168 h. Starvation for 2 h reduces the insulin binding of the nucleus to the half value after starvation for 24 h and this change in binding becomes settled by 48 h. Imprinting is not elicited either in the nuclear membrane. The down-regulation can be observed also after 48 h but it is not present in the 168-h measurements. The experiments emphasize the increased sensitivity of binding sites of the nuclear envelope and the role of it in the development of imprinting.

The hormonal imprinting develops in higher classes of animals at the perinatal period while in *Protozoa* at the first encounter with the hormone [1]. In higher classes the developing receptor and in *Protozoa* the not absolutely specific structures of recognition (binding sites) mature and win the final binding capacity [2]. By this time the cell receives its complete responsiveness [1, 3]. In unicellular cells the result of the hormonal imprinting is the receptor memory which embodied in a lasting alteration of the binding and response characteristics of the cell [4]. This change is transferred to several hundreds of generations of the daughter cells [3]. This has significance in the survival of the unicellular species as it makes easy to recognize the beneficial and dangerous molecules by the imprinted cells and their offspring [3].

In the development of hormonal imprinting the plasma membrane has a definitive role and its integrity is needed for the development of imprinting. In the last period some experiments demonstrated that the nuclear envelope contains binding sites similar to the plasma membrane [5, 6], like in mammals [7–13]. In the

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presence of hormones these binding sites are also imprintable (become stronger) like the binding sites of the plasma membrane. Simultaneously this will lead to the enhanced specificity of the receptor, in both cases.

At complete starving (in a physiological solution) the unicellular cell utilizes its own materials [14] and the enzyme substances produced by the mucocysts are released to the surface at the same time [15, 16]. Imprinting will not develop in *Tetrahymena* following 2 h of starvation probably because of the proteins of the membrane are utilized and digested. In our present experiments our goal is to study the role of the plasma membrane and nuclear envelope in the failure of imprinting and differences or similarities in the receptor's behaviour.

Materials and methods

Populations of Tetrahymena pyriformis GL cells, cultured in 0.1% yeast extract and 1% Tryptone containing medium (Difco, Michigan, USA) for 24 h at 28 °C were used in the logarithmic phase of growth. The maximal cell density of the suspensions was 2×10^5 cells/ml.

Experimental groups. (i) Untreated culture of *Tetrahymena* cells (C). (ii) One-day-old culture of *Tetrahymena* cells was transferred into 10 or 50 ml of Losina-Losinsky solution (0.001% KCl, $MgCl_2$, 0.01% NaCl, 0.02% $NaCO_3$) and incubated for 3 h without shaking at room temperature. Then the cells were replaced and maintained in culture medium for 24, 48 and 168 h (C–L). (iii) One-day-old culture of *Tetrahymena* cells was transferred into Losina-Losinsky solution. After 2 h the cells were treated with 10^{-6} M insulin (insulin, Semilente, MC, Novo, Copenhagen, Denmark) for 1 h. Following the treatment the cells were transferred and maintained in culture medium for 24, 48 and 168 h (I–L).

The FITC–insulin binding of the whole cell was measured parallel with the isolated nucleus in all groups of the experiment.

Isolation of the cellular nuclei. The cultures were cooled in ice bath for 10 min, centrifuged at 3 000 *g* for 5 min to remove the nutrient medium, washed in 10 mM NaCl solution, resuspended in 10 volumes of homogenizing buffer solution (0.04% deoxycholate; 0.04% NP 40; 6 mM $CaCl_2$; 37.5 m*M* NaCl; 1.5 m*M* Na-phosphate; 210 μg/ml spermidine tetrahydrochloride; pH 7.2 [17]) and returned to the ice bath for 10 min. By this procedure 80–90% of the cells could be disrupted without nuclear injury. The contents of the lysate were checked by light microscopic examination. The lysate was then rotated five times in a manual Potter homogenizer to disrupt the remaining cells for release of intact nuclei into the solution. The nuclei were washed in 5–15 volumes of raffinose-$CaCl_2$ solution (6 m*M* $CaCl_2$ + 0.28 *M* raffinose) and centrifuged at 3 000 *g* for 5 min. The sediment was resuspended in 1.7 *M* raffinose solution and was mounted on a sugar cushion (2.14 *M* sucrose) by centrifugation at 9 500 *g* for 30 min, using a VAC 602 ultracentrifuge. Thus the sediment yielded intact nuclei free from adherent details of cytoplasm. These were resuspended in a so-called "nuclear washing buffer solution" (0.25 M sucrose; 10 m*M* $MgCl_2$; 10 m*M* Na-acetate; pH 7.2) which was further on used as solvent in nuclear studies. All steps of the nuclear isolation procedure were performed at 0 °C.

Preparation for binding studies on isolated nuclei. The isolated nuclei were incubated in the presence of tenfold serial dilutions of insulin (range: 10^{-9}–10^{-4}) for 1 h at room temperature, fixed in 10% formalin solution (in nuclear washing buffer solution), centrifuged at 5 000 *g*, washed again, incubated in presence of FITC-insulin (FITC: protein ratio 0.30; protein concentration 0.21 mg/ml) for 30 min at room temperature for the purpose of labelling, washed twice in nuclear washing buffer, spread

on slides and dried. Similarly prepared nuclei from cells not pretreated with insulin (0 insulin concentration) served as control.

Preparation for binding studies on whole cells. Tetrahymena cells collected by centrifugation and reconstituted in 200 μl nutrient medium were pretreated with $10^{-9}-10^{-4}$ dilutions of insulin for 1 h at room temperature, as above. Cells not treated with insulin (0 insulin concentration) were used as control. After 1 h the experimental and control cells were fixed in 4% formalin solution (in PBS), centrifuged at 5 000 *g*, washed in PBS, labelled with FITC–insulin as described above, washed in two changes of PBS and once in distilled water, spread on slides and dried.

Assay of FITC–insulin binding. Cells were fixed in 4% formalin solution (in PBS); centrifuged at 2 000 *g*, incubated in the presence of 200 ml FITC–insulin (FITC–protein ratio 0.3; protein concentration 0.24 mg/ml) for 1 h. They were washed twice with PBS, then with distilled water and spread on slides and dried (the nuclei were treated on the same way, with a consideration of the applied special buffers).

The intensity of fluorescence was determined by Zeiss Fluoval cytofluorimeter. The signals emitted by the latter were registered and transformed to digital ones by a Hewlett Packard HP41CX calculator. Intensity of fluorescence was measured of 20 cells or nuclei in each group. The experiments were done in five replicas, which means that one bar of the figures represents the average of 100 measurements. The percent value is related to the untreated cells or nuclei. Statistical analysis of data was based on Student's *t* test and analysis of variance, performed by means of an IBM AT computer using the Harvard program.

Results and discussion

From the results of the experiments (Fig. 1) it is clear that starving for 2 h does not alter the binding capacity of plasma membrane of *Tetrahymena.* On the other side the insulin treatment of the starving *Tetrahymena* cannot develop imprinting according the binding capacity of the cell in the 24-, 48-, or 168-h samples. The binding capacity is not higher but significantly decreased compared to the control cells. This means that the development of imprinting requires the presence of glycoprotein structures associated with the plasma membrane. These results support our previous observations [14]. At the same time they direct our attention to the fact that in the plasma membrane afflicted by starving the down-regulation lasts longer than in the control cell. In control cells it is less than 24 h while in the present case, following 48 h, we registered low values identical to those found at 24 h, and after 168 h there is also a significant difference compared to the control.

The study of the nuclear envelope (Fig. 2) explicitly represents that 2 h starvation is enough for a 50% decreased of binding capacity in the nuclear envelope. However, the values measured after 48 and 168 h do not differ significantly from the control. After 24 h the insulin binding of the nuclear envelope of insulin pretreated *Tetrahymena* cells is similar to the only starving, untreated ones and after 48 h it remains on the same level. This points that there is no possibility to develop imprinting under circumstances of starvation. On the other hand it shows that the down-regulation induced by insulin is more durable as well in the nuclear envelope as in the plasma membrane. The values of 168 h are not significantly different from the control.

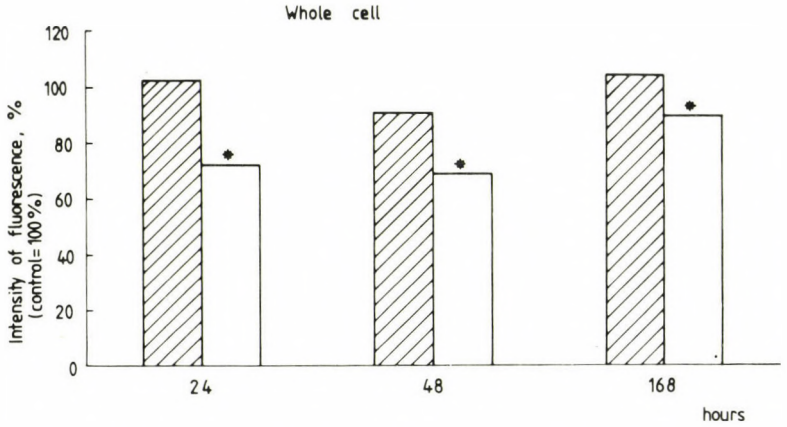

Fig. 1. Insulin binding by the plasma membrane of cells starved and treated (spotted columns) or not treated (shaded columns) with insulin related to the normally fed *Tetrahymena* (control = 100%); * $p < 0.005$

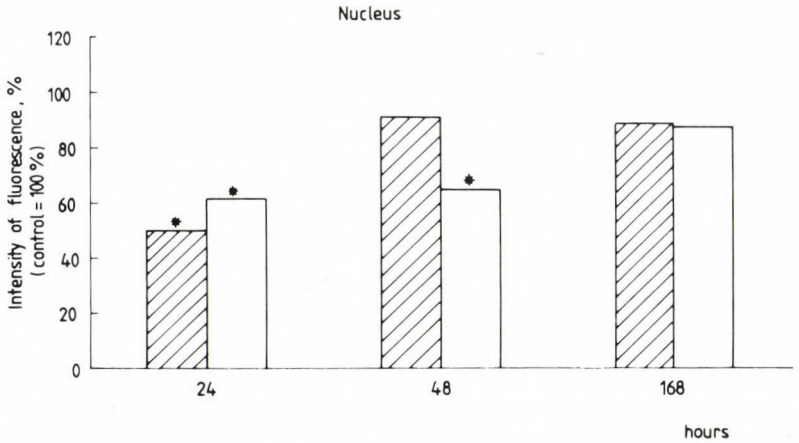

Fig. 2. Insulin binding by the nuclear envelope of cells starved and treated (spotted columns) or not treated (shaded columns) with insulin related to the normally fed *Tetrahymena* (control = 100%); * $p < 0.005$

From the results it is obvious that, (i) the starvation itself (up to 2 h) cannot disturb the binding of insulin to the plasma membrane, but inhibit the imprinting, (ii) the result of the insulin treatment is a durable down-regulation in the plasma membrane which is more or less present in 168 h after treatment, (iii) the nuclear envelope is much more sensitive to the starvation than the plasma membrane; its binding capacity reduced in the half even passing 24 h; (iv) insulin cannot develop

imprinting in starving cells; (v) the down-regulation evoked by insulin is prolonged also in the nuclear envelope.

In accordance with the results, the 2h starvation cannot change the binding capacity of the plasma membrane but it can be altered in nuclear level in the offspring generations, without provoking imprinting. This way it is possible that the imprinting of the nucleus is required for the development of imprinting in the plasma membrane. This is more conceivable as we know the relations of the nuclear envelope and the intracytoplasmic membrane pool resulting in a continuous exchange between this pool and the plasma membrane in *Tetrahymena* [18–21]. It is possible that the receptorial restitution of the nuclear envelope is followed by those of the plasma membrane, demonstrated by the receptorially restituted nuclear envelope and the lack of this in the plasma membrane following 168 h.

As the receptors of the nuclear envelope are present just as in mammalian and in the unicellular *Tetrahymena* the experiments call attention to the study of nuclear receptor function also in higher organisms.

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THE ROLE OF *PROPIONIBACTERIUM PROPIONICUS* IN CHRONIC CANALICULITIS*

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Delicate branching filaments, and cocci or rods, were seen by microscopic examination in the pus of the canaliculus lacrimalis in patients with chronic canaliculitis. *Propionibacterium propionicus* and different bacteria were cultivated and identified in the pus. After i.p. inoculation of mice with the *Propionibacterium* strains, the morphological changes characteristic of this microorganism were observed in the peritoneum, in the spleen and in the lung, giving an adequate explanation of the direct microscopic findings in the pus.

Inflammation of canaliculus lacrimalis may be caused by pyogenic cocci or rods, actinomycetes, blastomycetes or molds [1]. In samples obtained from the canaliculus by pressing out with a syringe, purulent granular discharge can be seen even macroscopically. The microscopic picture of the preparations shows a network of filaments, together with various bacterial forms. Sometimes, the granules grow to form calculi completely obstructing the canaliculus.

To treat patients successfully and prevent common relapses, it seemed necessary to perform a detailed microbiological analysis of the specimens.

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Materials and methods

Specimens from affected canaliculi of four patients suffering from chronic canaliculitis were examined.

Staining. Gram, Giemsa, methylene blue.

Cultivation was performed on nutrient agar containing 5% bovine blood with incubation under aerobic and anaerobic conditions in an atmosphere containing 10% CO_2; on Sabouraud agar, nutrient broth and Brewer's medium.

CFLP white mice were inoculated i.p. with 0.5 ml saline suspension of a strain (10^7 cells/ml) harvested from 48 h blood agar plates.

Macroscopic detection and re-isolation of *P. propionicus* was attempted from the peritoneum, the spleen and the lung, from overnarcotized animals 24, 48, and 96 h and 7 days postinoculation.

To determine antibiotic susceptibility, blood agar was inoculated with fresh saline of the strain. Resistest discs were used as prescribed by the manufacturer (Institute of Serobacterial Production and Research Human, Gödöllő, Hungary). Sensitivity to the following antibiotics was determined: penicillin, ampicillin, carbenicillin, methicillin, oxacillin, azlocillin, mezlocillin, cephotaxim, cephuroxim, cephalexin, cephtriaxon, cephoperazon, cephoxitin, cephamandole, vancomycin, lincomycin, erythromycin, oleandomycin, paromomycin, clindamycin, spiramycin, gentamycin, neomycin, kanamycin, streptomycin, tobramycin, amikacin, tetracycline, chlortetracycline, oxytetracycline, chloramphenicol, colistin, polymyxin B, ofloxacin, oxolinic acid, nalidixic acid, nystatin and co-trimoxazole.

Results

In the microscopic smears prepared from the specimens a network of Gram-positive branching non-sporeforming filaments, together with various forms of bacteria, was always seen (Fig. 1).

On blood agar plates incubated under aerobic conditions yellowish, dry colonies of granular surface appeared 48 h after inoculation; these colonies grew neither under anaerobic conditions nor in increased CO_2 atmosphere.

The isolates formed a membrane on the surface of the browth medium, from which filaments were growing out in stalactite form. After 48 h, a floccular sediment was found in the bottom of the tube; the medium itself was clear.

Microscopically, rods swollen at the ends were seen, which reminded us of diphteroids having neither spores nor capsule (Fig. 2).

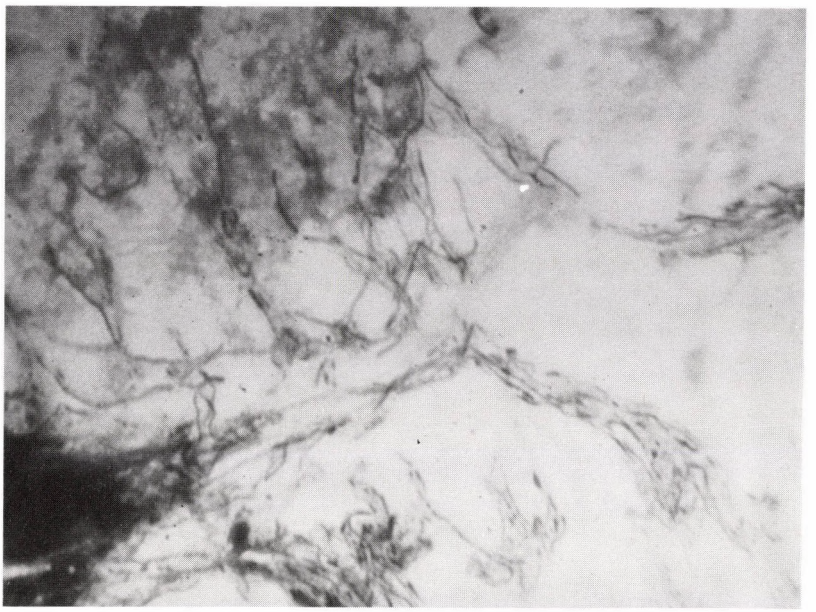

Fig. 1. Canalicular specimen from one of the patients. Gram stain. × 900

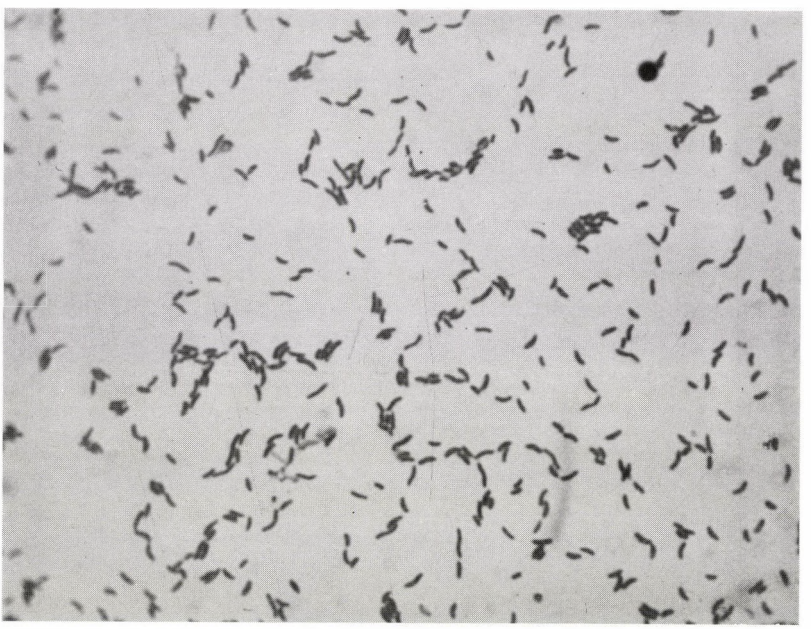

Fig. 2. Aerobic culture from blood agar. Gram stain. × 900

Table I

Characteristics of 4 Propionibacterium propionicus isolates

Catalase	–
Oxidase	–
DNase	–
Haemolysis	–
Nitrate reduction	+
H_2S	–
Gelatin	–
Indole	–
Urease	–
Pyrazinamidase	–
Pyrrolidonyl arylamidase	–
Alkaline phosphatase	–
Beta-glucuronidase	–
Beta-galactosidase	+
Alpha-glucosidase	+
N-acetyl-beta-glucosaminidase	–
Glucose	+
Ribose	+
Xylose	–
Mannitol	–
Maltose	–
Lactose	+
Sucrose	+
Glycogen	–
Esculin	–

The biochemical characteristics of *P. propionicus* isolates are similar to those of *Arachnia* [2, 3] (Table I).

In mice infected i.p., *P. propionicus* formed a network arranged in filaments and nodes on the peritoneum, in the spleen and in the lungs 24 to 48 h postinoculation. We succeeded in re-isolating the strain in pure subcultures. Figure 3 shows filaments that had been formed by the 24th h. In mice killed 4 to 6 days postinoculation, yellowish nodules and abscesses consisting of Gram-positive filaments developed on the peritoneum (Fig. 4).

The antibiogram of *P. propionicus* is characterized by high-grade sensitivity to penicillins, cephalosporins and the members of the tetracycline group. A moderate sensitivity was seen around the aminoglycoside discs. The strain was resistant to polymyxin B, colistin, nalidixic acid, oxolic acid, ofloxacin and nistatin.

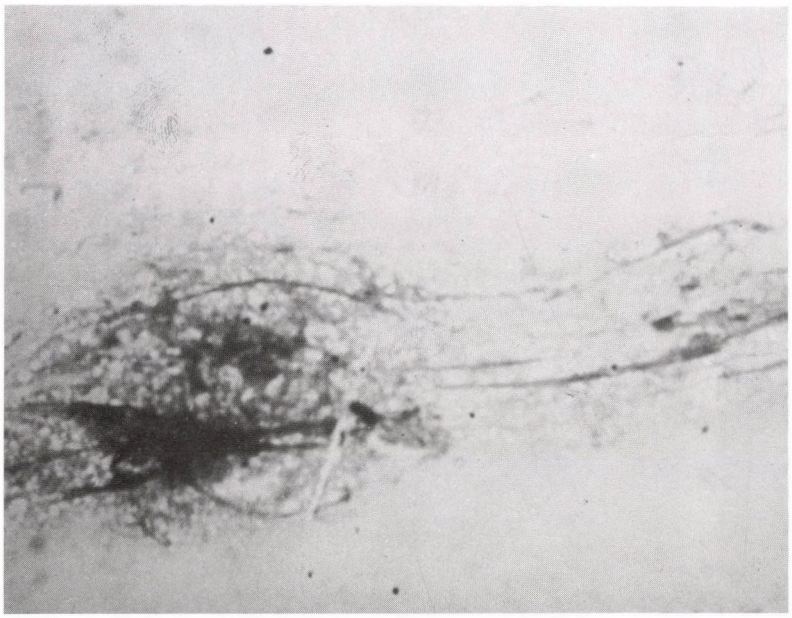

Fig. 3. Smear from the peritoneum, one day after i.p. inoculation of the mouse. Gram stain. × 900

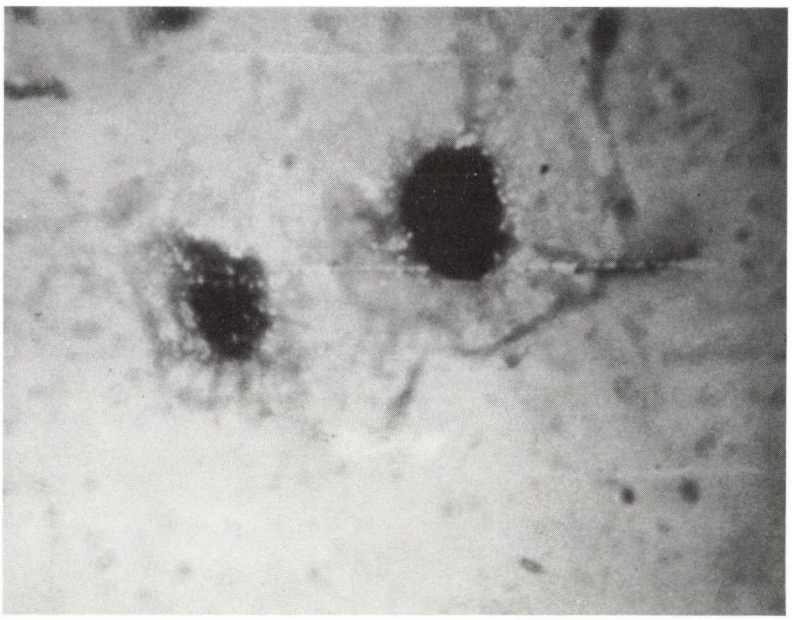

Fig. 4. Smear from the peritoneum, four days after i.p. inoculation of the mouse. Gram stain. × 900

Specimens from each patient contained one bacterial strain other than *P. propionicus*. From the four patients four different bacterial species were isolated, such as *Staphylococcus warneri, Actinobacillus lignieresii, Staphylococcus epidermidis* and *Micrococcus luteus*.

Discussion

Gram-positive short diphtheroids which may or may not have branched filaments were named *Arachnia propionica* according to their morphological and biochemical characteristics [2, 3]. This microorganism belongs to the normal microbial flora of the human oral cavity. It has not been isolated yet from other animals, though, experimental animals can be infected with it [2, 3].

In *Actinomyces* and *Propionibacterium* species the isolation of 16S rRNA and sequence determination by reverse transcriptase were performed and described by Charfreitag et al. [4]. On the bases of this analysis *A. propionica* was reclassified in the genus *Propionibacterium* as *P. propionicus* in 1991 [5].

Pine and Hardin [6], Brock et al. [7], Jones [8], Seal et al. [9], and Schaal et al. [10] proved the presence of *Arachnia* by cultivation or by immunofluorescence in purulent discharge of the ductus lacrimalis or in characteristic calculi. Brock and Georg [11] and Schaal and Pulverer [12] reported on *A. propionica* infections extending to the cervico-facial region; these showed a clinical picture similar to antinomycosis. Furthermore, we have found reports on lung, kidney, brain and bone processes caused by *A. propionica* [11, 13, 14].

In pus specimens of patients with relapsing canaculitis we met the characteristic filamentous structure of *P. propionicus* consistently, whereas the concomitant flora was variable. For this reason, we think that the endogenous *P. propionicus* infection coming from the oral cavity accounts for the process. In the human tissues the filamentous form of the *P. propionicus* develops similarly to the process in the infected mouse. The filaments grow into the canaliculus lacrimalis, then the canaliculus may be obstructed by the calculus having developed in this manner. Consequent stasis and secondary infection may develop. The epithelium of the canaliculus also can be infected by the filament and this kind of infection may cause relapses.

The treatment applied up to now, which consisted of cleaning and rinsing of the canaliculus, failed to prevent relapses. Knowing the basic etiological role of *P. propionicus* strains, microbiological examination seems to be of great importance. A successful therapy should consist of systematic use of antibiotics strongly effective on *P. propionicus* and reaching high levels in tissues.

In our four cases not only the canaliculitis healed but, during a 9-month period following a one-week oral treatment with doxycycline no relapses occurred.

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GENITAL HUMAN PAPILLOMAVIRUS (HPV) INFECTION IN HUNGARIAN WOMEN*

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The prevalence of genital human papillomavirus (HPV) infection in Hungarian female populations is not essentially different from that found in other countries of Europe and North-America. Using filter in situ hybridization (FISH), we found that, in a group of cytologically normal women some low risk HPV types (such as HPV 6 and 11) and the most important high risk HPV types (HPV 16 and 18) were present in 23% and 8%, respectively. Eighty-eight percent of condyloma acuminatum patients harboured HPV 6 or HPV 11 in their tumours. On the other hand, in precancerous lesions (cervical intraepithelial neoplasia, CIN) HPV 16 was the predominant type, being present in 29–48% of patients, depending on the detection method used (Southern blot hybridization vs. polymerase chain reaction). The detection rate of high risk HPV types was found to rise with the increasing severity of cervical neoplasia. Finally, 48% of invasive cervical carcinoma specimens were positive for HPV 16 DNA in a type-specific polymerase chain reaction. For patients with HPV 16 positive primary tumours, all but one lymph mode metastases and about 30% of histologically normal lymph nodes proved positive for HPV 16 DNA. Our results – in accordance with the numerous data found in literature – seem to confirm the hypothesis that certain HPV types are greatly involved in the development of cervical cancer.

More than 60 distinct types of human papilloma-virus (HPV) have been described so far, of which 27 are able to infect the genital mucosa, probably through sexual transmission [1]. "Low risk" genital types (such as HPV 6 and 11) may cause benign genital tumours (for example condyloma acuminatum). On the other hand, "high risk" HPV types (the most important of which are 16 and 18) are strongly associated with high grade cervical intraepithelial neoplasia (CIN) and invasive cervical carcinoma [2, 3]. Multiple types of HPV with varying oncogenic potential were found in low grade CIN [4] and in latent infections, where no morphological abnormality is present but HPV DNA is detectable [5].

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Genital HPV infection and HPV-associated neoplasia can be detected using cytology, colposcopy and histology. Detection of HPV DNA seems to be the most reliable way of diagnosing subclinical disease, while for latent infections it is the only way. The presence of HPV DNA can be demonstrated using various hybridization methods (Southern blot, dot blot, in situ and filter in situ hybridization) or the recently introduced polymerase chain reaction (PCR) [6].

The purpose of our investigations was to estimate the prevalence of the most important low risk and high risk genital HPV types in Hungary (i) in cytologically normal cervical specimens; (ii) in certain benign tumours (condylomata acuminata); (iii) in precancerous lesions (CIN) and (iv) in invasive cervical carcinomas and their metastases.

Patients and methods

Study patients and sample collection. Our study group (a total of 340 women) consisted of the following patients: (i) 172 women with normal cervical cytology; (ii) 26 women with condyloma acuminatum on the cervix, vagina or vulva; (iii) 67 cervical intraepithelial neoplasia (CIN) patients; (iv) 75 patients with invasive cancer of the uterine cervix.

Exfoliated cells from the cervix were taken from the cytologically normal women using a cytobrush, and were filtered onto two replicate nitrocellulose membranes (0.45 μm pore size, 25 mm diameter, Schleicher and Schüll, FRG). The filters were dried at room temperature; denaturation, neutralization, baking and filter in situ hybridization (FISH) took place according to Schneider et al. [7].

Tissue samples from condylomas, precancerous lesions and invasive carcinomas were collected by excisional biopsy or during radical surgery. DNA was prepared from biopsies by proteinase K digestion, phenol-chloroform-isoamilalcohol (25:24:1) extraction and ethanol precipitation. Purified DNA was subjected to either Southern blot hybridization or to a PCR specific for HPV type 16 DNA.

HPV-DNA probes. HPV 6, 11, 16 and 18 DNA sequences, each cloned into pBR 322 were provided by Dr. H. zur Hausen (Cancer Research Center, Heidelberg, FRG). Plasmid DNA was transfected into *Escherichia coli* K12 HB101 and amplified as described earlier [8]. After digesting with the appropriate restriction enzymes, viral sequences were separated from vector DNA by agarose gel electrophoresis and recovered by electroelution.

Purified DNA probes were labelled by nick translation or random priming using (α-^{32}P) dATP to a specific activity of $\sim 1 \times 10^8$ dpm/μg.

Filter in situ hybridization (FISH). Pretreated filters were cut in half and four half-filters containing cells from the same patient were hybridized with radioactively labelled HPV 6, 11, 16 and 18 DNA probes. Prehybridization was carried out at 42 °C for 3 h (50% formamide, 2 × SSC, 5 × Denhardt's solution, 100 μg/ml sonicated, denatured calf thymus DNA). Hybridization took place at 42 °C for 24 h, in a solution containing one of the four radioactively labelled HPV DNA probes (50% formamide, 5 × SSC, 80 m*M* sodium phosphate buffer pH 6.2, 100 μg/ml sonicated, denatured calf thymus DNA, 1 × Denhardt's solution and 0.1% SDS; 0.1 ml solution/cm^2 of filter). After high stringency washing (10 min in 2 × SSC at room temperature; 1 h in 2 × SSC at 65 °C; finally 1 h in 0.1 × SSC at 65 °C) the filters were autoradiographed at −70 °C for 7 days. Hybridization signals were compared to that obtained using positive and negative control filters (containing cloned HPV DNA and human fibroblast cells, respectively).

Southern blot hybridization. About 5 μg of cellular DNA was digested with selected restriction enzymes (BamHI, EcoRI, PstI) and separated by gel electrophoresis in 1.2% agarose gel. After denaturation and neutralization, the DNA was transferred and fixed to a nylon membrane (Hybond N, Amersham). Hybridization and washing were carried out under high stringency conditions as described for FISH.

Polymerase chain reaction (PCR). About 1 μg of sample DNA was mixed with PCR buffer (16.6 m*M* $(NH_4)_2SO_4$, 67 m*M* Tris–HCl pH 8.8 at 25 °C, 6.7 m*M* $MgCl_2$, 10 mM β-mercaptoethanol), dNTP-s at a final concentration of 50 μM of each, 0.08 μ*M* of each of the oligonucleotide primers and 2 units of Taq DNA polymerase (Perkin-Elmer Cetus) to a 50 μl reaction volume. Before applying the HPV specific oligomers, the presence of human DNA in the samples was checked with an internal reaction control using a β-globin primer pair (PCO_3/PCO_4) [6]. In the HPV specific PCR reaction each sample was subjected to 2 oligonucleotide primer pairs (16ME21/16ME49; 16ME60/16ME78) [9]. The following temperature cycling scheme was applied in 35 cycles: melting at 95 °C for 30 s; annealing primers at 55 °C, for 30 s; elongation at 70 °C for 30 s in a thermal cycler (TECHNE, Duxford, Cambridge, UK). Positive (cloned HPV) and negative (without DNA) controls were amplified along with the gynecological specimens throughout the amplification reactions.

In analysing amplified DNAs, characteristic sizes of the PCR products were evaluated by electrophoresis of 20 μl aliquots from each reaction mixture in ethidium bromide stained 1.2% SeaKem ME (MC Bio-Products, Rockland, Maine, USA) agarose gel. Bands were compared to the migration of a molecular weight standard $\phi \times 174$/HaeIII. All the amplified products were subjected to Southern blot hybridization as well. Blots (Hybond N nylon membrane; Amersham) were prehybridized for 1 h, then 2×10^6 dpm/ml ^{32}P labelled entire genome of HPV 16 (Multiprime DNA labelling technique Amersham, UK) was added into the same mixture for overnight at 42 °C (50% formamide, 5× SSC, 5× Denhardt's solution, 1 m*M* EDTA, 0.1% SDS, 100 μg/ml salmon sperm DNA). After washing (high stringency) the filters were autoradiographed for 48 h at −70 °C.

Results

Cervical scrapes from a total of 172 cytologically normal women (belonging to two different study groups) were examined for HPV 6, 11, 16 and 18 DNA using filter in situ hybridization (FISH). Combining the results of the two groups (Table I) we found an HPV positivity of 30% (52/172) for the HPV types tested. The detection rate of the low risk (HPV 6/11) and the high risk (HPV 16/18) types was 22% (38/172) and 8% (13/172), respectively.

Samples were analyzed by Southern blot hybridization from 26 women with condyloma acuminatum on the cervix, vagina or vulva (Table II). Twenty-five of the samples (96%) proved to be HPV-positive. Low risk HPV types (HPV 6 and 11) were found to dominate in this histological manifestation of HPV infection: we detected either of them in 23 samples (88%).

Table I

Detection of different human papillomavirus types by FISH in cytologically normal cervical specimens

No. of cases	Cases positive for HPV				Multiple positives	HPV positives
	6	11	16	18		
108 (women wearing an intrauterine device, average age: 34.5 ys)	11	9	4	1	3[a]	22
65 (colposcopically normal women not wearing IUD, average age: 38.4 ys)	8	19	8	3	7[b]	27
Total: 172	19	28	12	4	10	49

[a] Three cases were positive for both HPV 6 and HPV 11

[b] Four cases were positive for HPV 6 and 11, 1 case for HPV 16 and 18 and 2 cases for HPV 6, 11, 16 and 18

Table II

HPV types detected in condylomas by Southern blot hybridization

No. of cases	Cases positive for HPV				Multiple positives	HPV positives
	6	11	16	18		
26	9	16	2	0	2[a]	25

[a] Two cases were positive for both HPV 6 and HPV 11

DNA samples from patients with cervical intraepithelial neoplasia (CIN) were analyzed by either Southern blot hybridization or PCR. We examined 38 CIN biopsies by Southern blot hybridization and found 21 (55%) to be positive for the HPV types tested (Table III). Among the HPV positive cases, the proportion of high risk types (HPV 16/18) seemed to rise with the increasing severity of CIN: it was 1/6 in CIN I, and 13/31 in CIN II–III ($p < 0.01$).

Table III

HPV types detected in CIN lesions by Southern blot hybridization

Histology	No. of cases	Cases positive for HPV				HPV positives
		6	11	16	18	
CIN I	7	2	3	1	0	6
CIN II	14	1	0	4	1	6
CIN III	17	0	1	6	2	9
Total	38	3	4	11	3	21

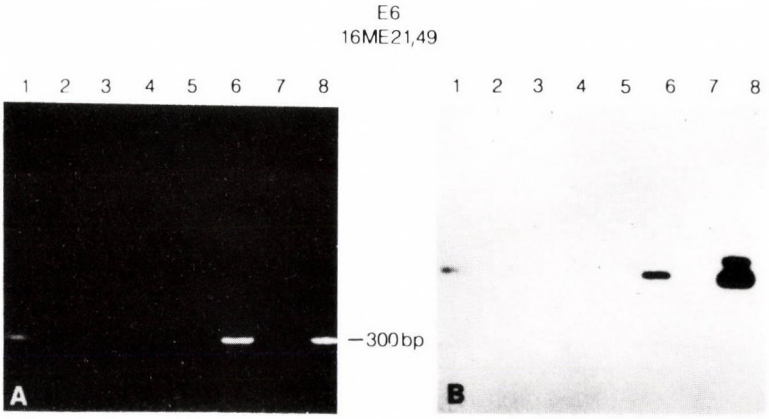

Fig. 1. A. Agarose gel analysis of human papillomavirus (HPV) 16-specific PCR amplification of DNA from CIN lesions: *lane 1* and *6* are HPV 16-positive samples; *lanes 2, 4, 5* and *7* are HPV 16-negative samples; *lane 3* is a PCR reaction mixture without DNA; *lane 8* is cloned HPV 16. B. Result of the Southern blot hybridization performed on the same amplified samples after gel electrophoresis. The entire genome of HPV 16 labelled with (α-^{32}P) dCTP was used as a probe

Biopsy samples from an other group of CIN patients were analyzed using a PCR specific for E6 and E7 ORFs of HPV 16 DNA. We targeted the primers of the PCR to these ORFs because they are essential for HPV induced transformation and are usually retained in cancer cells, while other regions of the viral genome may be lost during integration to the host cell DNA [9, 10]. One of the primer pairs used in the PCR (16ME21/16ME49) generates a 300 bp product from E6 ORF. The other primer pair (16ME60/16ME78) amplifies a 200 bp fragment from E7 ORF. An example of electrophoretic separation of amplified PCR products and corresponding patterns of subsequent Southern blot hybridization are illustrated in Fig. 1. The results obtained for 29 CIN patients using this method are summarized in Table IV.

Fourteen of the 29 CIN biopsies (48%) contained HPV 16 DNA. The results obtained with the two independent primer sets were in agreement. Southern blot hybridization performed on the products of the E7 specific PCR confirmed the presence of specific amplified DNA in all but 2 cases.

Table IV

Detection of transforming gene regions (E6 and E7) of HPV 16 DNA in CIN lesions by PCR and subsequent Southern blot hybridization

Histology	No. of cases	Amplified DNA detected by	
		Electrophoresis	Southern blot
CIN I	9	3	2
CIN II	10	5	4
CIN III	10	6	6
Total	29	14	12

Table V

Presence of HPV 16 DNA in invasive cervical carcinomas as detected by a PCR specific for E6 and E7 ORFs

Stage of cancer (FIGO)*	No. of cases	Amplified DNA detected by	
		Electrophoresis	Southern blot
I/B	34	16	16
II/A	19	8	8
II/B	22	12	12
Total	75	36	36

* FIGO: International Federation of Gynecology and Obstetrics

Using the same PCR based DNA detection system, we analyzed cervical biopsy samples from 75 patients with invasive cervical carcinoma who required radical surgery (Table V). Reactions specific for the E6 and E7 ORFs gave concordant results in all cases. Southern blot hybridization subsequent to E7 specific PCR confirmed the results obtained with the observation of ethidium bromide-stained agarose gels. Altogether 36 of the 75 cervical specimens (48%) contained HPV 16 DNA sequences. We also analyzed 46 pelvic lymph nodes from 16 patients with HPV 16 positive primary cervical tumours. Seven of the 16 patients had at least one pelvic

lymph node containing HPV 16 DNA. Nine out of ten metastasizing lymph nodes (originating from three women) were HPV 16 positive. It was interesting that 11 of 36 histologically negative lymph nodes (coming from four patients) also carried HPV 16 DNA which may reflect the presence of a few metastasizing cells undetected by histological examination. On the other hand, all the lymph node specimens from 19 patients with HPV 16 negative primary cervical tumours lacked HPV 16 DNA, which indicates the specificity of the detection method used.

Discussion

Our data obtained on the prevalence of representative genital HPV types in different clinical manifestations seem to be in agreement with the results of others [11–15]. We found the low risk HPV types (HPV 6 and 11) to dominate in benign genital tumours (condylomata acuminata), while the high risk HPV types (HPV 16 and 18) were characteristic of high grade CIN and invasive cervical cancer. On the other hand, multiple types of HPV (both low and high risk types) were found in low grade CIN and in latent infections.

The most important evidence for a role of high risk HPV types in the induction of cervical cancer, namely that they are more prevalent in cervical neoplasia than in normal cervices was confirmed by our results. The high risk HPV types examined in this study by hybridization methods (HPV 16 and 18) were present in 8% (13/172) of cytologically normal cervical samples, while their prevalence was 42% (13/31) in high grade CIN ($p < 0.001$). Using a more sensitive PCR-based method, the prevalence of the high risk prototype HPV 16 in cytologically normal cervices [16], in high grade CIN and in invasive cervical carcinoma was 9% (9/102), 55% (11/20) and 48% (36/75), respectively.

The lack of a significant difference in HPV positivity between high grade CIN and invasive cancer is in good agreement with the widely accepted model for the role of HPV in cervical carcinogenesis [17]. According to this model of multistep carcinogenesis, high risk HPVs may directly cause latent infections and low grade dysplasias, while additional factors (hormones, mutagens, infection with other pathogens) are required for the lesions to progress to malignancy. The fact that high risk HPV types are present in a significant proportion of cytologically normal women, yet only a minority of these women eventually develop invasive cancer also calls for the importance of these potential co-factors.

A further strong, though indirect evidence for the role of high risk HPVs in the induction and maintenance of cervical cancer is that we managed to demonstrate HPV 16 DNA in most lymph nodes containing metastatic cells from HPV 16-positive primary tumours. Thirty percent of histologically negative lymph nodes also carried HPV 16 DNA which may reflect the presence of a few metastasizing cells or of

scavenger leukocytes containing phagocytosed cellular material from the primary tumour. In order to clearly distinguish true metastatic cells from cellular debris of the primary tumour we are performing an analysis of HPV transcripts on primary cervical cancers and on regional lymph nodes filtrating them.

Further investigations are needed to answer the question why the incidence of cervical cancer is 2–3 times higher in Hungary than in highly industrialized countries, since we have not found a higher HPV detection rate than that reported from these countries.

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HTLV-I-RELATED RETROVIRAL MARKERS IN HUNGARIAN PATIENTS WITH MYCOSIS FUNGOIDES*

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Cell and serum samples from 7 Hungarian patients with mycosis fungoides were examined for the presence of HTLV-I-related DNA sequences and antibodies recognizing HTLV-I antigens. DNA sequences distantly related to the proviral DNA of HTLV-I were shown by Southern blot hybridization in 3 patients. Serum samples from these patients contained antibodies reactive with the internal core polypeptides of HTLV-I and HTLV-II, but not with the *env* gene encoded type-specific HTLV antigens. Restriction enzyme analysis with EcoRI, PstI, BamHI and SacI revealed structural similarity of the provirus(es) integrated in the DNA of mycosis fungoides cells to HTLV-I but not to HTLV-II. Data suggest that these proviruses and HTLV-I are similar to each other along *gag* and *pol* regions.

The first human retrovirus [1], associated with adult T-cell leukemia (ATL) was named human T-cell leukemia/lymphoma virus (HTLV), and later HTLV-I after the discovery of a new member of the family, HTLV-II [2]. Hints of presence of other retroviruses related to HTLV-s were indicated by serological cross-reactivities and by cross-hybridization with HTLV-I. In European areas, non-endemic for HTLV-I, antibodies cross-reacting with HTLV-I capsid proteins have occasionally been found in the sera of patients with mycosis fungoides [3] and Sézary syndrome [4], and the presence of the virus in a subset of mycosis fungoides (MF) was proven by cross-hybridization of neoplastic genomic DNA with HTLV-I probes [5]. In 1987, a new human retrovirus, distantly related to HTLV-I was isolated from a patient with MF in the leukemic phase [6]. In the present study we performed molecular

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biological and serological analyses on specimens from Hungarian patients with mycosis fungoides. In three of them, data showed the presence of retrovirus(es), similar but not identical to HTLV-I.

Materials and methods

Cell and serum samples. Tumour biopsy specimens from 7 patients with MF were minced, and the fragments passed through a stainless-steel wire mesh. Serum samples were stored at −70 °C until used.

Separation of peripheral blood mononuclear cells (PBMC). Mononuclear cells consisting predominantly of lymphocytes were isolated from peripheral blood by Ficoll-Uromiro buoyant density gradient centrifugation by conventional technique.

Cell lines. HTLV-I-producing MT−2 cells [7] and HTLV-II-producing C3−44 cells [8] were maintained in RPMI−1640 medium containing 10% fetal calf serum and antibiotics. For control purposes concanavalin (Con−A) stimulated normal human peripheral T lymphocytes [9] were used.

Southern-blot hybridization. High molecular weight DNAs were extracted from the cells by the proteinase K phenol-chloroform method. The DNAs digested with restriction endonucleases EcoRI, PstI, BamHI or SacI were separated on 0.8% agarose gels and transferred to Gene Screen (New England Nuclear) membranes [10]. Molecular sizes were determined with reference to phage λ DNA digested with HindIII. The pMT−2 plasmid containing full length HTLV-I proviral DNA [11] was labelled with ^{32}P by nick translation [12] and utilized as a probe. The filters were prehybridized overnight in 50% formamide, 3×SSC, 5× Denhardt solution, 0.5% SDS, 10% dextran sulphate and 200 μg/ml denaturated salmon sperm DNA. Hybridization of DNA filters was carried out at 37 °C for 24 h with ^{32}P-labelled HTLV-I probe in the same hybridization buffer as described above. Filters were then washed in 3×SSC, 0.5% SDS at 60 °C or in 1×SSC, 0.5% SDS at 65 °C.

Indirect membrane immunofluorescence assay (IFA). For the assay, 100 μl of sera diluted in PBS were incubated with 10^6 MT−2 or C3−44 cells. After incubation for 20 min at 37 °C the cells were washed three times. Then 100 μl of goat anti-human IgG conjugated with FITC was added. After incubation for 20 min at 37 °C the cells were washed again. Control targets included Con-A-stimulated normal human peripheral T lymphocytes.

Indirect cytoplasmic IFA. The target cells were fixed in acetone on slides. Twenty μl of serum dilutions were applied to 2×10^5 cells. Samples were incubated for 30 min at 37 °C. After washing, FITC-labelled anti-human IgG was added to the fixed cells for 30 min at 37 °C. IFA titres reflect the serum dilution at which 50% of target cells showed marked fluorescences.

Radioimmunoprecipitation assay (RIPA). The experimental conditions described by Kurth et al. [13] were utilized. The HTLV-I p24 antigen was supplied by Dr. R. C. Gallo (NCI, Bethesda, MD, USA) and labelled with ^{125}I by the chloramine-T method [14]. Anti-p24 titres are defined as ng of viral protein precipitated by 10 μl of serum diluted 1:10.

Results

Analysis of cellular DNA. Figures 1 and 2 show the Southern blot analysis of EcoRI digests under low and high stringency conditions, respectively. The MT−2 cell line was used as positive source for HTLV-I. PBMC from a healthy donor and Con-A-stimulated T lymphocytes of the same person served as negative controls. The

HTLV-I probe hybridized specifically, but only under low stringency conditions (3×SSC at 60 °C) with the DNAs from tumour cells of 3 patients with MF (Fig. 1). Under high stringency conditions (1×SSC at 65 °C), the pMT-2 probe hybridized only with the DNA from MT-2 cells, but not with any of the MF samples (Fig. 2). Restriction enzyme analysis with EcoRI revealed two bands in the DNA from MT-2 cells (9.0 and 5.0 kb) and only one band in the DNAs from MF cells (20.0, 23.0 and 20.0 kb).

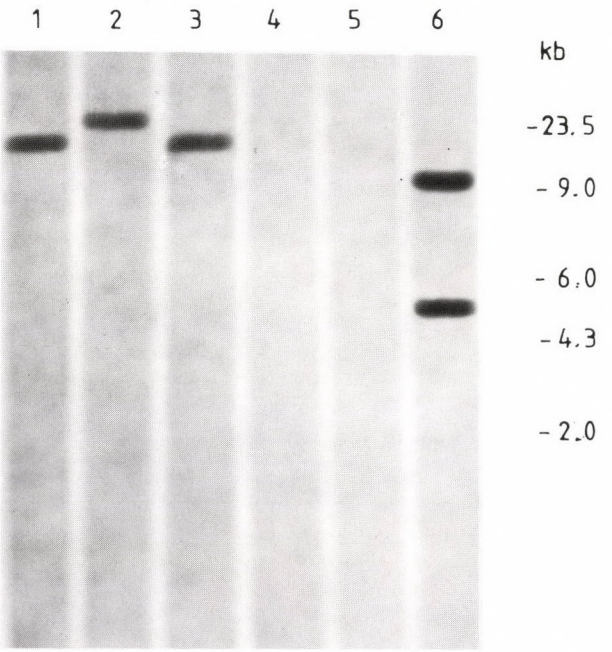

Fig. 1. Hybridization of HTLV-I probe with genomic DNAs digested with EcoRI under low stringency conditions. DNA samples: lane 1, MF No. 1; lane 2, MF No. 2; lane 3, MF No. 3; lane 4, normal PBMC; lane 5, Con-A-stimulated T lymphocytes; lane 6, MT-2 cells

For more detailed comparison, DNAs from MT-2 cells and MF samples were examined by Southern hybridization, using the enzymes PstI, BamHI and SacI. All proviral DNA sequences hybridized to the HTLV-I probe at low stringency conditions. The proviral PstI fragments are shown in Fig. 3. PstI digestion of the DNA from MT-2 cells generated 3 internal fragments of 2.5, 1.8 and 1.2 kb. Digestion of DNA from MF samples gave rise to hybridizing fragments of 1.8 and 1.2 kb, whereas the 2.5 kb fragment was absent. Restriction enzyme analysis with BamHI revealed an internal 1.0 kb fragment in the DNA from MT-2 cells (Fig. 4). In contrast, the proviral DNA sequences integrated in the DNA of MF cells were not

cleaved within the proviral genome. As shown in Fig. 5, an 8.0 kb hybridizing fragment generated after digestion with SacI was found in DNAs from MT-2 cells and the MF samples.

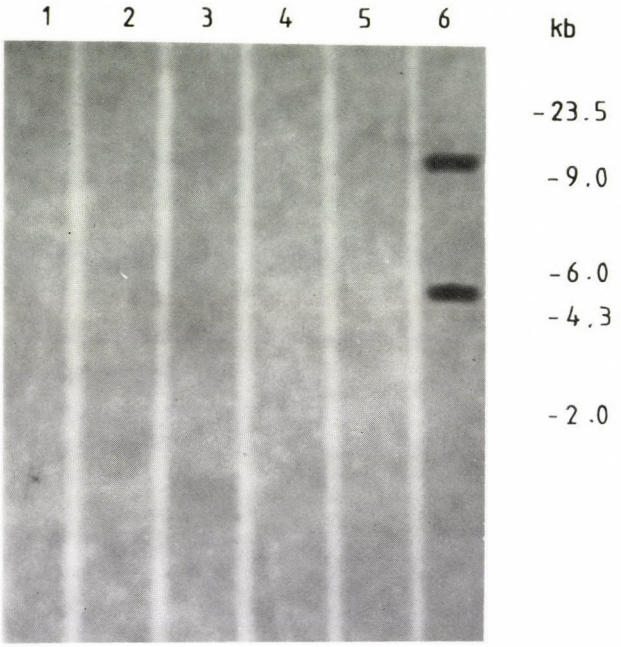

Fig. 2. Hybridization of HTLV-I probe with genomic DNAs digested with EcoRI under high stringency conditions. DNA samples are the same as in Fig. 1

Serological studies. Serum samples collected from the 7 MF patients and 10 healthy donors were investigated for reactivity with HTLV antigens. Goat immune serum raised against HTLV-I p24 polypeptide (from R. C. Gallo) was used as a positive control. In membrane IFA experiment all sera were negative. Sera taken from MF patients Nos 1-3 carrying HTLV-related proviral DNA were found to have antibodies reacting with HTLV-I- and HTLV-II-infected cells in cytoplasmic IFA but not in membrane IFA (Table I). None of the sera from MF patients negative for the presence of HTLV-related proviral DNA or from healthy donors contained antibodies reacting with HTLV-I- or HTLV-II-infected cells. The positive results were confirmed by anti-p24 RIPA, in which 8.4-24.2 ng of HTLV-I p24 were precipitated by 10 μl of sera diluted 1:10.

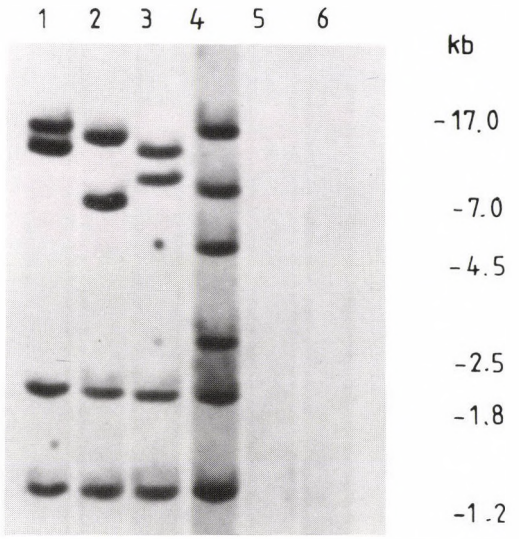

Fig. 3. Southern blot of genomic DNAs digested with PstI, and hybridized to HTLV-I probe under low stringency conditions. DNA samples: lane 1, MF No. 1; lane 2, MF No. 2; lane 3, MF No. 3; lane 4, MT-2 cells; lane 5, normal PBMC; lane 6, Con-A-stimulated T lymphocytes

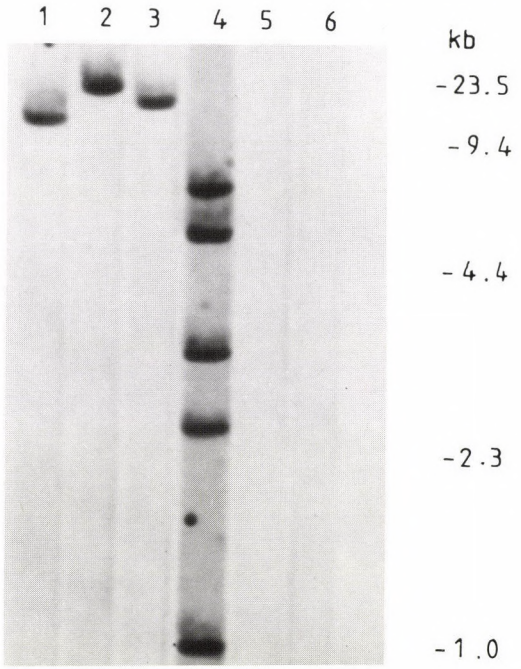

Fig. 4. Southern blot of genomic DNAs digested with BamHI, and hybridized to HTLV-I probe under low stringency conditions. DNA samples are the same as in Fig. 3

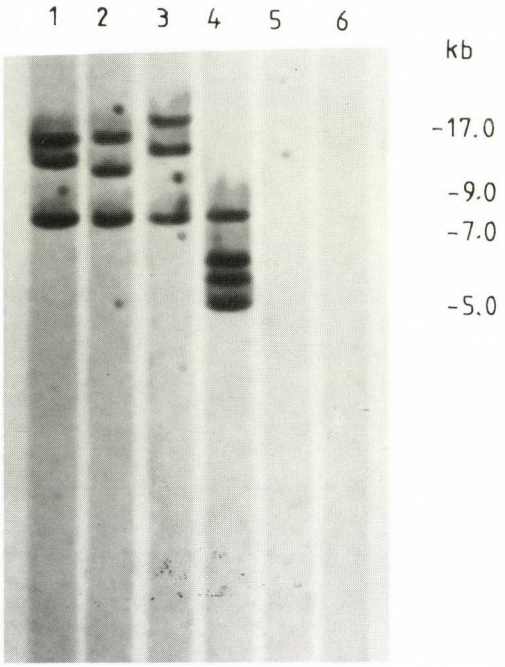

Fig. 5. Southern blot of genomic DNAs digested with SacI, and hybridized to HTLV-I probe under low stringency conditions. DNA samples are the same as in Fig. 3

Table I

Detection of antibodies to HTLV antigens in serum samples

Source of serum	Titres detected By IFA				By RIPA
	On MT-2 cells		On C3-44 cells		
	Membrane	Cytoplasm	Membrane	Cytoplasm	
MF patient No. 1	<1	48	<1	32	24.2
MF patient No. 2	<1	32	<1	16	18.6
MF patient No. 3	<1	16	<1	16	8.4
MF patients Nos 4-7	<1	<1	<1	<1	<0.3
Immune serum	<1	256	<1	128	146
Healthy donors	<1	<1	<1	<1	<0.3

Discussion

Our study documents the presence of DNA sequences related to, but not identical with HTLV-I provirus in three Hungarian patients suffering from MF. The genomic DNAs were cleaved with EcoRI that does not cut within the HTLV genome. Hence, the number of bands detected in this examination reflects that the MT−2 cells contained two copies of HTLV-I proviral DNA, whereas MF cells contained only one proviral copy. The 5.0 kb EcoRI fragment in the DNA of MT−2 cells represents a defective HTLV-I genome. The restriction enzyme PstI cleaves the HTLV-I genome at several sites and produces three large internal fragments of 1.2 kb from the *pol* region, 1.8 kb from the *gag* region, and 2.5 kb from the *env* region [15]. The *env*-specific fragment was absent in the MF samples. The additional bands present in MF samples include viral and cellular flanking sequences arising from the integration of proviral copies at various integration sites. BamHI cleaves HTLV-I within the 3' end of the *pol* region and the *env* region [16]. The absence of the 1.0 kb BamHI fragment in the proviral DNA from MF cells suggest that these proviruses and HTLV-I are different in the *env* region. SacI digestion yielded an 8.0 kb hybridizing fragment in each MF sample containing HTLV-related DNA. Most HTLV-I genomes have a unique SacI site in the LTR sequences [17]. Therefore, the observation of the 8.0 kb fragment shows the presence of full proviral copies in the three MF samples.

No structural similarity of the HTLV-like genomes in MF cells was found to HTLV-II. Restriction endonuclease EcoRI has two cleavage sites in HTLV-II generating an internal 3.9 kb fragment [18]. BamHI digestion of HTLV-II provirus produces a characteristic internal fragment of 3.6 kb [18, 19]. Digestion of proviral DNA by PstI yields an internal fragment of 2.4 kb from HTLV-IIa, but no internal fragment from HTLV-IIb [20]. SacI cleaves HTLV-IIa within the LTRs and *pol* region [21], whereas HTLV-IIb lacks the internal SacI site [20].

In IFA, sera from the three MF patients, positive for the presence of HTLV-related proviral DNA, had antibodies reacting with the group-specific HTLV antigens which were localized in the cytoplasm of HTLV-infected cells [22], but no reactivity was found to the type-specific antigens expressed in the cell membrane [23, 24]. The group-specific nature of the antibodies detected by IFA was confirmed by anti-p24 RIPA. Serologic data indicate that the three MF patients did not have genuine HTLV-I or HTLV-II infection.

In conclusion, our cases represent the first report of the presence of HTLV-like proviral DNA in Central European patients with mycosis fungoides. It still remains to clarify the relatedness of these proviral sequences to those detected in Central European patients with Sézary syndrome [25] and adult T-cell leukemia [26]. It should also be revealed if these proviral sequences are identical to HTLV-V [6] or not.

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SIGNAL TRANSDUCING MECHANISMS IN INTERFERON ACTION* (A BRIEF REVIEW)

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Experimental results suggest that protein kinase C (PK–C) may play a substantial role in the action of IFNs, but the precise biochemical pathway remains unknown. Recent evidences reveal the complexity of the mechanism of IFN-action and show that the IFN–α, –β and –γ induced pathways are overlapping. We briefly discuss what is known in this field and suggest a way in which the contrasting views might be reconciled.

In the last years we have studied the mechanism of interferon (IFN)-induced transmembrane signalling. In 1985, at the very beginning of this work, we summarized our knowledge and formulated a "working hypothesis" [1]. According to this hypothesis triggering of the inositol phospholipid-diacylglycerol-protein kinase C signal transducing pathway by IFN might be responsible for several effects of IFNs. Since then several publications dealt with the molecular mechanisms that mediate signal transduction by IFNs. In the present paper we try to sum up our experimental results and the data published by other authors on this field to see the relevance of the "working hypothesis".

Role of PK–C in the transmembrane signalling of IFNs

IFNs are a heterogeneous family of cytokines with antiviral, antiproliferative and immunomodulatory activities. IFNs elicit their pleiotropic effects by binding to the cell-surface receptors and activating distinct signal transduction pathways. Treatment of cells with IFNs stimulates the expression of several proteins which

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mediate their biological activities. The biochemical events following the binding of IFNs are poorly understood.

According to our "working hypothesis" IFNs utilize the well recognized mechanism of signal transduction that involves activation of PK-C [1]. In the classical scheme extracellular signals – interacting with cell surface receptors – activate the breakdown of inositol phospholipids resulting in the release of inositol 1,4,5-triphosphate and diacylglycerol [2]. Both of these molecules function as second messengers. Inositol triphosphate mobilizes Ca^{++} from intracellular stores and diacylglycerol activates PK-C.

The first observation calling our attention to the possibility that PK-C might be involved in the IFN-induced signal transduction mechanism was the finding that phorbol myristate acetate, which can substitute for diacylglycerol, inhibited the cytopathic effect of vesicular stomatitis virus (VSV) [3]. The role of PK-C in IFN action was confirmed by Hamilton et al. [4], who showed that IFN-γ stimulated PK-C activity in murine peritoneal macrophages. These findings were consistent with the results obtained by others. Yap et al. [5, 6] reported that IFN-α, -β and -γ caused a rapid and transient increase in the level of diacylglycerol in different cell types. McCoy and Strynadka [7] also measured an increase of diacylglycerol in IFN-α and -β treated cells. Later, many observations pointed to the possibility that PK-C is involved in the mechanism of IFN action [8-12].

We have found that treatment of human amnion cells with IFN-α or phorbol myristate acetate resulted in translocation of PK-C activity from the cytosol to the membrane fraction [13]. Popescu et al. [14] reported that phorbol myristate acetate and calcium ionophore A 23187 protected human embryo fibroblast cells against VSV infection when the cells were pretreated 24 h prior to the virus infection. IFN-α induced also a transient translocation of PK-C from the cytosol to the particulate fraction in U937 cells [15]. After IFN-γ treatment of U937 cells no significant change in the PK-C activity could be observed. In contrast with this result, recombinant murine IFN-γ induced a transient translocation of PK-C activity in a murine pre-B lymphocyte line (70Z/3) [16] and function of PK-C kinase was modulated by IFN in murine peritoneal macrophages [17]. Long-term modulation of PK-C activity was shown in human tumour cells after IFN-β and IFN-γ treatment [18]. In agreement with these results the role of PK-C in the priming effect of human IFN-α was demonstrated [19]. Phorbol esters and synthetic diacylglycerol (OAG) was described to prime cells for IFN production, and antiviral effect of OAG was shown, too [19, 10].

The major recent advances in this field were made by Pfeffer and his co-workers [21]. They have shown that activation of PK-C by phorbol myristate acetate mimicked the inhibitory action of IFN-α on HeLa cell proliferation and downregulation of PK-C blocked the IFN-α induced antiviral activity in HeLa cells.

In addition, it was demonstrated lately that isoforms of PK–C behave differently. Thus, IFN–α induces selective translocation of the β isoform in HeLa cells, while the subcellular distribution of α and ε isoforms remain unaffected. In Daudi cells IFN–α treatment results in a rapid and transient diacylglycerol production and in activation of the ε isoform [22], but the α isoform is unaffected. In an IFN-resistant subclone of Daudi cells IFN–α treatment had no effect on the diacylglycerol content of the cells and on the activation of PK–C–ε. In contrast with PK–C–α, the ε isoform appears to be resistant to downregulation in many cell types [22–24] and has no requirement for an elevation in $(Ca^{++})_i$ in order to be fully activated [25]. The Pfeffer group reported also that pretreatment of the cells with selective PK–C inhibitors (H7 and staurosporin) inhibited the action of IFN–α both in human fibroblasts and HeLa cells [21, 26].

It has been published that PK–C inhibitors blocked the INF–γ-induced transcriptional activation in U937 macrophage cell line [27], in a human eosinophilic leukemia cell line, Eo1–3 [28], in human Raji cells [29], in a hepatoblastoma cell line Huh 6 [30] and in astrocytes [31]. Consistently with these reports, it was found that induction of 2', 5'A synthetase by recombinant IFN–αA was sensitive for H7 treatment of Daudi cells [32].

H7 was also shown to inhibit activation of cytotoxic peritoneal murine macrophages by IFN–β [33]. Treatment of quiescent primary human fibroblasts with all three types of IFNs resulted in Egr–1 mRNA induction [34], which could be inhibited by H7 and staurosporin indicating the involvement of functional PK–C. Furthermore, it has been reported that inhibitors of PK–C block the activation of IFN-stimulated gene factor (ISGF3) in HeLa cells [26]. Similar results were found recently by Bandyopadhyay and Sen [35] in HeLa M cells. IFN–α and IFN–γ both activated the phosphorylation of ISGF3α subunit. Ten n*M* of staurosporin (at this low concentration the inhibitor is highly specific for PK–C) completely blocked ISGF3α activation. Induction of the other subunit (ISGF3γ) by IFN–α or IFN–γ was totally insensitive to staurosporine. The observation that downregulation of PK–C inhibits the induction of 2', 5'A synthetase mRNS also supports the role of PK–C in the effects of IFNs [32].

There are, however, studies in which authors failed to obtain evidence for the role of PK–C in IFN-induced signal transduction [36–38]. For example, in Swiss 3T3 cells IFN–β treatment did not stimulate the phosphorylation of an acidic Mr 80 000 cellular protein, which serves as a substrate for PK–C, in spite of the fact that in a parallel experiment bombesin treatment gave positive results. Moreover, downregulation of PK–C did not inhibit the mechanism by which IFN–β blocked cell growth [36]. These contradictory data may be explained by recently published results demonstrating that multiple isoforms of PK–C – which coexist within a single cell type – are differentially regulated by extracellular signals [39] and the

individual isoforms may phosphorylate different substrates [40]. Furthermore, it is known that some isoforms of PK-C may be resistant for downregulation in several cell types [23]. Interestingly, PK-C dependent signal pathway may be involved in those mechanisms by which IFN is able to downregulate the transcription of genes whose expression it initially induces [41].

On the basis of the above results definite role of PK-C in IFN induced signal transduction can be considered as proven.

Molecular mechanisms of PK-C activation

Although there are several reports suggesting that IFN-treatment increases intracellular diacylglycerol levels, the lipid source of this diacylglycerol is still uncertain. According to the classical model diacylglycerol reducing the calcium requirement for PK-C activates its translocation [2]. However, it has been shown that unsaturated fatty acids (oleic acid and arachidonic acid) can activate PK-C independently of Ca^{++} and phospholipids [42]. Since certain lysophospholipids, particularly lysophosphatidylcholine, are effective stimulators or inhibitors of PK-C [43], it has been proposed that PK-C is dually regulated [44]. One route is mediated by phospholipase C activation leading to elevation of diacylglycerol levels and the other by phospholipase A_2 activation resulting in elevation of cis fatty acids or lysophospholipids. Recently, a third pathway was suggested via the activation of phospholipase D. According to this scheme diacylglycerol is originated by the sequential action of phospholipase D and phosphatidic acid phosphohydrolase [45].

