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EDITORIAL NOTE

The name of Acta Microbiologica Academiae Scientiarum Hungaricae has been changed to *Acta Microbiologica Hungarica.*

Acta Microbiologica Hungarica is a journal of the Hungarian Academy of Sciences. The change does not affect the status and editorial policy of the journal.

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DNA-METHYLATING ACTIVITY OF MYCOBACTERIA

I. I. Nikolskaya, P. A. Somogyi, I. Földes and S. S. Debov

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(Received December 23, 1981)

The methylating activity of the four mycobacterium strains *Mycobacterium phlei*, *Mycobacterium smegmatis* strain Butyricum, *Mycobacterium smegmatis* strain Rabinowitz, lysogenic *Mycobacterium smegmatis* strain Rabinowitz was studied in vitro. All the four strains were found to have methylating activity; enzyme containing extracts of *M. smegmatis* strain Butyricum and *M. phlei* showed a stronger activity than those of *M. smegmatis* strain Rabinowitz and the lysogenic Rabinowitz strain. The methylases of all the four strains methylated the adenine and cytosine residues of the acceptor DNA-forming 5-methyl-cytosine and 6-methyl-aminopurine. The mycobacterial DNAs were methylated by the corresponding mycobacterial methylases in vitro only to 25–30% as compared to the methylation on phage Sd–DNA. This is comparable with the low in vivo methylation of mycobacterial DNAs. The kinetics of methylation indicated a low non-specific nuclease content of the mycobacterium cells.

Phenomena of modification and restriction ensuring host specificity of DNA are of utmost importance for the functions of microorganisms. Up-to-now about 40 genera of microorganisms have been studied [1]. A number of bacteria, among them the mycobacteria have not been investigated in this respect.

In this paper we described our results referring to the specificity and some characteristics of DNA-methylating enzymes of four mycobacterium strains.

Materials and methods

Mycobacterium strains. M. smegmatis strain Rabinowitz ("Rabinowitz"), *M. smegmatis* strain Butyricum ("Butyricum") and its phage ("Butyricum phage"), *M. phlei* and its phage ("Phlei phage") were used. These strains were obtained from Dr. Edith Vandra of the National Institute of Tuberculosis and Pulmonology Korányi, Budapest. A lysogenic *M. smegmatis* strain Rabinowitz ("lys. Rabinowitz") was isolated in our laboratory [2].

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(H^3) methyl-S-adenosyl-L-methionine (H^3–SAM) (Radiochemical Centre, Amersham) with a specific activity of 2.7 TBq/ml was used as methyl donor. Thymus DNA (Calbiochem), DNAs prepared from phages by the phenol method [3] and mycobacterial DNAs extracted as described earlier were used as methyl-acceptors [4].

Bacteria were grown at 37 °C by shaking in Sauton medium (4 g asparagine, 0.5 g KH_2PO_4, 2 g citric acid, 0.5 g $MgSO_4$, 0.05 g ferri-ammonium citrate, 6% glycerol per litre distilled water, pH adjusted to 7.4 with 10% NH_4OH) until the early stationary phase had been reached. Before harvesting the culture was treated with glycine (0.2 M, 2 h) to have bacterial spheroplasts. After centrifugation, the bacterial masses were kept at —20 °C for several months.

For preparing crude extracts with methylase activity, bacteria were suspended in 0.1 M K-phosphate of Tris–HCl, pH 7.2–7.5, disrupted in an MSE ultrasonic disintegrator for 5–10 min followed by centrifugation at 105 000 *g* for 90 min. For removing nucleic acids the supernatant was treated with streptomycin sulphate and then with ammonium sulphate. The protein fractions obtained after ammonium sulphate precipitation were tested for enzyme activity [5].

Methylating activity of the crude extracts was determined in incubation mixtures containing Tris–HCl or K-phosphate buffer 0.1 M to achieve the required pH, 100 μg of acceptor DNA, ammonium sulphate precipitated crude extract corresponding to 3–5 mg protein and 100 kBq/ml of (H^3)–SAM. Samples were incubated at 37 °C. For measuring radioactivity, samples were treated as described earlier [6].

Non-specific nuclease activity was determined by the change of relative viscosity (η rel.). η rel is given by the ratio t_1:t_0, where t_1 is the transit time for solution and t_0 is the transit time for solvent. Determinations were carried out in an Ostwald viscosimeter. The incubation mixture (0.5 ml) contained 400 μg of DNA and 3–5 mg of protein of the ammonium sulphate precipitated crude extracts.

Identification of methylated products was carried out by hydrolysis of the acceptor DNA with perchloric acid (70%) followed by paper chromatography in a solvent system of n-butanol–water–ammonia (60:10:0.1 v/v).

Results and discussion

DNA-methylase activity of crude extracts of mycobacteria

The DNA methylase level of microorganisms in the logarithmic or stationary phases is known not to differ markedly [7, 8], and cells from the stationary phase are generally used only because of the larger biomasses available in this phase.

To prove whether the same correlation is true for mycobacteria, we compared the methylase activity of crude extracts of strain Butyricum from cultures in the stationary and logarithmic phases of growth. Table I shows that no difference could be observed in this respect. Table I also shows that the majority (over 80%) of the enzyme activity could be found in the 0.4–0.8 ammonium sulphate fractions in all the four strains. (It is important to note that the bacterium masses did not change their enzymatic activity during 6–8 months, but the enzymatic activity of ammonium sulphate treated extracts decreased after 10–15 days). The highest methylase activity of strain Butyricum could be measured using Tris–HCl buffer, while phosphate buffer proved to be optimal for the other strains. Optimal pH was 7.4; 7.2; 7.5; and 7.5 for strains Butyricum, *M. phlei*, Rabinowitz and lys. Rabinowitz, respectively.

Table 1

DNA-methylating activity of mycobacterial extracts prepared from cells in stationary and logarithmic phase; distribution of methylating activity in ammonium sulphate precipitation fractions

Bacterial strains	DNA-methylating activity*				
	of extracts prepared from cells in		of extracts precipitated with		
	stationary phase	log. phase	0.4 saturation of ammonium sulphate	0.8 saturation of ammonium sulphate	1.0 saturation of ammonium sulphate
M. smegmatis strain Butyricum	100	97	3	87	10
M. phlei	ND	ND	7	85	8
M. smegmatis strain Rabinowitz	ND	ND	11	86	3
M. smegmatis strain lys. Rabinowitz	ND	ND	10	86	4

* in percent of methylating activity by the extracts prepared from cells in stationary phase

ND = not done

The optimum protein content of the extracts for methylation was also determined. Results are shown in Fig. 1. The minimum protein content causing the highest methylation of the acceptor proved to be 3 mg for strains Butyricum and *M. phlei*, and about 5 mg for lys. Rabinowitz and Rabinowitz. It will be shown later that this difference is the consequence of the lower methylating activity of the Rabinowitz strains.

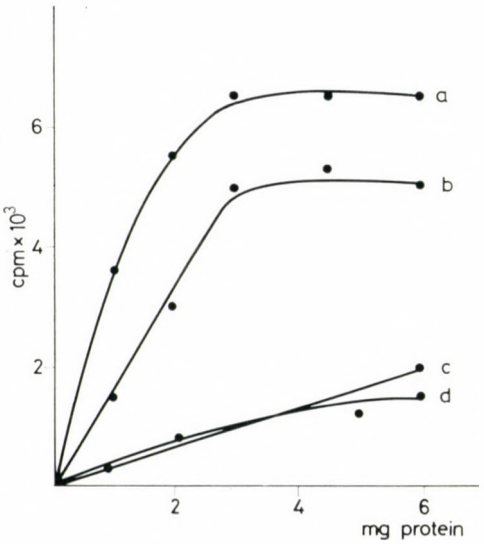

Fig. 1. Methylation of acceptor DNA and protein content of crude mycobacterial extracts. 0.5 ml incubation mixture contained 10 μg thymus DNA, 100 kBq/ml H^3–SAM, and different quantities of proteins of the crude extracts. Two hours incubation at 37 °C. a = strain Butyricum; b = *M. phlei*; c = strain Rabinowitz; d = strain lys. Rabinowitz

Figure 2 shows the kinetics of the methylase activity of the four mycobacterium strains. After 2 h incubation, maximum methylation of the acceptor was observed. The methylation levels using Butyricum and *M. phlei* extracts (curves *a* and *b* in Fig. 2) did not change during 22 h of incubation. This type of kinetics of enzymatic methylation is principally different from that of *Escherichia coli* SK and *Shigella sonnei* 47843 [7, 8]. Using these latter bacteria a sharp fall of the curves could be observed after 6 h of incubation and after 18 h the radioactivity of the acid insoluble fraction of the acceptor DNA was only 60 and 10% of the maximum levels, respectively. This phenomenon proved to be in correlation with the nuclease content of the extracts used, which was shown by special experiments determining the viscosity of phage Sd–DNA incubating it with enzyme containing extracts prepared from *E. coli* SK, and *S. sonnei* 47843.

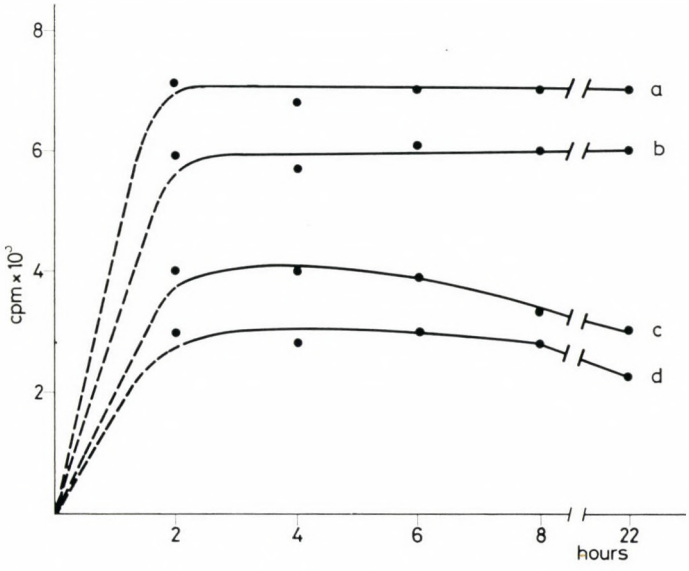

Fig. 2. Kinetics of thymus DNA methylation by extracts of mycobacterium strains. Experimental conditions and symbols are the same as in Fig. 1. (Broken line means that between 0–2 h no measurements were performed)

Figure 3 shows the relative viscosity of phage Sd–DNA incubated with extracts of different microorganisms. The relative viscosity of phage-DNA did not change during 60 min incubation with Butyricum and *M. phlei* extracts. On the contrary incubating the phage-DNA with *E. coli* SK-containing extract, the viscosity began to decrease after 20 min, and incubating it with *S. sonnei* 47843 extract, after 10 min. These results point to a correlation between the methylation of the acceptor DNA and the nuclease activity of the bacterium extracts studied. On the basis of these results the peculiar kinetics of methyla-

tion by Butyricum and *M. phlei* extracts can be explained with their very low nuclease activity.

As it is seen in Fig. 2, the lower radioactivity of acceptor DNA in a later period of incubation with Rabinowitz and lys. Rabinowitz extracts corresponds to that observed with *E. coli* and *S. sonnei* 47843 extracts, thus it can be assumed that in these cases the nuclease activity, too, influenced the methylating process.

On the basis of our experiments the optimal conditions of enzymatic methylation in vitro are: 100 μg acceptor DNA, crude mycobacterium extract corresponding to 3 or 5 mg protein. 100 kBq/ml H^3–SAM in Tris–HCl or phosphate buffer, pH 7.2–7.5, and 2 h incubation.

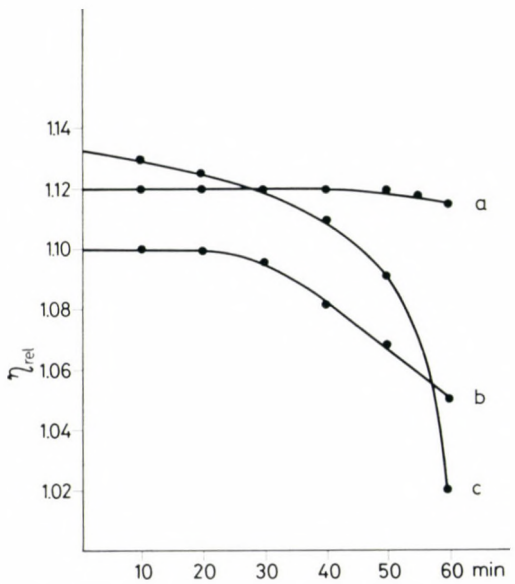

Fig. 3. Viscosity of Sd phage DNA under the effect of bacterial extracts. a = strain Butyricum; b = *E. coli*; c = *S. sonnei* 47843

Comparison of DNA-methylase activity of crude extracts of mycobacteria in homologous and heterologous systems

To determine methylase activity, several types of DNAs can be used as substrate, among these DNAs having no methyl groups at the site of recognition of the corresponding enzymes. In addition to DNAs prepared from mycobacteria and mycobacteriophages we have used also thymus and Sd phage-DNAs as acceptors. Thymus DNA being far in evolution from bacterial DNAs, thus the probability of the presence of common recognizing sites of methylases of animal and bacterial origin is very low. Phage Sd–DNA is not methylated

in vivo [9] but contains susceptible sites for bacterial enzymes and proved to be suitable for determining the activity of different methylase preparations.

Table II

Comparison of the DNA-methylating activity in homologous and heterologous systems

Bacterial strains	Radioactivity of acceptor DNA (cpm)					
	thymus	Sd phage	strain Butyricum	Butyricum phage	*M. phlei*	Phlei phage
M. smegmatis strain Butyricum	6300±200	8100±100	1800±150	1800±200	5250±150	5800±200
M. phlei	5000±150	6300±150	600±100	650±100	2000±200	1700±100
M. smegmatis strain Rabinowitz	2900±100	3700±150	2850±300	2200±150	2700±150	3000±250
M. smegmatis strain lys. Rabinowitz	3300±100	4000±150	2750±150	2900±250	3000±200	2500±200

Our results are summarized in Table II. The DNA of phage Sd proved to be the most sensitive substrate for the methylases of the bacterium strains studied. Thus, mycobacterial methylases are different from those of *E. coli* and *S. sonnei* 47843 for which thymus DNA is the most sensitive substrate. Table II also shows that the methylating activity of strain Butyricum and *M. phlei* is more expressed than that of Rabinowitz and lys. Rabinowitz, when thymus and phage Sd–DNAs are used as substrates. As no difference could be observed between the methylation of substrates by the Rabinowitz and lys. Rabinowitz extracts, a prophage induced methylation mechanism can be rejected. The same conclusion can be drawn from the fact that Butyricum and *M. phlei* DNA, as well as the DNAs of Butyricum and Phlei phages similarly accept the methyl groups by the effect of the enzymatic activity of all the four mycobacterium strains studied both in homologous (self DNA-self enzyme) and heterologous (self DNA-non self enzyme) systems. This can be explained by assuming that phage DNA is methylated during replication only by the enzymatic activity of the host cell.

It was an interesting and unexpected observation that *M. phlei* extract showed a very low methylating activity when Butyricum DNA and Butyricum phage DNA were used as substrates. This can be explained by the low number of susceptible sites on these substrates for *M. phlei* methylase, though these two mycobacteria belong to the same genus.

The intensive methylation in homologous systems, i.e. when self-DNA was used as substrate, is worth mentioning. The methylase activity of *M. phlei* and Butyricum extracts on self-DNA reached 25–30% of the methylase

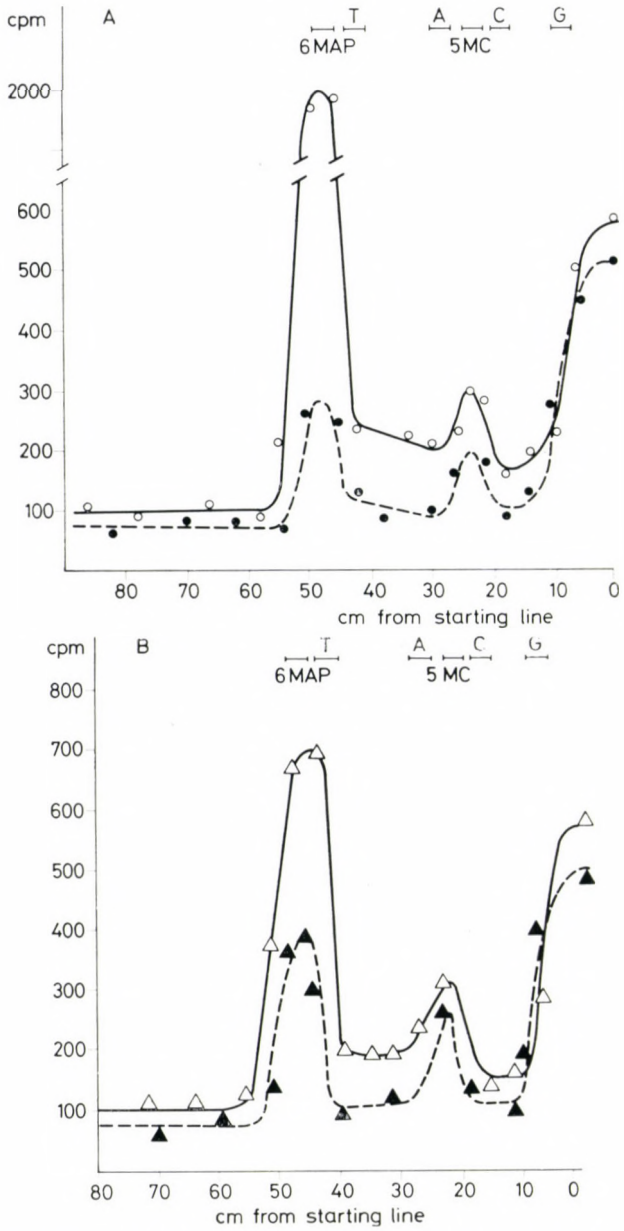

Fig. 4. Chromatography of hydrolysates of thymus DNA after in vitro methylation by mycobacterial extracts for identification of the methylated bases. Incubation mixtures (as described in Material and Methods) were treated with 2% perchloric acid after deproteinization and RNA removal. The acid-insoluble fraction was hydrolysed with a carrier in 70% perchloric acid. The hydrolysate was streaked on Whatman 1 paper and chromatographed inbutanol–water–ammonia (60:10:0.1 v/v). The chromatogram was cut into 2 cm wide bands, eluted with 0.1 M HCl, then radioactivity was determined. (A) Methylation by extracts of strain Butyricum ○————○, and Rabinowitz ●- - - - - - - -●. (B) Methylation by extracts of *M. phlei* △————△ and strain lys. Rabinowitz ▲- - - - - - -▲. T = thymine; A = adenine; C = cytosine; G = guanine; MAP = methyl-aminopurine; MC = methyl-cytosine

activity measured using the most sensitive substrate, which means that mycobacterial DNAs are less methylated in vivo than DNAs of *S. sonnei* 47843, *E. coli* and *Bacillus brevis* [5, 10, 11].

We have also studied which nucleic acid bases become methylated by the effect of the enzyme-containing extracts of mycobacterium strains. Thymus DNA was incubated in the presence of H^3-SAM with our enzyme-containing preparations and after perchloric acid hydrolysis paper chromatography was performed as described in Materials and methods. Figure 4 shows that 6-methyl-aminopurine and 5-methyl-cytosine were detected in every case.

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STIMULATION OF HUMAN PERIPHERAL LYMPHOCYTES WITH ENDOTOXIN AND RADIODETOXIFIED ENDOTOXIN

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(Received December 23, 1981)

The effects of parent endotoxin and radiodetoxified endotoxin on human peripheral lymphocytes were compared in experiments in vitro. Radiodetoxified endotoxin is able to exceed the degree of the stimulation induced by parent endotoxin and its stimulatory effect. At the same time, no change was observed in the presence of autologous serum. Radiodetoxified endotoxin did not inhibit the phytohaemagglutinin-induced proliferative response.

To our present knowledge, activation of B-lymphocytes and immunoglobulin synthesis is induced by polyclonal B-cell activators [1]. Endotoxins (LPS) of Gram-negative bacteria are known to have various effects on the immune system. They stimulate the B-lymphocytes, they can play an essential role in adjuvancy, and might elicit even a small synthesis of specific antibody [2–4].

In mice both spleen and peripheral lymphocytes respond to LPS stimulation by blastogenesis and antibody production. In humans the best stimulation by LPS was found in lymphoid cells from the spleen, lymph nodes, tonsils and the bone marrow, but it was ineffective in stimulating human peripheral lymphocytes (HPL) to blast transformation [4–7].

The finding that LPS treatment might increase the antibody secretion, in spite of its slight effect on blast transformation in lymphocytes from human tonsils and in HPL points to an effect of LPS on a subpopulation of B-lymphocytes present among the lymphocytes.

LPS is known to be toxic, a fact greatly inhibiting its practical use. Promising are, however, the results obtained in experiments with radiodetoxified endotoxin (rdLPS), in vivo. Apart from a significant adjuvant activity, enhanced regeneration of the immune system could be observed under the influence of radiodetoxified endotoxin following radiation injury [8, 9]. The complement inactivating effect of parent endotoxin was much weaker when rdLPS was applied; its hypotensive effect was also considerably weaker

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[10]. The above mentioned advantageous changes together with other favourable features of rdLPS have been summarized by Bertók [11].

The present paper describes the stimulation of HPL in vitro, by parent LPS and rdLPS, detoxified with ionizing radiation.

Materials and methods

Cell suspension. Venous blood from healthy donors was used in the experiments. Samples were collected in 500 ml sterile blood transfusion bottles containing 10 000 U/litre heparin (National Institute for Haematology and Blood Transfusion). The mononuclear cells were obtained from blood diluted 1:1 with physiological saline and centrifuged in Ficoll-Uromiro gradient.

Cell culture, maintenance fluid and reagents. Lymphocytes were washed in PBS 3 times and cultured in Parker's 199 medium supplemented with penicillin (100 000 U/litre), 10% inactivated AB serum or autologous serum, and glutamine (100 mg/litre) in Falcon tubes in air containing 5% CO_2 for 3–6 days at 37 °C. Each culture contained 5×10^6 cells in 5 ml volume.

The sera were obtained from healthy humans of blood group AB. Following inactivation at 56 °C, they were stored at —20 °C.

Endotoxin preparation. Westphal extraction [12] from *Escherichia coli* O89 was used (LPS).

Radiodetoxified endotoxin. Westphal extraction was irradiated by a 50 kGy ^{60}Co gamma dose (rdLPS) [10, 11].

Phytohaemagglutinin. (PHA–P) Difco Bacto.

Stimulation. In 5 ml Parker's 199 medium 5×10^6 lymphocytes were incubated together with 10–100 μg LPS and rdLPS in the presence of 10% AB or autologous sera. Four hours before concluding the incubation, the cells were labelled with 1 μCi ^3H thymidine/culture (Prague, Czechoslovakia, 1 Ci/mmol). After labelling, the cells were washed in physiological saline, 5% TCA and 96% ethanol, then after extraction by hyamine-hydroxyde the radioactivity incorporated in the lymphocytes was measured in a Diotol cocktail, using a liquid scintillation spectrometer. Results and the standard error are given in dpm.

Results

The kinetics of LPS stimulation did not indicate any significant increase in DNA synthesis before the 5th day. After the 5th day the DNA synthesis enhancing effect of the tested LPS increased (Fig. 1). Stimulation by both unirradiated and irradiated LPS measured on the basis of the incorporation of tritiated thymidine given in dpm, reached its maximum at a dose of 10 μg LPS/culture and in the case of rdLPS at a dose of 50–100 μg/culture on day 6, examined in the presence of AB serum (Fig. 1).

A study of the stimulatory effect of detoxified and non-detoxified LPS in the presence of AB and autologous sera (Table I) showed that it was highly dependent on the type of serum in the case of parent LPS. The stimulatory value obtained with AB sera was higher than in the presence of autologous serum in the case of parent LPS, while the stimulation by rdLPS seemed to be independent of the type of serum. Thus, in the presence of autologous serum, rdLPS in a dose of 100 μg/culture elicited a stronger response than LPS in a dose of 10 μg/culture.

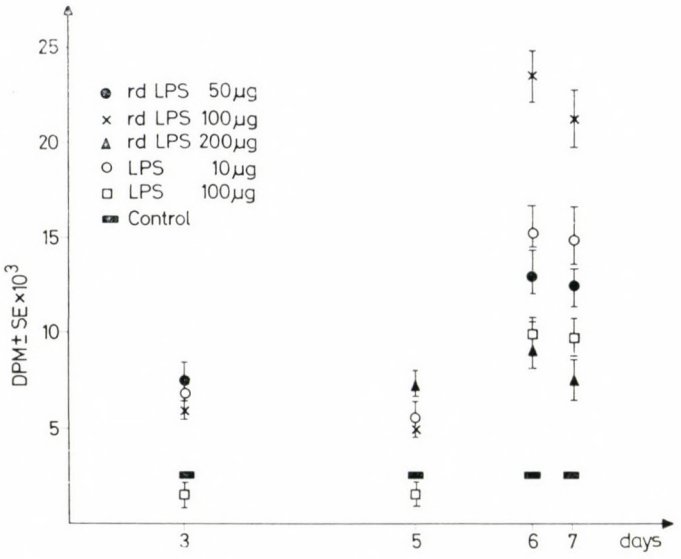

Fig. 1. Kinetics of the stimulatory effect of LPS. DNA synthesis by human peripheral lymphocytes in the presence of 10% AB serum was measured by ^{3}H-thymidine incorporation with different LPS doses

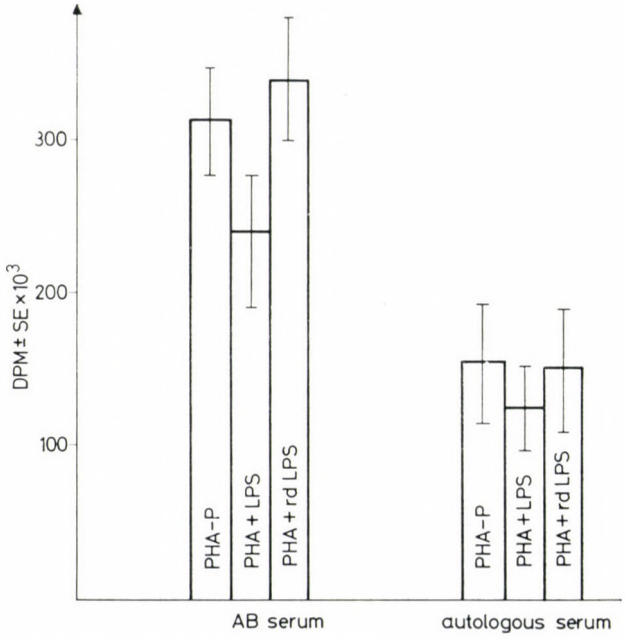

Fig. 2. Combined effect of PHA and LPS on the stimulation of human peripheral lymphocytes

The effect of LPS on the mitogenic response of lymphocytes induced by PHA in the presence of various sera, showed that rdLPS decreased the tritiated thymidine incorporation neither in the presence of AB nor of autologous sera. LPS decreased the PHA induced stimulation if used with AB or autologous serum (Fig. 2).