Our analysis of ^{32}P incorporation into phospholipid fraction and studies of alterations in the fatty acid content of the major phospholipids of IFN-α treated human amnion cells suggest that both phospholipase C and A_2 are activated. Moreover, addition of neomycin (an inhibitor of phospholipase C) as well as mepacrine (an inhibitor of phospholipase A_2) to IFN-treated cells inhibited the antiviral activity of IFN-α [13]. These observations indicate that in human amnion cells diacylglycerol can be generated directly via phospholipase C and suggest that activation of phospholipase A_2 may be also an important step in the mechanism of IFN-α action. IFN-α, -β and -γ were equally reported to induce a transient increase of inositol phosphates in primary human fibroblasts [5]. These data are in harmony with the results published by Popescu et al. [14]. According to these authors monoclonal antibodies against phospholipase C blocked the antiviral effect of IFN-α and IFN-β in quiescent fibroblasts. Neomycin also inhibited the effect of α and β IFN [20]. In Daudi cells IFN-α and -β also activated inositol phospholipid turnover [6]. An other laboratory, however, has reported that IFN treatment of Daudi cells resulted is no measurable changes of inositol phospholipid turnover although they were unable to define the lipid source for diacylglycerol [22]. In IFN-α-treated

HeLa cells this group could detect increased hydrolysis of phosphatidylcholine and the production of phosphorylcholine and phosphatidic acid [21]. There are, however, no data proving that phosphatidyl-choline-specific C or D phospholipases are involved in the mechanism.

The observation that there is a rapid but transient stimulation of phospholipase A_2 activity in 3T3 fibroblasts after IFN-α treatment complicates the problem even more [46]. Inhibiting the function of phospholipase A_2 with p-bromophenacyl-bromide (BPB) specifically blocks the binding of IFN-induced nuclear factors to the IFN-stimulated response element (ISRE) of the 2', 5'A synthetase gene. BPB also inhibits ISRE-dependent transcription. Treatment of the cells with inhibitors of fatty acid cyclooxygenase or lipoxygenase enzymes resulted in amplification of IFN-α-induced ISRE binding and gene expression.

It is interesting that in human neutrophils IFN-α also activated arachidonic acid release and lipoxigenation [47] and stimulated prostaglandin synthesis by endothelial cells through interference with phospholipase A_2 and cyclooxygenase [48]. IFN-β was also observed to cause arachidonic acid release from phospholipid membrane pools in a human fibrobroblast cell line [49].

All these data do not exclude the possibility that all these mechanisms may have a function in IFN-induced signal transduction processes. The latter possibility is supported by the findings that cells in general can respond to any particular agonist through multiple pathways and it was shown that cross-talk between phospholipase C, phospholipase A_2 and phospholipase D do exist [45, 50, 51]. Since tyrosine kinase activity also was demonstrated recently to play a role in the IFN-α/β-induced signalling pathway the mechanism in question is even more complicated [52, 53]. It is not yet known whether changes in the concentration of diacylglycerol induced by IFN are primary or secondary responses or – similarly to the mechanism observed in T cell receptor-mediated signal transduction [54] – a dual signal transduction pathway is induced by IFN. Recent studies indicate that tyrosine phosphorylation may activate phospholipase C-γ [55, 56]. Such a model is currently supported by informations suggesting that the effects of other cytokines (IL2 and IL3) are also required for tyrosine kinase activity [57, 58].

PK-C as an activator of Na^+/H^+ exchange

PK-C is known as an activator of a Na^+/H^+ antiporter system [59]. Its role in the lymphokine-mediated cytoplasmic alkalization was first tested by the use of H7, an inhibitor of PK-C [60]. In rat astrocytes PK-C and Na^+ influx both are required for IFN-γ-induced class II MHC expression [31]. It was found that inhibitors of Na^+/H^+ exchange or PK-C activation equally blocked the IFN-γ-induced gene expression. Other studies demonstrated that IFN-γ-induces rapid Na^+/H^+

exchange in murine macrophages and this is important for mediating expression of class II MHC genes [61]. Mouse IFN-β (in L_B cells) and human IFN-α_{2a} (in HeLa cells) induced an increase in intracellular pH_i [62]. Addition of amiloride reversed the IFN-induced alkalinization. Similar increase in pH_i has been demonstrated in vivo in tumour cells of mice after IFN-α/β treatment [63].

However, in a murine B lymphoid cell line (70Z/3) the mechanism of the regulation appears to be more complicated [16]. Although recombinant IFN-γ induced association of PK-C with the membranes and stimulated Na^+/H^+ exchange across the plasma membrane, the presence of PK-C inhibitor (H7) did not block Na^+/H^+ exchange. Despite significant depletion of PK-C activity cytoplasmic alkalization in response to IFN-γ was not decreasing. These data show that in this cell line Na^+/H^+ exchange is independent of PK-C activation.

Effect of IFN on intracellular $(Ca^{++})_i$ concentration

Yap et al. [5] reported that human IFNs stimulate a transient two- to threefold increase in the concentration of inositol triphosphate in human diploid fibroblasts. Inositol triphosphate is known as an intracellular messenger which triggers the release of Ca^{++} from the endoplasmic reticulum [64]. However, the role of intracellular $(Ca^{++})_i$ in IFN-effects appear to be very contradictory. In diploid fibroblasts liberation of inositol triphosphates following IFN-triggering was not accompanied by measurable increases of cytosolic-free Ca^{++} [5]. In Daudi cells similar results were detected [6]. Using the latter cells no inositol phospholipid turnover was measured in an other laboratory [22] and similar results were obtained by the study of IFN-α-treated HeLa cells [21, 65, 66].

However, some role for intracellular Ca^{++} mobilization in IFN-action can be inferred from the results of studies with the Ca^{++}-ionophores A 23187 and ionomycin. It was demonstrated that Ca^{++}-ionophores evoke and potentiate antiviral activity of IFN in several cell types [14, 67] or elevate induction of 2', 5'-oligoadenylate gene expression [68]. In accordance with these observations it was shown that in a human colon cancer cell line (RPMI 4788) addition of IFN-γ and IFN-β resulted in a dose-dependent, rapid and transient elevation of $(Ca^{++})_i$ [69, 70]. The increase of $(Ca^{++})_i$ induced by IFN-γ proved to be independent of the extracellular Ca^{++} but that induced by IFN-β was partially dependent. In an other cell type, U 937, which is a monocyte like cell line, IFN-γ treatment significantly increased $(Ca^{++})_i$ but only one-third appeared to be derived from intracellular stores, two-third originated from the influx of extracellular Ca^{++} [71]. Addition of IFN-γ to murine macrophages induced an increased efflux of intracellular $(Ca^{++})_i$ [72].

On the basis of these contradictory data it is not possible to rule out the possible role of $(Ca^{++})_i$ exchange in IFN action.

Indirect evidences indicating the role of PK–C in IFN-action

As phorbol myristate acetate and diacylglycerol treatment proved to have antiviral [3, 14] and antiproliferative [73, 74] activities it seems to be reasonable to suppose that PK–C may be involved in IFN-signalling mechanism. Moreover, the results suggest that activation of PK–C by other substances should have an effect on cells similarly to that of IFN.

It was reported first by Blalock and Stanton [75] that noradrenalin induced an "IFN-like" antiviral state in mouse myocardial cells. On this basis they supposed common pathways of IFN- and hormonal stimulation. It was demonstrated only later that α_i-adrenergic agonists (e.g. noradrenalin) are able to stimulate inositol phospholipid breakdown and to activate PK–C [76, 77]. Antiviral activity of tumour necrosis factors (TNF) was also reported [78, 79]. As IFN-antibodies only slightly reduced the TNF mediated antiviral protection it was concluded that TNF has its own antiviral activity. The activation of phospholipase C – PK–C signal pathway by TNF was described, too [34, 80, 81]. Our results confirm these data. It was shown that triggering the breakdown of inositol phospholipids and activating the translocation of PK–C by different biological substances (phorbol myristate acetate, poly (I/:/C), vitamin A, cytodex 1, oxytocin) induce an antiviral state in human amnion cells [3, 82–85].

These observations illustrate that antiviral state of the cells can be induced not only by IFNs but by different substances which trigger the inositol phospholipid turnover and activate PK–C. Another line of evidences, demonstrating IFN-like antiproliferative activity with parallel induction of PK–C, is in good agreement with this conclusion [86–89].

Conclusion

The purpose of this article was to consider our recent knowledge of IFN-induced signal mechanisms. As it could be expected, the action of IFNs is regulated via a number of different pathways, but the results suggest that PK–C plays a substantial role in these mechanisms. There are, however, contrasting views in this area. According to one concept involvement of classical second message pathways – with activating PK–C isoforms [9, 24] or with stimulating of arachidonic acid release [90] – is of great importance. Other favour the direct activation of a transcription factor induced by IFN-receptor complex without any requirement for classical second messengers [91]. Quite recently this group, and others too, drew the attention to the

possibility that IFN-receptor complex could be associated with an intracellular tyrosine kinase activity [52, 53, 92, 93]. These opinions can be reconciled by the help of the informations rapidly accumulating due to intensive investigations of cellular functions regulated by neurotransmitters, hormones and a wide variety of regulatory and growth promoting factors. As it is discussed by Michell the same signalling reactions can be provoked by the classical way and by receptors that are tyrosine kinases or are associated with intracellular tyrosine kinases [94]. Furthermore, it is now clear that many of the protein components of these signal pathways exist in multiple isoforms with different tissue distribution and presumably with subtly different functions. On the basis of these works it can be supposed that different signal pathways induced by IFNs function in a concerted action and the details of the mechanisms are depending on the characteristics of the individual cells. These require, however, further studies.

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ANTIBACTERIAL ANTAGONISM BETWEEN FUSIDIC ACID AND CIPROFLOXACIN*

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A routine laboratory disk susceptibility testing of a resistant *Staphylococcus aureus* strain showed that around the ciprofloxacin disk, placed by chance in proximity to a fusidic acid disk, the inhibition zone was truncated. Follow-up of this observation by a planned disk approximation method showed that there is a real antagonism between these two antibacterial agents. The antagonism was observed while testing *S. aureus* isolates including the standard ATCC 25923 strain, with *Bacillus subtilis* ATCC 6633 spores and also with a mutant *Escherichia coli* made fusidic acid susceptible. The antagonistic property was found structure-specific, only associated with those fluoroquinolones containing the cyclopropyl substituent at the N_1-position: ciprofloxacin, enrofloxacin, sparfloxacin and WIN 57273. Fluoroquinolones without this substituent such as enoxacin, norfloxacin, pefloxacin and ofloxacin were not antagonized by fusidic acid, the steroidal Gram-positive active antibiotic.

During routine clinical laboratory susceptibility testing for selecting the most appropriate antibiotic, ciprofloxacin and fusidic acid disks were placed, by chance, next to each other on the agar surface seeded with a beta-lactamase-producing and methicillin-resistant *S. aureus* strain. On reading the test result after incubation at 37 °C for 18 h, the round inhibition zone of ciprofloxacin (C), was flattened (Fig. 1) on the side of the fusidic acid (F) disk. The inhibition zone of norfloxacin, placed on the other side of the fusidic acid disk did not show this altered shape of inhibition zone. This chance experimental result indicated the existence of antagonism between fusidic acid and ciprofloxacin, but not between fusidic acid and norfloxacin. The other known anti-staphylococcal antibiotics on the plate were rifampicin, erythromycin and chloramphenicol all with well-defined inhibitory zones, as well as ceftizoxime without inhibitory zone, as expected.

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This observation promted the study reported in this paper in order to elucidate the underlying molecular structural basis, as well as any possible genetic background for this antagonistic phenomenon. For this purpose, many other bacterial strains, mostly Gram-positive ones were tested with other known fluoroquinolones.

Materials and methods

Antibacterial agents. For most of the in vitro antagonistic tests using various bacterial strains, fusidic acid and ciprofloxacin susceptibility disks were used. However, for the study of the possible mechanism of the antagonism, other fluoroquinolones were included.

Fusidic acid is the only therapeutically used member of the antibiotics containing a steroid-like chemical structure (Fig. 2). Originally it was described as a rather narrow-spectrum orally absorbed antibiotic active mainly against Gram-positive bacteria [1–3]. Although its principal clinical use is in the treatment of staphylococcal infections, including those caused by methicillin resistant *S. aureus* strains [4], it has in vitro activity against *Plasmodium falciparum* [5], *Mycobacteria* [6–8] and probably against human immunodeficiency virus (HIV) [9–12]. Quite recently, a fusidic acid-susceptible *Escherichia coli* mutant strain was created in the laboratory [13].

The water-soluble crystalline sodium salt of fusidic acid is marketed and used clinically in many parts of the world (but not yet in the USA) under the trade name of "Fucidin" [2]. This was used in the present experiment, either as the crystalline powder or paper disks containing 10 μg of fusidic acid. They were received from Leo Pharmaceutical Products, Copenhagen, Denmark.

Ciprofloxacin is presently the most potent, extensively and effectively used member of the fluoroquinolone antibacterial agents which have been for the last decade one of the most important drugs against bacterial infections [14–19]. Ciprofloxacin was obtained from Miles Laboratories, Inc., West Haven, Connecticut. Its chemical structure is shown in Fig. 3. The cyclopropyl (encircled) substituent at the quinolone ring nitrogen (N_1) is probably responsible for its potent inhibitory action on DNA gyrase (topoisomerase II) and excellent therapeutic effect in various systemic infections [20–23]. Other newer fluoroquinolones used in the study with the same structural configuration (i.e., cyclopropyl group attached to the N_1-position) were: enrofloxacin [24], from Mobay Corporation, Shawnee, Kans., sparfloxacin [25] from Parke Davis, Ann Arbor, Mich., and WIN 57273 [26] from Sterling Research Group, Rensselaer, N. Y. Fluoroquinolones without the N_1-cyclopropyl substituent included in the study were norfloxacin [27] from Merck Sharp and Dohme, West Point, PA, ofloxacin [28] from Ortho Pharmaceutical Company, Raritan, N. J., enoxacin [29] from Dainippon Pharmaceutical Company, Osaka, Japan, and pefloxacin [30] from Rhône Poulenc, Paris, France.

Bacterial strains. Since fusidic acid has significant activity against Gram-positive strains, microorganisms used in this study were predominantly Gram-positive bacteria, such as *S. aureus* ATCC 25923, the specifically mouse-pathogenic Tour strain of *S. aureus* [31], and other laboratory isolates of staphylococci, as well as *B. subtilis* ATCC 6633. In addition, a Gram-negative *E. coli* strain made fusidic acid susceptible by mutagenesis with nitrosoguanidine [13] was also included. All strains were grown in and/or on trypticase soy media for preparing both the seed inocula and the seeded plates for assay.

The standard agar diffusion techniques [32, 33] were used as modified to investigate the interactions between fusidic acid and quinolones. One form used was the extensively applied "disk approximation test", also referred to as the "double-disk (diffusion)" test [34–42]. The other variation used was the "impregnated cross filter paper strips" method [43, 44].

The disk approximation test (double disk test) is performed by placing the antibiotic containing disks in established proximity on the surface of the agar medium seeded with the appropriately diluted

suspensions of the bacteria. The distance between the two disks should be strategically "optimal", usually twice the radius of the inhibition zone [39]. After incubation at 37 °C for 18 h, the inhibition zones are observed visually. The test indicates drug antagonism if an otherwise round inhibition zone is flattened (truncated, D-shaped, distorted, asymmetrical).

The "cross-paper strip" method [43, 44] is basically similar to the "disk approximation" test. In this case, drug-impregnated paper strips are placed on the seeded agar surface horizontally and vertically at a ninety-degree angle. Both strips develop inhibition zones. The test is positive for drug antagonism, if the inhibition zone becomes narrowed or abolished at the intersection of the two stripes.

Susceptibility disks were obtained from the relevant manufacturers for fusidic acid, ciprofloxacin and norfloxacin, containing 10 μg, 5 μg and 10 μg per disk, respectively. Disks containing conveniently 1–10 μg per disk, were prepared in our laboratory for those fluoroquinolones for which no established susceptibility disks were as yet available.

Results and discussion

The chance observation made during a routine disk susceptibility test on a clinical isolate of a resistant *S. aureus* strain, that the inhibition zone of ciprofloxacin facing that of the fusidic acid zone was flattened (Fig. 1), suggested an antagonism between the two drugs. Using the disk approximation test against *S. aureus* ATCC 25923, the antagonism between the two drugs was clearly demonstrated (Fig. 4). The identical antagonism was repeatedly shown against many other laboratory staphylococcal strains, as well as against fresh clinical isolates. The same occurred against *B. subtilis* ATCC 6633. Since fusidic acid is a narrow-spectrum steroidal antibiotic and is active mainly against Gram-positive bacteria, its combined action can be studied only on such microorganisms. However, Julian Davies in the Pasteur Institut, Paris, France, developed by mutagenesis an *E. coli* strain susceptible to fusidic acid [13]. This strain also showed a certain degree of antagonism with fusidic acid and ciprofloxacin. Figure 5 shows the slightly truncated inhibitory zone with a visible "halo", often referred to as "ambiguous degree of truncation".

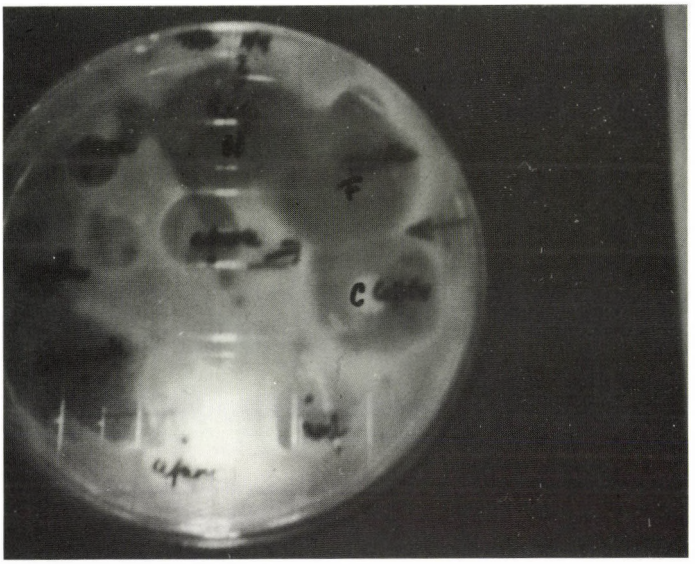

Fig. 1. Chance first observation of the antagonism between fusidic acid (F) and ciprofloxacin (C) against *S. aureus*. Flattening (arrow) of the inhibition zone around the ciprofloxacin disk is caused by fusidic acid. The inhibition zone of norfloxacin (N) is not affected by fusidic acid

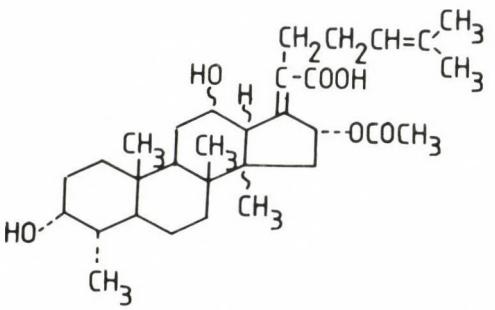

Fig. 2. The steroid-like chemical structure of fusidic acid

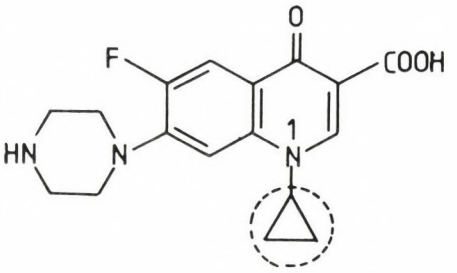

Fig. 3. Chemical structure of ciprofloxacin. Encircled is the characteristic N_1-cyclopropyl substituent

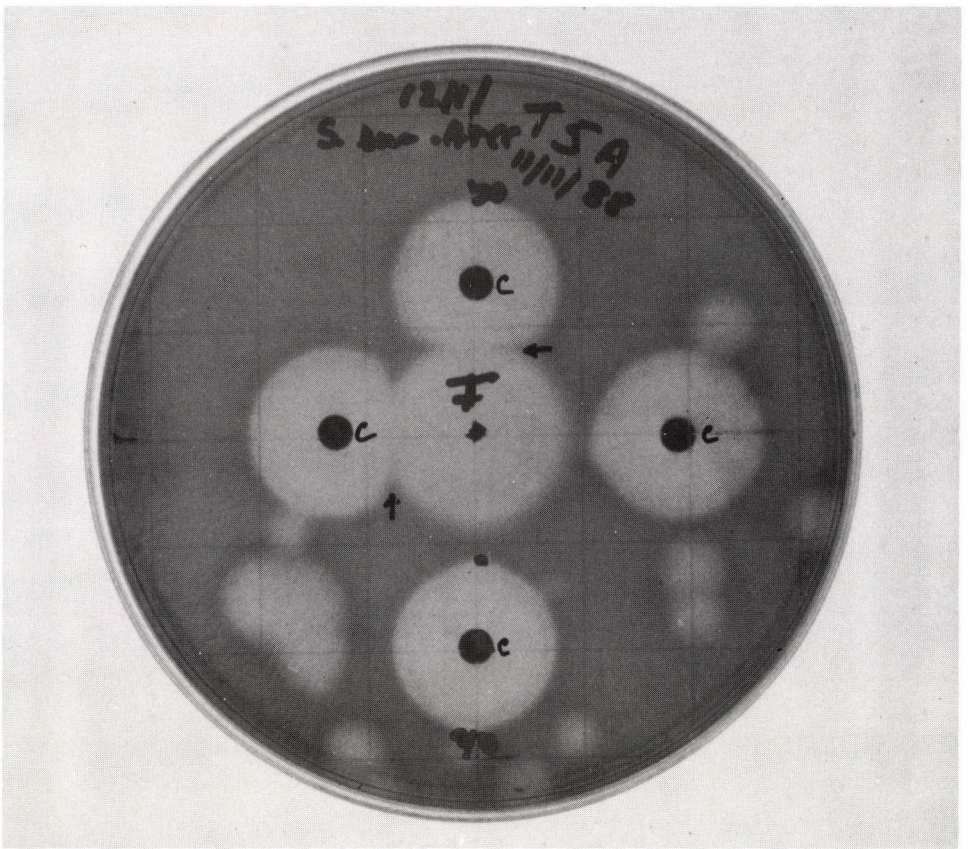

Fig. 4. The "disk approximation test" using *S. aureus* ATCC 25923. Flattening or truncation of the inhibition zones of optimally placed ciprofloxacin (C) disks by fusidic acid (F) is a clear sign of antagonism

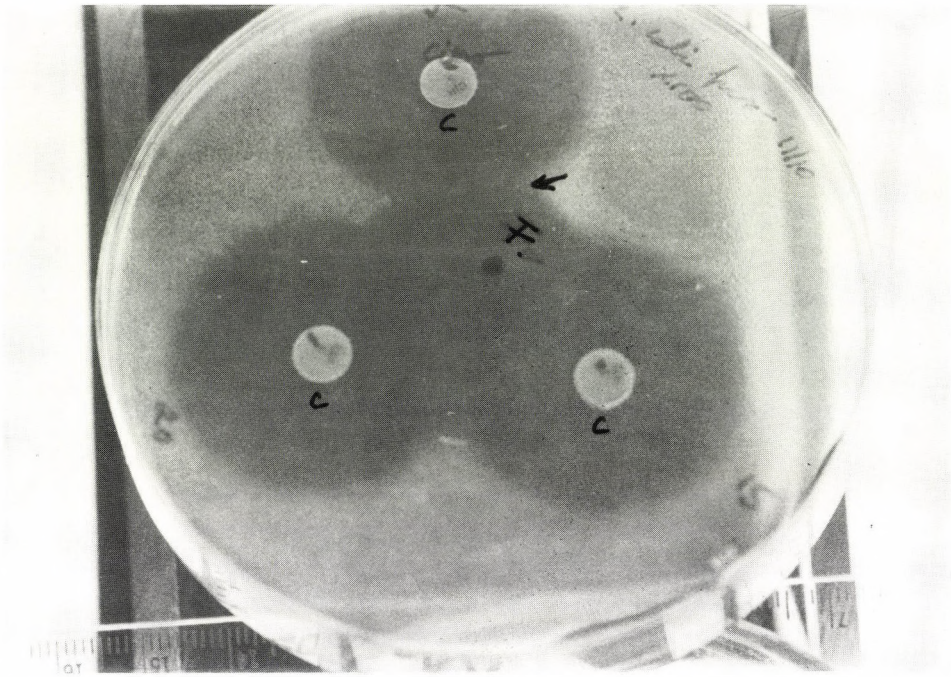

Fig. 5. Disk approximation test using the mutant *E. coli* strain susceptible to fusidic acid (F). "Halo" truncation (arrow) of the ciprofloxacin (C) inhibition zone indicated slight antagonism

By the time this study was completed, a literature search revealed a short meeting abstract mentioning that the combination of ciprofloxacin and fusidic acid exhibited antagonism against *S. aureus* and *S. feacalis* strains [45]. However, my independent study included also *B. subtilis* and an *E. coli* mutant and furthermore, expanded the study on *S. aureus* ATCC 25923 and by including additional fluoroquinolones with various molecular compositions. These experiments showed that a common chemical structure of the studied fluoroquinolones underlies the antagonism produced with fusidic acid. Table I shows that enrofloxacin, sparfloxacin and WIN 57273 exerted antagonistic effect, like that of ciprofloxacin. All these drugs have a common structural feature, namely, a cyclopropyl substituent at the N_1-position of the quinolone ring (Fig. 3). On the contrary, enoxacin, norfloxacin, pefloxacin and ofloxacin, which do not contain this cyclopropyl substituent, are not antagonized by fusidic acid. These results were obtained not only with the disk approximation test, but also with the "cross paper strip" method. A typical experiment with ciprofloxacin (two concentrations), enrofloxacin and norfloxacin is demonstrated in Fig. 6. It can be seen that norfloxacin (N) is not, whereas both ciprofloxacin (C) and enrofloxacin (E) are definitely antagonized by fusidic acid.

Table I

Effects of combination of fusidic acid with eight fluoroquinolones against S. aureus ATCC 25923

Antagonism	*Indifference*
Ciprofloxacin*	Enoxacin
Enrofloxacin	Norfloxacin
Sparfloxacin	Pefloxacin
WIN 57273	Ofloxacin

* Slight antagonism was also observed between fusidic acid and ciprofloxacin against a fusidic acid susceptible mutant *E. coli* strain [13].

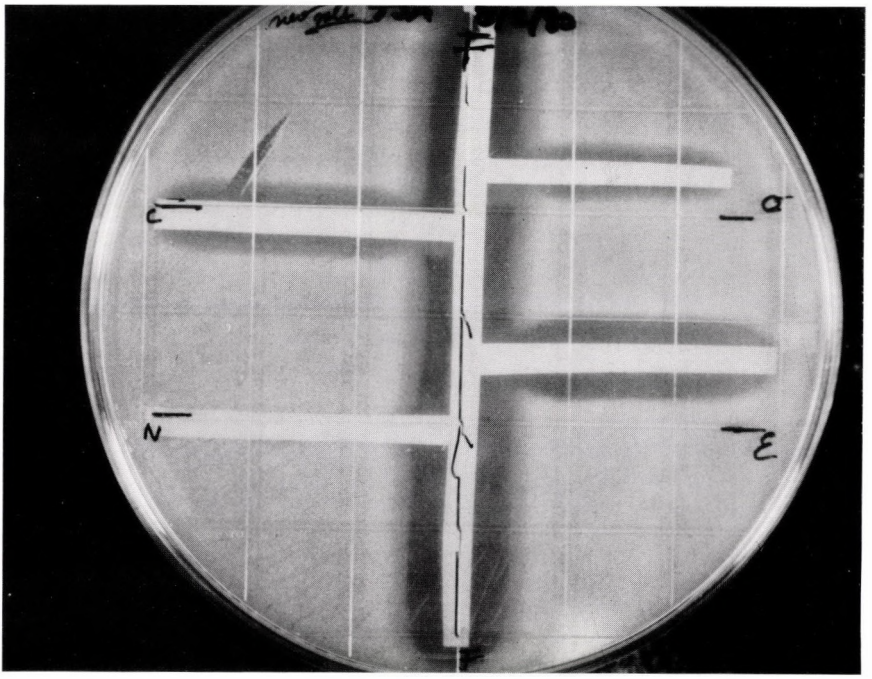

Fig. 6. The "cross paper strip method" demonstrating fusidic acid (F) antagonism toward low (C^-) and high (C) concentrations of ciprofloxacin, and toward enrofloxacin (E). Narrowing of the inhibition zones near intersections of the paper strips indicates antagonism. Activity of norfloxacin (N) is not antagonized

At present, the basis for this specific antagonism is unknown. On the molecular level, the mechanism of action of fusidic acid is the inhibition of protein synthesis in

whole bacterial cells and in cell-free extracts. The fluoroquinolones' mode of action is the inhibition of the function of DNA gyrase (topoisomerase II). These two different points of attack on the bacterial cells could theoretically result in synergism, rather than antagonism. Further studies including more fluoroquinolones with varied chemical structures may give answer to this problem and also to the role of the N_1-cyclopropyl substituent in this antagonism.

It is not known at present whether this in vitro antagonism has any influence on the therapeutic effect if the two drugs are given concomitantly. One study showed that the bactericidal effect of ciprofloxacin was suspended by fusidic acid on *S. aureus* phagocytized by human polymorphonuclear leukocytes, during the first 6 h of incubation [46].

In the past decade the fluoroquinolones represent one of the most innovative and important class of antimicrobial drugs. More than 200 derivatives have been synthesized, some are in use and many more are under study [47]. The total number of quinolones is presumed to be about 5000 compounds. In such a fast developing situation, it is expected that, from a therapeutic point of view, their interactions with other antimicrobials and other drugs will continue to be evaluated and reviewed [17, 48, 49].

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FOLLOW-UP OF THE EFFECT OF BCG VACCINE TREATMENT IN BLADDER TUMOUR PATIENTS*

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The effect of intravesical BCG vaccine treatment in 38 patients with superficial bladder tumour after TUR (Transurethral Resection) was followed by the lymphocyte transformation test (LTT) and the staphylococcus phagocytosis test of in vitro washed leukocytes. The results have confirmed the immunostimulating and hence anti-tumour effect of intravesical BCG vaccine. Monitoring of the cellular immune response is suitable for the continuous follow-up of the BCG effect. Comparing the tolerable side-effects with their favourable therapeutic results, BCG vaccine is considered to be effective for the prevention of recurrences in treating superficial bladder tumours.

While diagnosed, 70–80% of bladder tumours are still in the superficial stage [1]. It is known, that within one year after surgical removal of the tumour the ratio of recurrences is 20–70% [2, 3]. That is why it is necessary to apply a possibly effective therapy following the surgical removal of the visible tumour. Local chemotherapy has been used since 1961. As a result of the tumour-immunological researches of the 1970s, intravesical BCG treatment has been extensively employed in the last two decades [4]. Although BCG treatment has already been known for a decade, several questions, among them first of all its mechanism of action is still not satisfactorily clarified. According to some authors, intravesical BCG vaccine exerts a favourable effect through the intact or injured bladder mucosa, or through the interaction with fibronectin [5, 6]. As a distant effect, it increases interferon and interleukin production, i.e. the anti-tumour mechanisms by activation of macrophages, T-

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* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

Akadémiai Kiadó, Budapest

lymphocytes and K- (killer) cells [7–10]. Nor it is appropriately known when, beside the local effect of BCG vaccine, its immuno-stimulating effect appears and how long it lasts. Accordingly, it remains to be cleared whether BCG treatment is indicated and at what intervals, or else, it is contraindicated. To follow these parameters, the reactivation of the T-lymphocytes and the phagocytotic ability of leukocytes of tumour patients were investigated in vitro.

Patients and methods

Patients. In the period from January 1989 to April 1990, intravesical BCG vaccine was administered to 38 patients with superficial uroepithelial carcinoma (Ta, T1) according to the scheme of Morales [4]. Twenty healthy persons served as control. The examination was made prior to administering BCG, during the treatment and at weekly then, in general, at monthly intervals. Every three months cystoscopic and random bioptic checking were performed, in addition to the chest X-rays and laboratory tests. Average follow-up time was one year.

BCG vaccine. For treatment, 20 mg/ml lyophilized Pasteur vaccine obtained from the BCG Laboratory of the National Institute of Hygiene, Budapest was used (live bacterial count, 2.4×10^6/ml).

Lymphocyte transformation test (LTT). The method applied was described earlier [11]. One part of the separated lymphocytes was cultured without antigen (control). To the other part of T-lymphocytes Difco-P Phytohaemagglutinin (PHA) was given, which is a non-specific mitogenic substances. In addition, T-lymphocytes in some cases were cultured also directly in vitro with BCG vaccine. On evaluation, the ratio of cells transformed into lymphoblast was compared to the intact T-lymphocytes.

Phagocytosis. The immune effect was measured also by the phagocytosing ability of in vitro washed leukocytes [12]. The percentage of leukocytes capable of phagocytosing staphylococci, and the number of bacteria ingested by one leukocyte was assessed.

Results

Depending on the tumour stage, the PHA stimulability of T-lymphocytes as compared to the controls (mean 65–85%) in the patients with superficial bladder tumour was found to be reduced (46–60%). Following BCG vaccine treatment, this value – that expressed the cellular immune response – approached the normal (Fig. 1).

During the follow-up study, it was observed that favourable immunoreactivity following administration of BCG decreased after some months (as an average: 3.5 months), then by repeated administration (1×120 mg), the stimulability of T-lymphocytes again increased (Fig. 2).

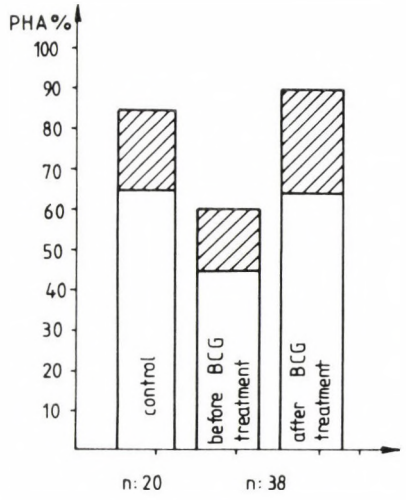

Fig. 1. Change in the stimulability with PHA of lymphocytes due to BCG treatment

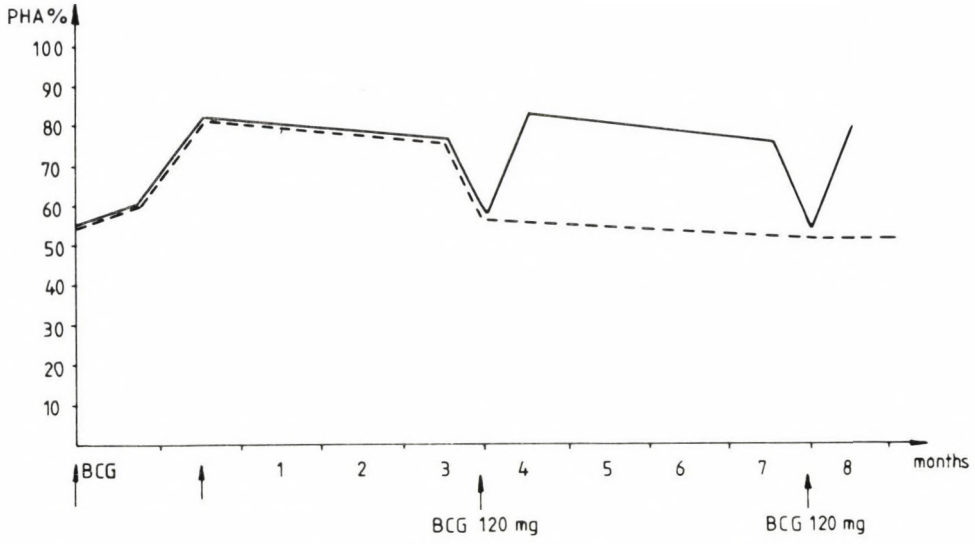

Fig. 2. Change in the stimulability with PHA of lymphocytes during BCG treatment. ----- 6-week maintenance therapy; - - - - without 6-week maintenance therapy

It is notable that in patients, who did not show an adequate immune response to repeated BCG administration, recurrences occurred more often (Table I). In other cases morphological and functional impairment of the cells were observed. In this patient group side-effects occurred at a higher rate. In such cases BCG vaccine was not administered.

Table I

Survey of results obtained with intravesical BCG treatment after TUR; mean follow-up time: 1 year

Total number of patients: 38			
Recurrence within one year		Free of recurrences	
12	31%	26	69%
Reduced PHA stimulability			
9 of 12	75%		

The bacterium phagocytosing ability of some patients, similarly to the LTT, diminished (30–35%) as compared to the control (70–75%). However, after treatment this value approached the normal value-like lymphoblast transformation (Fig. 3).

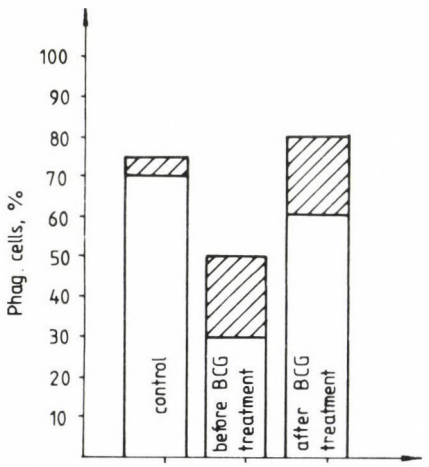

Fig. 3. Change in the phagocytosing ability of polymorphonuclear cells as an effect of BCG treatment

Discussion

It is known that stimulation of the weakened immune system in tumorous diseases favourably influences the disease [13, 14]. Besides levamisol, haemocyanin and interferon [15, 16], interest is now undoubtedly focussed on BCG vaccine as a non-specific stimulant in relation to bladder tumours [17]. Results obtained so far reflect that, in superficial bladder tumours, postoperative intravesical BCG vaccine treatment causes a more complete remission in a higher percentage of cases, even despite the more serious side-effects, than do Adriamycin, Thiotepa, Epodyl, Doxorubicin and Mitomycin-C [18–20].

Although recently a considerable amount of experience has accumulated concerning intravesical BCG treatment (the strain to be selected, treatment protocol, dosage etc.), its mechanism of action is still debated. A great part of authors agree that BCG, as a non-specific immunomodulator, has a local and general effect [3, 17, 21].

Studies with monoclonal antibodies of the diffuse inflammation arising after BCG treatment, as well as of the suburothelial granulomas appearing later have verified, as opposed to the advocated theory of cell-detachment after simple inflammation, the local immunostimulating effect of BCG. Besides the quantitative difference, an essential qualitative difference was also found in the immunohistochemical picture of the non-specific or cytostatic cystitis [22].

Following treatment, mostly lymphocytes (T-helper) suppressor (1:2–2:1) and plasma cells were found to be present in the mucosa and the submucosa, but also polynuclear giant cells, histiocytes and neutrophils, appeared. It is notable that these changes, supposed to be related with the effectiveness of BCG, were observed permanently if the patients received a maintenance therapy every three months after the 6-week treatment [23]. Other, however, question the necessity of maintenance therapy [24, 25].

The immune response of the host organism is elicited by the circulating macrophages. The macrophages, besides their anti-tumour cell-destructive ability, activate cytotoxic lymphocytes, and K-cells through the growth of interferon and interleukin production. There have been scarce reports on the changes in peripheral blood. Similarly experiments in animal, in patients after BCG therapy a significantly higher anti-BCG antibody titre was found in the blood and the changes were more sensitive indicators than the PPD (Purified Protein Derivate) skin test [26, 27].

The applicability of PPD skin test was also questioned by others. In 1987 Nissenkorn determined the monocyte activation of peripheral blood after BCG treatment. He found that in patients where the activation of monocytes was of a smaller extent, there were more recurrences. The isolated monocyte cytolytic factor from human lymphocytes treated with BCG elicited a considerable anti-tumour

effect against the bladder tumour cells [10]. Ruitenberg verified in animal experiments the macrophage and lymphocyte function-stimulating effect of BCG preparations. The maximal lymphocyte stimulation after BCG administration was found by him to be 14–21 days [28].

Our results have confirmed observations stating that, in addition to its local effect, intravesical BCG vaccine has an immunomodulatory effect. The effect in the immune system becomes evident immediately after starting treatment, increasing abruptly from the 2nd–3rd weeks onwards and lasting for some months (on average 3–4 months). On repeated administration of BCG vaccine (120 mg) the decreasing immune response can again increase. That is why maintenance therapy is considered necessary.

In addition to the generally favourable stimulating effect, BCG vaccine occasionally produces morphological and functional impairment of the immune cells. Therefore, in such cases, it is not recommended to administer BCG for the risk of more severe side-effects.

In cases where the patient's immune cells cannot be mobilized even on repeated BCG treatment, there is a greater risk of recurrence, and it may be an alerting sign for changing therapeutic strategy. Finally, it can be stated the study of cellular immune response is suitable for following the efficiency of BCG therapy and for the selection of the maintenance therapy.

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INFLUENCE OF THE THYMUS AND THE NORMAL MICROFLORA ON THE PLASMA FIBRONECTIN CONCENTRATION IN MICE*

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The plasma fibronectin (pFN) concentration (cc) of untreated genetically or artificially athymic mice, or treated with TP-4 (thymus hormone sequence analog synthetic preparation) showed no significant difference from their euthymic or untreated controls. In contrast, the pFN cc in mice with different microbiological state showed significant alterations; the highest level occurred in conventional mice and the lower level in germfree mice was increased by bacterial monocontamination. The alternation from SPF into conventional state in nude mice also resulted in the increase of the pFN cc. Based on these and earlier results, it was assumed that the pFN cc is independent from the presence or absence of the thymus, but it depends on the actual microbiological state of the macroorganism.

Plasma fibronectin (pFN) is a high molecular mass adhesive glycoprotein produced by several cell types. It is found in an insoluble form on the surfaces of various types of cells, associated with basement membranes and connective tissue, and it is present in a soluble form in plasma and in other body fluids [1–7]. The pFN is the ligand of VLA (very late antigen) – 5 and many other receptor molecules in the integrin superfamily [8–10]. The actual level of pFN depends among others on the age of the animal. According to our earlier results its concentration (cc) was higher in old mice than in young ones [11]. In contrast, in earlier experiments we proved that the pFN cc in germfree (Gf) mice is significantly lower, independently of

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* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

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age, than in conventional (Cv) animals, and their pFN level increased after stimulation with microbial immunomodulants [11–13]. We assumed that the low pFN level in Gf mice might be due to the lack of normal microbial flora and that the normal microbial flora could increase the pFN level.

It is well known that the immune system of Gf mice is undeveloped, their lymphocyte count in peripherial blood is low. The function of their thymus is insufficient, their cellular immune reactions are decreased [14–16]. Based on these results, we thought that the insufficient thymus function – beside the absence of normal microbial flora – might have a role in the low pFN level of Gf mice. Since it is not known whether the thymus has an influence on the cc of pFN, the present study was undertaken to examine whether the thymus takes part in the control of pFN level in mice.

To examine the role of the thymus, we determined the pFN cc in athymic nude mice, in neonatally thymectomized young adult mice, in SPF nude, in conventional normal mice, and in mice treated with a synthetic thymus hormone component (TP–4). To study the significance of the normal microbial flora, the pFN cc was determined in Gf monocontaminated with *Peptostreptococcus*, in Cv mice, in SPF nude and conventionalized nude mice.

Materials and methods

Experimental animals. The experiments were carried out in young adult mice of both sexes (LATI, Gödöllő). We used SPF and conventionalized Balb/c nude, SPF and normal Balb/c, Gf, monocontaminated, and Cv C3H, neonatally thymectomized and sham thymectomized young adult CFLP, TP–4 treated and untreated SPF nude and Cv CFLP mice. The Gf and SPF mice were kept in isolators, the Gf state and its control were provided according to references [17].

Monocontamination. Gf mice were inoculated orally with *Peptostreptococcus* that had been isolated from the coecum of SPF mice, and was cultivated anaerobically. Each mouse received approximately 10^6–10^8 viable organisms suspended in 0.5 ml PBS. The bacteria colonized the gut continuously and induced the reduction of coecum size.

Conventionalization. Two weeks before having determined the pFN cc, SPF mice were placed into the conventional animal room.

Neonatal thymectomy was carried out in the first 24 h after birth by Miller's method [18]. Sham thymectomized mice served as controls. The athymic state was controlled by autopsy at the end of the experiment, data of mice with thymus residuum were omitted from the results.

Treatment with TP–4. TP–4 is a synthetic product (Gedeon Richter Pharmaceutical Products Co., Budapest), tetrapeptide analogue of the natural thymopoietin sequence (Arg-Lys-Asp-Val) [20]. The treatment of mice with TP–4 was carried out by intraperitoneal injection in 2 mg/kg dosis, 7 times during two weeks. pFN cc was determined 3 days after the last injection.

Determination of mouse plasma fibronectin level. Blood was taken from the caudal vein using sodium citrate as anticoagulant. pFN cc was determined from individual plasma by electroimmunodiffusion [20].

Statistical evaluation. Student's *t* test was applied. The accepted significance level was $p = 0.05$.

Results

The pFN cc determined in Balb/c nude, and Balb/c normal, neonatally thymectomized and sham thymectomized, TP-4 treated and untreated Balb/c nude and CFLP normal mice are seen in Figs 1 and 2. Each group contained ten mice. According to the data, there were no significant differences in pFN cc between athymic SPF nude and euthymic (C) SPF Balb/c mice, between thymectomized (T) and sham thymectomized (ST) young adult Cv mice, between TP-4 treated and untreated control (C) SPF athymic mice and finally between TP-4 treated and untreated control (C) CPFL Cv euthymic mice.

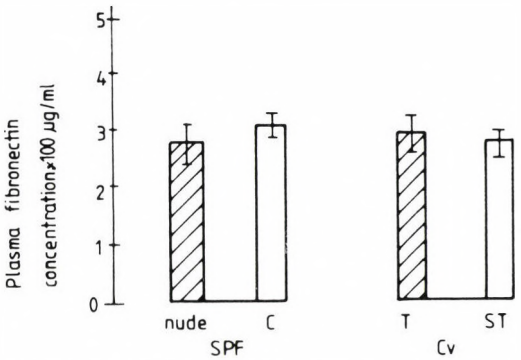

Fig. 1. Plasma fibronectin cc of athymic and euthymic mice. (nude: Balb/c n/nu, C: Balb/c euthymic; T: neonatally thymectomized, ST: neonatally sham thymectomized)

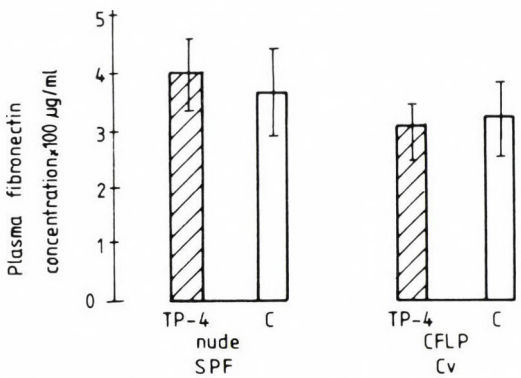

Fig. 2. Plasma fibronectin cc of TP-4 treated and untreated athymic (Balb/c nude) and euthymic (CFLP) mice. (TP-4: treated with TP-4, C: untreated)

The pFN cc of mice with different microbial flora are shown in Fig. 3. Each group contained 8 mice. Comparing the data of Fig. 3, the Gf mice had the lowest pFN level that could be stimulated by monocontamination with *Peptostreptococcus.*

There was a significant increase in pFN cc of nude mice after conventionalization from SPF into Cv state.

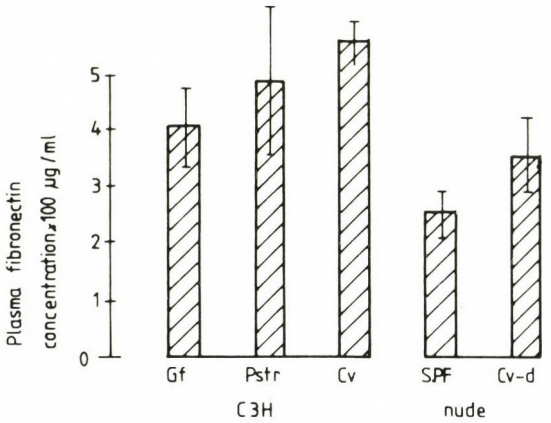

Fig. 3. Plasma fibronectin cc of mice with different microbiological state. (Gf: germfree; Pstr: monocontamined with *Peptostreptococcus*; Cv: conventional; SPF: Specific Pathogenic Free; Cv-d: conventionalized)

Discussion

We found no significant difference between the pFN cc values of mice without thymus, or stimulated with TP-4 and their proper controls.

The TP-4 preparation had no effect on pFN level, nevertheless, its cellular immune response stimulating effect could be proved by us [24] and by several other authors [21-23]. This means that neither the lack of thymus (genetic or artificial) nor the treatment with thymus-hormone fragment had pFN cc influencing effect in mice with the same microbial background.

Altering the microbial state, we could change the pFN level both in euthymic and athymic mice: monocontamination of Gf mice with *Peptostreptococcus*, or conventionalization of SPF nude mice resulted in a higher pFN cc. These results are in accordance with our earlier data connected with the pFN cc increasing effect of NDV vaccine in Gf mice, and of Zymosan (Mannozym) in Gf and Cv mice [11-13]. Consequently, the actual level of pFN is not associated with the presence or absence of the thymus, but depends on the actual microbial state, on the presence or absence of the normal microbial flora.

One of the most important biological effect of the pFN is its opsonic activity. Phagocytic cells with their FN-binding receptors could bind and phagocyted opsonized debris, digested tissue-elements, different blood-particles, bacteria and

bacterial proteins [5, 6, 25]. Furthermore, pFN also mediates the motility and soluble mediator production of phagocytic cells [5, 6, 26]. The effective operation of mononuclear phagocytic system depends on the concentration of pFN. The phagocytic activity of this system is impaired when its cc is reduced and improved if its level is increased [1, 2, 5–7, 27]. It means that the protection of the macroorganism against microorganisms depends on the actual level of pFN, and this actual level depends on the actual microbiological state. There is a positive correlation between the normal microbial flora, and the actual level of the pFN: lack of the normal flora resulted in low pFN cc, and this low level could be increased by the stimulatory effect of microorganisms and their products. This positive correlation appears both in athymic mice, indicating that the thymus has no influence on the pFN level.

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CONTENTS

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CYTOTOXIC HUMAN LYMPHOCYTES: FROM IN VITRO TESTING (1970s) TO IMMUNOTHERAPY (1990s)*

(A Review)

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(Received March 1, 1993)

The senior author was the recipient of a contract (1–CP3–3292) from the National Cancer Institute, USA (NCI) in the early 1970s. The aim of NCI's targeted research program was the establishment of a tumour-specific human lymphocyte-mediated cytotoxicity assay. Neither lymphocyte growth factors nor monoclonal antibodies for lymphocyte typing were available. Tumour-specific populations of lymphocytes could not be maintained but their presence in ficoll-hypaque preparations of blood buffy coats or in primary cultures of tumours was clearly recognized. Another indiscriminately cytotoxic population of lymphocytes had usually overridden the tumour-specific population. In contradistinction to the ruling doctrine of the era, indiscriminately cytotoxic lymphocytes were readily found in the blood of tumour-bearing patients and healthy individuals (the senior author's lymphocytes were shown to practice indiscriminate cytotoxicity in 1971, an observation first interpreted as "immune

* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992), director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

Based on presentations at "Specific Immunotherapy of Cancer with Vaccines" of the New York Academy of Sciences, Washington, D. C. January 21–24, 1993; and the "Second International Congress on Biological Response Modifiers" of Inter-American Society for Chemotherapy, San Diego, California, January 29–31, 1993.

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surveillance at work" in an individual daily exposed to patients with metastatic cancers). Instead of converting the subject matter of the contract from a tumour-specific to a non-specific cytotoxicity assay, the NCI prematurely "phased it out" (but continued the project as intramural research). Nevertheless, many functions of cytotoxic lymphocytes that had become by now well established were foreshadowed during the early 1970s with the limited support of that NCI contract and funds from other sources.

Here we recount those early observations; present the outlines of adoptive immunotherapy with various autologous lymphocyte populations and in a separate report in this volume give a technical description how these lymphocyte populations are prepared in the laboratory for therapeutic reinfusions into the patient.

I. An Overview of Early Contributions

Textbooks of the 1940s described the "reticulo-endothelial system" as the producer of antibodies. In the 1950s, antibody production in regional lymph nodes of antigen administration was discovered. In the 1960s, the dichotomy of the immune system was recognized and diversified functions of thymus-dependent (T) and bursa-dependent (B) lymphocytes were outlined. Cell-mediated cytotoxicity and lymphokine-production in delayed hypersensitivity reactions were attributed to T lymphocytes; antibody production remained the function of B lymphocytes maturing into plasma cells. In the 1970s, monoclonal antibodies permitted the recognition of subclasses of T and B lineages. Memory cells and suppressor cells were formally avowed. In the 1980s, the structure of B and T cell receptors and their genetic rearrangements were identified. Antigen processing and presentation to the T cell receptor (TCR) as peptides in the groove of MHC molecules were visualized and understood. It has become clear that natural killer (NK) cells acted without, and immune T cells with, MHC-restriction. Two major mechanisms of target cell destruction by lymphocytes were distinguished: tube-forming proteins (perforins) piercing through the target cell membrane and lysing the cytoplasm; and tumour necrosis factors alpha and beta triggering endogenous endonuclease synthesis leading to condensation and fragmentation of DNA in the nuclei of target cells: programmed cell death or apoptosis. In the 1990s, lymphokine-activated killer (LAK) cells and clones of immune T cells expanded by interleukin-2 (IL-2) have become treatment modalities of malignant tumours.

In the following, data from the early 1970s will be briefly recalled to show that by now well established features of lymphocyte function were then already conjectured in their early outlines [1, 2]:

(1) by morphological observation of dying target cells, the co-existence of different mechanisms of lymphocyte-mediated target cell destruction was surmised: the phenomena of cytoplasmic and nuclear lysis (illustrations published elsewhere) [1, 3];
(2) killer lymphocytes remained viable and could perform target cell destruction repeatedly (molecular mediators were suspected but the biotechnology of their documentation was beyond our capabilities);
(3) specifically and indiscriminately cytotoxic lymphocytes coexisted in the circulating blood (and there were no means to separate or subclassify them before the availability of monoclonal antibodies) [2, 4];
(4) patients with sarcomas, melanoma and other solid tumours (nasopharyngeal carcinoma, kidney carcinoma, lymphoma incl. Hodgkin's disease) possessed lymphocytes that attacked autologous malignant cells in primary tissue cultures while sparing their normal fibroblasts [5];
(5) these patients also possessed lymphocytes cytotoxic to tumour cells unrelated to their tumour types [6];
(6) healthy individuals circulated lymphocytes indiscriminately cytotoxic to various tumour cells of established cell lines [7, 8]: for example, see Fig. 1 showing the senior author's lymphocytes kill tumour cells on August 19, 1971 and Fig. 2 showing antitumour cell cytotoxicity of lymphocytes from a patient free of malignancy [2];
(7) larger lymphocytes with granular cytoplasm exerted cytotoxicity on allogeneic cells and frequently but not exclusively practiced cytoplasmic lysis;
(8) whereas smaller lymphocytes killed autologous tumour cells and frequently but not exclusively killed through nuclear lysis [1];
(9) cytotoxic lymphocytes could be generated in vitro by exposure to tumour cells (in ficoll-hypaque preparations which inadvertently contained macrophages), but these clones could not be maintained in permanent cultures or expanded without T cell growth factor discovered later and now known as IL-2 [2]; and
(10) serum factors (antibodies) existed that could either block or intensify lymphocyte-mediated cytotoxicity [2, 9ab].

An apparently non-immunological interaction between lymphocytes and autologous connective tissue cells in culture was also observed. In this relationship, lymphocytes divided and formed colonies in the cytoplasms of fibroblast-like cells (Fig. 3). Cytoplasmic bridges between lymphocytes and host cells were formed. Granules traveling through these bridges (seen in dark field microscopy) stained yellow with acridine orange [10-12]. A "feeder layer" mechanism was postulated.

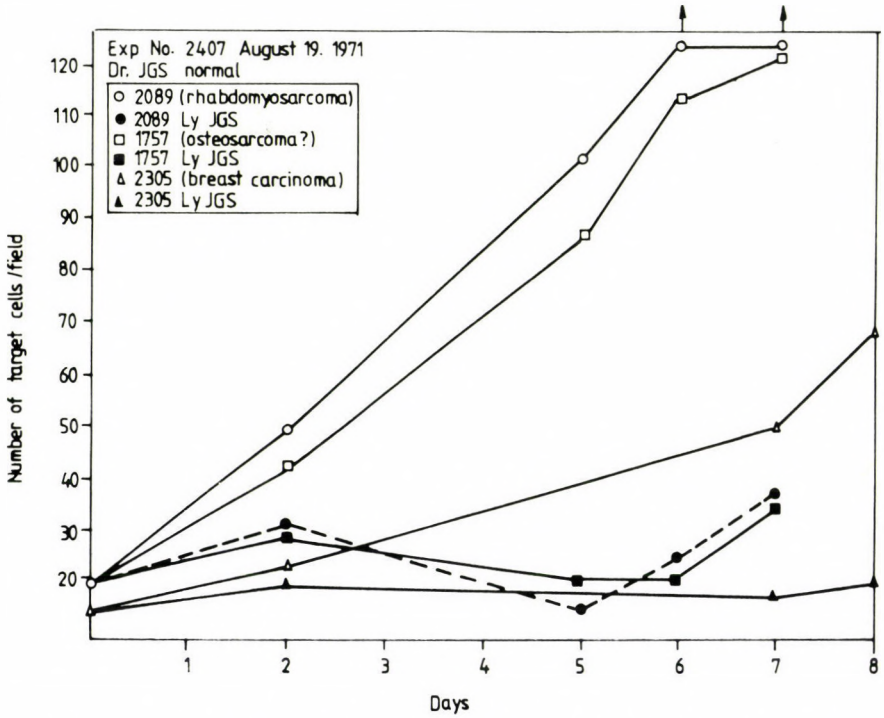

Fig. 1. In an experiment conducted on Aug. 19, 1971, the senior author's buffy coat lymphocytes inhibited the growth of three allogeneic tumour cell lines (rhabdomyosarcoma, osteosarcoma and breast carcinoma) [2]

In the mid-1970s, in patients immunized with X-irradiated tumour cells or viral oncolysates [13], an increase in the population of cytotoxic lymphocytes, a decrease in blocking, and an increase in intensifying antibodies (in terms of lymphocyte-mediated cytotoxicity) were observed. Correlation of these parameters with longer survival was suggestive but not fully proven: some patients relapsed despite good antitumour immunity demonstrable in vitro, and increased populations of cytotoxic lymphocytes were not always specific only to the patient's type of tumour [13].

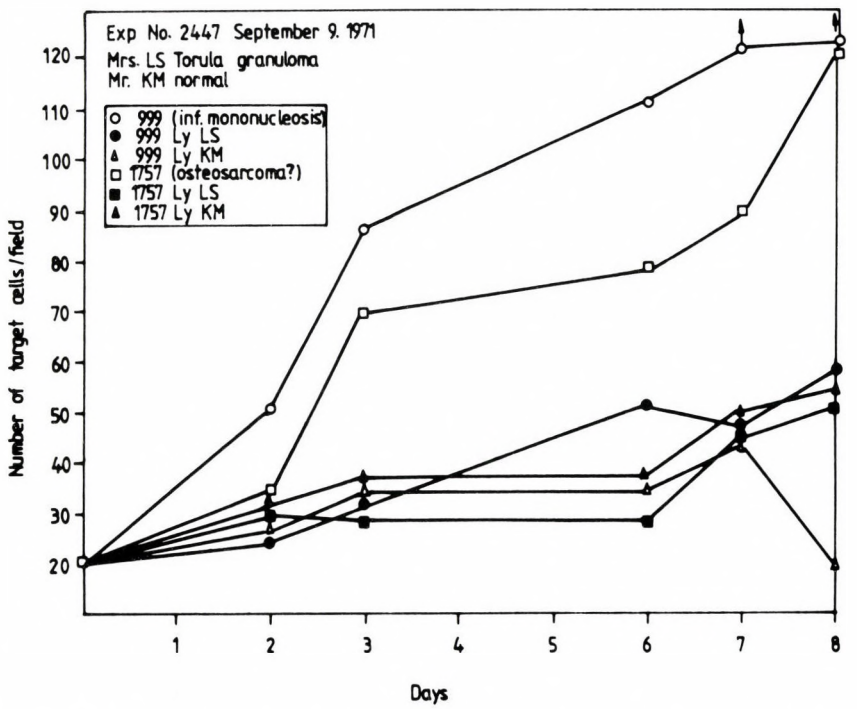

Fig. 2. In an experiment conducted on Sept. 9, 1971, the buffy coat lymphocytes of a patient (LS) with cryptococcus granuloma of the frontal bone but without malignancy inhibited the growth of two established human cell lines; the lymphocytes of a healthy laboratory technologist (KM) also inhibited the growth of these cell lines [2]

II. Neoplastic Growth of NK Cells

In 1969–70, a male patient with "lymphosarcoma cell leukemia" was attended [14]. He was emaciated, had low grade fevers, arthralgias and night sweats but his blood and urine cultures yielded "no growth". His neoplastic cells were large and possessed azurophilic cytoplasm with fine granulation. These cells were indiscriminately cytotoxic to a battery of cultured human cancer cells. His blood serum and the supernatant fluid of his cultured neoplastic lymphocytes were also cytotoxic to various target cells. His case was reported as "lymphocytic cytotoxins in wasting syndrome" [14] and as a new entity "cytotoxic lymphoma" [15]. It was concluded that neoplastic lymphocytes produced autonomously cytotoxins in vivo [14]. When this patient was attended, the terminology of "human NK cells" or tumour necrosis factors alpha and beta did not as yet exist.

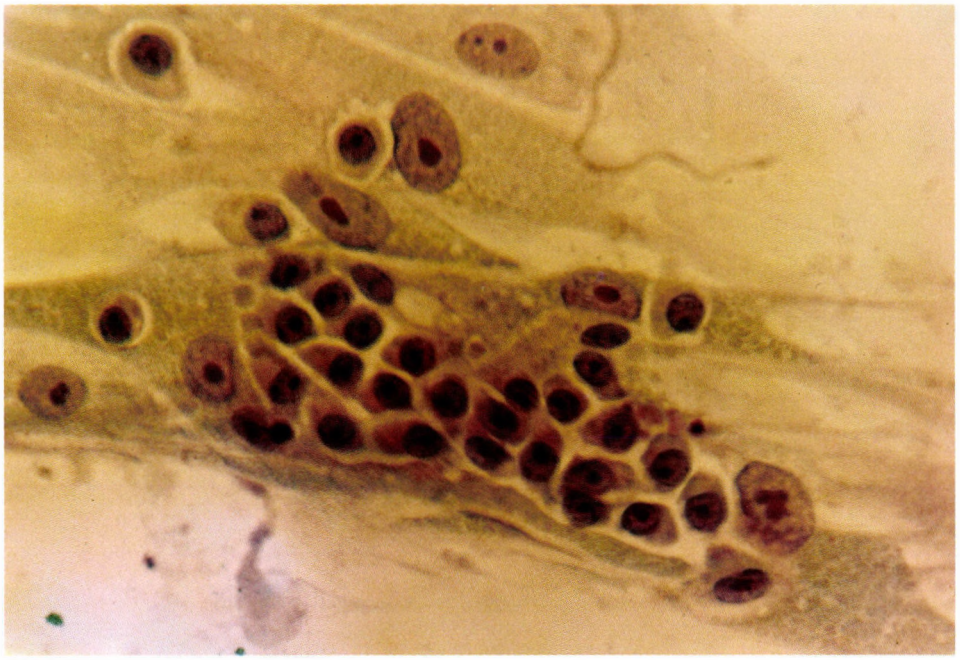

Fig. 3. A colony of small lymphocytes resides in a large cytoplasmic vacuole of an autologous fibroblast in a primary culture of lymph node from a patient with chronic lymphocytic leukemia: the "feeder layer principle" (similar pictures of the same series were published in "The Proliferation and Spread of Neoplastic Cells", Williams and Wilkins, Baltimore 1968. pp. 91–97)

Thereafter, cases of neoplastic transformations affecting the large, azurophilic granular lymphocytes, now known as NK cells, were described (for case reports from 1980–84 see previous review [2]). Even though our patient was presented in 1971 in Milan, Italy [15], the first report on Italian patients published in 1984 [16] did not contain a reference to our patient. Some of the Italian patients with this syndrome circulated antibodies to p19 and p24 antigens of HTLV–I and II [17]. No reagents were available to type the lymphocytes of our patient in 1971, but the morphology of his neoplastic lymphocytes and their indiscriminate cytotoxicity were clearly shown [15].

Recent reports subclassify the syndrome into type A (T) and type B (NK) categories [18]. The neoplastic cells of type A patients display $CD2^+$, $CD3^+$, $CD8^+$, $CD11b^-$, $CD16^-$ ($Leu-16/NKH-1^-$) and $CD57^+$ ($Leu-7^+$) phenotype. Rheumatoid arthritis and autoantibodies prominently feature in the clinical picture [18]. A patient with type A disease died rapidly after low dose cyclophosphamide treatment. At necropsy, polyclonal proliferations of EBV^+ lymphoblasts in various organs and ulcerations of the GI tract were found [19]. In another case, the

phenotype of the neoplastic cells was $CD3^+$, $CD4^-$, $CD8^-$, $TCR\alpha\beta^-$, $TCR\gamma\delta^+$ and $CD16^+$. These malignant cells exhibited NK cell cytotoxicity by killing ^{51}Cr-labelled K562 target cells [20]. Another patient with $CD3^+$, $CD8^+$, $CD57^+$ neoplastic large granular lymphocytes harboured HTLV-II genomic sequences *gag*, *pol*, *env*, and *pX* and antibodies to HTLV-II structural protein gag [21]. The neoplastic cells of type B (NK) patients display $CD2^+$, $CD3^-$, $CD8^-$, $CD11b^+$ ($OKM-1^+$), $CD16^-$ and $CD56^+$ phenotype [22]. Thus heterogeneity and overlap between these two types have been reorganized.

III. Therapeutic Use of Lymphocytes

Therapeutic administration of human lymphocytes without growth factors was doomed to failure in the 1970s (for review see previous publications) [23a]. Our Solid Tumour Service (now Melanoma-Sarcoma Service) at M. D. Anderson Hospital was also engaged in early clinical trials of "lymphocyte therapy" [23b]. Only after the discovery and clinical application of T cell growth factor (IL-2), could improved results be expected. All three major subclasses of lymphocytes (LAK cells; immune T cells; tumour infiltrating lymphocytes) now in use for adoptive immunotherapy respond to IL-2 with expansion of their clones [24].

Adoptive immunotherapy in animals achieved good results even without growth factor co-administration because lymphocytes could derive from syngeneic donors actively and specifically immunized against the tumour. Spleen and lymph node (or bone marrow) cells of donor mice actively immunized with photodynamically killed mouse leukemia virus (MLV) vaccine could readily transfer anti-leukemia viral immunity to recipients preinoculated with the mouse leukemia virus [25] or render mice resistant to challenge with MLV or leukemic cells [26].

In human practice, the donor cells are autologous and so far have not been collected after tumour specific immunotherapy. It is presumed, or shown by in vitro assays, that lymphocytes reacting to autologous tumour cells by undergoing blastogenesis or by exerting cytotoxicity on the tumour cells do exist in the population of lymphocytes expanded by IL-2 for reinfusion into the patient.

Human LAK cell precursors are nonadherent, E rosette negative with the phenotype of $OKM-1^-$, $OKT-3^-$, $Leu-7^-$, $Thy-1^-$, Ia^-, sIg^- and collected from blood (but are present in all lymphatic organs). LAK effector cells remain nonadherent, $OKM-1^-$, $OKT-3^-$, but express $Thy-1^+$. LAK cell cytotoxicity is not MHC-restricted and is operational against autologous and allogeneic tumour cells. Adenocarcinoma cells show mediocre susceptibility (20-24% lysis); lymphomas and sarcoma cells are susceptible (41-50% lysis); adrenal and esophageal carcinoma cells are highly susceptible (68-78% lysis). These in vitro data did not readily translate into clinical efficacy; in clinical trials, melanomas and kidney carcinoma

proved to be the most treatable tumours [27], whereas sarcomas and carcinomas remained resistant. Results recently tabulated show 6 CR and 25 PR in 31 patients with metastatic melanoma treated with autologous LAK cells and IL-2 [24]. In 43 patients with kidney carcinoma, 14 CR and 29 PR were induced by this therapy [24]. Toxicity of intravenously administered IL-2 can be substantial with pulmonary capillary leakage syndrome, pulmonary oedema, oliguria and prerenal azotemia. GI tract toxicity consists of nausea, vomiting and diarrhoea. Haematologic toxicity manifests in anaemia and thrombocytopenia. There are neurological and behavioural side effects. Erythema and desquamation of epidermis may occur.