Table I

Effect of sera on the stimulatory characteristics of LPS

Serum	DPM ± S.E.		
	LPS	rdLPS	Control
AB	22.773±5.803	22.301±4.674	2.500±480
Autologous	14.613±2.584	21.239±4.145	2.841±700

Discussion

Several authors have reported on stimulation induced by LPS and on the degree of transformability in various lymphocyte cultures [2–6]. The kinetics of lymphocyte stimulation differs according to the origin of the lymphocytes (mouse or human). In humans it depends on their origin from a lymphoid organ, or pheripheral blood, and on the experimental circumstances [13]. Results showed maximal stimulation of HPL on days 6–7, while no stimulatory effect could be measured before the 5th day. These findings agree with several data in the literature [9, 13, 14].

In the case of parent endotoxin (LPS) the stimulation measured on the basis of tritiated thymidine incorporation was maximal in the presence of AB serum. The stimulatory effect of rdLPS, however, was independent of the type of the applied serum. This was probably due to the different toxicity of the two substances.

It is important to determine the optimal culturing conditions, to select a suitable serum. In order to obtain maximum stimulation, human serum must be added [9]. The stimulatory effect of LPS was more intensive in the presence of inactivated deep frozen AB serum than with autologous serum. Most authors have used commercially available calf or human sera [1–9]. These sera can promote the cell transformation induced by mitogens to reach the required level. Our experiments showed that mitogens exerted their maximal effect in the presence of inactivated freshly frozen AB serum. It is favourable that rdLPS showed a good stimulatory effect with both the applied serum types, and they did not inhibit the PHA induced proliferative response.

It is not easy to explain the results. It is possible that the lymphocyte stimulation elicited by LPS does not reach a measurable degree in the presence of autologous serum, only in the presence of AB serum.

Our results with various sera agree with the observation of Miller et al. [9] in that a better stimulation can be reached in the presence of pooled inactivated human sera. Using LPS in the presence of AB serum under the effect of LPS an inhibitory substance might be released by the macrophages which can prevent even the strong mitogenic effect of the PHA. It is considered a prostaglandin like effect [15], and the released substances inhibit T-helper cell activity [16].

Previous experiments have shown that the PHA-induced mitogenic response of lymphocytes of patients suffering from pseudomonas infection depends on the condition of the patient and on the applied serum of probably different endotoxin content [17, 18]. Our model experiments on lymphocytes from normal healthy humans were devised to find the explanation of these results. We have compared the effect of toxic endotoxin and detoxified endotoxin on the PHA induced transformation of lymphocytes in the presence of various sera. The activity of radiodetoxified endotoxin showed that it reaches and might even exceed the rate of stimulation of HPL induced by LPS, while it does not inhibit the PHA-induced stimulation in the presence of autologous sera.

These in vitro results might support the in vivo studies of Skarnes and Harper [15] who reported on the prostaglandin release induced by LPS.

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THE COURSE OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN GERMFREE MICE TREATED WITH *BORDETELLA PERTUSSIS* VACCINE

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A single injection of *Bordetella pertussis* vaccine, applied intraperitoneally one day before intracerebral lymphocytic choriomeningitis virus infection depressed the immune response both in conventional and germfree adult mice, but the rate of the immunosuppressive effect differed. In adult mice with a normal immune system the vaccine only delayed the manifestation of fatal lymphocytic choriomeningitis, while it prevented its development in germfree mice with an underdeveloped lymphoid system, i.e. it inhibited the cellular immune response to the virus infection.

The course of intracerebral lymphocytic choriomeningitis (LCM) virus infection depends on the cellular immune responsiveness of the animals. Lymphocytic choriomeningitis develops in adult mice with a mature intact immune system, due to the cell-mediated immune response to the virus infection. These animals die on the 6th to 8th day after the infection [1]. The meningitis arises of a cell-mediated immune reaction to the leptomeningeal cells which display, due to virus reproduction, changed (viral) antigens. Cytotoxic T lymphocytes play a basic role in the disease [2, 3]. In mice with insufficient T lymphocyte function due to their impaired or immature lymphoid system, meningitis does not develop after the viral infection. They survive the infection and become virus carriers. On the other hand, substances which exert a stimulating immunomodulatory effect on the cellular immune response, contribute to the development of lymphocytic choriomeningitis [4, 5].

Bordetella pertussis vaccine is generally known to have immunomodulatory effects. These effects are not homogeneous. They may be stimulatory or suppressive depending on the given conditions. Previous experiments have indicated that the effect of pertussis vaccine on the course of LCM virus infection is age dependent, in that it decreases the cellular immune response

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to the infection in adult mice with mature immune system whereas it enhances it in suckling mice with undeveloped lymphoid system [6, 7].

Germfree mice have an underdeveloped lymphoid system even in adulthood due to the scarcity of antigenic stimuli in their environment [8]. Adult germfree mice did not develop meningitis following intracerebral LCM virus infection [9], their immune response was found similar to that of suckling mice with a likewise undeveloped lymphoid system. In both cases the absence of LCM indicates an insufficient T-lymphocyte function and the reduction of the cellular immune response.

The present experiments in conventional and germfree mice aimed at the exploration of the immunomodulatory effect of *B. pertussis* vaccine on the cellular immune responsiveness to LCM virus infection in adult germfree mice.

Materials and methods

Experimental animals. Germfree and conventional 5 to 6-week-old C3H mice of both sexes (LATI, Gödöllő, Hungary) were used.

Germfree state was maintained in plastic isolators (VELAZ PO-1, Czechoslovakia). These mice were fed gamma sterilized pellets and autoclaved water ad libitum. The germfree state of the outer and inner environment was controlled by culture media as suggested in the literature [10].

B. pertussis vaccine treatment. The vaccine contained 30×10^9/ml killed bacteria suspended in physiological saline (Institute for Serobacteriological Production and Research Human, Budapest). The mice received single intraperitoneal injections of 0.3 ml vaccine containing 9×10^9 bacteria/mouse. Control mice were treated with physiological saline administered in the same way and quantity.

LCM virus infection. The WE strain was maintained in serial mouse brain passages. The mice were infected by intracerebral inoculation with 100 LD_{50} of the pretitrated virus.

Recovery of the virus. The brain suspensions prepared from the surviving mice were inoculated intracerebrally to mouse groups in a 1:10 dilution. Presence of LCM virus was confirmed by the characteristic neurological symptoms in the inoculated mice.

Absolute lymphocyte count and lymphocyte-index were determined from blood taken from the caudal vein under standardized conditions. Lymphocyte index:

$$\frac{\text{mean absolute lymphocyte count in the test group}}{\text{mean absolute lymphocyte count in the control group}}$$

Relative spleen weight and spleen index of the sacrificed animals were determined as follows:

$$\text{Relative spleen weight} = \frac{\text{spleen weight, mg}}{\text{body weight, g}}$$

$$\text{Spleen index} = \frac{\text{mean relative spleen weight in the test group}}{\text{mean relative spleen weight in the control group}}$$

Histology. The brains were fixed in formalin, embedded in paraffin, and the sections were stained with haematoxylin–eosin.

Statistical evaluation of data. Evaluation of the results was carried out by Student's two sample t test. The accepted level of significance was $p = 0.05$.

Results

Thirty germfree and 30 conventional mice were treated with pertussis vaccine and, simultaneously, 30 germfree and 30 conventional mice received physiological saline. Two-thirds of both vaccinated and physiological saline treated mice were infected with LCM virus one day after the treatment. The rest of the mice received normal (virus-free) brain suspension in the same way.

Table I demonstrates the experimental groups, the number of mice in each, and the applied treatments.

The experiments were concluded on the 21th day after virus infection. The neurological symptoms characteristic of LCM and the death rate among the animals were registered in the virus infected groups. Recovery of the virus was performed using one half of each animal's brain that had survived the virus infection until the 21st day, when they were sacrificed. The other brain halves were prepared for histology.

Table I

Experimental mouse groups and treatments

Groups	No. of animals	Treatment	
		intraperitoneal	intracerebral
GF–P–LCM	20	pertussis vaccine	LCM virus
GF–LCM	20	physiological saline	LCM virus
GF–P	10	pertussis vaccine	×
GF	10	physiological saline	×
P–LCM	20	pertussis vaccine	LCM virus
LCM	20	physiological saline	LCM virus
P	10	pertussis vaccine	×
C	10	physiological saline	×

× = normal (virus free) brain suspension

The rate and time curve of death in the LCM virus infected groups during the experiment are presented in Fig. 1.

All virus infected conventional mice died with characteristic neurological symptoms on the 7th or 8th day after infection (group LCM). The mice in the groups GF–LCM and P–LCM died later, on the 7th to 13th day. In contrast, in the group GF–P–LCM 90% of the mice survived the infection. The virus could be recovered from the brain of each surviving animal in this group whereas the lymphocytic infiltration of the leptomeninx could not be demon-

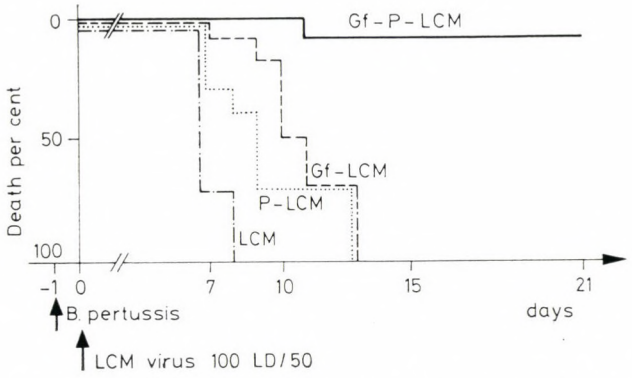

Fig. 1. Rate and time of death in groups infected with LCM virus

strated histologically. In group GF–P–LCM the surviving mice had, thus, become asymptomatic virus carriers.

The effect of pertussis vaccine on the lymphoid system was examined in the groups which had not been infected by LCM virus. Figure 2 demonstrates the mean absolute lymphocyte counts and relative spleen weights as determined on the 5th day after vaccine treatment. The mean absolute lymphocyte count and the mean relative spleen weight were significantly lower in the germfree mice (group GF) than in the conventional animals (group C), whereas the mean values obtained in the groups treated with pertussis vaccine (GF–P, C–P) were significantly higher. We found that pertussis vaccine had caused a lymphocytosis and spleen hypertrophy both in the germfree and conventional mice by the fifth day after treatment, and the alterations were of approximately the same extent (lymphocyte-index: Gf–P: Gf = 3.3; P:C = 3.2; spleen-index: Gf–P:Gf = 2.5; P:C = 2.6).

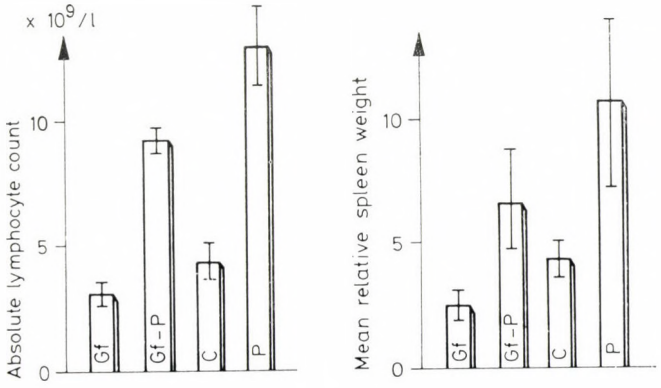

Fig. 2. Absolute lymphocyte count and mean relative spleen weight on the 5th day after treatment

Discussion

In our earlier experiments [11] the reaction to *B. pertussis* vaccine of the underdeveloped lymphoid system of germfree mice was similar as that of conventional mice, thus the lymphocytosis in peripheral blood and the spleen hypertrophy were of the same degree.

Simultaneously with the spleen hypertrophy and lymphocytosis due to the pertussis vaccine treatment, the cellular immune reactivity to LCM virus infection was suppressed in both conventional and germfree mice. Thus, an immunosuppressive effect was observable due to the immunomodulatory

Table II

Immunmodulatory effect of B. pertussis vaccine on the cellular immune reaction to LCM virus infection in mice

Mice	Immune system		Immunomodulatory effect of pertussis vaccine
	state	function	
Conventional adult	mature	normal	suppression (delay)
Conventional suckling	undeveloped	insufficient	stimulation
Germ-free adult	underdeveloped	insufficient	suppression (inhibition)

pertussis vaccine in both GF and conventional mice, but its rate differed in the two groups. The vaccine delayed the development of fatal lymphocytic choriomeningitis in conventional mice having a mature immune system, while it prevented the disease in germfree mice with underdeveloped lymphoid system, that is, it inhibited the cellular immune reaction to the virus infection.

Our previous results [9] showed that the immune response of adult germfree mice to LCM virus infection was similar to the immune response of conventional suckling mice with likewise undeveloped lymphoid system. In both cases the immune response was weaker than that of conventional adult mice. Nevertheless, the immunomodulatory effect of *B. pertussis* vaccine proved to be the opposite in mice with undeveloped lymphoid system due to various reasons: the vaccine had a stimulatory effect in suckling mice [7], while it was immunosuppressive in germfree adults (Table II).

Thus, the effect of pertussis vaccine on the lymphoid system was similar in its mode of action in germfree and conventional adult mice but it was different in degree. The effect, however, differed in the germfree and in the conventional suckling mice. In the germfree animals it had an opposite direction as compared to the sucklings.

Our results suggest that the presence or absence of the normal microbial flora may influence the reaction of the immune system to immunomodulatory effects.

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THE EFFECT OF MICONAZOLE ON ERGOSTEROL-LESS MUTANT OF *CANDIDA ALBICANS*

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The effect of miconazole in fungistatic concentration on an ergosterol-competent *Candida albicans* strain and its ergosterol-nonproducing mutant was investigated by scanning and transmission electron microscopy. The ergosterol-less mutant showed pronounced alterations by scanning electron microscopy. The increased sensitivity to miconazole of the ergosterol-less mutant was attributed to an altered plasma membrane composition and rigidity.

Detailed investigations have demonstrated that miconazole (1-[2,4-dichloro-β-/2,4-dichlorobenzyl oxy/-phenethyl] imidazole nitrate) is active against most pathogenic fungi and against Gram-positive bacteria [1]. It has been shown that the primary sites of action of miconazole at low concentration are the plasma membrane and cell wall. Induced permeability changes result in a selective inhibition of the uptake from the medium of some essential growth precursors and in the leakage of these intracellular components, the effects depend on the dose of the drug and the duration of treatment [2–4]. The drug has been shown to be a potent inhibitor of fungal ergosterol biosynthesis, preventing demethylation at C-14 and reduction of the 24/28 double bond [5].

We describe the effects of miconazole on an ergosterol-producing strain of *Candida albicans* and its ergosterol-less progeny.

Materials and methods

Strains. The ergosterol-producing, nystatin-sensitive strain of *C. albicans*, designated *33 erg*$^+$ (ATCC 44829), and its ergosterol-less, nystatin-resistant mutant, designated *erg-16* (ATCC 44830), were isolated and characterized as described earlier [6, 7].

Culture conditions. The minimal inhibitory concentrations were determined on the surface of media containing a two-fold dilution series of miconazole nitrate (donated by I. Kurutz, Chemical Works of Gedeon Richter Ltd., Budapest), as for nystatin [7]. It was also

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determined using shaken cultures [4] in complete liquid medium [7] containing 5×10^6 colony-forming units per ml. Twenty five hour cultures of treated and non-treated strains grown in the presence of 20 μg/ml miconazole at 30 °C were subjected to electron microscopic examination.

Electron microscopy. Scanning electron microscopy (SEM) studies were carried out according to Pugh and Cawson [8] .The objects were examined in a Tesla BS 300 scanning electron microscope at 15 KV and a 45° specimen tilt. For transmission electron microscopy (TEM), samples were prepared by the method of May [9], ultrathin-sectioned and studied in an EM 3–2 of VEB Werk für Fernsehelektronic, Berlin, at 60 KV.

Results and discussion

Fungistatic concentration of miconazole. The minimal inhibitory concentration of the nystatin-sensitive *33 erg*$^+$ strain and its nystatin-resistant *erg-16* mutant was 20 μg/ml; this resulted in complete inhibition of growth on the surface of media and in shaken cultures. This concentration of miconazole, however, did not cause a decrease of the colony-forming units but only blocked the multiplication of cells. Thus, both types of cell were able to survive this drug treatment but further cell propagation was totally inhibited.

Electron microscopic observations. Untreated log-phase cells of both strains were spherical to elongated in shape and smooth-walled, showing multipolar formation of buds on SEM (Figs 1, 3). The surface of cells of the *33 erg*$^+$ strain exposed to the fungistatic dose of miconazole (20 μg/ml) were slightly wrinkled and displayed randomly distributed bud scars (Fig. 2), in agreement

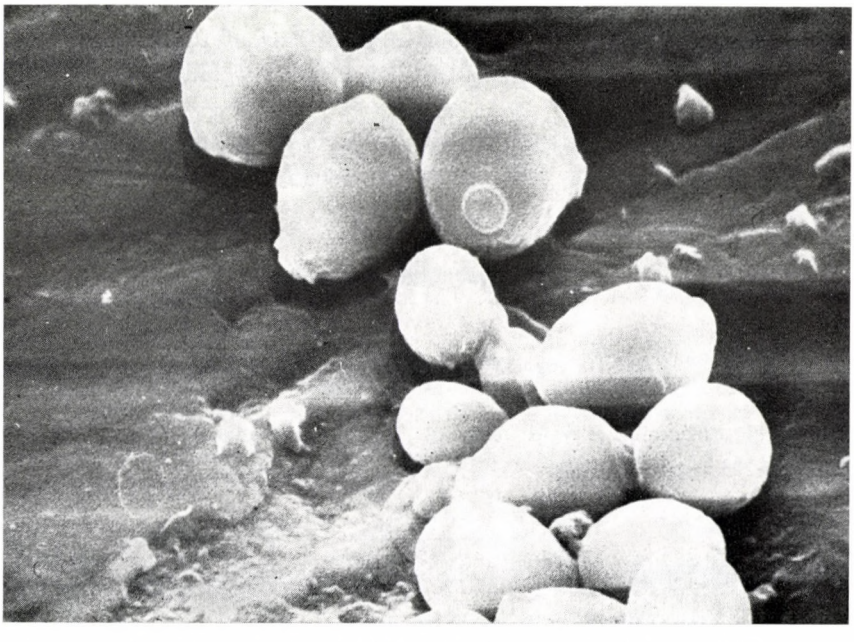

Fig. 1. Untreated cells of *C. albicans 33 erg*$^+$ strain. $\times$10 200

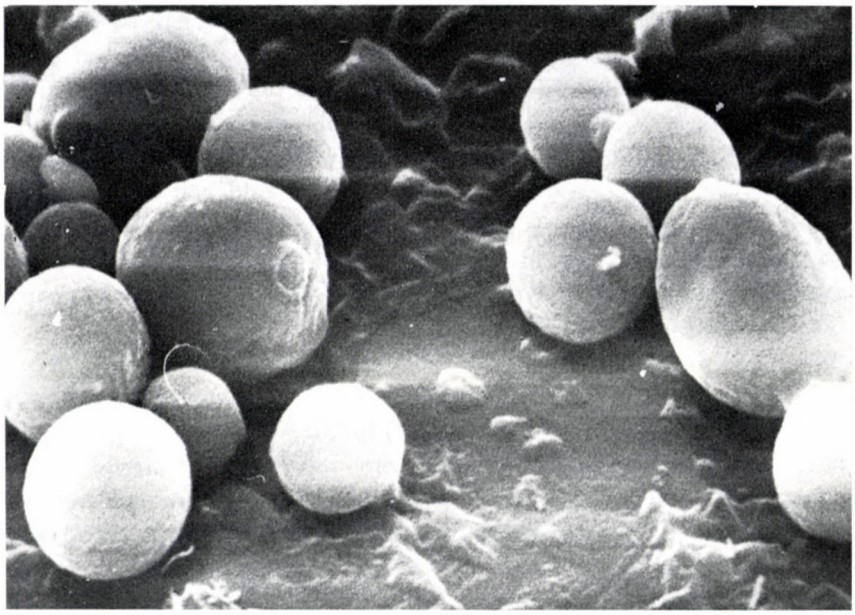

Fig. 2. Miconazole-treated (20 μg/ml, 30 °C, for 25 h) cells of *C. albicans 33 erg*$^+$ strain. ×10 100

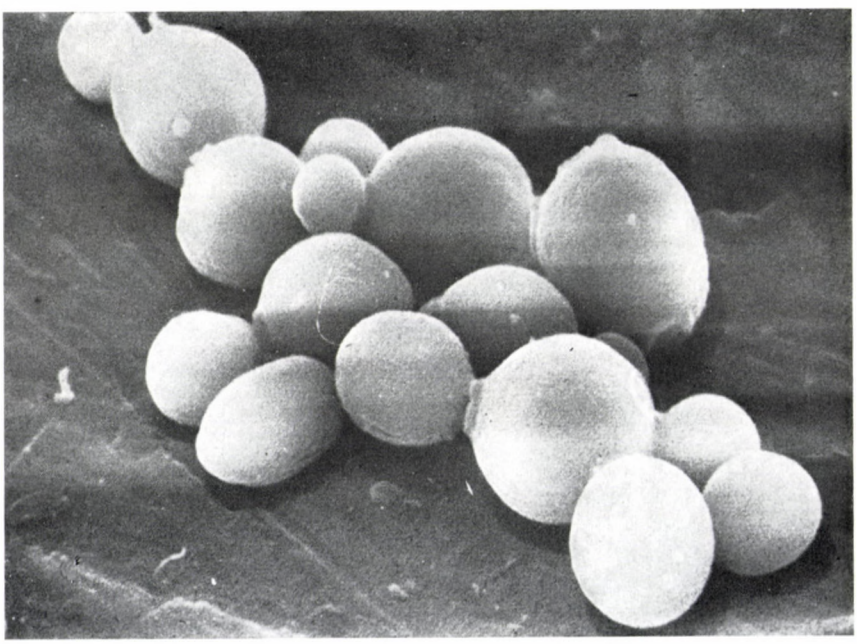

Fig. 3. Untreated cells of *C. albicans erg-16* strain. ×10 000

with the findings of De Nollin and Borgers [10]. Daughter cells seemed to be separated from the mothers in spite of the presence of the drug. The cells became more spherical and in agreement with earlier findings [11] appeared to have grown in size as a consequence of blocked ergosterol synthesis. The same was observed in sterol mutants of *C. albicans*, too [12, 13]. The hypothesis that the absence of ergosterol results in an increased cell volume [12] was proven by Pesti and Ferenczy [14] who produced complementing and non-complementing diploid hybrids for ergosterol synthesis of ergosterol-less mutants of *C. albicans*. It is worth mentioning that an increased ion leakage of these sterol mutants was observed.

The same treatment of *erg-16* cells resulted in pronounced alterations of the cell surface (Fig. 4) in comparison to the 24 h control of untreated *erg-16* cells (Fig. 3) and of treated *33 erg*$^+$ cells (Fig. 2): some cells had deep depressions in the cell wall, while most of them were deformed and thread-like formations were visible among them. These formations resemble the ornamentations of the cell surface of nystatin-treated *33 erg*$^+$ cells revealed by freeze-fracture electron microscopy [7].

No such significant differences between the two types of treated cells were observed by TEM. The alterations found were similar to those described by De Nollin and Borgers [2] using a fungistatic concentration of miconazole for 24 h.

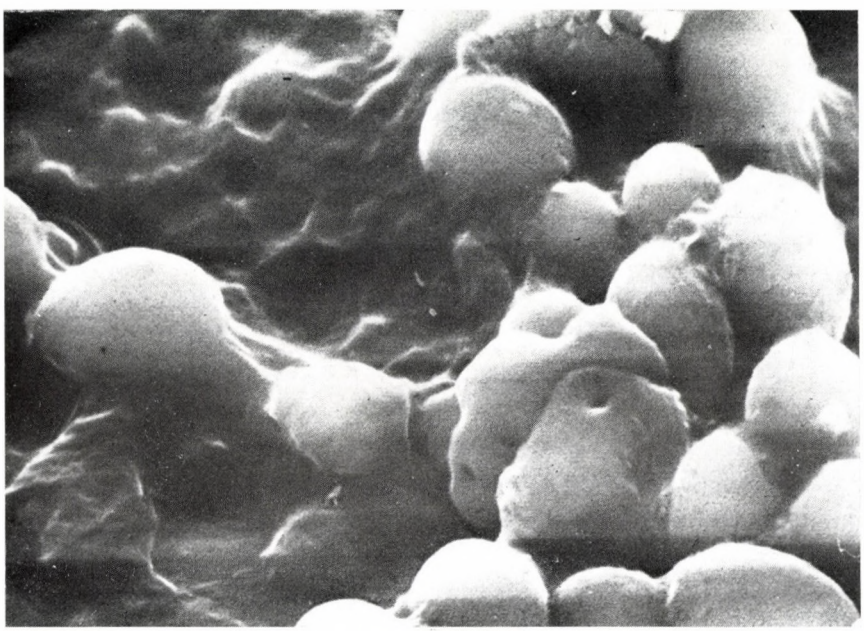

Fig. 4. Miconazole-treated (20 μg/ml, 30 °C, for 25 h) cells of *C. albicans erg-16* strain. ×10 000

It has been reported that the *erg-16* sterol mutant has a 16-fold level of resistance to nystatin and a significantly higher plasma membrane order parameter than that of the parental *33 erg*$^+$ strain [6]. This and other sterol mutants were twice as susceptible to chloral hydrate as the ergosterol-producing strains [14]. Sterol mutants of *Saccharomyces cerevisiae* were more permeable to crystal violet and mono-, di- and trivalent cations than their ergosterol-competent parents [15]. On the basis of these results it is presumed that the pronounced alterations of miconazole-treated cells of the *erg-16* strain revealed by SEM were a consequence of its ergosterol-less, altered, more rigid plasma membrane being more sensitive to the drug.