The traffic of intravenously administered LAK cells in the recipient is the subject of studies and controversies. An adherent subclass of LAK cells (A-LAK cells) is especially controversial as to their access to the liver or to tumour sites [28, 29].

Tumour infiltrating lymphocytes (TIL) are heterogeneous and consist of NK-related cells and MHC-restricted and non-restricted cytotoxic T cells. The majority of TIL extracted from melanoma, glioblastoma, breast, ovarian and renal carcinomas are $OKT3^+$, $OKT8^+$, $Leu-2^+$, $Leu-4^+$ ($CD3^+$) cytotoxic-suppressor T cells ($CD8^+$) in contrast to $Leu-3^+$ helper cells ($CD4^+$) and $Leu-11b^+$ or $Leu-19^+$ LAK cells [30-34]. When freshly isolated, TIL cells lyse autologous tumour cells almost exclusively and with higher efficacy than peripheral blood $CD8^+$ cells. Later after expansion by IL-2, the cytotoxic T cell population begins to react to allogeneic cells indiscriminately [35]. Recently tabulated results with TIL therapy utilizing also IL-2 (with or without low dose cyclophosphamide) are disappointing. Of 39 patients with metastatic melanoma, 15 responded; but of 11 responding patients only one achieved CR [24]. Of 11 patients with kidney carcinoma, three responded. Patients with breast, colon and lung carcinoma did not respond at all [24]. However, lysis of autologous melanoma cells correlated with clinical response. LAK cells lyse equally well tumour cells of clinically responding and nonresponding patients. When TIL lysed autologous melanoma cells in vitro, the patient also responded to TIL infusions [36].

There are two measures that may increase cytotoxicity of TIL to autologous tumour. One is stimulation of TIL growing in IL-2 with irradiated autologous tumour cells [37]. Cytotoxicity thus achieved and sustained resides in a $CD4^+8^+$ double positive population also exhibiting CD3 and is practiced in a MHC class I-restricted manner. Anti-CD3 monoclonal antibody inhibited cytotoxicity. Cytotoxic TIL produced interferon-gamma and TNF-alpha but not IL-1beta, IL-4 and IL-6. The target cells in this study were those of kidney carcinoma [37]. In contrast, TIL obtained from melanomas do produce IL-4 [38].

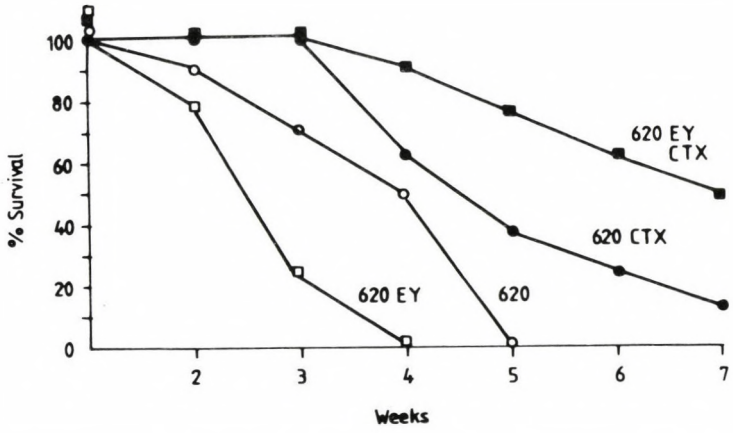

Fig. 4. Inhibition and delay of growth of mouse lymphoma #620 in mice pretreated with embryonic cells (EY) and low dose cyclophosphamide (CTX) as presented in 1978 [40]. Injection of Embryonic Tissue Accelerates Growth of Transplantable Mouse Lymphoma. Injection of Embryonic Tissue and Cyclophosphamide Retards Growth of Lymphoma. o Mouse lymphoma # 620 $10^{7.3}$ cells i.p.; • Mouse lymphoma # 620 $10^{7.3}$ cells i.p. cyclophosphamide (CTX) 1.5 mg i.v.; □ Mouse lymphoma # 620 $10^{7.3}$ cells i.p. Mouse embryo cells (EY) i.p.; ■ Mouse lymphoma # 620 $10^{7.3}$ cells i.p. EY i.p., CTX 1.5 mg i.v.

The second measure is the co-administration of low dose cyclophosphamide to patients receiving TIL and IL-2. This principle is usually led back to animal experiments in which lung metastases could be eradicated by this combination [39]. However, much earlier, another relevant observation in favor of low dose cyclophosphamide co-administration was reported (Fig. 4) [40]. An antigenic mouse lymphoma grew faster in mice pretreated with embryonic cells and extracts. Cyclophosphamide alone could not cure this tumour. In mice pretreated with embryonic cells and extracts and with low dose cyclophosphamide, complete rejection of the lymphoma was observed. The interpretation was offered that embryonic and tumour cells alike evoke the expansion of suppressor cells that protect the embryo (on account of its paternal antigen expression) and the tumour (on account of its "tumour antigens") against rejection. These suppressor cells when in the process of rapid expansion, are sensitive to low dose cyclophosphamide. Elimination of these suppressor cells allows for suppression-free tumour rejection [40]. This early concept is seldom cited in the modern literature (even when similar conclusion is reached 15 years later in the same institution where the original experiments where published from) [41].

Lymphocytes accumulate in malignant effusions. This subgroup of lymphocytes is best studied in ascites of ovarian carcinoma. The most prominent population of these lymphocytes express $CD3^-$ (or $CD3^+$), $CD2^+$ ($OKT-11^+$), $CD16^+$ ($Leu-11b^+$) and $Leu-M3^-$ phenotype [42], thus they belong to the NK-LAK class

of cells. Peritoneal exudate cytotoxic T lymphocytes express mRNA for perforin and exert their effect on the target tumour cells through the formation of pore-forming proteins [43]. It was in ascites of patients with ovarian carcinoma where an increase of NK cell cytotoxicity was demonstrated after viral oncolysate immunotherapy [44]. This population of lymphocytes expanded in IL-2-containing media but it did not practice MHC-restricted cytotoxicity [45]. A population of T cells specifically cytotoxic to autologous tumour cells also appeared in ascites and recognized in an MHC-restricted fashion peptide antigens individually specific for ovarian carcinoma [46]. Immune T cells can be extracted from tumours, regional lymph nodes of tumours [47] or generated in vitro. Table I summarizes the most prominent surface markers of cytotoxic human lymphocytes.

Table I

Surface markers of human lymphocytes cytotoxic to tumours

Lymphocytes	Markers	Tumours
NK cells	$CD3^-CD56^+$	Allogeneic (autologous)
T non MHC-restricted	$CD3^+CD56^+$	Autologous
T MHC-restricted or non-restricted	$CD3^+CD8^+$ $TCR\alpha/\beta^+ \gamma/\delta^-$	Autologous

A very large body of references exists showing the efficacy of such lymphoid cell populations in recognizing MHC-presented viral peptide antigens and in eliminating virally infected cells expressing these antigens. HIV peptide epitopes, including those of the envelope protein, can induce $CD8^+$ lymphocytes cytotoxic to HIV-infected cells in an MHC class I-restricted fashion [48-50]. $CD8^+$ lymphocytes specifically recognizing HIV pX protein circulate in the blood of AIDS patients [49]. HIV mutants resistant to CTL develop naturally in vivo [51]. Glycoproteins, neuraminidase, haemagglutinin, nucleoprotein and matrix proteins of influenza viruses generate such populations of lymphocytes which recognize and destroy virally infected target cells [52-57]. Naturally processed [58] or short synthesized peptides expressed on the cell surface by MHC class I molecules can replace the larger target molecules as they trigger recognition and reaction of $CD8^+$ cytotoxic T cells [59-61]. In the absence of $CD8^+$ lymphocytes, $CD4^+$ cells can also accomplish clearing of influenza virus-infected respiratory epithelial cells in the mouse [62]. Excess peptide epitopes interfere with the activity of specific cytotoxic $CD8^+$ T cells [63], thus overimmunization with a virus may be counterproductive.

Transforming gene product proteins of oncogenic DNA viruses are also subject to attack by cytotoxic MHC class I-restricted T lymphocytes. E1A-specific T

lymphocytes can destroy large adenovirus-induced tumours [64]. Point mutations of the large transforming (T) antigen of simian virus 40 result in the loss of CTL recognition sites from the antigen. Tumours with mutated T antigens become insensitive to CTL [65]. Point mutation in the antigen coding sequences of lymphocytic choriomeningitis virus leads to "escape mutants" of the virus [66]. Herpes simplex virus-infected fibroblasts resist CTL by inactivating CTL through a contact mechanism and not by a soluble molecular mediator [67]. These escape mechanisms of virally infected cells from CTL have broad implications in regard to virus or viral oncolysate therapy of cancer and to the use of expanded clones of immune T cells for lymphocyte therapy of cancer.

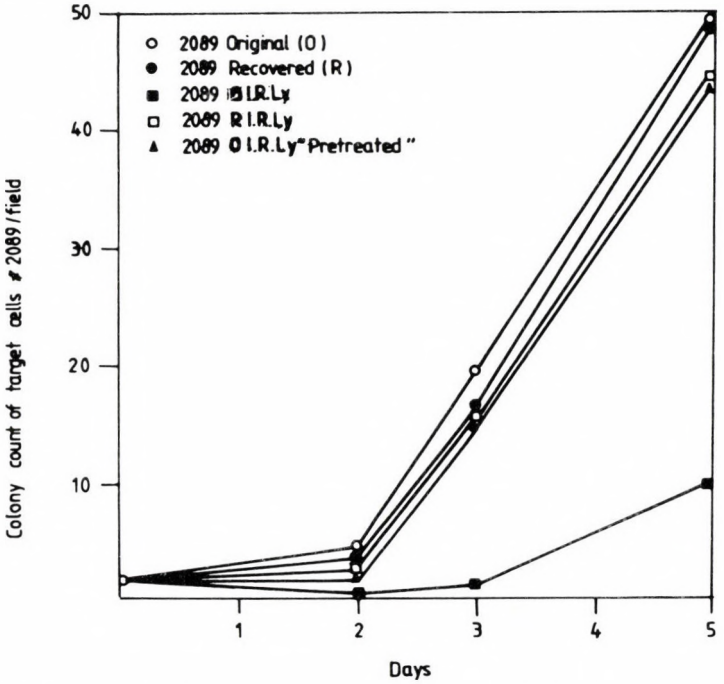

Fig. 5. Experiments with rhabdomyosarcoma cell line # 2089: O = the original cell line in passage; R = subline recovered from a culture exposed to cytotoxic lymphocytes; the recovered (R) cell line grew in the presence of cytotoxic lymphocytes. IRLy = lymphocytes of a patient with rhabdomyosarcoma stage IV who entered durable CR. IRLy inhibited the growth of # 2089 O, but fail to inhibit # 2089 R. IRLy fail to inhibit # 2089 O after pretreatment with supernatant fluid of # 2089 R [3]

It was in 1971 when resistance acquired in vitro of human tumour cells toward cytotoxic lymphocytes was first observed (Fig. 5) [3]. The phenomenon was elaborated on later [68] and it was attributed to solubilized tumour antigens that pre-empt antigen receptors of the lymphocytes before contact between the attacker and

target cells could be made. A special project was registered for the study of the phenomenon [69] and the National Cancer Institute approved the grant application; but again without funding. This early observation is seldom quoted in the modern literature [70]. Instead, examples of tumour cells' escape due to antigenic mutations or due to nonexpression of MHC molecules are cited. Indeed, susceptibility of tumours to CTL could be restored by retroviral vectors transfecting the cells with an excess dosage of MHC genes [71, 72].

The complexity of tumour cell eradication by immune T cells is evident from studies on the Friend leukemia model FBL-3 [73]. Tumour-specific $CD4^+$ cells activate macrophages through interferon-γ production; $CD8^+$ cells also secreted interferon-γ but only upon co-stimulation by both tumour cells and IL-2 [73].

While LAK cell therapy with IL-2 remains disappointing except for kidney carcinoma or melanoma (for example: in 30 patients with colorectal carcinoma, only 1 CR and 4 PR were noted) [74], a promising new development in the lymphocyte therapy of human cancers is the establishment of cloned autologous melanoma-specific T cell lines [74]. These are either $CD4^+$ or $CD8^+$ MHC class I-restricted T cell populations. Nude mice carrying hepatic metastases of human melanoma cells received melanoma-specific human T cells and IL-2: 24 of 25 control mice and one of 18 treated mice developed fatal metastases [75]. Clones of human cytotoxic T lymphocytes specific for autologous melanoma cells can be massively expanded in vitro and stored cryopreserved in a viable state [76]. Similar $Leu-1^+$, $Leu-2^+$, $Leu-3^-$, $HNK-1^-$, HLA class II clones of cytotoxic immune T cells could be developed when patients' lymphocytes were stimulated with irradiated autologous sarcoma cells and clonally expanded with IL-2 [77].

In a separate report in this volume we describe the laboratory technology for the development of human LAK cells and TIL populations for adoptive immunotherapy as practiced at our institution [78].

Abbreviations

CTL	cytotoxic T lymphocytes
CD	cluster of differentiation
CR	complete remission
HIV	human immunodeficiency virus
HTLV	human T cell leukemia virus
IL-2	interleukin-2
LAK	lymphokine-activated killer
NK	natural killer
MHC	major histocompatibility complex
PR	partial remission

TCR T cell receptor
TIL tumour infiltrating lymphocytes
TNF tumour necrosis factor

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A COMPARATIVE STUDY ON GROWTH IN SOFT-AGAR, ADHERENCE TO GLASS AND HAEMOLYSIS TYPES OF COAGULASE-NEGATIVE STAPHYLOCOCCI*

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Growth properties of coagulase-negative staphylococci in the presence and in the absence of human and rabbit serum in soft-agar prepared in modified Staphylococcus 110 broth were studied. The adherent growth was examined in modified Staphylococcus 110 broth and 1% glucose-meat broth. Of 100 strains examined 69% exhibited diffuse, 18% compact, 7% transient and 6% mixed growth. Compact type colonies were mainly characteristic of *Staphylococcus haemolyticus* strains. The presence of serum failed to influence the types of colony morphology in any of the strains. Sixty-three percent of the strains showed adherent growth; none of the *S. haemolyticus* strains produced adherent growth. The glucose-meat broth, unlike modified Staphylococcus 110 broth, was suitable to study adherence. The coincidence of the compact colony morphology in soft-agar and the absence of adherent growth seems to be a taxonomic sign for the species *S. haemolyticus* and differentiate it from the species *Staphylococcus epidermidis*.

* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904–1992) director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

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In recent years the frequency of coagulase-negative staphylococcus (CNS) infections has considerably increased worldwide [1–7]. This has partly been due to the fact that more and more "biomaterials" such as artificial valves, hip joint, catheters, cannula and other prosthetic devices have been implanted in humans, and CNS have an extraordinary high affinity to these biomaterials [2, 4, 10–14]. In the adhesion, the main role has been attributed to the slime produced by these bacteria [4, 8–10, 15–21]. The other cause of the higher incidence of CNS infections may be the fact that – due to the highly developed medical care – today a number of premature newborns, immune compromised patients, and very old people may be kept in life but these patients are extremely susceptible to the CNS colonizing their own skin and mucosal surfaces as members of the normal flora [2–4, 6, 7].

The pathogenesis of CNS infections is far from a full understanding, although a lot of work has been done [2, 4, 12, 13]. Presumably the infection is associated with a number of pathogenic factors produced simultaneously and/or concomitantly by the cocci on one side, and a special clinical stage on the patient's side. The slime production of CNS and its role in the pathogenesis have been studied by a number of researchers, however, the growth properties of CNS in serum soft-agar (SSA) and in soft-agar (SA) received less attention [22, 23]. Mainly the slime formation by *Staphylococcus epidermidis* has extensively been investigated but growth studies on a high number of strains of other CNS species cannot be found in the literature.

The aim of this work was to examine the growth properties of different CNS species in soft-agar in the presence and absence of human and rabbit serum, and the correlation between colony morphology, adherent growth and haemolysis types. The study was initiated by the fact that in case of *Staphylococcus aureus* the production of diffuse colonies in serum-soft agar (SSA) was taken as an evidence for slime positivity [23, 24].

Materials and methods

Sources of strains. The 100 coagulase-negative strains of different *Staphylococcus* species included in this study were isolated from inpatients treated at departments of the University Medical School of Debrecen, Debrecen, Hungary. Taxonomic identification was performed according to a combined scheme from Kloos and Schleifer [25] and Akatov et al. [26]. The strains were stored at –20 °C in Trypticase Soy Broth (TBS) (Difco Laboratories, Detroit, Mich, USA) containing 15% (v/v) of glycerol. Reference strains were *Staphylococcus saprophyticus* HNCMB 110008, *Staphylococcus haemolyticus* HNCMB 110013, *Staphylococcus hominis* HNCMB 110014, *Staphylococcus xylosus* HNCMB 110021, *S. aureus* HNCMB 110007 and HNCMB 112002 from the National Institute of Hygiene, Budapest, Hungary, *S. aureus* Smith diffuse and Smith compact, M and M-variant, *S. epidermidis* 6A, RP12 (ATCC 35983), RP62A (ATCC 35984), and SP2 (ATCC 35982) kindly donated by G. D. Christensen (Harry S. Truman Memorial Veterans Hospital, Department of Internal Medicine, University of Missouri at Columbia, MO, USA) and B. J. Wilkinson (Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, Il, USA).

Growth media. For general cultivation a glucose-meat broth was used consisting of 5 g Lab-lemco powder (Oxoid Ltd., Basingstoke, Hants., England), 5 g peptone siccum (Hungaropharma, Budapest, Hungary), 10 g glucose, 5 g NaCl and distilled water up to 1000 ml, pH 7.2. This broth was supplemented with 15 g agar-agar (So. Bi. Gel., Budapest) and 10% defibrinated bovine blood (Phylaxia, Budapest) to prepare blood agar. Growth studies were performed in modified Staphylococcus medium 110 (mS110) broth (Difco Laboratories, Detroit, Mich, USA).

Colony morphology in serum-soft-agar (SSA) and soft-agar (SA). A loopful bacteria from agar cultures of each strain was inoculated into 5 ml of TSB and incubated overnight at 37 °C. These cultures were then diluted in physiological saline solution up to 10^{-4}. Hundred µl of the final dilution were promptly inoculated into 10 ml SSA and SA tubes [22] supplemented with 1% (vol/vol) normal rabbit serum and normal human serum (Behring, FRG, and Human, Budapest, resp.) and 0.15% agar (Noble) with mS110 medium. Controls were made in mS110 broth without serum and agar, and in mS110 broth containing serum but free of agar. After thorough mixing, incubation lasted for 48 h at 37 °C in a normal incubator. Readings were made at 24 h and 48 h of incubation.

Estimation of adherent growth. This was made according to Christensen et al. [15]. One loopful of bacteria from overnight blood agar culture was used to inoculate glucose-meat broth and mS110 broth. Standard glass culture tubes previously cleaned with chromic acid were used to assay adherence. Two parallel cultures were performed for each strain in 5 ml broth and incubated for 24 and 48 h. The contents of the tubes were removed, then the tubes were rinsed with physiological saline and stained with safranin or methylene blue or trypan blue to demonstrate the adherent growth. A strain was considered to be strongly adherent if a dense film was present lining the inner surfaces of the tubes. Weak adherence was recorded if the adherent growth formed a less dense film. A strain was considered as non-adherent if no film or only a ring was visible at the liquid-air interface.

Determination of haemolysis type. The method of Adamczyk and Blaurock [27] modified by Meyer [28] was used.

Results

Growth properties in soft-agar. The colony morphology of CNS strains did not differ in the presence of only agar (SA) and in the presence of agar and serum (SSA) either rabbit or human serum was used. Likewise, the growth type of the individual strains was identical after 24 h and 48 h incubation. Table I shows the distribution of the colony morphology in mS110–SA according to CNS species. The same results were obtained in SSA, too. One can see that all strains of *S. epidermidis* grew in diffuse colonies. The diffuse growth types appeared in two forms. One well-known type was shown by all species, except *S. haemolyticus*. The other condense-band-like type of diffuse growth was a feature of *S. epidermidis* only.

Table I

Growth characteristics of different species of coagulase-negative staphylococci in soft-agar prepared with modified Staphylococcus 110 broth

Staphylococcus species	No. of strains tested	Colony morphology			
		Diffuse	Transient	Compact	Mixed
S. epidermidis	22	22	–	–	–
S. hominis	14	7	2	1	4
S. haemolyticus	16	–	2	12	2
S. warneri	5	5	–	–	–
S. capitis	6	4	2	–	–
S. saprophytiocus	12	11	1	–	–
S. xylosus	8	8	–	–	–
S. cohnii	6	4	–	2	–
S. simulans	3	3	–	–	–
S. sciuri	5	5	–	–	–
I. group*	3	–	–	3	–
Total number/%	100	69	7	18	6

* Interspecies group

Note. Colony morphology was identical in soft-agar and serum-soft-agar

In contrast, none of the *S. haemolyticus* strains formed diffuse colonies in SA and SSA. Most of them grew in compact colonies, and this growth feature was very pronounced in more than 75% of *S. haemolyticus* strains. To be sure that compact colony morphology in mS110–SA is characteristic of *S. haemolyticus*, the growth of 10 additional strains from different sources was also examined. These strains grew in compact colonies, too.

On the whole, 69% of the CNS strains formed diffuse colonies in mS110–SA and in mS110–SSA. A few strains of *S. hominis* and *S. haemolyticus* produced mixed colonies even in repeated subcultures, although they showed uniform colonies on blood agar. Some strains of *S. hominis* and *S. haemolyticus* produced mixed colonies even in repeated subcultures, although they showed uniform colonies on blood agar. Some strains of *S. hominis, S. haemolyticus* and *S. saprophyticus* exhibited slight tail colonies [22] which became more marked with time.

Control cultures of all strains in mS110 broth free of agar formed very viscous deposit sticking strongly to the bottom of the tubes after 24–48 h incubation. Above the deposit there was a filamentous growth. This property was not altered by the presence of any kind of serum.

Adherent growth. Cultivation in mS110 broth was not suitable to examine adherence because adherent growth was not present as a film. Instead, as mentioned

above, a mucous deposit was formed at the bottom of the tube. This deposit could only be homogenized by strong vortexing, but it sedimented again upon standing for a while. On the other hand, it slipped sometime out of the tube when the culture was poured out. The deposit of all strains could be stained with the dyes mentioned, but not the walls of the tubes thus cultivation in mS110 broth then staining the tubes seemed not to be an appropriate procedure to differentiate between adherence-positive and -negative strains.

On the contrary, growth of strains in glucose-meat-broth resulted in homogeneous suspensions and a portion of the strains adhered to the inner surface of the tubes, while another group failed to form film on the tube surfaces. The frequency of the adherent growth was identical after 24 h and 48 h incubation, but the film was thicker after 48 h growth and had more contrast when stained.

Table II

The frequency of adherence of coagulase-negative staphylococci in glucose-meat broth

Staphylococcus species	No. of strains tested	Adherence positive total	positive strong	positive weak	negative
S. epidermidis	22	21	15	6	1
S. hominis	14	5	1	4	9
S. haemolyticus	16	–	–	–	16
S. warneri	5	4	–	4	1
S. capitis	6	6	5	1	–
S. saprophyticus	12	12	9	3	–
S. xylosus	8	8	8	–	–
S. cohnii	6	4	2	2	2
S. simulans	3	2	1	1	1
S. sciuri	5	1	1	–	4
I. group*	3	–	–	–	3
Total number/%	100	63	42	21	37

* Interspecies group

Note. The incidence of adherent growth was identical in glucose-meat broth and in glucose-Trypticase soy broth

The incidence of adherence is shown in Table II. *S. epidermidis, S. warneri, S. saprophyticus, S. capitis* and *S. xylosus* strains usually produced adherent growth. In contrast, none of the *S. haemolyticus* strains and interspecies strains did so. On the whole, adherence occurred in 63% of CNS strains and 42% of them were strongly adherent. It is worth to mention that most strains of the *Saprophyticus* group. (*S.*

saprophyticus, S. xylosus and *Staphylococcus cohnii*) [4, 12] exhibited a strong adherence.

Haemolysis type. As Table III shows, 52% of 87 strains examined excreted haemolysins, and haemolysis type E was somewhat more frequent (58%) than type A/E.

Table III

Haemolysis types of coagulase-negative staphylococci

Staphylococcus species	No. of strains tested	Haemolysis type			Non-haemolysing
		All	A/E	E	
S. epidermidis	21	17	2	15	4
S. hominis	14	7	6	1	7
S. haemolyticus	13	13	8	5	–
S. warneri	5	–	–	–	5
S. capitis	6	–	–	–	6
S. saprophyticus	10	4	1	3	6
S. xylosus	4	1	1	–	3
S. cohnii	4	–	–	–	4
S. simulans	2	1	1	–	1
S. sciuri	5	–	–	–	5
I. group*	3	2	–	2	1
Total number	87	45	19	26	42
%	100	51.7	21.8	29.9	48.3

* Interspecies group

Association between colony morphology and adherent growth. As seen in Table IV, the majority of adhering strains grew diffusely in mS110–SA and mS110–SSA, and about 10% formed transient colonies. None of the adherent strains grew in compact or mixed colonies. However, all types of colony morphology appeared with non-adherent strains.

Relationship between adherent growth and haemolysis type. Recent works of Hébert et al. [29, 30] have suggested a correlation between haemolysis type and slime production of CNS strains. For this initiation, we compared the adherence to the haemolytic activity and summarized the results in Table V. A higher proportion of non-adherent strains showed haemolysis than the adherence positives. Haemolysis type A/E was more frequent in non-adhering strains. Of strong adhering and haemolysis strains, haemolysis type E was predominant.

Table IV

Association between colony morphology in soft-agar and adherence to glass in glucose-meat broth of 100 coagulase-negative staphylococcal strains

Colony morphology	Adherence		
	strong	weak	negative
Diffuse	38 (90.4%)	21(100%)	10 (27.1%)
Transient	4 (9.6%)	–	3 (8.1%)
Compact	–	–	18 (48.6%)
Mixed	–	–	6 (16.2%)
Total, number	42	21	37
%	100	100	100

Table V

Relationship between adherence to glass and haemolysis type of 87 coagulase-negative staphylococcal strains

Haemolysis type	Adherence		
	strong	weak	negative
A/E	2 (5.7%)	4 (22.2%)	13 (38.2%)
E	12 (34.3%)	6 (33.3%)	8 (23.5%)
No	21 (60.0%)	8 (44.5%)	13 (38.3%)
Total, number	35	18	34
%	100	100	100

Correlation between colony form and haemolysis type. In accordance with the previous statements, strains that formed compact colonies haemolyzed more frequently than the diffuse strains. Haemolysis type A/E occurred more frequently with the former, and type E with the latter strains.

Discussion

The pioneer observation of Finkelstein and Sulkin [22] that all CNS strains formed diffuse colonies in SSA has been accepted as a rule, and restricted scientists to reevaluate this statement [23]. Since a large majority of CNS strains belongs to *S. epidermidis* it may be supposed that strains examined by these authors belonged to this species, for which the statement is still valid. On the contrary, we have found a definitive difference in colony morphology exhibited in SSA and SA between *S. haemolyticus* and other species of CNS, mainly *S. epidermidis* strains. Namely, the vast majority of strains of the species *S. haemolyticus* have compact colonies in both

SSA and SA prepared with mS110 broth similar to the compact spherical types of coagulase-positive colony observed in serum medium [22].

The peculiarity of this growth is that neither human nor rabbit sera can influence the formation of the compact colony by *S. haemolyticus* in mS110–SA. At present the cause of this particular behaviour of the species *S. haemolyticus* is not known. One of the explanations may be that the bacteria of this species are strongly hydrophobic (Rozgonyi et al., unpublished data) and a hydrophobic interaction attaches the cocci the each other forming compact colonies. The other possibility may be that some polysaccharide-binding protein(s) exist(s) on the surfaces of the cocci and the molecules of agar serve as bridges between cocci to keep them in close contact resulting in compact colonies. A unique sedimentation property of *S. haemolyticus* in a hypertonic medium is unlikely since this species forms similar deposit to other species in mS110 broth free of agar.

It is possible that compact colony morphology is characteristic of taxonomically related strains of CNS. In this sense, compact growth in SA prepared with mS110 broth, a medium frequently used for a selective cultivation and enumeration of staphylococci seems to be a diagnostic sign for *S. haemolyticus*.

Another peculiar property of the species *S. haemolyticus* is non-adherence in glucose-meat broth and in TSB supplemented with glucose. These two properties sharply distinguish the species *S. epidermidis* (diffuse growth in SA and adherence to glass) from *S. haemolyticus* (compact growth and non-adherence to glass) and are additional taxonomic features to differentiate the two main species of the group *S. epidermidis* as required recently [12].

As the adherence is concerned, most strains of *S. epidermidis* proved to be adherent similarly to the finding of Righter [3]. The overall figure (63%) for the frequency of adherence by CNS is higher than that published by Hamilton-Miller and Iliffe [1] due probably to the difference of the growth medium used [14, 15] and to the fact that all strains were isolated from severe coagulase-negative staphylococcal infection in our cases.

All the adhering strains grew diffusely or some of them showed transient growth in mS110–SA and in mS110–SSA. However, only such a correlation exists between the two properties, since not all of the diffuse or transient colony forming strains adhered to glass. Furthermore, it is inaccurate to take the presence of diffuse growth automatically as evidence of slime production [23, 24]. These properties appear often together, but the diffuse colony morphology in SA or in SSA does not automatically mean that the strain is a slime producer or an adherent one. Finally, it is worth to mention that there is a negative correlation between the haemolysis type and the adherent growth of CNS strains. More non-adherent strains than adherent ones exhibit haemolytic activity, and haemolysis type A/E is more frequent among non-adhering strains. This observation gives some explanation why slime producing strains are less invasive than non-adherent ones [10, 12]. Namely, the possibility of a bacterial invasion is limited without host cell membrane damage and cell lysis.

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MONOCYTOTOXIC ANTIBODIES IN HIV-INFECTED PERSONS. TNF-ALPHA TREATMENT OF U937 CELLS INCREASES THE COMPLEMENT DEPENDENT CYTOTOXICITY*

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Sera of 40 intravenous drug addicts were tested for the presence of cytotoxic antibodies against uninfected and HIV-infected monocytic U937 cells. Twelve out of 31 seropositive samples proved to be cytotoxic for HIV-infected, untreated target cells in the presence of complement. The TNF-alpha treatment of HIV-infected U937 cells increased the detectability of cytotoxic effect of sera (21/31). The complement dependent cytotoxic activity of sera was reduced by pretreatment with recombinant HIV gp120. This reduction proved to be dose-dependent in the majority of cases. Immunofluorescence studies indicated that the cytotoxic sera interacted with antigens mostly localized on the cell membrane of HIV-infected TNF-alpha treated U937 cells. The specificity, the possible role and origin of monocytotoxic antibodies in HIV-infected persons is discussed.

The human immunodeficiency virus (HIV) has been clearly established as the primary aetiologic agent of AIDS and related disorders [1, 2]. However, the role of the anti-HIV antibodies in the pathogenesis of AIDS is still controversial.

* This paper was written in honour of the memory of Professor Zoltán Alföldy (1904 – 1992) director emeritus of the Institute of Microbiology, Semmelweis University Medical School, Budapest (Hungary)

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Furthermore, in addition to the virus-specific lymphocytotoxic antibodies [3], a large number of other (auto) antibodies were demonstrated during the course of HIV-infection, that showed reactivity with different types of blood cells, such as lymphocytes [4], erythrocytes [5] and thrombocytes [6]. Here we describe the detection and preliminary characterization of monocytotoxic antibodies in sera of HIV-seropositive drug addicts.

Materials and methods

Sera. Serum samples were obtained from 40 HIV-infected and uninfected drug addicts as well as from healthy control individuals. All persons were clinically asymptomatic at the time of blood sampling. The sera were stored at −20 °C until used. The ELISA tests were performed by using DuPont anti-HTLV-III kits and the results were confirmed by the Western blot method (Diagnostic Pasteur, Marnes La Coquette, France).

Cell cultures. The U937 monocytoid cell line [7] was infected with HTLV-III B strain of HIV. The details for HIV-infection and for cell culture procedures were described elsewhere [8]. HIV-infected and control uninfected U937 cells were maintained in RPMI 1640 medium supplemented with 10% FCS [8].

TNF-alpha treatment of cell cultures. The recombinant human TNF-alpha (specific activity 7×10^7 U/mg) was a generous gift of Professor W. Fries and Dr. J. Tavernier (Ghent, Belgium). Uninfected and chronically HIV-infected cells (5×10^5 ml) were incubated in the presence of TNF-alpha (1 μg/ml) for 24 h before performing the ^{51}Cr release assay.

Cytotoxic antibody assay. Complement-dependent antibody-cytotoxicity was detected by the ^{51}Cr release technique. Details were described elsewhere [9]. Uninfected and HIV-infected U937 cells (untreated or pretreated with TNF-alpha) were used as targets. The target cells were resuspended in serum-free RPMI 1640 medium and incubated with ^{51}Cr as sodium chromate (Amersham, Buckinghamshire, UK). For the study, 5×10^4 ^{51}Cr labelled target cells were added to 100 μl of heat-inactivated (56 °C, 30 min) serum samples diluted in PBS. After an incubation of 30 min at 37 °C, 100 μl of nontoxic guinea-pig serum were added as a source of complement and the incubation was continued for an additional 30 min. After centrifugation, the supernatant fluid was assayed for released radioactivity. Spontaneous release (SR) was determined by incubating the target cells in medium alone. Maximum release (MR) was determined by incubating the cells with 5% Zaponin. Experimental release (ER), SR and MR were determined in triplicate.

Results were expressed as:

$$\% \text{ cytotoxicity} = \frac{ER - SR}{MR - SR} \times 100$$

Inhibition of cytotoxic reactions by HIV gp120 antigen. Before adding to target cells, serum samples were incubated at 37 °C for 30 min with 10 μl of PBS containing increasing amounts of gp120. The recombinant HIV gp120 antigen was produced by Micro Gene System in Baculovirus vector (Westham, CT, USA).

Immunofluorescence technique. Indirect immunofluorescence assays were performed using TNF-treated and untreated HIV-infected U937 cells as well as their uninfected counterparts. Briefly, the spotted cells were fixed with methanol. Sera were diluted 1 in 50, whereas the FITC-conjugated sheep anti-human IgG serum (Hyland, Costa Mesa, VA, USA) was diluted 1 in 40.

Results

Data of Table I show that roughly two third (21/31) of seropositive sera exerted complement-dependent cytotoxic activity against HIV-infected U937 cells, whereas one third of them (10/31) proved to be negative for the presence of such antibodies. The cut-off level was 20%. The "positive" group can be further divided into three subgroups on the basis of effect of TNF-alpha treatment of target cells on cytotoxic activity. In the subgroup "a" (sera No. 11–16), the TNF-treatment did not increase the cytotoxic effect of sera. The subgroup "b" (sera No. 17–22) was characterized by the significant increase of cytotoxic activity after TNF-treatment of target cells. The sera of the subgroup "c" (sera No. 23–31) were characterized by conversion of cytotoxic activity. In fact, these sera did not exert cytotoxic effect on untreated, HIV-infected U937 cells, however, the TNF-treatment of target cells resulted in the appearance of cytotoxic activity.

Table I

Cytotoxic activity of human sera on HIV-infected U937 cells

Serum No.	% Cytotoxicity		Comment
	before TNF-alpha treatment	after TNF-alpha treatment	
1–10 (seropositive)	4.0	4.9	negative+
11–16 (seropositive)	44.3	42.1	positive (a)++
17–22 (seropositive)	28.6	81.3	positive (b)
23–31 (seropositive)	3.6	55.6	positive (c)
32–40 (seronegative)	5.0	4.0	negative

+ negative: cytotoxicity less than 20%

++ positive: cytotoxicity more than 20% (a): basal level (b): increase (c): conversion

The seropositive samples (No. 1–31) did not exert cytotoxic effect on uninfected U937 cells either before or after TNF treatment.

Sera of 20 healthy control persons showed no cytotoxic activity either against uninfected, or HIV-infected U937 cells. Similar negative results were obtained using TNF-alpha-treated target cells.

In order to investigate the virus-specific nature of cytotoxic antibodies, we have determined the percentage of the lysis after absorbing the antibodies from the sera

by incubation with different amounts of HIV gp120. Results of Figs 1, 2 and 3 show that, in the vast majority of cases, the incubation with 40 mg gp120 reduced the cytotoxic activity of sera against TNF-untreated HIV-infected U937 cells and in five cases this amount of competitor antigen proved to be sufficient to reduce the percentage of lysis under the cut-off level (sera No. 13, 15, 21, 27, 28). The increase of the competitor concentration (80 mg) resulted in further reduction of cytotoxic activity of sera and residual activity was detected only in 7 cases, very close to the cut-off level.

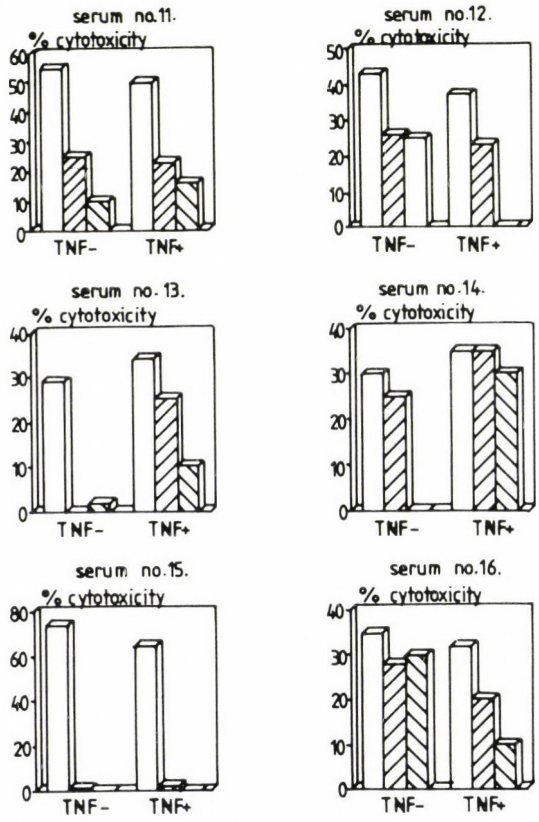

Fig. 1. Blocking effect on cytotoxic activity of sera of subgroup "a" by recombinant HIV gp120. The HIV-infected U937 cells (5×10^5/ml) were treated with TNF-alpha (1 μg/ml) for 24 h or left untreated. For details see Materials and methods. Open columns: untreated; leftwards shaded columns, 40 ng HIV gp120; rightwards shaded columns, 80 ng HIV gp120

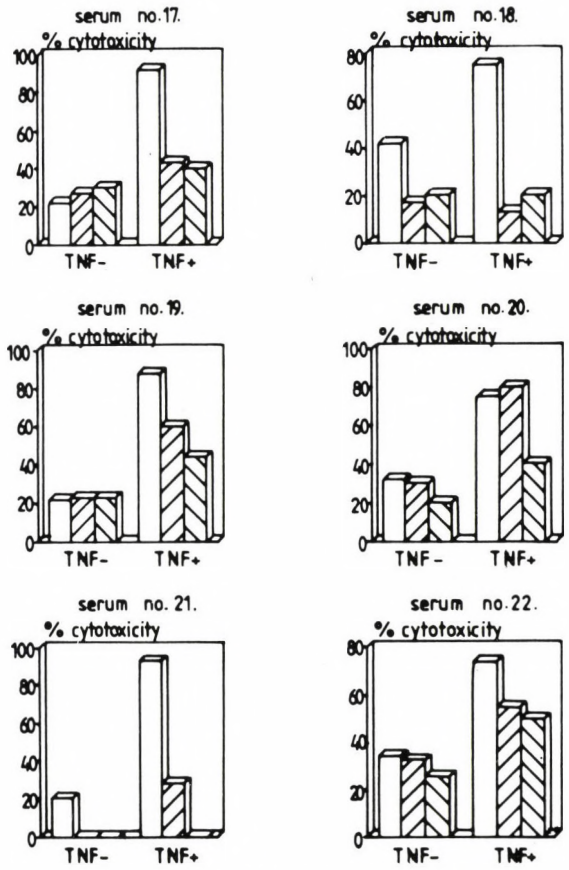

Fig. 2. Blocking effect of cytotoxic activity of sera of subgroup "b" by recombinant HIV gp120. Columns as in Fig. 1

The blocking experiments of TNF-alpha treated HIV-infected U937 cells showed a similar pattern (i.e. the HIV gp120 antigen inhibited the cytotoxic activity of sera, in the majority of cases, in dose dependent way). In the subgroup "a", the cytotoxic activity of sera against TNF-treated target cells could be practically eliminated by large amounts of competitor antigen (with exception of sample No. 14). On the contrary, subgroup "b" and "c" were characterized by a remarkable residual cytotoxic activity even after blocking with the largest amount of competitor. This phenomenon could be observed (with three exception, No. 20, 24, 25) in each case of this latter subgroup, (i.e. in those that were characterized by increase or by conversion of cytotoxic activity as a result of TNF-treatment of target cells).

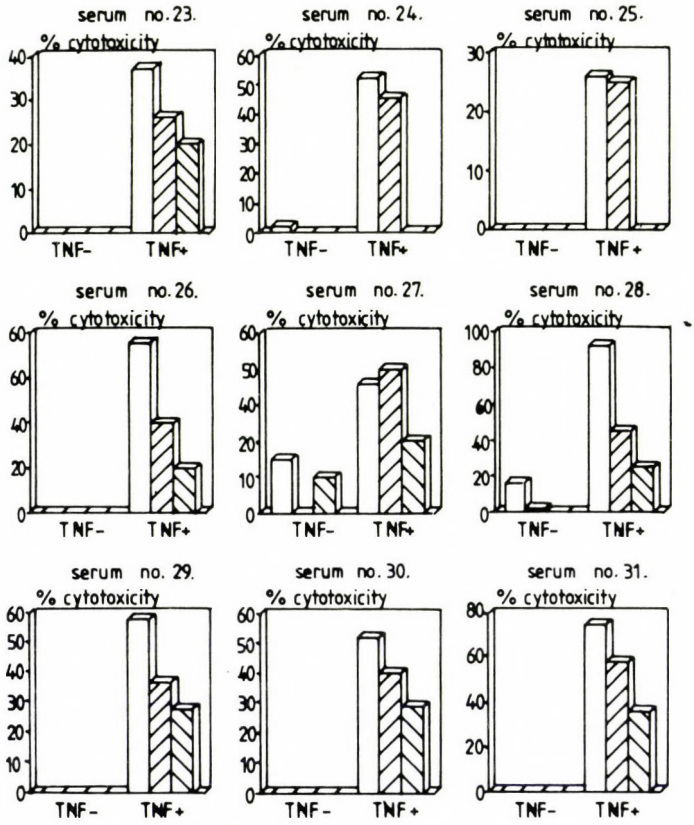

Fig. 3. Blocking effect of cytotoxic activity of sera of subgroup "c" by recombinant HIV gp120. Columns as in Fig. 1

Table II

Average percentage of peripherally localized fluorescence in the three different groups of sera

Serum No.	Percentage of peripheral fluorescence			
	U937 −		U937 +	
	TNF −	TNF +	TNF −	TNF +
1 − 10	0	0	0	0
11 − 31	0	0	30 ± 10	95 ± 22
32 − 40	0	0	0	0

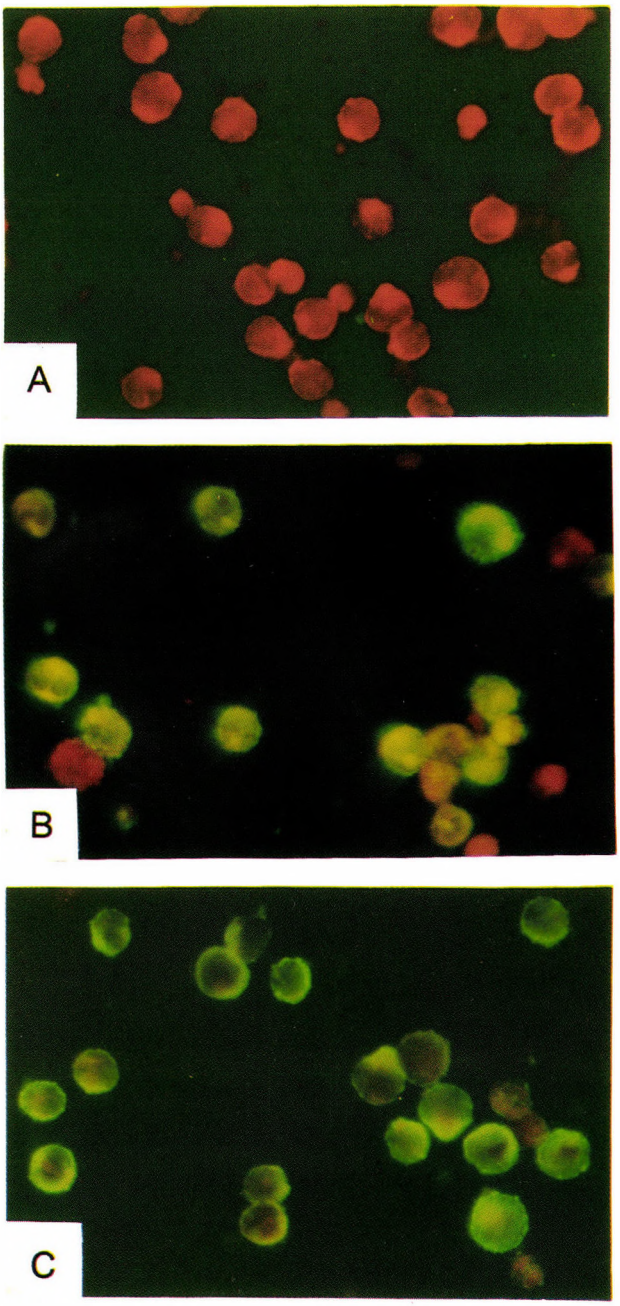

Fig. 4. Reactivity of sera to TNF-treated HIV-infected cells. Indirect immunofluorescence on methanol-fixed cells. The negative cells are stained by the red counterstain, whereas the positive cells exhibit the green colour of fluorescein isothiocyanate. For details see Materials and methods. A: HIV-infected U937 cells incubated with a negative serum; B: HIV-infected, untreated U937 cells incubated with a cytotoxic serum (No. 17); C: HIV-infected, TNF-treated U937 cells incubated with serum No. 17

We then evaluated the reactivity of these sera on untreated and TNF-treated U937 cells as revealed by indirect immunofluorescent techniques. Table II summarizes our results. Cytotoxic sera did not exhibit any immunofluorescence using uninfected target cells. On the contrary, HIV-infected U937 cells gave positive results: approximately 30% of these cells showed a membrane associated immunofluorescence. TNF-alpha treatment of the HIV-infected U937 cells resulted in a marked increase in the percentage of cells exhibiting a membrane associated fluorescence (from 30% to 95%). Figure 4, Panel C shows the characteristic morphology of the membrane-associated immunofluorescence. A diffuse fluorescence was observed using non-cytotoxic sera (No. 1–10) on either untreated or TNF-alpha treated virus-infected cells.

Discussion

A large variety of different types of antibodies reacting with blood cells are found in HIV-infected persons. The clinical relevance of anti-lymphocyte, anti-granulocyte, anti-thrombocyte, and anti-erythrocyte antibodies has been studied [10–13].

These antibodies can play a role in the pathogenesis of ongoing immune suppression, and possibly in that of the thrombocytopenia and anaemia. We have found antibodies cytotoxic for the HIV-infected monocytoid U937 cell line in one third of the sera of HIV-seropositive drug addicts. In the majority of cases, the percentage of cytotoxic activity exerted by the sera showed a remarkable increase after TNF-alpha treatment of target cells, furthermore, more than one third of the samples proved to be cytotoxic only against TNF-treated target cells. In is well known that TNF-alpha treatment increases the HIV-expression in different cell types (for a review see [14]), including the U937 monocytoid cell line [8]. Thus, it is reasonable to assume that the elevated density of HIV-envelope antigens on the surface of target cells is responsible for the increased cytotoxic activity. Results of immunofluorescence tests indicate that the cytotoxic sera exhibit a membrane-localized reactivity especially on TNF-treated HIV-infected cells (Fig. 4).

Blocking effect by HIV gp120 antigen on cytotoxic activity of sera was observed in the majority of cases, and this phenomenon proved to be roughly dose-dependent. However, in a remarkable percentage of sera, cytotoxic activity could not be totally eliminated even by using high doses of competitor HIV-gp120. This phenomenon was observed mainly in those subgroups of sera which showed increased cytotoxic activity after TNF-treatment of target cells (subgroup "b") or for those which were characterized by conversion of cytotoxic activity as a result of such a treatment (subgroup "c"). One possible explanation for the phenomenon of the residual activity

may be the high titre of anti-gp120 antibodies. However, the involvement of other HIV-antigens in the generation of a cytotoxic immune response cannot be excluded.

Furthermore, one has to keep in mind that, in the majority of anti-blood-cell activities in HIV-infected persons, certain types of autoantibodies are also involved. Stricker et al. [4] described a 18 Kd protein in the membrane of lectin-stimulated HIV-infected T-lymphocytes. This antigen is probably responsible for the generation of non-virus-specific autoantibodies which may contribute to the ongoing immune suppression. The expression of a cellular antigen in the membrane of HIV-infected U937 cells due to the cytokine treatment can also be hypothetized.

A cross-reactivity between HIV gp41 and the beta-chain of MHC II class antigens has been demonstrated [15] and evidence is being collected concerning the partial homology of this class of MHC molecules and the HIV envelope [16]. It might be possible to speculate, therefore, that some anti-HIV antibodies can also react with some epitopes on MHC class II antigens. The search for the target antigen responsible for the generation of monocytotoxic antibodies is still in course in our laboratory.

The biological significance of antibodies exhibiting monocytotoxic activity (either of viral or cellular specificity) is not clear. Antibodies against autologous monocytes have been reported by several authors in different types of diseases, mainly in disorders of uncertain or autoimmune origin, including sclerosis multiplex or thyreoiditis [17, 18]. It is well known that the HIV-infected monocytes/macrophages represent one of the major pathways of the dissemination of HIV in the infected individual [19]. Thus, the individual differences in the course of HIV-induced disease may depend on the early elimination of these cells by monocytotoxic antibodies whose effect could also depend on the individual differences of the serum levels of TNF-alpha [20], or the constitutive TNF-production. Follow up studies are in progress in our laboratories to clarify the potential role of monocytotoxic antibodies in the pathogenesis of AIDS.

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CARRIAGE, TRANSFER AND INTERACTION OF ORAL VIRUSES AND BACTERIA

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Carriage of antigens and infectious herpes simplex virus type 1 (HSV–1) and human adenovirus type 1 (Ad–1) by salivary leukocytes was compared with the antibacterial activity of oral polymorphonuclear leukocytes (PMNL) and with the spectrum of oral bacterial and fungal flora. Risk of iatrogenic infections by microbes was assessed by detecting these viruses and microbes after disinfecting dental instruments. The results indicate carriage of antigens and infectious viruses in each age group between 6 and 60 years. Phagocytic activity by PMNL of virus carrier persons was found to be decreased as compared to virus-free subjects. The species number and survival after disinfection of oral bacteria and fungi were significantly higher in virus carrier persons. Infectious viruses were also obtained after disinfecting instruments used in their dental treatment. It is concluded that, virus infection of immune cells can contribute to the oral suppression of phagocytosis by PMNL. Intracellular viruses hidden from disinfectants can also result in infection of other subjects, especially if contemporary immunosuppression exists.

Changes in the oral flora reflect the actual immune state of the organism. Whole saliva contains whole blood serum transudate from the gingival crevices around the teeth, nasopharyngeal secretions, mucosal epithel cells as well as secretions from the salivary gland, therefore mixed whole saliva is representative of the systemic as well as mucosal humoral immune responses to microorganisms [1]. A vast number of different types of peripheral blood leukocytes emigrate from the

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blood vessels of the gingiva [2, 3], of the tonsils and from other sources [4] into the mouth. Their main role is to protect oral mucosa against microbial infections [3, 4]. In healthy subjects, the phagocytic and killing activities by gingival and salivary PMNL and mononuclear phagocytes (MNPh) were found to be similar to that of blood derived cells [3, 5, 6]. Their functions were dramatically decreased with herpes simplex virus type 1 (HSV-1) or human adenovirus type 1 (Ad-1) emigrated to the sulcus gingivalis at the same time [3]. More recently, severe oral fungal [8] and bacterial infections [8, 9] in patients infected with human immunodeficiency virus type 1 (HIV-1) have drawn attention to the disturbed interaction between different parts of the immune system [1, 2, 8, 10, 11].

Several in vivo [5, 7, 12, 13] and in vitro [5, 14-17] viral infections are known to impair functions of PMNL. Life cycle and antimicrobial activities of PMNL are under control of several cytokines [18], especially of those secreted by T lymphocytes [19]. Altered production of mediators by virus infected immune cells might diminish activity of phagocytes [3, 5, 18, 19]. These mediators are diluted in the blood stream, but their effect could be more pronounced between different immune cells in close proximity in gingival space or saliva [3]. As a consequence of suppressing microbicidal activities, the number of oral microbes increase and more virulent strains dominate flora [8, 9, 20, 21]. The metabolic products of these variants have detrimental effect on periodontal tissue and oral mucosa [9, 10, 20, 22-24]. Besides, dental care of patients with uncotrolled microbial flora and/or higher number of virus carrying cells might exert an elevated risk to both medical personnel and other patients [5, 25-28].

Infection by HSV-1 and Ad-1 in salivary lymphocytes were, therefore, compared to the antibacterial activity of oral PMNL and to the spectrum of oral flora. Risk of iatrogenic infections was assessed by detecting viruses and microbes on dental instruments after common disinfection procedures.

Materials and methods

Saliva samples. Saliva from healthy subjects denying occurrence of herpes labialis or any other oral infection in the last 6 months was collected into sterile test tubes. Four to five ml specimens were diluted to 10 ml by phosphate buffered salt solution (PBS) pH 7.4, then washed by centrifugation at 1500 rpm for 10 min twice to remove mucus. The last pellet was resuspended in Parker's 199 minimal essential medium (MEM, obtained from the National Institute of Hygiene, Budapest, Hungary) containing 3% rabbit serum (Human Institute for Serobacteriological Production and Research, Budapest, Hungary), 50 U/ml penicillin (Biogal Pharmaceutical Co., Debrecen, Hungary) 50 μg/ml streptomycin (EGIS United Pharmaceutical Co., Budapest, Hungary) and 50 μg/ml nystatin (Chinoin Pharmaceutical Co., Budapest, Hungary) and the cell number was adjusted to 2×10^4/ml. Their viability was scored in a Buerker type hemocytometer by the trypan blue exclusion test [29].

Contaminating cells on dental instruments. Small dental instruments (forceps, Volkman spoons, Kerr needles, diamond drill heads, etc.) were sterilized by heat and after using in conventional dental practice in otherwise healthy subjects with no herpes or other oral lesions in the previous 6 months, were rinsed with tap water and immersed into different concentrations of disinfectants used widely: 1 to 4% of cetylpyridinium bromide, a quaternary ammonium derivate (Sterogenol, EGIS), or 0.02 to 0.2% of phenylmercury borate (Merfen, Reanal, Budapest, Hungary). After 5 min or its multiplications the instruments were immersed and forcefully rotated in test tubes containing MEM. Visible mucus was removed by sterile glass sticks into the medium. Cells were washed and their number adjusted as mentioned above.

Cell cultures and their infection. HEp-2 (obtained from the NIH, Bethesda, MD, USA and maintained at the Institute of Microbiology), VERO (obtained from the National Institute of Hygiene, Budapest, Hungary), SIRC (obtained from Dr. J. Rajčáni, Institute of Virology, Bratislava, Slovakia) monolayer cell cultures were maintained by regular trypsinization in our laboratory [3, 30]. Their growth medium consisted of MEM supplemented with 10% calf serum (Human), 50 U/ml penicillin, 50 μg/ml streptomycin. Primary human amnion cultures also were prepared by a standard trypsinizing procedure using 0.25% trypsin (Difco, Detroit, MI, USA) in Hanks' balanced salt solution (HBSS) pH 7.4. The cells were grown in HBSS supplemented with 10% human serum, 0.4% lactalbumin hydrolysate, 0.225% $NaHCO_3$, and the usual antibiotics [30]. After reaching confluency, the medium of 48-h-old cultures in test tubes was replaced by 0.1 ml oral cell suspension for 1 h, then their volume was adjusted to 1 ml with MEM containing 3% rabbit serum and antibiotics. Infected amnion cells were incubated for 3 to 4 weeks, but subcultures were prepared weekly from established other cell lines until week 6. As controls, 1000 tissue culture infectious $dose_{50}$ ($TCID_{50}$) of HSV-1 (obtained from Dr. I. F. Barinsky, Ivanovsky Institute of Virology, Moscow, USSR) or Ad-1 in 0.1 ml volume were added to parallel cultures. Cytopathology was scored in a light microscope, and supernatant fluid of cultures containing viruses of patients were transferred to other types of tissue cultures.

Immunofluorescent studies of oral cells and tissue cultures. HEp-2, VERO or SIRC cells from the 4th subcultures were grown on 1.6×0.8 mm coverslip placed into the medium of test tubes. At the end of incubation coverslip was removed, the cells were washed in PBS 4 times, dried in air and finally fixed in cold methanol for 30 min. Furthermore, from each salivary cell specimen or cells washed off dental instruments 2000 of them were plotted onto microscopic slides, dried and fixed. Cell preparations were covered with anti-HSV-1 or anti-Ad-1 rabbit sera conjugated by rhodamin B or flourescein-isothiocyanate (FITC) in a 22 °C moist chamber for 30 min, then excess sera were washed twice with PBS, rinsed in distilled water, finally scored in a Zeiss Fluoval UV microscope for the presence of viral antigens [30].

Electron microscopy. Some oral cell samples indicating presence of HSV-1 or Ad-1 were examined by electron microscopy. 2×10^4 cells were pelleted, fixed in glutaraldehyde and osmium tetroxide, then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and studied in a JEOL 100B electron microscope.

Bacterial and fungal contamination of saliva and dental instruments. One tenth ml of the unwashed saliva samples were transferred into broth and anaerobic cooked meat broth cultures. Similarly to virological tests, dental instruments also were washed in broth and cooked meat broth cultures. After their incubation at 37 °C for 24 h, subcultures were plated onto agar (Difco), blood agar, chocolate agar, Clauberg agar, deoxycholate agar, eosin-methylene-blue agar, brilliant green agar plates, egg yolk agar slants for 24 to 48 h at 37 °C. Fungal contamination was checked on Sabouraud dextrose agar plates at both 22 and 37 °C for three weeks. Characteristic colony morphology was coupled by Giemsa, Gram, acid-fast, alkaline methylene blue and Indian ink stains of smears [31]. All stains and

media used regularly for teaching purposes were obtained from the media laboratory of the Institute of Microbiology.

Phagocytosis by salivary leukocytes. From each sample 2000 oroleukocytes in 0.1 ml MEM containing 3% rabbit serum were mixed with 2×10^4 to 2×10^5 live *Staphylococcus aureus* (isolated from a patient, characterized by the Diagnostic Bacteriological Laboratory of the Institute of Microbiology, and grown in broth for 24 h and counted by standard human red blood cell suspension in Buerker's hemocytometer immediately before use) in 0.1 ml of the same medium at 37 °C for 2 h, then mixtures were vigorously shaken and washed in PBS by centrifugation three times to remove non-phagocytosed bacteria. Those bacteria, which had been phagocytosed in vivo preceeding the incubation with staphylococci were established in parallel samples to which nothing has been added. This number was then subtracted from the total bacterial count of the appropriate samples for figuring out exact in vitro phagocytosis [3].

Results

Microorganisms and their effect on leukocytes in the saliva. Probands were separated into three age groups (Table I). The leukocytes of a significant number of persons in each group contained viruses judged by their intracellular antigens. Occasionally, exfoliated epithelial cells of the oral mucosa exhibited positive fluorescence with antibodies to HSV-1 or Ad-1, mainly in those patients, whose lymphocytes proved to be positive (Fig. 1). Both herpes- and adenoviruses could be transferred to different tissue cultures. SIRC and VERO cells showed the highest susceptibility for HSV-1 and Ad-1, respectively, but transferring supernatant of one type of infected cultures to the other one resulted in appearing cytopathic effect characteristic for the same virus (Fig. 2 A and B). In few cases, the cytopathic effect characteristic of neither HSV-1 nor Ad-1 appeared; cytomegalovirus or another infectious agent might account for this phenomenon. The number of cultures containing virus antigens exceeded those of lymphocytes of patients. Extracellular viruses in the saliva or those in macrophages or epithelial cells also could be responsible for this phenomenon. Immunofluorescence of tissue culture cells (Fig. 2 C and D) and electron microscopy (Fig. 3) verified cytopathology by viruses.

Table I

Demonstration of viruses in the saliva of different age groups (%)

Type and number of persons	Age in years (mean)	Antigens in the lymphocytes of persons				Antigens in cell cultures in vitro				Infectious viruses in cell cultures in vitro				
		HSV-1	Ad-1	HSV-1 +Ad-1	Total virus	HSV-1	Ad-1	HSV-1 +Ad-1	Total virus	HSV-1	Ad-1	HSV-1 +Ad-1	Other virus	Total virus
Children N=25	6-16 (11.7±4.3)	5 (20.0)	NT	NT	5 (20.0)	3 (12.0)	NT	NT	3 (12.0)	3 (12.0)	NT	NT	0	3 (12.0)
Juvenile N=41	17-23 (18.5±1.4)	2 (4.9)	2 (4.9)	2 (4.9)	6 (14.6)	4 (9.8)	6 (14.6)	3 (7.3)	13 (31.8)	3 (7.3)	1 (2.4)	0	3 (7.3)	7 (17.0)
Adult N=41	24-60 (44.6±8.1)	6 (14.6)	NT	NT	6 (14.6)	5 (12.2)	NT	NT	5 (12.2)	3 (7.3)	NT	NT	2 (4.9)	5 (12.2)

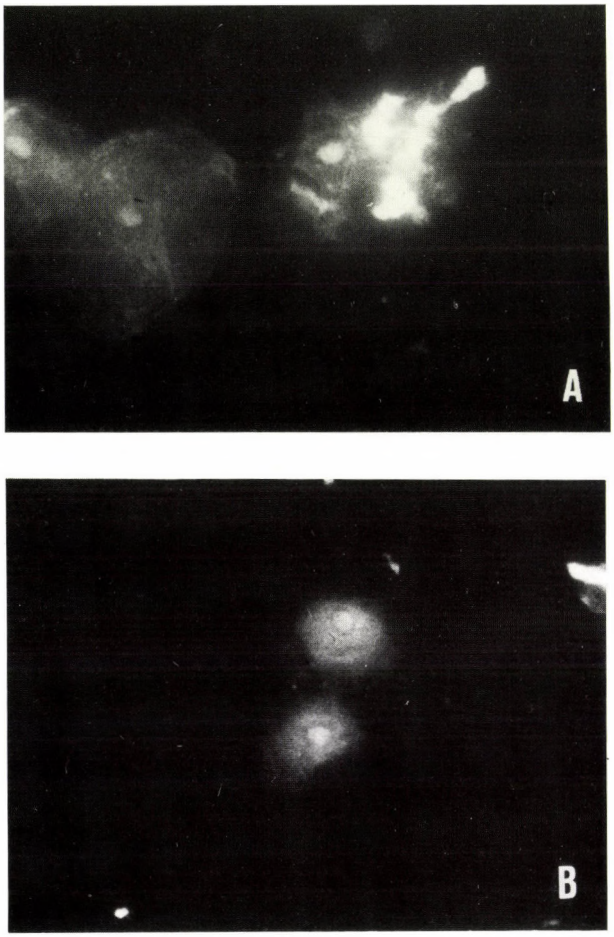

Fig. 1. A: Group of oral lymphocytes showing positive fluorescence for Ad – 1; epithelial cells are negative. B: positive fluorescence for HSV – 1 in small epithelial cells exfoliated early. × 1000

Several types of bacteria were isolated from the saliva, but their range did not show any significant difference among patient groups. Only the number of bacterial species isolated from the same individuals was characteristic of particular groups (Table II). Interestingly, this number was found to be higher in virus carrier persons, especially in adults, than in virus-free individuals. Higher number of adolescent persons proved to be active virus carrier, and the saliva of virus carrying persons contained leukocytes in a lower number and viability. Both in vivo and in vitro phagocytosis were diminished in virus carrier persons, mainly in youngsters (Fig. 4).