These results show that (i) nystatin-resistant sterol mutants have the same or a somewhat increased sensitivity to miconazole as the nystatin-sensitive, ergosterol-producing strains. Thus, miconazole and other clinically important imidazole agents may cure patients who harbour nystatin-resistant variants of *C. albicans*. (ii) To study the sterol-blocking effect of miconazole separately from other possible ones, one should not look for sterol mutants in which demethylation of the sterol ring is blocked.

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EFFECT OF *BORDETELLA PERTUSSIS* VACCINE ON THE SENSITIVITY TO A LYMPHOTROPIC CYTOSTATIC AGENT IN GERMFREE MICE

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A single injection of 15 mg/kg dianhydrodulcitol caused death at a higher proportion of germfree, specific pathogen free, and conventional mice with lymphocytosis and spleen hypertrophy induced by *Bordetella pertussis* vaccine pretreatment, than of unvaccinated controls.

Previous studies have shown an increased sensitivity to the lymphotropic cytostatic agent dianhydrodulcitol (DAD) in mice with impaired lymphoid system due to various causes [1–3], in newborn and suckling mice [4], and in aged mice with thymic involution [5]. Otherwise the sensitivity of germfree mice to DAD was found to be decreased [6].

In mice treated with *Bordetella pertussis* vaccine, spleen hypertrophy and lymphocytosis develop under both conventional and germfree conditions [7, 8]. Our previous experiments have revealed an increased sensitivity to DAD in conventional mice with their lymphoid system hypertrophic due to vaccine treatment [9].

The present experiment was undertaken to examine the effect of pertussis vaccine on the DAD sensitivity of germfree mice.

Materials and methods

Experimental animals. The experiments were carried out on 5–6-week old, germfree (GF), specific pathogen free (SPF), and conventional (CV) C3H mice of both sexes weighing 20–25 g, purchased from the Zootechnical Institute of Laboratory Animals (LATI, Gödöllő, Hungary). The germfree state was maintained with plastic isolators (VELAZ–PO-1, Czechoslovakia). Microbiological control of the germfree state was performed as described in the literature [10]. The germfree mice were fed with gamma ray sterilized pellets and autoclaved water *ad libitum*.

Treatment with dianhydrodulcitol (NSC-132313). Dianhydrodulcitol is a lymphotropic cytostatic agent of the alkylating group. The substance (Chinoin Chemical and Pharmaceutical Works Ltd., Budapest) was dissolved in distilled water and then used within 30 min. The mice

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were treated with a 15 mg/kg DAD dose intraperitoneally. The control mice received sterilized physiological saline administered in the same way and quantity.

Treatment with B. pertussis vaccine. The vaccine contained 30×10^9/ml killed bacteria suspended in physiological saline (Institute for Serobacteriological Production and Research Human, Budapest). The mice received a single intraperitoneal injection of 0.3 ml containing 9×10^9 bacteria. The control mice were treated with physiological saline administered in the same way and quantity.

Examination of the lymphoid system. Absolute lymphocyte counts were determined from blood taken from the caudal vein under standardized conditions.

The relative spleen weight and spleen index of mice which had died or were sacrificed during the experiment, were determined as follows:

$$\text{Relative spleen weight} = \frac{\text{spleen weight, mg}}{\text{body weight, g}}$$

$$\text{Spleen index} = \frac{\text{mean relative spleen weight in the test group}}{\text{mean relative spleen weight in the control group}}$$

Statistical evaluation of data. Evaluation of the results was carried out by Student's two-sample t test. The accepted level of significance was $p = 0.05$.

Results

Mouse groups were inoculated with *B. pertussis* vaccine or physiological saline. Then 6 days later part of both the vaccinated and the physiological saline treated mice received DAD in a dose of 15 mg/kg. The rest of the mice received physiological saline. Table I demonstrates the experimental groups, their size and the treatments.

The mouse groups were observed till the 21st day after DAD treatment.

Figure 1 shows the rate and time curve of mortality due to DAD treatment.

In accordance with our previous results, a 15 mg/kg DAD dose caused no death among GF mice, while it induced a mortality rate of 45% in SPF mice, and 70% in CV mice (DAD groups). The same dose of DAD caused a 20% mortality in GF mice pretreated with pertussis vaccine, and 100% mortality in the vaccinated SPF and CV mice. There was no difference in the time of death of the different mouse groups.

Table I

Mouse groups and treatments

Group	Type and number of mice			Treatments	
	GF	SPF	CV	0 day	6th day
P–DAD	20	20	20	*B. pertussis* vaccine	DAD
DAD	30	20	20	Physiological saline	DAD
P	12	12	12	*B. pertussis* vaccine	Physiological saline
C	12	12	12	Physiological saline	Physiological saline

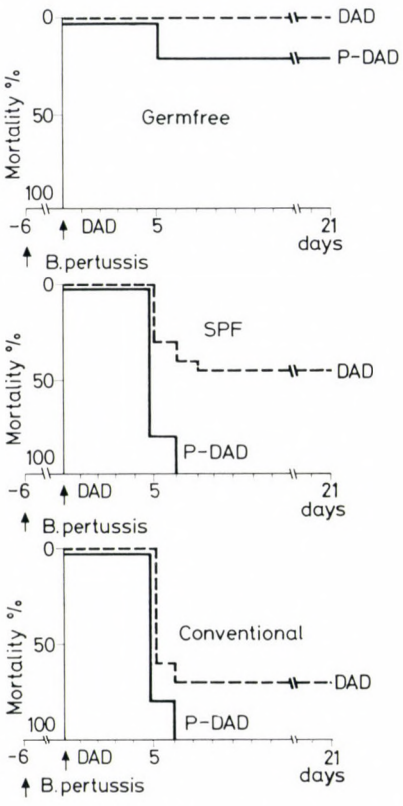

Fig. 1. Rate and time of death after *Bordetella pertussis* vaccine and DAD treatment

Table II

Data concerning the lymphoid system on the 6th day after DAD treatment

	Groups	Mean absolute lymphocyte count $\times 10^9$		Mean relative spleen weight		Spleen index DAD/C	Spleen index P-DAD/P
	P–DAD	—		1.8 ± 0.1			
	DAD	—		1.5 ± 0.6	$0.1 > p > 0.05$		
GF	P	2.2 ± 0.7	$p > 0.5$	8.5 ± 1.8	$p < 0.001$	0.49	0.23
	C	1.8 ± 0.6		3.0 ± 0.2			
	P–DAD	—		2.3 ± 0.4	$0.05 > p > 0.02$		
	DAD	—		1.8 ± 0.5		0.51	0.18
SPF	P	1.9 ± 0.5	$p > 0.3$	12.0 ± 2.7	$p < 0.001$		
	C	2.9 ± 1.2		3.5 ± 1.6			
	P–DAD	—		2.0 ± 0.5	$0.05 > p > 0.02$		
	DAD	—		1.4 ± 0.1			
CV	P	11.8 ± 3.5	$p < 0.001$	12.8 ± 3.8	$p < 0.001$	0.40	0.15
	C	2.5 ± 0.5		3.7 ± 0.7			

In order to examine the effect of pertussis vaccine on the lymphoid system, half of the mice in groups P and C, and one third of the germfree mice (10 mice) in the DAD group were sacrificed after blood sampling. Relative spleen weight and spleen index were determined in the mice which had died spotaneously or had been sacrificed. The obtained data are shown in Table II. The data in Table II show that the effect of pertussis vaccine is still very expressed on the relative spleen weight in groups P and C at the time of death due to DAD treatment. The absolute lymphocyte count was significantly higher only in conventional mice (groups P and C). DAD treatment caused grave lymphoid atrophy in the DAD and P–DAD groups, but the hypertrophic spleen of pertussis vaccine treated mice was more sensitive to the atrophy-inducing effect of DAD than the spleen of untreated mice, as indicated by their respective spleen index values.

Discussion

In accordance with our previous results [6, 9] in the present experiment we could find a decreased sensitivity to DAD in germfree mice as compared to conventional mice (Fig. 1, DAD groups), and a higher sensitivity to DAD in pertussis vaccine treated conventional mice than in untreated mice (Fig. 1). Pertussis vaccine treatment increased the sensitivity to DAD of both germfree and conventional mice. The data obtained led to the assumption that DAD caused lethal lymphoid atrophy in more animals treated with pertussis vaccine than in unvaccinated animals, independently of the presence or absence of a normal bacterial flora.

Spleen atrophy due to DAD treatment was approximately as grave in germfree mice as in the SPF and CV mice, but no death occurred among the germfree animals owing to the absence of a bacterial flora. The absence of a bacterial flora was responsible also for the lower mortality rate of germfree mice due to pertussis vaccine and DAD treatment.

Thus, in GF, SPF and CV mice with different sensitivity to DAD, the sensitivity to DAD had changed in the same direction, the change being an increase in sensitivity if spleen hypertrophy and lymphocytosis had been caused by the pertussis vaccine. The altered DAD sensitivity of the various mouse groups differed from the sensitivity of unvaccinated mice.

This observation is in accordance with our previous results concerning the effect of *B. pertussis* vaccine on the different stress sensitivity of germfree and conventional mice. This sensitivity changes in a similar direction if the mice receive pertussis vaccine and, consequently, develop lymphoid hypertrophy and lymphocytosis [8].

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EFFECT OF GLUTAURINE ON LIVER TUMOUR DEVELOPMENT AND ACUTE LEUKAEMIA INDUCED BY MC29 VIRUS IN TURKEY POULTS

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The effect of glutaurine (gamma-L-glutamyl-taurine, Litoralon®) on the take and development of hepatoma and acute leukaemia induced by MC29/L avian oncorna-virus has been investigated in turkey poults. Glutaurine significantly decreased the incidence of hepatoma, but had no significant effect on the lethality of MC29/L infected birds. The number of primitive myeloid cells was lower in the peripheral blood of glutaurine treated birds than in the untreated controls. Reverse transcriptase determinations in turkey fibroblast cell cultures indicated that glutaurine delays MC29/L virus expression.

Glutaurine (gamma-L-glutamyl-taurine, Litoralon®), a recently isolated hormone of parathyroid glands, has a wide range of physiologic effects [1, 2]. It acts synergistically with vitamin A [3, 4], antagonizes the effect of cortisone, and activates mesenchymal and macrophage cells [5, 6]. Glutaurine has a radioprotective effect, increasing the survival and mitotic activity of bone marrow cells of irradiated rats [7].

Vitamin A is known to stimulate the immune system, to increase the resistance to infection, to have antiviral properties [8] and to inhibit the manifestation of some epithelial tumours [9]. As glutaurine acts like vitamin A it seemed interesting to study its effects on virus induced tumours.

The avian RNA tumour virus MC29 induces primary hepatoma [10, 11] and acute leukaemia [12] in chicken and turkey [13, 14]. The liver variant of the virus (MC29/L) separated in our laboratory [15] has an increased hepatoma inducing activity [16] and thus appeared to be a suitable model for studying the effect of glutaurine in experimental tumour and leukaemia.

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Materials and methods

Experimental birds. Two weeks old British bronze-turkeys (Szentes, Hungary) of both sexes weighing 180–200 g were used. They were kept at 30 °C and their food was complemented with vitamins and chamomile-tea.

Cell culture. Primary turkey embryonic fibroblast cultures were prepared according to standard methods. Cells were plated into Falcon flasks (75 cm^2) using Parker's 199 medium supplemented with 10% fetal calf serum.

Viruses. The liver variant of myelocytomatosis virus 29 (MC29/L) was used throughout the *in vivo* and *in vitro* experiments [15]. It was inoculated intravenously (i.v.) into one to two-day-old turkey poults. Plasma from hepatoma-bearing birds was collected, pooled, stored at —70 °C and used as the stock of MC29/L virus.

In the experiments turkeys were inoculated i.v. with the 0.2 ml of the diluted plasma pool containing 10^5 LD_{50} virus.

Turkey fibroblast cultures were infected with the same stock of MC29/L virus (m.o.i. = = 100 virus particles per cell).

Glutaurine treatment. Glutaurine (synthetic preparation of 99% purity) was provided by Chinoin Pharmaceutical and Chemical Works (Budapest).

Turkey poults were inoculated intramuscularly (i.m.) with 100 μg/kg body weight of glutaurine dissolved in 0.9% NaCl solution 24 h before virus infection. After infection 400 μg/kg/24 h glutaurine was administered in the drinking water up to the end of the experiment. Consumption of drinking water was checked.

Control groups of turkeys were inoculated only with MC29/L virus and no glutaurine was added to the drinking water.

Primary turkey fibroblast cultures were treated with 100 μg/ml of glutaurine 24 h before and for six days after virus infection. At different intervals cell culture media were collected and changed for fresh media containing the same quantity of glutaurine.

Control cultures of turkey fibroblasts were infected with MC29/L virus but no glutaurine was added.

Reverse transcriptase assay. In the media of turkey fibroblast cultures the presence and relative titre of MC29/L virus were estimated by reverse transcriptase assay. Media were clarified at 10 000 *g* for 30 min and virus particles were sedimented at 100 000 *g* at 4 °C for 90 min. The pellet was resuspended in a buffer containing 0.01 M Tris–HCl pH 7.5, 0.1 M NaCl and 0.001 M EDTA. The standard exogenous reaction was performed as described earlier [17].

Blood cell analysis. To follow the development of leukaemia, blood smears were prepared at different intervals after infection, fixed with methanol and stained with Giemsa solution. The percentage of primitive myeloid cells characteristic of MC29 virus infection [15] was determined by counting 400 white blood cells per smear. Mean values for six birds were calculated.

Statistical analysis was performed by the variation analysis test (δ).

Results

Tumour incidence. The effect of glutaurine on the incidence of liver tumour and lethality of turkeys infected with MC29/L virus was investigated. Table I shows that glutaurine applied in a single i.m. injection before and orally after virus infection significantly decreased the incidence of hepatoma.

In Experiment 1 (49 days of observation) and in Experiment 2 (56 days of observation), liver tumour developed in less than half of the birds in the glutaurine-treated groups than in the non-treated groups. The difference proved to be significant ($\delta > 3$). Besides, in the glutaurine-treated birds only single hepatomas 1–2 mm in diameter developed, while numerous tumours 8–12 mm in diameter were found in the liver of the control turkeys.

Table I

The effect of glutaurine on the incidence of hepatoma induced by MC29/L virus in turkey poults

Experiment	Treatment of turkey poults	No. of birds	Period of observation (days)	Birds with liver tumous***	
				No.	%
1	Glutaurine*	25	49	7	28
	—**	22	49	16	73
2	Glutaurine	20	56	5	25
	—	26	56	12	46

* Glutaurine (100 μg/kg) was injected i.m. into turkey poults; 24 h later birds were infected i.v. with MC29/L virus (10^5 LD_{50} per 0.2 ml), then 400 μg/kg/24 h glutaurine was administered in the drinking water up to the end of the experiment.

** Turkeys were infected i.v. with the same dose of MC29/L virus as in the glutaurine treated group.

*** In variation analysis test $\delta > 3$ in both experiments.

Table II

Percentage of primitive myeloid cells in peripheral blood of turkeys infected with MC29/L virus

Treatment of turkey poults*	Per cent of primitive myeloid cells			
	14	24	34	44
	days after infection			
Glutaurine	31.1 ± 6.2**	21.8 ± 6.0	20.6 ± 4.0	27.5 ± 9.5
—	34.4 ± 7.8	34.0 ± 4.7	39.8 ± 5.1	43.7 ± 6.3

* For virus infection and glutaurine treatment see the notice under Table I

** Mean values ± SD for 6 birds

Figure 1 shows the dynamics of tumour incidence in Experiment 1. At any interval after virus infection the number of tumour bearing birds in the glutaurine-treated group was about one half of those in the control group. Figure 1 also shows the lethality of the birds of the two groups. It is obvious that lethality was higher than the incidence of tumour as a number of birds succumbed to acute leukaemia, so practically there was no difference in lethality in the two groups.

Development of leukaemia. As to leukaemia after MC29/L virus infection, Table II shows the percentage of primitive atypical mononuclear myeloid cells in the peripheral blood of infected turkeys. Within 14 days acute leukaemia developed in all the infected birds with the appearance of primitive myeloid cells characteristic of MC29 infection. In the untreated control group the num-

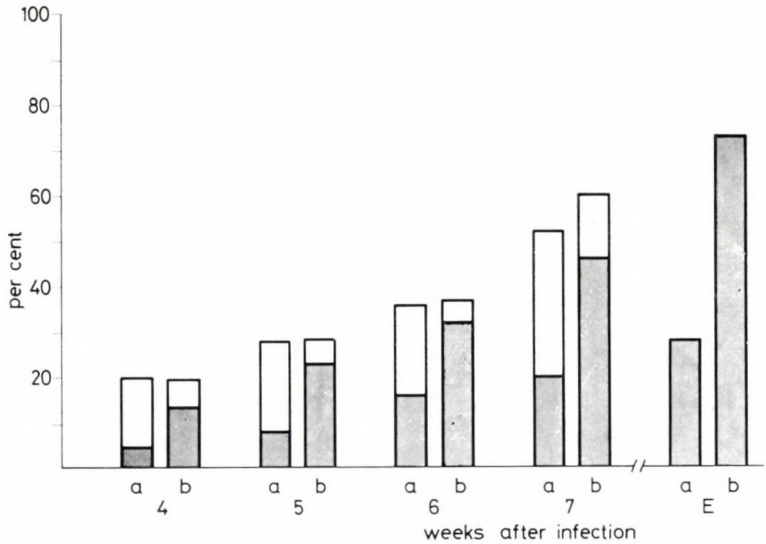

Fig. 1. Incidence of hepatoma and lethality of turkeys infected with MC29/L virus (a) glutaurine treated; (b) without treatment. E = hepatoma incidence at the end of the experiment. Open columns: lethality; shaded columns: hepatoma. For glutaurine treatment and virus infection see the notice under Table I

ber of primitive myeloid cells (myeloblasts have not been found among them) increased continuously up to the end of the experiment, indicating a serious leukaemia. In the peripheral blood of turkey poults treated with glutaurine the number of primitive myeloid cells remained lower throughout the experiment.

Virus expression in vitro. Virus expression in MC29/L infected turkey fibroblast cultures was controlled by reverse transcriptase assay. The reverse transcriptase activity detected in the media of glutaurine-treated and untreated cells is shown in Fig. 2. In the first six days the activity was low in both media. At 10 and 13 days after virus infection in the media containing no glutaurine the activity was markedly higher than in the media of cells treated with glutaurine for six days. The difference was most expressed at 13 days after infection.

On the 16th day after virus infection practically the same activity was measured in the media with glutaurine as in the media without glutaurine on the 13th day. All these indicate that glutaurine delayed MC29/L virus expression in the turkey fibroblast cultures.

Discussion

Glutaurine treatment significantly decreased the incidence of MC29/L virus induced hepatomas. This effect was observed at any interval after infection indicating the inhibitory effect of glutaurine on hepatoma development.

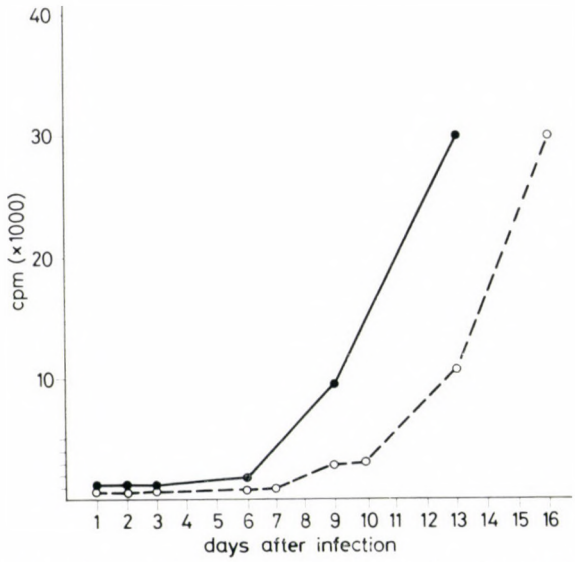

Fig. 2. Virus expression of turkey fibroblast cell cultures infected with MC29/L virus determined by reverse transcriptase assay. Cells were in contact with glutaurine (100 μg/ml) for six days after virus infection. ●——● MC 29/L, ○- - - - -○ MC 29/L + glutaurine.

The same glutaurine doses did not prevent the development of acute leukaemia, while due to the leukaemia there was no difference in lethality between the treated and untreated groups. Still, some restricting effect manifesting with a lower percentage of primitive myeloid cells in the glutaurine treated birds could be observed.

The mechanism of the glutaurine effect on virus-induced malignant processes has not been clarified. The known properties of glutaurine such as its vitamin A like action [3, 4], macrophage activation and glucocorticoid-antagonistic effect [5], prolongation of the survival of irradiated rats by stimulation of mitotic activity of bone marrow cells [7, 18], its influence on the permeability of biomembranes [1], might jointly be responsible for its inhibitory effect.

The results of the reverse transcriptase assay clearly proved the ability of glutaurine to delay virus expression in MC29/L infected turkey fibroblast cell cultures. As virus induced leukaemias are virus-dose dependent and the inhibitory effect of glutaurine on virus expression detected in cell culture might play a role in virus infected birds, it is assumed that the reduction of virus expression was also responsible for the phenomena observed. In this respect the immunoregulating and immunostimulating effects of glutaurine have also to be taken into consideration.

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THE LYMPHOID SYSTEM OF TOXOPLASMA INFECTED MICE

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(Received March 31, 1982)

Acute toxoplasmosis was induced in CFLP mice by intraperitoneal infection with the virulent RH strain of *Toxoplasma gondii*. The relative lymphoid organ weights and absolute lymphocyte counts were determined in mice killed in regular intervals after the infection. Characteristic changes of the lymphoid system were found in the course of acute toxoplasma infection: spleen hypertrophy began at 6 h after infection and its rate increased continuously till death. The thymus showed first hypertrophy, then it gradually decreased with grave atrophy at the time of death. The absolute lymphocyte count had decreased by the 6th h to reach a significantly low value by the time of death.

In the last decade several authors reported on the immunosuppressive effect of some protozoa (plasmodium, trypanosoma) [1–3], then immunosuppression was observed also in mice with experimental toxoplasmosis [4–6]. Reports published later suggested that the toxoplasma infection altered the morphology and function of the lymphoid system [7–9]. Our previous experiments demonstrated that in mice with acute toxoplasmosis significant spleen hypertrophy and grave thymus atrophy developed by the time of their death [10]. The present experiment was undertaken to examine the lymphoid system in mice infected with the RH strain of *Toxoplasma gondii*.

Materials and methods

Experimental animals. Six-week-old CFLP mice of both sexes were used (LATI, Gödöllő, Hungary). They were fed conventional pellets.

Toxoplasma strain. The standard international *T. gondii* RH virulent strain was used. The strain was maintained in serial mouse passages by weekly intraperitoneal inoculations. Experimental infection was done with a toxoplasma suspension prepared from the abdominal exudate of mice which had shown the symptoms of acute toxoplasmosis. The exudate was subjected to bacteriological sterility examination for 24 h.

Lymphoid system. Absolute lymphocyte count was determined under standardized conditions in blood samples from the caudal vein of 10 infected and 5 control mice in different intervals following the infection. After blood sampling, the animals were weighed and sacrificed in order to determine their relative lymphoid organ weights:

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$$\text{relative organ weight} = \frac{\text{organ weight, mg}}{\text{body weight, g}}$$

The spleen and thymus indices were determined on the basis of the relative organ weight.

Results

Ninety mice were infected with 25×10^3 germs of toxoplasma. This number of germs causes acute toxoplasmosis with fatal outcome on the 6th to 9th day after infection as observed in previous experiments.

After blood sampling, 10 infected and 5 control mice were sacrificed at 6, 12 and 24 h and on the following days after infection and at the time of the first spontaneous death among the animals. In order to determine the lymphoid organ weight, the spleen and thymus were removed and weighed. Data on the lymphoid system of infected and control mouse groups are presented in the Tables and Figures indicating the mean relative organ weights and absolute lymphocyte counts.

Figure 1 shows that the relative spleen weight did not change significantly in the first 24 h after the infection, though some increase could be observed after 12 h. From the second day, spleen weights were gradually increasing until death.

As indicated in Fig. 2, relative thymus weight increased till the 12th h after infection and from the 24th h it gradually decreased to a low value at death.

The mean absolute lymphocyte counts are presented in Fig. 3. It is seen that while the absolute lymphocyte count decreased already by the 6th h after infection, the values were varying till the 4th day. Subsequently the count gradually decreased; one day before spontaneous death its value amounted to 85% of the controls.