Table II

Biological activity of salivary leukocytes

Type of persons	Virus carrier state (%)	Leukocyte/ml in the saliva (10^6)	Viability of leukocytes (%)	Phagocytosis (bacteria/phagocyte)			Number of bacterial species in the saliva
				In vivo	In vitro	Total	
Juvenile	Total N=41 (100)	1.26±0.2	59.1±5.5	5.1±0.5	4.2±0.8	9.3±0.8	5.4±1.4
	Carrier N=15 (36.6)	1.18±0.3	41.4±8.3	3.9±0.4	0.8±0.4	4.7±0.7	5.8±1.7
	Free N=26 (63.4)	1.31±0.2	69.3±3.9	5.8±0.5	6.1±1.1	11.9±0.8	5.1±1.2
Adult	Total N=41 (100)	1.38±0.3	66.8±8.9	4.8±0.7	6.5±1.1	11.3±1.7	5.6±2.0
	Carrier N=8 (19.5)	1.44±0.4	62.1±0.6	3.9±0.5	3.9±0.9	7.8±1.4	6.9±2.6
	Free N=33 (80.5)	1.37±0.3	67.9±8.7	5.0±0.7	7.2±1.1	12.2±1.8	5.3±1.9

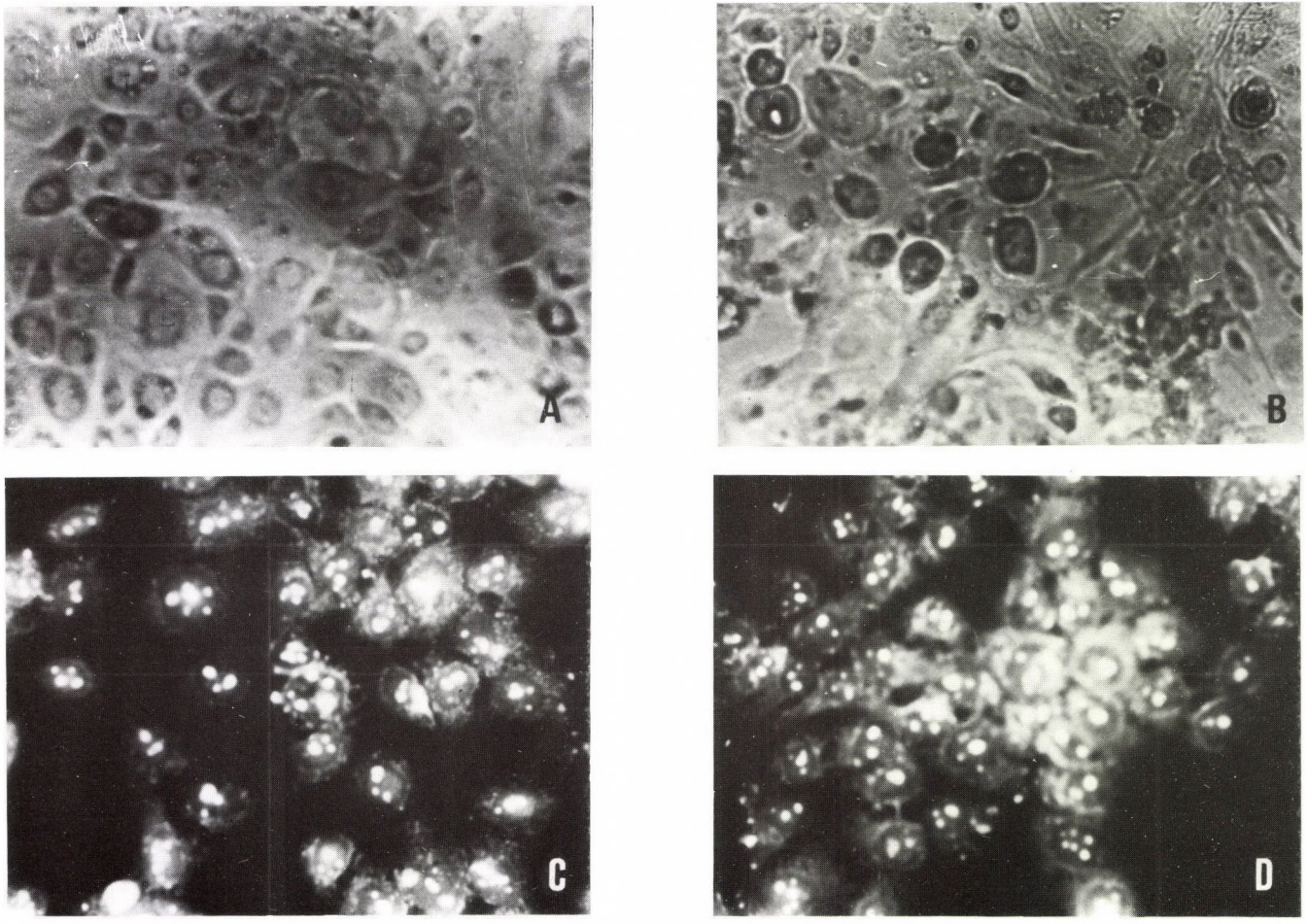

Fig. 2. Micrographs of 72-h-old cell cultures. A: normal amnion monolayer. B: amnion monolayer split with enlarged, rounded cells and nuclear inclusions indicate the presence of an infectious agent. C: uninfected HEp-2 cells with no immune fluorescence. D: a cluster of HEp-2 cells showing positive fluorescence for Ad-1 after transferring supernatant of amnion cells mentioned above. × 1000

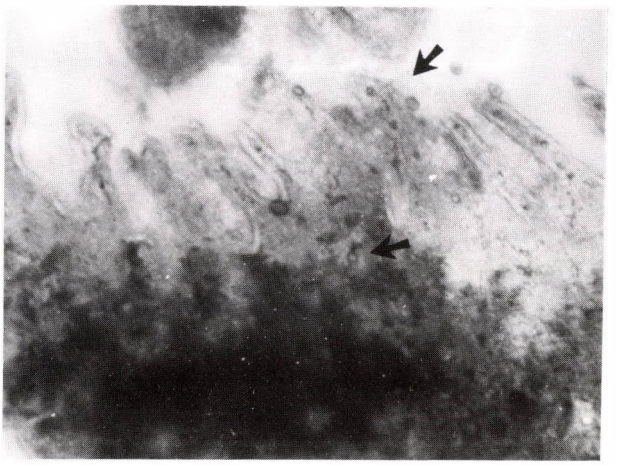

Fig. 3. Electron micrograph of HSV-1 particles (arrows) inside cytoplasm and microvilli, and those attached to microvilli of an exfoliated oral epithelial cell. × 10 000

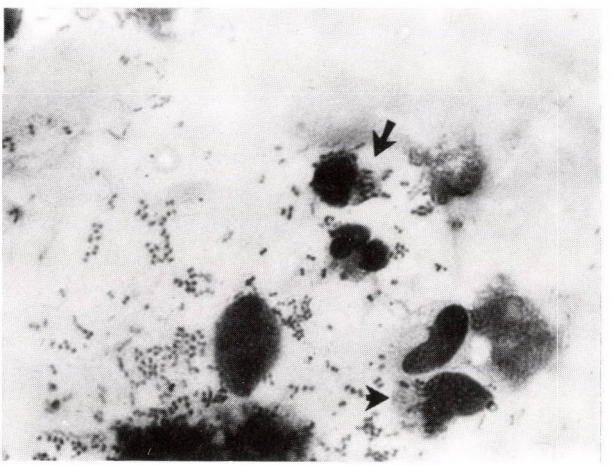

Fig. 4. Micrograph of phagocytosed bacteria in oral PMNL (arrow) and MNPh (arrowhead). × 1000

Difference in survival of microbes on dental instruments. A great number of cells remained microscopically intact after disinfection procedure. These cells could harbour viruses as immunofluorescence showed antigens of single or multiple types of viruses (Table III). These viruses resulted in a productive infection of different tissue cultures and their antigens were verified by immunofluorescence. No agent causing cytopathic effect was transferred to the tissue cultures from persons not carrying viruses by their cells. If their saliva contained extracellular viruses, these latters were efficiently destroyed during disinfection.

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Table III

Surviving viruses on dental instruments after disinfection (%)
(Cetylpyridinium bromide 4 ‰, 22 °C, 15 min)

Type and number of persons (%)	Antigens in the lymphocytes of persons				Antigens in cell cultures in vitro				Infectious viruses in cell cultures in vitro				
	HSV-1	Ad-1	HSV-1 +Ad-1	Total virus	HSV-1	Ad-1	HSV-1 +Ad-1	Total virus	HSV-1	Ad-1	HSV-1 +Ad-1	Other virus	Total virus
Total N=98 (100)	2 (2.0)	3 (3.1)	4 (4.1)	9 (9.2)	1 (1.0)	2 (2.0)	3 (3.1)	6 (6.1)	1 (1.0)	1 (1.0)	2 (2.0)	2 (2.0)	6 (6.1)
Virus carrier N=11 (11.3)	2 (18.2)	3 (27.3)	4 (36.4)	9 (81.9)	1 (9.1)	2 (18.2)	3 (27.3)	6 (54.5)	1 (9.1)	1 (9.1)	2 (18.2)	2 (18.2)	6 (54.5)
Virus-free N=87 (88.7)	0	0	0	0	0	0	0	0	0	0	0	0	0

Several bacteria and fungi survived the disinfection procedures (Table IV).

Table IV

List of surviving microbes on dental instruments after disinfection
(Cetylpyridinium bromide 4 °/₀₀, 22 °C, 15 min)

Species or genus	Virus carrier persons N = 11	Virus-free persons N = 87	Total number of identification N = 98
Staphylococcus epidermidis	2	4	6
Staphylococcus aureus	5	3	8
Streptococcus pyogenes	1	1	2
Streptococcus mitis	2	1	3
Streptococcus pneumoniae	2	2	4
Sarcina	1	0	1
Corynebacterium	0	1	1
Bacillus	4	8	12
Clostridium	0	1	1
Lactobacillus	0	1	1
Escherichia coli	2	1	3
Klebsiella pneumoniae	2	1	3
Proteus	3	0	3
Pseudomonas	3	1	4
Neisseria	0	1	1
Haemophilus influenzae	1	2	3
Bacteroides	3	3	6
Fusobacterium	1	0	1
Borrelia	1	0	1
Other aerobic bacteria	1	1	2
Other anaerobic bacteria	5	3	8
Total	39	35	74
mean/person	3.54	0.40	0.75
Fungi	4	7	11
mean/person	0.36	0.08	0.11
Total number of microbes	43	42	85
mean/person	3.91	0.48	0.87

The microbicidal efficiency on dental instruments exerted by cetylpyridinium in recommended concentrations was less than the effect of phenylmercuric borate in generally used concentrations (Table V). All equipments remained contaminated in 1 or 2‰ cetylpyridinium after 10 min immersion, while only 77% of instruments became microbe-free in 4‰ cetylpyridinium after 30 min.

Table V

Bactericidal and fungicidal efficiency of disinfectants on contaminated dental instruments (% of 105 tests)

Cetylpyridinium bromide				Phenylmercuric borate					
Concentration	Incubation (min)			Concentration	Incubation (min)				
(‰)	10	15	30	(‰)	5	10	15	20	30
1	0	38	32	0.02	35	62	72	75	70
2	0	51	35	0.04	38	74	78	83	100
3	40	62	61	0.1	53	71	74	100	100
4	68	75	77	0.2	66	79	91	88	100

Discussion

Our results clearly demonstrate that saliva is a source of viruses carried by lymphocytes and of other microbes. Similar results have been published after studies on lymphocytes and epithelial cells from the gingival sulcus of healthy subjects and patients with gingival disorders. However, detectability of HSV−1 and Ad−1 antigens and infectious viruses in saliva was lower, than it was in gingival fluid and cells [3], although, the source of mononuclear cells in any body fluid is the blood [2, 32]. Similar differences were found with several viruses in different body fluids and blood, in spite of using far more sensitive polymerase chain reaction (PCR) to detect viral nucleic acids [26, 32, 33]. Virus carrier cells could be more fragile and disintegrate easily, furthermore, antibodies and enzymes found in saliva destroy free viruses and damage virus carrier cells [2]. Most infections and subsequent establishment of latent virus carriage occur already in childhood, but our demonstration of HSV−1 and Ad−1 by their antigens and infectivity reflecting some form of activation was the highest in the juvenile group. It is not surprising, because hormonal changes in that age are known to activate latent viruses [30]. In adults, other activating factors (trauma, irritation, immunosuppression, etc.) can trigger shedding HSV−1 as it has been shown world-wide [34], and the frequency of isolations shows similar percentage to our earlier data [35].

Reactivation of viruses does not per se lead to disease [11], and interaction with the host and other environmental factors are important in this respect [16, 17]. Killing of bacteria, fungi, protozoa and even viruses [14, 15] by neutrophils is increased through the respiratory burst by soluble factors produced by activated T cells [18, 19]. Viral infection of T cells might alter T cell-neutrophil interaction by damaged signal transduction [20]. Generalized virus infections (measles, influenza, infectious mononucleosis, Newcastle disease virus) result in neutropenia of many patients [16], which is the consequence of temporary suppression of haematopoiesis known to be under control by several cytokines [18]. In contrast, gastrointestinal adeno- and rotavirus infections hardly affecting the immune system do not induce neutropenia [13]. Virus-free supernatants of lymphocytes infected in vitro by HSV−1 or different adenovirus types decreased phagocytosis and intracellular killing of *Staphylococcus aureus* in a dose dependent manner [5]. In contrast to the circulating cells in blood, the close proximity of virus carrier immune cells and phagocytes in saliva or gingival space for relatively longer time allows enhanced transmission of cytokines between them [4]. We found that, both salivary and gingival [3] PMNL of virus carrier persons exhibited significantly diminished antimicrobicidal functions as compared to virus-free subjects. Non-viraemic feline leukemia virus (FeLV) challenged cats expressed depressed PMNL functions [16, 17], and the activity of PMNL was also found to be decreased in HIV−1 infected persons in spite of their

small fraction containing proviral DNA compared to macrophages [12]. As the result of diminished phagocytosis, the oral cavity of AIDS patients contains greater number of different types of bacteria and fungi. The majority of HIV-1 infected individuals without overt clinical symptoms already possesses significantly higher salivary and mucosal yeast cell counts [8]. Strong association found between low $CD4^+$ T lymphocyte number in their blood ($<200 \times 10^6/l$) and risk for oral candidiasis and *Pneumocystis carinii* pneumonia [11] reflects the lack of appropriate stimulus of phagocytes by intact T cells. Immunodeficiency can result in the activation of latently carried heterologous viruses [9, 11, 34], which in turn can further diminish antimicrobial and antiviral activities of PMNL [14, 15].

Weakened phagocytosis and intracellular killing is followed by uncontrolled growth of both normal residents and invading species in oral flora. Similarly to our recent findings, others also established that in virus infected persons Gram-negative bacteria and fungi dominate the oral flora, and these are more virulent, invasive and resistant to other salivary host defence systems [7–9, 36]. Beside those identified in our tests, others found *Bacteroides gingivalis* and *B. intermedius*, *Fusobacterium nucleatum, Candida albicans, Histoplasma capsulatum, Cryptococcus neoformans, Myobacterium tuberculosis* and *M. avium-intracellulare, Neisseria gonorrhoeae, Treponema palladium, Actinobacillus actinomycetemcomitans* in the oral lesions of HIV infected persons [7, 9], *Porphyromonas gingivalis* and *Treponema denticola* and others [24, 37], different spirochetes, *Actinobacillus* and *Capnocytophaga sputigena* [21] in other types of patients and *Eikenella corrodens, Pseudomonas cepacia* in experimental rats [20]. Similar microbes probably fell into the unidentified group of bacteria in our studies. These microbes, especially those producing proteolytic enzymes [37] induce periodontitis [7, 21, 22] and consequently alveolar bone resorption at well defined anatomical sites [20, 23] as well as oral mucosal ulcerations [9]. Such disorders in aggrevated forms are frequently seen in immunocompromised animals [20] and in AIDS patients [9, 10].

The high prevalence rate of more virulent microbes and of virus carrier cells may have important implications regarding medical and dental practice [32]. Appropriate decontamination of instruments is essential [25], because more virulent microbes can have factors resistant to conventional disinfection [36]. Quaternary ammonium compounds are practically non-toxic and non-irritant, therefore they are widely used for disinfecting surgical instruments [38]. They have high bactericidal power against Gram-positive bacteria, but are less effective against Gram-negatives. Many pseudomonas-type bacteria are resistant to them. They are also active against fungi and protozoa, but viruses appear to be more resistant [39]. In common with other disinfectants, the antibacterial activities of the quaternaries are markedly suppressed in the presence of organic matter of any sort. Mention must be made of the adverse effect of certain metallic ions, a point of some importance, when

quaternaries are diluted with tap water before use. Calcium, magnesium and ferric ions are particularly detrimental [38]. Several organic mercury compounds are also generally used in treating surgical instruments. Organic matters particularly plasma and the whole blood severely depress and may even abolish the activities of the mercurials. Mucus, different proteins, blood [22] and especially cells surrounding viruses protect microbes efficiently against disinfection as we also demonstrated here. Therefore the equipments must be more vigorously washed clean before immersion in the solution and the disinfecting period must be not less than 15 min [38]. A recent analysis conducted in private surgeries in the UK showed that 22% of high risk instruments were inadequately decontaminated. Furthermore, half of the general practitioners had no autoclave or pressure cooker system [25]. In the practice of a Florida dentist with apparent symptoms of AIDS five of his patients were infected through procedures including tooth extraction and root canal therapy. Although the precise mode of HIV transmission to the patients could not be identified, transmission might have occurred by contamination of dental equipments with blood from the dentist or a patient already infected by the dentist [26]. In general, the knowledge level about transmission of microbes and about risk associated with treating special patients is acceptable [25]. Willingness of dentists and other health care personnel to treat HIV positive patient can be prejudiced because of fear of declination of their private practice if HIV positive patients were seen in their surgery by other patients [27]. Use of autoclaves and of dispensable small equipments would help resolve these problems partially.

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Note added in proof. The importance of possible transmission of HIV-1 and other viruses in dental practice was emphasized in a recent Editorial of the Lancet (Risk of HIV transmission during dental treatment, **340**, 1259, 1992). Another paper in the same issue (Lewis, D. L. et al.: Cross-contamination potential with dental equipment **340**, 1252, 1992) showed that, tissue fragments, HIV, hepatitis B virus in air-driven high speed handpieces used for drilling were not removed by disinfectant detergent treatment as shown by polymerase chain reaction. Bacteriophages used to contaminate equipments experimentally remained infectious after disinfection. The authors conclude that all devices should be cleaned and heat-treated between each patient to kill microbes safely.

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COMPUTERIZED COMPLEX TYPING OF *ESCHERICHIA COLI* STRAINS FROM DIFFERENT CLINICAL MATERIALS*

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A multivariate analysis of 3334 *Escherichia coli* strains originating from different clinical materials revealed that 50.2% of isolates belonged to the most common 12 (O1, O2, O4, O6, O7, O8, O15, O18, O45, O75, O78, O83) out of 133 serogroups. Haemolysin (Hly) production, mannose resistant haemagglutinating activity for human erythrocytes (MRHA) and colicinogenicity (Col) were recorded in 30, 30 and 36%, respectively. Antigens K1 and K5 were present in 11% and 6.6%, respectively. Association were found among certain serotypes and virulence markers (O1, H^-, H7, K1, MRHA, Col; O2, H^-, Kl, Col; O4, H^-, H5, MRHA, Hly; O6, H^-, H1, MRHA, Hly; O6, K5, MRHA, Col; O7, H^-, H4, K1, MRHA, Col; O18ac, H7, K1, Col; O18ac, H^-, K5, MRHA, Hly; O78, H^-, Col (V-type); O83, H^-, K1, Col). There were associations among clinical specimens, age of patients, nosocomial group of diseases, serogroups and virulence markers, too (cerebrospinal fluid – CSF – O7, O18ac, O45, O83 – K1 – newborn meningitis; O78 – ColV – meningitis, sepsis, inflammatious diseases of premature babies; CFS – O6, MRHA, Hly – adult-meningitis, sepsis, urinary tract infection – UTI –, pneumonia, other inflammatory diseases; blood – O2, O4, O6, O18ac, ONT, K5, MRHA, Hly – sepsis, UTI, hepatic diseases; urine – O1, O2, O4, O6, O18ac, O75, virulence markers fall to differ among upper and lower UTI; faeces – O1, O4, O6, O18ac, O78, virulence markers rare). Associations were also found among animal pathogenicity tests, specimens, serogroups and virulence factors: highly virulent group strains (i.e. LD_{50} below 10^6) belonged to serogroups O2, O6, O18ac, possessed antigen K1 (less frequently the presence of MRHA, Hly, K5) and originated mainly from CSF. With mouse lung toxicity test correlations of serogroups (O4, O6, O18ac), antigen K5, MRHA, Hly and specimens (blood) were also shown. However, association was found between the lack of virulence factors and phage insensitivity and also between K5 positivity and sensitivity to phages 16, 17, there were no correlations between serogroups and phage patterns. On the basis of the above-described associations one can find correlations among virulence markers, serotype, and nosological group of diseases. Animal pathogenicity tests give additional data in understanding the

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pathomechanism of diseases. Correlations between phage patterns and serogroups reveal certain epidemiological relatedness and also virulence of strains.

"Farewells"

In 1903 H. J. Webber first employed the "clone" definition for designation of bacteria deriving from the same progenitor; now it is postulated that a limited number of clones with special pathological properties have been spread over large geographical areas [1]. In *Escherichia coli* certain strains characterized with well-defined serotypes, and virulence markers can be isolated from pathological conditions rather than from normal intestine [2]. Many methods have been used by microbiologists to characterize *E. coli* strains. They were tested for colicin (Col) and haemolysin (Hly) production, their O, K, H and F antigens were determined [3, 4]. Studying their adhesins and colonization factors, cell surface components [5, 6] it was possible to recognize that various virulence factors (Hly, K1, K5 antigens, mannose resistant haemagglutinating activity – MRHA) were associated with certain diseases (i.e. newborn's meningitis, septicaemia, urinary tract infection). Moreover, it could be assumed that any feature associated with diseases was implicated in the diseases processes [3].

In our earlier studies we have found that *E. coli* isolates deriving from human extraintestinal infections harboured certain well-defined virulence factors [7, 8]. That was the reason why we were interested to study the characteristics of 3334 *E. coli* strains isolated in Hungary between 1979–1990 from different clinical materials (blood culture – 282, cerebrospinal fluid – 72, urine – 906, cervix or vaginal swabs – 180, bile – 105, pus or wound – 185, nose or throat – 349, faeces – 860, or other clinical materials – 405). In the research reported here, a multivariate analysis of strains was performed by employing computer program. Association of serogroup, phage pattern, virulence markers (MRHA, Hly, detection of K1 and K5 antigens, colicinogenicity), LD_{50}, mouse lung toxicity, and site of infection were analysed.

Materials and methods

Bacterial strains. E. coli strains were isolated from clinical samples in 6 County Public Health Stations and 7 hospitals.

Classification. According to serological examination of O and H antigens, virulence markers, phage sensitivity, colicinogenicity, animal pathogenicity tests were carried out as described previously [7, 9, 10].

Computerized analysis was made using the EPI-INFO computer programme (Version 5, CDC, Atlanta).

Results

A total of 133 serogroups represented O antigens 1 to 170. The most common O serogroups among the isolates were O1 (112 strains), O2 (205), O4 (251), O6 (400), O7 (67), O8 (64), O15 (49), O18 (175), O45 (29), O75 (83), O78 (197) and O83 (24). The isolates fell in 50.2% in the above-mentioned 12 serogroups. In addition, 177 strains were spontaneously agglutinable (Sp, 5.3%) and 782 ones (23.4%) were O-non-typable (NT); 719 strains (21.0%) belonged to one of the remaining 119 serogroups, each represented by 1 to 17 isolates. Haemolysin production was recorded in 30% of the isolates. The frequency of mannose resistant haemagglunating activity among the strains was 30% and 36% of them were colicinogenic. Antigens K1 and K5 were possessed by 11% and 6.6% of the isolates, respectively. Characteristics of the most common 12 O serogroups were further analyzed (Table I).

Associtation of the most frequently occurring serogroups with the origin and virulence markers of strains

Serogroup O1

Almost all 112 strains (82%) were either non-motile or possessed antigen H7. H1, H33, H34, H55 or not typable (NT) H antigens were present in few strains only. Twenty percent of strains were Hly^+ and 60% were $MRHA^+$. Hly and MRHA properties occurred simultaneously in 17% of the isolates. The frequency of MRHA positivity was higher (86%) among Hly^+ strains than the rate of Hly positivity (28%) among $MRHA^+$ strains. Colicin production was recorded in 67% of the isolates.

The frequency of $MRHA^+$ (77%) and Col^+ (81%) among strains with $K1^+$ was higher than among strains with other/or without K antigens (32% and 42%, respectively). It is remarkable that $K1^+$ or $K1^-$ strains occurred at an equal rate in blood and urine, however, those isolated from faeces were more frequently $K1^-$ than $K1^+$. $K1^+$ isolates originating from cervix and vagina showed higher incidence than those without K1 antigen. Strains belonging to serogroup O1 also occurred in nose, throat and wound. There was no correlation between the incidence of virulence markers and the diagnosis of the patients when strain originated from blood or urine.

Table I

Characteristics of strains belonging to the most frequently occurring serogroups

O antigen group	K antigen type	No, % of strains		H antigen (%)	Hly+%	MRHA+%	Hly, MRHA+%	Col+%	Incidence in clinical samples (%)
1		112	3.3	-(54),7(28)	19.8	61.3	16.9	67	
	1	72	64	-(58),7(36)	25.4	77.5	86.0*	80.6	2(8.3),3(22.2),4(15.3),9(11.1), 5(9.7),7(6.9),13,14(5.6)
	Others	40	36	-(47),7(15)	10.0	32.5	28.0#	42.5	2(10),4(40),3(17),5,6,12,13(5), 9,11,18(2)
2		205	6.1	-(45)	32.7	32.7	14.6	43.9	
	1	66	32	-(32),4(17)	9.1	34.8	44.8*	71.2	2(4.5),3(27.3),9,14(10.6),4(9.1),5(7.6), 8(4.5),6
	5	19	9	-(84)	21.1	15.8	44.8#	15.8	3(68.4),4(21.1),15(5.3)
	Others	120	59	-(37)	47.5	34.1		44.4	2(9.3),3(43.9),4(7.8),5,14(5.4),6,7(3.9), 9(3.4)
4		251	7.5	5(53),	79.3	85.3	68.5	8.8	1(2.0),2(13.9),7(15.5),4,6(14.7),3(12.4) 5(5.6)
							86.4*		
							80.3#		
6		400	11.9	1(63)	85.2	64.8	58.2	21.0	
	1	19	4.8	1(50)	78.9	42.1	68.2*	15.8	1(5.3),2(10.5),12(21.1),3,14(15.8),5, 7(10.5),6,9(5.3)
	5	47	11.8	1(50)	83.0	80.9	89.9#	42.6	1(2.1),2,4(14.9),7(19.1),3,14(10.6), 6(6.4)
	Others	334	83.5	1(75)	85.8	63.8		21.0	1(3.3),2(13.5),3(28.0),4(11.3),7(9.3), 5(8.0)
7		67	2.0	-(57)	9.0	29.9		58.2	
	1	30	44.8	-(94)	13.3	56.7		86.7	1(23.3),2(20.0),3,4(16.7),9(10.0)
	Others	37	55.2	-(27),4(30)	5.4	8.1		35.1	2(2.7),3(43.2),4(27.0),12,13(5.4), 7,14(2.7)
8		64	1.9	-(26)	7.8	6.3		26.6	2(9.4),3(40.6),4(35.9)
15		49	1.5	-(28),18(26)	30.6	51.0		24.5	2(20.4),4(40.8),3(14.3)
18ac		175	5.2	-(61),7(27)	50.3	52	94*	41.4	
	1	64	36.6	7(62)	0	0		87.5	1(32.8),2(17.2),3(10.9)
	5	85	48.6	-(100)	85.9	89.4	92#	12.9	2(7.1),4(34.1),3(12.9),7(10.6),14(8.2)
	Others	26	14.8	-(57)	53.8	57.7		20.0	1(3.8),2(7.7),3(42.3),4(23.1)
45		29	0.86	7(34),-(21)	3.4	65.5		73.3	
	1	21	72.0		0	85.7		100	1(14.3),2(38.1),3(28.6)
	Others	8	28.0		12.5	12.5		11.1	2(11.1),3(44.4)
75		83	2.5	-(53)	65.0	32.5	25.3	18.1	
	1	4	4.8	-(25)	100	25	38*	25	7(50),3,14(25)
	5	37	44.6	-(81)	83.8	62.2	77.7#	24.3	2(10.8),3,7(27),4(24)
	Others	42	50.6	-(31)	45.2	7.1		11.9	2(2.4),3(38.1),4(26.2)

Table I

(cont.)

O antigen group	K antigen type	No, % of strains		H antigen (%)	Hly+%	MRHA+%	Hly, MRHA+%	Col+%	Incidence in clinical samples (%)
78		197	5.9	-(89.8)	1.5	3.6		91.4	1(4.1),2(3.0),4(52.8),6(16.2),7(15.7)
83		24	0.71	-(67)	12	4		66.7	
	1	19	79	-(79)	5.3	5.3		73.7	1(15.8)2,(5.3),3(42.1)
	Others	5	21		40.0			40.0	3(60.0),4(20.0)

* Hly+ strains MRHA+%, # MRHA+ strains Hly+%

Samples: 1 = cerebrospinal fluid, 2 = blood, 3 = urine, 4 = faeces, 5 = wound, 6 = nose, 7 = throat, 8 = umbilicus, 9 = cervix, 10 = others, 11 = skin, 12 = bile, 13 = ear, 14 = vagina, 15 = abdominal cavity, pus, 16 = lochia, 17 = amniotic fluid, 18 = vulva

Serogroup O2

Almost half (45%) of the 205 strains were non-motile, the remaining possessed 12 kinds of H antigen (H1, H2, H4, H5, H6, H7, H12, H25, H34, H39, H42, H49) or were non-typable. Hly^+ and $MRHA^+$ were present in 32.7%. Both properties occurred simultaneously in 45% of strains. One third of the 66 strains with K1 antigen (32%) were non-motile and 17% of them harboured antigen H4. Col positivity of $K1^+$ strains was higher (71.2%) than those with antigen K5 (15.8%) or with other K antigens (44.4%). Non-motile strains were frequent among isolates with $K5^+$ (16/19). Haemolysin production occurred frequently among strains possessing K antigens other than K1 or K5 (47.5%). Among isolates from blood, the presence of K1 or other K antigens were frequent (see Table I), whereas among those from urine $K5^+$ strains had a higher incidence (68.4%). $K5^+$ isolates were found frequently in inflammatory diseases, too. Among strains from urinary tract infection (UTI) 36 (from 25 patients) were cultured from lower UTI and 33 (from 30 patients) from upper UTI. Those strains that were isolated from upper UTI possessed rather frequently antigen K1 (11/33) or were more frequently Hly^+ (14/33) than those from lower UTI (4/36 and 6/36, respectively).

Serogroup O4

More than half (53%) of the 251 strains harboured antigen H5, the other half of them (45%) were non-motile or had either H1 or NT flagellar antigens. Two percent of them had H3, H4, H7, H14 and H16. Hly^+ (79.3%) and $MRHA^+$ (85.3%) were frequent in serogroup O4. They occurred together in 68.5% of strains. Their simultaneous occurrence were frequent mainly among isolates in blood (83%) but also in nose and throat (76.3%). Only 8.8% of strains were Col^+.

Strains of serogroup O4 could be isolated from CSF (2%), blood (14%) nose and throat, faeces, urine and wound. CSF strains originated from infants younger than one week (4 cases) or 1 month (1); two infants had pneumonia and enteritis beside meningitis. Thirty of the 35 strains isolated from blood (25 patients) were from adults (19 to 75 years old) and 2 of the remaining from the above-mentioned newborns with meningitis. Clinical diagnoses were known in 11 of the 25 adult patients. Sepsis was associated with UTI in 4 cases, with hepatic diseases (cirrhosis hepatis, tumour hepatis, cholecystitis) in the other 4 patients. In the remaining 3 cases other diseases were in the background of sepsis. Diagnosis was known in 17 of 31 patients with UTI: 7 of them had lower and the other 10 upper UTI. Ten out of 14 Hly^+, $MRHA^+$ urinary strains belonged to the following groups: pyelonephritis (6), cystitis (4).

Serogroup O6

Main part (63.5%) of 400 strains possessed H antigen H1 or were non-motile (19%). Strains belonging to the remaining 18% had antigens H4, H5, H6, H12, H16, H21, H31, H42, H52 or were NT. Hly production was recorded in 85.2%, MRHA positivity was also frequent (64.8%). K1 was shown in 4.8% and K5 in 11.8% of the strains. MRHA positivity of $K5^+$ strains was more frequent (80.9%) than that of $K1^+$ isolates (42%). $K5^+$ strains were also more frequently characterized by Col^+ (42.6%) than those having other K antigens (15% and 21%, respectively). Simultaneous occurrence of Hly^+, $MRHA^+$ (58.2%) was frequent in serogroup O6. Among $MRHA^+$ strains the Hly^+ character occurred more frequently (89.9%) than among Hly^+ strains the $MRHA^+$ character (68.2%). Strains in CSF, blood and urine had both types of K antigen at an equal frequency, whereas $K1^+$ strains were not isolated from faeces. $K1^+$ strains occurred frequently in bile and vaginal specimens. Strains of serogroup O6 were found also in nose, throat, cervix. Four out of 7 patients represented by 13 strains in CSF were younger than 1 week. One of these strains had K1 capsule, whereas the strain isolated from one of the remaining 3 adults harboured antigen K5. Only 3 out of 45 patients with sepsis (54 strains in blood) were infants (1–2 weeks old). Twenty out of 28 septic cases with known diagnosis were associated with UTI. Of the 112 strains in urine (103 patients) 48 came from lower and the other 32 from upper UTI. Strains from lower UTI showed Hly^+ and $MRHA^+$ properties (out of 48 39 and 22, respectively) nearly at an equal rate as compared to those from upper UTI out of 32 28 and 11, respectively.

Serogroup O7

More than half (38) of the 67 isolates were non-motile, 12 had antigen H4. Nearly half (30) of them possessed antigen K1. As a rule, $K1^+$ strains were non-

motile (28/30) and were characterized by MRHA$^+$ (56.7%) and Col$^+$ (86.7%) properties, in contrast to those having other K antigens (8% and 35%), respectively. K1$^+$ strains were frequent in CSF (23.3%) and blood (20%), however, they could be isolated also from urine, faeces and cervix. Strains with other K antigens occurred in blood, but they were found most frequently in urine. Seven strains originating from CSF represented 4 patients (3 of them were infants younger than 2 weeks). Seven strains (6 of them were K1$^+$) were isolated from the blood of 6 patients, 3 of them having meningitis. In case of 21 urinary strains the diagnosis for 10 patients was known: 6 of them had lower and another 4 upper UTI.

Serogroup O8

Twenty-six percent of the 64 strains were non-motile, while H antigens of 22 were not typable. The following H antigens occurred in O8 strains: H1, H3, H4, H9, H16, H20, H21, H30, H38, H40, H49 and H55. None of Hly, MRHA, K1 and K5 properties were characteristic of this serogroup. One of the 6 strains in blood (5 patients) came from a newborn, the others from adults. Strains of serogroup O8 occurred in urine and faeces.

Serogroup O15

Main part (40) of the 49 strains were non-motile, or had H antigen H18 and HNT. One third of them were Hly$^+$ (16), 25 were MRHA$^+$ and 12 Col$^+$. Five out of 10 strains from blood (O15: H18) were associated with an outbreak among newborns, 3 of whom developed meningitis. All these 5 strains were Hly$^+$, MRHA$^+$ and none of them produced colicin. Strains of this serogroup occurred also in faeces and urine.

Serogroup O18ac

More than half (61%) of the 175 strains were non-motile and 27% of them had antigen H7. Antigens H1, H14, H19, H52, H55 and not typable were also found. Hly$^+$ and MRHA$^+$ occurred in 50% and 52% of strains, respectively. Simultaneous occurrence of these properties was very high: 94% and 92%, respectively. Antigens K1 and K5 were present in 36.6% and 48.6%, respectively. Fourty out of 64 K1$^+$ strains possessed antigen H7, none of them had Hly and MRHA but 87.5% of them were Col$^+$. K1$^+$ strains were isolated mainly from CSF (21), blood (12), however, they also occurred in urine.

All of the 85 strains with K5 antigen were non-motile, whereas 86% and 89% of them were Hly$^+$ and MRHA$^+$, respectively. Their Col$^+$ (13%) was less frequent than those in K1$^+$ group. These strains occurred in blood (7.1%), faeces (34%), and frequently in urine, throat and vagina (see Table I).

The incidence of the above-mentioned properties in $K1^-$ and $K5^-$ strains (26) varied. They were isolated from CSF, blood, urine and faeces.

Twenty-one out of 22 CSF strains were $K1^+$, 19 of them were isolated from infants below 4 weeks. Eleven out of 20 strains in blood possessed also K1 antigen, 8 of them were isolated from the blood of infants with meningitis younger than one month. Five out of 6 $K5^+$ strains came from the blood of adults. Eleven out of 31 urinary strains had K5 antigen and 9 of $K5^+$ strains were $MRHA^+$, too. Other 7 out of 31 urinary strains were $K1^+$. Half (24) of the faecal strains (43) were isolated from infants younger than one year, among them 12 were 1 to 2 months old. Main part of the faecal strains (29) possessed antigen K5. Two and 7 out of 10 strains in vagina had K1 and K5 antigens, respectively. Only 17 strains belonged to serogroup O18ab.

Serogroup O45

Eleven out of 29 strains harboured H antigen H7, another 6 were non-motile. The rest had H antigen H1, H21, H45 or were not typable. Antigen K1 was possessed by 21 isolates. $K1^+$ isolates were mostly $MRHA^+$ (86%), Col^+ (100%) and none of them produced Hly. Virulence markers of $K1^-$ strains varied. Half of the $K1^+$ strains were isolated from CSF or blood, and third of them from urine. Three out of 4 strains from blood were associated with meningitis; the CSF of two infants younger than 2 weeks yielded the same agent.

Serogroup O75

More than half (53%) of the 83 strains were either non-motile or possessed antigen H7. Other H antigens were: H5, H43, H55 and HNT. Hly^+ and $MRHA^+$ occurred in 65% and 32% of the strains, respectively. Among $MRHA^+$ strains the Hly^+ character occurred more frequently (77%), than among Hly^+ strains the $MRHA^+$ character (38%). Antigen K1 occurred only in few strains (4) whereas nearly half of strains (37/83) possessed antigen K5. $K1^+$ or $K5^+$ strains were more frequently Hly^+ (100% and 83.2%, respectively) than those having other K antigens. MRHA positivity of $K5^+$ strains was also more characteristic (62.2%) than those possessing other K antigens (25 and 7%, respectively). It is remarkable, that all the 20 Hly^+ $MRHA^+$ strains were $K5^+$ and non-motile. Four out of 5 strains in blood and 10 out of 27 urinary strains were $K5^+$. Diagnosis of urinary infections was known in 20 patients: 16 had lower and only 4 of them upper UTI.

Serogroup O78

Most part (90%) of the 197 strains derived from repeated nosocomial outbreaks during the observation period in a hospital ward. The overwhelming

majority of strains were non-motile (89.8%), Hly$^-$ (98.5%), MRHA$^-$ (96.4%) and Col$^+$ (91.4%). Nearly half of the colicinogenic strains (42.5%) produced V-type colicin. Three out of 8 CSF strains were associated with one of the above-mentioned outbreaks, and all of them derived from infants younger than 3 weeks. Blood isolates came from bacteraemia, associated with meningitis, bronchopneumonia, UTI, sinusitis. Epidemic strains occurred also in nose, throat and faeces of contacts.

Serogroup O83

Sixteen out of 24 strains were non-motile, and 19 possessed antigen K1. Non-motile and Col$^+$ strains were more frequent K1$^+$ than among isolates having other K antigens. Four strains from CSF and blood were K1$^+$ and 3 of them came from newborn meningitis.

Association of clinical specimens with the serogroups and virulence markers

In cerebrospinal fluid *E. coli* serogroups O18ac (30.6%), O6 (18.1%), O78 (11.1%), O7 and spontaneously agglutinable (Sp) – (9.7% each), O4 (6.9%), O45, O83 (4.2% each) were most frequently found (Table II). Antigen K1 occurred in 55.6% and antigen K5 only in 2.8%. Among K1-positive specimens CSF participated in 10% whereas in all specimens CSF represented only 2.2%. From the 72 CSF strains 49 derived from infants younger than one month, another 10 came from 1 to 6-month-old babies and only 13 were isolated from adults. Characteristics of 49 strains originating from newborns were as follows: they belonged to serogroups O7, O18, O45, O78, O83 and 34 of them possessed antigen K1. Seven out of 10 strains from 1 to 6 months old infants belonged to serogroups either O18 or were Sp, and 6 of them were K1$^+$. However, only 3 were K1$^+$ among those 13 strains deriving from adults and 9 of them belonged to serogroup O6.

Among strains in blood serogroups O6 (19.1%), O4 (12%), O18ac (7.1%), O2 (6.7%) and NT (18.8%) showed the highest incidence. Blood strains possessed antigen K1 less frequently than those in CSF, but they had a higher rate of antigen K5 (6.7%) than CSF strains. Hly (46.8%) and MRHA (48.9%) virulence markers were rather characteristic of blood than of CSF isolates. Colicinogenicity occurred less frequently among blood than CSF strains (Table II). Virulence markers were present one and a half times more among blood strains than among all specimens: participation of blood isolates in the total material was 8.5% while they occurred more frequently among virulence marker positive specimens (in K1-positive specimens their participation ratio was 12.8%, in Hly$^+$ 13.3%, in MRHA$^+$ 14.1%). Antigen K5 and Col$^+$ are exceptions as blood strains of K5$^+$ (8.6%) and Col$^+$ (10.5%) groups occurred nearly as frequently as in the total material.

Table II

Association of the different clinical samples with serogroups and virulence markers

Clinical sample (No.)	Most frequent serogroups (%)	Incidence of virulence markers (%)	Incidence of clinical samples in total material (%)	Incidence of clinical samples in virulence marker positive material (%)
CSF (72)	O18ac(31),O6(18)	K1(55.6)		10
	O78(11),O7,Sp(10)	Col(64)		3.8
	O4(7),O45,O83(4)	Hly(29)	2.2	2.1
		MRHA(33)		2.5
		K5(2.8)		1.0
Blood (282)	O6,NT(19),O4(12)	K1(16.7)		12.8
	O2,O18ac(7)	Hly(46.8)		13.3
		MRHA(48.9)	8.5	14.1
		K5(6.7)		8.6
		Col(45)		10.5
Urine (906)	O6(12),O2(10)	Hly(24.5)		22.4
	O1,O4,O18ac,	MRHA(25.9)		24.0
	O75(2–3)	Col(33.1)	27.2	24.9
		K1(9.2)		22.6
		K5(5.8)		24.1
Faeces (860)	O78(12),O6,O18ac	K1(4.1)		9.5
	(5),O4(4),O1(3)	K5(6.3)		24.5
		Hly(18.4)	25.8	15.4
		MRHA(20.3)		17.3
		Col(32.9)		23.4
Total (3334)		K1	11	
		K5	6.6	
		Hly	30	
		MRHA	30	
		Col	36	

CSF = cerebrospiral fluid; K1, K5 = antigens; Hly = haemolysin; MRHA = mannose resistant haemagglutination; Col = colicinogenicity

In urine the typable strains belonged to serogroups O6 (12.4%), O2 (9.9%), and O1, O4, O18ac, O75 (2.5–3%). Antigen K1 and K5 were met with in 9.2% and 5.8%, respectively. Comparing the frequency of urinary $K1^+$ (22.6%) or $K5^+$ (24.1%) isolates to their rate in the total material (27.2%), there was no demonstrable difference between these two groups. This was true for the incidence

of Hly (22.4%), MRHA (24.0%) and Col (24.9%) properties. Moreover, MRHA activity rate failed to differ in upper and lower UTI.

Among faecal isolates serogroups O78 (12.1%), O6, O18ac (5.2%), O4 (4.3%), O1 (3.1%) occurred most frequently. The incidence of $K1^+$ and $K5^+$ isolates (4.1% and 6.3%, respectively) was very low. Other virulence markers (Hly, MRHA, Col) occurred also rarely (18.4%, 20.3% and 32.9%, respectively). Faecal strains in the total material amounted to 25.8% while their incidence in virulence marker-positive groups was lower than the above rate (Table II).

Association of pathogenicity tests with the specimens, serogroups and virulence markers

Correlations of LD_{50} with other phenotypic characteristics of strains are presented in Table III. In the highly virulent group (i.e. LD_{50} below 10^6) strains belonged frequently to serogroups O18 (43%), O2 (18%) and O6 (14%). On the other hand, in the avirulent group (i.e. LD_{50} over 10^6) the most frequently occurring serogroups were O1, O8, O4, O6 (4–6%) or were non-typable (31%), and Sp (5%). Among virulence markers, antigen K1 was mostly responsible for the low LD_{50} values: in the highly virulent group 57% of the strains were $K1^+$ while this ratio was only 4% in the avirulent group. The difference was lower in case of antigen K5 (16% and 2%, respectively), however, $K5^+$ strains occurred more frequently in the highly virulent than in the avirulent group. Regarding Hly, MRHA and Col positivity the same was found, although the dimension of the difference in the two LD_{50} groups never reached that of the antigen K1 or K5.

CSF strains occurred significantly more frequently in the highly virulent (17%) than in the avirulent (1%) group. The difference in the incidence of strains isolated from blood was not as high as of CSF strains. On the other hand, urinary and faecal isolates had higher incidence in the avirulent (22% and 44%, respectively) than in the highly virulent (12% and 16%, respectively) group.

Correlation of mouse lethality test with other phenotypic characteristics of strains are presented in Table IV.

Serogroups O4 (29%), O6 (16%) and O18ac (31%) caused most frequently haemorrhagic pneumonia in mice with lethal outcome. Presence of antigen K5 in serogroup O18 was characteristic of strains that caused lung oedema, as 12 out of 18 $K5^+$ strains belonged to serogroup O18. Beyond K5, these strains were Hly^+, $MRHA^+$, too (10/12).

Table III

Association of LD_{50} with clinical sample, serogroup and virulence marker

LD_{50}	Most frequently occurring sample (%)	serogroup (%)	Incidence of virulence marker positive strains (%)
I $\leq 10^6$ virulent group	1*(17.0), 2(19.0), 3(11.6), 4(15.6), 5(4.8), 9(6.8)	O2(18),O6(14) O18ac(43), NT(6)	K1(57),K5(16), Hly(25),MRHA(36) Col(70)
II $\geq 10^6$ avirulent group	1(1.0), 2(12.6), 3(21.7), 4(44.3), 9(3.0)	O1,O8(4), O4,Sp(5), O6(6),NT(31)	K1(4),K5(2), Hly(15), MRHA(19), Col(30)

* See Table I

Table IV

Association of mouse lung toxicity with origin, serogroup and virulence markers of strains

Mouse lung positive No.	Clinical sample (%)	Serogroup (%)	Virulence marker positive (%)
55	1*(1.8),2(5.5), 3(12.7), 4(41.8),17(3.6)	O4(29),O6(16) O18ac(31)	Hly(85.5), MRHA(70.9), K1(3.6), K5(34.5), Col(34.5)

* See Table I

In relation to virulence markers, in other serogroups Hly and MRHA positivity were also characteristic of mouse lung positive strains: 85% and 70.9% of lung positive strains had these virulence factors. K5 positivity was also more frequent among mouse positive (34.5%) than among all specimens (6.6%). The other markers (Col, K1) could hardly be blamed for lung toxicity as their incidence in the lung

positive material (34.5% and 3.6%, respectively) did not differ significantly from those in the total material (36% and 11%, respectively).

Association of phage sensitivity patterns with other phenotypic characters of strains

Fourty percent of strains belonged to 6 main phage patterns or were non-typable by any of phages (NT). The most frequently occurring phage patterns were: 4a, 4b (398 strains), 4a (113), 12 (72), 17 (62), 15 (61), 4a, 4b, 16, 17 (56), 16, 17 (42), NT (517). There could not be shown any correlation among serogroups and phage patterns, however, strains in certain serogroups were more frequently sensitive to certain phages than in the others (Table V). Thus 44 out of 177 strains lysed by phage 12 belonged to serogroup O2. In serogroups O1 and O2 strains were also frequently sensitive to phage 13 (8 and 18 strains out of 56, respectively). Sensitivity to phage 15 was also associated with these serogroups (out of 90 strains 16 belonged to serogroup O1 and 32 to O2). Thirty-two strains were sensitive to phage 23; 14 of them belonged to serogroup O1.

Association of phage patterns and virulence factors of strains are analysed in Table VI. Those strains that were devoid of virulence markers were more frequently non-typable than those having one or the other virulence markers. There were only 5% NT among Hly^+ strains whereas 19% among Hly^- ones. It was the same among $MRHA^+$, $K5^+$ and $K1^+$ strains. Colicinogenicity was the only exception as there was no difference in the incidence of NT strains between Col^+ and Col^- groups. It is remarkable, that 80% of $K5^+$ strains, whereas only 16% of $K1^+$ and 13% of $K1^-$, $K5^-$ strains belonged to phage patterns 16; 16, 17 or 17.

Table V

Association of phage sensitivity and serogroup

Designation of phage	No. of strains sensitive to phage	Serogroup (No.)
4*	1417	O6(326),O4(227), O78(176),ONT(141) O18(118),O75(68), Sp(48)
12	177	O2(44),ONT(36), O18(21)
13	56	O18(8),O2(18), O45(7)
15	90	O1(16),O2(32), ONT(11),O22(9)
23	32	O1(14),ONT(5)

* Sensitive to phage 4, independently of sensitivity to other phages

Table VI

Association of phage resistant strains with virulence markers

Virulence marker	Virulence marker positive (%)	Virulence marker negative (%)
Hly*	5	19
MRHA	5	19
K1	7	16
K5	3	17
Col	17	15

* See Table II

Discussion

The importance of defining clones is emphasized in many publications [1, 3, 11]. It is a question, however, how to determine a clone? In some of the studies serotypes, fimbriation and other phenotypic character of strains were examined [11] while others used outer membrane protein (OMP) patterns in defining clones [12]. In

spite of the fact that plasmid profile may be characteristic of a clone [11], it should not be used as a primary criterion [3]. Others classify clones on the basis of their enzyme electrotype and LPS properties [13–15] beside O, K antigens, and OMP pattern. It is also a question to be answered to what extent are bacterial strains of identical serotype of common origin? Anyway, the more is known about the phenotypic characters of bacterial strains the best they can be characterized.

The next question frequently asked is whether virulence determinants are clonally associated? According to Kauffmann and his coworkers certain serological types belonging to the frequent O groups possess a particular pathogenicity [16]. Ørskov and Ørskov [17] suggested that certain virulent clones reflected nature's selection. Similarly, Korhonen et al. [18] found that characteristics of strains were attributed to the association of certain patterns (i.e. K1 capsule was seen together with O2, O7, O18 etc.).

In our collection including more than 3000 isolates we found that about half of the strains belonged to a relatively small number of *E. coli* O groups (i.e. 12). As a large number of isolates represented one serogroup, it was possible to analyse the association of different phenotypic characters within one serogroup. It was found that possession of antigen K1 was associated with MRHA and Col properties in serogroups O1, O7, O45, only with Col in serogroups O2, O18 and O83 and with Hly in serogroup O75. Those strains that harboured antigen K5 were characterized by MRHA and Hly properties and lack of flagella in serogroups O18, O75, with MRHA, Hly and Col in serogroup O6, with lack of virulence factors and H antigen in serogroup O2 and only with Col in serogroup O78. In serogroup O4 antigen H5, MRHA, Hly properties occurred frequently simultaneously. Thus there are associations of O, K antigens and virulence markers of strains.

In the literature we have met with the existence of an association between certain virulence markers and serogroups, however, such strains occurred less frequently or sometimes originated only from certain samples (either urine or blood [18]), whereas findings of other authors [19] were inconsistent with ours. Result of Korhonen et al. [18] agreed with ours (P specific fimbriae were often in serogroups O7: K1, O6: K2, O18: K5, none of the 11 strains of serogroup O18ac: K1: H7 produced Hly, three of six O18 strains had K5 capsule and P fimbria, as well as two of them produced Hly etc.). Our results support these findings in a wider scale of clinical samples and larger number of strains belonging to one serogroup. We can add to data of Marild et al. [20], that in serotype O1: K1: H7, beside MRHA positivity, Col production is also a characteristic property of strains (81%). Our results are consistent with data of Selander et al. [11] and also of Hacker et al. [21] whose O18 $K5^+$ Hly^+ P^+ strains represented a homogeneous group independently of the origin of strains. However, in contrast to data of Zingler et al. [19] we found that only 5 of the 38 O6: K5 strains of $MRHA^+$ failed to produce Hly. We suppose

that this difference might come from the different methods using for the detection of Hly production (we employed overlayered medium, which is more sensitive than a common blood agar).

On the basis of our results it may be assumed that serotypes, and virulence markers are closely associated in *E. coli* thus knowledge of this association may be helpful in the detection of certain *E. coli* clones.

As to the association between O antigens, virulence markers and sites the strains were isolated from, the following may be concluded. Strains belonging to serogroup O1, and possessing antigen K1 occurred frequently in vaginal and cervical swabs, wounds, as well as in umbilicus, nose or throat of infants. Their occurrence in blood and urine was independent of the presence of antigen K1, whereas faecal strains lacked antigen K1. Strains of serogroup O2 harboured mostly antigen K5 if they were isolated from urine or pus. However, their frequency in blood was not associated with the presence of K antigens. *E. coli* O4 strains could be isolated from cerebrospinal fluid only of those newborns who had pneumonia, and/or meningitis associated with enteritis. Serogroup O4 and O6 blood strains were isolated from adults suffering from urinary tract infection (30–70%), and from hepatic disorders beside sepsis. Urinary strains of serogroups O4, O6 were isolated at equal rate both from patients with lower UTI and from those with pyelonephritis. In these serogroups MRHA and Hly positivity was about the same in both groups of patients. Serogroup O6 CSF strains were cultured mainly from adults. Serogroup O7: K1 strains occurred frequently in CSF, blood and cervix. Most meningitis strains were isolated from the CSF and/or blood of newborns under one week of age. Strains belonging to serogroup O18: K1 occurred in CSF, blood, urine and faeces of infants. Blood isolates from newborns with meningitis possessed frequently antigen K1. On the other hand $K5^+$ strains were isolated mainly from adults. Vaginal and urinary strains had antigen K5 more frequently than K1. Urinary strains behaved similarly. Strains of serogroup O45 were isolated mostly from cerebrospinal fluid, blood and urine mainly of infants. Strains were frequently associated with newborns' meningitis. Strains belonging to serogroup O75 were present in blood and urine and mostly had antigen K5. Urinary strains caused rather lower UTI than pyelonephritis. *E. coli* O78 strains occurred in CSF, blood and also in nose, throat and faecal samples. In this serogroup most part of strains originated from endemic cases of meningitis among premature babies in a perinatal intensive care unit. Beyond meningitis, bronchopneumonia, urosepsis, sinusitis were also present. They produced colicin of V type. Strains belonging to serogroup O83 possessed antigen K1 and were frequently isolated from cases of newborns' meningitis.

Summarizing, it may be assumed that there are two different groups of *E. coli* in extraintestinal infections. One of them is characterized by antigen K1, serogroups O7, O18, O45 and O83 and ability to cause meningitis among newborns. *E. coli* O78

isolates are exceptions as they lack antigen K1, but they produce colicin V known to be associated with virulence [22, 23]. The other group of *E. coli* cause local inflammation of different organs (UTI, pneumonia, cholecystitis etc.) in adults and – entering the bloodstream – they may be responsible for septicaemia, too. Serogroups belonging to the latter group are: O1 (with or without K1), O2 (with K5, with or without K1), O4, O6 (with MRHA and Hly positivity), O18, O75 (with K5).

The significance of our findings described above is, that knowing the serotype, we can foretell the virulence markers. On the basis of their serotype and virulence markers we can usually forecast the nosological group they belong to. For example, strains of serotype O18: K5: H^- will have MRHA, Hly and will cause UTI, pneumonia or inflammation etc. of adults. Whereas strains of serogroup O18: K1 will produce colicin, and are of a great danger to cause meningitis among newborns.

Association of certain O serogroups, neonatal septicaemia and capsular antigen K1 has been described by several groups of researchers [8, 14, 24]. According to Korhonen et al. [18] and Kusecek et al. [13] K1 strains having a special virulence property are capable of invading the newborn's blood and CSF. Among their collection most strains belonged to serogroups O7 and O18 or harboured R antigen and were associated with infections in age group 0–21 days. In contrast, their O1: K1, O18: K5 isolates were capable of colonizing but not of invading. The difference of these two groups in their virulence corresponded to their different incidence of newborns meningitis. Probably O7: K1, O18: K1 virulence is explained by their property according to which they are able to cause bacteraemia in newborn rats after oral feeding [3]. Neonatal meningitis is preceded by bacteraemia thus in some studies bacteraemia and neonatal meningitis are regarded as one group of disease [17]. All of these clones were very infrequent among *E. coli* strains from other sources. These data are consistent with ours or some of them help to understand our results. According to Minshew et al. [25], and Evans et al. [26] antigen K1, is an additive virulence factor in UTI. In our material they occurred in urine less frequently. However, we can confirm the finding of Robbins et al. [27]: our $K1^+$ strains occurred frequently in CSF of age group under one week. *E. coli* K1 bacteria are of course present in faecal samples of healthy individuals – faeces may be a potential source of infection and can serve a reservoir of K1 strains.

As to an other nosological group of strains, responsible for sepsis and local infections in adults, according to Ørskov [17] bacteraemia does not occur only in newborns, and bacteria can get in the blood frequently from the urinary tract. These results are in accordance with ours. Ørskov's [28] findings – that more than half of the O groupable *E. coli* isolates from blood belonged to serogroups O2, O4, O6 or O75 – is also in good agreement with ours. In their material, like in ours, O18: K5 was a common cause of UTI and was rare in newborn meningitis, while presence of antigen K1 had no significance in causing bacteraemia. According to Brauner et al.

[29] almost half of the strains isolated from blood were P fimbriated, on the other hand MRHA activity was not associated with the site of infections. We found similar results: as 48.9% of our blood strains gave MRHA agglutination (89.2% of them reacted with P kit – not published) and we could not show any association between upper or lower UTI and the incidence of MRHA positivity of strains. The importance of haemolysin in strains causing UTI is also significant: Hly positivity of strains from sepsis mostly associated with UTI was characteristic.

There is another question to be answered: what is the significance of animal pathogenicity tests? Various animal models are employed to confirm the virulence of *E. coli* [8, 30–34]. In these tests different routes of infection were employed. Mouse lethality rate (i.e. LD_{50}) after intraperitoneal infection is one of the commonest model for detecting virulence property of a strain [35, 36]. According to our former experience we found some associations among virulence markers (Hly, MRHA, antigens K1, K5) and LD_{50} values [36]. Our previous observations and conclusions are supported and completed by our recent studies. We could show an association between certain serogroups and virulence of strains in LD_{50} model. Namely, those isolates that belonged to serogroups O2, O6, O18 had higher virulence (i.e. their LD_{50} was below 10^6) than those belonging to serogroups O1, O8 and being spontaneously agglutinable. Similarly, there was an association between harbouring certain virulence markers and LD_{50} of strains (i.e. those isolates that possessed antigen K1 belonged significantly more frequently to a highly virulent group than strains without K1). Frequency of strains with antigen K5 failed to differ significantly in two groups (virulent-avirulent) of LD_{50}. On the other hand the presence of other virulence markers (Hly, MRHA) was not so expressed by different LD_{50} values. In highly virulent group we found frequently strains originating from CSF. This was probably due to the association of the presence of antigen K1 and newborns' meningitis. Interestingly, blood isolates failed to belong significantly more frequently to highly virulent than to the non-virulent group (i.e. LD_{50} values were over 10^6). Probably this is due to the fact that Hly and MRHA activities – which are characteristic of strains originating from septic infections – fail to influence this type (i.e. LD_{50}) of animal pathogenicity. As expected, faecal and urinary strains proved to be avirulent.

There are studies concerning the lethal effect of haemolysin in mice [37] and the production of haemorrhagic lung oedema in mice infected intranasally [32, 38]. According to Ørskov [17] this effect is due to a condition when iron is available to bacteria by lysis of the erythrocytes. In our former examinations we found that, *E. coli* strains of serogroups O4 and O18ac were more frequently responsible for mouse lethality than those belonging to other serogroups. (Association of human enteric pathogenicity and mouse lung toxicity of *E. coli* [39]). In recent analysis we found that strains belonging to serogroup O6 beside serogroups O4 and O18 also cause

frequently mouse toxicity. As to the virulence factors concerned, we can complete the former findings [38] since beside haemolysin positivity, MRHA activity and possession of antigen K5 can also strengthen the virulence of strains in mouse lung model, beside haemolysin positivity. The other virulence factors (antigen K1, colicinogenicity) fail to influence the virulence of strains in mouse lung test.

The correlation between phage sensitivity and O antigen was studied by Kauffmann and Vahlne [16]. Schmidt [40] demonstrated that the lipopolysaccharide could be characterized by phage pattern. Nicolle et al. [41] as well as Eörsi et al. [42] classified enteropathogenic *E. coli* (EPEC) serogroups into different phage types. The phage typing method was further developed by Milch and Deák [43] and by Milch and Gyenes [44]. Milch [45] found that serogroups could be divided further on the basis of phage sensitivity (i.e. strains belonging to serogroup O4 were sensitive most frequently to phages 4; 4, 22; 4, 20, 22; 4, 12, 13, 22, while those of serogroup O18 to phages 4, 12, 13, 16, 17 and 18 were the most characteristic patterns).

On the basis of our recent work we can complete former results [45]. However, lysis to certain phages occurs more frequently in certain serogroups than in others, different phage patterns may belong to one serogroup and one kind of phage pattern may be present in different serogroups. Thus it must be emphasized that phage sensitivity pattern is useful for providing the common origin of strains (i.e. in the course of an outbreak) but not for drawing conclusion to the serogroup, or for replacing serotyping with phage typing. Interestingly, there was an association between the lack of certain virulence factors and not typable strains. Namely, those isolates that failed to have one of the virulence markers were more frequently not typable by phages than those having one or the other virulence factors. Colicinogenicity – as virulence marker – is an exception of the rule, as colicin production was not associated with phage resistance of strains. Another remarkable finding is in our study that there is an association between K5 positivity (i.e. lysis to K5 specific phage) and sensitivity to certain phages (phages 16 and 17). A similar association was shown in some of our $K5^+$ strains in a former study [46].

We believe that knowledge of the associations analyzed above will help microbiologists – cooperating with the clinicians – in the recognition and therapy of extraintestinal infections caused by *E. coli*, moreover, in the prevention of new diseases and spread of infection.

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QUALITY ASSURANCE IN CLINICAL BACTERIOLOGY – A CONTINUOUS DEVELOPMENT IN HUNGARY SINCE 1927*

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In Hungary, uniform methods and quality assessment date back as early as 1927, when the National Institute of Hygiene and regional public health laboratories were established. The National Microbiology Committee organized in 1984 was replaced in 1992 by the National Clinical Microbiology Board and the Joint Quality Assurance Committee of Clinical Microbiology for which the National Institute of Hygiene acts as an operating centre. A long-range programme of proficiency testing and on-site inspections had a beneficial effect on performance in public health laboratories: in 1989 20 out of 24 of them were scored in the excellent or good degree. Hospital laboratories, which joined this programme only in 1984, exhibited less satisfactory results.

Quality control in clinical microbiology diagnosis had its beginning in the first part of this century with the development of organized laboratory services. The importance of establishing written guidelines for equipment, reagents, procedures and safety measures was first recognized in the USA [1–3]. More recently, in many countries there has been a trend toward legal requirements that regulate the operation of laboratories not only in view of accuracy of procedures but also with concern for medical care costs. Accordingly, much study has been gives to assess the quality of laboratory performance. Methods used for this purpose have been summarized by Bartlett [4–6], Keitges [7] and many others. One of the most elaborate regulations on clinical laboratory standards and requirements has been published by the US Centers for Disease Control [8]. A quality assessment scheme has been published by the Quality Assurance Laboratory, Colindale, UK [9].

In Hungary, traces of the establishment of uniform methods and quality assessment data back as early as 1927, when the National Institute of Hygiene was

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organized by Johan [10]. By 1942 fifteen regional public health stations, each having a laboratory department, had been created. At that time a great majority of microbiological examinations was carried out in order to diagnose and prevent the "classical" communicable diseases. The National Institute of Hygiene was responsible for quality assurance in the microbiology laboratory network – its activities included guidance for a uniform, up-to-date performance of procedures and education of personnel.

As far as it can be estimated from early documentation, apart from daily controls, the first quality program was applied by Rauss [11] for the evaluation of the Weil-Felix test, then by Alföldy [12] on the effectiveness of Lovrekovich's bismuth sulphite agar [13].

In 1954 the Hungarian Public Health Service was reorganized so that public stations were established for each county and for the city of Budapest. As a consequence of the introduction of antimicrobial therapy, immunosuppression, widespread use of sophisticated medical procedures and devices etc., an increasing need arose for the laboratory diagnosis of diseases caused by opportunistic pathogens. In the 1950s the overwhelming majority of clinical bacteriology examinations were performed in public health station laboratories, then in the 1960s part of the hospital laboratories also became interested in bacteriology. Whereas under the guidance of the National Institute of Hygiene, public health laboratories used standardized methods and participated in proficiency testing and on-site inspection programmes, microbiology activities of hospital laboratories were not regulated, except that they had no licence to process specimens taken in connection with notifiable communicable diseases.

In 1984 a Government Act delineated the operation of microbiology unites of hospital laboratories, and appointed the National Institute of Hygiene and the newly established National Microbiology Committee as supervisors. Accordingly, from 1984 onwards inspections and proficiency testing were extended to hospital laboratories. This regulation established four categories of bacteriology laboratories according to the complexity of examinations performed by them. Two categories were established for hospital laboratories (I) those performing tests of moderate complexity, e.g. microscopic examination, isolation and identification of aerobic bacteria; (II) those performing tests of high complexity, e.g. isolation and high-level identification of aerobic and anaerobic bacteria. Regional public health laboratories falling in category III were accredited to make enteric bacteriology in addition to all kinds of test listed above, whereas the National Institute, performing all tests on reference level, was categorized as IV. The microbiological diagnosis and prevention of notifiable communicable diseases were restricted to categories III and IV.

In 1992 an important change occurred that made quality assurance more efficient. To replace the Microbiology Committee, the National Clinical

Microbiology Board (in Hungarian terminology "College") was established to act as an advisory council to the Minister of Health and Welfare. For improving the management of quality assessment, the Board organized the Joint Quality Assurance Committee of Clinical Microbiology. The National Institute of Hygiene acts as an operating centre of the national quality assessment programme. Attempts have recently been made to institute a governmental regulation of the licensure of microbiology laboratories.

Materials and methods

Methodical basis. Quality assessment schemes for clinical microbiology laboratories have been based on uniform methods described in Hungarian handbooks and their supplements on bacteriology [14, 15] and on epidemiological typing and parasitology [16] as well as internationally used handbooks and periodicals.

Analysis of annual reports and computer print-outs of data successfully contributes to the interpretation of competence.

Proficiency testing. Freeze-dried ampoules of simulated clinical specimens, usually containing more than one organism, and request forms are prepared in, and distributed by, the Department of Bacteriology, National Institute of Hygiene. The laboratories have to report as accurately and as quickly as possible the presence of a clinically significant bacterium and its in vitro antibiotic susceptibility. The procedure and scoring of laboratories have been described in detail in reference [17].

Announced on-site inspection is performed by members of the National Clinical Microbiology Board and those of the Joint Quality Assurance Committee. In addition to checking the routine performance, culture and identification media are tested with reference strains distributed by the National Institute of Hygiene. Workloads, space, laboratory environment and equipment are recorded.

Unannounced on-site inspections will be conducted by experts of the National Clinical Microbiology Board and of the Joint Quality Assurance Committee. The purpose of unannounced inspection is to review in-house quality control and specimen records, surveying the collection and handling of specimens, checking the quality of routine performance and interpretation of the results.

Table I

Proficiency testing in Hungarian clinical bacteriology laboratories

Year and reference	Specimens			Performance		
	kinds prepared	kinds distributed to each laboratory	Category	Degree*	No. of labs** PH	H
1958 [18]	3005	3005	Patient sera for agglutination test	E	-	
				G	-	
				M	3	-
				P		
1964 [19]	2	2	Simulated sera for agglutination test	E	8	
				G	6	
				M	3	-
				P	5	
1964 [19]	2	2	Simulated sera for agglutination test	E	8	
				G	5	
				M	6	-
				P	3	
1964 [19]	8	8	*Enterobacteriaceae* strains for identification	E	8	
				G	7	
				M	2	-
				P	5	
1965 [19]	6	6	Strains for antibiotic susceptibility testing	E	7	
				G	7	
				M	3	-
				P	5	

Table I

(cont.)

Year and reference	Specimens			Performance		
	kinds prepared	kinds distributed to each laboratory	Category	Degree*	No. of labs** PH	H
1971 [20]	13	13	Simulated clinical specimens for isolation, identification and antibiotic susceptibility testing of pathogens	E	6	
				G	4	
				M	10	-
				P	5	
1989 [17]	32	5-6		E	9	1
				G	11	6
				M	4	10
				P	-	6

* Degree: E, excellent; G, good; M, medium; P, poor - see reference [20]

** PH, public health laboratories; H, hospital laboratories

Results and discussion

Proficiency testing. As shown in Table I, in Hungary the first well-assessed proficiency testing was carried out in 1959 [18]. Patient sera were to be tested in three laboratories for the serological diagnosis of typhoid, paratyphoid and typhus fever and brucellosis. There were considerable errors in the readings of agglutination test: a more than one dilution step difference between the reported and standard results occurred in 26.9 to 50.0% varying with the tests and laboratories. Since the tests were performed with standardized bacterial suspensions distributed by the National Institute of Hygiene, the way how to determine correct agglutination endpoints had to be improved. Proficiency testing with simulated sera in 1964 [19] gave somewhat better results: erroneous findings were reported in 4.0 to 28.0%. These findings inspired us to provide the laboratories with uniform agglutinoscopes.

Identification and antibiotic susceptibility testing of pure cultures in 1964–1965 was considered as an easy task, however, out of the 22 public health laboratories only 13 and 15, respectively, fell into the first two ranges of score (Table I).

By 1971, the diagnostic ability of public health bacteriology laboratories had developed considerably, and it seemed advisable to distribute simulated clinical specimens. In each of these usually two or three different kinds of organism were incorporated. Because of a failure to detect fastidious aerobic and anaerobic agents in specimens containing abundantly growing or swarming bacteria, and of erroneous identification and antibiotic susceptibility testing, the results obtained in 1971 were still not satisfactory [20].

In 1989, with similarly prepared specimens, public health laboratories exhibited a much better competence than in earlier assessments. For the first time, in 1989 the test specimens were also distributed to hospital laboratories. For reasons described above, the majority of hospital laboratories fell into the medium or poor degree of performance (Table I and [17]).

Inspections. Table II shows the results of inspections made in the years 1985–1992. As public health laboratories are visited, as a rule, at three-year intervals, the data represent at least two inspections in each laboratory (the number of public health laboratories being 24). In contrast, data for hospital laboratories stand, in majority, for one inspection in each of them. It is evident that, in all criteria considered, public health laboratories were superior to hospital laboratories. However, floor space, equipment and number of personnel failed to meet the optimum requirement not only in most hospital, but also in part of public health laboratories.

Table II

Announced on-site inspections in Hungarian clinical bacteriology laboratories in the years 1985-1992

Year	Public health laboratories					Hospital laboratories				
	Total No.	No. of laboratories with excellent and good degree of performance[1]	floor space[2]	equipment[2]	personnel[3]	Total No.	No. of laboratories with excellent and good degree of performance[1]	floor space[2]	equipment[2]	personnel[3]
1985-1989	26	25	16	16	17	55	22	8	13	15
1990-1992	24	23	13	11	13	20	5	2	-	2
Total	50	48	29	27	30	75	27	10	13	17
Percent of total	100.0	96.0	58.0	54.0	60.0	100.0	36.0	13.3	17.3	22.6

[1] Estimated on the basis of processing test bacteria and routine clinical specimens

[2] Considered on the basis of laboratory standards of the National Institute of Hygiene

[3] With consideration of number, qualification and experience of personnel as compared to the category of laboratory and annual number of specimens processed

The question arises, why had public health laboratories a much better competence in proficiency testing and in general laboratory performance as compared to hospital laboratories. Beside their personnel qualified in microbiology, equipments and facilities appropriate for up-to-date diagnostics, this can be partly attributed to a close connection than has developed for decades among public health laboratories and with the Department of Bacteriology, National Institute of Hygiene. The use of uniform methods described in handbooks, supplements and circulars, as well as courses and individual trainings in the National Institute of Hygiene greatly contributed to the improvement of performance in the public health laboratory network. Many of the hospital laboratories that achieved good results have had a special interest in bacteriology and maintained connection with the regional public health laboratories.

Looking to the future, the National Clinical Microbiology Board believe that proficiency testings and inspections should be continued to develop bacteriology on a unified and collaborative basis. For this reason even more emphasis should be placed on education and training of personnel. Improvement of equipment and increasing of floor space may be considered important for an efficient service. Research and development activities should also be supported more powerfully than in the last decades.

As to the usefulness of unannounced inspections to be introduced in 1993, we expect to get a better insight in the everyday operation of laboratories.

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MODIFYING EFFECTS OF THE PARENT AND RADIO-DETOXIFIED ENDOTOXINS ON CELL-MEDIATED CYTOTOXICITY AND CYTOKINE RELEASE*

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The cytotoxic effects of the parent and radio-detoxified lipopolysaccharides (LPS and RD-LPS, respectively) were studied at various concentrations (0–50 μg/ml) upon cultured human lung carcinoma target cells. There was no significant difference between the effects of the two endotoxins. LPS and RD-LPS at the same concentrations, however, modified the cytolytic activity of human leukocytes (effector cells) and their mediators in different extent. The most remarkable difference was found at 10 μg/ml. A much higher cytotoxic activity of effector cells was observed in the case of RD-LPS as compared to the parent LPS. This concentration used for the treatment of X-irradiated and unexposed effector cells at 24 h incubation resulted in elevated release of some cytokines as measured by ELISA. For increasing the natural defence, RD-LPS as immunomodulator may be of practical value.

In spite of wide-scale stimulatory effects of endotoxins on various biological processes and their abilities to strengthen the natural defence of macroorganisms, the practical use of these lipopolysaccharides in medicine is limited because of several noxious properties. Radiation treatment diminished the toxic effects of the endotoxin from *Escherichia coli* O89 providing a lipopolysaccharide that possessed tolerance-inducing, immuno-adjuvant, shock-preventing, non-specific resistance-enhancing capacities as well as a certain radio-protective effect [1–5].

Earlier, a difference has been revealed concerning cell membrane effects of parent and radio-detoxified endotoxins [6]. As a consequence of different alterations at plasma membrane level, the final reaction of cells was expected to be also different. In the present series of experiments in vitro, the effects of LPS and RD-LPS on the cytolytic activity of human leukocytes (effector cells) against human lung

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carcinoma cells (target) were studied. Beside the detection of cell-mediated lysis of tumour cells, the contents of various cytokines released from the effector cells into the culture medium were measured. Through the changes of functional conditions of the cells playing an important role in the development of natural resistance, the effects of toxic and radio-detoxified endotoxins were compared. The main question behind these studies was: how the cytotoxicity test for human leukocytes reflects the changes provoked by endotoxin treatments; the main purpose was to provide experimental evidence on the possible use of beneficial properties of RD-LPS in medical practice for stimulation of natural defence, for example, in the course of radiotherapy of oncological patients.

Materials and methods

Parent and radio-detoxified endotoxins. Preparation and detoxication of endotoxins were performed according to methods described earlier [7, 8]. In the experiments 0.1, 1, 10 and 50 μg/ml concentrations were used.

Effector cells. The unfractionated population of human leukocytes from healthy persons was used as effector cells. The cells were separated from heparinized blood of 20-30-year-old males by centrifugation on Ficoll-Uromiro gradient of 1.077 g per ml density. After washing out the gradient, the effector cells were resuspended in complete RPMI – 1640 medium (Sigma) and used at 10^6 cells per ml.

Target cells. Calu-1 cell line originally established from human lung carcinoma was kindly supplied by the Memorial Sloan Kettering Cancer Center, New York. For the experiments, 24 h cultures grown in 24-well plates (Bellco) were used. The nutrient medium consisted of RPMI – 1640 completed by 10% foetal calf serum, L-glutamine and antibiotics. It also contained 0.5 μCi per ml ^{3}H-thymidine (UVVVR, Prague) for radio-labelling of the target cells.

Treatment of the effector cells with LPS and RD-LPS. Freshly separated human leukocytes were treated with various concentrations of parent and radio-detoxified endotoxins for 1 h at 37 °C and 5% CO_2. Then both the cells and their supernatants were used in the cytotoxicity test. In other series of the experiments the unirradiated and 2 Gy-irradiated (THX – 20 X-ray machine, 200 kV, 20 mA, SSD 60 cm, 1 mm Cu filter, dose rate 0.331 Gy per min) effector cells were incubated first for 24 h then treated with LPS and RD-LPS of 10 μg/ml concentration for 1 h. This was followed by the separation of supernatants, and the contents of gamma-IFN and TNF-alfa released from the effector cells into the supernatants were measured by use of ELISA kits.

Cytotoxicity test. The cytolytic capacity of the effector cells and the effector cells' supernatants against tumour target cells was determined according to the methods already published [9 – 11]. After washing out the radioactivities related to the killed target cells, previously labelled with ^{3}H-thymidine, the values of "remained" radioactivities were measured in a liquid scintillation spectrometer. Calculation of the natural and modified cytotoxic activities was made according to the formula earlier described and expressed as a cytotoxic index in per cent [10, 11].

Determination of various cytokines. The amount of TNF-alfa and gamma INF released from the effector cells into the supernatants were measured by ELISA with the kits purchased from Endogen (Boston).

Results

Cytotoxic effects of LPS and RD-LPS on the target cells. The cytotoxicity of various concentrations (0.1–50 μg/ml) of LPS and RD-LPS were tested on Calu-1 human lung carcinoma cells (Fig. 1). It was obvious that the effects of two endotoxins were very similar. The highest toxic effect above 20% was observed for 50 μg/ml concentration as compared with the untreated control. The lower concentrations caused sell toxic effect. The concentration of 10 μg/ml usually used for in vivo and in vitro experiments destroyed about 13–15% of target cells. The latter values, however, proved to be much lower than the spontaneous (natural) cytotoxicity of human leukocytes (33 ± 5%).

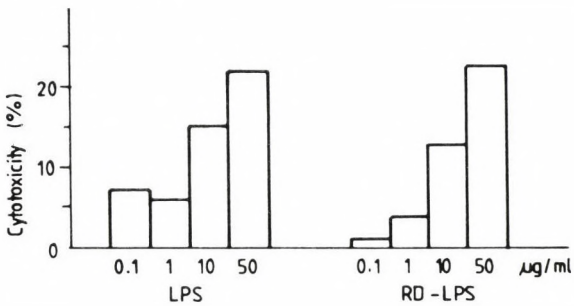

Fig. 1. Cytotoxic effects of LPS and RD-LPS at various concentrations as detected on cultured human lung carcinoma cells (target). The results represent mean values of three measurements

Effects of LPS and RD-LPS on the cytolytic capacity of effector cells. By the treatment of human leukocytes with various concentrations of LPS and RD-LPS, changes in cytolytic capacity could be achieved (Fig. 2). The lowest concentration of 0.1 μg/ml for both LPS and RD-LPS did not modify the natural cytotoxicity of the effector cells. As for 1 μg/ml concentration, LPS remained uneffective; at the same time, RD-LPS caused an increase above 7%. The increasing tendency appeared more clearly in the case of 10 μg/ml RD-LPS. At the highest concentration (50 μg/ml), the cytotoxicity index for LPS increased remarkably, whereas it decreased slightly in the case of RD-LPS (as compared to the effect of 10 μg/ml).

Effects of LPS and RD-LPS on the cytolytic activity of supernatants. For both endotoxins, 0.1 μg/ml concentration did not change the levels of natural cytotoxicity, whereas LPS and RD-LPS equally increased the cytotoxicity of leukocyte supernatants at 1 μg/ml (Fig. 3). The difference between the actions of two endotoxins occurred at 10 μg/ml concentration: it did not modify the natural cytolytic capacity in the case of LPS, and there was further elevation of cytotoxicity (as compared with the increased effect at 1 μg/ml) after the treatment with RD-LPS.

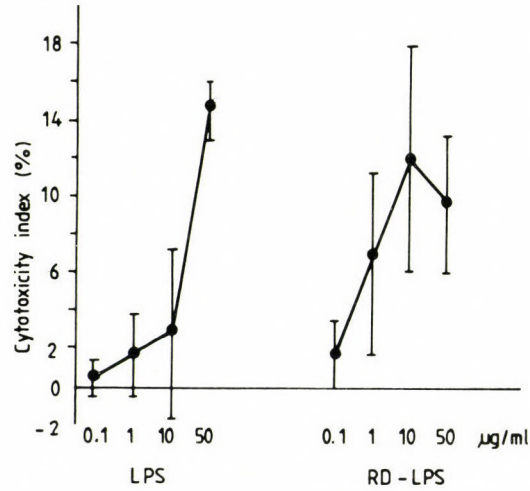

Fig. 2. Effects of various concentrations of LPS and RD-LPS on the cytolytic capacity of human leukocytes (effector cells) as tested against cultured human lung carcinoma cells (target). The results represent mean values ± SD

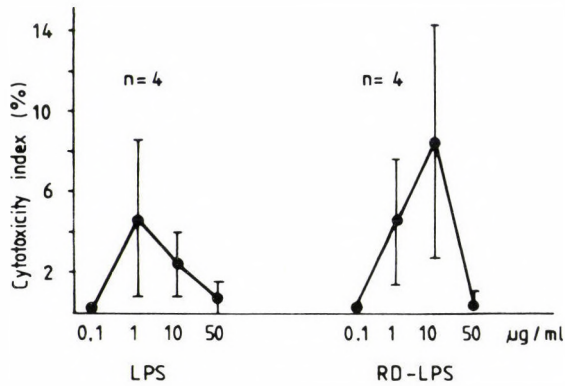

Fig. 3. Effects of various concentrations of LPS and RD-LPS on the cytolytic capacity of supernatants from human leukocytes (effector cells) as tested against cultured human lung carcinoma cells (target). The treatment of the effector cells with the lipopolysaccharides lasted for 1 h. The results represent mean values ± SD

Release of TNF-alfa and gamma-IFN upon the LPS and RD-LPS treatments. The unirradiated and 2 Gy-irradiated leukocytes were used after 24 h incubation (Fig. 4). The treatment of these cells with 10 µg/ml LPS or RD-LPS lasted for 1 h followed by a separation of supernatants. As for TNF-alfa release, the unirradiated leukocytes did not respond either to the LPS- or RD-LPS treatments, whereas the

response of X-irradiated cells to RD-LPS was significantly higher as compared to that of the LPS-treated leukocytes. The latter effect did not differ from the control level which slightly increased after the exposure. The stimulatory effect of RD-LPS could also be observed in the case of gamma-IFN. It was already pronounced for the unirradiated cells. This enhanced effect for the supernatants of irradiated cells was indistinguishable from the radiation-related control level which increased considerably.

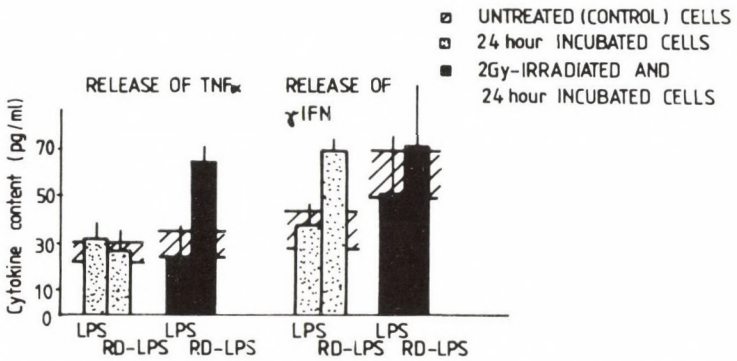

Fig. 4. Cytokine release into the supernatants from human leukocytes treated with LPS or RD-LPS at concentration of 10 μg/ml for 1 h. The treatment of unirradiated and 2 Gy-exposed cells was performed after 24 h incubation. The results represent mean values ± SD. Shaded areas: untreated (control) cells; spotted columns: cells incubated for 24 h; solid columns: 2 Gy-irradiated cells incubated for 24 h

Discussion

The toxic effects of LPS and RD-LPS were similar for the same concentrations as detected on the target cells (cultured human lung carcinoma cells). At the same time there was a difference between the actions of two endotoxins on the natural cytolytic capacity of the effector cells (human leukocytes). The stimulatory effect of RD-LPS was higher than that of LPS used in the same concentration (10 μg/ml). A greater effectiveness of RD-LPS was also observed for the supernatants of lipopolysaccharide-treated leukocytes.

The effects of lipopolysaccharides are realized through the induction of a battery of various cytokines. For example, the radioprotective effect of LPS is related to an enhancement in vivo by cytokines IL-1 and TNF which stimulate one another, and both stimulate IL-6 [12, 13]. Our attention was paid to the spontaneous and LPS-related release of TNF-alfa and gamma-IFN from human leukocytes because of their significant impact in the development of cytolytic effects on tumour cells [14-17]. The contents of these cytokines were measured by ELISA in the supernatants of control (natural) human leukocytes and after treatment with LPS or

RD-LPS. Although the viability of unirradiated and 2 Gy-irradiated cells was approximately 93–98% at the end of incubation, they were exhausted functionally since the cytolytic capacity of such cells decreased from 30–50% to 0–10% (unpublished data). One-hour treatment with RD-LPS increased the release of TNF-alfa into the supernatants of irradiated effector cells as well as the gamma-IFN release from both unirradiated and 2 Gy-exposed leukocytes, whereas the LPS-result were similar to those of the controls. Presumably, the difference between actions of two endotoxins may be explained by their different effects on the plasma membranes of effector cells [6].

For practical purposes, RD-LPS may be used for the monitoring in vitro of the reactivity of immune competent cells. It is also suggested that RD-LPS provides possibilities for better management of patients with decreased immune capacity (for example of those receiving oncological treatment), through its stimulatory effect on the host defence mechanisms.

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COMPARISON OF TWO *SALMONELLA ENTERITIDIS* PHAGE TYPING METHODS*

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In the last 10 years several phage typing methods were developed for *Salmonella enteritidis*, leading to confusion in the predominant phage types (PT) reported from different countries. We made comparative examinations on 1487 *S. enteritidis* strains isolated in Hungary in 1990–1991, using two phage-sets: a modified version of the method elaborated by László et al. (here in after Hungarian method) and the system of Ward et al. (here in after Colindale method). Typability of the strains was nearly the same: 98.0% and 98.3%, the isolates belonging to 18 and 19 phage types, respectively. The Hungarian method revealed 6 (1, 1c, 1b, 1d, 7, 18), the Colindale method 5 (1, 6, 8, 21, 26) frequent phage types. In Hungary PT 1 has been predominant since 1981 and using the Colindale method 64% belonged to this type; using the modified Hungarian method this type could be divided into PT 1, PT 1c, PT 1b and PT 1d. Other frequent phage types (PT 18, PT 7) were nearly identical with Colindale types PT 26 and PT 21.

The first *Salmonella enteritidis* phage typing scheme was proposed by Lilleengen in 1950 [1]. Anderson [2] also worked out a system but it was not introduced into practice. In Poland, Macierewicz et al. [3] elaborated a phage typing system, which was modified on several occasions [4, 5]. A phage typing method was published by Gershman [6]. Later a single phage typing set, consisting of 50 phages was developed by him to differentiate 58 serovars belonging to 9 serogroups [7]; the method was modified [8], the number of type phages being reduced to 27. In Israel a phage typing method was elaborated; using 12 phages 32 types were recognized by typing 829 cultures between 1971 and 1975. The most frequent types were F1 (37.6%), F2 (28.9%) and F3 (5.4%) [9]. The method was slightly modified and now 10 phages are used. In Hungary we introduced on the basis of the method of Lalko

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and Macierewicz [5] a phage typing method [10], that we modified in 1989 (see Materials and methods). A phage typing scheme for *S. enteritidis* was described by Ward et al. [11]. The scheme differentiates 27 types using 10 typing phages. In Spain a phage set consisting of 5 wild and 1 lysogenic phage was developed by Alonso et al. [12]. Out of the examined 1500 strains the majority (74.6% and 19.7%) belonged to phage types A and B. Vieu et al. [13] typed 890 *S. enteritidis* strains by the use of 14 *S. dublin* phages and found 42 types; the 611 human strains belonged to PT 33 in 63.5%. A set of 25 phages for typing and distinguishing 857 strains of different serogroups of *Salmonella* was described by Castro et al. [14]; they established 75 phage types. Some phages were serotype and serogroup specific.

Material and methods

Bacterial strains. Phage types were determined by using 18 phage type standard strains of László et al. [10] and 34 phage type strains of Ward et al. [11] (received from the WHO International Centre for Enteric Phage Typing, Colindale). A total of 1487 *S. enteritidis* cultures isolated from 1200 human, 262 animal and food, and from 25 water samples in Hungary between 1990 and 1991, were examined.

The S. enteritidis phage typing method of László et al. [10], here in after the Hungarian method, described in 1985 was modified in the addition of phages 1 and 2 of the set of Alonso et al. [12], to subdivide the frequent phage type 1 (PT 1). (The phage-set was received from Laboratorio de Enterobacterias, Madrid). The differentiation of PT 1 (determined by the 8 Hungarian phages) is presented in Table I.

A new phage (PT 18) that appeared in Hungary in 1989 was added to the scheme, strains of this type were lysed by phage 4 (+ +) and phage 8 (SCL).

The S. enteritidis phage typing system of Ward et al. [11], here in after Colindale method. The 10 phages described by Ward et al. [11] were obtained from the WHO International Centre for Enteric Phage Typing. The preparations represented 100 × RTD and were sent unrefrigerated.

Results

Table II shows the phage typing of the Hungarian phage type standard strains by the Colindale method. Out of the 18 strains 16 were typable, two strains were not typable. The Hungarian phage type 1 (HPT 1) corresponded to Colindale phage type 1 (CPT 1); HPT 4, HPT 1a, HPT 1c were also typed as CPT 1. HPT 1b, HPT 1d corresponded to CPT 21, HPT 6 to CPT 4 and HPT 7 to CPT 8. Strains of HPT 3, 9, 18 failed to give a characteristic lytic pattern, being lysed only by phage 6; we named that type 26.

Table I

Subdivision of phage type

Phage types	Spanish phage	
	1	2
1	SCL	SCL
1b	–	–
1c	SCL	+ + or +
1d	–	+ +

Table II

Phage typing of Hungarian reference strains by the Hungarian and Colindale methods

Strain designation	Hungarian phage type	Colindale phage type
7115	1	1
9396	2	20
9381	3	26*
7068	4	1
7761	5	15
885	6	4
708/86	7	8
4296	9	26*
10562	11	11
2480	16	Not typable
1122	17	Not typable
3197/89	18	26*
214/83	1a	1
1368/83	3a	13
514/83	10d	23 degraded**
326/89	1b	21
39/91	1c	1
1952/91	1d	21

* The strains was lysed only by phage 6, named by us 26.
** Lysed by phages 2, 4, 9

Table III shows the correspondence of Hungarian and Colindale phage types. The lytic patterns of five strains were not characteristic. Lytic patterns described by Ward et al. [11] of strains 4a, 15, 25 were not reproducible (strain 4a was not lysed by phage 10; strain 15 by phages 3, 8, 10; strain 25 proved to be not typable). We were not provided with the lytic patterns of strains 5a, 9a, 9b, 11a, 13a, 14b, and 20a,

therefore these strains were omitted from the Table. CPT 1 and CPT 17 were identical with HPT 1, and CPT 8, 13, 16 and 19 corresponded to HPT 7.

Table III

Correspondence of Colindale and Hungarian phage types

Colindale phage type	Hungarian phage type
1	1
2	3
3	Nc
4	6
5	4
6	4
7	Nc
8	7
9	16
10	7a
11	Nc
12	10d
13	7
14	Nc
16	7
17	1
18	4
19	7
20	Nc
21	1a
22	3
23	16
24	17

Table IV presents the phage type distribution of 1200 strains of human origin isolated in Hungary. The typability of the strains was nearly the same with both methods: 98.9% (H) and 98.6% (C). Though, the strains belonged into 18 Hungarian and 19 Colindale phage types, only 7 Hungarian and 5 Colindale types were more frequent than 1%. The majority of the strains belonged to PT 1 with both methods, but at a different ratio. The Hungarian method was able to reduce the rate of PT 1 by distinguishing PT 1b, 1c and 1d. HPT 1b was divided between CPT 1 (34.3%) and CPT 21 (60.6%).

Table IV

Phage type distribution of human S. enteritidis strains using both phage typing methods

Hungarian phage types	Colindale phage types							Total	
	1	6	8	21	26	O+	Nt,Nc,Pr	No	%
1	495	–	–	–	–	5	–	500	41.7
1b	47	–	–	83	1	5	1	137	11.4
1c	194	–	–	4	–	2	–	200	16.7
1d	5	–	–	43	–	–	–	48	4.0
7	–	1	90	–	–	4	–	95	7.9
17	–	–	–	–	9	–	3	12	1.0
18	–	–	–	–	152	–	1	153	12.7
O++	6	13	–	7	1	15	–	42	3.5
Nt,Nc,Pr	–	–	–	–	–	1	12	13	1.1
No.	747	14	90	137	163	32	17		
Total								1200	100.0
%	62.3	1.1	7.5	11.4	13.6	2.7	1.4		

O+ = other Colindale phage types (3, 4, 5, 7, 9, 11, 12, 14, 17, 20, 24, 25, 34) occurred in less than 1.0%
O++ = other Hungarian phage types (1a, 2, 3, 3a, 4, 4a, 6, 7a, 10d, 12, 16) occurred in less than 1.0%
Nt = Not typable
Nc = Not characteristic
Pr = Phage resistant

Table V shows the phage type distribution of strains of animal, food and water origin. The frequencies of the predominant phage types are very similar in the different groups.

Figure 1 present the phage types of the 1487 examined strains by the two methods. The strains belonged to CPT 1 in 64% and this type was distributed into HPT 1, 1c, 1b and 1d by the Hungarian method. In Hungary PT 1 is the predominant phage type since 1981 and the Hungarian method proved to be more effective for epidemiological tracing. As to the other frequent Hungarian phage types: HPT 18 was nearly identical with CPT 26, whereas HPT 7 belonged to CPT 21 in 96.4%.

Table V

Phage type distribution of S. enteritidis strains of animal food and water origin using both phage typing methods

Hungarian phage types	Colindale phage types: No. of strains from animal food							No. of strains from water				Total	
	1	6	8	21	26	O+	Nt,Nc,Pr	1	8	O++	Pr	No	%
1	120	-	-	-	-	-	1	10	-	-	-	131	45.7
1b	10	-	-	9	2	-	-	1	-	1	-	23	8.0
1c	51	-	-	-	-	-	-	5	-	-	-	56	19.6
3a	-	5	-	-	1	-	-	-	-	-	-	6	2.1
4a	-	5	-	-	-	-	-	-	-	-	-	5	1.7
6	6	-	-	-	-	2	-	-	-	1	-	9	3.1
7	-	-	13	-	-	1	-	-	4	-	-	18	6.3
18	-	-	-	-	20	-	-	-	-	1	-	21	7.3
O+++	1	-	-	1	-	2	-	-	-	1	-	5	1.7
Nt,Nc,Pr	-	-	-	-	-	1	11	-	-	-	1	13	4.5
No.	188	10	13	10	23	6	12	16	4	4	1		
Total												287	100.0
%	65.5	3.5	4.5	3.5	8.0	2.1	4.2	5.6	1.4	1.4	0.3		

O+ = other Colindale phage types (4, 9, 11, 27); O++ (6, 11, 21, 26) occurred in less than 1.0%

O+++ = other Hungarian phage types (1a, 3, 4, 10d) occurred less than 1.0%

Nt = Not typable

Nc = Not characteristic

Pr = Phage resistant

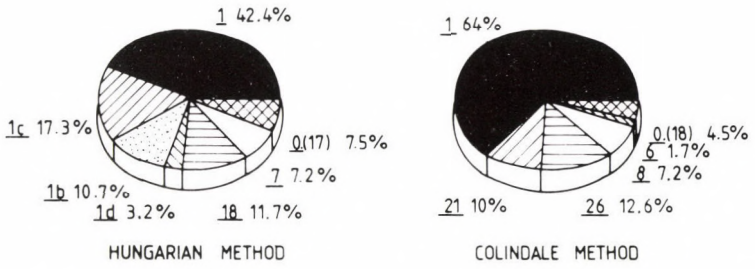

Fig. 1. Incidence of *S. enteritidis* phage types by the Hungarian and the Colindale methods. No. of strains, 1487 (Hungary 1990–1991)

Discussion

The occurrence and epidemiological significance of salmonellae increased throughout the world and also in Hungary. On the basis of data of the Hungarian National Salmonella Centre the incidence of human *Salmonella* isolates was 6152 in 1976, reached a peak (21347) in 1987 and had gradually decreased to 16273 by 1991. (In the number of salmonellae *S. typhi, S. paratyphi-A, S. paratyphi-B, S. paratyphi-C* are not included and one isolation means one isolation per person for a given serotype.) The ratio of *S. enteritidis* increased from 1980–1982 onwards and now it is the most frequent *Salmonella* serotype in Hungary, constituting 67.9% of all human *Salmonella* isolates in 1991.

The increase in the incidence of *S. enteritidis* was observed worldwide. Rodrigue et al. [15] reported that during the past 5 years *S. enteritidis* infections in humans increased on both sides of the Atlantic Ocean. The WHO salmonella surveillance data for 1979–1987 show that *S. enteritidis* was increasing on the continents of North America, South America and Europe and presumably in Africa.

S. enteritidis was the most common *Salmonell*a serotype in England and Wales in 1988 [16]. According to the report of the Public Health Laboratory Service in England the number of *S. enteritidis* isolates increased from 1087 to 6858 between 1981 and 1987. The increase continued in 1988, 15427 isolates were identified, of which 12522 were phage type 4 [17].

Data of the Austrian Salmonella Centre revealed that the total number of *Salmonella* isolates increased from 1725 to 6047 and the rate of *S. enteritidis* increased from 17.6% to 63.2%, between 1984 and 1989 [18].

The rate of *S. enteritidis* isolations increased by 610% in Switzerland, between 1979 and 1987; the strains belonged to PT 4 and PT 8 [19].

Among the 3914 *Salmonella* isolates of human origin the incidence of *S. enteritidis* was 9.1% in Israel in 1989; among strains isolated from chicken this was the second most common serotype [20].

The predominant *S. enteritidis* phage type in the United Kingdom was Colindale PT 4, while in the United States PT 8 and PT 13a occurred the most frequently [15].

Since 1985 in England and Wales there has been a striking increase in the number of human infections due to PT 4; up to the end of 1988, 12522 isolates were identified [21].

In Austria PT 4 was predominant, 71% of the isolates belonged to this type in 1989 [18].

In Belgium [22] among *S. enteritidis* strains of animal origin type 33 was frequently isolated between 1987 and 1989 (according to Vieu et al. [13], this type is identical with PT 4 of Ward et al. [11]).

In the United States 573 *S. enteritidis* strains, collected in 1988 and 1989 were typed. The most common types were PT 8 (48.2%), 13a (20.1%), 13 (7.8%) and 14b (7.8%) [23].

In Canada out of 674 strains PT 8 showed the highest incidence in human (69.9%) and non-human (72.0%) sources between 1976 and 1989 [24].

In Maryland the most frequent types were PT 8 and PT 13a [25].

In the Central African Republic the lytic spectra of the examined *S. enteritidis* strains were not characteristic, the strains were lysed by phages 6 and 7 (by Ward's phages) [26].

In Russia [27] out of the 1142 strains isolated between 1985 and 1986, 86.7% belonged to phage type 1, of the Hungarian system [10].

In Israel [20] the most frequent phage types were F2 and F3 (using the method described by Cahan et al. [9]), PT E2 is identical with the Hungarian PT 1 and F3 with PT 6 (Sechter, I. personal communication).

Though, in this comparative examination the 1487 Hungarian strains tested were randomly chosen, their phage type distribution was in good agreement with our yearly statistical observations. In 1990 and 1991 the 2723 and 2144 human *S. enteritidis* strains were phage typed. In 1990 and 1991 the percentage frequency of HPT 1 was 57.0% and 33.8%, of HPT 1b 7.1% and 19.9%; of HPT 1c 3.5% and 16.4%; of HPT 7 7.2% and 9.6%; of HPT 18 16.1% and 10.5%. HPT 6 corresponding to CPT 4 was found in 0.7% both in 1990 and 1991. However, the spread of this type was observed in 1992, and its frequency increased to 7.8% among 2708 strains (unpublished data).

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EDITORIAL

Dear Reader!

It is our privilege to announce a new and promising development in the life of Journal: we are going to change its name and from 1994 it will be published as ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA. The change in the main heading covers a basic broadening of our scope. This kind of expansion stands on rather solid ground, as one of the major source of immunology goes back to old age, when microbiologists and physicians started and developed vaccination. Moreover, nowadays infectology represents a complex approach simultaneously involving microbiological and immunological aspects of infectious diseases. We are convinced that large and wide scope of immunology, both basic and clinical may attract many interesting papers and more readers.

Our second aim is to renew the microbiological side, introducing more molecular biology and biotechnology. Our board is ready to start some new columns, like invited reviews, technical leaflets, all-round inquiry on a selected problem, centre-page (a removable figure reviewing one issue). We should like to publish the abstracts of annual National Conferences for Microbiology and Immunology, too.

We have plans to initiate an entirely new series. "Outstanding Papers in Microbiology and Immunology" in which leading scientists will be asked to remember favourite scientific papers affecting their concept fundamentally during their scientific career.

Standing on valuable traditions our board will try to make efforts to refresh the spirituality of ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA. We are intended radically shorten the passing through time for the manuscripts.

The Editorial Board of the Journal accepts regular papers from every topics of basic and clinical microbiology and immunology. Obviously we welcome both experimental and clinical papers in biotechnology and molecular genetics, as well.

Please do not hesitate to ask for further information and honour our Journal with your distinguished attention and personal contribution with your manuscripts.

On behalf of the Editorial Board

Dr. I. NÁSZ
Editor in Chief

Acta Microbiologica Hungarica, 40 (4), pp. 265–302 (1993)

NEUROENDOCRINE DEFENCE IN ENDOTOXIN SHOCK

(A REVIEW)

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Mild infection or sublethal dose of endotoxin elicits a brief elevation of GH and PRL in the serum. These hormones have proinflammatory and immunostimulatory effect. In severe trauma, sepsis and shock, GH and PRL are suppressed, whereas glucocorticoids and catecholamines are elevated. Under these conditions an acute phase response is initiated by tissue derived (cytokine) hormones, namely IL-1, IL-6, TNFα, and several others, which elicit a neuroendocrine response and initiate major metabolic alterations. There is fever and catabolism prevails, whereas the synthesis of acute phase proteins in the liver, cell proliferation in the bone marrow, and protein synthesis by leukocytes is elevated. This is an emergency reaction to save the organism after the local immune/inflammatory response has failed to contain and eliminate the infectious agent. During sepsis and endotoxin shock the systemic activation of the complement system and of leukocytes releasing enzymes and highly toxic cytokines seriously threaten survival. Glucocorticoids suppress proinflammatory cytokine production and potentiate the secretion of acute phase proteins. Some of these proteins, such as C reactive protein, or LPS binding protein, are designed to combine with microorganisms and trigger their destruction by the activation of complement system and of phagocytes. The increased production of some complement components also helps host resistance. The rise in serum fibrinogen promotes blood clotting which can serve to isolate the invading agent by

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triggering thrombosis in infected tissues. A number of enzyme inhibitors are produced as acute phase proteins, which are likely to serve to curb the nonspecific damage inflicted by enzymes released from activated phagocytes and from damaged cells into the circulation during sepsis and shock. Catecholamines are also elevated, which serve to inhibit inflammatory responses and to promote, even initiate, the acute phase response. If the acute phase reaction fails to protect the host, shock will develop. Patients with subclinical adrenal insufficiency succumb to septic shock almost invariably if glucocorticoid therapy is not given. However, glucocorticoid treatment of septic patients with normal adrenal function has not been helpful. The use of antibiotics to control infection did not lead to spectacular success either because of the emergence of resistant bacterial strains or the enhanced release of endotoxin by this therapy. The new approaches to prevent and treat septic shock involve the use of antibodies capable of neutralizing LPS and of cytokines and the inhibition of cytokine action by antagonist agents.

Introduction

The first observations that glucosuria occurs during infectious disease were made at the end of the 19th century. This was followed by the demonstration of hyperglycaemia and insulin resistance during infection. It was also observed that fever is due to endogenous pyrogenic and that a leukocyte endogenous mediator (LEM)* induces the synthesis of a number of proteins called acute phase reactants in the liver during severe infection. The endogenous pyrogen was also shown to induce ACTH release. By 1975 a substantial amount of information was available with regards to the metabolic response to infection and the accompanying hormonal alterations that involve glucocorticoids, mineralocorticoids, thyroid hormones, insulin, glucagon, and growth hormone [1–3]. Recent advances in our understanding of the immune system, the cytokine network and the mutual regulatory interaction of these systems with the neuroendocrine system makes it possible to give a general outline of the regulatory interactions that operate during endotoxin shock. Although it seems clear that hormones, cytokines and neurotransmitters are the chief mediators of the pathophysiological response to endotoxin, there are many gaps in our knowledge of this process at the present time.

The response to infection and endotoxin

1. *Cytokine and endocrine response to infection*

Infections frequently occur in immunocompromised individuals that include that elderly, patients on immunosuppressive therapy, cancer patients, and those suffering from severe trauma or shock. The immune system has evolved to control infections regionally so that the spread of pathogenic microbes is prevented while

* A list of abbreviations used is given at the end of this text.

systemic immunity is developed. If, however, the local control mechanisms fail, viraemia or bacteriaemia may develop, which invariably triggers and acute phase reaction (APR) with the elevation of tumour necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6), and a number of cytokines in the blood which function as acute phase hormones. The neuroendocrine and metabolic changes are the result of the interaction of acute phase hormones with the central nervous system and with other tissues and organs [4-7].

Of 18 patients suffering from intraabdominal infection (pancreatitis, abscess, peritonitis) 5 died with multiple organ failure. The deceased patients showed significantly lower mean plasma levels of TNFα and IL-6 and a high rate of unstimulated thymidine uptake by peripheral mononuclear blood cells. T lymphocytes were depressed with lower helper/inducer subset cell numbers and lower PHA stimulation values [8]. In patients with severe acute pancreatitis high levels of LPS in the blood and the early presence (on day 1) of TNF correlated with poor prognosis and mortality [9].

The systemic response to Gram-negative or Gram-positive bacteria to viruses and even to protozoa (malaria) has virtually identical characteristics. Although pathogenic microbes stimulate the immune system through their antigenic determinants, it is obligatory for them to resort to various tricks in order to escape destruction by immune mechanisms. A detailed discussion of the diverse mechanisms that are used by pathogens to avoid immune destruction is beyond the scope of this review. Of major importance to our subject matter is the phenomenon of polyclonal lymphocyte activation by numerous pathogenic microorganisms [10]. This leads to the excess production of toxic cytokines such as TNF, IL-1 and IL-2, which in turn elicit a powerful neuroendocrine response leading to immunosuppression as detailed later. An interesting recent observation relevant to this question is that the glycoprotein, Gp120, of the human immunodeficiency virus stimulates IL-1 production in the brain but not in the periphery, which activates the ACTH-adrenal axis and the induced glucocorticoids (GC) elicit a profound immunosuppression [11].

The liver plays a major role in insulin resistance present during infection. Gram-negative infection enhances the non-insulin mediated glucose uptake by tissues that harbor lymphoid and phagocytic cells such as the spleen, ileum, lung and liver, but not by muscle. Insulin, glucagon, adrenalin, noradrenalin, and cortisone are all elevated during infectious disease [12-15].

During acute infectious disease the increase of GC occurs shortly before the onset of fever and the circadian rhythm is lost. If the infection becomes subacute or chronic, GC levels generally fall to values below normal [3, 16]. Sibbald et al. [17] studied plasma cortisol levels in 26 septic patients. Four patients with greatly increased plasma cortisol were judged agonal and died within 5 days. Seven patients with elevated plasma cortisol (19.2±6 μg/dl) responded adequately to ACTH

stimulation. The remaining 5 patients had 13.8±3.3 μg/dl (normal 8–18 μg/dl) average cortisol level and showed a poor response to ACTH stimulation. Only one of these five patients survived after receiving pharmacological doses of GC immediately after testing. It was suggested that patients with severe sepsis who were not responding adequately to standard therapy should be suspected of having adrenocortical insufficiency and tested accordingly. Rothwell et al. [18] found 13 patients out of a total of 30 with septic shock with poor cortisol response to ACTH stimulation, all of whom died. In contrast, of 19 patients with adequate response, 6 died. Steroid replacement in severe sepsis with physiological doses of cortisol has been suggested as a possible treatment of value in contrast with high doses of methyprednisolone.

In sheep bacteriaemia caused a surge of plasma β-endorphin/β-lipotropin, followed immediately by increases of prolactin (PRL) and growth hormone (GH) and a depression of luteinizing hormone (LH). Only the GH response was inhibited by treatment with opiate receptor antagonist, naloxone [19].

Basal insulin levels were suppressed and glucagon was elevated in 5 septic patients. These patients did not respond to treatment with human GH by elevated serum levels of insulin-like growth factor I (IGF–I), whereas normal individuals did [20]. Leprosy patients could be divided into two groups on the basis of LH response to LHRH: (1) the ones showing an exaggerated response, (2) those with normal response. Basal PRL levels were normal in both groups, but in the first group there was an increased PRL response to thyrotropin releasing hormone (TRH) and to metoclopramide stimulation, whereas in group 2 the response was normal. Both groups had increased thyroid stimulating hormone (TSH) response to TRH in the presence of normal basal T4 and T3 levels. The PRL response to TRH correlated with basal and peak follicle stimulating hormone (FSH) response to LHRH, but not with LH, estradiol (E2) or testosterone. The TSH response did not correlate with either gonadotropins, E2 or thyroid hormone levels [21].

In mice infected with *Pseudomonas aeruginosa* the fall of serum T3 concentrations present in normal animals after fasting could not be induced [22].

2. *The response to bacterial endotoxin*

The lipopolysaccharide (LPS) component of the cell wall of Gram-negative microorganisms, which is released after the death of bacteria, is capable of inducing lethal shock in higher vertebrate animals, but has very little ill effects in fish and frogs. The sensitivity to LPS varies considerably even among various species of mammals [23]. Parenterally administered LPS induced similar pathological changes to Gram-negative bacterial infections, whereas orally it was ineffective [24–26]. Normally, LPS is detoxified by bile acids that are present in the gastrointestinal tract

which prevents oral intoxication. If, however, the bile is diverted biologically active LPS will absorb from the gastrointestinal tract [27].

LPS activates B lymphocytes polyclonally for proliferation and immunoglobulin secretion which frequently contains autoantibodies [28]. Monocytes/macrophages are stimulated by LPS to secrete TNFα, IL-1, IL-6 and other biologically active molecules as shown in Table I. LPS is also capable of triggering mediator release from mast cells and basophils (Table II) and from endothelial cells (Table III) and platelets (Table IV). Neutrophilic granulocytes are also affected by LPS. LPS activates the complement and coagulation cascades and affects directly or indirectly many other tissues and organs in the body [29, 30].

Table I

*Mediators produced by activated monocytes-macrophages**

Cytokines/hormones	Enzymes/proteins
Angiogenesis factor	arginase
Fibroblast growth factor	cathepsin
G-CSF, GM-CSF, M-CSF	coagulation factors
IP-10	collagenase
IFN-α, -β	complement components
IL-1, 6, 8	elastase
Leukotrienes	fibronectin
MCAF	inhibitors of enzymes & cytokines
PDGF	lipoprotein lipase
PGE2	lysozyme
PAF	plasminogen activator
Sterol hormones	reactive oxygen and intermediates
TGF-β	tissue factor
Thromboxane A2	
TNF-α	

* An exhaustive discussion of monocyte/macrophage activating agents and of mediator release is beyond the scope of this article. For reviews on the subject, please see references 223-225 and text for further references. Ag-Ab= immune complex; G-CSF= granulocyte colony stimulating factor; GM-CSF= granulocyte macrophage colony stimulating factor; IFN= interferon; IL= interleukin; IP-10= chemotactic and proinflammatory cytokine; MCAF= macrophage chemoattractant and activating factor; M-CSF= monocyte colony stimulating factor; PAF= platelet activating factor; PDGF= platelet derived growth factor; PGE2= prostaglandin; TGF= transforming growth factor; TNF= tumour necrosis factor

Table II

*Cytokines and enzymes released by tissue mast cells and basophils**

Cytokines/hormones		Enzymes
ECFA	NECF	cathepsin G
GM-CSF	PAF	proteases
Heparin	PGD2, -D4, -E4	
Histamine	serotonin	
IL-3	TNF	
LTC4		

* It is beyond the scope of this review to discuss all aspects of basophil/mast cell activation and mediator release. Instead the reader is referred to recent reviews on the subject (refs 226-228). ECFA= eosinophil chemotactic factor; IL-3= interleukin-3; LTC4= leukotriene C4; NECF= neutrophil chemotactic factor; PAF= platelet activating factor; PGD2, -D4, -E4= prostaglandins. Please see previous table for other abbreviations

The mechanism by which LPS exerts its wide spectrum of biological effects has not been fully elucidated. An LPS binding protein (LBP) of 60 kD molecular weight has been isolated from human and rabbit serum which is capable of opsonizing LPS bearing particles, indicating that it plays a role in the activation of complement system. LBP-LPS complexes were found to be 1000-fold more active than LPS alone in the induction of TNF or IL-1β. The 55 kD glycoprotein, called CD14, serves as the receptor for LPS-LBP complexes on monocytes and macrophages. LBP in the serum may rise up to 100-fold (from 0.5 to 50 μg/ml) during an acute phase response [31]. Others identified an 80 kD LPS binding receptor expressed on murine macrophages and monocytes which is capable of inducing tumouricidal activity [32]. Several other receptors for LPS have been proposed [33]. Signal transduction pathways and nuclear proteins that may be involved in LPS induced gene expression by a variety of cells are currently under investigation but not yet conclusive.

The B lymphocytes of C3H/HeJ mice cannot be activated by LPS and such mice respond poorly to infection with Gram-negative bacteria [34]. Recently LPS was shown to exert an antiproliferative effect on B and T lymphocytes of C3H/Hej mice and to inhibit PGE2 synthesis by macrophages [35].

Table III

*Cytokines produced by the endothelium**

Cytokines/hormones		Other molecules
Endothelin	PAF	factor VIII
GM-CSF	PDGF	fibrinolysis inhibitor
IL-1	PGE2	plasminogen activators
IL-8	Prostacyclin (PGI2)	von Willebrand factor
IP-10		

* For review, please see references 224, 229-232. Please see previous tables for abbreviations

Table IV

*Platelet derived mediators**

Cytokines/Hormones	Other mediators
β-Thromboglobulin	acid hydrolases
Chemotactic factor	ATP, ADP, GTP, GDP
IL-8	calcium
Mitogenic factor	factors V, VIII
PF-4	fibrinogen
PAF	fibronectin
PDGF	proteins
Prostaglandins	thrombin
Serotonin	von Willebrand factor
TGF-β	
Thromboxanes (A2)	

* The data listed in this table are for the purposes of this discussion only. Readers interested in more information are referred to reviews (refs 224, 233, 234). PF-4= basic platelet factor. Please see previous for more abbreviations

(i) *Cytokine and acute phase hormone response to endotoxin.* The intravenous injection of LPS to rats induced little or no increase in whole organ TNF mRNA in the spleen, liver and small bowel up to 4 h, whereas serum TNF levels were elevated significantly at 1 and 2 h. Pretreatment with dexamethasone (DEX) suppressed the LPS induced serum TNF concentration without affecting TNF mRNA. A second LPS dose 2 h after the first injection did not induce a second peak of serum TNF. LPS induced IL-1α mRNA readily in the spleen, beginning at 1 h, peaking at 2-4 h, and disappearing at 6 h. IL-1β mRNA was induced at 0.5 h, peaking at 1 h and disappearing by 6 h. DEX pretreatment inhibited the induction of IL-1α and β mRNA by LPS. The injection of TNF or IL-1 i.v. also induced IL-1 mRNA expression [36].

In murine bone marrow derived macrophages LPS induced the accumulation of one TNF mRNA species, which was amplified 7-fold by pretreatment with cycloheximide, and resulted in the synthesis and release of TNF. Murine TNF induced the synthesis of two distinct TNF mRNA species in marrow derived macrophages which was not affected by cycloheximide and did not result in the synthesis of TNF protein even when IFN−γ was used as an additional stimulus, which enhanced TNF mRNA synthesis induced by both agents [37].

Hydrocortisone inhibited significantly TNF and IL−1 secretion and TNF, IL−1 and IL−6 mRNA levels induced in murine adherant peritoneal exudate cells by LPS. The secretion of IL−6 was only slightly diminished. ACTH and insulin increased TNF secretion in response to LPS while both hormones decreased TNF mRNA, suggesting that TNF protein production was modified at the posttranscriptional level. LPS induced IL−6 production was also increased by ACTH and insulin without altering mRNA levels [38].

Peripheral administration of endotoxin to rats induced immunoreactive IL−1β in macrophages and ramified microglial cells located in the meninges, choroid plexus, brain blood vessels, and in the brain parenchyma [39].

High but transient TNFα levels were observed in mice after bolus i.v. injection of LPS or live bacteria (*Escherichia coli* O111). The cytokine levels (TNFα, IL−1, IL−6) during lethal peritonitis (produced by *E. coli* O111) increased progressively, but remained 50−100-fold lower than the peak values observed after i.v. injections and remained constant until death [40].

Adrenalectomized (ADX) mice show an extreme sensitivity to the lethal effect of LPS and respond with an exaggerated TNF production, which is maximal at 2 h after injection and returns to baseline levels by 4 h (Fig. 1). The inhibition of GC synthesis in intact mice by metyrapone has a similar though less dramatic sensitizing effect to LPS. Treatment with DEX inhibited excess TNF production and protected ADX mice from the lethal effect of LPS [41]. Parant et al. [42] described a similar kinetics for the serum TNF response in mice to LPS and found that in addition to ADX, pregnancy and treatment with BCG also sensitized the animals to the lethal effect of LPS. BCG treatment was associated with elevated levels of TNF, whereas pregnancy has no effect on TNF production. Zuckerman et al. [43] described a similar kinetics for the serum TNF response in mice to LPS, but found that in ADX or hypophysectomized (Hypox) mice TNF levels remained elevated 5 h after LPS injection. He also measured the IL−1 response, which was maximal at 4 h and remained elevated at 24 h. Pretreatment with DEX prevented the TNF response.

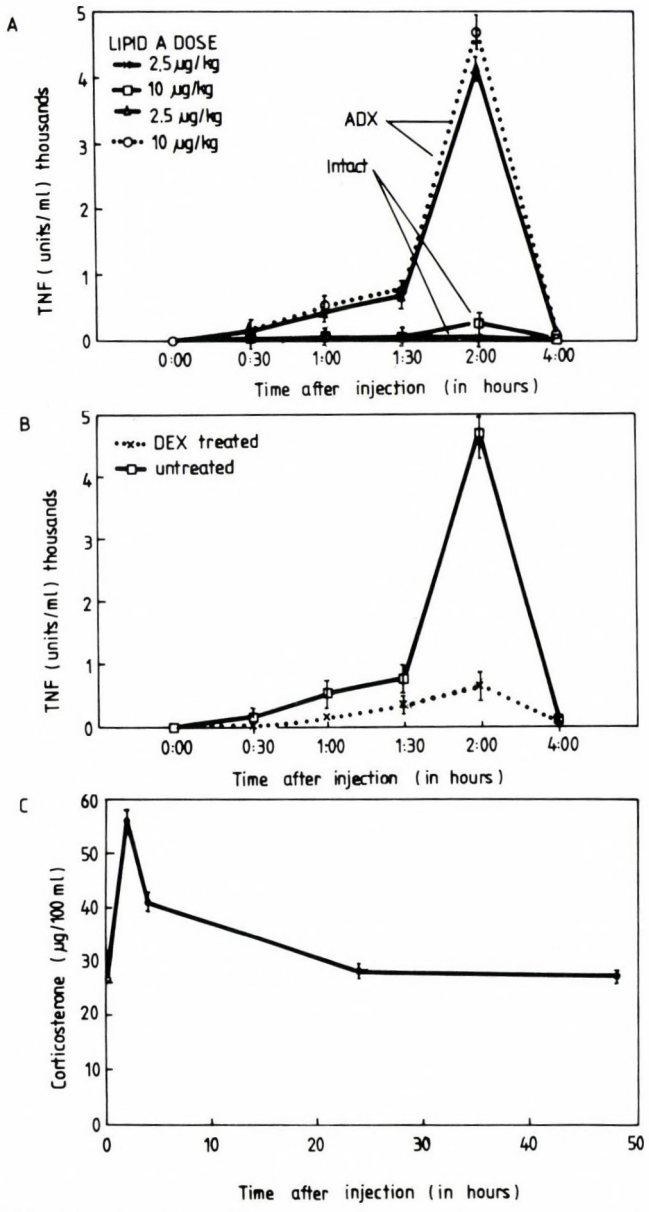

Fig. 1. (A) TNF response to lipid A in intact and adrenalectomized (ADX) mice. Statistical analysis (Student *t*-test): the levels of intact and ADX mice were significantly different ($P < 0.001$) 2 h after LA injection. (B) Suppression of the TNF response in ADX mice by dexamethasone. A group of four ADX mice was administered dexamethasone (3.0 mg/kg i.p.) once a day for 5 days. Lipid A was given at 10 µg/kg i.v. 30 min after the last DEX injection. Statistical analysis: TNF levels of DEX treated mice were significantly lower ($P < 0.001$) when compared to the untreated group. (C) Corticosterone response to lipid A in intact mice. The LA dose was 10 000 µg/kg i.p. in this experiment. Statistical analysis: A highly significant elevation of plasma corticosterone ($P < 0.001$) was found 2 h after LA injection [41]

In rats, the TNF response to LPS was maximal at 60–90 min and showed a half-life of 27±7 min. The TNF response to a second LPS injection 3 days later was 15% lower. Pretreatment with DEX 5 h or more prior to LPS injection reduced the concentrations by 70–90% [44]. Macrophages prepared from Hypox rats showed an 83% reduction in the TNF response to LPS in vitro. This deficiency could partially be corrected by the treatment of Hypox animals with GH, however, GH did not prime the macrophages for TNF synthesis in vitro. The macrophages from Hypox rats responded to stimulation with rat IFN–γ by increased TNF production [45]. LPS induced TNFα production by porcine alveolar macrophages was inhibited by porcine $TGF\beta_2$, cortisol or DEX and enhanced by porcine IFN–γ, which also reversed the inhibitory effect of the above hormones [46].

Normal mice do not respond to repeated LPS injections with increased serum levels of TNF (endotoxin tolerance), whereas ADX mice do, which is associated with an increase in splenic TNF mRNA. Treatment with glucocorticoids restores the ability of ADX mice to develop tolerance. Mice trated with galactosamine lack similarly the ability to develop endotoxin tolerance even though there is a normal elevation of serum glucocorticoids in response to LPS. Galactosamine is known to act on the liver and on this basis the authors suggested that both glucocorticoids and some liver derivated factors, possibly acute phase proteins, may be required for the development of endotoxin tolerance [47].

The continuous infusion of nonlethal dose of LPS to rats for 2 weeks compromised the ability of their spleen cells to produce IL–1 and TNF after stimulation in vitro with LPS. There was also a moderate suppression of Con A induced IFN–α, –β, and –γ production, which indicates that suppression was not restricted to LPS, but the response to a nonspecific plant mitogen is also affected [48].

Cortisol (10^{-7}–10^{-6} *M*) and DEX (10^{-8}–10^{-6} *M*) suppressed LPS induced TNF production by human monocytes in a dose dependent manner, being most effective when 48 h pretreatment was done. The suppressing effect of DEX varied greatly from donor to donor [49]. Treatment of human macrophages with IFN–γ increased the number of TNF receptors, whereas treatment with epinephrine, insulin, glucagon, somatostatin, ACTH and angiotensin decreased receptor numbers. Insulin, epinephrine and somatostatin also inhibited the ability of macrophages to kill *Mycobacterium avium* [50].

In 12 healthy human volunteers plasma TNF was significantly elevated 75–90 min and epinephrine at 1 h and cortisol 1–5 h after LPS infusion. A profound monocytopenia was observed at 1 h with the reversal of the early granulocytopenia to granulocytosis and development of lymphocytopenia which lasted up to 6 h. The change in leukocyte subsets was attributed to the acute adrenocortical response to LPS [51]. The plasma of 6 human volunteers treated with endotoxin inhibited IL–1

induced PGE2 release from fibroblasts at 3 h, which coincided with the development of fever. This activity tended to disappear with declining body temperature. The plasma inhibitory activity of TNFα induced PGE2 release was also increased during endotoxemia. This was maximal at 120 min when circulating TNFα levels peaked [52]. Serial samples of plasma from human volunteers obtained after i.v. LPS administration contained soluble TNF receptors (STNFR type A – p75, – type B – p55) at 3 h. STNFR – A, but not – B, correlated with peak TNF levels. The amount of STNFR liberated was insufficient to block TNF-mediated cytotoxic effects [53].

LPS induced IL – 1 secretion by human monocytes, but not endothelial cells, was reduced by 10^{-7} *M* DEX [43]. The induction of IL – 1 mRNA in human peripheral monocytes and in peritoneal macrophages was inhibited by progesterone and estradiol [54]. Histamine release by human basophilic leukocytes induced by anti-IgE, specific antigen, or the calcium ionophore A23187, was enhanced by LPS which depended on surface binding of LPS to the basophils. This potentiation could be abolished or reduced by galactose and N-acetylglucosamine, which acted by binding to the basophil cell membrane [55].

Mice injected with LPS showed a significant elevation of colony stimulating factor (CSF) in the serum at 45 min which was further increased after 2 h [56].

The pretreatment of human monocytes with IfN – γ for 24 h followed by exposure to LPS potentiated significantly the release of PGE when compared to LPS alone [57]. LPS stimulated the release of PGE2 from isolated cerebral microvessels of cats which suggests a direct effect of blood borne LPS on the central nervous system (CNS) [58].

LPS stimulated the release of IL – 6 from rat medial basal hypothalamus and enhanced IL – 6 mRNA in both the hypothalamus and pituitary tissue. IL – 1β mRNA was also increased in the pituitary gland by treatment of rats with LPS [59, 60].

In rats LPS stimulated the synthesis of platelet activating factor (PAF), particularly in the lung. DEX pretreatment inhibited PAF synthesis in the lung but had no effect on LPS induced PAF increase and vascular permeability in the gastrointestinal tract [61]. LPS induced the production of nitric oxide (NO) synthase by rat aorta in vitro which could be prevented by glucocorticoids [62].

(ii) *Neuroendocrine response to endotoxin.* Wexler et al. [63] observed first the stimulation of ACTH release in rats as detected by the depletion of ascorbic acid and cholesterol in the adrenal glands. Such depletion could not be observed after LPS treatment in Hypox rats.

Table V

Neuroendocrine changes during infection, endotoxicosis and the acute phase response

Hormone	Infection[1]	Endotoxin[1]	APR[2]
Adrenocorticotropic hormone		↑	↑
Glucocorticoids	↑	↑	↑
Growth hormone	↑↓	↑	↑↓
Prolactin	↑	↑↓	
Luteinizing hormone	↓		
Estrogen			↑↓
Androgen			↑↓
Thyroxine		↓	↑↓
Triiodothyronine	0↑		↑↓
Insulin	↑	↑	↑↓
Glucagon	↑	↑	↑
α-Melanocyte stimulating hormone		↑	
Endorphin, enkephalin	↑	↑	
Epinephrine	↑	↑	↑
Norepinephrine	↑	↑	↑
Dopamine		↑	
Arginine-vasopressin		↑	↑
Aldosterone			↑

[1] Please see text for references. [2] References 235, 236.

↑= increased serum/plasma level; ↓= decreased level; ↑↓= level may be either increased or decreased; 0= no change. APR= acute phase response.

In mice the LPS induced ACTH release could be blocked by treatment with IL−1 receptor antibodies and the induction of ACTH release by human IL−1β but not IL−1α was blocked partially by treatment with α-melanocyte stimulating hormone (α−MSH) [64].

Subpyrogenic doses of LPS given to rats i.p. elevated ACTH and cortisone concentrations 2 h later, whereas plasma PRL, LH, epinephrine and norepinephrine levels were not altered. Only at 6 h post-LPS was a small elevation of norepinephrine. ACTH and cortisone response to subpyrogenic, but not pyrogenic, doses of LPS could be prevented by macrophage depletion [65].

The paraventricular nucleus which is the source of hypophyseotropic peptides (e.g. CRF−41, AVP) was lesioned in rats. The hypophyseal-adrenal axis was still activated in such animals by LPS as indicated by elevated plasma ACTH and corticosterone levels. Thus, LPS could have a direct effect on the pituitary gland, or perhaps it may act through an extrapituitary nonparaventricular pathway to release ACTH [66].

Newborn rats responded to LPS with corticosterone production which was lost between ages 5 and 14 days and resumed at 21 days. The refractory period to endotoxin correlated with a depressed responsiveness of the adrenal cortex to ACTH [67]. Adrenal cells isolated from endotoxaemic rats showed an impaired steroidogenic response to ACTH at 2 h post-LPS when corticosteroid serum levels were maximal [68].

LPS injection or immobilization stress increased serum ACTH and corticosterone levels in rats. LPS, but not stress induced a significant increase in serum corticosterone in Hypox rats. The injection of histamine also provoked a corticosterone response in Hypox rats, however, neither histamine nor LPS had appreciable effect on corticosterone release by cultured adrenal cells. It was concluded that LPS induced glucocorticoid secretion is largely dependent on the release of ACTH, but a pituitary independent mechanism is also operative [69].

Glucocorticoid binding by leukocytes of dogs during endotoxin shock decreased significantly 2 h after LPS injection in conscious and at 6 h in anesthetized animals. In conscious dogs the specific binding returned to normal by 6 h. There was no correlation between the changes of serum cortisol and ^{3}H-DEX binding [70].

Circulating α-MSH increased significantly in rabbits after LPS injection and α-MSH given i.v. to LPS treated animals inhibited fever but had no effect on the acute phase reaction [71].

Serum PRL levels increased 5-fold 1 h after LPS injection to mice which returned to normal by 90 min. Adrenalectomy had no influence on this reaction [41]. Low doses of LPS given to sows 2 days postpartum caused a decrease in PRL plasma levels, whereas a 5-10-fold increase was observed in cortisol levels. The PRL concentration also declined in primapartus gilts if injected with LPS on postpartum day 2, no PRL change on postpartum days 5, 6 and 7 was seen in gilts exposed to LPS the first time or second time on day 6 postpartum. Treatment with TRH increased PRL in all animals, but the increase was significantly lower in gilts treated with endotoxin on day 2 postpartum. Cortisol concentrations increased after endotoxin exposure on postpartum days 2 and 6 [72, 73]. The binding of ovine PRL by liver membranes was increased 2-fold within 15 min after LPS injection to responder (C3HfB/Hen), whereas no such increase was found in nonresponder (C3H/HeJ) mice. Insulin binding was not affected by LPS. Scatchard analysis revealed that a receptor of different binding affinity appeared within 15 min of LPS injection which disappeared by 1 h post-LPS [74].

Human volunteers injected with 4 ng/kg endotoxin responded with elevated growth hormone and cortisol secretion. The magnitude and kinetics of the clinical symptoms was altered when γ-irradiated LPS was used. Irradiated endotoxin was more effective in decreasing the platelet count than was untreated LPS [75].

The injection of non-lethal doses (0.01–2 mg/kg) of LPS to male Wistar rats i.v. induced 3–9-fold increases in estrogens, 4-fold increase in progesterone, 2–3-fold increase in corticosterone, and decreased serum testosterone 2-fold, with maximal responses at 2 h. Serum estrone and estradiol was not increased, however, in ADX or orchidectomized animals [76].

Treatment of newborn rats with a single subtoxic dose of LPS decreased the level of serum T4 and led to an impaired thyroid response to TSH in adulthood, which was associated with somatic retardation. Treatment of adult rats with a shock inducing dose of LPS led to a similar decrease in serum T4 and the inhibition of the T4 response to TSH. In contrast, radio detoxified endotoxin did not inhibit the TSH response and decreased serum T4 to a lesser extent [77, 78].

LPS induced hyperglycaemia is associated with elevated insulin levels. Both LPS and IL–1 increased the sensitivity of isolated pancreatic islets to glucose, but neither affected the maximum secretory response. The LPS induced insulin secretion may be partially mediated by IL–1 [79]. Glucose mobilization is induced by LPS through the stimulation of adrenergic mechanisms while glucose utilization is promoted by insulin [80]. The inhibitory effect of epinephrine (EP) on insulin levels is decreased by LPS, which is more pronounced in the fed than in the fasted rat, and this is potentiated by anesthesia [81]. Insulin binding and endocytosis by cultured rat hepatocytes was decreased by LPS treatment in a time and temperature dependent manner [82].

DEX given to rats 1 h prior to LPS pevented hyperinsulinaemia and hypoglycaemia, but there was no significant influence on hyperinsulinaemia if DEX was given jointly with LPS [83]. In LPS-treated dogs plasma met-enkephalin and β-endorphin (β-END) increased prior to the onset of hyperinsulinism, whereas the elevation of plasma leu-enkephalin occurred later. Plasma insulin was elevated 100-fold by 6 h after treatment with LPS and glucose. Hyperinsulinism was reduced markedly by treatment with naloxone. However, naloxone given with glucose appeared to have a stimulatory effect on insulin [84].

LPS decreased adrenal EP and norepinephrine (NEP) in both newborn and 20-day-old rats. This response was prevented in 20-day-old, but not in 1-day-old animals by ganglionic blockage, indicating that the release of catecholamines in the neonatal rat was non-neurogenic [85]. Plasma levels of NEP and EP increased in adult conscious rats with both the dose of LPS and with time. Significant increases in plasma catecholamine and heart rate occurred after various doses of endotoxin in the absence of hypotension which suggests that factors other than blood pressure mediated the sympathetic outflow in endotoxicosis [86].

In dogs given a large bolus of LPS (5 mg/kg) followed by a continuous infusion at 2 mg/kg/h, there was marked increase of EP and NEP in the adrenal vein and in the circulation. Immunoreactive met-enkephalin levels followed closely the change in

catecholamines in the adrenal vein, whereas arterial levels rose progressively, indicating that the adrenal medulla is not the only source of circulating met-enkephalin [87].

The levels of EP and NEP were depleted in the adrenal glands of LPS treated rats, whereas dopamine was increased. LPS also decreased the NE content of the heart, spleen, liver and kidney. Treatment with indomethacin kept the catecholamine levels constant in endotoxic rats, whereas naloxone had no effect. PGF2α tissue levels were increased 2-fold by LPS treatment and PGE2 content was moderately increased in the spleen and liver. In the kidneys the increase of PGF2α and PGE2 paralleled with NEP content. It was suggested that prostaglandins participate in the control of norepinephrine tissue levels in endotoxaemia [88].

Sympathetic nervous system activity was increased in the heart, liver, brown adipose tissue and gastrocnemius muscle of rats 24 h after endotoxin injection [89]. LPS activated noradrenergic, dopaminergic and epinephrine containing neurons in the hypothalamus and dopaminergic neurons in other regions of the brain in rats. Prostaglandins played an important role in the catecholamine response to LPS in all brain regions examined [90].

LPS induced a 35% decrease in the number of β-adrenergic binding sites of splenic lymphocytes and a 20% reduction in the number of β-adrenergic sites in peripheral lung tissue of guinea pigs 4 days after treatment. The deterioration of the β-adrenergic system in the lung could be prevented completely by splenectomy [91]. In dogs 5 h after the injection of LPS, β-adrenergic receptor number was decreased on lymphocytes and in the myocardium which was caused by an increase in circulating catecholamines. NaF-stimulated cAMP accumulation was also reduced in lymphocytes [92].

In rats treated with LPS the blood pressure was significantly reduced and plasma levels of endothelin (ET)-1 were increased 7-fold and ET-3 levels 2-fold. Human TNFα induced a similar increase in plasma ET levels without affecting blood pressure. Pretreatment with indomethacin attenuated significantly endotoxin induced increase of plasma ET-1 and ET-3 without affecting blood pressure [93]. Endothelin-like immunoreactivity increased significantly in arterial blood and in pulmonary lymph of sheep 8 h after the start of LPS infusion [94].

(iii) *Endotoxin shock.* Geller et al. observed first in 1954 [95] that the administration of cortisone to mice prior to or simultaneously with a lethal dose of LPS protected the majority of the animals from death, but there was no protection when cortisone was administered after endotoxin. Some animals developed a transient bacteriaemia a few h after LPS injection, which could be suppressed by antibiotic treatment without influence on mortality.

The infusion of a lethal dose of endotoxin to rabbits caused leukopenia, thrombopenia, increased serum levels of β-glucuronidase and LTB4 and decreased

levels of complement, CH50 and tissue plasminogen activator (t-PA) activity. Methylprednisolone (MP) prevented death which correlated significantly with the decrease of the granulocyte release product β-glucuronidase. Leukopenia and thrombopenia were not prevented by this treatment, but the cell numbers returned to normal more rapidly. LTB4, CH50 and t-PA were not altered by treatment indicating that MP did not affect the activation of complement, arachidonic acid products and the fibrinolytic system. Protection was afforded by the inhibition of leukocyte activation and low dose of MP (1 mg/kg) was as effective as the high dose (40 mg/kg) which is most frequently recommended [96].

In dogs given a bolus of 5 mg/kg LPS followed by a continuous infusion of 2 mg/kg/h no significant haemodynamic or neuroendocrine changes were demonstrated by treatment with MP (30 mg/kg) administered 15 min after LPS [97].