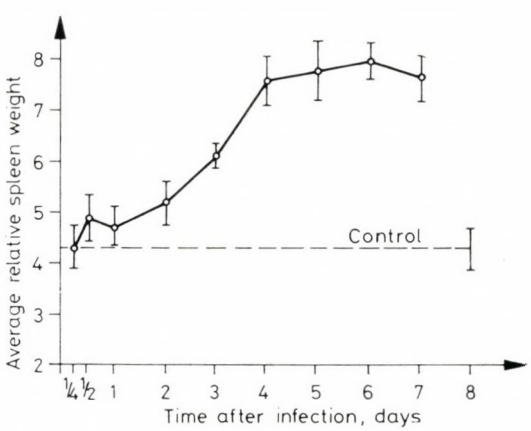

Fig. 1. Mean relative spleen weights at different times after infection

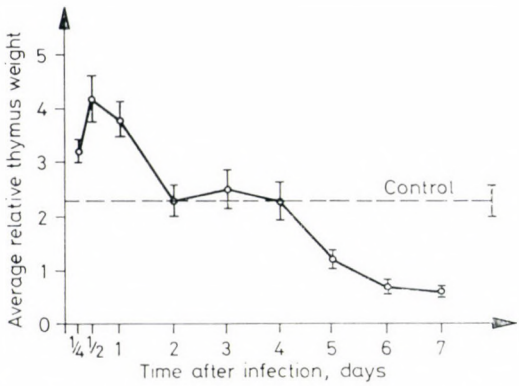

Fig. 2. Mean relative thymus weights at different times after infection

Table I

Mean spleen and thymus indices at different times after infection

	Control	Time (days)								
		¼	½	1	2	3	4	5	6	7
Spleen index	1.00	1.00	1.14	1.09	1.21	1.42	1.77	1.81	1.60	1.63
Thymus index	1.00	1.39	1.78	1.65	1.00	1.09	1.00	0.52	0.30	0.26

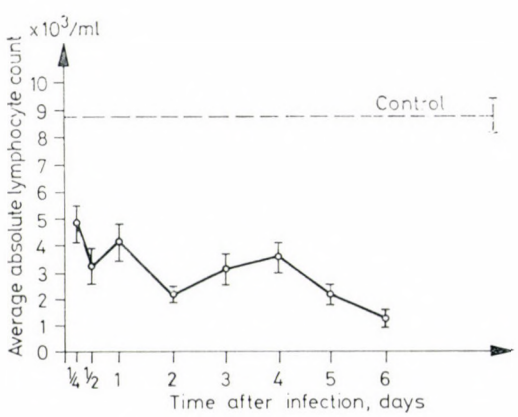

Fig. 3. Mean absolute lymphocyte count at different times after infection

Discussion

The results showed that the pathological processes develop very early in mice after infection with a virulent strain of *T. gondii*, which leads to a considerable spleen hypertrophy and grave thymus atrophy.

The results might explain the not unfrequent observation in humans suffering from a tumourous disease and after being treated with cytostatics and irradiation, die with acute toxoplasmosis [11, 12]. Immunosuppression, an unwanted side effect of these treatments, together with the immunosuppressive effect of the activated toxoplasmas, probably lead to a rapid and total prostration of the immune system. The situation might be the same in the case of immunosuppressive treatment applied during transplantation [13–16].

Our data on the absolute lymphocyte counts confirm that in human toxoplasmosis a 50–75% decrease of the lymphocyte count may take place in the first stage of the disease [17].

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CELL-MEDIATED IMMUNOREACTIVITY IN ACUTE HUMAN TRICHINELLOSIS

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Twenty patients were examined for studying specific cellular immunoreactivity in human trichinellosis. Serial leukocyte migration inhibition tests were carried out using trichinella larvae as antigen. The mean migration indices of the patients differed significantly from that of the healthy controls. As the most distinct immunoreactivity was found during the acute phase of the disease, it is suggested that the cellular immune response may be of some importance in the development of symptoms. It is, however, stressed that the diagnostic value of the LMI method is less than that of the serological tests in individual cases.

As shown previously, determination of the serum immunoglobulin levels provides valuable data for studying the pathogenesis of human trichinellosis [1]. On the other hand, some animal experiments indicated that investigation of the cellular immune response might contribute to our knowledge of this subject [2, 3]. The present study was undertaken to examine the specific cellular immunoreactivity and its possible relation to the development of the clinical picture in human trichinellosis.

Materials and methods

Patients. Twenty trichinellosis patients who fell ill during an epidemic were examined. Controls were 10 healthy persons having negative trichinella serological test results. On hospital admission patients were treated with 50 mg/kg/day of tiabendazol for 5 to 14 days. In addition 8 of them were also given prednisolone for 3 to 14 days.

Tests. Cellular immune response was tested by the leukocyte migration inhibition (LMI) test as described by Søborg and Bendixen [4]. The antigen was a suspension of live trichinella larvae in Hanks' solution at a concentration of 2×10^3 larvae/ml. Migration indices (MI) were calculated by comparing the mean migration area in 3 chambers with antigen with that in 3 chambers without antigen. MI values less than 0.8 were considered significant.

Patients were tested on 3 occasions, (i) during the acute phase of the disease (i.e. 20 to 38 days after infection), (ii) 40 to 60 days, (iii) 70 to 80 days after infection. Controls were tested only once.

Along with the LMI tests serological tests were carried out. Larva microprecipitation [5] and CF tests [6] were applied.

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Table I

Laboratory data of patients with trichinellosis

Patient	Days after the infection	Trichinella microprecipitation	CF	MI
	28–38	neg.	1:64	0.65
1. K.G.	40–60	pos.	1:256	0.80
	70–80	pos.	1:128	0.71
	28–38	neg.	1:64	0.67
2. S.K.	40–60	pos.	1:32	0.62
	70–80	pos.	1:16	0.81
	28–38	neg.	1:1024	0.82
3. M.Á.	40–60	pos.	1:512	0.86
	70–80	pos.	1:512	0.73
	28–38	neg.	1:16	0.63
4. B.J.	40–60	pos.	1:64	0.66
	70–80	pos.	1:128	0.63
	28–38	neg.	1:8	0.81
5. K.S. +	40–60	pos.	1:128	0.70
	70–80	pos.	1:128	0.88
	28–38	neg.	1:8	0.77
6. T.Gy.	40–60	neg.	1:64	0.85
	70–80	pos.	1:128	0.70
	28–38	neg.	1:256	0.86
7. K.I.	40–60	neg.	1:2048	0.90
	70–80	pos.	1:1024	0.85
	28–38	pos.	1:512	0.68
8. H.J. +	40–60	pos.	1:1024	0.79
	70–80	pos.	1:1024	0.73
	28–38	pos.	1:128	0.65
9. Sz.F.	40–60	pos.	1:512	0.69
	70–80	pos.	1:512	0.69
	28–38	pos.	1:256	0.59
10. K.L. +	40–60	pos.	1:512	0.68
	70–80	pos.	1:256	0.82

+ tiabendazole and steroid treatment

Patient	Days after the infection	Trichinella microprecipitation	CF	MI
	28–38	pos.	1:32	0.70
11. K.S. II. +	40–60	pos.	1:128	0.72
	70–80	pos.	1:256	0.70
	28–38	pos.	1:64	0.58
12. K.G. II.	40–60	pos.	1:1024	0.74
	70–80	pos.	1:1024	0.73
	28–38	neg.	1:256	0.85
13. K.L. +	40–60	pos.	1:128	0.84
	70–80	pos.	1:256	0.87
	28–38	pos.	1:64	0.76
14. D.I.	40–60	pos.	1:512	0.84
	70–80	pos.	1:512	0.72
	28–38	neg.	1:16	0.64
15. H.J.	40–60	pos.	1:128	0.84
	70–80	pos.	1:256	0.71
	28–38	pos.	1:256	0.84
16. M.J. +	40–60	pos.	1:256	0.93
	70–80	pos.	1:1024	0.79
	28–38	neg.	1:4	0.82
17. E.J.	40–60	pos.	1:1024	0.94
	70–80	pos.	1:2048	0.88
	28–38	neg.	1:512	0.81
18. E.J. II.	40–60	pos.	1:256	0.85
	70–80	pos.	1:1024	0.85
	28–38	neg.	1:256	0.70
19. Sz.F. +	40–60	pos.	1:256	0.92
	70–80	pos.	1:256	0.83
	28–38	pos.	1:64	0.66
20. K.L. II. +	40–60	pos.	1:2048	0.92
	70–80	pos.	1:512	0.70

Results

Data are presented in Fig. 1 and in Table I. As seen in Fig. 1, the mean MI values observed in each of the three examinations were invariably lower in the patient group than in the controls. The differences proved significant in Student's *t*-test ($p < 0.001$). The lowest mean was found in the acute phase of the disease while after a rise it decreased once more.

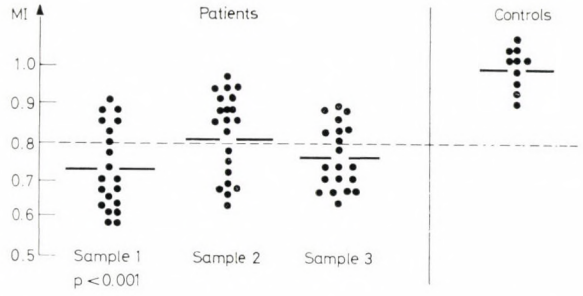

Fig. 1. Leukocyte migration indices of patients with trichinellosis and of control subjects

In Table I the individual MI values are compared with the serologica results. During the period of clinical symptoms, the microprecipitation test was positive in 8 cases and the CF test in 14 cases. Later, positive serological results were found with both methods in all the cases. The LMI was consistently negative in 4 patients. Four patients were positive only once and 7 patients only twice. Five patients were found positive with all the 3 tests.

No correlation between the results of the serological tests and LMI could be observed.

Discussion

Taking into account the LMI results as a whole it can be stated that a cellular reactivity against trichinella larvae develops in patients with trichinellosis. Similar observations were made in experimental trichinellosis of the guinea-pig [3] and the rat [2]. Reactivity was most distinct during the acute period of the disease; later the reactivity decreased then it rose again. Since, however, the LMI tests were done in treated patients, the treatment might have a role in the transitory decrease of immune reactivity.

Although the simultaneous occurrence of different phenomenas permits no conclusion as to their causual relationship, both the present and previous observations [1] suggest that the cellular immune response must be of some importance in the development of human trichinellosis.

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RESULTS OF SPACE EXPERIMENT PROGRAM "INTERFERON"

I. PRODUCTION OF INTERFERON IN VITRO BY HUMAN LYMPHOCYTES ABOARD SPACE LABORATORY SOLYUT-6 ("INTERFERON I") AND INFLUENCE OF SPACE FLIGHT ON LYMPHOCYTE FUNCTIONS OF COSMONAUTS ("INTERFERON III")

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The results of the biological space experiment "Interferon" performed by two international cosmonaut crews aboard the space laboratory Solyut-6 are reported. Human lymphocytes separated from the blood of healthy donors and placed into "Interferon I" equipment could be kept for 7 days in suspension culture under spaceflight conditions. Interferon production could be induced in human lymphocytes by preparations of different origin, such as virus, synthetic polyribonucleotides, bacterial protein and plant pigment.

An increased lymphocyte interferon production was observed in the space laboratory as compared to the ground control. A decrease of induced interferon production and natural killer cell activity was observed in the cosmonauts' lymphocytes on the 1st day on Earth after 7 days spaceflight.

A space experiment program named "Interferon" was elaborated within the frames of the international scientific cooperation "Intercosmos". The program was carried out during the flight of the Hungarian—Soviet cosmonaut team (Experiment 1, May 26–June 3, 1980) and repeated during the speaceflight of the Romanian–Soviet crew (Experiment 2, May 16–22, 1981).

The experiment had the following aims.

In experiment "Interferon I" the effect of the complex spaceflight conditions on interferon production in vitro by human lymphocytes isolated from healthy donors was investigated.

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The study was based on the hypothesis that spaceflight conditions, i.e. zero-gravity, may change the functions of cell membranes and thus modulate interferon production.

In experiment "Interferon II" the influence of spaceflight on the biological activity of human interferon preparations and interferon inducers was studied. The results of this investigation could be essential for recommendation of these preparations in medication of cosmonauts during long-term orbital flights.

In experiment "Interferon III" we investigated the effects of extreme circumstances of spaceflight on the interferon producing capacity and natural killer activity of blood lymphocytes of cosmonauts during spaceflight. These examinations render a basis for the estimation of the condition of non-specific resistance of the organism, especially of protection from viral infections.

In this paper the results of experiments "Interferon I" and "Interferon III" are presented.

Materials and methods

Description of the "Interferon I" equipment. The small and light equipment constructed by Medicor Works, Budapest, for the space experiment "Interferon" was a metal box with a metal rack keeping 10 plastic tubes. Eight tubes were provided with head-receptacles and plungers, two tubes were closed by screw caps. The head-receptacle was separated from the tube by a one-way valve opening into the direction of the tube on rotating the plunger head.

Application of "Interferon I" equipment. The experiments were performed under spaceflight conditions and on the Earth simultaneously (ground-control) using materials of identical origin.

Seven hours before launching, the tubes were filled with human lymphocyte suspension, and the head-receptacles with interferon inducers. The flight equipment was transported to space laboratory Solyut-6 by the cosmonauts. Interferon induction was carried out by the cosmonaut in the first hour following arrival to the space laboratory, and synchronously on the ground. Interferon inducers were administered to the lymphocyte suspensions by rotating the plunger heads. Then the "Interferon I" equipments were kept in thermostats at 37 °C, in experiment 1 for 6 days, in experiment 2 for 4 days. As the flight thermostat was switched off for 8 h daily (period of rest of cosmonauts) the ground device was switched off at the same time. After 6 days aboard Solyut-6 the flight equipment was brought back to Earth.

Lymphocyte separation for experiment "Interferon I". Fresh heparinized blood from healthy donors was centrifuged on a Ficoll (Pharmacia)-Uromiro(Bracco) gradient [1] and a lymphocyte suspension of 5.1×10^7 cells/3 ml Parker's medium supplemented with 2% fetal calf serum (Difco) was prepared and filled into every tube of the equipments. Cell viability was controlled by rapid counting of lymphocytes stained with 0.2% trypan blue in a haemocytometer.

Interferon inducers. In Experiment 1, the following drugs were applied in 0.3 ml volume: Polyriboinosinic-polyribocytidylic acid (poly I:C) (Calbiochem), 600 μg; polyriboguanylic-polyribocytidylic acid (poly G:C) (Institute of macromolecular compounds, USSR Academy of Sciences, Leningrad), 600 μg; gossipol (cotton plant, *Gossipium herbaceum*, pigment) (Institute of Bioorganic Chemistry, Tashkent, USSR), 500 μg; purified protein derivative (PPD) of tuberculin (Institute of Vaccines and Serum Control, Moscow), 500 μg.

In Experiment 2, two inducers were used: poly I:C (Calbiochem), 600 μg; and Newcastle Disease Virus, UV-inactivated (NDV–UV), $10^{7.3}$ LD_{50}/ml. NDV, Hertfordshire strain, was inactivated by UV-rays of a Germicid bulb (100 W, 32 cm distance) for 4 min.

Interferon induction in cosmonauts' lymphocytes in vitro (experiment "Interferon III"). Venous blood from the cubital vein of the cosmonauts was taken in Experiment 1, twelve days before spaceflight, and 1 and 6 days after returning to Earth. In Experiment 2, blood was taken 1 month before and 1 day after the spaceflight.

The blood was centrifuged on a Ficoll-Uromiro gradient and a suspension of 5×10^6/ml lymphocytes in Parker's medium containing 2% fetal calf serum was prepared. NDV–UV was used as inducer in a dose of $10^{6.3}$ LD_{50}/100 μl. The tubes were kept at 37 °C; supernatants were taken at different intervals and kept at —20 °C till assay.

Assay of interferon. In "Interferon I" experiment the tubes from both equipments (flight and ground) were centrifuged immediately after spaceflight; cell viability of the pellet was controlled. Supernatants of lymphocyte suspensions were kept at 4 °C till assay.

Antiviral activity of lymphocyte supernatants from "Interferon I" experiments as well as titration of the samples of lymphocyte supernatants from the cosmonauts ("Interferon III" experiments) was carried out by the adapted cytopathic effect-inhibition test described in detail earlier [2], using permanent human lung fibroblast cultures Wi-38. We included in each assay a standard interferon preparation (MRC Research Standard, B 69/19 for human interferon, titre 5000 units). Interferon titres were expressed in international units (IU).

The identity of the antiviral activity of the substance produced by lymphocytes with IFN-α activity was controlled according to Tálas et al. [3].

Natural killer cytotoxicity assay. Natural killer (NK) activity of lymphocytes was determined by the method of Rykova et al. [4]. Lymphocytes were separated from venous blood of the cosmonauts by centrifugation on a Ficoll–Uromiro gradient. K-562 human myeloid cells labelled with ^{3}H-uridine (3μ Ci/ml, specific activity 24 Ci/mM) were used as targets. The cytotoxicity test was carried out in plastic trays (Flow Laboratories) in 0.2 ml volumes. In every well 1×10^4 target cells and 2×10^5 lymphocytes were placed. Target cells were resuspended before assay in RPMI 1640 medium supplemented with pancreatic ribonuclease (concentration 20 μg/ml), lymphocytes were resuspended in the same medium complemented with 10% fetal calf serum. Incubation time was 14 h at 37 °C. After incubation the contents of the wells were rinsed on Millipore filter (pore diameter 2.5 μ) with Parker's medium, then treated with cold 5% trichloroacetic acid and 80% ethanol. The radioactivity of cells was determined in a Packard 3320 scintillation counter.

The cytotoxicity index (C.I.) was calculated as follows:

$$\text{C.I.} = \left(1 - \frac{\text{cpm of test sample}}{\text{cpm of target cells}}\right) \times 100$$

Results

Table I shows the results obtained in Experiment 1. As interferon inducers poly I:C, poly G:C, gossipol and PPD (in two parallels each) were used in doses marked in Table I. Assay of antiviral activity of flight and ground-control samples showed that in every tube where inducer had been added, interferon production could be demonstrated. In tubes where lymphocytes had been incubated without inducer no interferon was found. Comparing the antiviral activity of flight samples to that of their corresponding ground controls, an increased interferon production could be observed. Interferon titres of the flight samples were 4–8 times higher than those of controls, independently of the inducer used.

Viability of lymphocytes in flight specimens on the 1st day after landing was 50%, in the synchronous ground controls 70% (mean of 10 samples), in comparison to the 98% viability of cells in suspension before filling the tubes.

The results of Experiment 2 are presented in Table II. As interferon inducers poly I:C (600 μg/0.3 ml) and NDV–UV ($10^{7.3}$ LD_{50}/ml, 0.3 ml), each in 4 parallels, were applied. The tubes were incubated at 37 °C for 4 days.

Similarly as in Experiment 1, interferon production was found in every sample (flight and ground-controls) where inducer was administered. En-

hancement of interferon synthesis in flight samples compared to that of controls could be seen. Antiviral activity measured in flight specimens was found to be 4 times higher than in ground controls.

Lymphocyte viability (mean of 10 samples) after landing was 64%, in synchronous ground samples 70%; viability of lymphocytes before spaceflight was 98%.

Interferon production in vitro by lympocytes of cosmonauts investigated before and after spaceflight (Experiment 1) is presented in Table III. Interferon could be detected in samples taken 48 h after induction.

Twelve days before spaceflight 250–500 IU of interferon were found in the samples of both cosmonauts. One day after landing a decrease of interferon production was seen; in samples of both cosmonauts IFN levels were found to be considerably lower than before flight. Six days after return to Earth in lymphocytes of one cosmonaut (B.F.) a tendency to normalization of interferon production was observed as the interferon level was almost the same as 12 days before flight. Lymphocytes of the second cosmonaut (V.K.) produced low quantities of interferon 6 days after flight; the level was the same as 1 day after landing.

Table I

Interferon production by human lymphocytes placed in "Interferon I" equipment under spaceflight and ground conditions. Experiment 1. (May–June, 1980)

Tube number	IFN inducer	Dose μg/0.3 ml	IFN-α titre (IU)	
			Flight sample	Ground control
1	poly I:C	600	2500	312
2	poly I:C	600	2500	312
3	poly G:C	600	2500	312
4	poly G:C	600	2500	312
5	gossipol	500	624	156
6	gossipol	500	624	156
7	PPD	500	2500	312
8	PPD	500	2500	312
9	—	—	<8	<8
10	—	—	<8	<8

Each tube was filled with 5.1×10^7 lymphocytes in 3 ml Parker's medium 199 with 2% fetal calf serum

poly I:C = polyriboinosinic-polyribocytidilic acid
poly G:C = polyriboguanilic-polyribocytidilic acid
gossipol = pigment of cotton plant *Gossipium herbaceum*
PPD = purified protein derivative (tuberculin)

Interferon inducers applied in the doses indicated did not induce detectable interferon levels in cells of the interferon assay system used

Table II

Interferon production by human lymphocytes placed in "Interferon I" equipment under spaceflight and ground conditions. Experiment 2. (May, 1981)

Tube number	IFN inducer	Dose 0.3 ml	IFN–α titre (IU)	
			Flight sample	Ground control
1	poly I:C	600 μg	312	80
2	poly I:C	600 μg	312	80
3	poly I:C	600 μg	312	80
4	poly I:C	600 μg	312	80
5	NDV–UV	$10^{6.7}$ LD_{50}	1248	312
6	NDV–UV	$10^{6.7}$ LD_{50}	1248	312
7	NDV–UV	$10^{6.7}$ LD_{50}	624	312
8	NDV–UV	$10^{6.7}$ LD_{50}	624	312
9	—	—	<8	<8
10	—	—	<8	<8

Each tube was filled with 5×10^7 lymphocytes in 3 ml Parker's medium 199 with 2% fetal calf serum

poly I:C = polyriboinosinic-polyribocytidilic acid

NDV–UV = Newcastle Disease Virus, Hertfordshire strain, inactivated by UV-rays for 4 min

Interferon inducers applied in the doses indicated did not induce detectable interferon levels in cells of the interferon assay system used

Table III

Interferon production in vitro by lymphocytes of cosmonauts before and after spaceflight. Experiment 1

Time of blood sampling	Time after induction h	IFN–α titre (IU)	
		B.F.*	V.K.**
12 days before spaceflight	4	<8, <8***	<8, <8
	10	<8, <8	<8, <8
	48	250, 250	250, 500
1 day after spaceflight	4	<8, <8	<8, <8
	10	<8, <8	<8, <8
	48	62, 62	62, 31
6 day after spaceflight	4	<8, <8	<8, <8
	10	<8, <8	<8, <8
	48	250, 125	62, 62

As interferon inducer, NDV–UV (10^7 LD_{50}/0.1 ml) was used.
Each sample contained 5×10^6/ml lympocytes
* B.F., Hungarian cosmonaut
** V.K., Soviet cosmonaut
*** IFN–α titres of two parallel samples

Table IV

Interferon production in vitro by lymphocytes of cosmonauts before and after spaceflight. Experiment 2

Time of blood sampling	Time after induction h	IFN–α titre (IU)		
		L.P.*	D.P.**	N.D.***
1 month before	48	31	31	250
spaceflight		31	62	250
1 day after	48	31	31	250
spaceflight		31	31	250

As interferon inducer NDV–UV (10^7 LD_{50}/0.1 ml) was used.
Each sample contained 5×10^6/ml lymphocytes
* L.P., Soviet cosmonaut
** D.P., Romanian cosmonaut
*** N.D., doubler pilot

Interferon production by lymphocytes of cosmonauts examined in Experiment 2 is shown in Table IV. Lymphocytes of the two cosmonauts L.P. and D.P. separated 1 month before spaceflight produced low interferon levels. No alteration in the interferon producing capacity of lymphocytes could be observed 1 day after orbital flight. As a control to the flight experiment lymphocytes of the doubler pilot N.D. were investigated simultaneously. High interferon titres—250 IU—were measured in both assays.

Natural killer activity of the cosmonauts' lymphocytes isolated before and after spaceflight is shown in Table V. In Experiment 1, marked individual differences could be seen in the cytotoxicity index (C.I.). Comparing the C.I. measured 1 month before orbital flight to that of 1 day after landing, an about

Table V

Natural killer activity of cosmonauts' lymphocytes before and after spaceflight

Time of blood sampling	Cytotoxicity index, %				
	Experiment 1 (May–June 1980)		Experiment 2 (May 1981)		
	V.K.*	B.F.*	L.P.*	D.P.*	N.D.**
30 days before launching	84.1	22.3	30.5	27.7	49.2
1 day after landing	19.0	5.5	17.9	32.6	46.2

* Cosmonauts
** Doubler pilot

4 times lower value was observed in both pilot's samples independently of the levels before flight.

In Experiment 2, NK activity of lymphocytes of two cosmonauts and a doubler pilot was determined. The cytotoxicity of lymphocytes of cosmonaut L.P. was about 2 times lower 1 day after spaceflight than 1 month before launching.

C.I. values of pilot D.P. showed no difference before and after orbital flight. C.I. of the doubler pilot N.D. was almost the same on the two occasions when blood samples were taken.

Discussion

In earlier spaceflight experiments, aboard biosatellites of the Cosmos series [5], in Skylab-3 mission [6] and in biological experiments carried out on the Soviet space station Solyut-6 [7–9], the effects of spaceflight conditions on living cells were studied.

Montgomery et al. [6] reported the results of a detailed study on Wi-38 diploid human embryonic lung monolayer cultures exposed to weightlessness for 1 to 59 days. Cell growth and morphological characteristics, chromosome analysis and chemical analysis of the nutrient media were studied. Exposure to zero-gravity did not influence the duration of the cell cycle and mitoses. No differences were observed in vacuole formation, cell movement, in the size of cells and cell organelles, in confluence of cells when flight and control samples were compared. Similarly, no differences could be observed when the nutrient medium was analysed after flight and compared with the ground control.