Unanesthetized sheep were given either 0.5 mg/kg LPS i.v. alone, or on a different experimental day, an identical LPS dose preceded by a 1 g bolus of MP plus a 1 g/h continuous infusion. LPS induced the release of cytokines into lung lymph that were able to stimulate normal sheep neutrophils to aggregate, migrate and release superoxide. The cytokine appeared within 1 h of LPS injection and persisted for a least 4 h. Pretreatment with MP did not prevent the early activity but did significantly reduce cytokine activity at 3-4 h when the permeability defects caused by LPS were most pronounced [98]. LPS given to rats i.p. caused lung injury and increased the half-life of PGE2 in isolated perfused lungs. The adverse effects of LPS were reversed by MP administered 20 min after intoxication. Budenoside given 1 h prior to LPS prevented lung injury and leukaemia partially, but did not affect the half-life of PGE2 [99]. Acute lung inflammation was induced in guinea pigs by aerosol exposure to LPS. Budenoside decreased the number of neutrophils in the airways at 24 h but did not influence neutrophils either in airways or lung interstitium at 4 h after LPS exposure nor did it affect the secretion of chemotactic factors by alveolar macrophages at that time. The antiinflammatory effect of budenoside was attributed to its protection of the epithelium which prevented the delayed influx of serum fluid and neutrophils into the airways [100].

One hundred and two patients suffering form bacterial meningitis were treated with DEX (0.5 mg/kg every 6 h for 4 days) while 98 patients received placebo. One patient died in the placebo group. DEX treated patients became afebrile earlier and were less likely to acquire moderate or more severe bilateral sensorineural hearing loss than patients in the placebo group [101].

Mice pretreated with rabbit anti-mouse TNF antibodies were protected against a lethal dose of LPS, whereas the febrile response was unaffected. Protection could be overcome by a high dose of LPS as the medium lethal dose of LPS in mice given 50 μl of anti-TNF serum was approximately 2.5 times greater than the dose for controls [102].

Anesthetized baboons treated with anti-TNF monoclonal antibodies were subsequently infused with *E. coli* bacteria (LD_{100}). The $F(ab')_2$ fragments of neutralizing monoclonal anti-TNF antibodies given 1 h before bacterial challenge protected against shock, but did not prevent renal and pulmonary failure. The protection was complete, however, against shock, organ dysfunction, stress hormone release and death if the antibodies were given 2 h prior to bacterial infusion [103].

Anti-TNF antibody protected mice fully against i.v. challenge with 1000 LD_{50} of LPS which also reduced serum IL-1 and IL-6 levels. However, anti-TNFα antibodies did not improve the survival of mice nor reduce serum IL-1 and IL-6 levels after i.p. bacterial challenge. On the other hand, mice suffering from peritonitis were protected by anti-LPS antibodies which was associated with a striking reduction of bacterial numbers and TNF, IL-1 and IL-6 levels in the serum. However, the levels of these cytokines were only marginally affected in the peritoneal lavage fluid [40].

Cecal ligation and puncture (CLP) was performed in mice, whereas other groups were treated with LPS either i.p. or i.v. Plasma LPS was detectable in the CLP group within 4 h reached a peak at 8 h (136 ± 109 μg/ml). TNF bioactivity peaked at 12 h and after LPS given i.p. at 90 min and IL-6 at 12 h after the induction of CLP. Anti-TNF antibody treatment of CLP mice reduced significantly serum TNF bioactivity, but had no effect on mortality or on pulmonary neutrophilic infiltration. Anti-TNF antibody given to mice at the time of i.v. LPS administration reduced plasma TNF activity but there was no protection against the lethal effect. This was also true for i.p. administered LPS. Mortality was not affected even when the antibody was administered 4 h prior to a lethal i.v. LPS dose [104].

In rats there was no correlation between the lethal effect and serum TNF levels induced by different types of LPS. Upon repeated administration of endotoxin, late tolerance was associated with progressively lower levels of TNF and increasing titres of anti-LPS antibodies. In the later tolerant state a direct correlation between the LPS dose and peak TNF serum levels was observed [105].

TNF given to conscious rats through indwelling jugular vein cannules at 1 mg/kg dose killed 5 out of 6 animals within 6 h and induced comparable pathophysiological changes to a lethal dose of LPS (30 mg/kg). Cotreatment with TNF + LPS led to a rapid shock like state similar to those induced by TNF or LPS alone and all animals died within 4 h [106]. Recombinant human TNF or its fragment ($TNF_{114-130}$) given i.v. to mice did not have a lethal effect but a dose-dependent mortality was observed when combined with a nonlethal dose of LPS [107].

Treatment of rats with a low i.v. dose of TNF prevented death from a lethal dose of TNF administered 24 h later and provided protection also against a lethal dose of endotoxin or against CLP. TNF treatment given 6 h prior to CLP still had a significant protective effect, but was ineffective if applied 24 h after the insult [108].

Treatment of mice with the dual inhibitor of arachidonic acid metabolism, SK & F 105809, 30 min prior to LPS + D-galactosamine reduced serum TNF and protected the animals against mortality. Similar results were obtained in *Proprinobacterium acnes* sensitizes mice [109].

Naloxone given to anesthetized dogs that were treated with LD_{80} doses of LPS i.v. 15 min earlier, attenuated the haemodynamic changes induced by LPS alone. Naloxone also prevented the characteristic diarrhoea and reduced mortality in endotoxin treated animals [110]. Naloxone given intracerebroventricularly to dogs increased the mean arterial pressure, cardiac output and left ventricular performance in endotoxin shock, but was ineffective in haemorrhagic shock [111]. The δ opiate receptor antagonist (M154,129) reversed endotoxin shock in rats, whereas a μ receptor antagonist (β-funaltrexamine) was ineffective [112]. Naloxone significantly augmented the pressor response and its duration to EP in edotoxin treated but not in normal rats [113].

Plasma AVP levels in rats given LPS i.v. increased 100 times within 1 h. The infusion of AVP V_1 receptor antagonist agent started 15 min prior to LPS injection and continuing for 6 h did not prevent the cardiovascular changes or mortality [114].

Indomethacin reduced the mortality of rats suffering from endotoxin shock which was further reduced by additional dopamine infusion, but dopamine alone was ineffective [115]. Nine of 10 rabbits treated with 100 mg/kg of IL-1 receptor antagonist survived an LD_{80} dose (0.5 mg/kg) of LPS and showed full recovery by day 7. At 24 h the lung pathology (exudation, cellular infiltration) was grossly diminished although some surface bleeding was present. When the treatment with IL-1 receptor antagonist was delayed for 1 or 2 h after LPS injection, 7 or 8 animals survived for 7 days each group, whereas 3 of 4 animals treated with LPS only died within 48 h [116].

Treatment of mice with IFN-γ prior to LPS resulted in nearly 5-fold increase in serum TNF, whereas IFN administered after LPS had no such effect. TNF mRNA did not increase correspondingly in pretreated animals. In mice pretreated with monoclonal antibodies to IFN-γ mortality was significantly reduced while there was only a minor effect on serum TNF levels. Anti-IFN-γ administered 2 h after LPS was similarly protective [117]. Mice treated with antibodies to murine TNFα or IFN-γ were protected against a lethal dose of LPS administered 6 h later. In mice receiving anti-TNF antibody serum IFN levels were lower at 2 h post LPS compared to those receiving non-immune IgG. Serum TNFα levels at 1 h post LPS were more than 4-fold higher in mice receiving anti-IFN-γ antibody compared to controls. Doses of TNFα (300 μg/kg) and IFN-γ (50 000 U) which were well tolerated when given individually were lethal when combined and induced higher serum levels of IL-6 than did either cytokine alone [118]. Combined treatment of rabbits with IFN-γ and LPS led to significant decrease in cardiac output, in oxygen pressure and

white blood cell counts and an increase in plasma exudation into the lung, whereas neither agent alone had these effects [119].

Platelet activating factor plays a pathogenic role in endotoxin shock. Treatment of mice with PAF antagonist (SR27417 or BN52021) provided complete protection against mortality. Similar protection was achieved by treatment with indomethacin or by the dual cyclo-oxygenase/lipoxygenase inhibitor, BW755C. However, mortality induced by low dose LPS + human TNF was unaffected by BN52021, indomethacin or BW755C [107].

The acute phase response

A proteinaceous secretion from leukocytes termed leukocytic endogenous mediator (LEM) was described by Wannemacher et al. [120]. LEM stimulated the uptake of amino acids by the liver in ADX, Hypox, thyroidectomized or diabetic rats. Such stimulation could not be duplicated by a large variety of hormones. LEM augmented RNA synthesis and enhanced the hepatic production of a number of acute phase plasma globulins. Thus LEM stimulated hepatic protein synthesis directly without the mediation of other hormones though it was noted that the physiological quantities of adrenal corticoids may be necessary.

The acute phase response (APR) may be triggered by infectious disease or by a variety of agents capable of causing injury. APR is mediated by cytokines which appear in the circulation and function as acute phase hormones affecting the central nervous system, the neuroendocrine system and virtually the function of every other tissue and organ in the body. IL-6, TNF and IL-1 have been identified as major mediators of the endocrine and metabolic changes characteristic of APR. Recently several new cytokines have been found as inducers of acute phase proteins [121-124]. ACTH, GC, EP, NEP, glucagon, AVP and aldosterone are all elevated during the acute phase response, whereas GH, estrogens, androgens, insulin and thyroid hormones may be either elevated or suppressed depending on the severity of the condition [125].

The Kupffer cells (KC) in the liver appear to play an important role in the initiation of APR. Sepsis induced in mice (C3H/HeN and C3H/HeJ) by CLP elicited an early (at 1 h) IL-6 response in KC, but not in splenic macrophages, irrespective of endotoxin susceptibility. However, the release of IL-1 or TNF was not markedly different from KC of mice with CLP or of sham operated animals. At 24 h post-CLP, KC no longer showed a cytokine response, whereas splenic macrophages were suppressed [126]. Human KC produce IL-6 after exposure to LPS in vitro [127].

During APR the liver synthesized new proteins (acute phase reactants), whereas the synthesis of some normal serum constituents such as albumin or transferrin was decreased. The concentration of acute phase serum proteins (APR)

increase dramatically, as much as 1000-fold in man in the case of C reactive protein (CRP) and serum amyloid A (SAA). Fibrinogen, α1 antitrypsin and certain complement and properdin components (factor B and C3) show a more moderate increase.

CRP has the capacity to combine specifically with phosphocholine and possibly also with some polysaccharides containing galactose, and with some biologic polycations such as protamine, poly-*L*-lysine, myelin basic protein. These moieties are frequently present on the surface of bacteria, fungi, parasites and damaged cells. CRP, after having combined with the specific ligand, will active complement by the classical pathway and induces chemotaxis and enhanced phagocytosis by neutrophils and monocytes and tumouricidal activity in macrophages, which is complement dependent. CRP stimulates the synthesis of IL-1, TNFα, potentiates the cytotoxic activity of T lymphocytes, NK cells, and platelets. CRP binds PAF and blocks its activity, and was found to localize in vivo at sites of inflammation. Therefore, CRP is an important resistance factor defending the host against infectious agents. IL-6 induced CRP synthesis by human liver cells in vitro which could be modulated by IL-1, TNFα, TGFβ1 and IFN-γ. DEX potentiated the production of both CRP and SAA. The clinical determination of CRP has diagnostic value for infectious and inflammatory disease. Similar proteins have been identified in mammals, chicken, fish and even crab [128].

The LBP which has been isolated from human and rabbit serum also shows a 100-fold increase during APR. LBP is capable of opsonizing LPS bearing particles and thus may participate in the activation of complement by LPS. LBP-LPS complexes are potent stimulators of cytokines from monocytes and macrophages [31].

A number of other acute phase proteins which are abundant in the rat are proteinase inhibitors. These include α2-macroglobulin, α1-acid glycoprotein, antithrombin III, α1-acute phase globulin, and α1-proteinase inhibitor. Turpentine injection elicited an enhanced secretion of acute phase proteins in normal, but not in Hypox rats. Turpenine plus DEX did induce APR in Hypox animals. Tissue culture medium conditioned by Kupffer cells induced a dose dependent stimulation of α2-macroglobulin by hepatocytes in the presence of 10^{-9} M DEX [129].

Some of the acute phase proteins promote blood clotting (fibrinogen) and healing. Alpha-macrofetoprotein (α-MFP) is a strong inhibitor of inflammatory mediators, including histamine, bradykinin, serotonin, PGE2, and it also inhibits polymorphonuclear chemotaxis. Catecholamines and GC induce α-MFP in normal rats. Catecholamines induce very high levels comparable to those observed in the host injury phase, whereas the effect of GC was moderate. In ADX rats the effect of catecholamines on α-MFP synthesis was greatly diminished, whereas the moderate effect of GC remained. The combination of GC and catecholamines induced

extremely high levels in ADX animals. Other APP, such as haptoglobulin and α1-major acute phase protein, were affected differently by these hormones [130].

Treatment of mice with LPS, TNF or IL-1 decreased cytochrome P-450 in the liver, increased plasma fibrinogen and induced hypoferremia. IL-1, but not TNF or LPS, depressed cytochrome P-450 in cultured hepatocytes. Pretreatment with DEX protected against the depression of liver cytochrome P-450 by LPS or TNF, but not IL-1 and such treatment had no influence on the increase of plasma fibrinogen levels and decrease of plasma iron [131]. IL-1 and TNF can initiate a full range of APP in vivo but only a limited number of APP were induced by the cytokines in cultured liver cells and compared with a crude cytokine preparation from macrophages. This observation led to the discovery of IL-6 as a major inducer of APP synthesis. Recently additional cytokines, namely interferon-γ, leukaemia inhibitor factor, transforming growth factor β, and oncostatin M were found to be inducers of APP from the liver [124]. A protein isolated from rat sciatic nerve and named ciliary neurotrophic factor induced APP in human hepatoma cells and in primary cultures of rat hepatocytes [132].

IL-6 activates the genes of APP through the DNA binding protein called NF-IL-6, which may be a pleiotropic regulator of many genes induced during immune and inflammatory responses, similarly to another DNA regulatory protein, NFkB. Both NF-IL-6 and NfkB binding sites are present in the genes of IL-6, IL-8 and several APP genes [124].

Adrenalin induces a high level of IL-6 in rats which can be blocked by propranolol. The fast reacting APP, α2-macroglobulin and cisteine protease inhibitor, are strongly repressed by such blockage. Isoprenalin, which is a β2-adrenergic receptor agonist, also causes very high levels of IL-6, indicating the involvement of β2 receptors [133].

In human volunteers treated by hydrocortisone infusion (3 μg/kg/min) for 12 h, and given a bolus injection of *E. coli* LPS (2 ng/kg) at 6 h after the initiation of infusion, the IL-6 response was attenuated and CRP and fibrinogen synthesis decreased. The TNF response was near normal as compared to controls [134].

Rats treated with LPS for 14 days showed signs of APR, the abrogation of the cytokine response (TNF, IL-6) to LPS and resistance to subsequent infected thermal injury, as revealed by food intake, fibrinogen levels and mortality [135].

The host defence against endotoxin shock

1. *The role of acute phase hormones*

Severe infection, endotoxicosis and other forms of injury will elicit the appearance in the circulation of cytokines that function as acute phase hormones.

The acute phase hormones initiate the defence response of the host through interactions with the neuroendocrine system and with other tissues and organs in the body. The nature of the injury has an influence on the response elicited. For instance, in patients undergoing elective surgery IL-1 appeared first briefly in the circulation which was followed by IL-6, but other cytokines such as TNFα or IFN-γ were not detected. Similarly, in burn injury TNFα and PGE were elevated only if sepsis was also present [136, 137]. TNF is invariably elevated, however, if endotoxemia is associated with injury. In patients with burn injury, both serum IL-2 and IL-2 receptor α are elevated for long periods of time [138, 139], whereas in trauma there is a consistent depression of IL-2 [140]. Colony stimulating factors (GM-CSF) may also be elevated, especially during infections and endotoxaemia. Following is a brief overview of the major cytokine hormones that play a role in endotoxin shock:

(i) *Interleukin-1α and* β: initiate the immune response, induce fever, release ACTH and other pituitary hormones, antagonize opioid receptors in the brain, promote slow wave sleep, decrease appetite, stimulate acute phase proteins in the liver, promote proteolysis in muscle, inhibit thyroglobulin gene expression, stimulate thyroid growth, insulin release (inhibitory in high concentration), inhibit steroid synthesis in the gonads and adrenals, stimulate bone resorption [141-145].

(ii) *TNFα (cachectin) and TNFβ (lymphotoxin)*: TNFα has many overlapping functions with IL-1, including pyrogenicity, promotion of slow wave sleep, a strong catabolic effect, release of ACTH and GnRH, blocking of GH release, T and B cell activation, stimulation of bone resorption. Neutrophils, eosinophils and macrophages are activated by TNF. TNF is cytotoxic for certain tumour and other target cells, it causes inflammation, haemorrhage and shock if produced in excess. TNF plays a major role in the multiple endocrine and metabolic changes associated with trauma and sepsis. IL-1 and TNF inhibit the β-adrenergic responsiveness in several tissues [144, 146-152].

TNF, IL-1 and other LPS induced mediators enhance nonspecific resistance to bacterial, viral and parasitic infections, autoimmune disease and radiation sickness [153-155]. IL-1 injected to rats i.v. or into the cerebrospinal fluid inhibited gastric injury induced by a variety of experimental agents such as stress, aspirin and ethanol. Protection was mediated by the inhibition of acid secretion and PG release [156].

(iii) *Interleukin-6*: IL-6 promotes the differentiation of B lymphocytes, supports the multipotential colony formation of hemopoietic stem cells, induces APP in the liver, plays a role in the differentiation and activation of T cells and macrophages, and contributes to neural differentiation [124]. IL-6 released ACTH and β-END from mouse pituitary adenomas in vitro [157, 158], stimulated the release of ACTH, inhibited TSH release by acting on the hypothalamus in rats, and inhibited ACTH release by acting on rat pituitary glands in vitro [159, 160]. IL-6 acts as a locally produced cytokine in the pituitary gland in rats and may function as a

paracrine regulator of PRL release [161]. Nevertheless, IL-6 injected peripherally to rats stimulated ACTH and corticosterone output [144].

(iv) *Interleukin-2*: IL-2 is produced by T lymphocytes and its primary function is growth stimulation of T cells, activation of NK cells and of lymphokine activated killer cells. High doses of IL-2 given to animals or patients exerted a neurotoxic effect which in rats coincided temporarily with the induction of serum TNF [162, 163]. IL-2 inhibited GH, LH and FSH release and promoted ACTH, TSH and PRL release from pituitary glands of rats and mice [164, 165]. In man, IL-2 treatment increased ACTH, β-END, GH and PRL in the circulation [166, 167].

(v) *Interferon-γ*: IFN-γ is the product of T lymphocytes and macrophages and was recognized first for its anti-viral effect. Now it is clear that IFN-γ is a major regulator of cytokine production by macrophages and T lymphocytes, and it also activates NK cells for cytotoxicity. IFN-γ has the capacity to induce glucocorticoid receptors in monocytes and acts in conjunction with GC to initiate the expression of FcRγI receptors on human monocytes [168, 169]. IFN-γ release ACTH and inhibited GH and TSH release in rats by acting on the hypothalamus. Some investigators found the inhibition of ACTH, PRL and GH release by rat pituitary glands exposed to IFN-γ in vitro, whereas others observed increased release of ACTH, LH, FSH, GH and PRL under similar conditions. IFN-γ released ACTH from human pituitary adenomas [160, 161, 170-173].

(vi) *Colony stimulating factors*: G-CSF, M-CSF, GM-CSF play important roles in the maturation of granulocytes, macrophages in the bone marrow, and they are also important activators of these cells during infection and inflammation [174]. G-CSF was shown to release ACTH in vitro from human pituitary adenomas [173].

(vii) *Platelet activating factor*: PAF is a lipid mediator produced by most mammalian cells during inflammation. It has vasoactive properties and plays a role in arterial thrombosis, endotoxin shock, allergic disease, embryo implantation, parturition and fetal lung development [175]. In rats PAF released ACTH and β-END by acting on the hypothalamus and GH and PRL through direct action on the pituitary gland [176-178].

(viii) *Other factors*: Platelet derived growth factor released PRL from rat pituitary adenoma in vitro [179], bradykinin stimulated PRL release from rat pituitary glands [180, 181]; histamine released ACTH, β-END and PRL in rats by acting on the hypothalamus [182, 183]. TGFβ stimulated the basal secretion of GH and inhibited basal PRL secretion and TSH mediated PRL release from rat pituitary glands. PRL gene expression was also inhibited by TGFβ in rat pituitary adenoma [184, 185].

It is not known whether the appearance of acute phase hormones in the circulation is the result of release from various tissues after infection or rather they are secreted by leukocytes activated in the circulation. Observations in mice that TNF

produced locally during bacterial peritonitis did not readily enter the circulation, whereas LPS did [40, 104] suggests that the pathogenic agent (e.g. bacteria, bacterial products, or viruses) and not the cytokines enter the circulation under these conditions. Conversely, circulating cytokine hormones may not extravaste readily to the brain where a vascular barrier exists, or into tissues in general. Cytokines, such as IL-1, IL-6, TNF, etc., are produced in the central nervous system readily [186]. Even though the entry of cytokines into the CNS is debated, the fact remains that after the peripheral injection of the above discussed cytokines they have the capacity to alter brain function and hormone secretion by the pituitary gland, and to influence other organs and tissues in the body.

The normal function of cytokines is to promote host defence and to help recovery and healing. For instance, IL-1 is the initiator of the immune response and acts in conjunction with the antigen on B and T lymphocytes. IL-2, -4, -5, and -6 are also essential growth factors for mounting an immune response. TNF and IFN-γ protect the host against bacterial and viral infections nonspecifically, probably through their ability to induce cytokines and activate cells possessing phagocytic and/or killing activity. The colony stimulating factors do not only enhance the activity of phagocytes, but also stimulate their production by the bone marrow during infection.

Inhibitory cytokines are also produced during infection and trauma. These include the IL-1 inhibitor, IL-1 receptor antagonist, TNF inhibitor, soluble TNF receptors, IL-4 which inhibits the action of IL-6 and of TGFβ, in turn TGFβ antagonizes TNFα. IL-5 and IL-10 have antiinflammatory properties. Our observation that the kinetics of TNF production in ADX mice is not different from that in intact animals after LPS injection, even though ADX animals show an exaggerated response, may be explained by the existence of these regulatory cytokines (Fig. 1) [41]. The fact that TGFβ promotes local inflammation, whereas systemically it has an antiinflammatory effect, is important in relation to the localization of damage due to infection or other forms of injury. A complement split product, C3a, shows similar local and systemic regulatory effects [187, 188].

Recent observations indicate that the elaborate internal regulatory pathways of the immune system, which involves soluble mediators and suppressor cells, is inadequate to control excess cytokine production after stimulation with LPS and by a number of other agents that include the so-called "superantigens". The extreme susceptibility of ADX animals to endotoxin or even to stimulation with complete Freund's adjuvant [189] emphasizes the protective role of the neuroendocrine system which is the ultimate regulator of immune and inflammatory activity and coordinates these responses in the interest of host defence and survival.

2. *The role of the neuroendocrine system*

The major pathophysiological pathways of endotoxin shock are illustrated in Fig. 2. The neuroendocrine response to infection, endotoxin and neuroendocrine alterations during the acute phase response are summarized in Table V. LPS stimulates the release of both GH and PRL although the inhibition of PRL secretion has also been observed. The GH and PRL response is short lived and the level will return to baseline within a matter of hours and later on the level of these hormones may become subnormal in severe infections, or during the acute phase response. Because both GH and PRL stimulate the immune response and also potentiate the production of IL-1, TNFα, IFN-γ, one may suggest that during infection a sharp but brief increase of these cytokines contribute to host defence as discussed earlier. For instance, treatment of mice with PRL elevated the antibody titres against retrovirus in serum and milk [190]. In the case of severe sepsis or endotoxicosis, however, when excess cytokine production is life threatening, the subnormal levels of GH and PRL appear to serve as one of the control mechanisms to limit the production of inflammatory mediators.

The hypothalamus-pituitary-adrenal axis (HPA) serves as the ultimate neuroendocrine control pathway of immune/inflammatory reactions in the hierarchy of regulators. In general the level of glucocorticoids increase in the circulation proportionally to the severity of infection, endotoxicosis or shock. Under these conditions most of the response is mediated by the HPA, but there is also indication of direct release of glucocorticoids from the adrenal gland by inflammatory mediators. The extreme sensitivity of ADX animals to LPS, TNF or IL-1, which can be reversed by glucocorticoid treatment, illustrates the vital importance of this neuroendocrine regulatory system. Glucocorticoids are powerful suppressors of the immune response and of inflammation which is due to their ability to inhibit the production of most cytokines with the possible exception of IL-4 and IL-6 [191].

A significant number of pathogenic microorganisms have the capacity to activate the immune system nonspecifically. This leads to the excess production of cytokines which is life threatening. The activation of autoimmune lymphocyte clones is an additional problem. Invariably, this exaggerated cytokine response must elicit a powerful glucocorticoid response, if the host is to survive. That this may help the pathogen is illustrated by the early observation that LPS treatment triggered bacterial invasion in animals [95]. Even the infusion of sublethal LPS to rats over long periods of time interferes with the induction of cytokines, not only by LPS but also by unrelated stimulants [48]. The implications of these facts in relation to pathogen-host interaction deserves further attention.

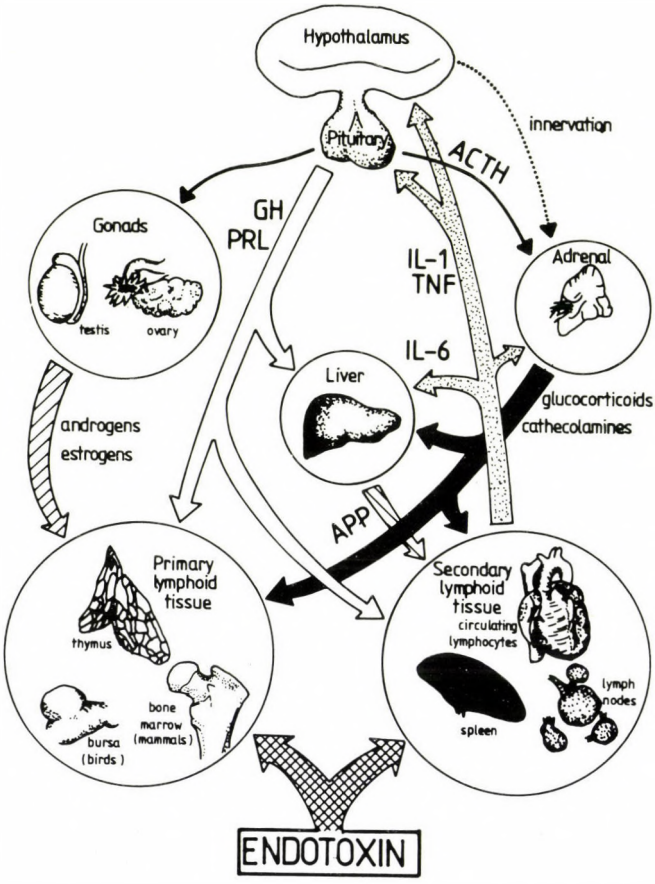

Fig. 2. Major pathophysiological pathways in endotoxin shock. Endotoxin induces the production/release of cytokines, including IL-1, IL-6, TNFα, from monocyte/-macrophages and from other cells. The cytokines released activate other leukocytes, act on the brain to elicit fever, induce pituitary hormone release, and also act on other organs (adrenals, liver) and tissues (muscle). There is an early rise in serum growth hormone (GH) and prolactin (PRL), the levels returning to normal in a few hours and may become subnormal in severe intoxication. Both GH and PRL are known to enhance immune/inflammatory reactions and nonspecific resistance. In severe endotoxaemia these cytokine initiated interactions culminate in the acute phase response, which is characterized by general catabolism, the elevated synthesis of acute phase proteins (APP) by the liver at the expense of other serum proteins (albumin, transferrin) and increased metabolic activity of leukocytes. Circulatory collapse may occur due to the excess release of vasoactive substances. The most important neuroendocrine defence is provided by glucocorticoids and catecholamines released from the adrenals. A "sympathetic outflow" from other sources may also play a role. Glucocorticoids suppress cytokine production and act on the liver to promote APP secretion. Catecholamines inhibit the inflammatory response and induce APP synthesis. Specific immune reactivity is suppressed, whereas nonspecific resistance to infection and other noxious agents (e.g. endotoxin) is increased as the result of the acute phase response

Memory T lymphocytes are resistant to glucocorticoids and are capable of profilerating in an autocrine fashion after exposure to the specific antigen. On this basis it is possible that some anamnestic immune responses may develop during infection, although in severe shock no anamnestic reaction is detectable. Preformed antibodies directed against infectious agents or their toxins are functional during infectious disease. Moreover, some cytokines have the ability to antagonize the antiproliferative effect of GC on T lymphocytes [192–194].

Glucocorticoids upregulate IL–1 receptors on monocytes and fibroblasts, IL–6 receptors on hepatocytes and epithelial cells, IFN–γ receptors and FcγI receptors on monocytes [168, 195–197]. By these effects GC may potentiate the specific immune and nonspecific host defence mechanisms during infection/intoxication.

The fact that GC are required for the induction of APP synthesis by cytokines in the liver is an additional example of boosting host defence by these steroids. The stimulation of CRP, LBP and the like, and of complement components serve the destruction of microorganisms. The elevation of fibrinogen contributes to the isolation of the infectious agent by thrombosis of the microvessels in affected tissues. The excess production of enzyme inhibitors are to curb the damage caused by inadvertently released enzymes by dying cells and by phagocytes at sites of injury and also in the circulation during systemic disease. Alpha-macrofetoprotein serves to curb inflammation. Finally, the development of endotoxin tolerance is also dependent on GC and liver function as discussed earlier.

Catecholamines are invariably elevated during severe infection or endotoxin shock. Haemodynamic and metabolic changes under these conditions may contribute to the catecholamine response. Beta-adrenergic agents inhibit asthma and anaphylactic shock. Catecholamines have an inhibitory effect on other immune phenomena, including neutrophil activation and macrophage cytotoxicity, whereas the effect is variable on antibody formation [198, 199]. Finally, EP is capable of stimulating IL–6 production and it appears that catecholamines, in conjunction with GC, can induce an acute phase response without the presence of infection or injury.

Insulin is elevated in endotoxicosis and infection and may be either elevated or decreased during APR. Glucagon is always elevated, usually in proportion to the severity of the condition. Insulin has an influence on the production of cytokines such as IL–6 and TNFα, and in turn cytokines (IL–1, TNF) regulate insulin secretion. However, the role of these hormones is likely to be more important in the metabolic response to infection than in the direct regulation of cytokines. Thyroid function is invariably suppressed by infection and LPS. This again may be related to metabolic alterations. The lasting impairment of thyroid function induced in newborn rats by LPS [77, 78] may point to the developmental interaction of the endocrine system with cytokines.

Beta-endorphin is invariably elevated during infection and in endotoxin shock, β-END boosts IL-1 which should be beneficial for host defence. However, this hormone has a variable effect on most other immune reactions and it is reasonable to propose that endorphins are capable of amplifying both stimulatory and suppressive signals to lymphocytes, but the mechanism(s) for this are poorly understood. Opioids seem to be involved in the development of haemodynamic changes and diarrhoea during endotoxin shock.

Arginine vasopressin is consistently elevated during endotoxin shock and may play a regulatory role of blood vessels under these conditions. AVP also has an antiinflammatory effect and it may be helpful in the control of excess production of inflammatory cytokines during severe infection/endotoxin shock.

The metabolic alterations during infection are the result of the coordinated action of acute phase hormones with the nervous and endocrine systems. In general, catabolism prevails which is manifested in the loss of muscle protein, body fat, decrease of the synthesis of regular proteins such as albumin in the liver. However, the activity of leukocytes and of bone marrow and the synthesis of APP primarily by the liver, are enhanced. Fever, which is the result of the action of several acute phase hormones on the brain, is beneficial to the host as elevated temperatures enhance the activity of immune mechanisms and is likely to accelerate also the production of APP which are in high demand under these conditions. The metabolic and febrile response to infection is under the ultimate control of the central nervous system [200-206].

3. *Severe sepsis and shock*

The acute phase response may be regarded as an emergency reaction where the host defence system is still trying to boost nonspecific immune and inflammatory reactions while controlling the systemic activation of destructive immune/inflammatory mechanisms. This emergency reaction occurs after the local and specific immune mechanisms have failed, and still has a good chance in assuring host survival. The danger is, however, that the pathogenic microbes take advantage of the general suppression of specific immunity and invade the organism, usually from the gastrointestinal tract.

After a critical injury, the patient is normally stable or shows improvement for 2-3 days, then a septic response may develop with the failure of several organs, especially the lung around day 10 and bacteriologic evidence of sepsis develops which is refractory to antibiotic treatment and the patient usually succumbs to pneumonia. Endotoxin and tissue degradation products may cause diffuse intravascular coagulation and the systemic activation of the complement system, the cytokine network. The cytokines in turn release hormones into the circulation. A systemic release of C3a causes immunosuppression and may impair chemotaxis. The

activation of phagocytes, especially neutrophils by C3a and C5a, may lead to vascular damage, increased permeability with oedema, impaired microvascular flow, and ultimately contribute to multiorgan failure. The release of cytokines and other biologically active substances from basophils, mast cells and platelets may further aggravate the situation. The production of IL-1, IL-2, IFN-γ are depressed, whereas the level of PGE2 is excessively elevated throughout the course of disease. TNF was found in the plasma of 27 out of 74 patients with septic shock and in 1 of 12 patients with shock due to other causes. TNF was detected with equal frequency in patient with shock from Gram-negative or from Gram-positive bacillary sepsis. The levels of TNF were highest in the initial sample and decreased significantly over the subsequent 24 h. The rise in serum glucocorticoids and catecholamine levels is normally proportional to the severity of the septic condition. Patients with subclinical hypocortisolism are more susceptible to death during sepsis than those with normal adrenal function. In severely traumatized or septic patients, there is a lack of skin reactivity to recall antigens, monocyte/macrophage function is depressed coupled with the increased production of PGE. Chemotaxis, phagocytosis, chemiluminescence and intracellular killing activity of neutrophils are also suppressed. There is a decreased NK and LAK function, and long-term T cell depression with increased T suppressor cell activity and reversal of the T helper/T suppressor ratio may be detected [4, 140, 188, 207-210].

The pathological process during severe trauma, sepsis and shock may start by various pathways and snowball into the septic state, multiple organ failure and death. In resuscitated patients some organs such as the gut or liver may be critically injured at the beginning by oxygen free radicals. In severely injured patients devitalized tissue may activate the complement system and phagocytic cells if left in place. The complement and coagulation systems may also be activated by infectious agents, along with massive cytokine production. There is a correlation between intensity of complement activation and the blood levels of elastase with the severity of injury, the development of adult respiratory distress syndrome, multiple organ failure and fatal outcome. Septic patients with detectable TNF also had a higher incidence and severity of the adult respiratory distress syndrome and a higher morality rate [4, 208, 209].

Animal experiments showed that TNF, IL-1 IFN-γ, PAF, opiates and prostaglandins all contribute to the fatal outcome of endotoxin shock. During infectious disease the destructive effect of microorganisms growing in various tissues or in the blood, the consumption of essential nutrients by these pathogens, and the toxicity of their metabolic byproducts are additional factors that complicate the situation. By the time septic shock develops, enough damage has been done to kill the individual and for this reason it is not surprising that treatment with glucocorticoids has no beneficial effect [4, 211-213]. However, glucocorticoid

therapy could be expected to be beneficial in a minority of patients with glucocorticoid deficiency. In those patients where there is an adequate glucocorticoid response to infection, additional high dose glucocorticoid therapy may inflict further damage by aggravating immunosuppression or the inhibition of the rapid growth of intestinal cells, for instance [214]. Early enteric protein feeding and the prompt removal of devitalized tissue from severely traumatized patients is important for the prevention or decrease of the severity of septic state [209, 210].

Treatment of septic patients with antibiotics has not been excessively useful because of the development of resistance and, in the case of Gram-negative organisms, such treatment accelerated the release of LPS which in turn aggravated the disease [215, 216]. Pharmacological treatment with cortisol, lidocain and calcium blockers reduced the release of bioactive complement components. Plasmapherisis has been useful for the removal of anaphylatoxins from the blood [188].

Ideally one would like to prevent the systemic activation of complement and coagulation systems and the excess serum levels of cytokine-hormones during trauma and infection. A simple and inexpensive measure to prevent the absorption of LPS from the gastrointestinal tract, especially if liver function is impaired, may be to apply bile acids in order to detoxify LPS in the gut [27]. Although such treatment has long been proven experimentally, so far it has not been introduced to clinical practice in a significant way. The administration of antibodies to LPS or to its toxic moiety, lipid A, may also be beneficial (for review see 217). This approach is being studied experimentally at the present time and there are ongoing clinical trials. Human and murine anti-lipid A monoclonal antibodies improved the survival rate and facilitated the recovery of patients suffering from Gram-negative bacteriaemia and septic shock [218, 219].

In animal studies lipid A specific monoclonal antibodies were capable of protecting the host against lethal endotoxin shock only if given prior to or simultaneously with LPS. No protection can be achieved if the antibody is administered 2 h after LPS injection [220]. The reason for this is that the initiation of excess TNF/cytokine production takes place shortly after LPS injection. TNF reaches maximum levels within 2 h, which in turn initiates the cytokine cascade, and therefore, LPS is not required for the development of the shock beyond this time [41]. Once the cytokine response is underway, TNF is not required either, as it returns to baseline levels within 4 h or so and the host is resistant to respond with elevation of TNF to additional LPS injection. Therefore the use of specific TNF antibodies [102] also has limitations as discussed earlier. Treatment with specific antibodies to IL-1 and TNF coupled with IL-1 receptor antagonist, with soluble TNF and IL-1 receptors as additional inhibitors may have therapeutic advantages. The use of PAF antagonists, analogs of LPS and its receptor(s) are additional possibilities [217, 221, 222].

Although the above therapeutic approaches should be tested in the interest of improving patient care, there is an imminent danger that they are based on a gross oversimplification of the pathomechanism of sepsis and lethal shock. We have just begun to understand the principles by which the complex interaction of the immune and neuroendocrine systems provide defence against infection and endotoxin shock. As we progress with our understanding of this defence mechanism our insights into the pathomechanism of trauma, sepsis and shock, and possibly also other diseases with underlying immune/inflammatory mechanisms, will improve which should result in better approaches for treatment.

Abbreviations

ACTH = adrenocorticotropic hormone; ADX = adrenalectomy; APP = acute phase protein; APR = acute phase response; AVP = arginine vasopressin; BCG = bovine tubercle bacillis (Bacille Calmette Guérin); CLP = cecal ligation and puncture; CNS = central nervous system; CRF = corticotropin releasing factor; CRP = C reactive protein; DEX = dexamethasone; E2 = estradiol; β-END = β-endorphin; EP = epinephrine; ET = endothelin; FSH = follicle stimulating hormone; GC = glucocorticoid; GH = growth hormone; HPA = hypothalamus-pituitary-adrenal axis; IFN = interferon; IGF-I = insulin-like growth factor-I; IL- = interleukin; KC = Kupffer cells; LBP = lipopolysaccharide binding protein; LEM = leukocytic endogenous mediator; LH = luteinizing hormone; LHRH = luteinizing hormone releasing hormone; LPS = lipopolysaccharide; LT = leukotriene; α-MFP = alpha-macrofetoprotein; MP = methylprednisolone; α-MSH = alpha-melanocyte stimulating hormone; NEP = norepinephrine; PAF = platelet activating factor; PG = prostaglandin; PRL = prolactin; SAA = serum amyloid A; STNFR = soluble TNF receptor; T3 = triiodothyronine; T4 = thyroxin; TNF = tumour necrosis factor; TRH = thyrotropin releasing hormone; TSH = thyroid stimulating hormone.

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CLINICAL PICTURE AND EPIDEMIOLOGY OF ADENOVIRUS INFECTIONS*

(A REVIEW)

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Adenoviruses produce a variety of serious diseases in people of all ages. The mode of transmission of adenovirus infections includes respiratory, fomite, droplet, venereal, and faecal-oral routes. They have been shown to spread with ease in AIDS patients, in young children and in hospitalized patients. A constant rate of about 8% of world-wide reported virus infections were observed to be due to adenovirus infections. In the military, it can cause serious respiratory disease (ARD) of epidemic proportion in new recruits. The present commercial vaccine is prepared in unique triple-layered tablets containing live lyophilized virus. This vaccine has been taken by more than ten million subjects during the past 25 years with no adverse reaction and with near total eradication of ARD epidemics among new rectuirs at training centers. As the epidemiology of adenovirus diseases becomes more clearly defined, the need for and the possible role of potential vaccines, is becoming more evident.

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Introduction

Various disorders have been attributed to infections with adenoviruses. The most common sites of infection are the respiratory and gastrointestinal tracts, the ocular system and the urinary bladder. In addition, strains of adenovirus were found, on occasion, in the central nervous system, in individuals with hepatic disorders [1–3] and from herpes-like genital lesions [1, 4–6]. Other illnesses, more often associated with generalized diseases, in which adenoviruses have been isolated from blood or peripheral leukocytes include exanthems of different types, juvenile rheumatoid arthritis, thyroiditis, and neonatal sepsis [6, 7–12]. Adenovirus infections are often incriminated in nosocomial infections; they may often be fatal in immunocompromised individuals, as well as be life-threatening in children particularly infants [1, 13–15].

Although diseases probably caused by adenoviruses were described 100 years ago [16], the virus was not isolated and classified until the mid-1950s [17–20]. Rowe et al. [20] observed that a transmissible cytopathogenic agent was responsible for the spontaneous degeneration of cells in tissue cultures, tonsils and adenoidal tissues removed surgically from children. During the same period Hilleman and Werner [18] reported the recovery of new agents from patients (military personnel) with acute respiratory illness, and Huebner et al. [19] also reported on adenoidal-pharyngeal conjunctival agents. These viruses were first known as adenoid degeneration (AD), adenoid-pharyngeal conjunctival (APC), and acute respiratory disease (ARD) agents [21]. In 1956, Enders et al. [17] proposed the group name "Adenovirus" for these new respiratory tract viruses.

By the 1960s, The Commission on Acute Respiratory Diseases of the US Armed Forces recognized that these adenoviruses were the cause of epidemic-type occurrences of ARD among military recruits [22]. At times, as many as 80% of new military recruits could be affected by ARD within the first three weeks of training. Of these, perhaps 20% required hospitalization. Studies with live oral vaccines led to the development of commercial vaccines for use in the military. Although adenovirus vaccines have been used with great success in North America, there has been no effort to introduce elsewhere. This is somewhat surprising since European virologists have long recognized the importance of these agents in causing serious and even fatal diseases. Van der Veen et al. [23–25] reported on the incidence of adenovirus respiratory disease in military recruits in the Netherlands and in NATO forces in the early 1960s.

Adenoviruses belong to the family *Adenoviridae* which is divided into two genera: *Mastadenovirus* and *Aviaadenovirus* [26]. These genera have structural proteins that differ immunologically from each other. Human, simian, bovine,

equine, porcine, ovine, canine and opossum varieties are found in the *Mastadenovirus* genus. Birds (fowl, turkey, goose, pheasant, duck) harbour *Aviaadenovirus*.

Clinical description

At present 47 serotypes of human adenovirus associated with a variety of clinical syndromes have been described (Table I).

The most common serotypes causing particular diseases (Serotypes 1–8, 11, 21, 35, 37, 40, 41) have been well documented and reviewed [1, 27–31]. The mode of transmission of adenovirus infections includes respiratory, fomite, droplet, venereal, and faecal-oral routes. They can spread with ease in AIDS patients, in young children and in hospitalized patients [9, 29]. It has been suggested, that since adenoviruses are stable viruses, indirect, water-borne, or food-borne transmission should also be considered [32].

Illnesses due to adenovirus infection are prevalent throughout the year except ARD which occurs mostly during the winter months. Many such illnesses are common in infants, although up to age 5–6 months they may benefit from maternal antibodies to adenovirus [33]. The infections are asymptomatic in approximately 50% of affected children. While some disease entities are generally mild, adenovirus pneumonia in infants may have a fatal outcome [34–38]. Some infections with adenoviruses have been observed to persist for months, even years. The possibility that the lymphoid cells are reservoirs for these persistent infections has been raised [39]. Sequelae, such as bronchiectasis which, in some cases, has manifested itself years after the primary adenovirus infection, have been observed in those patients who have recovered from lower respiratory infections [40–42]. Rare complications such as blindness can be of major concern [43]. Other extrapulmonary manifestations such as renal involvement, hepatomegaly, myocarditis, encephalomeningitis, haemorrhagic tendency, peripheral oedema, gastroenteritis and exanthema can occur alone or in combination and can be fatal in infants [1].

Approximately 5% of respiratory infections (mostly upper respiratory diseases) in children under 5 years of age are due to adenovirus infections [29]. Although they may appear to cause many symptoms of the common cold, these viruses more often produce pharyngitis and tonsillitis [44, 45]. It is important to note that the virus persists, in a latent state, in adenoidal and tonsillar tissues in about 50% of the infected children. Furthermore, the virus may continue to be excreted in the stools for several months after infection although the children can become asymptomatic [28].

Lower respiratory infections such as bronchitis and pneumonia are common complications of adenovirus infections in children. A number of neonatal deaths associated with adenovirus serotypes 7 and 35 have been documented [36]. Those

who survive run a high risk of developing bronchiectasis or other chronic pulmonary problems [41]. Extrapulmonary involvements can be seen, particularly in infants and immunocompromised patients – the outcome is often fatal [1].

Table I

Clinical picture of adenovirus infections

Clinical picture	Adenovirus serotypes
Respiratory illnesses	
ARD (Acute Respiratory Disease)	3, 4, 7, 14, 21, 35
Upper respiratory illness	1–6, 11, 21, 29, 31
Lower respiratory illness	1–5, 7, 21, 35
Pertussis-like syndrome	1–3, 5
Epidemic keratoconjunctivitis	3, 4, 7, 8, 10, 11, 19, 21, 37
Acute haemorrhagic conjunctivitis	1–11, 37
Pharyngoconjunctival fever	1–4, 7, 11, 14, 16, 19, 37, 21/H21 + 35
Cystitis	7, 11, 21, 34, 35
Gastroenteritis	1, 2, 5, 7, 21, 29, 31, 32, 35, 40, 41
Reye's syndrome*	1–3, 7
CNS disorders	1–3, 5–7, 32
Sexually related disease	1, 2, 5, 7, 11, 19, 31, 37
Infections in immuno-compromised patients	1–7, 11–12, 21, 29, 31, 34, 35, 37–39, 43–47
Nosocomial infections	3, 4, 7, 8, 19, 21, 21/H21 + 35, 23, 31, 37, 40, 41

* Recent evidence favours the strong possibility that Reye's syndrome is related to aspirin use and not directly caused by adenovirus or any other specific infectious agent [83, 84]

Of special interest for the use of vaccines are the epidemic infections (ARD) of military recruits by adenoviruses (serotypes 3, 4, 7, 14 and 21). These produce typical upper respiratory tract and constitutional symptoms. In some instances, infections

may extend to the lungs with a prolonged and extensive course that is occasionally fatal [46]. In the absence of vaccine, the incubation period of ARD in the military is usually 5 to 10 days, with the gradual development of a variery of respiratory symptoms beginning with fever and chills. The duration of infectivity is short – the virus is not demonstrable in the respiratory tract after 4 days of illness. Routes of infectivity for the ARD causing adenoviruses were reported to be airborne (aerosolized virus inhaled into the lungs) [47]. Since the routine administration of the adenovirus vaccines to US military recruits began, ARD in this population has been substantially reduced [48, 49].

Whooping-cough-like syndrome has been reported with adenovirus infection leading to speculation that adenoviruses rather than *Bordetella pertussis* have caused many reported cases of whooping-cough [29, 50]. Isolates of adenovirus 5 from multiple organs of a patient who died from severe whooping-cough with lymphocytosis raised a question about the true causation of pertussis associated lymphocytosis [29, 51]. Recent reports of a large number of adenovirus isolates recovered from cases with whooping-cough [52, 53] may be explained as due to conditions favourable for the reactivation of latent adenovirus from tonsillar tissues during *Bordetella pertussis* infection [29]. Synergism between the adenovirus and the pertussis infection has also been proposed [54].

Special attention has been given recently to viral infections in children with severe combined immune deficiency, transplant patients, cancer patients undergoing chemotherapy, and AIDS patients [2, 3, 55–58]. In about 10% of these patients, such infections cause generalized illness which often leads to death [1]. The viruses were recovered from brain, throat, leukocytes, lung, urine, stool, cerebrospinal fluid, kidney, liver and pancreas. Approximately 12% of AIDS patients have an adenovirus (various genotypes) in their urine, stools, and rectal swabs [28, 29]. Based on his own observations and those of other investigators, Horwitz [29] has concluded that immunocompromised patients are not more commonly infected with adenoviruses than normal hosts, but that the outcome of such infections is more serious, even fatal. Recent studies point to a difference in adenoviruses isolated from AIDS patients and those from transplant recipients. Five of the new human serotypes described recently (serotypes 43–47) were isolated from AIDS patients, and have not yet been encountered in the general population [9]. In addition, Horvath et al. [59] suggests that AIDS patients are not only more susceptible to the serious consequences of adenovirus infections, but to Epstein-Barr virus infections as well. Their work further suggests that complementation between the two viruses might help the survival of adenovirus mutants and promote the formation of variants and possible new virus serotypes after multiple mutations.

Approximately 50% of hospital-acquired infections are reported to be caused by viruses [60]. Adenovirus infections are responsible for about 10% of the cases of

pneumonia in hospitalized children, cases that are occasionally fatal [1, 13, 61, 62]. As recently as 1988, an immunocompromised woman died of disseminated infection due to adenovirus serotype 3. During her hospitalization and after her death, 38 hospital personnel developed acute serotype 3 respiratory illness (23 of them had confirmed infections – documented by culture and seroconversion) [63]. Other nosocomial outbreaks include adenovirus enteritis in hospitalized children, keratoconjunctivitis, and pharyngoconjuntival fever in eye clinics or hospitals [30, 55, 64–74]. Recently, an outbreak of viral gastroenteritis was reported in a home for the aged. Both residents and staff developed illness. Adenovirus was identified in vomitus and faecal specimens. It was suggested that the most likely mode of transmission was environmental contamination by vomitus [75].

Since adenoviruses are easily recovered from stools of patients without gastrointestinal disease, it was not possible to correlate adenovirus growth from stools with clinical illness [29]. While adenovirus can be detected in faeces for prolonged periods of time following adenoviral respiratory tract infections, such detection offers no proof of adenoviral gastroenteritis. New laboratory techniques identify two serotypes of adenovirus (serotypes 40 and 41) which have been mainly associated with gastroenteritis [76]. Adenoviral gastroenteritis occurs mostly in children under the age of two, particularly in the first year of life, and seems to have no seasonality. Adenoviruses are, after rotaviruses, the most common viruses identified in stool specimens from children with serious gastroenteritis [57, 70, 76–81]. Severe dehydration has been reported to be as common as with astroviruses [76].

Fatty degeneration of the liver and a clinical and pathological picture characteristic of Reye's syndrome have been observed in several infants with severe adenovirus infection. Serotypes 1, 2, 3 and 7 were isolated; some of the infants died [34, 35, 82]. Evidence now relates Reye's syndrome to aspirin use during viral infections and not directly caused by adenovirus or other infectious agents [83, 84].

A number of reports link adenoviruses to aseptic meningitis, meningoencephalitis, and encephalitis, some more carefully documented than others [5, 43, 85–92]. Occasionally, an otherwise healthy person may develop adenovirus-associated meningoencephalitis [90]. Central nervous system inflammation caused by adenovirus has also been reported in immunocompromised hosts or those already infected with another virus [86, 92]. Neurological complications of adenovirus infections have been reported to be associated with severe respiratory conditions in children [5, 93]. Other reports include an adult patient with terminal lymphoma who developed subacute encephalitis – adenovirus was isolated from the brain [94]. Adenovirus encephalitis was seen in a child with leukaemia, and in a child recovering from a cerebellar astrocytoma [93]. One report of a case of sudden unilateral deafness links it to an adenovirus infection of the nasopharynx [95]. Anders et al. [96]

studied a four-year-old child with AIDS who, two months before death, developed an encephalopathy and spinal fluid abnormalities consisting of protein of 6 g/l, glucose of 2.87 nmol/l and 50 cells/μl (all mononuclear). Necropsy revealed a necrotizing ependymitis with periventricular gliosis and oedema. Adenovirus type 7 was isolated from the spinal fluid. This is a case of particular interest as the lesions are similar to those produced in monkeys by intracerebral inoculation of adenovirus serotypes 1–5 and 7.

Adenoviruses have been recognized for their persistence in a latent state in adenoidal and tonsillar tissues [59]. This persistence in normal human hosts extended up to 24 months after initial infection [46, 97]. This propensity of adenoviruses may explain the acquisition of disease by immunocompromised individuals by reactivation of latent virus from an endogenous source [29].

The oncogenic potential of adenoviruses has been reported [98] but with no definite proof as yet that these agents are associated with malignancies in humans [99–101]. One disputed study in 1982 reported adenovirus RNA sequences in human neurogenic tumours [102].

Diagnosis

Establishment of a definitive diagnosis of an adenoviral infection may be impossible unless a laboratory is available with personnel experienced in handling this group of agents. Procedures to be followed and pitfalls to be avoided are discussed in detail by Horowitz [29] and Hierholzer [1, 28] with the following recommendations for collection and preparation of specimens, laboratory tests, isolation and identification of the virus.

1. Avoid laboratory-acquired infections. No hand-to-eye contact should occur during processing of specimens or performance of diagnostic tests. Frequent hand-washing, the use of laminar-flow hoods, and periodic disinfection of the work surface will reduce the risk of laboratory-acquired infections.

2. Specimens should be collected from affected sites early in the illness.

3. Specimens may be stored at −70 °C, if immediate inoculation is not possible.

4. Paired blood samples are needed to establish or confirm a diagnosis by serological methods. The first specimen should be collected as soon as possible, and the second should be collected 2 to 4 weeks later. After clotting, serum is separated under sterile conditions and stored at −10 to −20 °C.

5. Throat swabs, nasal washes, conjunctival swabs or scrapings, and anal swabs should be placed in standard tissue culture media to which 0.5% gelatin or bovine serum albumin has been added with antibiotics (penicillin, streptomycin and amphotericin). Protein stabilization with whole serum should be avoided.

6. Approximately 20 ml of urine should be sedimented at 2000 *g* for 5 min to form a pellet of the exfoliated cells. Both cells and supernate are cultured.

7. Blood cultures are prepared by heparinizing blood, fractionating on Ficoll-Hypaque gradients, and culturing the washed cells in human embryonic kidney cells in Roswell-Park Memorial Institute (RPMI) medium with fetal calf serum. Portions of these cells should also be co-cultivated on such cells as human embryonic kidney (HEK) cells, which are usually susceptible to adenovirus infection. Similar techniques have been utilized in the US Army surveillance program for ARD identification.

Positive laboratory diagnosis is achieved by: (*a*) Direct detection in clinical specimens which use several methods such as electron microscopy (EM), immunoelectron microscopy (IEM), immunofluorescence assay (IFA), time-resolved immunofluorescence assay (TR-IFA), enzyme immunoassay (EIA), radioimmunoassay, counterelectrophoresis, restriction enzyme analysis (RE), and DNA probe. Since it is important to diagnose a viral infection quickly and accurately, research in this area is ongoing and newer methods are constantly being described. (*b*) Virus isolation in cell cultures of human origin (HEK, HeLa, KB, Hep-2, also after adaptation HELF, WI-38, and MRC-5). All of the human adenoviruses except serotypes 40 and 41 replicate and produce cytopathic effect (CPE) in some of these cell lines. Serotypes 40 and 41 grow to consistent (but low) titres in tertiary cynomolgus monkey kidney cells and in the Graham-293 adenovirus serotype 5-transformed secondary HEK cell line.

Identification of adenovirus in cell cultures inoculated with specimens taken from the patients can be accomplished using the tests for direct detection listed below, but none is simple, easy or foolproof: (*i*) IEM, IFA, TR-IFA, and RIA are genus-specific tests. (*ii*) Counterelectrophoresis, EIA, and DNA hybridization assays are genus-specific but can be made serotype-specific under certain conditions (if selected antisera, monoclonal antibodies (Maabs) or DNA fragments are used). (*iii*) RE analysis is serotype specific if the electrophoretic patterns matches that of a prototype strain. (*iv*) Haemagglutination inhibition (HI) and neutralization (SN) tests with selected antisera are serotype specific.

Serological diagnosis is performed using the acute-phase along with the convalescent-phase sera (collected at 2 to 4-week interval). It is critical to take the acute-phase serum early in the illness. Usually genus-specific tests are performed which can include the adenovirus antigen as a part of a battery of antigens. In serious cases of ARD or pharyngoconjunctivitis type-specific tests may be performed to pinpoint the serotype involved. The complement fixation test (CF) is widely used for serodiagnosis by the Army laboratories, but the EIA is much more sensitive and is increasingly practiced. HI and SN tests are capable of measuring type-specific antibody rises.

Epidemiology

Adenovirus infections in humans and animals have been reported from various parts of the world. The human adenovirus serotypes are generally not pathogenic to animals although asymptomatic simians have been observed to have antibodies to human adenovirus 12 [103]. Upon evaluation by neutralization tests, antibodies to bovine, simian, and canine adenoviruses have been seen in humans [29].

World incidence of adenovirus infections in man has been estimated from data of the World Health Organization (WHO) [32]. The data are unfortunately flawed because of the uneven distribution of reported cases, and of the paucity of data originating from less developed countries.

From available information Wigand and Adrian [32] calculated that, over the years, a constant rate of about 8% of all reported virus infections were adenovirus infections. When considering the respiratory viruses, the incidence of adenovirus infection is equal to that of Respiratory Syncytial virus infection and second to that of Influenza A virus. The distribution according to age was: 26% in children less than one year of age, 35.5% in children 1 to 4 years of age, 16.5% in children 5 to 14 years of age, and 22% in adults (15+ years). Diseases of the respiratory tract were the most frequently encountered adenovirus infections, followed by gastrointestinal diseases (occurring approximately 50% less often than the respiratory infections up to 1979). Since 1980, the number of gastrointestinal infections is approaching that of respiratory tract infections. This is possibly a reflection of the increased recognition of adenovirus serotypes 40 and 41 causing gastroenteritis in infants.

According to the WHO data covering the period of 1967–1976 and evaluated by Schmitz et al. [104] the epidemic serotypes of adenovirus show a periodicity over the years: adenovirus 3 in 1967 and 1970, adenovirus 7 in 1973 and 1974, adenovirus 4 in 1970 and 1971, adenovirus 8 in 1967, 1971, 1975. No such differences were observed for the "endemic" types 1, 2, 5, and 6. The "endemic" group of adenovirus was observed to cause a preponderance of infections of the respiratory tract in late winter and spring which is consistent with the report of Brandt et al. [105]. Adenovirus 3 and 7 showed an incidence peak in late summer, consistent with the observations of Spigland et al. [106]. Almost all viruses showed age predilections: adenovirus 1, 2, 5, and 6 for infants and children up to 4 years of age, adenovirus 3 for school age children (5–14 years of age), adenovirus 7 for school children and adults (> 15 years of age), and adenovirus 4, 8, and 19 for adults. There was a significantly higher affinity of most adenoviruses for males. Upon comparison of reports from the Northern and Southern Hemispheres, the very low or missing occurrence of certain viruses in the Southern Hemisphere was striking.

Wadell et al. [15] analyzed the numbers of adenovirus 7 recoveries in Sweden (1958–1977) and in England and Wales (1971–1977) and found that adenovirus 7

was epidemic in Sweden during 1959 and 1964, whereas 1958, 1961, 1962 and 1971–1973 were periods of minimum isolation of the virus. In England and Wales, there was and epidemic of adenovirus 7 between 1973 and 1974; prior to 1971 this serotype had rarely been recovered (since the late 1950s). By 1976, the numbers of recoveries had begun to decline. When Wadell further studied adenovirus 7 epidemic patterns, he noted three distinct patterns:

(1) Infants were affected, as in Paris in 1956 and in Peking in 1958 and possibly in 1953, in Holland in 1962, in Finland in 1967, and in Montreal in 1970. It is of interest that during the outbreak in Finland, military personnel was also affected. The epidemics occurred in the winter and caused severe illness with a mortality rate of up to 23%. The 1958 Peking epidemic was preceded by a measles epidemic and was very severe with a high mortality rate (57% in infants).

(2) Children (5–7 years of age) were affected, as in Stockholm in 1955, in Helsinki in 1959, in London in 1974. This epidemic pattern usually lasted about two years with peak incidence from August to October. In Sweden, adenovirus 7 epidemics occurred in 1959, 1964, and 1969 (combined adenovirus 3 and adenovirus 7). In the interval from 1971 to 1976, there was minimal adenovirus activity. From these observations, Wadell suggests that relatively benign adenovirus 7 outbreaks should be expected every 4 to 6 years in populations of sufficient size.

(3) Infections in semiclosed communities of military recruits have been associated with adenovirus 4 and 7, but at other times serotypes 3, 14, and 21 were found to be the cause of such epidemics. These epidemics, according to Horwitz [29] are usually observed in the winter months, but seasoned or civilian personnel in close contact with recruits appear to be resistant to the infection.

Interestingly, the disease does not occur in similarly congregated college students, suggesting that other conditions or stress and fatigue associated with basic training are contributing factors. Furthermore, in recruits congregated during the summer months no adenovirus epidemics were observed until the onset of the colder months.

Wadell [31] studied the global distribution of adenoviruses by analyzing the prevalence of antibodies and/or the frequency of isolation of adenovirus strains. He used the report of the WHO for the period 1967 to 1976 [107] which had been previously analyzed by Schmitz et al. [104] and D'Ambrosio et al. [108] as well as numerous studies from the late 1950s to the 1980s. Wadell [31] found that in an early study of Cleveland children, antibodies to adenovirus 1 and 2 were observed early in life and that antibodies to adenovirus 3 and 7 were acquired later. In the Netherlands adenovirus 7-specific antibodies were observed in sera from umbilical cords, in 4 to 5-year-old children, and in adults in 40%, 15%, and 30% of cases, respectively. In Japan, adenovirus 7 antibodies were detected in 30% of children and in 50% of adult sera. Adenovirus 3-specific antibodies were detected in 70% of the children and in

90% of the adults in Japan. In the United States, adenovirus 3 antibodies were observed in 30% of the children and in 50% of the adults.

Antibody prevalence and frequency of adenovirus 7 isolations can be highly discordant. In Japan, from the adenovirus isolates which were typed from 1966 to 1979 only 2% were typed as adenovirus 7 whereas 52% were typed as adenovirus 3. All of the adenovirus 7 isolates in Japan were obtained from healthy carriers or from sporadic cases of pharyngoconjunctival fever. In West Germany, of the adenovirus isolates typed and reported during the period 1967 to 1978, adenovirus serotypes 3 and 7 accounted for 11% and 25% of cases, respectively.

A further analysis of the molecular epidemiology of adenovirus serotypes 3 and 7 by Wadell [31] demonstrated regional differences in the distribution of adenovirus genome types. Differences in virulence may have contributed to the discordance between antibody prevalence and isolation frequency of adenovirus 7 in Japan.

The distribution of the different adenovirus 7 genome subtypes obtained since 1958 has been analyzed in isolates from different continents. In Europe, a preponderance of the adenovirus 7c and 7b genome types was noted. Longitudinal studies on adenovirus 7 and adenovirus 3 strains collected in the Netherlands and Sweden from 1958 to 1980 revealed that adenovirus 7c circulated from 1958 to 1969, whereas adenovirus 7b genome was not isolated until 1969 in the Netherlands and 1972 in Sweden. The study on adenovirus 3 strains showed that the adenovirus 3 prototype was consistently found in Europe, with the exception of a substrain (designated adenovirus 3a) isolated in 1963 and 1979. In Australia, the pattern seen above with a preponderance of the adenovirus 3 prototype and adenovirus 7b was noted, and the shift from adenovirus 7c to 7b taking place in 1975. The adenovirus 7 prototype was detected in Australia as well as in Japan. In China, a fifth adenovirus 7 genome type, adenovirus 7d, was identified; this genome has not been found elsewhere. In South Africa, one adenovirus 7b strain was isolated in 1967 and then adenovirus 7c strains. It is important to note that Africa, China, and Japan are the only regions where adenovirus 7b has not been detected in recent years. The adenovirus 3a genome type was detected both in China and Japan; the only other region where it predominated was North America. It can be assumed that adenovirus 3a was introduced to North America by immigrants of Asiatic origin.

Adenovirus serotypes 1, 2, 5 and 6 are considered endemic but distinct epidemics do occur [31]. Bruckova et al. [109] described an outbreak of a virulent adenovirus 5a genome type in Czechoslovakia. Antibody serology analyzed by Wadell [31] in Junior Village (The Seattle Virus Watch Study) indicated that the antibody prevalence was 50% and 70%, respectively, for adenovirus 1 and 2 by 2 years of age. Maximum antibody prevalence reached 80% for adenovirus serotypes 1 and 2 and 50% for adenovirus serotypes 5 in the 6 to 9 age group. In Panama, the antibody prevalence was 48%, 35%, and 27% for adenovirus serotypes 2, 1 and 5, respectively,

in the 1 to 4 age group and 75%, 70% and 40% in a teen-age group. In Stockholm, a slow build-up of immunity was noted. The antibody prevalence for adenovirus 2, 1 and 5 was 27%, 15% and 8%, respectively, in the 1 to 3 age group and 71%, 24%, and 27%, respectively, in the 9 to 12 age group. Wadell [31] further reports that a longitudinal study of adenovirus strains isolated in the Netherlands from 1958 to 1981 indicated that, during that period, the adenovirus 5 prototype was not isolated, and that the adenovirus 5a genome type accounted for 60% of all isolates.

Enteric adenovirus (serotypes 40 and 41) infections have been shown, in some studies, to be second to rotavirus infections as the most common cause of serious pediatric viral gastroenteritis [69, 110]. Since 1980, the frequency of gastrointestinal infections due to adenovirus is similar to that reported for adenovirus-caused respiratory tract infections. These infections occur mostly in children under two years of age. Data from around the world show that adenoviruses 40 and 41 are responsible for 2 to 22% of pediatric diarrhoea [57, 76, 78, 81, 111]. Controls, in some studies, show an infection incidence of 2% in asymptomatic children [57, 69, 112]. Adenoviral gastroenteritis was reported to show no seasonality [76]. Nosocomial outbreaks have been observed. Although the infection appears to spread to other children, the disease does not seen to affect adults [76]. Adenovirus 40 and adenovirus 41 have been detected in stool specimens from Europe, North America and Asia. Around 50% of 6 to 8-year-old children from Africa, Asia and Europe display antibodies to either adenovirus 40 or adenovirus 41, with no clear-cut difference in prevalence between the different geographic areas [31].

Epidemics of ocular adenovirus infection (conjunctivitis and keratoconjunctivitis) can occur sporadically or cause disease in large groups of contacts including family members. Sources may be swimming pools or small lakes or medical contacts [29, 113]. The common-source water-borne conjunctivitis outbreaks would naturally occur during the summer and are mostly due to adenovirus 3 and 7 [29]. Nosocomial transmission of the more serious disease, keratoconjunctivitis, in medical settings have led to large outbreaks (due to serotypes 8, 19, 37] that were difficult to control [30, 64, 74, 113]. Serotype 8 was the first identified cause of adenovirus keratoconjunctivitis. From 1973 to 1976, adenovirus serotype 19 was common; since 1977 adenovirus serotype 37 was found to be the predominant cause of this infection [29]. World-wide occurrences include a late summer and fall epidemic in certain parts of Japan, Taiwan and Vietnam, and a report of concurrent incidence with endemic trachoma in Saudi Arabia. About 50% of Japanese and Taiwanese children have antibodies to serotype 8 adenovirus, whereas antibodies to this type are uncommon in American school children [29]. The determination of antibodies to adenovirus serotypes 8, 19, and 37, as reported by Wadell [31], indicated that in children living in Rome, 2% or less had antibodies against adenovirus serotype 8 or serotype 19. In Germany, 15% of adults were immune to

adenovirus 19. In Zaire, however, the occurrence of antibodies against adenovirus 19 was 85%.