Tixador et al. [7] studied the multiplication of *Paramecium aurelia* in suspension aboard the orbital laboratory Solyut-6. They reported that spaceflight resulted in a stimulation of cell proliferation. Further, an augmentation of cell volume of *Paramecium tetraaurelia* at different times of the experiment (12–96 h) was described. The phenomenon was clearly seen in paramecia in division [8]. In connection with this observation the authors presumed a modification in cell membrane functions. It was shown [9] that the increase of cell volume is a consequence of the changed membrane permeability associated with cellular hyperhydratation and loss of K, P, Ca and Mg ions. In experiment "Interferon I" it was shown that human lymphocytes could be kept in suspension culture under conditions of spaceflight for seven days. Their viability after exposure to extreme conditions of orbital flight (acceleration, zero-gravity) did not decrease considerably in comparison with synchronous ground controls. Moreover, in these cultures interferon production could be induced, and independently of the inducer used (virus, polyribonucleotides, bacterial

protein or plant pigment) higher levels of interferon were observed than in ground controls.

Our results concerning the possibility of keeping living cells in culture under spaceflight conditions are in agreement with the results of Montgomery et al. [6], Gurovsky and Ilyin [5] and Tixador et al. [7]. The fact that interferon production could be induced in human lymphocytes during orbital flight may be considered a new contribution. The augmentation of interferon production in orbital samples could be in relationship with the presumed alterations in the lymphocyte membrane functions, similarly to those shown in suspension cultures of paramecia [9]. This question needs further studies.

Investigation of interferon production by lymphocytes of cosmonauts after spaceflight (experiment "Interferon III") rendered some new data about the nonspecific defence reactions of the organism.

Guseva and Tashpulatova [10] described a marked inhibition of the immunological reactivity of the crew of Solyut-6 spaceship after a 49 day flight: a decrease in the immunoglobulin content of saliva and tonsillary lacunae, a decline of the bactericidal activity of the serum and a decrease of the albumin content of blood were observed.

In experiment "Interferon III" (Experiment 1) a marked decrease of interferon production by lymphocytes of both pilots (B.F. and V.K.) was shown 1 day after landing. Six days after landing interferon induction in pilots' lymphocytes was repeated. A tendency to normalization was seen in the samples of one cosmonaut.

In Experiment 2, such a decrease of lymphocyte interferon production was not expressed 1 day after flight, since before flight the interferon levels were very low. The cause of this phenomenon remains unexplained. It is worth mentioning that such low interferon production can be observed in normal blood donors, too.

The natural killer activity of lymphocytes separated from cosmonauts' blood was also investigated.

As reported earlier [11], the activity of natural killer cells can be modulated by interferon. Gidlung et al. [12] and Djeu et al. [3] showed that interferon and interferon inducers augmented the natural killer cell activity of experimental animals. Santoli and Koprowski [11] and Benczur et al. [2] reported that administration of human interferon and interferon inducers enhance the NK activity of human lymphocytes. In certain pathological conditions such as multiple sclerosis and systemic lupus erythematosus a decrease of NK cell activity and synchronously a deficient interferon producing capacity of lymphocytes was described [2, 14–16].

NK activity and IFN producing capacity of human lymphocytes after cosmic flight have not been investigated up-to-now.

In two experiments 1 day after landing, a depression of NK cell activity in three out of four cosmonauts was observed. In two cosmonauts (B.F. and V.K.) this phenomenon ran parallel with the decrease of interferon producing capacity of lymphocytes.

It would be of importance to elucidate the role of different factors of cosmic flight (stress, extreme acceleration, weightlessness) influencing the regulatory mechanisms of the immune system.

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RESULTS OF SPACE EXPERIMENT PROGRAM "INTERFERON"

II. INFLUENCE OF SPACEFLIGHT CONDITIONS ON THE ACTIVITY OF INTERFERON PREPARATIONS AND INTERFERON INDUCERS ("INTERFERON II")

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The influence of spaceflight conditions on the biological activity of HuIFN-α preparations (lyophilized, in solution and in ointment) and interferon inducers was studied. In antiviral activity no difference was observed between the samples kept aboard the spaceship and the controls kept under ground conditions. The interferon inducers poly I:C, poly G:C and gossipol placed in the space laboratory for 7 days maintained their interferon-inducing capacity. The circulating interferon level in mice was the same irrespective of the induction being performed with flight or ground-control samples of inducers.

In Part I of this paper the production of interferon in vitro by human lymphocytes aboard the space laboratory Solyut-6 and the effect of spaceflight on cosmonauts' lymphocyte functions were reported ("Interferon I" and "Interferon III").

The aim of the "Interferon II" experiment was to investigate the influence of orbital flight on the biological activity of different human interferon preparations and interferon inducers. Taking into consideration the antiviral action of interferon and interferon inducers, the results of these investigations might promote the application of these preparations during long-term spaceflights.

In this paper the results obtained in experiment "Interferon II" are presented.

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Materials and methods

"Interferon II" equipment. The "Interferon II" equipment constructed by Medicor Works, Budapest, consists of a small and light metal box with a lid, containing a metal rack with 16 plastic ampoules. Experiments performed using this equipment were carried out synchronously in the ground laboratory and in the space laboratory Solyut-6 (May–June, 1980).

Two weeks before launching of spaceship Soyuz-36, the plastic ampoules of the equipment "Interferon II" were filled with the preparations to be investigated and kept at 4 °C. The following preparations were used: lyophilized HuIFN-α, HuIFN-α in ocular ointment (white petrolatum) and HuIFN-α in physiological (0.85%) saline solution; poly I:C pure substance, poly I:C in physiological saline solution, poly G:C in physiological saline solution, gossipol in physiological saline solution and poly (rA) · $d(pT)_{10}$ in physiological saline solution. Their doses are given in the Tables.

HuIFN-α preparations were provided by the Laboratory of Interferon Biosynthesis of the Gamaleya Institute of Microbiology, Moscow. Polyriboinosinic-polyribocytidylic acid (poly I:C) and polyriboadenylic-oligodeoxythymidylic acid (poly(rA) · $d(pT)_{10}$) were purchased from Calbiochem. Polyriboguanylic-polyribocytidylic acid (poly G:C) was provided by the Institute of Macromolecular Compounds, USSR Academy of Sciences, Leningrad. Gossipol (cotton plant pigment) was a product of the Institute of Bioorganic Chemistry, Tashkent, USSR.

The flight equipment was taken by the crew of spaceship Soyuz-36 to the space laboratory Solyut-6, where it was kept at room temperature (20 °C) for seven days. During the spaceflight the ground-control equipment was also kept at 20 °C. After landing of the spaceship, both equipments were stored at 4 °C until assay of the biological activity of the preparations.

Assay of antiviral activity of HuIFN-α preparations. Lyophilized samples were dissolved in Parker's medium 199 before assay. HuIFN-α in ocular ointment was treated as follows: to every tube 1.2 ml Parker's medium was added, stirred with a glass rod and kept at 4 °C for 24 h. Next day the fluid phase was assayed for antiviral activity. All HuIFN-α preparations were assayed according to the method described by Benczur et al. [1], using permanent human lung fibroblast cultures Wi-38.

Interferon induction in vivo and assay. Interferon inducers poly I:C (100 μg), poly G:C (100 μg) and gossipol (200 μg) were administered after spaceflight to inbred BALB/c mice of 20 g weight intraperitoneally. Each inducer was injected to 10 mice. They were exsanguinated by punction of the orbital plexus at the time of the peak circulating interferon level determined in earlier experiments: 2 h after injection of poly I:C, 18 h after poly G:C, and 24 h after induction with gossipol. Mouse serum interferon was assayed individually in microtest using L-929 permanent mouse fibroblast cultures by means of the CPE-inhibition method [2]. Encephalo-myocarditis virus (EMC) was used as challenge. The laboratory mouse interferon standard was compared to WHO International Reference Preparation, Mouse IFN, 12 000 IU/ml, prepared and distributed by NIAID–NIH, Bethesda, USA. Interferon titres were expressed in international units (IU).

Reverse transcriptase assay. Activity of the exogenous template poly (rA) · $d(pT)_{10}$ placed in the flight and ground equipments "Interferon II" was determined by the standard reverse transcriptase test using MC29 avian myelocytomatosis virus as described earlier [3].

Results and discussion

Lyophilized HuIFN–α, HuIFN–α in physiological saline solution and HuIFN–α in white petrolatum kept at room temperature (20 °C) aboard the space laboratory Solyut-6 and on Earth (controls) were titrated simulateneously after the return of flight samples. Each material was assayed in duplicate.

The antiviral activity of the different HuIFN–α preparations is presented in Table I. Comparing the interferon levels of flight specimens with those of the controls, no difference was observed.

Interferon inducers poly I:C, poly G:C and gossipol placed in the "Interferon II" equipments were injected intraperitoneally into BALB/c mice 7 days after the return of Soyuz-35. Interferon production in vivo induced by preparations exposed to conditions of spaceflight and by control samples is presented in Table II.

Titres of circulating interferon of mice injected with flight samples of inducers did not differ from those of mice injected with the control inducers. Table III shows the results of the RNA-directed DNA-polymerase assay using MC29 virus as enzyme source and flight and ground samples of poly (rA) · $d(pT)_{10}$ templates. Practically no difference in activity was found between the two samples of template.

The results obtained by the assay of antiviral activity of various HuIFN-α preparations showed that the conditions of spaceflight did not influence their antiviral properties.

Taking into consideration the prophylactic activity of human interferon in acute viral respiratory infections [4–6] and the therapeutical effect of HuIFN preparations in localized viral eye infections [7, 8], it would be useful to recommend these preparations for the medication of cosmonauts during long-term flights.

There are reports referring to application of interferon inducers in the therapy of localized viral infections. Poly I:C [9] and bacteriophage double-stranded RNA [10] were reported to be effective in the therapy of acute

Table I

Antiviral activity of HuIFN-α preparations placed in flight and ground equipment "Interferon II"

Content of ampoule	No. of ampoules	Interferon titre (IU)	
		Flight sample	Ground control
HuIFN-α in physiological saline solution	1	1600	1600
	2	800	800
HuIFN-α in physiological saline solution	3	25600	25600
	5	12800	12800
HuIFN-α in white petrolatum	4	6400	6400
	8	1600	1600
	12	1600	1600
	16	3200	3200
HuIFN-α lyophilized	9	12800	12800
HuIFN-α lyophilized	11	400	800

herpes simplex infections of the eye. Our data showing the unchanged interferon-inducing capacity of inducers after exposure to orbital flight for 7 days allow to presume that poly I:C could also be applied in form of eye-drops in acute eye infections of cosmonauts if virus infection were introduced by cargo--spaceships, expeditions visiting the space laboratory, or in the case of activation of pilots' persistent viruses.

Table II

Interferon production in mice induced by preparations placed in "Interferon II" equipments

No. of ampoules	Inducer	Inducer dose (μg/0.2 ml)	Serum IFN titre (IU)			
			Flight sample		Ground control	
			Mean	s.e.	Mean	s.e.
6	poly I:C in physiological saline solution (pool 1)	100	280	27.3	272	24.4
7	poly I:C in physiological saline solution (pool 2)	100	432	58.7	368	48.0
10	poly G:C in physiological saline solution	100	288	21.3	304	16.0
13	poly I:C pure substance	100	160	20.6	148	23.9
14	gossipol in physiological saline solution	200	8	1.1	8	1.3

Inducers were applied intraperitoneally to 10 mice each; blood was taken 2 h after poly I:C, 18 h after poly G:C and 24 h after gossipol injection

Table III

Biological activity of template poly (rA) · d(pT)$_{10}$ placed in "Interferon II" equipment (ampoule No. 15)*

Source of template	Origin of MC29 virus	cpm	mean
Ground control	Turkey embryo tissue culture supernatant	2342 1986	2164
Flight sample	ditto	2101 2295	2198
—	ditto	276	
Ground control	Plasma of infected turkey poults	1491 1027	1259
Flight sample	ditto	1037 836	937
—	ditto	437	

* polyriboadenylic-oligodeoxythymidylic acid

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SURVIVAL OF *CAMPYLOBACTER JEJUNI* IN DIFFERENT MEDIA AND FAECES AT DIFFERENT TEMPERATURES AND TIMES OF PRESERVATION

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Nineteen *Campylobacter jejuni* strains survived best in suspensions with approximately 10^9 bacteria in 4% albumin solution at —60 °C for 17 months (18 out of 19). At the same temperature and during the same period they survived less well in milk (11 out of 19). Recovery of campylobacters after 7 weeks was abundant in albumin, and somewhat less in milk. At —20 °C survival in milk was worse; 17 out of 19 cultures did not survive 5 weeks in milk and 7 died in albumin. At 4 °C all but one strain survived two weeks in milk and none died in albumin, but after 3 weeks only 7 survived in milk and 2 in albumin. At room temperature campylobacters did not survive 10 days in any of the media used. Peptone water (4%) and peptone-saline proved unsuitable for preservation. From 20 positive stool samples kept at 4 °C, on the day after the first sampling *C. jejuni* could not be recovered from 10 samples; bacteria survived for 2–7 days in 8 samples, for 12–20 days in 2 samples.

It is only since 10 years that *Campylobacter jejuni* has been studied and found to represent an important enteric pathogen [1–5]. This fact has aroused interest in the microorganism and some of its principal properties such as its survival in different media. Many methods have been used to maintain and ensure the survival of this organism [6–9].

The purpose of the present study was to compare 4 different media: milk [10], 4% albumin solution, peptone water, and peptone–saline at 22 °C, 4°, —20° and —60 °C, during different periods of time to find suitable way to preserve *C. jejuni* for further investigations. A study of the survival of *C. jejuni* in stool samples has also been carried out in order to determine the time of survival of campylobacter in faeces and to obtain information concerning the possibilities of its transport.

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Materials and methods

Bacterial strains. Nineteen *C. jejuni* strains were used from the lyophilized collection of one of the authors.

Cultivation was performed on campylobacter agar (Ca_0) consisting of agar base [10] for chocolate agar with 5% lysed ox-blood without antibiotics [11, 12] in adequate atmosphere and temperature for 48 h [3, 11].

Cultivation of faecal samples. Twenty *C. jejuni* positive stool samples received from infants under 1 year with enteritis [12] were cultured on Skirrow's modified selective campylobacter agar (Ca) made up of agar base [10] with 5% lysed ox-blood and Skirrow's antibiotics [11], by streaking out directly on the day of sampling. Subsequently the samples were kept at 4 °C and cultures were set up succesively every day until they had become negative.

Bacterial suspensions for survival. The growth of two plates of each strain was recollected and suspended in 1 ml of nutrient broth [10] (40 mg of bacteria, wet weight/ml). From the suspensions 0.2 ml was added to 1.2 ml of each medium used for survival.

Media for survival. Peptone water with 4% Bacto-peptone; *peptone water–saline* with 4% Bacto-peptone and 0.85% NaCl; *milk* from skimmed milk for anaerobic diagnostics [10]; *albumin* solution with 4% Bovine albumin (Bovine Plasma Albumin, FA V; Browning Chem. Corp., New York).

The bacterial suspensions were distributed in 0.1 ml aliquots in 4.5×5 mm tubes, then the tubes sealed by cotton plug were stored at 22 °C, 4 °C, —20 °C and —60 °C. Subcultures were made under adequate conditions of atmosphere and temperature (42 °C) on Ca_0-agar at the following points of time: 22 °C: after 10 days; 4 °C: 2, 3 and 4 weeks; —20 °C: 3, 4 and 5 weeks; —60 °C: 5, 7 weeks and 17 months.

Results

None of the 19 strains tested in the four media and stored at room temperature for 10 days was able to grow.

As shown in Table I, in peptone water after 2 weeks storage at 4 °C 11 strains could be recovered; at —20 °C only one survived. After 3 weeks storage in the same medium none was recovered at 4 °C, and only 7 survived at —20 °C. In peptone–saline at —20 °C survival was poor, as only 4 and 3 strains were recovered in the 2nd and 3rd week, respectively. At 4 °C for 2 weeks 15 strains survived, but after 3 weeks only one could be recovered. At —60 °C for 7 weeks, 15 strains were able to grow from peptone water, but the number of colonies was poor. In peptone–saline only 9 strains survived for 7 weeks. At —60 °C for 17 months the survival was very poor in both media.

From Table II it is seen that in both milk and albumin at 4 °C good growth could be obtained after 2 weeks. In the same media at —20 °C (Table II) strains could easily be maintained for 3 weeks, but after 4 weeks the growth became poor, especially in milk.

Survival was best at —60 °C in both milk and albumin (Table II). After 17 months one strain failed to survive in albumin and 8 in milk. The one strain that failed to survive in albumin as well as in milk, had in the initial nutrient browth suspension the lowest density i.e. its transmittance in 1:50 dilution was 99% in contrast to the 82% average transmittance of the other 18 suspensions.

Table I

Survival of C. jejuni in peptone water and peptone-saline, Number of strains, 19

Weeks	Growth*	Peptone water			Peptone-saline		
		4 °C	—20 °C	—60 °C	4 °C	—20 °C	—60 °C
	4+	3	0		3	0	
	3+	3	0		4	0	
2**	2+	3	1		2	0	
	1+	2	0		6	4	
	±	0	0		0	0	
	—	3	18		4	15	
	4+	0	0		0	0	
	3+	0	2		0	0	
3	2+	0	1		0	0	
	1+	0	3		0	3	
	±	0	1		1	0	
	—	19	12		18	16	
	4+	0	0		0	0	
	3+	0	0		0	0	
4	2+	0	0		0	0	
	1+	0	0		0	0	
	±	0	0		0	0	
	—	19	19		19	19	
	4+		0	9		0	3
	3+		0	1		0	2
5	2+		0	3		0	6
	1+		0	0		0	3
	±		0	2		0	3
	—		19	4		19	2
	4+			0			0
	3+			1			0
7	2+			2			2
	1+			7			0
	±			5			7
	—			4			10
	4+			0			0
75	3+			0			1
(17 months)	2+			1			0
	1+			3			0
	±			1			0
	—			14			18

* 4+ to 1+ relative number of colonies; ± few colonies; — no growth

** Survival of 5 strains in peptone water at 4 °C could not be evaluated because of contamination

Table II

Survival of C. jejuni in milk and albumin solution. No. of strains, 19

Weeks	Growth*	4 °C		—20 °C		—60 °C	
		Milk	Albumin	Milk	Albumin	Milk	Albumin
	4+	9	13				
	3+	2	2				
2	2+	3	3				
	1+	4	1				
	±	0	0				
	—	1	0				
	4+	0	0	12	17		
	3+	0	0	1	0		
3	2+	0	0	2	2		
	1+	6	2	1	0		
	±	1	0	0	0		
	—	12	17	3	0		
	4+	0	0	0	9		
	3+	0	0	0	3		
4	2+	0	0	3	2		
	1+	0	0	3	2		
	±	0	0	3	1		
	—	19	19	10	2		
	4+			0	0	13	15
	3+			0	0	0	0
5**	2+			0	4	0	1
	1+			1	6	0	2
	±			1	2	2	1
	—			17	7	0	0
	4+					9	12
	3+					4	0
7	2+					2	4
	1+					2	3
	±					2	0
	—					0	0
	4+					5	8
75	3+					2	0
(17 months)	2+					2	3
	1+					0	3
	±					2	4
	—					8	1

* 4+ to 1+ relative number of colonies; ± few colonies; — no growth

** Survival of 4 strains in milk at —60 °C could not be evaluated because of contamination

As it can be seen in Table III, *C. jejuni* could not be cultured again from the faeces of patients on the day after sampling in ten cases. Longer than 3 days survival was found in 7 cases.

Table III

Campylobacter jejuni: recovery in 20 positive faecal samples kept at 4 °C

Days after storage at 4 °C	No. of samples	
	positive	negative
0	20	0
1	10	10
2	8	12
3	7	13
5	6	14
7	2	18
12	1	19
20	0	20

Discussion

As the present results showed, room temperature is not suitable for the recovery of *C. jejuni* after ten days storage, although Amos [13] reported good recovery at that temperature using Amies medium [14], and Lecce [8] reported similar results using thioglycolate with 0.3% agar. Other authors, such as Blaser et al. [6] failed to obtain a good survival in human bile, urine and bovine milk at room temperature. Neither seems peptone water (4%) or peptone-saline suitable for the purpose at any of the temperatures studied. Milk and 4% albumin solution gave the best results, although we have to consider the differences in storage temperature. While at 4 °C recovery was adequate only for 2 weeks, at −20 °C it was satisfactory for 3 weeks. Our results showing the best recovery at −60 °C are in agreement with the results of Walder [15] and King [5] who used defibrinated rabbit blood at −40 °C and Chow [16] who used milk at −75 °C with success. We found albumin solution as the best medium for the preservation of *C. jejuni;* its use in small quantities with dense suspensions can be recommended in combination with cooling for laboratories where lyophilisation is not available.

Campylobacters differ in survival not only in pure cultures and under known circumstances, but particularly under unknown conditions, like in stool samples. It is known that survival of *C. jejuni* is better at 4 °C than at room temperature [6]. This is a warning against the transport of stools lasting

longer than one day, since campylobacter could not be recovered on the day after the first culturing from half of the samples kept at 4 °C.

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NOTE

ISOLATION AND IN VITRO ANTIMICROBIAL EFFICIENCY OF *BUTEA MONOSPERMA* SEED OIL ON HUMAN PATHOGENIC BACTERIA AND PHYTOPATHOGENIC FUNGI

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The in vitro antimicrobial efficiency of seed oil of *Butea monosperma* was studied by the filter paper disk method against several human pathogenic bacteria and fungi. The oil showed a significant bactericidal and fungicidal effect.

Medicinal plants have been found to possess different chemical constituents active against a number of bacteria and viruses. The antimicrobial activity of many plant extracts [1] and fatty oils of different seeds [2–5] has been reported. The seeds of different medicinal plants are rich in fatty oils having good antibacterial activity [3–7]. The present investigation deals with the isolation and testing of in vitro antimicrobial activity of the fatty oil of *Butea monosperma* seeds on human pathogenic bacteria and phytopathogenic fungi.

The seeds of *B. monosperma* (palas) belonging to the family *Leguminaceae* are used in the treatment of various diseases [8, 9] as they possess anthelminthic and antifertility properties [10–12]. The oil extracted from the seeds was found to contain different saturated and unsaturated fatty acids such as palmitic, stearic, linoleic, linolic, oleic, behenic, arachidic, crucic and cerotic acids [6, 7]. In addition to fatty acids, the oil contained glycosidic sterols namely ceryl alcohol, β-sitosterol-α-amyrin, β-sitosterol-α-D-glycosides, and sucrose [13].

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Materials and methods

The dried and powdered seeds were extracted with light petrol; after removal of the solvent by suction, a yellow oil (34%) was obtained.

For the study of antibacterial and antifungal activity in vitro we used Oxoid nutrient broth and Sabouraud broth solidified with 2.5% agar. The agar diffusion technique [14, 15] was followed by using special microbial filter paper disks [16].

The plates were flood with broth cultures grown at 37 °C for 18 h (bacteria) or at 26 °C for 72 h (fungi). After removing the excess liquid, the filter paper disks 4 mm in diameter were moistened with the oil (0.15 μg) and placed over the seeded medium. Control microbial susceptibility testing was performed with biodisks [17] of penicillin, streptomycin and tetracycline for antibacterial, and with Hamycin for antifungal activity. The plates were incubated at 37 °C for 24 h, and at 26 °C for 72 h in case of fungi. The experiments were performed in triplicate and the average diameter of the inhibition zone was recorded.

Results

As seen in Table I, the most susceptible test organisms were Staphylo*coccus aureus*, *Escherichia coli*, *Klebsiella* sp., *Bacillus subtilis*, *Bacillus anthracis*, *Salmonella pullorum*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Aspergillus sydowi*, *A. luchuensis*, *A. flavus*, *Fusarium solani*, *F. semitectum*, *F. oxysporum*, *F. moniliforme* and *Cladosporium*.

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Table I

Antimicrobial activity of the seed oil of Butea monosperma

Microorganisms*	Diameter of zone of inhibition (in mm)
Staphylococcus aureus	7
Staphylococcus epidermidis	7
Bacillus subtilis	12
Bacillus anthracis	8
Klebsiella sp.	8
Salmonella newport	.
Salmonella pullorum	10
Proteus vulgaris	10
Escherichia coli	9
Pseudomonas aeruginosa	8
Aspergillus niger	9
Aspergillus ustrus	9
Aspergillus sydowi	14
Aspergillus luchuensis	13
Aspergillus flavus	10.5
Fusarium solani	10.5
Fusarium semitectum	13
Fusarium oxysporum	7
Fusarium moniliforme	12
Cladosporium	6
Cladosporioides	4
Periconia spp.	16

· Not done

* Test organisms were obtained from the College of Veterinary Sciences and Animal Husbandry, Jabalpur (bacteria) and from the School of Studies in Botany, Ujjain

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NOTE

PURIFICATION AND ELECTRON MICROSCOPY OF A LARGE PLASMID OF *RHIZOBIUM MELILOTI* 41

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(Received June 14, 1982)

A large plasmid DNA molecule was purified from *Rhizobium meliloti* 41 by CsCl–ethidium bromide density gradient centrifugation. Electron microscopic and agarose gel electrophoretic data suggest that addition of alkali effectively removes the chromosomal DNA, the plasmid DNA can be precipitated from the cleared lysate and no gradient centrifugation is needed for plasmid purification.

Material and methods. DNA was purified for density gradient centrifugation by the slightly modified method of Currier and Nester [1]. One and half litre exponential phase culture of *Rhizobium meliloti* 41 was pelleted and resuspended in 0.5 litre TE buffer (0.05 M Tris–HCl, 0.02 M EDTA, pH 8).

Lysis of cells was achieved by adding 30 ml 20% sodium dodecylsulphate solution. Sixty ml Proteinase K (5 mg/ml) was added to the lysate and incubated at 37 °C for 30 min. The clear, very viscous lysate was sheared at 500 rpm for 10 s, then stirred at 150 rpm for 10 min with a magnetic stirrer.

Linear DNA was removed by adding 3 N NaOH solution drop by drop to the cell lysate until the pH reached the value of 12.3. Solid NaCl was then dissolved in the lysate by stirring up to 3% concentration. An equal volume of freshly distilled phenol saturated with 3% NaCl solution was mixed by stirring for further 10 min. The DNA content of the aqueous phase was precipitated by 0.7 vol. ethanol at —20 °C overnight. The precipitated DNA was pelleted in a bench-top centrifuge, vacuum dried and dissolved in TES buffer (0.05 M Tris–HCl, 5 mM EDTA, 0.05 M NaCl, pH 8.5).

Solid CsCl to a refractive index of 1.39 ($\varrho = 1.598$ g/cm^3) and ethidium bromide up to 0.5 mg/ml concentration were dissolved. Centrifugation was carried out in a Beckman L2 65B type ultracentrifuge in an SW41 rotor at 35 000 rpm for 50 hr. After centrifugation two DNA bands were found under UV light.

The aqueous method was applied [2] for spreading DNA for electron microscopy. The specimen grids were shadowed with platinum.

Agarose gel electrophoresis [3] of the *Hind*III digested DNA [4] was carried out in a vertical slab gel electrophoretic apparatus. Ethidium bromide (0.5 μg/ml) was dissolved in the hot gel. The DNA bands were visualized by 366 nm UV light illumination.

Results and discussion. Two DNA bands appeared after CsCl–ethidium bromide density gradient centrifugation suggesting the presence of linear and circular bacterial DNA. The bands were separated and extracted three

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times with CsCl saturated i-propanol to remove the dye and dialysed in sterile dialysis tubes against TES buffer. The DNA was precipitated with ethanol, pelleted and vacuum dried, dissolved in 50 μl sterile TES buffer and kept refrigerated at 4 °C in sterile Eppendorf tubes.

The electron microscopy of DNA showed that the lighter band contained linear DNA of 60 to 120 kilobasepairs (kbp) in length. The second, denser band consisted of circular DNA. Both relaxed and "supercoiled" forms were found (Figs 1 and 2). We use the term "supercoiled" for the category of plasmid DNA molecules shown in Fig. 2 which presumably contained less single stranded breaks than did the open circle form presented in Fig. 1. The presence of a few linear molecules was due to mechanical breakage during preparation of the specimen grids.

Fig. 1. Relaxed (OC) form of *Rm* 41 plasmid DNA molecule. ×6700; platinum shadowed

The molecular weight of the plasmid DNA was calculated by measuring the contour lengths of eight molecules using a conversion factor of 3.2 kbp = = 1 μm, since we did not spread marker DNA as internal standard. This calculation resulted in a value of 210 kbp which was in good agreement with that found by Casse et al. [5].

*Hind*III digestion of the DNAs from the two bands resulted in an identical pattern (not shown). The gel pattern of the DNA originating from the linear band had, however, a mediate background smear because of the random breakage of the undigested plasmid DNA and the presence of some chromosomal DNA contamination which was not totally removed by the alkali treatment. The molecular weight of the plasmid DNA was not calculated by gel electrophoresis.

The results indicate that the bulk of the DNA pelleted before CsCl–ethidium bromide centrifugation mainly consisted of plasmid molecules. We could not exclude the presence in the pellet of single stranded DNA or RNA as contaminating nucleic acids. Still, there was no sign of them either in the bottom of the gradient or as a precipitate after UV illumination of the gradients. The purification method itself guarantees a good quality and yield of this large plasmid and the time-consuming and expensive CsCl gradient centrifugation can be omitted.

Fig. 2. "Supercoiled" form of *Rm* 41 plasmid DNA molecule. ×10 000; platinum shadowed

There are indications that *R. meliloti* 41 has another, bigger plasmid, too [6]. Due to its large size (more than 400 kbp) it could only be detected by gel electrophoresis [7].

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STRUCTURE AND AGGREGATION OF ADENOVIRUS HEXON PROTEINS

(A REVIEW)

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(Received: November 12, 1982)

Introduction

Nearly 30 years have passed since the discovery of adenoviruses [1] and since then more than 100 adenovirus types (species) of human and animal origin were recognized [2–8]. The amount of 5000–20 000 virus particles produced per cell [9] during multiplication in infected cells is of great advantage in their study. The fact that only a small quantity of the virus specific proteins is used for the formation of the complete virion and 80–95% of them may be recovered in soluble form from the infected cells and the medium [10] and then purified, provides means for physical, chemical, immunological and morphological studies.

The cohesive force among the polypeptide subunits constituting the hexon capsomer of the protein coat is in the case of adenoviruses such a strong though not covalent bond that the hexons may be recovered in intact form from the infected cells and each of their characteristics correspond to the hexons obtained by destruction of the virion [11].

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Hexon capsomers are present in the largest amount in the adenovirus capsid. Of the 252 capsomers (morphological units) building up the capsid of icosahedral symmetry, 240 are the hexons [12] representing approximately 53% of the total bulk of the virion. Only about 10% of the hexon proteins produced in the course of virus multiplication are built into the virion. This high rate of excess production and the possibility of extraction and purification might be the cause of the fact that the adenovirus hexon is probably one of the most intensively studied and best known virus specific protein.

Morphology of the hexon

The first electron microscopic examinations of the adenovirus hexon described the capsomer as a globular formation [12]. Studying the hexons obtained by the destruction of adenovirus type 5 they were found to be polygonal rod-like formations with a maximum size of 8.0 nm and a hole of 2.5 nm in diameter in their centre [13]. The increased resolution power of electron microscopes and the introduction of freeze-etching and freeze-drying techniques [14] resulted in a more detailed recognition of the hexon structure. Thus, the structure resembles a triangular prism with a base of 8.5 × 9.9 nm, with a smaller end of 7.5 nm and a hole of 2.5–3.5 nm in diameter at the top of the hexon.

Upon heat treatment the adenovirus capsid disrupts rapidly to nonamers [15]. These formations which consist of nine hexons (GON = group of nine) staying together in a defined formation, decompose into monomers upon more drastic treatment. Reconstruction of the capsid in vitro showed that the nonamers were adsorbed to the grid in two positions, in right-handed or left-handed form [16]. According to the examinations of Nermut and Perkins [17] the hexons are stained on the electron microscopic grid from below in a width of 2–3 nm, thus in electron microscopic examinations only those hexon-end profiles can be studied which are adsorbed to the grid and the image is actually independent of the particle "height". Ninety percent of the nonamers adsorb in left-handed position to the hydrophobic carbon-formvar-coated grids, nonamers of right-handed array are demonstrated only if hydrophilic or positively charged grids are used.

A new three-dimensional model of the hexon has been elaborated on the basis of electron micrographs with computer modelling [17]. According to the model the internal end of the hexon facing originally the virion core in the virus capsid measures 7.5–8.0 nm and that of the nearly round hole in its centre, 2.5—3.0 nm in diameter. The diameter of its external end measures 8.5–9.0 nm and there is a Y-shaped slit between the three polypeptide subunits. Owing to this, the two ends of the hexons are different in profile. A very thin channel

runs through the hexon and widens slightly towards the basis. The middle part (waist) of the hexon between the roundish internal end and the approximately triangular external end is hexagonal [17–20], with a diameter of 8.5–9.6 nm. The top of the hexon is twisted by 30° with respect to the hexagonal middle and internal part. Burnett et al. [20] demonstrated by a 6 Å resolution electron density map that the hexon has a pseudohexagonal base and a triangular top. The top is about 10° offset in the anti-clockwise sense from the mid-points of three basal edges. The hexon is about 11 nm in height [8].

The structure of the capsid as determined from the hexon model constructed by the X-ray diffraction studies of Burnett et al. [18, 19] and by computer modelling [17] was more close and stable than that assumed on the basis of the earlier hexon model [14]. According to earlier considerations the capsid was more porous, the space among the hexons was permeable for larger molecules and the linkage was considerably looser than in the structure with hexons having a hexagonal middle part wider than the two ends. This structure only assumably allows ion migration between the core of the virion and the environment [21].

Different physical and chemical methods revealed that the three polypeptide subunits constituting the hexon were identical [22] and that the hexon polypeptides displayed a 3-fold symmetry in the complete hexon [23]. For adenovirus type 5, two hexon profiles were described in the nonamers [17]. In our studies undertaken for the determination of the structure of the two-dimensional hexon crystalline array (see later) the existence of two hexon profiles was shown with the adenovirus type 1 hexon [24, 25]. Though the three chemical subunits building up the hexon, i.e. the three polypeptides, are seldom clearly visible on negative staining owing either to the distribution of the negative stain or to the deviation or dislocation of one of the polypeptide ends in consequence of the flexibility of the polypeptides, we could often observe hexons with ring-shaped walls displaying three electron dense spots corresponding to the polypeptides. By direct examination of two-dimensional hexon crystalline array we found also capsomers with clearly visible three polypeptides. The two different hexon profiles became expressed using Markham's rotational integration technique [26] for the study of the symmetry of the polypeptide subunits building up the complete hexon. In one of the hexon profiles demonstrated in the two-dimensional hexon crystalline array, the polypeptide ends showed slightly bent oblong forms enclosing an approximately round hole (Fig. 1a) and in the other hexon profile a Y-shaped slit was discovered among the polypeptides (Fig. 1b). This was a direct electron microscopic proof of the correctness of the computer made hexon model. The second hexon profile mentioned was seldom seen in the crystalline array. Corresponding to the charge within the hexon, they adsorb to the grid by their internal end in the two-dimensional crystalline array. This end of the hexon can be stained and is

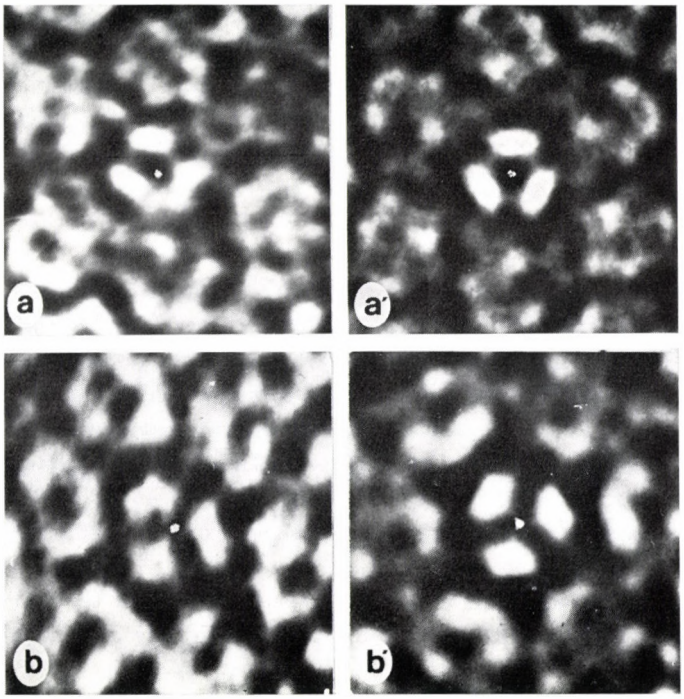

Fig. 1. Electron micrograph (a) showing a small part of a two-dimensional hexon crystalline array processed with Markham's rotational integration technique (a'). Note the three slightly rounded, oblong polypeptide subunits constituting the central hexon, with intrahexonal connective elements among them. Y-shaped slit among the three polypeptide subunits of the central hexon in a small part of a two-dimensional crystalline array (b) processed with rotational technique (b'). Angle of rotation: 120°

visible under the electron microscope. An occasionally inverse situation of the hexon means an irregularity in the structure of the crystalline array.

Taking into consideration the hexon ends, owing to the 3-fold symmetry of the hexon polypeptides, the adenoviruses represent such a special case of capsids with hexagonal structure which are built up of macromolecules of 3-fold symmetry [17]. Hexagonal packing similar to the capsid was demonstrable in the two-dimensional hexon crystalline array, too [27].

Crystallization of the hexon

Pereira et al. [28] were the first to report on the three-dimensional crystal which was obtained from the purified hexon preparation of adenovirus type 5. Then a few working groups crystallized hexon preparations of adenovirus types 2, 5 and 6 [29–32]. The crystal displayed in almost each case a tetra-

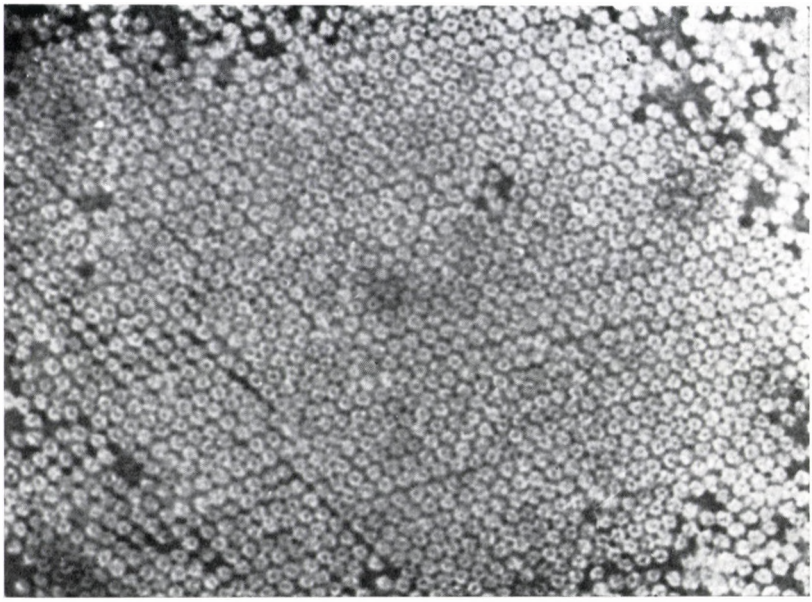

Fig. 2. Electron micrograph of a two-dimensional crystalline array developed during crystallization of the hexon. Approx. × 250 000

hedral form. We have elaborated a routine-like crystallization method by dialysing the hexon preparation (extracted and purified from adenovirus type 1 infected cells) against 0.5 mol/l acetate buffer [33]. The adenovirus hexon was the first virus subunit protein which was successfully crystallized.

With X-ray diffraction both adenovirus type 2 [30] and type 5 [29] hexon crystals were found to belong to crystals of the space group $P2_13$, the unit cell had an a = 14.99 nm and contained 4 hexons per unit cell. Similar results were obtained by the electron diffraction study of adenovirus type 1 hexon crystals, though the size of the unit cell was larger by a few nm-s [34]. Since there are 12 asymmetric units per unit cell in $P2_13$ there must be three crystallographic asymmetric units in each hexon and the axis of the hexon runs parallel with the axis of symmetry [35].

Two-dimensional hexon crystals

To follow the crystallization process, serial electron microscopic examinations were performed in the course of crystallization of the adenovirus type 1 hexon [27]. A few hours after dialysis which induced the aggregation of the hexons, small regular groups of hexons were found and with the progress of the process two-dimensional crystalline arrays consisting of several thousands of

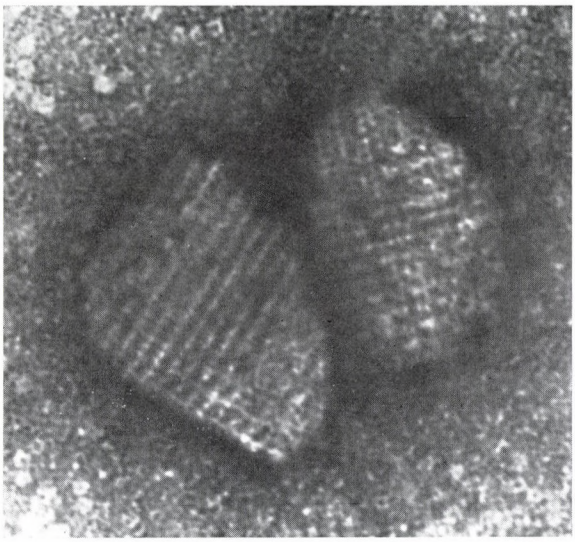

Fig. 3. Electron micrograph of hexon microcrystals with linear internal structure. Approx. × 200 000

hexons were seen to develop (Fig. 2). Electron microscopic studies also showed the development of "three-dimensional" microcrystals of multilayer appearance (Fig. 3) presenting a regular internal structure [36, 37]. This observation was the first one in this field. Detailed analysis of the structure of the two-dimensional crystalline array revealed besides the hexagonal regularity also irregularities in the structure [27]. The average values of the lattice constant measured in non-parallel directions of the hexon rows showed significant deviations. The hexons form a slightly skew and irregular hexagonal structure in the two-dimensional hexon crystalline array. Burnett et al. [20] demonstrated that the pseudo-hexagonal base and waist of the hexons facilitated the packing of the virus capsid. Due to its structure, the two-dimensional hexon crystalline array is suitable for optical diffraction studies. With the procedure adapted to the light microscope we could demonstrate that the intensity of the diffraction maxima characteristic of the structure was diverse; they appeared in relatively few number of orders, which referred to the short and long-range disorders of the crystal lattice [37, 38]. The values of the lattice constant calculated from the distance of the reflection maxima confirmed in accordance with the results of direct analysis the slightly skew hexagonal structure of the two-dimensional hexon crystal.

The two-dimensional crystalline array is especially suitable for the study of the mutual orientation and mode of linkage of the hexon polypeptide subunits, as the periodically repeating information can be found in it over a large area. A direct study of the electron microscopic picture and the introduction

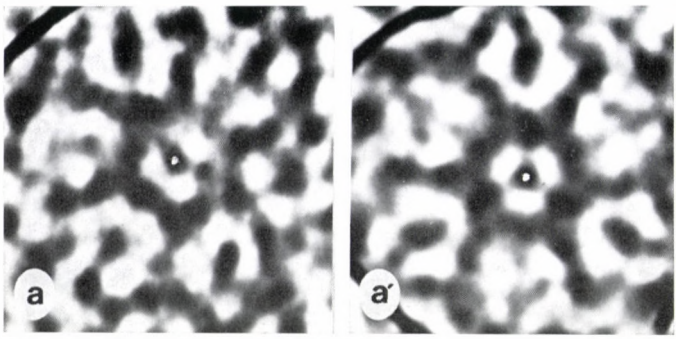

Fig. 4. Electron micrograph (a) of a small part of a two-dimensional hexon crystalline array processed with Markham's rotational integration technique (a'). The polypeptide subunits constituting the central hexon are well visible. Note that each of the polypeptide subunits are nearest to the two polypeptide subunits of the neighbouring hexons. "One-to-two" orientation. Angle of rotation: 120°

of different image improving procedures (Markham's rotational integration and computer image-analysis) provided means for the recognition of the structure of the two-dimensional crystalline array at the level of hexons and of the polypeptides constituting a complete hexon [24, 25, 39]. The "one-to-two" orientation system demonstrated between the polypeptides of the neighbouring hexons in small distinct parts of the crystalline array means that one of the polypeptide subunits of a given hexon is situated nearest to the two other polypeptides of the neighbouring hexon (Fig. 4). Thus in the two-dimensional crystalline array the complete hexons and their polypeptide subunits are in an equivalent environment corresponding to the hexagonal packing and 3-fold symmetry. This orientation may be universal not only in the two-dimensional crystalline array but also in the adenovirus capsid [34].

Studying the structure of the two-dimensional crystalline array which developed from adenovirus type 1 hexons we have demonstrated the existence of interhexonal connective elements [25]. These connective elements produce the "one-to-two" linkage system by being located between each polypeptide subunit of each hexon and two polypeptide subunits of the neighbouring hexon i. e. by linking them. That means that in a regular case six-times two approximately parallel connective elements run from the central hexon to its neighbouring hexons (Figs 5 and 6). Electron micrographs have confirmed the existence of interhexonal connective elements also in the capsid of adenovirus type 1 [34]. Fine connective elements can be discovered not only between the hexons but also among the three polypeptide subunits in the complete hexon (Fig. 2a) [25]. Introduction of the underfocussing technique [40] was of great help in the distinction of the connective elements from the background granulation and Markham's technique contributed to the recognition of the symmetry conditions [26].

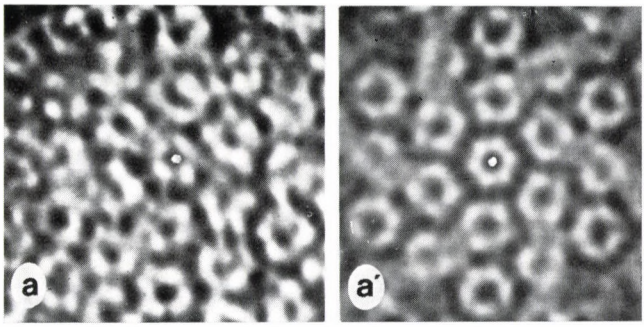

Fig. 5. Electron micrograph of a small part of the two-dimensional crystalline array (a), processed with Markham's rotational integration technique (a'). Six-times two parallel connective elements run from the central hexon to the six surrounding hexons. Due to the six-fold rotation the profiles of the three polypeptides of the central hexon with 3-fold symmetry are blurred. Angle of rotation: 60°

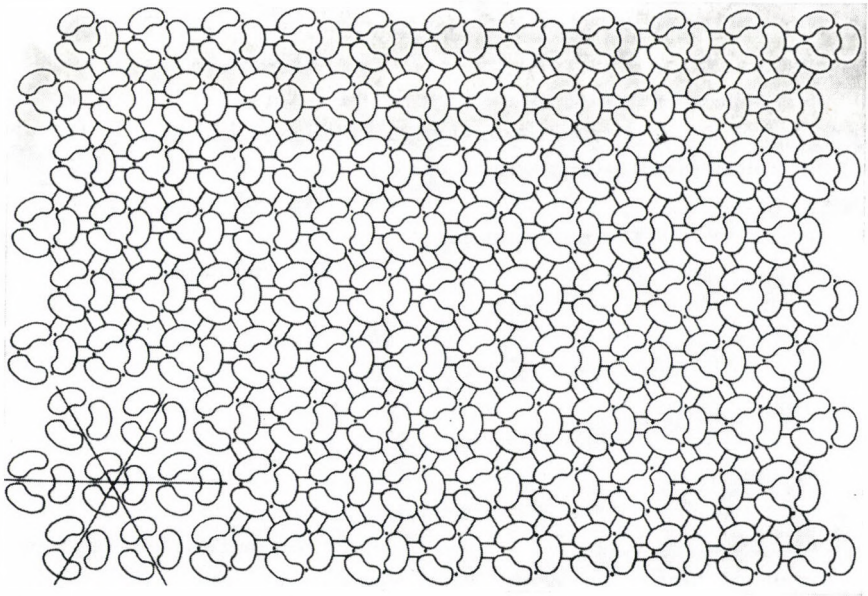

Fig. 6. Tentative diagram of the structure of the two-dimensional hexon crystalline array showing the possible mutual orientation of hexon polypeptide subunits and the connective elements. It demonstrates the "one-to-two" linkage of the hexon polypeptide subunits between and within the complete hexons. The orientation corresponds to the 3-fold symmetry and hexagonal packing. Lines indicate the interhexonal connective elements between the hexons and spots the intrahexonal connective elements between the hexon polypeptides. The axis corresponding to the three non parallel hexon rows (drawn in the lower corner on the left) runs between two polypeptide subunits of a given hexon and halves the third one

Molecular weight of the complete hexon

Examinations of the molecular weight of the hexon failed to give identical results even with identical types. The molecular weight of different adenovirus hexon types varied between 282 000 and 413 000 with analytical ultracentrifugation [41], with determination on the basis of density subjecting the crystallized hexon to ultracentrifugation in density gradient [29, 30] as well as in gel filtration and acrylamide-gel electrophoresis [42]. Differences exist not only in the molecular weight of different human adenovirus hexons but also in the human and bovine adenovirus hexons [43]. The differences found between the molecular weights of complete hexons may naturally be detected also in the molecular weight of their polypeptides. Thus in adenovirus type 5 the value calculated on the basis of the hexon crystal density was 282 000–315 000 for the complete hexon [29] and using the same method this value was 363 000–413 000 for type 2 complete hexon [30]. Studying the polypeptide subunits of the same types, the molecular weight of type 5 hexon polypeptide was lower by 15 000 than the value determined for the identical polypeptides of type 2 [11, 44].

Differences in molecular weight were reflected also by the values of the sedimentation coefficient [22].

Isoelectric point of the hexon

Owing to the structure of the virion not each hexon is located in identical environment within the adenovirus capsid. Hexons around the vertex capsomers, the so called peripentonal hexons [45] are neighbours of pentons on one side and of hexons on the other. Viewing a single triangular face of the icosahedron, three different hexons are discernible [46] of which one is the mentioned peripentonal hexon, the second is a non-peripentonal one situated on the edges and the third is located in the middle of the triangular face. Destruction examinations revealed the strongest bond among the latter. Their isoelectric point was found to be pI 4.7. The pI of the peripentonal hexon is 3.1 and that of two adjacent hexons in the middle of the edges is 3.7 with adenovirus type 5. The isoelectric point of hexons extracted and purified from cells infected by adenovirus type 1 was found to be pI 4.54 [47] which is near to the pI value determined for the hexons in the triangular face in the case of adenovirus type 5. This indicates that this hexon is dominant in the soluble hexon population. In the course of multiplication of adenovirus type 2 a hexon population was detected which could be found only in the infected cells or in their supernatant and not in the virion capsid and whose isoelectric point was pI 4.9 being thus higher than the above described pI values [48].

Antigenic structure of the hexon

The hexon protein being present in the largest amount in the virion capsid carries genus-specific antigen determinants. Antibodies produced against the common complement fixing antigen gives a cross reaction with adenoviruses belonging to the *Mastadenovirus* genus [8, 3, 49, 50]. No such cross reaction was observable with the members of the *Aviadenovirus* genus [3]. The serological cross reaction between the members of the *Mastadenovirus* genus is significantly stronger in the individual subgroups (subgenera) than between the members of the different subgroups [51]. This difference of reactivity strength within the same subgroup and between the subgroups is called intra- and inter-subgroup specificity [52]. Besides the genus-specific part a type specific (species specific) component can also be discovered in the hexon. Variety of epitopes related to all these antigens has been differentiated by hybridoma techniques [8]. In the virion capsid only the type specific antigen determinant is accessible for the antibodies because different regions of the hexon carry the corresponding antigen components. Genus-specific antigen was found in the internal end of the hexon facing the core of the virion while the external end contained type-specific antigen [11]. The serological reaction thus provides means for a topological recognition. In soluble hexons genus-specific antigens are naturally also accessible for the antibodies besides the type-specific part [50], however only type specific antigen reacts with antibodies in the virion capsid [54]. In this way antibodies produced against the type-specific components of the hexon are able to neutralize the infectivity of the virus [53]. Hexons of different adenovirus types are able to induce the production of neutralizing antibodies at different rates [55].