As early as 1964, it was reported that adenovirus 4 may cause conjunctivitis without respiratory symptoms. Several outbreaks of adenovirus 4-associated eye disease have been described in the literature in the past two decades [114–116]. It is of particular interest that adenovirus 4a has been identified in three outbreaks of eye disease [31]. Acute haemorrhagic conjunctivitis with a subconjunctival haemorrhage was observed in Rome in 1974. In 1977, a 5-year-old boy died with disseminated adenovirus 4 infection after treatment in an intensive care unit in Buffalo, NY. A nosocomial outbreak of pharyngoconjunctival fever affected hospital personnel primarily involved in caring for this boy. In 1981, a similar nosocomial outbreak of adenovirus 4 conjunctivitis was reported from Chicago. The index patient died of adenovirus serotype 4 pneumonia. In Brazil, an outbreak of pharyngoconjunctival fever caused by adenovirus occurred in 1976 in Belem (Northern Brazil). It was shown that the serotype responsible was adenovirus 4a. An epidemiological study of children with respiratory disease in Rio de Janeiro and Belem from 1977 to 1986 indicated that all adenovirus 4 strains recovered were also 4a indicating that this serotype, circulating in South America, can cause both ocular and respiratory diseases [117].

The propensity of adenoviruses to cause persistent infections may create a health hazard for children with severe combined immunodefficiency, immunocompromised individuals and those with AIDS [31]. Yolken et al. [118] conducted a prospective study on the aetiology of severe enteric infections in 78 bone marrow transplant recipients (mean age 21 years) from September 1980 to June 1981. Adenoviruses were recovered in 12/31 patients in whom enteric pathogens could be identified. The comparative mortality among infected and uninfected patients was 55% and 13%. A retrospective study of 15 immunocompromised patients in whom adenovirus was isolated at the UCLA Center for Health Sciences during 1967–1978 [12] indicated that all had a high temperature, 80% had pneumonia, 73% had elevated liver enzymes and 33% had diarrhoea. Nine of the adenovirus-infected patients died. Adenovirus 4 was the most common species isolated from 5/9. Adenovirus 34 and 35 were also recently isolated from 10/10 patients with AIDS [119]. Johansson et al. [14] compared adenovirus 31 isolates obtained from immunodeficient and immunocompetent individuals during 1963–1987. Isolates from 15 immunodeficient patients, 12 of whom (four with hepatitis and eight with lower respiratory tract infections) died during their infections, were investigated to determine if the severe outcome was due to infection with particularly virulent strain(s). No highly virulent genome type was identified. Wadell [31] suggests that, based on some of the above studies, adenovirus infections may substantially contribute to morbidity and mortality in immunocompromised

patients. There is, therefore, an urgent need for prospective studies to ascertain the rate of primary or reactivated infections with adenovirus in the immunocompromised population.

The combined data from many studies suggest that clinical adenovirus disease in humans can be divided roughly into four groups. The first group, which includes serotypes 1, 2, 5, 6, 40, and 41 usually affects children. By one year of age, at least 50% of all children have antibodies to one or more of these agents. By age 3, about 80% of United States children have such antibodies, and in some less developed countries all children age 3 years or older have such antibodies. The second group, including serotype 3, 4, 7, and 8 and occasionally 11, 14, 19, 21, and 37, is associated with epidemic ARD, pharyngoconjunctivitis, and keratoconjunctivitis in young adults. The third group, which includes serotypes 1, 2, 5, 6, 7, 11, 12, 18, 31, 34, 35, 43–47, is associated with severe illness and contributes to death in immunocompromised patients [31]. The fourth group causes sporadic illness. The rare adenovirus isolations made from urine and cerebrospinal fluid usually belong to serotypes 11, 14, 16, and 21. Almost all known adenoviruses have been found in outbreaks of gastroenteritis, coryza, conjunctivitis, respiratory diseases, and a variety of other infections.

Vaccines

Prophylaxis with adenovirus vaccines was and is still considered to be of enormous value and an essential requirement in the military. Although the vast array of adenovirus serotypes and the seriousness of their effects are evident in all populations, it has been difficult to introduce adenovirus vaccines for use in the general population. One problem has been the difficulties of preparing pediatric forms of the current vaccine. As the epidemiology of adenovirus disease becomes more clearly defined, the need for and the possible role of potential vaccines, is becoming more evident.

The first type of vaccines developed and tested between 1958 and 1962 was the inactivated adenovirus vaccines. They consisted of tissue culture-grown (monkey kidney tissue) virus inactivated with formalin. Subunit preparations of inactivated adenovirus were tested in animals, particularly guinea pigs [120–126]. The effects were described in terms of neutralizing antibody produced. Although several methods for the isolation of antigenic fractions were devised, none was as effective as the inactivated whole virus [120]. Field trials of inactivated adenovirus preparations produced a wide range of results. Efficacy varied from 70% to essentially nil [127]. These early trials were conducted by the military with vaccines made from adenovirus serotypes 3, 4, and 7. The vaccines were considered "useful but not completely satisfactory". By 1963, it became evident that the adenovirus seed stocks used to prepare these vaccines were contaminated with SV–40. Further

investigations showed that adenovirus needed a "helper" virus, usually SV-40, in order to reproduce in monkey kidney tissue culture [128]. Subsequently, it was also demonstrated that some adenovirus types were themselves oncogenic [98].

Soon after, in 1963, it became clear that inactivated adenovirus vaccines were unreliable and perhaps inadvisable, and alternative live virus vaccines were explored. It was observed that, similar to polioviruses, adenoviruses could proliferate in the gut and that protective antibodies could then be produced [129]. Early studies concentrated on tests with adenovirus serotype 4 (shown not to produce tumours in any animal species), grown in HEK cell culture. Later serotype 7 was required, and it was evaluated [130]. Vaccine containing adenovirus serotype 7 were developed using HEK cells and human diploid cells (WI-38). Design for the dosage form for oral adenovirus vaccines was much more complex than that for poliovirus immunization. Although many current reference describe the adenovirus vaccine as a "capsule", this preparation was not a practical dosage form for large scale production. Studies of other live virus vaccines clearly showed the importance of maintaining virus viability under less than ideal conditions. This viability was frequently achieved by lyophilization in the presence of additional serum proteins or other metabolizable organic materials, such as amino acids, peptides and carbohydrates. Stabilization of the vaccines was obtained by the rigid exclusion or the very prompt elimination of bacterial contaminants and their enzyme products [131]. Stability studies of adenovirus preparations also revealed that lyophilization itself did not insure sufficient stability in field use. Thus the adenovirus vaccines currently prepared on a large scale for commercial use are now made in the form of enteric-coated tablets. The tablet is stable at ordinary refrigeration temperature for a period of at least two years. The tablet consists of three layers: the innermost core contains specially lyophilized live virus of the pretested vaccine strain (vaccine strains used in the tablets are stored in a seed lot system so that each production lot consists of the same virus passage). The inner core is then covered with inert materials which protects the virus. The double-layered tablets are sprayed with an enteric-coating solution. This coating protects the virus from gastric fluids.

Vaccines containing adenovirus serotypes 4, 7, and 21 can be given together with no mutual interference [49, 132, 133]. During clinical evaluation of adenovirus vaccines in military recruits, it was observed that early administration of vaccine was of critical importance. The preferred practice is to give the vaccine to military recruits within the first two hours of arrival at the training base. Protection failure may occur when administration of vaccine is delayed. The rapid loss of efficacy when active immunization is delayed clearly indicates that passive immunization or antibody therapy would have no value unless antibodies are developed before infection. The vaccine strains were observed not to spread from vaccinated individuals to susceptible unvaccinated recruits barracked in close quarters.

Furthermore, although viruses were excreted in great quantity, vaccinated individuals showed no signs of disease or infection, however, when exposed to the wild virus strains, the vaccinated recruits remained susceptible to infection (as evidenced in throat cultures) but not to disease.

According to an ARD surveillance program encompassing the years 1966 to 1971 at two military bases where recruits had been vaccinated with adenovirus vaccine [133], adenovirus diseases were reduced by about 95%. More recent reports [134, 116] indicate that after initiation of year-round adenovirus vaccine administration in 1984 (prior to 1984 adenovirus vaccines were given during the presumed high-risk fall-winter seasons October through March) there were no ARD epidemics among new recruits at training centres.

Two recent reports [135, 136] from Walter Reed Army Institute of Research on safety and immunogenicity of conventional immunization during early HIV infection indicated that significantly fewer HIV-infected subjects responded to adenovirus 4, although HIV-infected subjects who did respond produced functional antibodies comparable to those of normal subjects. Postimmunization, no clinically apparent adverse reactions to vaccination were detected.

Safety of vaccine

There is no serious question about the efficacy of the present adenovirus vaccines, however, there is continuing concern about their safety. While the oncogenesis question (in humans) has been extensively explored [99, 100, 137–142] with negative results, there are many new studies that bear on the genetic aspects of cell transformation and tumourigenecity. How this relates to diseases in humans is not established. It may be important to note that transformation by nononcogenic strains does not result in tumourigenicity. The strains selected for use in the vaccine are nononcogenic even in hamsters. Current texts describe the adenovirus vaccine as containing unattenuated virus [143, 144]. This view seems to be based on the lack of attenuation "markers" such as exists for poliovirus strains. While the lack of markers remains an unsolved, genetic analyses are available that can show differences between the DNA of the vaccine strains and the original isolates [142]. Moreover, an abundance of convincing epidemiological evidence is available that bears on this question.

There is mounting evidence that many adenovirus strains are highly contagious. Nosocomial spread has been repeatedly reported. In contrast, there is no evidence of spread of vaccine strains and no evidence of respiratory, pathogenicity – even in recruits with less than fastidious hygiene or in their outside (family) contacts. Some early adenovirus vaccine studies, using NIH produced capsules, showed some spread in household contacts.

When serotype 4 unattenuated vaccine strains were given to a small number of children during an early original NIH clinical trials in 1967, the infection spread to household contacts and resulted in a few clinically manifest illnesses [145]. Similar NIH vaccine (unattenuated) administration to adults commonly spread to sexual partners but infrequently to other adults housed together [146].

The risk of spread of vaccine virus in immunized children has limited the large-scale testing of vaccines against the common endemic serotypes 1, 2, and 5. Oral vaccines containing these serotypes have been successfully administered to adult volunteers lacking homologous serum antibodies [147]. However, such tablets cannot be used in young children. Crushed tablets, a frequent practice in pediatric patients, would defeat the purpose of the dosage form design. Microspherules containing the live virus were prepared. Such microspherules cannot be enteric coated without destroying the virus. If live oral adenovirus vaccines are to be considered for pediatric use, it is necessary to reexamine the possible use of uncoated microspherule or even liquid preparations.

No reports of adverse reaction has ever been received either during clinical trials or during the ongoing routine administration of the vaccine to all new recruits.

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THE INDUCTION OF SPECIFIC PROTECTION AGAINST *ACTINOBACILLUS PLEUROPNEUMONIAE* INFECTION BY SPECIFIC DLE

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Immunoprophylactic and immunotherapeutic effects of specific actinobacillus DLE (dialysable leukocyte extract) were studied in model pigs experimentally infected with *Actinobacillus pleuropneumoniae*. Specific DLE used for immunoprophylactic purposes was administered intramuscularly to pigs either in two doses in combination with one dose of actinobacillus vaccine (1st group) or in a single dose (2nd group). All animals of the two experimental groups survived the intranasal *A. pleuropneumoniae* challenge (2×2 ml $2-5\times10^{10}$ c.f.u.) while 3 of 5 animals of the control group died of haemorrhagic-necrotic fibrinous pneumonia. DLE administered to pigs for immunotherapeutic purposes was applied intravenously in two doses 1 and 5 h after the actinobacillus challenge (1st group), and in a single dose 5 h following the challenge (2nd group). All animals of the first group survived the challenge while 3 out of 5 animals of the second group and 4 out of 5 animals of the control group died during the experiment.

Actinobacillosis presents an important epidemiologic and economic problem in pig rearing. For that reason considerable attention has been paid to the prevention of this disease. Several types of vaccines [1–3] have been developed and used in practice.

Due to anaphylactic states elicited by oil adjuvants an effort has been made recently to use biologically active substances (lymphokines, monokines), the nonimmunoglobulinic secretory products of sensitized lymphocytes, to prevent infectious diseases in animals. One of such substances is the transfer factor (TF) contained in the dialysable leukocyte extract (DLE), which comprises more than 200

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various low molecular weight substances [4]. DLE components mediate the transfer of immune reactions of the antigen-specific T-lymphocytic response [5]. Veterinary medicine, too, tends to use these properties of DLE in prophylaxis of the occurrence of infectious and invasive diseases of diverse aetiology [6–9]. We investigated the immunoprophylactic and immunotherapeutic effects of specific DLE in model experiments.

Materials and methods

Experimental animals. Thirty-five hysterectomized pigs of live weight 20–25 kg were assigned to seven groups, each comprising 5 pigs. The pigs were clinically healthy, were not immunized previously against any infection and their serological examination failed to show the presence of specific antibodies against *Actinobacillus pleuropneumoniae* or *Bordetella bronchoseptica*.

Preparation of DLE. DLE was prepared from lymph nodes and spleen of pigs immunized twice within 14 days with a commercial inactivated *A. pleuropneumoniae* vaccine (Hemikol, Mevak, Nitra). Pigs were killed on day 14 after the revaccination and their lymph organs were removed and deep-frozen to −70 °C. The procedure of DLE preparation was described in the paper by Mikula et al. [10]. DLE was lyophilized and stored at −70 °C.

Determination of biological activity of DLE. Biological activity of DLE was determined on the basis of a number of potential units (P.U.) in 1 ml of the preparation [11]. The migration-inhibition test (MIT) [12] in the presence of *A. pleuropneumoniae* was used for this purpose. Test conditions were described previously [10].

Characterization of DLE. Molecular weight of DLE determined by PAGE [13] was below 10 000. DLE was adjusted so that 1 to 1.5 ml of preparation corresponded to the amount of low molecular weight substances obtained by processing of $2-5\times10^8$ lymphocytes. The prepared preparation corresponded on average to an optical density 15 at 260 nm (1.5 at the 10-fold dilution). Biological activity tested by MIT amounted to 50 P.U. in 1 ml of the preparation. Three ml of DLE were mixed with 3 ml of pig albumin for i.v. application.

Preparation of albumin. Albumin intended for intravenous application was prepared using the method according to Mikula et al. [14].

Immunoprophylactic use of DLE. Five pigs of the 1st group were administered 3 ml of the DLE preparation i.m. and 2 ml of the vaccine s.c. to two different body locations. The i.m. DLE application was repeated 11 days after the first dose. Pigs of the 2nd group received placebo (2 ml of incomplete Freud's adjuvant s.c. to two different body areas). Three ml of DLE were applied to pigs i.m. 11 days later. The 3rd group served as a control. Pigs of this group received placebo in two doses (3 ml of saline i.m. + 2 ml of adjuvant s.c.). Pigs of the 3rd group were vaccinated s.c. with two doses, 3 ml each, of the Hemikol vaccine within 11 days. Pigs of all four groups were challenged intranasally (i.n.) with the *A. pleuropneumoniae* culture at a dose $2-5\times10^{10}$ colony forming units (c.f.u.) into each nasal cavity on day 4 after the last application.

Immunoprophylactic use of DLE. Fifteen pigs divided into three groups were challenged i.n. with *A. pleuropneumoniae* at a dose $2-5\times10^{10}$ c.f.u. into each nasal cavity.

Pigs of the fifth group were injected i.v. with 3 ml of specific DLE stabilized with pig albumin (3 ml) 1 h after the infection. Application of the preparation was repeated 5 h after the challenge.

Pigs of the sixth group were injected i.v. with placebo (3 ml of saline + 3 ml of albumin) 1 h after the infection and with 3 ml of LcD + 3 ml of albumin i.v. 4 h later.

Pigs of the seventh group, which served as a control, were injected i.v. with placebo (3 ml of saline + 3 ml of albumin) 1 and 5 h after the challenge.

Immunoprophylactic use of Hemikol vaccine. The inactivated Hemikol vaccine was used in 2×3 ml doses within 14 days to induce the specific protection [2]. The vaccine contained serotypes 1 and 2 of *A. pleuropneumoniae*.

Isolation of A. pleuropneumoniae from postmortem material. Samples were taken from characteristically altered foci of pulmonary tissue, trachea, tonsils, intrabronchial and peribronchial lymph nodes, blood, spleen and liver and were processed within 2 h after the sampling or within 24 h after deep freezing to −70 °C. The isolation itself was carried out using the method of cocultivation with *Staphylococcus aureus* and subsequent biochemical diagnostics according to Biberstein et al. [15].

Testing for antibody titre after vaccination. The test-tube agglutination test according to Mittal et al. [16] was used to determine the titre of specific serum antibodies after the application of Hemikol vaccine.

E-rosette test. The number of E-rosette-forming-T-lymphocytes (ER) was determined employing the method of Binns [17].

Results

Immunoprophylactic use of DLE

Results summarized in Table I point to the optimal application of DLE in the 1st group of pigs. The double application of DLE within 11 days, together with a single application of vaccine in this group, elicited a fairly good state of immune protection confirmed by both the clinical course of the disease and the pathologic-anatomical findings. Moderate apathy with hyperventilation was diagnosed 16 h after the intranasal infection and the average body temperature in this group was 39.8 °C. Clinical changes disappeared within 24 h after the infection and the physiologically normal state was restored. All animals of this group survived the infection and were killed after 8 days. Postmortem examination did not reveal any specific pathologic-anatomical findings indicating actinobacillous pneumoniae. Cultivation examination provided negative results.

Double application of LcD within 11 days without the vaccination (group 2) failed to induce protection analogical to that observed in the 1st group. Hyperventilation, overall apathy and muscle tremor were observed in pigs of this group 16 h post-infection and the average body temperature in this group increased to 41.2 °C. These clinical symptoms persisted as long as 24 h after the infection. In spite of persistent clinical symptoms of the disease none of the pigs of this group died. Animals were killed on day 8 after the infection and the postmortem examination revealed hyperaemia of lungs, interstitial oedema and 1 to 2 necrotic foci of the size 2×2 cm in the pulmonar parenchyma. Cultivation examination provided negative results (Table I).

Table I

Immunoprophylactic use of DLE

Group of pigs n=5	% of E-rosettes X	1st application	2nd application 11th day after the 1st dose	% of E-rosettes X	I.n. infection[a] on day 4 after the 2nd dose	Clinical symptoms 16 h p.i.	Clinical symptoms 24 h p.i.	Pathoanatomical findings	Death[b]	Cultivation findings
1	27.3	3 ml of DLE + 2 ml of vaccine[c] s.c.	3 ml of DLE i.m.	36.8	2 x 2 ml 2-5 x 10^{10} c.f.u.	moderate apathy, hyperventilation T=39.8 °C	physiological normalization	killed 8 days p.i. without specific pathoanatomical finding of *Actinobacillus pleuropneumoniae*	0	lungs-neg., trachea-neg., tonsils-neg., intrabronchial and peri-bronchial lymph nodes-neg., spleen-neg., liver-neg.
2	25.9	placebo 3 ml of saline i.m. 2 ml of adjuvant s.c.	3 ml of DLE i.m.	31.5	2 x 2 ml 2-5 x 10^{10} c.f.u.	hyperventilation, overall apathy, muscles tremor T=41.2 °C	persistence of symptoms	killed 8 days p.i. hyperaemia, interstitial edema, 1-2 necrotic foci 2 x 2 cm	0	lungs-neg., trachea-neg., tonsils-neg., blood-neg., liver-neg., spleen-neg., intrabronchial and peri-bronchial lymph nodes-neg.
3	N	3 ml of vaccine s.c.	3 ml of vaccine s.c.	N	infection	absence of clinical changes T=39.7 °C	absence of clinical changes T=39.5 °C	killed 8 days p.i. without specific pathoanatomical findings	0	lungs-neg., trachea-neg., tonsils-neg., intrabronchial and peri-bronchial lymph nodes-neg., spleen-neg., liver-neg.
4 control	28.1	placebo 3 ml of saline i.m. 2 ml of adjuvant[d] s.c.	placebo 3 ml of saline i.m.	26.7	2 x 2 ml 2-5 x 10^{10} c.f.u.	vomiting, shallow spasmodic hyperventilation, dyspnoea, severe cyanosis, overall apathy, anorexia	muscle tremor, epistaxis T=41.8 °C	extensive double acute haemorrhagic-necrotic fibrinosis, pleuropneumonia, interstitial edema	3	lungs-pos., trachea-pos., tonsils-pos., intrabronchial and peribronchial lymph nodes-pos., blood-neg., spleen-neg., liver-neg.

[a] experimental infection *A. pleuropneumoniae*
[b] death of animals within 24 h
[c] inactivated vaccine *A. pleuropneumoniae* "Hemikol" (Mevak, a.s.)
[d] incomplete Freund's adjuvants
DLE = dialysable leukocyte extract
N = untested

p.i. - post infection
s.c. - subcutaneous application
i.m. - intramuscular application
i.n. - intranasal application
c.f.u. - colony forming units

Pigs of the third group immunized with inactivated Hemikol vaccine did not show any clinical symptoms of the disease and their pathologic-anatomical and cultivation examinations were negative (Table I). The average titre of specific antibodies after two doses of vaccine against serotype 1 reached the value 1:360 and against serotype 2 the value 1:40 (Fig. 1).

Pigs of the control group (group 4) subjected to i.n. actinobacillus challenge showed characteristic clinical and pathologic-anatomical symptoms of actinobacillosis and 3 of 5 pigs of this group died. Cultivation of lungs, trachea and tonsils provided positive results (Table I).

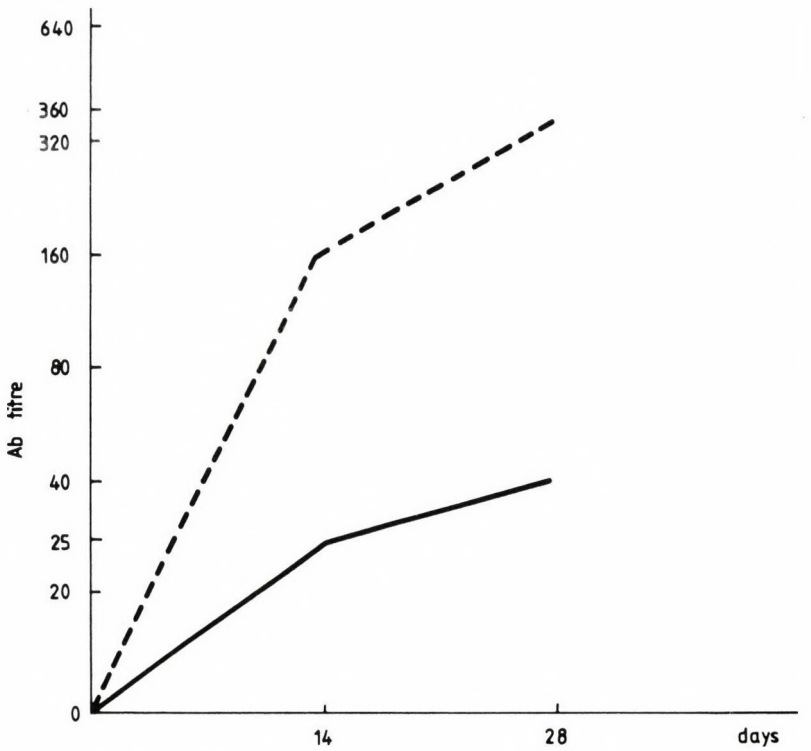

Fig. 1. Humoral immune response after immunization of hysterectomized pigs using the Hemikol vaccine. No. of pigs, 10, two doses of vaccine. Titre of antibodies was determined by the method of test tube agglutination. Titre against serotype 1: - - - - -, serotype 2: ——

Table II

Immunotherapeutic use of DLE

Group of pigs n=5	Infection[a] i.n.	Application of DLE i.v. 1 h p.i.	Application of DLE i.v. 5 h p.i.	Clinical symptoms 16 h a.i.	Clinical symptoms 24 h a.i.	Pathoanatomical findings[b]	Death[c]	Cultivation findings
5.	2 x 2 ml 10^{10} c.f.u.	3 ml i.v.	3 ml i.v.	hyperventilation apathy, anorexia, dyspnoea T=40.8 °C	persistence of symptoms	killed 8 days p.i. In 2 pigs: serofibrinous, haemorrhagic-necrotic foci in diaphragm lobes In 3 pigs: absence of specific pathoanatomical findings	0	lung-neg., trachea-neg. tonsils-neg., intrabronchial and peribronchial lymph nodes-neg., spleen-neg., liver-neg.
6.	2 x 2 ml 10^{10} c.f.u.	Placebo[d] 3 ml i.v.	3 ml i.v.	hyperventilation, apathy, anorexia, dyspnoea, vomiting T=41.2 °C	epistaxis, muscle tremor	Serofibrinous haemorrhagic-necrotic pleuropneumonia	3	lungs-pos., trachea-pos. tonsils-pos., blood-neg. liver-neg., spleen-neg., intrabronch. and peri-bronch. lymph nodes-pos.
7. control	2 x 2 ml 10^{10} c.f.u.	Placebo[d] 3 ml i.v.	Placebo[d] 3 ml i.v.	hyperventilation, vomiting, expiration dyspnoea, cyanosis, apathy, anorexia	epistaxis, muscle tremor T=41.9 °C	extensive double acute haemorrhagic-necrotic fibrinosis pleuropneumonia, interstitial edema	4	lungs-pos., trachea-pos. tonsils-pos., intrabronch. and peribronchial lymph nodes-pos., blood-neg., spleen-neg., liver-neg.

[a] experimental infection *A. pleuropneumoniae*
[b] dead pigs and pigs killed on day 8 post infection were subjected to postmortem examination
[c] death of animals within 24 h
[d] 5% albumin solution suitable for i.v. application

DLE = dialysable leukocyte extract
i.v. = intravenous application
i.n. = intranasal application
c.f.u. = colony forming units
p.i. = post infection

Immunotherapeutic use of DLE

All animals of the fifth group, which received two doses of specific actinobacillus DLE 1 and 5 h after the challenge, survived the infection. Non-specific symptoms of infection (apathy, anorexia, increased body temperature) were observed in these animals (Table II). When they were killed 8 days after the challenge, 3 pigs did not show any specific pathological-anatomic symptoms of actinobacillosis and additional two had necrotic foci in diaphragm lobes. Cultivation provided negative results (Table II).

Actinobacillus DLE was applied to pigs of the sixth group 5 h after the challenge. Apathy, anorexia, dyspnoea and vomitus were observed in animals 16 h after the infection. The average body temperature of animals of this group was 41.2 °C. Muscle tremor and epistaxis occurred 24 h after the infection. Focal haemorrhagic-necrotic pneumonia of the diaphragm lobes was detected in killed animals. Serofibrinous haemorrhagic pleuropneumonia was found in animals which died during the experiment. Positive findings were obtained by cultivation examination of lungs, trachea and tonsils.

In the control group (group 7) four of five animals died of infection. The average body temperature in this group reached 41.9 °C 24 h after the challenge. Pathologic-anatomical changes characteristic of actinobacillus pleuropneumonia were observed in animals which died and in one killed animal cultivation findings from lungs, trachea and tonsils were positive (Table II).

Discussion

Although various kinds of inactivated cellular vaccines have been developed so far, they do not fully resolve epizootics of the occurrence of actinobacillus infections of pigs. The problem consists in geographic redislocation of the occurrence of serotypes producing the infection. Vaccination with cellular inactivated vaccines does not provide an answer to the still pressing problem of the incidence of carriers of infection among immunized animals. This is the reason why an effort has been made to use subcellular pathogen structures to prepare effective immunogenes [18].

An additional trend of induction of specific protection consists in utilization of a low molecular weight non-antigenic substance isolated from leukocytes obtained from immunized animals [6, 7, 9, 19, 20]. Li Zailian [21] ascribed not only prophylactic but also important therapeutic effect to DLE in animals with clinical symptoms of swine plague and Marek's disease of poultry.

Wilson et al. [22] tested DLE from colostrum and milk of cows immunized with viral and parasitic antigens. The onset of the effect of bovine DLE on poultry was observed as early as on the first day after the application and persisted over the

period exceeding 25 days. Similarly, we have recorded in our experiments a very rapid development of resistance of pigs to *A. pleuropneumoniae*. DLE applied 1 and 5 h after the infection prevented the development of pleuropneumoniae and the death of pigs. Because of the absence of specific method of testing of DLE, applicable to standardization of the effective dose and testing of the biological effect of DLE, the physical-chemical methods as well as methods utilizing cells of the immune system [23] have been used for this purpose.

The migration-inhibition test at the presence of actinobacillus antigen [11] was employed in our study to determine the content of P.U. in 1 ml of the preparation, which was used as a basis fór determination of biological activity and specificity of DLE. While the specific actinobacillus DLE contained 50 P.U. in 1 ml, the non-specific one, obtained from non-immunized fattening pigs, displayed negative MIT reaction. Actinobacillus DLE prepared from pigs immunized with the Hemikol vaccine showed similar physical-chemical properties as DLE of bovine origin [10]. These properties were comparable with those of DLE preparations prepared by other authors [6, 24].

A number of authors prepared DLE of porcine origin with a wide spectrum of specifities [8, 25, 26], however, there has been no mention of specific actinobacillus DLE in the available literature.

Specific actinobacillus DLE used in our study prevented the death of subsequently challenged pigs and alleviated considerably the clinical symptoms of the disease when applied to infected animals. Pathoanatomical examinations did not reveal fibrinous pneumonia.

Our results indicate that pigs vaccinated with inactivated actinobacillus vaccine from a full-value resource for the preparation of DLE with a specific effect.

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TOXIC AND ADHESIVE PROPERTIES OF *ESCHERICHIA COLI* STRAINS BELONGING TO CLASSIC ENTEROPATHOGENIC SEROGROUPS

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Twenty-three strains belonging to classic enteropathogenic *Escherichia coli* (EPEC) serogroups were investigated for the production of heat-labile (LT) and heat-stable (STa) enterotoxins, verotoxins (VT), cytotoxic necrotizing factors CNF1 and CNF2, α-haemolysin (Hly), necrosis and modification of permeability in rabbit skin, lethal activity to mice, mannose-resistant (MRHA) and mannose-sensitive (MSHA) haemagglutination, relative cell surface hydrophobicity and the expression of P fimbriae. Of 23 EPEC strains, 7 (30%) belonging to serotypes O18ac: H7 (Hly^+), O20: H26 (lethal), O26: H^- (Hly^+), O44: H18 (Hly^+), O55: H^- ($CNF2^+$, necrotic and lethal), O119: H27 (VT^+ and Hly^+) and O142: H6 (lethal) produced toxic factors. Seven (30%) of 23 EPEC strains were $MRHA^+$, 17 (74%) were $MSHA^+$ and only 2 possessed high hydrophobicity. Two strains belonging to serotypes O18ac: H7 and O44: H18 that showed MRHA type IVa were fimbriated when grown on CFA medium.

Classic enteropathogenic *Escherichia coli* (EPEC) were associated with sporadic and severe outbreaks of diarrhoea which occurred in Europe and the USA in the 1940s and 1950s and have been incriminated as pathogens on the basis of epidemiological studies [1–3]. When virulence factors such as enterotoxin production and invasiveness were discovered the majority of strains previously described as EPEC were found to be negative for these pathogenic mechanisms [1–3]. During the 1970s, a controversy arose over whether EPEC are really enteropathogens [4, 5], but

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Levine et al. [6] demonstrated that EPEC are capable of causing diarrhoea in adult volunteers.

The mechanism by which EPEC cause disease remains to be defined. Although, some EPEC strains produce verotoxins (VT1 and/or VT2) or cytolethal distending toxin (CDT), the majority were found not to be toxigenic [7–11]. EPEC adherence factor (EAF) responsible for localized adherence to HEp–2 and HeLa cells encoded in a plasmid appears to be essential for an EPEC strain to cause diarrhoea in adult volunteers [12, 13]. Nevertheless, the role of adhesins discovered in the pathogenesis of diarrhoea caused by EPEC is not clear.

We have established recently [14, 15] the existence of two types of cytotoxic necrotizing factors (CNF1 and CNF2) in human and animal clinical isolates of *E. coli*. In this study, we have investigated the production of CNF1 and CNF2, and the expression of other virulence factors in 23 *E. coli* strains belonging to different enteropathogenic serogroups.

Materials and methods

Bacterial strains. A total of 23 reference *E. coli* strains belonging to classic enteropathogenic serogroups obtained from F. Ørskov and I. Ørskov (World Health Organization Collaborative Centre for Reference and Research on Escherichia, Statens Seruminstitut, Copenhagen, Denmark) were investigated. As controls were used the *E. coli* strains: m452–C1 (O63: K–: H–, LT^+STa^+), H19 (O26: H11, VT^+), MR249 (O6: K–, $CNF1^+$) and B26a (O123: K–, $CNF2^+$).

Toxicity assays. Sonic extracts [16], extracellular fluids [16] and filtrates of cultures treated with mitomycin C [14] were obtained as previously described. These three types of preparations were inoculated on Vero and HeLa cells for the detection of heat-labile enterotoxin (LT), verotoxins (VT), and cytotoxic necrotizing factors CNF1 and CNF2 [14]. Furthermore, the sonic extracts were assayed for necrosis and modification of permeability in the rabbit skin test [14] and for lethality in the mouse intraperitoneal test [16]. Extracellular fluids were also assayed for STa enterotoxin by the infant mouse test [16]. Seroneutralization assays in HeLa cells [14] and the rabbit skin test [15] were carried out with CNF1 and CNF2 antisera obtained by DeRycke et al. [15]. All cited assays were comprehensively described in previous papers [14–16]. Briefly, Vero and HeLa cell assays were performed on cell monolayers grown nearly to confluence in plates with 24 wells. At the time of assay, the growth medium was changed (0.5 ml per well) and 50 μl of undiluted sonic extract, extracellular fluid or filtrate of culture treated with mitomycin C was added [14]. For necrosis and modification of permeability 0.1 ml of undiluted sonic extract were intradermally injected into four rabbits. Twenty-four hours after inoculation, two rabbits were killed and the existence of necrosis was evaluated. The other two rabbits were injected with Evans blue dye at 2% (w/v) in 0.85% (w/v) NaCl to a final concentration of 2 mg of dye by Kg of weight. After 30 min of dye diffusion, the rabbits were killed and the permeability zones were measured [14]. Lethality in the mouse intraperitoneal test [16] was recorded during a period of 7 days following intraperitoneal administration of 0.5 ml of the undiluted sonic extract. The infant mouse test was performed by oral inoculation of 0.1 ml diluted extracellular fluid containing Evans blue dye [16]. Haemolysin production was detected after 24 h of growth on blood agar base medium containing 5% (v/v) washed sheep erythrocytes.

Haemagglutination test, cell surface hydrophobicity and P. fimbriae. Bacteria were inoculated into Mueller-Hinton broth (Difco, USA) and incubated statically at 37 °C for 5 days until a pellicle was formed on the surface. From this pellicle, bacteria were recovered, inoculated on the CFA [17], Minca-Is [16] and Blood agar (Tryptone soy agar with 5% sheep blood, Becton Dickinson, UK) solid media. Mannose-resistant (MRHA) and mannose-sensitive (MSHA) haemagglutination were carried out by the rocktile method with human groups A and O, calf, guinea-pig, adult chicken, sheep and pig erythrocytes. Strains were grouped according to their MRHA patterns in the six MRHA types (I–VI) of our classification [17]. Relative cell surface hydrophobicity was measured by the improved salt aggregation test (ISAT) [18]. The final molar ammonium sulphate concentrations used were 2.0, 1.4, 1.0, 0.4, 0.1, 0.06 and 0.02. Detection of P fimbriae was performed by a particle agglutination test (PF test, Orion Diagnostica, Espoo, Finland) using dense suspensions of bacteria grown on CFA medium [19].

Results

Toxic properties. A total of 7 (30%) from the 23 *E. coli* with classic enteropathogenic serogroups investigated produced toxic factors (Table I). Four strains of serotypes O18ac: H7, O26: H−, O44: H18 and O119: H27 produced α-haemolysin, one *E. coli* O55: H− synthesized CNF2 and was necrotic in rabbit skin, and an other strain of serotype O119: H27 was positive for production of VT. Furthermore, the sonic extracts from 3 strains of serotypes O20: H26, O55: H− and O142: H6 were lethal to adult mice after intraperitoneal injection. None of the 23 reference strains belonging to classic enteropathogenic serogroups were positive for production of LT, LTa of CNF1. Specific cytopathic effect on HeLa cells and necrotic activity caused by CNF2-producing strain of serotype O55: H− were neutralized by CNF2 antiserum diluted to 1:100 but not by CNF1 antiserum diluted to 1:10.

Adhesive properties. All 23 *E. coli* strains with classic enteropathogenic serogroups, grown on CFA, Minca-Is and Blood agar media, were tested for MRHA, MSHA and relative cell surface hydrophobicity. The results were as follows: 7 (30%) were MRHA$^+$, 17 (74%) were MSHA$^+$ and only 2 possessed high hydrophobicity, aggregating in $\leq$ 0.4 M ammonium sulphate concentrations. Furthermore, 2 strains belonging to serotypes O18ac: H7 and O44: H18 that showed MRHA type IVa were P fimbriated when grown on CFA medium (Table I).

Of the 7 MRHA$^+$ strains, 2 of type IVa expressed MRHA when grown on the three media, 3 of types IVb were MRHA$^+$ only when grown on Blood agar and Minca-Is media, and 2 of type IVb were MRHA$^+$ only when grown on Minca-Is medium. *E. coli* strains of MRHA type IVa haemagglutinated human groups A and O, chicken, sheep and pig erythrocytes. MRHA strains of type IVb were positive with human groups A and O, calf, guinea-pig, chicken and pig erythrocytes. Strains of type VI agglutinated human groups A and O and calf erythrocytes in the presence of D-mannose.

Table I

Toxic and adhesive properties of E. coli strains belonging to classic enteropathogenic serogroups

Strain	Serotype	α-haemo-lysin	Rabbit skin test[1]	Mouse intrape-ritoneal test[2]	Infant mouse test[3]	Cell assays		Haemagglu-tination[4] MRHA			MSHA			Hydrophobicity[5]			PF[6]
						Vero	HeLa	CFA	BA	M-Is	CFA	BA	M-Is	CFA	BA	M-Is	CFA
D-M3219-54	O18ac:H7	+	0/4	0/5	0.070	-	-	IVa	IVa	V	-	+	+	>2.0	>2.0	>2.0	+
CDC 2292-55	O20:H26	-	0/4	3/5	0.066	-	-	-	-	-	+	+	+	>2.0	>2.0	>2.0	-
F41	O26:H-	+	0/4	1/5	0.076	-	-	-	-	-	-	-	-	>2.0	>2.0	>2.0	-
5306-56	O26:H46	-	0/4	0/5	0.068	-	-	-	-	IVb	-	-	-	>2.0	>2.0	>2.0	-
KATTWIJK	O28ac:H-	-	0/4	1/5	0.066	-	-	-	-	-	-	-	-	>2.0	> 2.0	>2.0	-
H 702c	O44:H18	+	0/4	0/5	0.072	-	-	IVa	IVa	IVa	-	-	-	>2.0	>2.0	>2.0	+
SU3912-41	O55:H-	-	4/4 (N)	4/5	0.068	CNF2	CNF2	-	-	-	+	+	+	AA	1.4	2.0	-
ABERDEEN 1064	O55:H6	-	0/4	0/5	0.074	-	-	-	-	-	-	-	-	2.0	>2.0	>2.0	-
E990	O86:H-	-	0/4	0/5	0.070	-	-	-	-	IVb	+	+	+	>2.0	>2.0	>2.0	-
BP 12665	O86:H34	-	0/4	0/5	0.067	-	-	-	-	-	+	+	+	>2.0	2.0	>2.0	-
STOKE W	O111:H-	-	0/4	0/5	0.062	-	-	-	IVb	IVb	+	+	+	>2.0	>2.0	>2.0	-
E2808	O114:H2	-	0/4	0/5	0.065	-	-	-	-	-	+	+	+	>2.0	>2.0	>2.0	-
26W	O114:H32	-	0/4	0/5	0.073	-	-	-	-	-	+	+	+	0.1	2.0	0.4	-
C881-62	O119:H-	-	0/4	1/5	0.067	-	-	-	-	-	+	+	+	>2.0	>2.0	2.0	-
34W	O119:H27	+	0/4	0/5	0.069	VT	-	-	-	-	+	+	+	>2.0	>2.0	>2.0	-
EW227	O124:H30	-	0/4	0/5	0.070	-	-	-	-	-	-	-	-	>2.0	>2.0	AA	-
CANIONI	O125ab:H19	-	0/4	1/5	0.071	-	-	-	-	-	+	+	+	1.0	2.0	1.0	-
EW2129-54	O125ac:H6	-	0/4	0/5	0.064	-	-	-	-	-	+	+	+	0.4	2.0	0.4	-
E611	O126:H2	-	0/4	0/5	0.070	-	-	-	-	-	+	+	+	>2.0	>2.0	>2.0	-
4932-53	O127a:H-	-	0/4	0/5	0.071	-	-	-	VI	VI	+	+	+	>2.0	>2.0	>2.0	-
CIGLERIS	O128:H2	-	0/4	0/5	0.062	-	-	-	-	-	+	+	+	>2.0	>2.0	>2.0	-
C771	O142:H6	-	0/4	3/5	0.071	-	-	-	VI	VI	+	+	+	>2.0	>2.0	>2.0	-
E1020	O158:H23	-	0/4	0/5	0.069	-	-	-	-	-	+	+	+	1.4	1.0	2.0	-

[1] Number of rabbits positive / number of rabbits injected. N = necrosis

[2] Number of mice that died during a 7-day period of observation / number of mice inoculated

[3] Coefficients upper 0.100 are indicative of enterotoxigenicity by heat-stable enterotoxin (STa)

[4] Mannose-resistant (MRHA) and mannose-sensitive (MSHA) haemagglutination expressed bacteria grown on colonization factor agar (CFA), blood agar (BA) and Minca-Is (M-Is) solid media. Strains were grouped according to their MRHA patters in six MRHA types (I-VI) of our classification [17]

[5] Minimal molar ammonium sulphate concentration in which bacteria aggregated in the improved salt aggregation test (ISAT)

[6] P fimbriated strains grown on CFA agar

Discussion

Recent studies from several developing countries have shown enteropathogenic *E. coli* (EPEC) to be either the first or second most important bacterial cause of diarrhoea in children [13, 20]. However, the mechanism by which EPEC cause disease remains to be defined.

EPEC strains do not usually produce LT or STa enterotoxins and are not enteroinvasives [1–3]. This finding raised doubts in some authors about the pathogenicity of EPEC [4, 5]. Confirmation of the enteropathogenicity of EPEC led to an intensification of the search for new toxins produced by these bacteria. In 1977 Konowalchuk et al. [21] reported that some EPEC strains produced a substance that was cytotoxic for Vero cells. Although some verotoxigenic *E. coli* (VTEC) isolates belong to classic EPEC O:H serotypes (e.g., O26: H11 or H–, O55: H6, O111: H2, H8 or H– and O128: H2, H12 of H–), only a minority of EPEC strains produce verotoxins (VT) [9]. In 1978, Klipstein et al. [22] tested EPEC LT^- STa^- and reported that the bacteria synthesized products which induced net fluid secretion in a rat jejunum perfusion model. A year later, Kétyi et al. [23] described an altered LT, termed LT' produced by an EPEC strain of serogroup O55. The LT' was reported to cause CHO cell elongation without cytotoxicity and crude LT' showed some skin necrotic activity. Recently, Johnson et al. [24] detected a cytolethal distending toxin (CLDT) active in CHO, Vero, HeLa and HEp–2 cells in EPEC of serogroups O44, O55, O86, O111, O114, O119, O126, O127 and O128. This toxin did not show necrosis in rabbit skin and did not cause fluid secretion in the rabbit ileal loop assay. In our study, none of the 23 reference strains belonging to classic enteropathogenic serogroups were positive for production of LT and STa enterotoxins or necrotic factor CNF1. But, one strain of serotype O119: H27 was VT^+ and another of serotype O55: H– produced CNF2.

We have recently described that necrotizing *E. coli* (NTEC) strains produce two cytotoxic necrotizing factors (CNF1 and CNF2) [15]. These two toxins cause enlargement and multinucleation of Vero and HeLa cells and necrosis in rabbit skin. However, only CNF2 induces necrosis in the mouse footpad and moderate fluid accumulation in the rabbit ileal loop test [15]. CNF1 and CNF2 are heat-labile proteins of 115 and 110 kilodaltons, respectively [25, 26]. Whereas CNF2 is encoded by transmissible plasmids, the genes encoding CNF1 production appear to be chromosomally inherited [27]. Both necrotic toxins are cell associated products easy to detect when bacteria are sonicated or when they are grown in the presence of mitomycin C [14]. NTEC were associated with human extraintestinal infections [17, 28, 29], and isolated from calves with diarrhoea or septicaemia [15, 16]. Furthermore, it has also been suggested that NTEC might be opportunistic pathogens causing diarrhoeal diseases in humans [30, 31]. NTEC from human origin usually produce

CNF1, whereas bovine NTEC generally synthesize CNF2. Thus, 266 (99%) of 269 human NTEC strains isolated in Spain between 1979 and 1991 were CNF1 positive. In contrast, 123 (97%) of 127 bovine NTEC produced CNF2 [32]. Although bovine NTEC producing CNF2 belonged to 28 different serogroups, only six of them (O1, O3, O15, O55, O88 and O123) account for 60% of strains [32]. Bovine *E. coli* producing CNF2 of serogroup O55 belonged to serotypes O55: H4, O55: H21 and O55: H− [33]. In this study, the one strain that produced CNF2 was of serotype O55: H−. This is of interest, since little information exists on the pathogenic mechanisms of classic enteropathogenic *E. coli* strains. Nevertheless, further studies are necessary to establish the role of CNF2 in human diarrhoea. We think that the LT' toxin with necrotic activity detected by Kétyi et al. [23] in an EPEC O55 could to be really CNF2.

The adherence off EPEC to the small bowel mucose is an important step in the pathogenesis of diarrhoeal diseases. In our study 7 (30%) of 23 *E. coli* strains belonging to enteropathogenic serogroups showed mannose-resistant haemagglutination (MRHA) with some of the seven types of erythrocytes species. Bearing in mind the MRHA types expressed by strains cultured on CFA agar, Blood agar and Minca-Is medium, we conclude that the composition of the growth medium has an important influence on MRHA expression as well as on erythrocyte types agglutinated. Curiously two strains of serotypes O18ac: H7 and O44: H18 that showed MRHA type IVa expressed P fimbriae. It has been reported that the virulence of *E. coli* causing pyelonephritis is mediated by P-fimbriae [17]. All 12 bacteraemic *E. coli* strains of MRHA type IVa detected in a previous study were also P fimbriated [17]. It would be interesting to study the patterns of adherence to HeLa or HEp−2 cells of the 23 *E. coli* strains investigated in this study, and establish the relation of their MRHA types with the mannose-resistant haemagglutinins recently detected in EPEC strains of serotypes O44: H18 [12], O86 [34], O111: H12 [35], O111: H21 [12] and O126: H27 [12]. In contrast to Wadström et al. [36], we found that EPEC strains did not possess an elevated level of cell surface hydrophobicity. Taking in mind that many of the *E. coli* strains investigated in this study have been isolated several years ago and storaged at the laboratory, it is feasible that some of their virulence factors have been lost during this time.

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A SIMPLE IDENTIFICATION SYSTEM FOR SLOWLY GROWING MYCOBACTERIA II. IDENTIFICATION OF 25 STRAINS ISOLATED FROM SURFACE WATER IN VALENCIA (SPAIN)

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Based on a twenty-year experience, a simple identification system for slowly growing mycobacteria has been presented for clinical laboratories not specialized in this work. With this system (12 tests: tolerance to 0.02% picric acid, colony pigmentation in the dark, nitrate reduction, resistance to ethambutol, tween hydrolysis at 7 and 14 days, resistance to hydroxylamine, PAS degradation, tolerance to p-nitrobenzoic acid, production of nicotinic acid and colony morphology) we have identified 15 strains of *Mycobacterium gordonae* and 10 of *Mycobacterium avium-intracellulare* complex, isolated from surface water in Valencia, Spain.

Within the genus *Mycobacterium*, the importance of slowly growing mycobacteria has greatly increased in recent years for new species with clinical significance [1–4]. The identification of slowly growing mycobacteria presents a considerable difficulty, since the organisms show negative matches in many biochemical tests which are useful for differentiating between other microorganisms. In contrast to time consuming and laborious biochemical, cultural and immunological methods, based on our 20 years experience of our laboratory, a simple identification system for slowly growing mycobacteria is presented for clinical laboratories which are not specialized for this work. This system consists of 12 tests, including the niacin test, to start the process of identification to distinguish between the tubercle bacilli (*M. tuberculosis* and *M. bovis*) and other mycobacteria.

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Materials and methods

Bacterial strains. A total of 25 strains of slowly growing mycobacteria were identified. They were isolated in this laboratory from a variety of surface waters of Valencia, Spain.

Test and media. The tests and media used are listed in Table I. Ogawa egg medium consisted of: basic solution (1% sodium glutamate and 1% KH_2PO_4), 100 ml; whole eggs, 200 ml; glycerol, 6 ml; 2% solution of malachite green, 6 ml; the medium was poured in 8 ml quantities into tubes, 17 mm by 170 mm, and made as slopes by heating at 90 °C for 60 min. Ethambutol, hydroxylamine monochlorhydrate, sodium p-aminosalicylate and p-nitrobenzoic acid were added to the medium before heating.

Inoculation of media. A bacterial suspension (10 mg wet weight per ml) was prepared from a 14–21 days old Ogawa egg medium slant culture. One loopful sample (0.01 ml) of the suspension was inoculated onto each medium, and the media were stoppered by rubber cap with a 3 cm cut-line in its bottom and incubated at 37 °C exposed to light (a 40 W tungsten bulb, 30 cm). An Ogawa egg medium was incubated in the dark (control).

Reading of results. After incubation for 14 days, the growth on the control Ogawa egg medium incubated in the dark, was observed. If abundant membraneous growth occurred after 14 days, the results of all tests were read after incubation for 21 days. If no growth of only scanty growth occurred after 14 days, the results of all tests were read after incubation for 21 to 28 days. Ogawa egg medium with p-nitrobenzoic acid (PNB) was read after 21 days of incubation. Niacin test and nitrate reduction were read after 24 h, Tween hydrolysis after 7 and 14 days. Growth on PNB, HA, ETH and on PAS medium similar to the growth on the control medium was considered as "positive".

Results and discussion

The simple identification system for slowly growing mycobacteria (or differentiation of *M. tuberculosis* and *M. bovis* from other mycobacteria) has an increasing importance, as report on infection with these mycobacteria are continuously increasing (*M. shimoidei*, *M. farcinogenes, M. malmoense*, etc). The results of the test are show in Table II; all results obtained with this identification system were read after incubation at 37 °C for 21 days. From these data, an identification table was constructed (Table III).

All 25 strains isolated from surface water, 15 strains of *M. gordonae* and 10 strains of *M. avium-intracellulare* complex could be identified by using this system (Table IV). All strains of *M. gordonae* presented colony pigmentation in the dark while *M. avium-intracellulare* complex failed to do so.

Table I

Tests and media for the simple identification system

Tests	Media
1. Tolerance to 0.02% picric acid	A modified Sauton agar medium containing 0.02% of picric acid [5]
2. Colony pigmentation in the dark	Ogawa egg medium (incubated in the dark) [6]
3. Photochromogenicity	Ogawa egg medium
4. Nitrate reduction (24 h)	Basal medium with $NaNO_3$ and Erlich's aldehyde reagent [7]
5. Resistance to ethambutol	Ogawa egg medium containing ethambutol (5 μg/ml) [8]
6. Tween hydrolysis (7 days)	Basal medium with Tween 80 (0.5%) and neutral red as indicator [9]
7. Tween hydrolysis (14 days)	Basal medium with Tween 80 (0.5%) and neutral red as indicator [9]
8. Resistance to hydroxylamine	Ogawa egg medium containing hydroxylamine monoclorhydrate (0.5 mg/ml) [10]
9. PAS degradation	Ogawa egg medium containing sodium p-aminosalicylate [11]
10. Tolerance to 0.5 mg of p-nitrobenzoic acid per ml	Ogawa egg medium containing 0.5 mg/ml of of p-nitrobenzoic acid [12]
11. Niacin (production of nicotinic acid)	CN Br at 10% [13]
12. Colony morphology (S and R)	Observation in Ogawa egg medium

Table II

Characters useful for differentiating between slowly growing mycobacteria (number of strains showing positive reaction) [1, 2]

	Number of strains	Picric acid tolerance	Colony pigmentation	Photochromogenicity	Nitrate reduction	Resistance to ethambutol	Tween hydrolysis (7 days)	Tween hydrolysis (14 days)	Resistance to hydroxylamine	PAS degradation	PNB tolerance	Niacin test	Colony morphology
M. tuberculosis	125	0	0	0	125	125	0	0	0	0	0	111	R
M. bovis	100	0	0	37	0	0	0	0	0	0	0	9	S
M. microti	5	0	0	0	5	0	0	0	0	0	0	5	S
M. africanum	12	0	0	0	0	0	0	0	0	0	0	9	R
M. ulcerans	5	0	0	0	0	5	0	0	0	5	0	5	R
M. simiae	4	4	0	4	0	4	0	0	4	0	4	4	S
M. kansasi	16	0	0	15	16	4	13	15	0	0	15	0	R
M. marinum	18	0	0	18	3	16	4	9	17	0	16	0	S
M. asiaticum	6	0	0	6	0	6	0	0	6	0	6	0	S
M. szulgai	5	0	5	0	5	0	0	2	5	0	5	0	R
M. gordonae	30	0	30	0	0	0	24	29	15	0	30	0	S
M. scrofulaceum	12	0	12	0	0	12	0	1	10	0	12	0	S
M. xenopi	4	0	3	0	0	4	0	0	0	0	4	0	S
M. farcinogenes	3	0	3	0	3	3	0	0	0	3	3	0	R
M. triviale	11	0	0	0	11	0	11	11	10	0	11	0	R
M. avium -intracellulare complex	40	0	0	0	0	40	0	0	39	0	40	0	S
M. shimoidei	4	0	0	0	0	0	0	4	0	0	4	0	R
M. nonchromo-genicum-terrae complex	37	0	0	0	11	0	37	37	37	0	37	0	S
M. gastri/ /M. novum	15	0	0	0	8	0	14	15	15	0	15	0	S
M. haemophilum	3	0	0	0	0	0	3	3	0	0	3	0	S
M. malmoense	3	0	0	0	0	3	0	0	0	0	3	0	S

(1) All results were read after incubation at 37 °C for 21 days; (2) The strains used were partly received from culture collections (see Acknowledgements), partly isolated in this laboratory from a variety of sources (surface water, soil, urine and sputum)

Table III

Identification table for slowly growing mycobacteria

	Picric acid tolerance	Colony pigmentation	Photochromogenicity	Nitrate reduction	Resistance to ethambutol	Tween hydrolysis (7 days)	Tween hydrolysis (14 days)	Resistance to hydroxylamine	PAS degradation	PNB tolerance	Niacin test	Colony morphology
M. tuberculosis	−	−	−	+	+	−	−	−	−	−	+ −	R
M. bovis	−	−	−	− +	−	−	−	−	−	−	− +	S
M. microti	−	−	−	+	−	−	−	−	−	−	+	S
M. africanum	−	−	−	−	−	−	−	−	−	−	+ −	R
M. ulcerans	−	−	−	−	+	−	−	−	+	−	+	R
M. simiae	+	−	+	−	+	−	−	+	−	+	+	S
M. kansasi	−	−	+ −	+	− +	+ −	+ −	−	−	+ −	−	R
M. marinum	−	−	+	− +	+ −	− +	− +	+ −	−	+ −	−	S
M. asiaticum	−	−	+	−	+	−	−	+	−	+	−	S
M. szulgai	−	+	−	+	−	−	+ −	+	−	+	−	R
M. gordonae	−	+	−	−	−	+ −	+ −	+ −	−	+	−	S
M. scrofulaceum	−	+	−	−	+	−	− +	+ −	−	+	−	S
M. xenopi	−	+ −	−	−	+	−	−	−	−	+	−	S
M. farcinogenes	−	+	−	+	+	−	−	−	+	+	−	R
M. triviale	−	−	−	+	−	+	+	+ −	−	+	−	R
M. avium -intracellulare complex	−	−	−	−	+	−	−	+ −	−	+	−	S
M. shimoidei	−	−	−	−	−	−	+	−	−	+	−	R
M. nonchromo-genicum-terrae complex	−	−	−	− +	−	+	+	+	−	+	−	S
M. gastri/M. novum	−	−	−	+ −	−	+ −	+	+	−	+	−	S
M. haemophilum	−	−	−	−	−	+	+	−	−	+	−	S
M. malmoense	−	−	−	−	+	−	−	−	−	+	−	S

+ 95 − 100% positive; + − more than 50% positive; − + more than 50% negative; − 95 − 100% negative

Table IV

Results obtained with the identification of 15 strains of M. gordonae and 10 strains of M. avium-intracellulare complex

	M. gordonae No. of strains		*M. avium-intracellulare* No. of strains	
	+	-	+	-
Picric acid tolerance	0	15	0	10
Colony pigmentation in the dark	15	0	0	10
Photochromogenicity	0	15	0	10
Nitrate reduction (24 hours)	0	15	0	10
Resistance to ethambutol	0	15	10	0
Tween hydrolysis (7 days)	12	3	0	10
Tween hydrolysis (14 days)	15	0	0	10
Resistance to hydroxylamine	9	6	10	0
PAS degradation	0	15	0	10
PNB tolerance	15	0	10	0
Niacin test	0	15	0	10
Colony morphology	15S		10S	

The main differential characters are as follows:

- The growth on PNB medium clearly differentiates slowly growing mycobacteria from *M. tuberculosis* and *M. bovis*. Nitrate reduction and nicotinic acid production differentiates *M. tuberculosis* and *M. bovis*.

(A) *Photochromogenic species*

- Within photochromogenic species (*M. kansasi, M. marimun, M. simiae* and *M. asiaticum*) only *M. simiae* grows on Sauton agar with 0.2% picric acid; *M. kansasi* is the only of the four species that reduces nitrate; *M. marinum* differs from *M. asiaticum* in tween hydrolysis.

(B) *Scotochromogenic species*

- Among scotochromogenic species (*M. scrofulaceum, M. gordonae, M. szulgai, M. xenopi* and *M. farcinogenes*) only *M. szulgai* reduces nitrate to nitrite; *M. gordonae* is the only species that hydrolyzes tween; *M. scrofulaceum* differs from *M. xenopi* in the resistance to hydroxylamine; *M. farcinogenes* is the only species with PAS degradation.

(C) *Nonphotochromogenic species*

- Among nonphotochromogens (*M. triviale, M. avium-intracellulare* complex, *M. shimoidei, M. nonchromogenicum-terrae* complex, *M. gastri/M novum, M. haemophilum* and *M. malmoense*) differs in nitrate reduction, resistance to ethambutol, tween hydrolysis, resistance to hydroxylamine and colony morphology (smooth and rough). *M. triviale* is the only species that reduces nitrate nitrite. *M. avium-intracellulare* complex and *M. malnoense* differs in the resistance to

ethambutol. *M. nonchromogenicum-terrae* complex differs from *M. gastri* in the resistance to hydroxylamine. *M. shimoidei* produces rough colonies on Ogawa egg medium. Only *M. haemophilum* hydrolyses tween.

This system offers the three factors required for identification: accuracy, rapidity and simplicity.

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EFFECT ON INHIBITORS OF GLYCOPROTEIN SYNTHESIS (SWAINSONINE, 1-DEOXYNOJIRIMYCIN) ON HORMONAL IMPRINTING AND LECTIN BINDING IN *TETRAHYMENA PYRIFORMIS*

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Glycoprotein synthesis inhibitors (swainsonine = SW and 1-deoxynojirimycin = DNJ) influenced the insulin binding, insulin provoked hormonal imprinting and lectin binding of *Tetrahymena*. Insulin binding was increased and lectin binding decreased by both of them immediately after treatment, however, SW decreased insulin binding and both of them increased lectin binding after 24 h. SW inhibited, DNJ allowed the development of insulin imprinting. This means that the disturbance of glycosylation in general does not influence, but the blocking of mannosidase II disturbs the process of imprinting.

For the free living unicellular organisms it is essential to react properly to the environmental changes. During evolution of unicellulars, the clones which had the capacity of an adequate reception or had the adequate response to the signals possessed an advantage in selection over those cells which had not so sensible responsiveness. It may be assumed that the readiness of response to the changes of the environment is based on genetically coded informations. These informations could be transmitted from generation to generation. In our department we use this response to investigate the effects of imprinting evoked by new, biologically active substrates appearing in the environment [1].

The hormonal imprinting takes place at the first encounter of the target cells and the hormone. If the first encounter happens during the ontogenetical development of multicellular organisms (e.g. mammalians) we speak about the ontogenetical aspects of the hormonal imprinting [2]. There is also the possibility to form hormonal imprinting following the encounter with the hormone in unicellular organisms [3]. In the latter case we can speak about the phylogenetical aspects of hormonal imprinting.

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There are several ways to follow the development of hormonal imprinting. It is possible to measure the changed – generally increased – binding of the hormone [4]; the alteration of the intensity of physiological reactions induced by the hormone [5] etc. These alterations are present following more progeny generations; e.g. after several hundreds of generations there are also significant differences between the untreated and imprinted cells of *Tetrahymena pyriformis* [6].

The fact of presence of hormonal imprinting is demonstrated in wide range, but its molecular mechanism is not clarified completely. It is obvious that in the development of imprinting those biochemical steps play definitive role which participate in the binding itself and in the induced reactions of response of hormone. Among others the physiological state of the membrane [3]; the integrity and undisturbed machinery of second messenger system [7]; the normal work of protein syntheseis and lysosomal activity are fundamental conditions of development of hormonal imprinting.

Glycoproteins have a very important role in the normal function of the cell membrane. Structures of several hormone-receptors have a glycoprotein character. In our previous works we examined the effect of lysosomotrop substances (e.g. bacitracin, chloroquine) on the hormonal imprinting [8]. According to these results the hormonal imprinting of insulin does not develop in the presence of the mentioned materials. There is the possibility that this is due to the fact that the altered signalization of oligosaccharides could form structurally altered glycoproteins. This change could result in an altered function of the glycoprotein or these molecules will be transferred to a different compartment following the sorting of Golgi complex because of the altered signals. It is possible that by the disturbance of signalization the enzyme secretion and activity are also affected as the lysosomal enzymes are also glycoproteins. This effect can also influence the development of hormonal imprinting. To have an exact exposure of this problem we set up the next experiments using two enzyme inhibitors.

(i) Swainsonine (SW), an indolizidine alkaloid of *Swainsona canescens*, is a potent inhibitor of the lysosomal mannosidase and able to inhibit totally the activity of Golgi mannosidase II which is responsible for the -3,6- mannosidase action during the processing in the Golgi complex [9].

(ii) 1-Deoxynojirimycin (DNJ) (1,5-dideoxy-1,5-imino-D-glucitol) can inhibit the activity of Glc3- (glucosidase I) and Glc1/Glc2- (glucosidase II) during the processing of glycoproteins [10].

Both substances are able to influence the oligosaccharide-processing at the points of the glycoprotein synthesis, function, signalization and transport.

Materials and methods

Determination of FITC-labelled insulin- and lectin-binding. Populations of *T. pyriformis* GL strains in the logarithmic phase of growth were cultured in 0.1% yeast extract broth containing, 1% Bacto Tryptone (Difco, Michigan, USA) at 28 °C.

The hormonal imprinting was developed by 1 h treatment of 10^{-6} *M* insulin (Insulin Semilente MC, Novo, Copenhagen, Denmark). Following the treatments the cells were washed with and transferred into fresh medium and they were cultured for a consecutive 24 h. After this the cells were fixed in 4% neutral formaldehyde (diluted in PBS pH 7.2) for 5 min. The cells were washed with PBS twice. The cells were then incubated with FITC-insulin at room temperature for 1 h. The FITC (fluorescein isothiocyanate, Isomer I, BDH, Poole, England) insulin molar ratio was 0.42, the protein content was 0.05 mg/ml. Following the incubation the cells were washed three times with PBS, than placed onto slides of glass. The intensity of fluorescence was measured by a Zeiss Fluoval cytofluorimeter. The analogous signals of the instrument were transformed by a digital processor and these signals were registered with a Hewlett Packard HP 41 CX calculator. We analysed the mean values of groups, the standard deviation and significance of inter-gruop differences using Student's *t*-test by the help of the mathematical-programs of the calculator. In each group 20 cells were assayed. All the experiments were repeated three times, this way the results represent mean values of 60 – 60 cells.

Insulin imprinting was tested in the presence of (a) 10 μM swainsonine (SW, Boehringer, Austria), and (b) 2 mM 1-deoxynojirimycin (DNJ, Boehringer, Austria). Control groups were treated by (c) only SW, and (d) only DNJ. The FITC-insulin binding was measured not only on the subsequent day of the treatment but also immediately after the treatments.

In the above-mentioned groups we determined the binding of three lectins with different sugar specificity, immediately after the treatments and 24 h later. The applied lectins were: (i) Concanavalin A (Con A) (Serva, Heidelberg, Germany), sugar specificity: alpha-*D*-Man; alpha-*D*-Glc. FITC/protein: 1.4; (ii) *Helix pomatia* (purified in our laboratory as described in [11]; sugar specificity: *D*-GalNac). FITC/protein: 1.55. (iii) *Pisum sativum* (purified in our laboratory according to [12], sugar specificity: *D*-Man). FITC/protein: 1.77.

Lysosomal enzymes. The activity of acid phosphatase, glucosidase and glucosaminidase was determined by substrates containing methylumbelliferyl according to Suzuki's [13] method (slightly modified).

Enzyme activity was determined for control populations, 2 h 10 μM SW- or 2 mM DNJ-treated populations immediately after the treatment and 24 h later. At these time points the cells were harvested from the medium by centrifugation and the enzyme activity was determined in the cell-free medium and in the cells alike. First the cells were frozen at −40 °C and after melting they were sonicated. The protein concentration of the homogeneity was measured by spectrophotometer at 280 nm. The values of enzyme-activity are referred to "mg" of protein.

The substrates (Koch Light Laboratory Ltd. Colnbrook, England) were applied in 5 mM concentration: (a) 4- methyl-umbelliferyl for phosphate-acid phosphatase; (b) 4-methyl-umbelliferyl-beta-*D*-glucopyranosid for glucosidase; (c) 4-methylumbelliferyl-2-acetamido-2-deoxy-beta-*D*-glucopyranosid for glucosaminidase.

The methylumbelliferon liberated by the enzymes was determined by a Cliniflour 85 – M fluorimeter (Izinta, Budapest) with 365 nm excitation and 448 nm emission filters. One unit corresponded to the amount of enzyme which liberated 10^{-4} mg methylumbelliferon from the substrate, in pH 4.5, 0.2 M citrate buffer, at 28 °C in 30 min.

Results

The one hour treatment of insulin decreased the immediately measured FITC-insulin binding ($p < 0.05$ referred to control group); one day later there was a significant increase ($p < 0.01$) (Fig. 1).

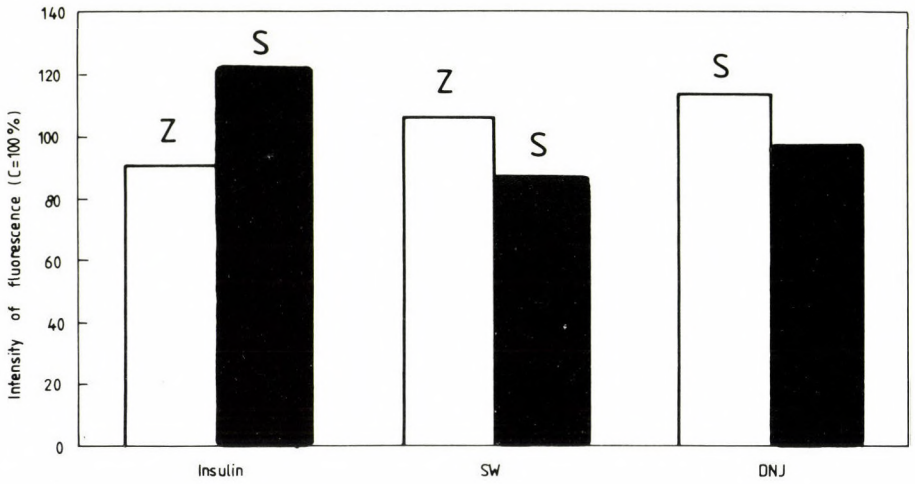

Fig. 1. Effect of insulin, SW or DNJ on FITC-insulin binding immediately (open columns) and 24 h after treatment (solid columns); S = $p < 0.01$; Z = $p < 0.05$ in each figure

Following the treatment both the SW ($p < 0.05$) and DNJ ($p < 0.01$) resulted an increased insulin binding; while one day later there was a considerable decrease ($p < 0.01$) of FITC-insulin binding capacity of the SW treated groups. After 24 h the effect of the DNJ treatment became almost undetectable (Fig. 1).