Experiments with adenovirus type 2 and 5 revealed [56] at least two hexon populations in the infected cells of which only one is able to induce type-specific antibody production. The two hexon populations were found to have different electrophoretic mobilities. This phenomenon was assumed to be due to a special polypeptide component being present only in the slower migrating hexon. This "slow" hexon exerted an approximately 50-times stronger neutralizing activity. The polypeptide subunit of the "slow" hexon is shorter by 50–60 amino acids than the other polypeptide of 115 000 molecular weight which is the component of the "normal" hexon present both in the virion capsid and in the supernatant. No "slow" hexon may be found in the virion capsid. Boulanger et al. [48] demonstrated that the "slow" hexon contained twice as many type-specific antigen determinants than did the hexon being built in the capsid. Their isoelectric points are also different.

According to digestion experiments carried out with different enzymes only the genus-specific part was affected by the treatment while the type-specific part remained intact [56]. Trypsin treatment however failed to change

the immunological activity and morphology of the hexon, in spite of the fact that the enzyme cleaved the polypeptide at several points [57, 58].

Döhner and Dieckmann [59] demonstrated that the hexon contained at least 20 determinant groups. Half of them was type-specific and the other half genus-specific antigen determinant. Monoclonal antibodies produced by hybridoma cells furnished further evidence of the fact that the hexon carried both genus- and type-specific antigen determinants [60].

Soluble antigens of adenoviruses were used also for human immunization [61]. The development of neutralizing antibodies i. e. the reactivity of the hexon is stronger in the presence of liposomes or Freund's adjuvant than in aequous solution [62, 63]. Immunizing volunteers with crystallized adenovirus hexon, Couch et al. [64] reported on a significant increase in neutralizing antibodies.

Polypeptides

The double stranded linear DNA of adenoviruses is of 20–25 $\times$ 10^6 molecular weight [65] and its genetic information content furnishes enough information for coding 20–40 proteins of medium size [11]. Only those will be mentioned here which are in connection with the hexon.

Polypeptides demonstrable in adenoviruses are designated by Roman numbers. Of them polypeptide II builds the hexon in such a way that one complete hexon contains three polypeptide II [22]. Besides the capsomer building polypeptides the virus capsid is composed of several other components [66]. Two of them demonstrated in connection with the hexon [67] are polypeptides VI and VIII. Antibodies produced against polypeptides VI and VIII fail to react with the intact virion, thus these polypeptides provide the connection between the hexons at their base facing the virion core. Polypeptide VI is demonstrable not only between the hexons located on the triangular faces and edges but also between the peripentonal hexons and pentons [68], whereas polypeptide VIII is located between the hexons only on the edges and triangular faces [66]. Polypeptide IX has been demonstrated among the hexons in the nonamers [69, 70] and its number was found to be 15 per nonamer. Minor peptides are playing presumably a role in the stabilization of the virus capsid [71]. Antibodies against polypeptide IX are able to precipitate the intact virion to a small extent, which refers to its location among the hexons near the virion surface [68].

Polypeptide IIIa is connected only with the peripentonal hexons being situated between the penton trimer [72] and the peripentonal hexons [68]. The protein 100K plays a scaffolder role in hexon morphogenesis, in the course of the formation of the complete hexon [68]. It is therefore not a structural protein. SDS polyacrylamide-gel electrophoresis of the main capsid polypeptide

with designations II, III, IIIa and IV gave a further possibility for the classification of adenoviruses [73].

Polypeptide synthesis, hexon formation

Polypeptides constituting the capsid appear as soon as 4–8 hours following the infection i.e. the DNA replication in infected cells [74–76] with the exception of polypeptide IX [77] whose mechanism of synthesis differs from that of the late polypeptides [78–80]. This polypeptide is demonstrable in the infected cells before the appearance of capsid proteins and is being produced even if the DNA replication is blocked by AraC [81].

Experiments performed with different mutants (ts mutants) showed that temperature influenced the assembling of the chemical subunits (polypeptides) into morphological units (capsomer) [82, 83]. Hexon polypeptides developing at 42 °C are unable to form trimers, complete hexons [84]. Studies with other ts mutants revealed the formation of hexons which though immunologically reactive were unable to get into the cell nucleus [85]. According to other experiments hexons constituted by polypeptides synthetized at non-permissive temperature though capable to find entrance into the nucleus are unable to incorporate into the virion capsid [11, 86]. In mutants where only the synthesis of the non-structural 100K protein is disturbed, the hexon polypeptides are unable to form trimers. This proves the scaffolder role of 100K proteins played as helpe in the process of assembling or may refer to their modifying role which contributes to the development of the hexon.

Garon et al. [87] demonstrated the similarity of the genetic structure of adenovirus hexons belonging to identical subgenus. Thus differences in the nucleotide sequence in the DNA-s of adenovirus type 2 and 5 was found only between the 0.50 and 0.60 genome units in the region coding the hexon. According to experiments performed with different restriction enzymes the hexon gene of adenoviruses studied so far is between the 0.50–0.60 genome units [76, 78, 88–92].

Amino acid composition of the hexon and the amino acid sequence of the hexon polypeptide

The amino acid composition of the hexon of adenoviruses belonging to different serological types was found to be similar according to examinations carried out in different laboratories [47, 56, 93, 94]. The hexon possesses a high number of amino acids resulting in a hydrophobic bond and they possibly play a role in the stabilization of the multimer structure. The presence of free

sulfhydril groups has been detected by analytical methods of high precision [11, 44, 95]. Jörnvall et al. [96] were the first to demonstrate that the N-terminal amino acid was acetylated and determined the sequence of the terminal peptide. Recently the primary structure of the adenovirus hexon protein has been determined and its connection with the hexon gene was established [97]. Separation of the CNBr fragments of the adenovirus hexon, the analysis of their location in the hexon and the chemical analysis of the carboxy-methylated CNBr fragments [98–100] contributed not only to the recognition of the amino acid composition of the adenovirus hexon but also to that of the amino acid sequence of adenovirus type 2 hexon protein which means the exact order of 966 amino acids in the hexon polypeptides.

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USE OF REDUCING COMPOUNDS IN THE CULTIVATION OF *AZOSPIRILLUM* SP.

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Attempts were made to grow the micro-aerophilic N_2-fixing bacterium *Azospirillum* sp. in complete liquid medium by incorporating some reducing agents. Ascorbic acid, glutathione and Na-thioglycollate stimulated while methylene blue inhibited the growth and N_2-ase activity. In complete liquid medium, Na-thioglycollate and ascorbic acid increased the N_2-ase activity with increasing concentration up to 800 μg/ml. With glutathione, growth of the bacterium was increased markedly but N_2-ase activity was repressed below 200 μg/ml. The possibility of employing these compounds for the cultivation of *Azospirillum* in complete liquid medium seems to be indicated.

With the isolation from the roots of tropical grasses of a N_2-fixing bacterium, *Azospirillum* sp. by Döbereiner and Day [1], attempts were made by several workers [2–4] to study the nutritional and physiological characteristics of the organism. It was reported by Day and Döbereiner [2] that it requires microaerophilic conditions (0.005 PO_2) for its growth when fixing atmospheric nitrogen and hence they suggested a semi-solid medium containing 0.175% agar. However, when nitrogen was supplied as ammonium chloride, the bacterium could grow aerobically but the N_2-fixation was repressed [3]. While semi-solid media are not ideally suitable for large scale multiplication, addition of ammonium chloride in liquid medium may lead to contamination by non N_2-fixing organisms. Hence, an effort was made in the present study to grow the organism in liquid medium devoid of agar and combined nitrogen but by incorporating some reducing agents.

Materials and methods

Azospirillum sp. used in the present study was isolated from the roots of *Opuntia microdays* [5] and identified as described by Tarrand et al. [6]. A semi-solid N-free malate medium [2] was used for growing the organism and the different reducing agents sterilized separately by filtration were added to the medium after sterilization. Initially all the four chemicals were tested at one particular concentration (100 μg/ml for Na-thioglycollate, glutathione and ascorbic acid and 5 μg/ml for methylene blue) in complete liquid medium and also in combination with two concentrations of agar (0.05 and 0.1%). Later methylene blue was omitted and the rest of the chemicals was tested at varying concentrations for their effect on the N_2-ase activity in complete liquid medium.

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The organism was grown in 8 ml test tubes containing 3 ml of the medium. Inoculation of these tubes was done with 0.1 ml of the 4-day-old culture grown in N-free semi-solid malate medium containing 0.08% agar. The tubes were incubated at 32 ± 1 °C for 48 h. The growth recorded as optical density was measured in a Spectronic-20 apparatus at 620 nm and the viable count of the population was estimated by the dilution plate technique using potato sucrose agar medium [7].

The culture tubes after 48 h incubation were closed with a cap and 10 % of the air from the tubes was replaced by acetylene. The ethylene produced during the 24 h incubation was estimated by an Aimil-Nucon gas chromatograph employing a poropak-T column of 2 × 0.003 m with a flow rate of 30 ml min^{-1} of N_2 as carrier gas. The activity was expressed as n moles of C_2H_4 per tube h^{-1}.

Results and discussion

Reducing agents have long been used in culture media to counteract the effect of oxygen and enable the growth of anaerobic and microaerophilic bacteria under low oxidation-reduction potential. In the present study the possibility of using some reducing agents in the culture medium for *Azospirillum* was investigated. It was found that the addition of even small quantities (100 μg/ml) of Na-thioglycollate, glutathione and ascorbic acid to the liquid medium allowed the *Azospirillum* to grow aerobically without combined nitrogen, but methylene blue (5 μg/ml) was found to be inhibitory. Although there was an increase in N_2-ase activity upon the incorporation of these chemicals, it was significant only with Na-thioglycollate (Table I). Addition of low amounts of agar (0.05 and 0.1%) along with the reducing agents even at 100 μg/ml level caused a significant enhancement in N_2-ase activity. Further, the activity obtained with 0.1% agar in combination with 100 ppm Na-thioglycollate or glutathione was nearly equal to the activity (159 n moles h^{-1}) obtained with the recommended agar concentration of 0.175% in the semi-solid medium for *Azospirillum*, indicating that O_2 levels maintained in both treatments might be the same. This indicated that microaerophilism in *Azospirillum* can partially be overcome by incorporating reducing agents in the liquid medium. Bowdre et al. [8] have also shown that aerotolerance in a related species, *Spirillum volutans*, can be enhanced by incorporating alkaline tyrosine and 3.4-hydroxyphenylalanine in the growth medium.

When these chemicals were used in higher concentrations (> 100 μg/ml) even in complete liquid medium, there was a significant increase in growth and acetylene reduction. While Na-thioglycollate and ascorbic acid enhanced growth and N_2-ase activity with increasing concentrations up to 800 μg/ml, in the case of glutathione the growth was increased but N_2-ase activity was totally inhibited below 200 μg/ml (Table II). Enhancement of the growth with glutathione was more marked than with two other compounds. The organism growing like an aerobic organism formed a dense pellicle both on the surface and at the bottom of tubes. The optical density of the culture was 0.69 with 900 μg/ml of glutathione as compared to 0.24 in the control. When the viable

Table I

N_2-ase activity of Azospirillum sp. as influenced by reducing agents at different concentrations of agar

Reducing agent	N_2-ase activity (n moles C_2H_4/h) at agar concentration			
	0%	0.05%	0.1%	0.175%
Control	3.4	69.2	93.8	159.0
Ascorbic acid 100 μg/ml	4.2	79.2	122.0	—
Glutathione 100 μg/ml	3.5	113.2	166.0	—
Methylene blue 5 μg/ml	N. D.	15.4	52.4	—
Na-thioglycollate 100 μg/ml	8.1	115.0	136.5	—

LSD (P = 0.05); reducing agents: 4.6; agar concentration: 3.6; interaction: 8.0
N. D.: not detectable

count of *Azospirillum* was determined by the dilution plate technique, a 65-fold increase in the population, i. e., from 250×10^5 ml^{-1} in the control to 164×10^7 at 500 μg/ml was observed. The inhibition of N_2-ase activity at higher concentrations (>200 μg/ml) of glutathione (α-glutamyl-cysteinyl--glycine) may, however, been due to the repression caused by glutathione or its metabolites. The repression was found temporary as was observed by Okon et al. [3] in *Spirillum lipoferum* with ammonium chloride. When the organism was transferred from the glutathione tubes into a fresh medium devoid of glutathione, the N_2-ase activity was regained (Fig. 1). The regained activity was directly proportional to the initial growth measured as OD obtained with increasing concentrations of glutathione.

Table II

Effect of reducing agents at different concentrations on N_2-ase activity of Azospirillum sp.

Concentration μg/ml	N_2-ase activity (n moles C_2H_4 h^{-1})		
	Ascorbic acid	Glutathione	Na-thioglycollate
0	8.2	3.2	3.2
100	4.6	3.5	6.9
200	6.1	20.2	8.2
400	6.6	N. D.	9.9
600	8.9	N. D.	10.9
800	13.2	N. D.	14.6
1000	17.0	N. D.	11.6

LSD (P = 0.05); reducing agents: 1.8; concentrations: 1.3: interaction: N. S.
N. D.: not detectable

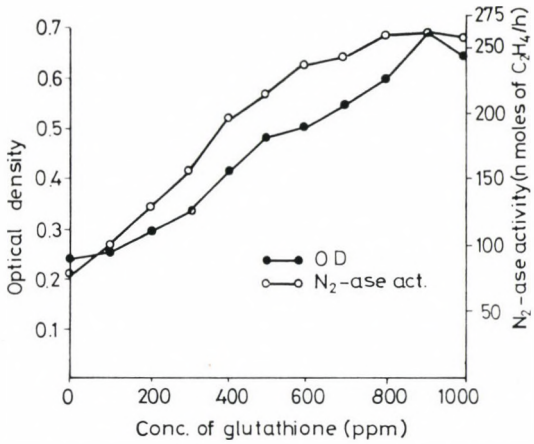

Fig. 1. Growth of *Azospirillum sp.* (OD) as influenced by glutathione and its derepressed N_2-ase activity in fresh medium

From these results it has been concluded that glutathione may be functioning as a reducing agent and nitrogen source. Salle [9] indicated that glutathione serves as a growth factor in microorganisms by acting as a hydrogen carrier or by preventing the inactivation of -SH groups. On the other hand, the moderate increase in growth obtained with Na-thioglycollate and ascorbic acid can solely be attributed to their role as reducing agents. These preliminary results indicated the possibility of using some of these reducing agents for the cultivation of *Azospirillum* in complete liquid medium. More thorough studies would, however, be needed for using the reducing agents in large scale multiplication of *Azospirillum*.

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PHAGE TYPING OF *LISTERIA MONOCYTOGENES* IN HUNGARY

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Ninety-eight (39.6%) out of 247 *Listeria monocytogenes* strains isolated from a variety of sources were typable by 27 phages. Of the 31 human strains only 3 belonged to phage types occurring in cattle, sheep and surface waters. A close correlation existed between serotype and phage type of the strains. Serotype 1/2 and 4 strains isolated in Hungary were less frequently typable than cultures originating from France. Phage typing is a useful tool for epidemiological tracing but, for a more effective differentiation, the number of phages should be increased and the method should be standardized.

Although the problem of phage typing of listeriae has extensively been studied [1–7], an internationally recognized and used method has not been developed. In 1979 Audurier et al. [8] described a procedure based on 15 phages isolated from lysogenic *Listeria* strains. Audurier et al. [9] elaborated the "octal code" system which allowed a more easy evaluation and computerization of the results. By the use of 20 phages, Audurier et al. [10] classified 823 strains isolated in France. In cooperation with the French authors, Ralovich et al. [11] at the Madrid symposium in 1981, pointed out the usefulness of the method and proposed it to the ICSB Subcommittee on Listeria for worldwide application. The present work summarizes further experience with the method.

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Materials and methods

A total of 247 *L. monocytogenes* strains was studied. They were isolated from patients, human carriers, animals and surface water. The faeces of healthy persons, animals and the membrane filters (0.2 μm) of water samples (250 ml) were seeded into Holman's cooked meat broth, incubated at 4 °C for 3 months and subcultured on trypaflavine–nalidixic acid–serum agar monthly [12]. The isolates were identified as described in reference [13]. Serotyping was made with slide agglutination using absorbed sera. The phage typing method described in reference [10] was extended to 27 phages and the new octal-code was employed. The phages used were 1967, 2685, 4477, 575, 1652, 12029, 1806, 2671, 1444, 2425, 3551, 3552, 1317, 2389, 3274, 1090, 1807, 184, 16, 4277, 5337, 4211, 4295, 4207, 4276, 4292 and 4286.

Results

Table I shows the number of typable and untypable strains according to their source. The number of typable strains (98, 39.6%) was rather low. The remaining 149 strains failed to show a sufficient degree of lysis with any of the 27 phages.

Table I

Distribution of L. monocytogenes strains according to source and typability by phages

Source	Total No. of strains	No. of strains typable	No. of strains untypable
Patients	17	8	9
Healthy human carriers	80	23	57
Sick cattle	3	3	—
Healthy cattle	67	31	36
Sick sheep	21	12	9
Healthy sheep	53	18	35
Other sick animals	2	2	1
Surface water	4	1	3
Total	247	98	149

Table II presents the phage type distribution of the 98 typable strains. Out of the 31 human strains 28 belonged to phage types occurring only in man. The remaining 3 human strains had phage patterns of cultures isolated from animal and environmental sources. Only 15 out of the 60 animal strains belonged to phage types occurring only in one animal species, the rest fell into phage types present in different sources.

Table III shows phage types that occurred in two or more kinds of sample. Type 40 000 0000 showed the widest distribution (man, animals and water); type 02 000 0000 occurred in man and animals. The remaining eight phage types were encountered only in cattle and sheep. The 3 human strains belonging

Table II

Distribution of 98 L. monocytogenes strains according to source and phage type

Source	Phage type (code)	No. of strains	Total no. of strains
Patients	*00 400 0004*	1	
	00 211 0000	1	
	00 201 0000	3	8
	00 200 0000	1	
	00 001 0004	1	
	00 001 0000	1	
Healthy carriers	40 000 0000	2	
	20 000 0000	2	
	13 000 0000	7	
	02 000 0000	1	
	00 201 4000	1	
	00 201 0000	1	23
	00 200 0000	1	
	00 060 0004	1	
	00 000 0420	3	
	00 000 0400	1	
	00 000 0010	3	
Sick cattle	74 000 0000	1	
	02 000 0000	1	3
	00 253 0000	1	
Healthy cows	*54 000 0000*	1	
	40 000 0000	2	
	34 000 0000	1	
	24 000 0000	1	
	10 000 0000	1	
	04 000 0000	3	31
	02 000 0000	4	
	01 000 0000	3	
	00 000 3232	1	
	00 000 2737	2	
	00 000 1012	1	
	00 000 0040	5	
	00 000 0004	6	
Sick sheep	74 000 0000	1	
	73 100 0000	1	

Source	Phage type (code)	No. of strains	Total no. of strains
	41 000 0000	2	
	40 000 0000	4	
	34 000 0000	1	12
	02 000 0000	2	
	00 253 0000	1	
Healthy sheep	40 000 0000	3	
	10 000 0000	3	
	04 000 0000	1	
	02 000 0000	1	
	00 000 3737	2	18
	00 000 2707	1	
	00 000 2004	1	
	00 000 0200	1	
	00 000 0040	1	
	00 000 0004	4	
Other sick animals	01 000 0000	1	2
	00 605 0040	1	
Surface water	40 000 0000	1	1

Italicized codes: host-specific phage types

Table III

Phage types found in two or more different sources

Phage type (code)	No. of strains				
	human carriers	cattle	sheep	water	other animal
40 000 0000	2	2	7	1	
02 000 0000	1	5	3		
74 000 0000		1	1		
34 000 0000		1	1		
10 000 0000		1	3		
04 000 0000		3	1		
00 253 0000		1	1		
00 000 0040		5	1		
00 000 0004		6	4		
01 000 0000		3			1

Table IV

Association between serotype (serovar) and phage type of L. monocytogenes

Serotype (serovar)	Phyge type (code)	No. of strains
	77 100 0000	1
	74 000 0000	2
	73 100 0000	1
	54 000 0000	1
	41 000 0000	2
	40 000 0000	8
	34 000 0000	1
1/2a	33 000 0000	1
I, II, (III)	20 000 0000	2
	13 000 0000	6
	10 000 0000	3
	04 000 0000	1
	02 000 0000	3
	01 000 0000	4
	ut	52
4ab	00 000 0400	1
(III), V, VI, VII, IX		
	00 605 0040	1
	00 400 0004	1
	00 253 0000	2
4b	00 211 0000	1
(III), V, VI	00 201 4000	1
	00 201 0000	4
	00 200 0000	1
	00 001 0004	1
	00 001 0000	1
	ut	2
4d		
(III), (V), VI, VII	ut	2
5	00 000 3737	2
(III), (V), VI, VIII, X	00 000 3714	1
	00 060 0004	1
	00 000 3232	1
	00 000 2737	1
6a*	00 000 2707	1
	00 000 0040	2

Serotype (serovar)	Phage type (code)	No. of strains
	00 000 0004	3
	ut	7
	00 000 2737	1
	00 000 2004	2
6b*	00 000 0420	2
	00 000 0200	1
	00 000 0010	3
	00 000 0004	2
	ut	9
V, VI, XIV, XV**	00 000 0040	2
	00 000 0004	2
	ut	10
V, VI, IX, XIV, XV**	00 000 0040	1
V, XIV, XV**	ut	1
V, VI, IX, X**	00 000 0420	1
V, VI, X, XIV**	00 000 0004	1

ut = untypable
* Strains with antigenic structure described by Seeliger and Schoofs [14]
** Unclassified serotypes (serovars)

to phage types present in other sources, were isolated from healthy human carriers. No such strains were isolated from patients.

Table IV shows the association between phage type and serotype of the isolates. Out of the 88 serotype 1/2a strains 36 (40.9%) could be classified into 14 phage types. Serotype 4b strains were mostly typable (14 out of 18). In serotype 5, two strains of identical phage pattern were isolated in Hungary, the remaining one of a different phage type, in Bulgaria. Serovars 6a and 6b were rather frequent; out of the 36 strains 20 were phage-typable. In addition to those listed, 21 unclassified serovars were studied; 7 out of them belonged to phage types found in serovars 6a or 6b. In view of O antigen components and phage pattern of these strains, they were assumed to belong to serovars 6a and/or 6b. According to our data there is an association between serotypes (serovars) and phage types.

Discussion

The epidemiology of listeriosis is far from being clear [15]. Its basic features have not been fully elucidated and methods for the tracing of the causative agent have not been internationally settled. Biotyping of listeriae by carbohydrate decomposition spectrum [16] has not widely been employed. The phage typing method used in our work needs to be improved in view of the high ratio of untypable strains (60.4%).

Table V

Typability by phages of strains isolated in Hungary and France

	Serotype							
	1/2 a		4		5		other	
	H	F	H	F	H	F	H	F
Typable	36	131	14	499	2	5	27	10
(ratio)	(0.40)	(0.57)	(0.77)	(0.88)			(0.47)	(0.41)
Untypable	52	96	4	68	—	—	30	14
Total	88	227	18	567	2	5	57	24

$\chi^2 = 7.186$ $p < 0.01$ $\chi^2 = 1.691$ $p > 0.05$ $\chi^2 = 0.221$ $p > 0.05$

H = Hungarian isolates
F = French isolates

Table V shows a comparison between Hungarian and French strains. Although the French strains were examined only with 20 phages as compared to 27 phages used in Hungary, the latter were less frequently typable than the former. Thus, although the French and Hungarian strains are similar in biochemical and serological properties, some other differences may exist between them. These may account for the fact that the epidemiological features of listeriosis differ in the two countries [15].

For a further improvement in standardization of the phage typing method, Ralovich has proposed that the 10 reference strains distributed for biochemical and serological control examinations by the ICSB Subcommittee on Listeria should be used also for checking phage typing; Audurier does not agree with this idea. We have examined 7 of these strains and found 3 of them phage-typable (Table VI). A few strains selected for speciel features may be added to this control set. Finally, it may be mentioned that the usefulness of the phage typing method applied by us has been confirmed by Taylor et al. [17], too.

Table VI

Characteristics of Listeria strains distributed by the ICSB Subcommittee on Listeria in 1979

Designation	Antigenic structure	Phage type	Name	Other designation
C_{52}	I, II	ut	*L. monocytogenes*	NCTC 7973
C_{201}	I, II	04 000 0000	*L. monocytogenes*	NCTC 10357
C_{212}	—	.	*L. denitrificans*	Prevot 55134
C_{214}	V, XII, XIV	ut	*L. grayi*	Seeliger L 332/64
C_{644}	V, VI, VII, IX, XV	ut	*L. innocua* 6a	Seeliger
C_{645}	V, VI, VII, IX, X, XI	00 000 2004	*L. innocua* 6b	Seeliger
C_{663}	VIII, IX, X	00 000 3714	*L. monocytogenes* 5	Ivanov (L72)
C_{664}	V, VI, X	.	*L. monocytogenes* 5	Ivanov (L173)
C_{670}	—	.	*L. denitrificans*	ATCC 14870
G_{44}	V, XII, XIV	ut	*L. murrayi*	ATCC 25401

ut = untypable
. = not examined

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CHANGES IN PHAGE-SENSITIVITY OF *SHIGELLA FLEXNERI* STRAINS

I. CHANGES IN VIRULENCE AND PHAGE-SENSITIVITY

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In the course of repeated passaging of virulent *Shigella flexneri* cultures in liquid media, avirulent variants and variants of reduced virulence appeared. They were easily recognizable by their colonial morphology. The avirulent variants became sensitive to certain phages not lysing the original virulent strains. The change of phage-sensitivity among the variants with no or decreased virulence was significant: $\chi^2 = 64.25$; $P < 0.001$. The amplification of phage-sensitivity developed in 15% of the originally avirulent cultures. No association was found between the loss of virulence and a specific phage-sensitivity pattern. In the majority of the cases sensitivity to phage Ms2 developed during the passages, but it was observed in some of the original strains, too. With few exceptions, sensitivity to phage Ms2 was associated with the total or partial degradation of the specific antigen and the antigenic structure detectable by *S. flexneri* factor sera. These exceptions were cultures with maintained or decreased virulence.