Immediately after the treatment, both SW and DNJ decreased the binding of the three lectins compared to the control group ($p < 0.01$ in each case except one SW treated group, where the binding difference of *Pisum* lectin was not significant (Fig. 2). One day later – except the binding of *Pisum* lectin of DNJ treated groups – the quantity of binding showed a significant increase ($p < 0.01$; SW/Pisum $p < 0.05$ (Fig. 2)).

On the following day of treatment the insulin imprinting manifested in a significant increase of FITC-insulin binding ($p < 0.01$); and there was a significant increase of Con A binding ($p < 0.01$) in the imprinted groups. The binding of *Helix* lectin did not present change, while the binding of *Pisum* lectin, that is similar in sugar specificity, also showed an increase but it was not so definite ($p < 0.05$) as the Con A group (Fig. 3).

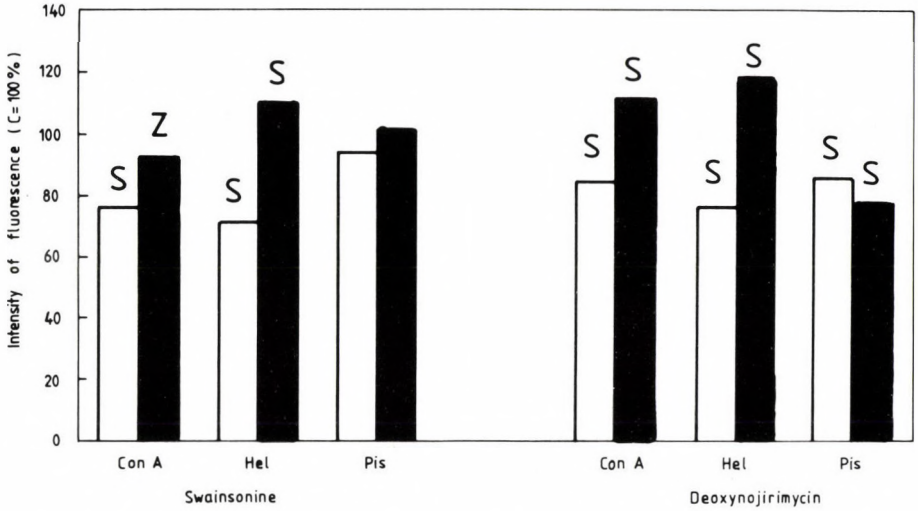

Fig. 2. FITC-lectin binding immediately (open columns) or 24 h after SW or DNJ treatment (solid columns); Con A = concanavalin A, Hel = *Helix pomatia*, Pis = *Pisum sativum*, C = control

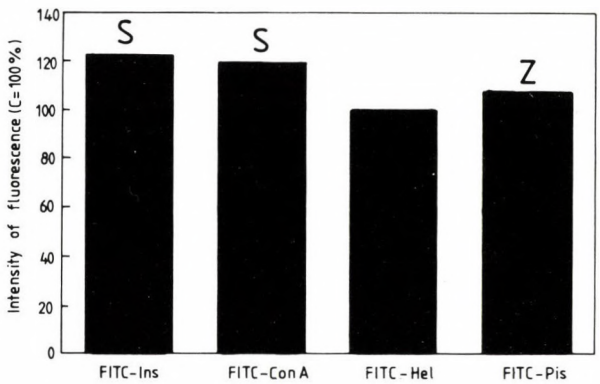

Fig. 3. FITC lectin and insulin binding 24 h after DNJ treatment

In addition, SW not only decreased the FITC-insulin binding on the following day, but inhibited the development of imprinting, too. On the next day DNJ was practically ineffective on the binding of FITC-insulin, but did not inhibit the development of insulin imprinting (Fig. 4).

Neither the SW nor the DNJ changed the amount of the secreted phosphatase (Fig. 5a) and glucosaminidase (Fig. 5c) in a significant manner following the treatment immediately or passing one day. Just after the treatment the amount of the

glucosidase secreted to the medium was diminished by the 2 h DNJ treatment. The SW was ineffective at this point. But one day later we could measure increased enzyme activity in the medium of the SW treated cells ($p < 0.05$); in the medium of the DNJ treated cell-populations the enzyme activity was identical to the control (Fig. 5b).

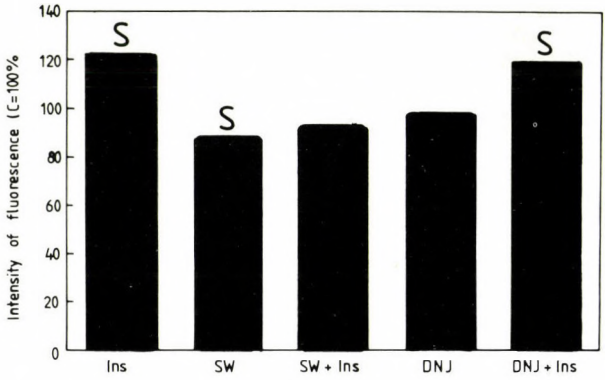

Fig. 4. Effect of insulin, SW, DNJ or combined treatments to FITC-insulin binding 24 h after treatment

The enzyme activity of the three enzymes of the cells was decreased significantly by the SW treatment, and one day later there was a considerable increase compared to the control cultures (Fig. 6a, b, c). The DNJ treatment also resulted a strong decrease of enzyme activity, but it was not significant in the case of glucosaminidase. One day after this activity remained under the control level.

Discussion

In the development of the insulin induced hormonal imprinting of *Tetrahymena* there is an increase of insulin-binding capacity of the cell membrane. There are also alterations in the activity and quantity of coupled second messengers following the binding of insulin molecules [4, 7]. Changes in the intensity of physiological responses which are stimulated by the systems of imprinting, had been observed, too. These symptoms refer to that the development of imprinting means really an amplification of the functionally active binding sites of insulin.

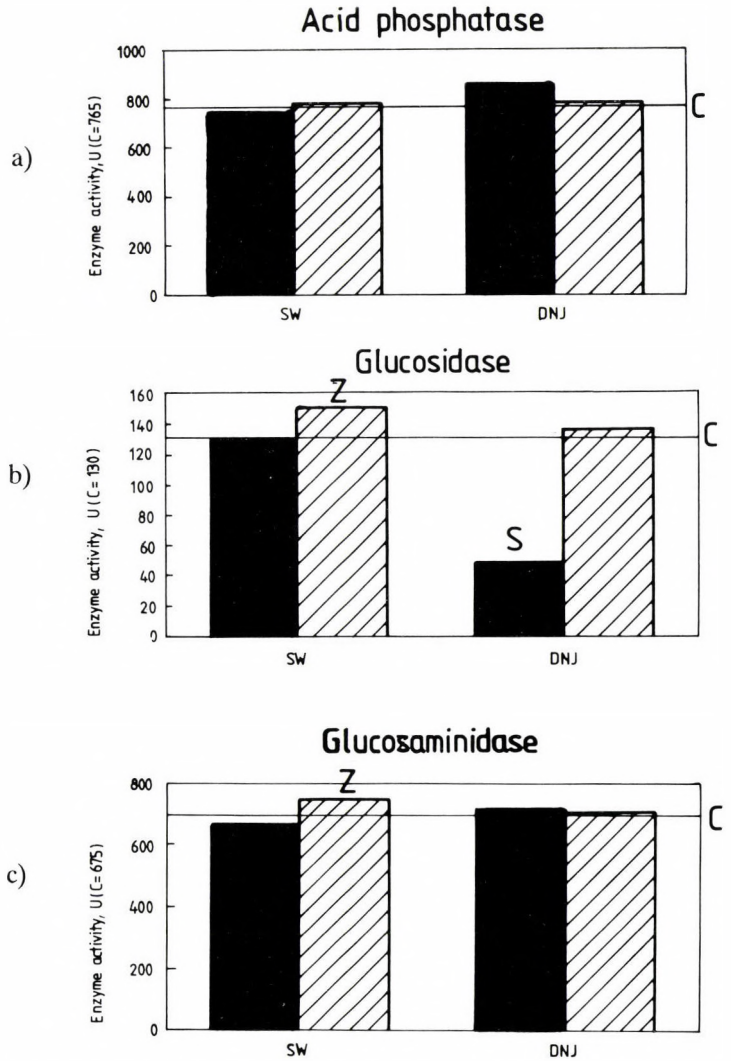

Fig. 5. Effect of SW or DNJ on the secreted enzyme quantity immediately (solid columns) or 24 h after treatment (shaded columns). *a* = phosphatase, *b* = glucosidase, *c* = glucosaminidase

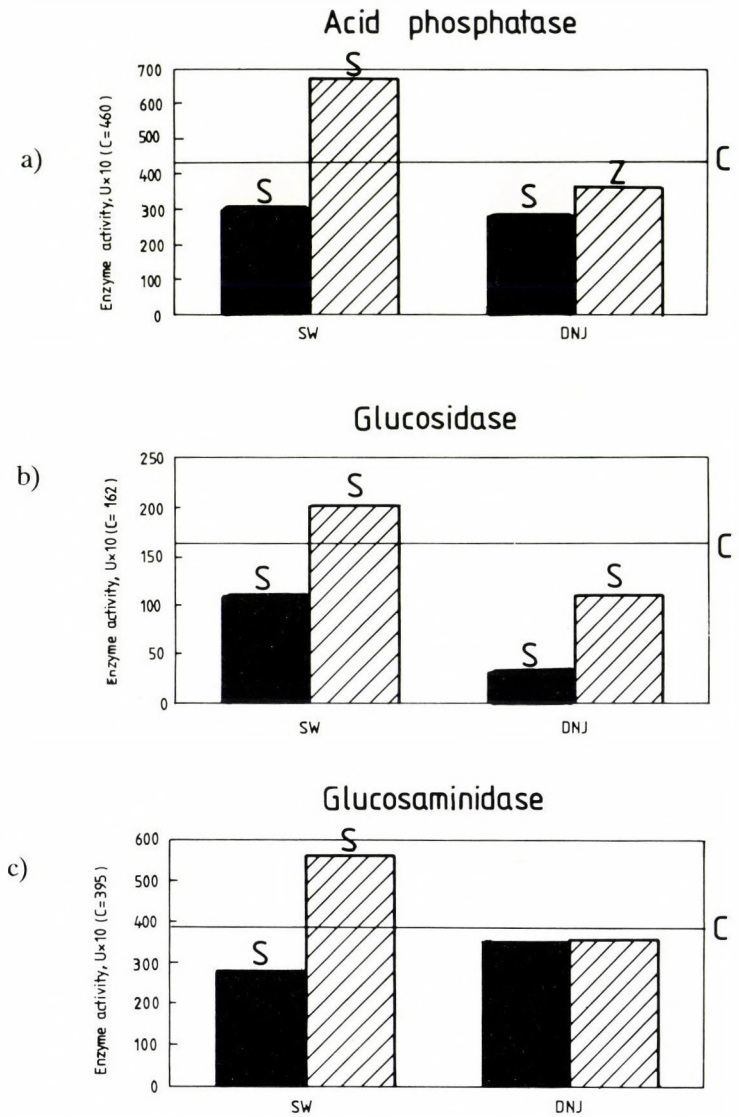

Fig. 6. Effect of SW or DNJ on the cells' enzyme content immediately (solid columns) or 24 h after treatment (open columns). *a* = phosphatase, *b* = glucosidase, *c* = glucosaminidase

The development of imprinting can be inhibited at several levels. This way it is possible to inhibit by alteration the membrane composition, fluidity and saccharide components [14, 15], by the modification of the functions of second messenger system, by inhibition of the lysosomal activity and degradation of proteins [8], etc.

It may be supposed that in *Tetrahymena* similar receptor-level alterations (e.g. down regulation) take place in the membrane as e.g. in mammalian cells. This way, following the receptor-ligand binding, the complex may arrive to the endosomal compartment. There the ligand will dissociate from the receptor and after this the receptor will be transferred to the intracellular receptor pool or to the surface membrane. The third way is the degradation of the receptor. The harmonious course of these processes could be the proviso of the normal function of surface membrane and this way proviso of the hormonal imprinting, too, which is established by our previous experiments [1, 3, 14]. The degradation of the ligand has also role in the later processes. For example several effects of insulin are assigned to the fragments of degradation developed during this process [16].

These presumed facts are supported by our previous works where in the presence of inhibitors (like insulin- and protein-degradation in general) and lysosomal enzyme activity (as bacitracin) we could not develop imprinting [8]. This antibiotic interferes also with the processes of the glycosilation. This way it is possible that beside the above-mentioned effects it can express its inhibitor effect on the imprinting through disturbance of glycoprotein formation. Either in the presence of the lysosomotrop chloroquine it is impossible to develop imprinting. This fact supports our assumption that the inhibition of lysosomal activity can resent failures in those systems which are responsible for the imprinting. Moreover, chloroquine is able to influence the function of the ion channels of *Tetrahymena* membrane which will result in an altered membrane potential. This may be responsible for the troubles of membrane function and the disturbed imprinting alike.

The glycoproteins of the cell membrane as markers, binding sites and receptors fulfill a very important role in the completion of the connection between the cells and their environment. In unicellulars these processes of recognitions are essential, by the help of them the cells can choose the adequate response to the new substances just appearing in the environment (e.g. chemotaxis). As the lifespan of the unicellular organism is short, the imprinting helps to conserve the signal recognition. Considering the relatively short individual life of a unicellular organism the repeated recognition of new materials is easier, at the same time the imprinting transfers to progeny generations a lasting "memory". As the binding sites formed by imprinting are presumed to be glycoproteins it was essential to investigate the role of the synthesis and processing of these complexes in the development of hormonal imprinting.

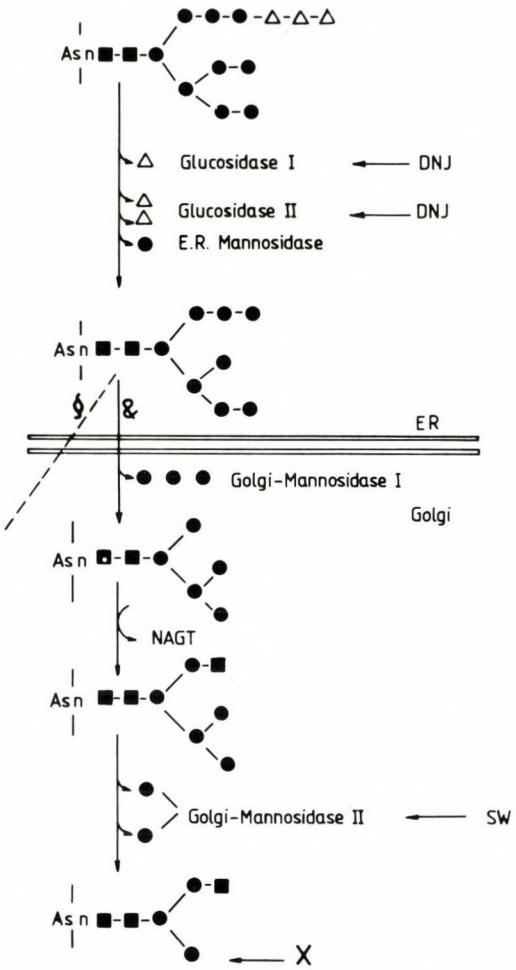

Fig. 7. The oligosaccharide processing in the endoplasmic reticulum (er) and Golgi complex. The sites of DNJ and SW effects

In mammalian cells the synthesis and the processing of glycoproteins start in the rER (Fig. 7) where the so-called acceptor asparagin of the newly synthesizing polypeptide chain receives a Glc3-Man9-GlcNAc2 oligosaccharide chain, transferred by pirophosphoryl-dolichol. Subsequently, the 3Glc will be broken down by glycosylase I enzyme [17]. This enzymatic breakdown is inhibited by DNJ (Elbein 1984). As the removal of the three glucose will promote the transport to the Golgi complex, in the DNJ treated cells this transport is very slow and the sensibility of the concerned glycoproteins will change in the Golgi complex towards special enzymes (e.g. endoglucosaminidase-H).

In the Golgi complex the "trimming" of the oligosaccharide chain goes on. At first the different mannosidase enzymes remove the differently bound mannose molecules. Swainsonine can inhibit totally the activity of mannosidase II [9], which is able to remove the 3,6-bound mannoses.

In *Tetrahymena* there are differences in the glycoprotein synthesis compared to mammalian cells. The polypeptide chain under the synthesis gets a Man5-GlcNac2 containing core region from a lipid donor [18].

During the mentioned processing the glycoproteins receive their oligosaccharide components that effects their function and signalisation. The two inhibitors (SW:DNJ) are suitable to test the importance of the processes belonging to the hormonal imprinting, too.

In the present experiment the presence of SW did not render the development of hormonal imprinting, however, DNJ had no such effect. We can explain this difference than DNJ decreases the speed of translocation between the rER and Golgi complex and does not interfere with the further processes of trimming [19]. At the same time SW makes impossible the action of mannosidase II and inhibits the two GlaNAc residues to become resistant toward the strong effect of endoglycosidase. Moreover, it is possible that the new glucosamine and GlcNac components cannot join each other because the enzyme of the next step will not recognize the molecule. All these events could elicit the segregation of the whole oligosaccharide chain from the protein. Therefore partly the protein (receptor) will remain without sugar signals and partly these signals will not appear on the surface of the cell. In high-level cells the conformation formed by mannoses and the number of mannoses seems to be definitive concerning the future of proteins. The role of glucose molecules segregated in the rER does not seems to be important during the processing in the Golgi complex. This makes understandable that the effect of SW was much some distinct than the effects of DNJ. This makes probable that the mannose groups have more significant role than the glucose groups in the processing of glycoproteins of unicellular *Tetrahymena*. Independently of the above-mentioned opinion, the ineffectiveness of DNJ is true for only the imprinting. It was able to disturb the binding of lectins and there is no difference in the tendencies of SW and DNJ effect. This could mean that the disorder of the oligosaccharide chain will not absolutely affect the imprinting because it requires a special type of the disorder which is characteristic of the inhibition of mannosidase II. The abnormality of sugar oligomers is shown by insulin and Con A binding. This runs parallel in untreated and treated cells but the treatment with SW and DNJ resulted a significant and consistent difference. The binding of insulin immediately after the treatment increased while 24 h later decreased. The binding of lectins is decreased in the promt assays and increased 24 h later (according to the main tendencies of the experiment, Figs 1 and 2).

In the biology of *Tetrahymena*, the importance of the secretion of synthesized enzymes [21] is interesting but has not been clarified in a proper way. It may be reasonable that secretion of hydrolases has a role in the breakdown of food materials of environment, thought the great quantities of the enzyme secreted to inorganic salt solution could query this explanation. Recent results show that large amounts of the enzyme secreted to the surface are embedded into the outer surface of the membrane. In this way *Tetrahymena* possesses three pools of hydrolases. These are (i) intracellular; (ii) covalently bound to the membrane and (iii) secreted to the medium (so-called soluble hydrolase pool, [20]).

In one of our previous experiments using a secretory mutant of *Tetrahymena thermophila* we found difference between the cells of the mutant and the secretory "wild" strain, regarding to the imprintability by insulin [21]. By the reason of this and other experiments where the secretion, synthesis of enzymes, processing were inhibited it is presumable that the secreted enzymes have function in the formation of readiness of membrane to environmental signals, and this way in the development of imprinting, too.

The intracellular enzyme level represents the ratio of the activity of enzyme production, the accidental inhibition of elimination and the spontaneous intracellular breakdown. The extracellular enzyme level represents the secretory activity of the cell, the membrane permeability, and in special cases the pretension of the environment. The conversion of intracellularly synthesized enzymes into extracellular ones requires the presence of signals which are essential for the transport. This depends on the oligosaccharide chain on a large scale. That is why the inhibition of sugar processing can considerably influence this.

In this aspect the two enzyme inhibitors showed a significant difference. When DNJ had effect at all, this effect was negative. The SW had a prompt negative effect on the intracellular enzyme content, but 24 h later the effect turned into positive. The secretion of enzymes also was positive following 24 h.

The length of the one hour treatment with the cell cycle of a *Tetrahymena*, may be considered as its half lifespan time. For this period the presence of the inhibitors of glucosidase or mannosidase can inhibit the enzyme production. Twenty-four hours later the daughter cells' enzyme production will affect the registered amount of enzymes and this represents the differences of the two enzyme inhibitors. Thought there was no DNJ effect on the imprinting but it had the capacity to inhibit permanently the enzyme production. This inhibition irradiates to the enzyme secretion as here the only negative result emerged from DNJ. Certainly, it seems that this effect manifested in enzyme production and secretion has no correlation with the development of imprinting.

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ENTEROHAEMOLYSIN PRODUCTION IN *ESCHERICHIA COLI* STRAINS

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Enterohaemolysin production was found in 11 (20.3%) out of 54 *Escherichia coli* strains isolated from stools of infants with dyspepsia and in 3 (2.3%) out of 130 *E. coli* strains isolated from urinary tract infections. Enterohaemolysin producing *E. coli* strains isolated from stools belonged to O groups 25 and 111 and the strains from urine to O groups 1 and 15. None of the enterohaemolysin-producing strains isolated from dyspepsia was shown to cause any damage on Vero cells.

In *Escherichia coli* several factors are known to play an important role in the pathogenesis of intestinal and extraintestinal infections [1–3]. Recently Beutin et al. [4] described in *E. coli* O26 and O111, both isolated from stools of infants with gastroenteritis an enterohaemolysin, and assumed that the haemolysin might play a role as a virulence factor in enteric infections of humans. The term "enterohaemolysin" was drawn from the finding that synthesis of the product was mainly found in enteropathogenic *E. coli*. Moreover, it was revealed that enterohaemolysin production in *E. coli* was often associated with the production of verotoxin (VT) VT1 and/or VT2 in VT$^+$ *E. coli* strains of bovine and human origin [5–8]. Owing to the frequent association of enterohaemolysin with VT$^+$ *E. coli*, its

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evaluation could be an useful epidemiological marker for the detection of potential VT$^+$ *E. coli*. The aim of this work was to evaluate enterohaemolysin production in *E. coli* strains isolated from various infections.

Materials and methods

Strains. Four groups of *E. coli* and control *E. coli* strains were tested: (*i*) strains isolated from stools of infants with haemolytic-uraemic syndrome (HUS); (*ii*) strains isolated from stools of relatives of HUS infants; (*iii*) strains isolated from stools of infants with dyspepsia; (*iv*) strains isolated from urine of infants and adults with urinary tract infections. Ten control *E. coli* strains were used (one O157:H?, four alpha-haemolytic, two beta-haemolytic, two gamma-haemolytic *E. coli* strains and *E. coli* C600 Rif). Altogether 226 strains were tested (Table I).

Production of enterohaemolysin in *E. coli* strains was evaluated by Beutin et al. [4, 5] on blood agar containing erythrocytes washed three times in phosphate buffer saline (PBS), pH 7.2. Human, beef and sheep erythrocytes were used. The strains were cultivated parallel on blood agar containing erythrocytes not washed. Evaluation was done after 3 h and 24 h cultivation at 37 °C.

Production of VT was evaluated according Košiarová and Hoštacká [9].

Table I

Production of enterohaemolysin in E. coli strains of various groups

Source	Number of strains	Strains producing enterohemolysin	
		No.	%
Stools (child with HUS[1])	22	–	–
Stools (relatives)	10	–	–
Stools (neonates dyspepsia)	54	11	20.3
Urine (UTI[2])	130	3	2.3
Control strains			
E. coli	1	–	–
alpha-haemolytic *E. coli*	4	–	–
beta-haemolytic *E. coli*	2	–	–
gamma-haemolytic *E. coli*	2	–	–
non-haemolytic *E. coli* C600 Rif	1	–	–
Total	226	14	

[1] HUS – haemolytic-uraemic syndrome;
[2] UTI – urinary tract infection

Results

From 226 of the strains tested, enterohaemolysin production was found in 14 isolates. Most enterohaemolysin producing strains were found among those isolated from stools of infants with dyspepsia (11 out of 54; 20.3%). The remaining three enterohaemolysin producing isolates were found among those isolated from urine in urinary tract infection (Table I). Among enterohaemolysin-producing strains isolated from stools in dyspepsia, eight belonged to serogroup O26 and three to serogroup O111. Enterohaemolysin-producing strains isolated in urinary tract infections belonged to serogroups O1 and O15, and one strain could not be grouped with O-antisera available (Table II). Enterohaemolysin producing *E. coli* strains isolated in dyspepsia were not found to produce VT.

Table II

Serogroups of E. coli producing enterohaemolysin

Source	Serogroup	Number of strains
Neonates dyspepsia	026	8
	0111	3
Urinary tract infections	01	1
	015	1
	NT	1

NT – not determined

Discussion

Using of various screening methods to demonstrate VT$^+$ *E. coli* is influenced by problems concerning both the detection of VT in stools and/or the VT-producing isolates. Screening methods are considered of improving the diagnosis of serious clinical cases associated with *E. coli* strains producing VT [10–15]. The methods for screening VT production could be successfully used also in any microbiology laboratory lacking other means needed for the demonstration of VT production. The majority of screening methods is based on isolation of *E. coli* O157, however, it is known that VT is produced by *E. coli* strains of other serogroups, too [16–18]. This is the reason, why for the demonstration of VT production other methods are used, e.g. the assay of evaluation of enterohaemolysin production. From the four *E. coli* groups tested, production of enterohaemolysin was found in strains isolated both

from dyspepsia and urinary tract infections. No one from 22 strains isolated in clinically demonstrated HUS was shown to produce enterohaemolysin as well as it was in the 10 strains isolated from relatives of child with HUS.

Production of enterohaemolysin was not found either in *E. coli* isolated from clinically demonstrated HUS or in *E. coli* O157. It is interesting, that none of the 11 enterohaemolysin-producing *E. coli* strains of O-groups 26 and 111, isolated from dyspepsia was shown to cause cytopathic effect on Vero cells. When these results are compared to those of Beutin [7], it should be stressed that the number of strains investigated by us was less. Finally, we realized that in case of our work, production of enterohaemolysin was not associated with production of verotoxin. It indicates that this haemolysin per se may play a role in the pathogenesis of *E. coli* infections and this remains to be studied further.

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MECHANISMS OF ANTIMOTILITY ACTION OF TRICYCLIC COMPOUNDS IN *PROTEUS VULGARIS*

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Tricyclic compounds were able to inhibit the motility of *Proteus vulgaris*. The effectiveness of antimotility action was related to the physicochemical properties of the molecules, i.e. energy of HOMO, Log P, total surface. The antimotility action of the compounds was due to their reversible inhibition on the proton pump of the bacterium. Phosphate anion antagonized the antimotility, and potassium cation enhanced the action of phosphate anion on the antimotility effect induced by the agents. Glucose reversed the antimotility action of the compounds. Factors directly increasing the bacterial proton-motive force (PMF) could change bacterial motility and the antimotility action of the tricyclic compounds.

Phenothiazines and structurally related tricyclic compounds were shown to affect a wide range of tissues and organisms. They have been used as psychopharmacons for decades and also they have been reported having the potential for adjuvant medical treatment to antibacterial therapy [1, 2], protection from endotoxic shock [3–7] prevention of atherosceloris lesion [4], and treatment of burns [5]. A substantial body of evidence suggest that the possible molecular targets of the compounds may locate in the membrane of cells [7–14].

The flagellar rotation of bacterium is considered to be driven solely by the PMF in general, although there are a number of instances in halo- or alkalo-tolerant

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bacteria of transmembrane sodium gradient energized rotation [15–18]. The lowered motility rate can be simply interpreted either as the decrease of PMF or the dissociation of the coupling between the membrane proton pool and the bacterial flagellar rotation machinery through which the inward proton currents return to the cytoplasm and complete the proton circuit while perform the mechanical work [19–21]. Therefore, we have established a prokaryotic model for in vivo evaluation of the antimotility effect of tricyclic compounds [22]. Our preliminary study revealed that the tricyclic compounds are active in inhibition of the motility of *Proteus vulgaris*. Furthermore, the antimotility action of compounds was demonstrated being antagonized by the extracellular ions. Similar results were obtained by Pitney and Doetsch [23] in *Leptospira interrogans* with some other drugs sharing common physicochemical properties of weak basic tricyclic molecules. The ionic factors which might be responsible for the antagonistic actions have been thought to be potassium and sodium cations. However, a more detailed study seemed to be worthwhile to analyse the interaction between the tricyclic compounds and the ionic environments on the bacterial motility molecular targets and the interference.

Materials and methods

Compounds and chemicals. Promethazine, chlorpromazine, 7-hydroxy-chlorpromazine, 3,7,8-trihydroxy-chlorpromazine, 5-oxo-chlorpromazine, imipramine, acridine orange, methylene blue, omeprazol, abscinic acid (Sigma), monensin (Sigma), quinidine, ethionamide, isoniazide, tetraethylammonium (Sigma), 2-aminopyridine (Sigma), inorganic salts as listed in Table I, glucose.

Bacterial strain and growth conditions. A clinical isolate of *P. vulgaris* employed in the previous study [22] was used. The culture medium of MTY broth [24] and conditions were the same as previously described [23].

Solutions. A comparable group of solutions were used to clarify the effect of ionic factors responsible for the antagonistic action and to have a better understanding about the mechanism. The solutions used are listed in Table I.

Motility inhibition test. As it was described previously, any drug could be used as control. Practically all the solutions without drug always gave similar results with no significant difference. PBS, therefore, was used as the control. Sample was taken after incubation for 15 min at 37 °C. Under dark field microscope, 200–300 cells were counted from 5–10 fields. Triple studies were conducted for each experimental case. The motile and non-motile cells were counted separately.

Determination of motility inhibitory rate. The motility inhibitory rate was calculated as follows: Motility inhibitory rate (%) = (1-Em/Et/Cm/Ct) × 100% where, Em is the number of moving cells and Et the number of total cells counted in experimental tube containing the test drug(s). Cm and Ct were termed in the same fashion for control. The results were expressed as average (plus and minus range) of three different experiments.

Calculation of the correlation between motility inhibitory effect and the physicochemical properties of the compounds. The correlations were obtained by computer-assisted calculation on the basis of the Hansch equation:

$$\log/1/c/ = B_1X_1....B_nX_n + C$$

where, c is the value of antimotility effect, B_1, B_2,.... B_n and the coefficients, X_1, X_2,....X_n the physicochemical parameters of a compound, which include energy of HOMO, energy of LUMO, log P, dipole moment, non-polar saturated surface, non-polar unsaturated surface, polar surface and total surface, and C the contant.

Results

Antimotility action of nine tricyclic compounds in different ionic environments is shown in Table II. Generally, the compounds were more effective in KCl and NaCl than in the other four solutions, namely PBS, K–B, Na–B and H–PO, all of which contained inorganic phosphate. This, therefore, may suggest that the phosphate anion should be of major importance for the decreased motility inhibition of the test

Table I

Composition of solutions employed in the study

Solution	Composition	Concentration (*M*)	pH (adjusted with)
PBS	NaCl	1.5×10^{-1}	7.0 (NaOH, HCl)
	KH_2PO_4	1.3×10^{-3}	
	Na_2HPO_4	5.3×10^{-3}	
K–B	K_2HPO_4	5.0×10^{-3}	7.0 (KH_2PO_4)
	$MgSO_2$	5.0×10^{-4}	
	EDTA	3.8×10^{-5}	
Na–B	Na_2HPO_4	5.0×10^{-3}	7.0 (NaH_2PO_4)
	$MgSO_4$	5.0×10^{-4}	
	EDTA	3.8×10^{-5}	
H_3PO_4	H_3PO_4	5.0×10^{-3}	7.0 (TRIS)
	$MgSO_4$	5.0×10^{-4}	
	EDTA	3.8×10^{-5}	
KCl	KCl	5.0×10^{-3}	7.0 (KOH)
	$MgSO_4$	5.0×10^{-4}	
	EDTA	3.8×10^{-5}	
NaCl	NaCl	5.0×10^{-3}	7.0 (NaOH)
	$MgSO_4$	5.0×10^{-4}	
	EDTA	3.8×10^{-5}	
$MgCl_2$	$MgCl_2$	5.0×10^{-3}	7.0 (TRIS)
$CaCl_2$	$CaCl_2$	5.0×10^{-3}	7.0 (TRIS)
$LiCO_3$	$LiCO_3$	5.0×10^{-3}	7.0 (TRIS)

agents. Among the four phosphate-containing solutions K–B was the most effective one in antagonizing the actions of the compounds on the bacterial motility. The results suggest that the potassium cation enhances the antagonizing effect of phosphate anion. The impacts of different solutions on the antimotility effects of the compounds was possible due to a common mechanism. Promethazine, chlorpromazine and methylene blue were the most effective and 5-oxo-chlorpromazine was the least effective of all the solutions.

To investigate the impact of certain ionic circuit pattern on the bacterial motility inhibitory effects of the test compounds, some agents blocking certain ion channels were tested in PBS, K–B and Na–B. The results (Table III) shows that phosphate ion channel blockers had little impact on the motility of the proteus strain while the other agents displayed mild motility inhibition. When combined with promethazine, the proton pump inhibitors and the sodium channel blocker demonstrated synergistic or additive effects on the antimotility of promethazine with the exception of omeprasol in PBS.

Table II

Motility inhibitory rate of tricyclic compounds in various solutions in P. vulgaris

Composition	Concentration (*M*)	Motility inhibitory rate (average ± range) in percent in solution					
		PBS	K–B	Na–B	H PO	KCl	NaCl
Promethazine	1.5×10^{-4}	40.7 ±4.8	5.8 ±3.1	30.4 ±4.2	31.0 ±3.1	40.7 ±3.2	74.6 ±5.4
Chloropromazine	1.5×10^{-5}	63.4 ±5.0	22.8 ±3.2	49.3 ±3.4	44.4 ±4.0	71.4 ±6.0	68.4 ±6.2
7-Hydroxy-chlorpromazine	1.5×10^{-3}	68.3 ±7.6	33.7 ±5.2	60.0 ±4.9	42.1 ±4.2	90.1 ±6.1	88.8 ±5.5
3,7,8-Trihydroxy-chlorpromazine	1.5×10^{-3}	25.9 ±3.1	15.2 ±1.4	39.4 ±2.1	12.7 ±3.2	92.7 ±7.1	66.6 ±7.0
5-Oxo-chlorpromazine	1.5×10^{-3}	18.8 ±3.3	17.6 ±3.1	23.7 ±2.8	16.6 ±2.1	40.8 ±4.5	36.0 ±3.9
Imipramine	1.5×10^{-3}	57.8 ±6.0	57.2 ±5.3	84.4 ±4.8	74.3 ±7.1	83.2 ±6.1	79.0 ±5.0
Acridine orange	1.5×10^{-3}	83.6 ±7.2	57.6 ±6.5	94.1 ±5.6	62.3 ±3.3	93.9 ±6.7	100.0 ±4.9
Methylene blue	1.5×10^{-4}	86.7 ±8.6	70.6 ±5.1	36.9 ±4.1	33.7 ±3.5	62.1 ±3.6	87.7 ±7.2

On the contrary, sodium-ionophore, phosphate and potassium channel blockers exerted antagonistic actions upon the antimotility of promethazine in PBS. However, in Na–B solution the above three combinations showed synergism (or difference) with promethazine and in K–B solution they showed indifference.

Table III

Effects of ionic circuit effectors on the motility of P. vulgaris and on the antimotility of promethazine in different solutions

Compound	Concentration (*M*)	Motility inhibitory rate (average ± range) in percent in solutions		
		PBS	K–B	Na–B
Promethazine	1.5×10^{-4}	40.7 ± 4.8	5.8 ± 3.1	30.4 ± 4.2
Proton pump blocker				
Omeprasol	1.5×10^{-3}	16.8 ± 6.3	41.5 ± 7.2	51.8 ± 6.0
		(11.6 ± 5.8)	(43.4 ± 3.2)	(56.5 ± 4.2)
Abscinic acid	1.5×10^{-3}	40.9 ± 5.3	18.9 ± 7.1	74.8 ± 8.5
		(52.6 ± 7.6)	(89.3 ± 7.5)	(92.6 ± 5.6)
Sodium channel blocker				
Quinidine	1.5×10^{-3}	23.9 ± 2.1	20.6 ± 3.2	45.0 ± 5.2
		(43.1 ± 4.5)	(76.3 ± 6.8)	(52.1 ± 2.3)
Ionophore				
Monensin	1.5×10^{-3}	39.3 ± 3.3	35.2 ± 3.2	34.0 ± 3.7
		(14.8 ± 3.2)	(31.2 ± 2.6)	(49.8 ± 4.3)
Potassium channel blocker				
Tetraethyl-ammonium	1.5×10^{-3}	38.2 ± 2.7	12.8 ± 2.5	38.2 ± 3.1
		(22.1 ± 3.1)	(9.1 ± 2.0)	(53.5 ± 4.3)
2-Aminopyridine	1.5×10^{-3}	42.9 ± 3.5	2.8 ± 2.1	23.6 ± 2.1
		(0.0 ± 3.1)	(17.1 ± 2.5)	(32.0 ± 3.2)
Phosphate channel blocker				
Ethionamide	1.5×10^{-3}	0.0 ± 2.1	0.0 ± 4.0	10.8 ± 2.1
		(0.0 ± 2.5)	(0.0 ± 3.1)	(11.2 ± 3.1)
Isoniazid	1.5×10^{-3}	0.0 ± 3.3	0.0 ± 2.7	0.0 ± 4.1
		(28.9 ± 4.0)	(6.2 ± 2.2)	(55.5 ± 5.1)

Numbers in parenthesis indicate the motility inhibitory rates of the compounds combined with promethazine

The demonstration of enhancing effect of potassium and the antagonizing action of phosphate against antimotility of tricyclic compounds prompted us to extend the investigation to other cations. The effects of $MgCl_2$, $CaCl_2$, and $LiCO_3$ on the motility inhibitory action of promethazine are shown in Table IV.

Table IV

Impacts of $MgCl_2$, $CaCl_2$ and $LiCO_3$ on the antimotility action of promethazine on P. vulgaris

Solution	Motility inhibitory rate (average ± range) of promethazine (1.6×10^{-4} *M*) in percent
H_3PO_4	31.0 ± 3.1
$MgCl_2$	50.1 ± 4.1
$MgCl_2 + H_3PO_4$	33.0 ± 3.9
$CaCl_2$	87.6 ± 7.1
$CaCl_2 + H_3PO_4$	29.3 ± 5.6
$LiCO_3$	72.3 ± 6.5
$LiCO_3 + H_3HPO_4$	27.5 ± 3.4

Magnesium ion had moderate effect on antagonizing the activity of promethazine; that might be associated with a competition between magnesium ions and the drug [8], whereas calcium and lithium ions were not effective.

Energy metabolism is the basis for generating PMF and hence for supporting the bacterial flagellar rotation. The impact of a metabolizable substrate on the motility inhibitory action of promethazine was tested using glucose (Table V): 20 m*M* glucose reversed the antimotility action of the drug in all the solutions to a different extent.

Correlations were found between the antimotility action of the tricyclic compounds and three of their eight physicochemical parameters, i.e. energy of HOMO, log P and total surface.

Table V

Reversal effects of 20 mM of glucose in different solutions on the antimotility of promethazine on P. vulgaris

Glucose (M)	Motility inhibitory rate (average ± range) in percent of promethazine (1.5×10^{-4} M) in various solutions					
	H_3PO_4	PBS	K-B	Na-B	KCl	NaCl
0	31.0 ± 3.1	40.7 ± 4.8	5.8 ± 3.1	30.4 ± 4.2	40.7 ± 3.2	74.6 ± 5.4
2.0×10^{-2}	23.3 ± 2.1	11.3 ± 1.5	0.0 ± 2.0	17.4 ± 3.1	17.2 ± 2.9	46.7 ± 2.3

The correlation in PBS system was R = 0.964. Concerning the three parameters log P was directly proportional, and energy of HOMO and total surface were indirectly proportional to the antimotility effects of the compounds as predicted by the Hansch equation with coefficients of HOMO energy 0.486 coefficient of log

P −0.151 and total surface 0.016 in PBS. In K−B solution the R value was 0.956, HOMO energy 0.653, log P −0.164 and total surface was 0.019.

Discussion

In the present work it has been revealed that the antimotility action of the tricyclic compounds is related to some of their physicochemical properties, suggesting that smaller, more stable and higher lipophilic molecules of the compounds could more efficiently interact with the molecular targets of the bacterial cells.

In general, PMF is the unique energy source for flagellar rotation. Therefore, bacterial motility could directly reflect the magnitude of PMF or the coupling state between the membrane proton pool and bacterial flagellar rotation machinery [19−21]. The repression of the bacterial motility by the tricyclic compounds (Table II) clearly indicates their interference with PMF or the coupling state.

For survival from such environmental insults as fluctuations of external pH, bacterial cells have evolved distinct systems to maintain a relatively stable internal pH [25−27]. Among them are the proton pump, the Na^+/H^+ and K^+/H^+ antiporters. At acidic external pH as in the present study, K^+/H^+ antiporter and external potassium were proposed to function for stabilizing the cytosolic pH [26, 27]. The external potassium allows more proton to be extruded by the primary proton pump and thus alkalizes the cellular interior and to help the generation of the internal homeostasis of pH, the "side-effect" of which is the enhancement of PMF. This might be the basis for the antagonism of potassium on antimotility actions of the tricyclic compounds (Table II) in the presence of phosphate.

PMF is a combination of two separate forces: (*i*) a membrane potential gradient and (*ii*) a concentration gradient of protons. The membrane potential gradient, primarily produced by the bacterial protonic generators, can be converted to the transmembrane potassium proton gradient of the opposite direction by means of an electrophoretic potassium influx. The membrane pH gradient also can be partly transformed as transmembrane sodium gradient through electroneutral Na^+/H^+ antiporter. Changes in both membrane potential and proton gradient can be buffered by gradients of respective potassium and sodium ions. The K^+ and Na^+ buffer regulation can retard dissipation of PMF only after the proton pumps are switched off. Inhibition of sodium channel by its blocker increased antimotility of promethazine (Table II) suggesting the further decrease of PMF by the blocker. However, for potassium channel blockers the situation appeared more complicated.

According to the above hypothesis, blockade of potassium channel can result in the inability to compensate the decreased membrane potential and hence PMF, and therefore decreases the bacterial motility. In Na−B and K−B solutions the blockers

showed additive effect with promethazine on the bacterial motility (Table III). In PBS the antagonistic action of potassium channel blockers on the antimotility or promethazine may imply that the physiological concentration of sodium compensate the decreased PMF.

The physiological concentration of sodium seems also important for Na^+/H^+ exchange by the ionophore [28] and for expression of the effects of phosphate channel blockers. Phosphate transport could phenotypically symport proton into cells [29]. Therefore the blockage of the phosphate channel may save the PMF. This could account for the results in Table III. It is easy to understand the synergistic effect of proton pump inhibitors on the antimotility action of promethazine since the inhibition of the proton pump directly lower PMF of bacterial cells.

The glucose in the test system reversed the antimotility action of the promethazine (Table V). Various ions decreased or increased the bacterial motility of the drug may suggest that: (*i*) the tricyclic compounds inhibit the bacterial motility through a reversible inhibition of proton pump other that the dissociation of the coupling between the proton pool and the flagellar rotation machinery. (*ii*) Factors increasing or decreasing PMF exert antagonistic or synergistic effects against the antimotility actions of the tricyclic compounds, respectively.

It is of interest that the presence of external phosphate displayed antagonistic action on the antimotility of the drugs. However, the mechanism is unknown. It may results from direct inhibition on the interaction between tricyclic compounds and proton pumps or from decreased PMF by direct ionic exchange in the presence of phosphate.

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INULIN FORMATION OF PENICILLIN PRODUCING INDUSTRIAL *PENICILLIUM CHRYSOGENUM* STRAINS

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Conidia of certain penicillin producing *Penicillium chrysogenum* industrial strains produced polyfructose. Two types of polyfructoses were formed by conidia of *P. chrysogenum* B10 from sucrose and with less yield from raffinose. Ten percent of fructans were in water insoluble form attached to the outer wall of conidia. The other, ethanol precipitable fructan formed a colloid opalescent solution. The latter had inulin type β (2 → 1) bonds – identified by ^{13}C NMR spectroscopy – between fructose molecules and had a molecular weight of 217 000 Daltons. The K_M value of sucrose hydrolysis – the first step of inulin production – was 0.86 M. The invertase hydrolysed about 70% of sucrose on the second day. Optimal conditions for inulin formation were: pH 6.0, 25–45 °C, 100 mg/ml sucrose, 10^7 spore/ml. The maximum conversion rate of fructose from sucrose into precipitable inulin was about 10% after 48 h incubation. The inulin production could be inhibited by glucose.

Fructans, the polymers of fructose, are common polysaccharides in nature. They are classified into two groups by the type of linkage between fructose molecules. Levan (Fig. 1a) is primarily microbial product (*Bacillus subtilis, Bacillus polymyxa* [1], *Zymomonas mobilis* [2], *Aerobacter levanicum* [3]) having β (2 → 6) bonds. Inulin a β/2 → 1) is a linkaged fructose polymer (Fig. 1b) can be found in various plants [4]. It was separated for the first time in 1804 from *Dahlia* tubers. At present this plant is the main source of inulin.

While the molecular weight of inulin is less than 10 000 Daltons, levan is sometimes more than one million D. Although there are a lot of publications on microbial levans [1–6] and plant inulins [4, 5, 7], relatively little is known about inulin, produced by fungi [8]. This communication present some results of inulin production by penicillin producing industrial *Penicillium* strains.

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Materials and methods

Microorganisms. P. chrysogenum B14, B13, B12, B10, LAR, URCM and UBF industrial strains were maintained on rice medium.

Condition of fructan production. In 50 ml 100 mg/ml sucrose containing pH 6.0 MES buffer 5×10^8 spores were incubated at 25 °C, without stirring for 2 days, unless different conditions are indicated.

Precipitation of fructan. Spores were removed by centrifugation. The opalescent supernatant was poured slowly into absolute ethanol containing one drop of 1% $CaCl_2$ (at volume ratio 1:3). After 1 h, the precipitated fructan was sedimented at 2000 *g* for 10 min. In order to purify the product, it was dissolved in warm water, precipitated in ethanol and centrifuged again. For further purification of fructan gel filtration on Sephadex G-10 column was applied. After subsequent precipitation it was dried at room temperature for two days.

Thin-layer chromatography. Fructan was prehydrolyzed with 1.2 M HCl at 90 °C for 30 min. Plate: HPTLC-NH_2 (Merck, 15647); eluent: acetonitrile-water (70:30); sample volume: 1-5 µl. After heating at 150 °C the chromatograms were investigated under UV light [9, 10].

Assay of fructan. The fructose content of precipitated fructan was measured after its hydrolysis (see above). (*i*) Spectrophotometrical determination based on the carbazol method of Dische et al. [11]. (*ii*) Fructose on thin-layer chromatograms was evaluated with Shimadzu CS-9000 spectrodensitometer at 310 nm.

Assay of glucose. Glucose content was measured by means of a biosensor [12] prepared from an oxygen electrode. The tip of electrode was covered with glucose oxidase (from *Aspergillus niger*, Sigma Chem. Co.) absorbed on nitrocellulose membrane (Sartorius SM 11325). Another oxygen electrode was used as a reference element and the difference signal between the enzyme covered and enzyme-free electrode was measured by a potentiometer. Samples were diluted with 100 mM phosphate buffer at pH 6.8 to obtain a concentration range of 0.01-1 mM where linear response was observed.

^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of inulin was recorded using a Bruker WP20054 type spectrometer at 50.1 MHz, in D_2O solution at 30 °C.

Molecular weight determination. Inulin solution (6 mg/ml) prepared from the purified material was investigated in a MOM 3170/B analytical ultracentrifuge at 20 000 and 44 000 r.p.m. Molecular weight was calculated using the Svedberg equation.

Results and discussion

Polyfructose production by conidia of high penicillin producing industrial *P. chrysogenum* B10 strain has been found. From the tested sugars (sucrose, glucose, fructose, invert sugar, lactose, galactose, mannose, raffinose), only sucrose and with far less yield raffinose were utilized for inulin production.

The fructan appears in two forms. One of them can be seen as a water insoluble capsule around the spores after about 30 min following inoculation. Its thickness grows only for some hours. During further incubation the supernatant becomes opalescent caused by other - colloid form - fructan. The ratio between these two forms is 1:10 on the second day. Both of them consist of only fructose molecules determined by thin-layer chromatography. French described glucose units

at the ends of the chains of a typical inulin having about 35 fructose residues [14]. We could not find glucose among the hydrolysis products of inulin by TLC, presumably because of the small glucose: fructose ratio of our high molecular weight inulin.

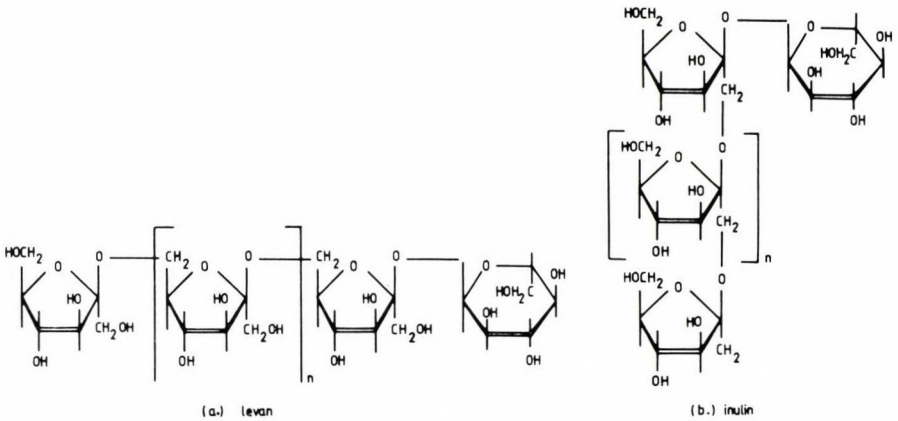

Fig. 1. Chemical structure of levan (*a*) and inulin (*b*)

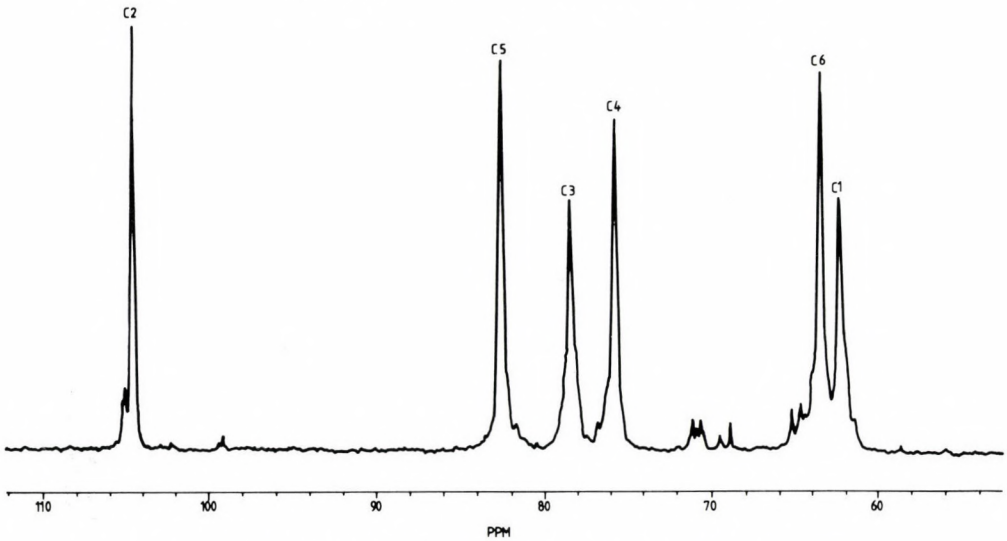

Fig. 2. ^{13}C NMR spectrum of precipitable inulin produced by *P. chrysogenum* B10

The colloid fructan was precipitated and purified by gel filtration. ^{13}C NMR spectrum of fructan (Fig. 2) shows six main resonances at 104.6, 82.6, 78.4, 75.7, 63.5 and 62.3 ppm, that are almost the same values found by Shimamura et al. [13] for inulin. Accordingly, the bonds between fructose units are β (2 → 1) inulin type.

The molecular weight of precipitated inulin was 217 000 ($D_{20,w} = 38.6 \times 10^{-7}$ cm^2) sec; $s_{20,w} = 137.9 \times 10^{-13}$ s; $\overline{V} = 0.6$ cm^3/g), that was much higher than that characteristic of plant inulins (about 6300 D) [14] and fructooligosaccharide produced by *A. niger* (< 1000 D) [8].

The first step of polyfructose production must be sucrose hydrolysis. By measuring increasing amounts of glucose liberated from 100 mg/ml sucrose by conidia, we proved the invertase activity (Fig. 3). From the Lineweaver-Burk plot of invertase activity 0.86 M K_m value can be calculated at pH 6.0, 25 °C (Fig. 4).

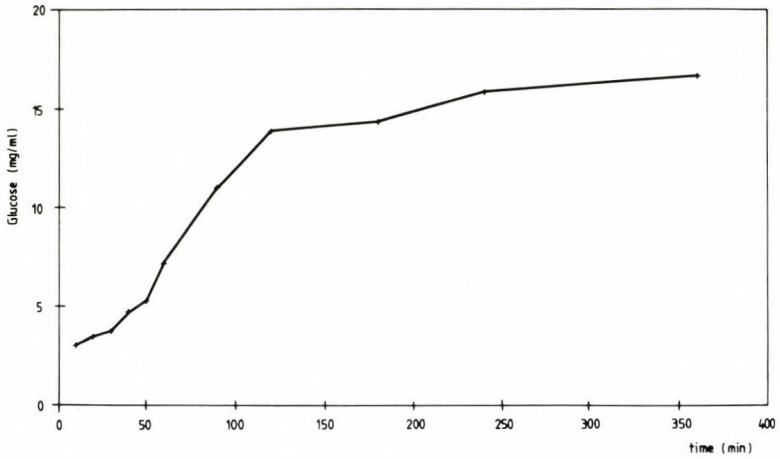

Fig. 3. Time course of glucose accumulation arising from hydrolysis of 100 mg/ml sucrose by *P. chrysogenum* B10

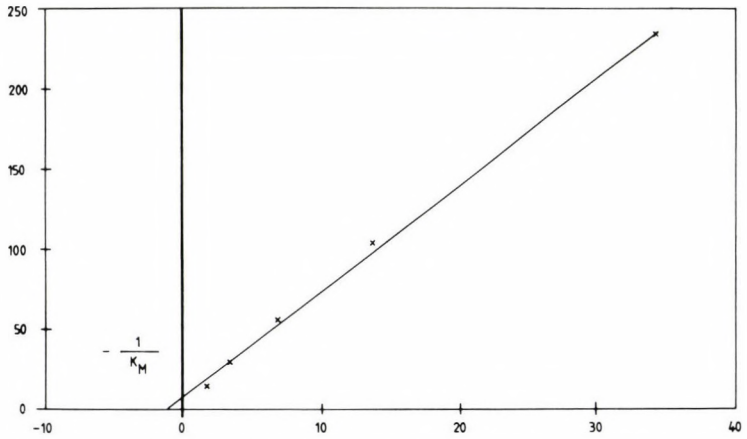

Fig. 4. K_M value determination from the Lineweaver-Burk plot of sucrose hydrolysis by *P. chrysogenum* B10

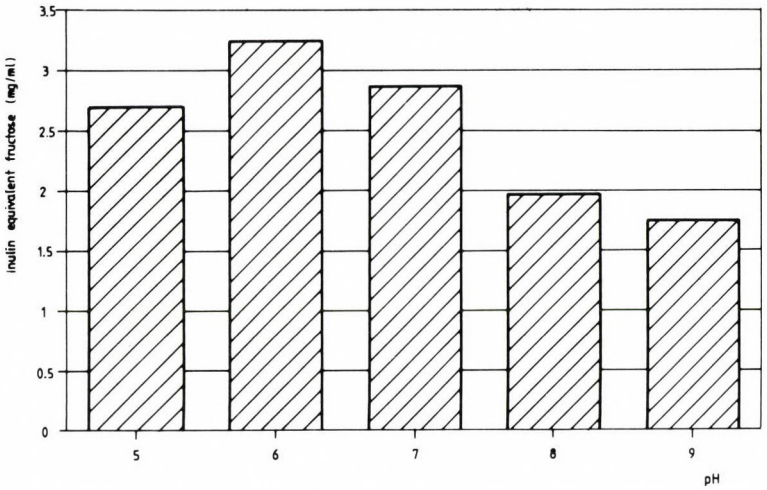

Fig. 5. Inulin production of *P. chrysogenum* B10 at different pH

We investigated the course of inulin production at a pH range of 5–9 (Fig. 5). The optimal pH is about pH 6.0. The temperature has no significant influence on inulin production in the range of 25–45 °C (Fig. 6). It is produced at a quite broad temperature scale of 5–70 °C.

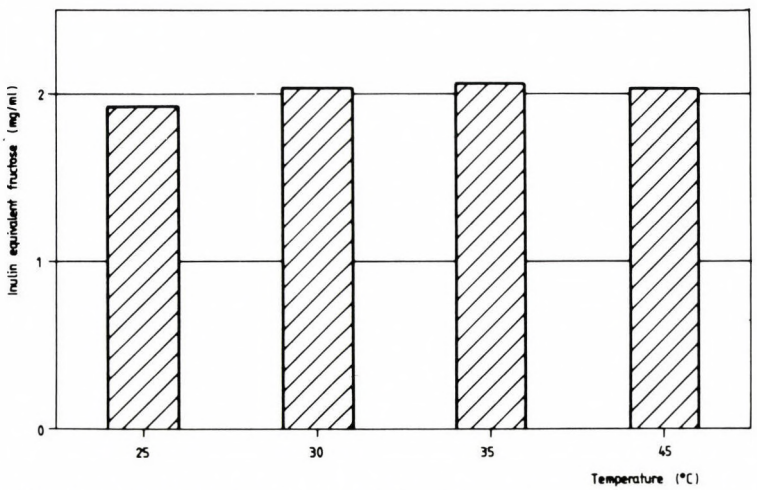

Fig. 6. Inulin production of *P. chrysogenum* B10 at different temperatures (measured at 24 h)

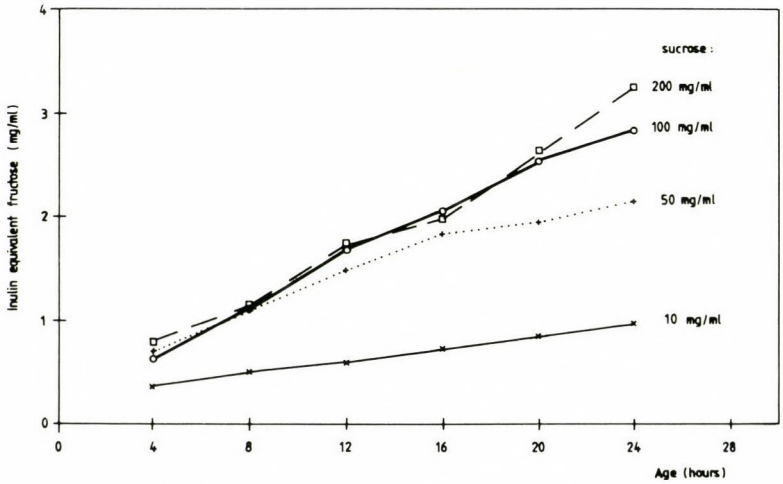

Fig. 7. Inulin production of *P. chrysogenum* B10 at different sucrose concentrations

Increasing the sucrose concentration the inulin production becomes higher (Fig. 7), but the yield of fructose conversion from sucrose to inulin is lower. After 24 h incubation the conversion of fructose to inulin in 10 mg/ml and 200 mg/ml sucrose containing media was 18% and 3%, respectively.

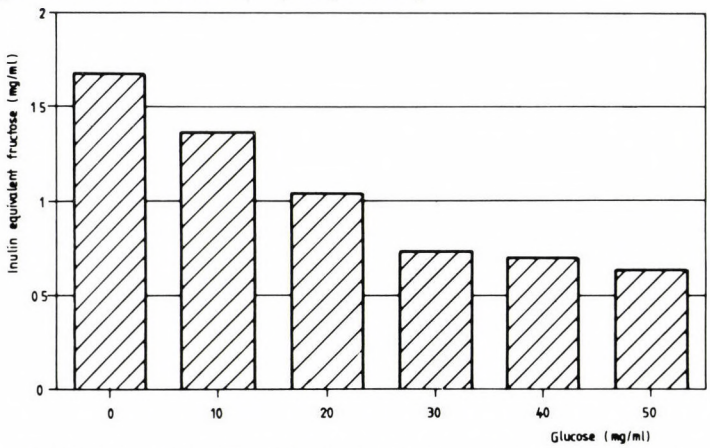

Fig. 8. Inhibition of inulin production by glucose (media contained 5% sucrose)

Inulin formation can be inhibited by glucose. Glucose at 50 mg/ml decreased the inulin production by about 60% in 50 mg/ml sucrose solution during a 6 h incubation period (Fig. 8). We assume that the half of inulin formation of conidia may be due to glucose accumulation, because the sucrose hydrolysis seems to be faster than polysaccharide synthesis from fructose molecules (Figs 3 and 7). Another

reason for the comparatively low inulin levels perhaps is the thick polyfructose capsule around the spores. This serves as a barrier between substrate molecules and enzyme(s) attached to the outer wall of conidia.

The ability of inulin formation is characteristic only of conidia. After the appearance of the germination tube only the opposite end of this structure with spore wall can produce inulin for some hours. There was not any inulin production in the presence of mycelia. This is a difference, too, between polyfructose production of *P. chrysogenum* B10 and other microorganisms, which form polyfructose levan in growing cultures [1, 2, 6]. So far any correlation between inulin production and the start of spore germination was found.

Although about 70% of sucrose are hydrolyzed on the second day, only 10% of liberated fructose molecules are converted into precipitable inulin. The main part of fructose probably forms kestoses [14] consisting of only some fructose units.

We investigated the polyfructose producing ability of other industrial *Penicillium* strains, too. *P. chrysogenum* B14, B13, B12, B10 – originating from the same strain line – and LAR produced polyfructose capsule and colloid inulin at about the same yield. Conidia of *P. chrysogenum* URCM and UBF strains could not form any polysaccharide (Table I).

Table I

Polyfructose production by P. chrysogenum *industrial strains (10^7 spores/ml in 100 mg/ml sucrose solution, at 25 °C)*

Strain	Polyfructose capsule after 3 h incubation	Inulin equivalent fructose in colloid fructan after 24 h incubation
B14	+	2.4 mg/ml
B13	+	2.8 mg/ml
B12	+	2.9 mg/ml
B10	+	2.9 mg/ml
LAR	+	2.6 mg/ml
URCM	–	–
UBF	–	–

The above-described phenomenon can be exploited:

– for quick counting of living *Penicillium* spores in sucrose solution (only living spores can produce fructose capsule that can be seen microscope after 30 min)

– as a marker for protoplast fusion

– since not all of our penicillin producing Penicillium strains posses this activity, this feature can be applied to establish the relationship between various strains.

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IN VITRO SUSCEPTIBILITY OF SELECTED NON-PROTOZOA TO MEFLOQUINE

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Mefloquine, an antimalarial, was tested against several bacteria and a yeast. It had moderate activity against *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Escherichia coli* and no measured activity against *Pseudomonas aeruginosa* and *Candida albicans*.

The emergent drug-resistant microbes are seriously challenging both medical practice and the pharmaceutical industry [1–4]. Physicians find that their time-honoured armamentarium is not adequate to treat some of these pathogens. Drug companies are realizing how burdensome in time and money is the process of synthesizing, modifying, testing and producing antimicrobial agents [3, 5, 6]. Consequently, industry is pursuing many other strategies: the retesting of "older" antimicrobials for eventual new uses, the application of combination therapy, the design of specific enzyme inhibitors, among others [3, 6].

Therefore, we report the in vitro activity of mefloquine, a recently introduced antimalarial drug, on selected bacterial strains and a yeast. The microorganisms were chosen because they represent major or unique clinical and/or epidemiologic problems.

Materials and methods

Microorganisms. Standardized strains were used for the following microorganisms: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* 759 [7]. *Staphylococcus saprophyticus* was originally isolated from a patient.

Broth macrodilution for bacteria. To determine bacteriostatic and bactericidal actions, the broth macrodilution method was employed according to NCCLS procedure [8]. Mueller-Hinton Broth (Difco 0757–01–4), at pH 7.2, was used to obtain the overnight cultures and to measure the minimum

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inhibitory concentrations (MIC's). Mueller-Hinton Medium (Agar) (Difco 0252-01-4), at pH 7.2, was employed for measuring the minimum bactericidal concentrations (MBC's) and for counting colonies from the 0.5 McFarland standards. Dilutions for colony counts were performed in phosphate buffered saline (FTA Haemagglutination Buffer, BBL 11248), at pH 7.2.

Broth macrodilution for yeast. To determine fungistatic and fungicidal actions, the broth macrodilution method was employed according to [9]. Sabouraud Liquid Broth Modified Antibiotic Medium 13 (BBL 10986) was used for the 1:100 dilution of the adjusted cell suspension and for the dilutions of mefloquine. Sabouraud Dextrose Agar (BBL 11584) was used for the 48-h growth culture and for minimum fungicidal (MFC) determination.

Mefloquine solution. A stock solution of mefloquine (at 400 μg/ml) was prepared by first dissolving 4 mg of mefloquine hydrochloride (Hoffmann LaRoche) in 0.5 ml of DMSO, then bringing the volume to 10 ml with the addition of 10 mM HEPES buffer plus saline (pH 6.2).

Results and discussion

From Table I, mefloquine has moderate activity against *S. saprophyticus, S. aureus* and *E. coli*: the MIC's are 12.5 μg/ml, 25 μg/ml and 50 μg/ml, respectively; the MBC's are 25 μg/ml, 50 μg/ml and 100 μg/ml, respectively. The staphylococci seem more susceptible. Mefloquine has no measured activity against *P. aeruginosa* and *C. albicans*: both the MIC's and the MBC (or MFC) are ≥ 100 μg/ml.

Table I

In vitro *activities of mefloquine against selected microorganisms*

Microorganism	MIC[a]	MBC[b] (MFC[c])
S. aureus ATCC 25923	25	50
S. saprophyticus	12.5	25
P. aeruginosa ATCC 27853	>100	>100
E. coli ATCC 25922	50	100
C. albicans 759	100	>100

[a] The MIC in μg/ml is the lowest concentration of mefloquine giving a culture turbidity equal to the negative control [8]

[b] The MBC in μg/ml is the lowest concentration of mefloquine yielding less than 11 colonies upon subculture [8]

[c] The MFC in μg/ml is the lowest concentration of mefloquine yielding less than three colonies upon subculture [9]

Mefloquine had originally been licensed as an antimalarial drug [10]. However, due to its structural similarity to quinine, its antibacterial activity was not completely unexpected. It is knows from the literature that, following a single one-gram dose of

mefloquine, a peak plasma level of 540 to 1240 ng/ml is reached within 24 h [11]. Following a continuous application of the drug for a week, the plasma level can increase to a therapeutic level reflecting the data in Table I. Furthermore, since protein binding is very high (98%) and elimination is slow (median of 18 days) [10], a lasting therapeutic effect is predicted.

Further molecular modification may extend this modest antibacterial activity to other species. Modification may also lower the MIC/MBC values recorded in Table I. Chemically modified mefloquine derivatives in progress could be in use in the near future after completion of toxicity studies.

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