It was reported previously [1] that avirulent *Shigella* variants easily recognizable by their colonial morphology on agar plates, had been obtained after a few passages in peptone water. Under oblique transmitted light depending on the angle of incidence of light the virulent colonies were glistening greenish (G), while the avirulent ones reddish (R). Dense colonies, opalescent (OP) at oblique light were also avirulent. Subsequent examinations showed [2, 3] that alterations in virulence and in colonial morphology were accompanied by changes in physicochemical structure of the bacterial cell surface. This was a possible explanation of the finding [4] that in consequence of the loss of virulence the sensitivity decreased to certain T phages, lysozyme and some chemical substances in invasive intestinal bacteria. Finally, determining phage types of a great number of *S. flexneri* strains it was observed [5, 6] that virulent *S. flexneri* cultures of given serotypes belonged to characteristic phage types. On the basis of these observations it was interesting to study whether the loss of virulence caused changes in the phage types of *S. flexneri* serotypes.

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Materials and methods

Bacterial cultures. Examinations were carried out on the one hand with virulent strains freshly isolated from patients and their avirulent derivatives chosen on the basis of colonial morphology from passages in liquid media, and on the other hand with avirulent laboratory strains and the analogous morphological variants obtained from passages.

Media. The strains were subcultured simultaneously in broth, peptone water and modified Erlandson–Mackey synthetic medium [7, 8] consisting of KH_2PO_4, 0.067 M; K_2HPO_4, 0.067 M; NaCl, 0.85%; $MgSO_4$, 0.05%; nicotinic acid amide, 0.01 M; distilled water to 1000 ml; pH 7.2.

Colonial morphology was examined with a hand lens under oblique transmitted light on agar plates [1, 9].

Estimation of virulence was carried out by means of guinea-pig conjunctival infection as described by Serény [10].

Phage sensitivity was determined as described by László et al. [5, 6].

Results

Changes in phage-sensitivity. The frequency of associated changes in virulence and phage sensitivity are presented in Tables I and II. Table I shows the results of passages of virulent cultures and Table II of the avirulent cultures. Colony morphological variants of the virulent cultures which had lost their virulence or had a decreased one showed a change in phage-sensitivity in 75%. The variants which maintained their virulence during passaging showed an unchanged phage type in 75% of the cases. When starting cultures were avirulent, variants of changed phage sensitivity were also observed after subculturing, but only in 15% of the isolated colonies. The change of phage-sensitivity among the variants of lost or decreased virulence was significant: $\chi^2 = 64.25$; $P < 0.001$. Phage types changed to other well-defined phage types [5] only in part of the cultures, in the majority of cases the changes resulted in uncharacteristic phage patterns. There was not one specific phage which would have lysed all of the cultures which had lost their virulence. Though all the avirulent variants were sensitive to type phage F2, the strains of serotype 1b and 4a were originally sensitive to this phage. Among the different serotypes, by getting avirulent variants from 1b and 2a, the variants became sensitive to phages F10 and F12, avirulent variants of 3a serotype acquired sensitivity to phages F6 and F12, and variants of X became sensitive to phage F6. Phage types of strains belonging into serotype 4b have not changed during the passages, but these cultures had originally been avirulent.

Sensitivity to phage Ms2. Some of the cultures having a changed phage sensitivity became sensitive to phage Ms2 [11, 12]. All of the examined serotypes showed this phenomenon except serotype 4b. Forty-two avirulent variants which comprised the majority of Ms2 sensitive cultures, were isolated as virulent cultures, only 4 variants originated from originally avirulent cultures. Spontaneous appearance of Ms2 sensitivity was observed in the first culture in two cases.

Table I

Changes in virulence and phage sensitivity of virulent cultures after special passages

Serotype	No. of strains	No. of variants	Virulence			
			unchanged		lost or decreased	
			Phage sensitivity		Phage sensitivity	
			unchanged	increased	unchanged	increased
1a	1	7	3	0	3	1
1b	1	6	2	0	1	3
2a	12	148	42	13	18 (5)	50 (20)
3a	5	57	15	7	4 (1)	29 (1)
3b	2	9	2	0	3	4
X	33	19	9	2	1	7
Total	54	246	73	22	30 (6)	94 (21)

The number of variants of decreased virulence to be added are in brackets

Table II

Changes in phage sensitivity of avirulent cultures after serial passages

Serotype	No. of strains	No. of variants	Phage sensitivity	
			unchanged	increased
1a	2	8	7	1
1b → 1b*	1	12	12*	0
1b → Y*	1	8	8*	0
2a	2	5	3	2
3a	2	8	4	4
4b	4	19	19	0
X	2	8	6	2
Total	14	68	59	9

* Culture 1b dissociated spontaneously into serotypes 1b and Y and remained unchanged during the passages

Five persons from one family excreted *S. flexneri*. The strains of four patients were of serotype 3b and were sensitive to phage Ms2 and the strain of the fifth subject belonged to serotype 3a and was not sensitive to Ms2. The two kinds of strain also differed in sensitivity to other phages.

In the other case, from a *S. flexneri* culture of serotype 1b which was not sensitive to Ms2, after long storage on Dorset medium in the refrigerator and

subculturing on agar plates, avirulent colonies of serotype Y were found and these were sensitive to Ms2. This dissociation process from 1b to Y could be repeated but only in the opposite direction, and not from Y to 1b. The different phage sensitivity patterns of the two serotypes did not change during passaging.

Correlation between Ms2 sensitivity and colony morphology. Among the 42 variants obtained from passages in liquid media, 35 were of the "OP" type and only 7 "R" type colonies were found.

The strains isolated from the above mentioned family, both the 3b–Ms2 sensitive and 3a–Ms2 non-sensitive ones, were virulent and of colony type "G".

In the case of dissociation from 1b to Y, after the first agar plate subculture among the 1b–Ms2 non-sensitive "R" type colonies there appeared colonies of serotype Y, Ms2-sensitive and of type "G". In the course of further passaging these properties proved to be constant.

Correlation between Ms2 sensitivity and antigenic structure. The Ms2 sensitive "OP" colonies did not agglutinate in any of the *S. flexneri* factor sera. There was a single exception, a variant from a *S. flexneri* 3a strain, which agglutinated in factor serum 7, 8. The "R" variants of a *S. flexneri* 2a strain retained the original antigenic structure while acquiring Ms2 sensitivity; their virulence was not lost, it only was decreased. Sensitivity to Ms2 was associated with a change in antigenic structure in case of the dissociation from 1b to Y and in the 3a–Ms2 non-sensitive and 3b–Ms2 sensitive variants, which caused the family infection. The biochemical reactions of the variants, not identifiable by serological tests, were identical with that of the original strains.

Discussion

Analysing the results it was found that phage sensitivity was increased among avirulent *S. flexneri* cultures obtained from 75% of serial subcultures of virulent cultures. The change in virulence and in sensitivity to new phages was significant: $\chi^2 = 64.25$, $P < 0.001$.

In other experimental systems e. g. by passaging originally avirulent cultures, the correlation was not without contradictions, and phage sensitivity was not functionally connected in the vital processes of the bacterial cells.

The receptor sites for the employed phages except for phage Ms2 are not known. Probably they are not the O-specific side-chain or its modifications by lysogenic conversion. Changes of the outer membrane, the loss of certain antigens might perhaps serve as an explanation for the variety of the changed phage-sensitivity patterns. In most cases the appearance of sensitivity to phage Ms2 was associated with a total or partial degradation of the specific antigenic

structure or the total degradation of the antigenic structural pattern detectable by *S. flexneri* factor sera.

It is not clear whether the sensitivity to phage Ms2 had developed as a consequence of a change in the antigenic structure or of the presence of an F-like plasmid? This will be the subject of a further study.

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CHANGES IN PHAGE-SENSITIVITY OF *SHIGELLA FLEXNERI* STRAINS

II. THE F-LIKE CHARACTER OF Ms2-SENSITIVE VARIANTS

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Variants sensitive to male specific phage Ms2 appeared among Ms2 non-sensitive *Shigella flexneri* cultures in the course of liquid medium passages. Sensitivity to Ms2 was lost on acridine orange treatment and was transferable into Ms2 non-sensitive variants. The Ms2 sensitive variants had fimbrial antigen. Electron microscopy showed that Ms2 phages were adsorbed on the F-like fimbriae of these variants. It was assumed that F-like plasmids determining F-like fimbrial antigen were carried by these variants.

Earlier [1] it was found that certain *Shigella flexneri* cultures on becoming avirulent developed sensitivity to phage Ms2, but this also occurred without any change in virulence. With few exceptions, the antigenic structure of the Ms2 sensitive variants was degraded. Further examinations were needed to establish whether Ms2 sensitivity was connected with the presence of a plasmid or it appeared only as a consequence of the alteration of the surface antigen. To prove the presence of a plasmid in the Ms2 sensitive variants we had to examine whether (1) Ms2 sensitivity was lost after plasmid curing experiments; (2) the supposed plasmid was transferable to F^- cultures; (3) it was possible to demonstrate fimbrial antigen by serological means; and finally (4) sex-fimbriae and attached Ms2 phage particles were visible by electron microscopy.

Materials and methods

Strains. The origin of the Ms2 sensitive and non-sensitive variants was described previously [1]. Ms2 sensitive and non-sensitive variants of *S. flexneri* 583 (serotype 3a) and 517 (serotype 2a) and Ms2 sensitive variant (var. Y) and Ms2 non-sensitive variant (serotype 1b) of strain 7/68 were used.

Reference strains were *Escherichia coli* PA 309 $Str^rLac^-F^-$, *E. coli* W58–161 $Str^sLac^+F^+$ and *E. coli* C3000 F^+.

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Acridine curing. Medium. 1000 ml broth, 5×10^{-2} M ($K_2HPO_4 + KH_2PO_4$), 5 μg/ml or 10 μg/ml acridine orange, pH 7.6. A 0.1 ml aliquot from a 10^{-4} dilution of an overnight culture of the Ms2 sensitive variant was inoculated into acridine orange broth and incubated at 37 °C for 24 h. A dose of 5 μg/ml acridine orange was ineffective, while 10 μg/ml killed the culture. Therefore the cultures were subcultured into 5 μg/ml fresh acridine broth daily at least seven times, then 0.1 ml was inoculated onto agar-plates and after 24 h incubation 50–100 colonies were tested for Ms2 phage-sensitivity by spot-test [2].

Plasmid transfer. Two-hour broth cultures were inoculated from the overnight cultures of the Ms2 sensitive and Ms2 non-sensitive variants; 0.5 ml Ms2-Str sensitive and 1.0 ml Ms2 non-sensitive Str R culture were mixed in 2 ml broth, incubated at 37 °C for 4 h and diluted to 10^{-3}. For selection, streptomycin agar (500 μg/ml) was used. Colonies were tested for Ms2 sensitivity after 24 h incubation [3]. As a control, Ms2 sensitive and Ms2 non-sensitive cultures were used separately.

Detection of fimbrial antigen. Rabbit immune serum was prepared with the 24 h living culture of the Ms2 sensitive variant and absorbed with the living culture of the Ms2 non-sensitive variant of the homologous strain. The titre of the remaining antibody was determined by tube agglutination with the living suspension of the Ms2 sensitive variant [4]. Agglutination was performed with *E. coli* C3000 F^+, and with the Ms2 non-sensitive variant of the homologous strain as control.

Electron microscopy. The 2 h broth culture (10^6 cell/ml) of the Ms2 sensitive *S. flexneri* variant and the Ms2 phage-lysate were mixed in equal volumes, dropped on grids coated by carbonated collodion film and after 10 min stained with 2% phosphotungstic acid pH 7.0. The preparations were examined in the JEOL model JEM 100 C transmission electron microscope [5, 6]. As a control *E. coli* C3000 F^+, treated in the same way, was examined.

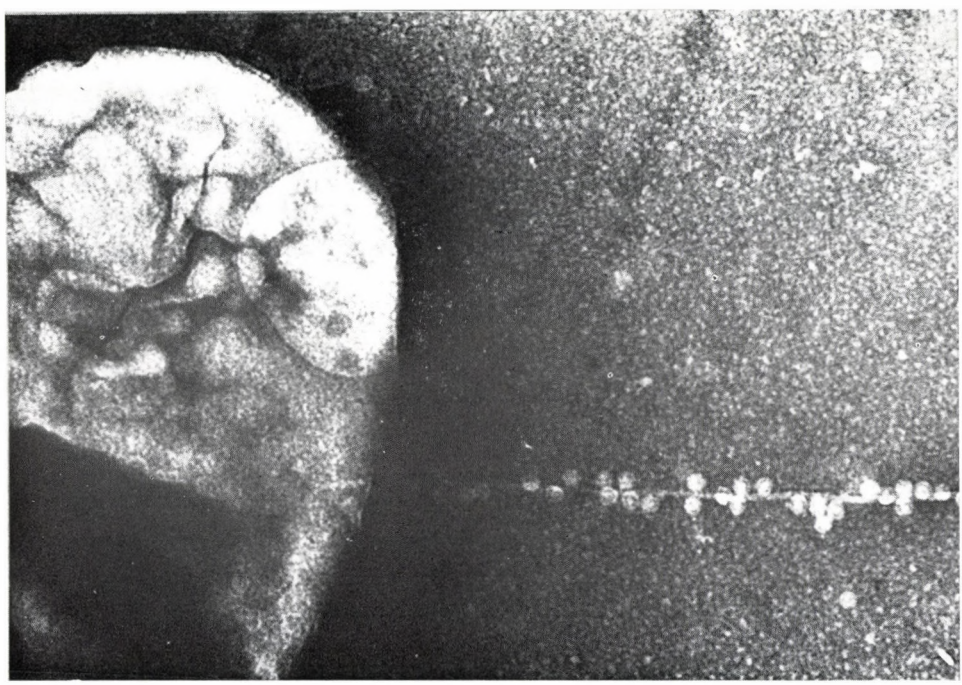

Fig. 1. Electron micrograph of negatively stained preparation of *S. flexneri* bacterial cell, fimbria and adsorbed Ms2 phages × 84 500

Results

1. *Elimination.* The Ms2 sensitive variants were treated with acridine orange to cure the supposed plasmid carrier state. *S. flexneri* 538 (3a) variants *S. flexneri* 517 (2a) and 7/68 (Y) variants have lost their sensitivity to phage Ms2 after acridine orange treatment in 3% and 6%, respectively. The eliminants of the antigenically degraded Ms2 sensitive *S. flexneri* 538 variants regained their original agglutinability corresponding to serotype 3a after the loss of Ms2 sensitivity. The cured *S. flexneri* 7/68 (var. Y) colonies failed to restore to serotype 1b. There occurred no alteration in the antigenic structure of *S. flexneri* 517 variants.

2. *Plasmid transfer.* As a model experiment, the frequency of F-plasmid transfer was examined in *E. coli* strains. Ms2 sensitivity was established in 20% when *E. coli* W58–161 Str^s F^+ was crossed with *E. coli* PA 309 Str^r F^-.

(a) Crossing *S. flexneri* 7/68 (Y) Str^s Ms2-sensitive and *S. flexneri* 517/16 (2a) Str^r Ms2 non-sensitive variants resulted in development of Ms2 sensitivity n 15% of *S. flexneri* 517/16.

(b) Crosses were carried out with *S. flexneri* 538 (3a) sorbitol$^+$ Ms2 sensitive and *S. flexneri* 517 (2a) sorbitol$^-$ Ms2 non-sensitive strains. Sorbitol fer-

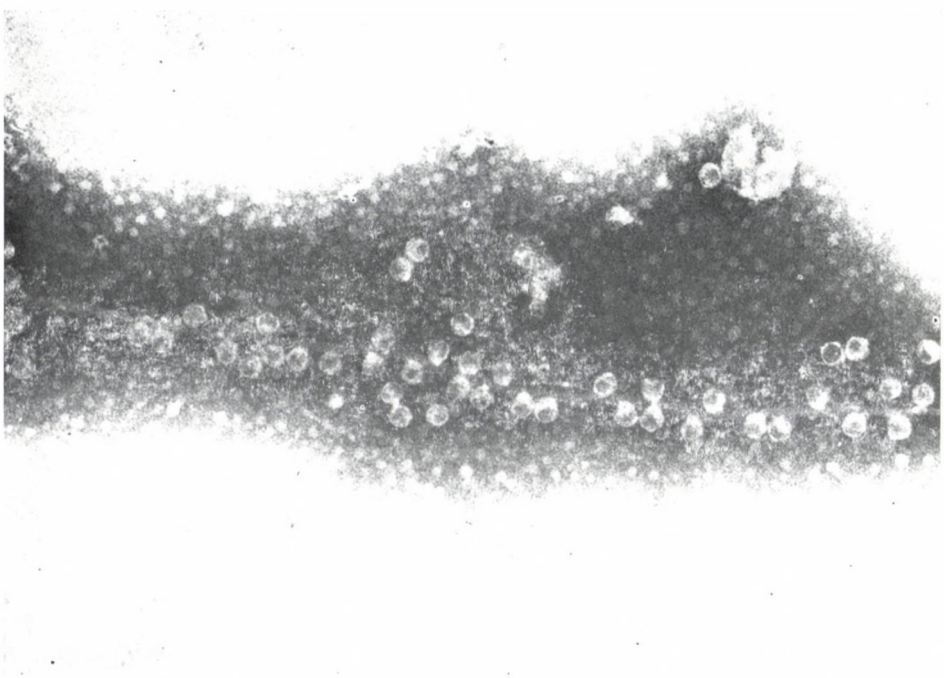

Fig. 2. Electron micrograph of negatively stained preparation of *S. flexneri* fimbria with adsorbed Ms2 phages × 115 500

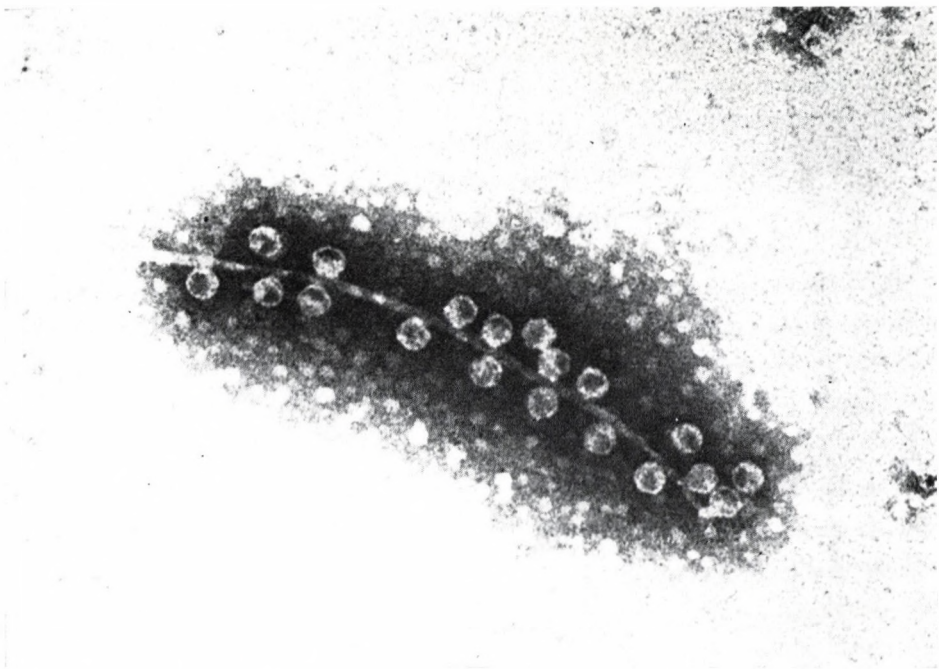

Fig. 3. Electron micrograph of negatively stained preparation of *E. coli* fimbria with adsorbed Ms2 phages × 161 700

mentation was applied for selection on agar plates containing 0.5 g/100 ml of the substrate. *S. flexneri* 517 (2a) strain became sensitive to phage Ms2 in 50%.

3. *Demonstration of fimbrial antigen.* The presence of fimbrial antigen was demonstrated serologically [4]. Table I shows the serological analysis by tube agglutination of the immune serum of the Ms2 sensitive *S. flexneri* 517/17 living culture absorbed with the Ms2 non-sensitive *S. flexneri* 517/16 living culture.

4. *Electron microscopy.* Figures 1 and 2 show Ms2 phages adsorbed to the fimbriae of *S. flexneri* 517/17 (2a) cells, Fig. 3 shows adsorbed Ms2 phages to sex fimbriae of *E. coli* C3000 F^+ cells.

Discussion

Our studies revealed that Ms2 sensitive *S. flexneri* variants carried F-like plasmids and possessed sex fimbriae adsorbing phage Ms2. Kétyi [7] observed the acquisition of the K-12 F^+ male state and the f^+ antigen by *S. flexneri* strains in studies of the antigenic structure of sex-fimbria determined by plasmids [8]. On the basis of our experiments it was assumed that Ms2 sensitivity developed as a result of derepression, though the mechanism of derepression

Table I

Titre of f⁺-like antigen in the serum of Ms2 sensitive variant

Serum	Antigen	Titre of f⁺-like fimbrial antigen
S. flexneri 517/17 with absorbed	517/17 (Ms2+)	1290 (+)
S. flexneri 517/16	517/16 (Ms2−)	5 (±)
	E. coli C3000 F⁺(Ms2+)	160 (+)

was not clear. Identification of plasmids and determination of the structure of fimbrial antigen needs further examinations.

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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES TO EPSTEIN-BARR VIRUS ANTIGENS*

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Enzyme-Linked Immunosorbent Assay (ELISA) was standardized for measurement of antibody activity of reference human and baboon *(Papio hamadryas)* sera to soluble Epstein–Barr virus (EBV) antigens. A comparison with the immunofluorescent (IF) method showed that ELISA detects antibody specifically and sensitively. In ELISA, Herpesvirus Papio (HVP) nuclear antigen (HUPNA) positive baboon serum reacted with EBV nuclear antigen (EBNA), as a further indication of the antigenic similarity between HVP and EBV. Forty-two baboon sera were tested with EBV antigens in both ELISA and IF test. The results showed an agreement between the two methods and also that by the use of EBV antigens, ELISA measures anti-HVP activity of baboon sera. ELISA did not reveal significant difference in antibody activity of 23 baboons with lymphoma and that of 24 healthy baboons. Results provide further data that ELISA can be used effectively in the field of EBV serology.

Epstein–Barr virus (EBV) is a unique herpesvirus with a capacity to immortalise B lymphocytes [1]. It has been established that this ubiquitary EBV is closely associated with two human cancers, African Burkitt's lymphoma and nasopharyngeal carcinoma [2] and more or less involved in the pathogenesis of other human diseases [3–5]. Immunological studies provided a considerable bulk of information on the biology of EBV and its role in human pathology; one of them was the disease related pattern of host antibody response to EBV antigens.

Predominantly the immunofluorescence (IF) test is used in detecting antibodies to various EBV-related antigens [6]. Still, as the IF technique is jeopardised by its subjectivity and relative insensitivity; introduction of an alternative, specific and sensitive method in the field of EBV serology promised further to facilitate pathogenetic investigations. Standardisation of ELISA [7] for measurement of EBV-specific antibodies seemed to be a feasible alternative to the IF method.

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The aim of the study was to establish ELISA for detecting antibodies to EBV viral capsid antigen (VCA), early antigen (EA) and nuclear antigen (EBNA), and to use this antigen panel for assessing antibody activity of baboon *(Papio hamadryas)* sera to the lymphotropic herpesvirus papio (HVP) closely related to EBV [8–10].

Materials and methods

Human reference sera. EBV negative (RK-3), positive (RK-1) and the serum named Jaboulin were provided by Dr. G. B. de Thé (International Agency for Research on Cancer, Lyon, France).

As baboon reference sera one HVP negative (No. 17602) and two positive sera (No. 12343 and No. 13912) were used. The antibody pattern of reference sera was determined by the indirect IF test (for VCA and EA) and the anticomplement IF test (for EBNA and HUPNA). Details of these techniques were described earlier [10].

Baboon sera. Altogether 47 sera were tested, 23 were collected from baboons with lymphoproliferative disease (sick animals) and 24 samples from age and sex matched healthy ones. In 13 of the 23 sick baboons malignant lymphoma was diagnosed at autopsy. The other ten baboons alive at the time of the study had clinical symptoms of malignant lymphoma.

Antigens. Extracts of the prototype EBV positive human cell lines P3HR-1 (P3), Raji (R) as well as R superinfected with P3 (R + P3) were used as antigens. Cell extracts were prepared as described [10].

ELISA was carried out in Dynatech Microelisa plates [11]. From the antigens diluted 1 : 5 or 1 : 20 in coating buffer, 0.1 ml was added to each well. Plates were kept at 4 °C overnight, then washed three times with phosphate buffer solution containing 0.05% Tween 20; 0.1 ml of human or baboon sera diluted twofold from 1 : 100 to 1 : 800 was added to the wells. After one hour incubation at 30 °C, plates were washed again and 0.1 ml of the conjugate (gamma chain specific rabbit anti-human IgG coupled with peroxidase, Dako Immunoglobulin Ltd.) was added to the plates, then they were incubated and washed. Subsequently 0.15 ml of substrate solution (10 mg o-phenylenediamine dihydrochloride in 20 ml phosphate–citrate buffer pH 6) was added to the wells. After 30 min incubation at 30 °C the reaction was stopped by adding 0.05 ml of 4 H_2SO_4. Absorbance values (extinctions) were recorded by a Dynatech Elisa Reader. Reference positive and negative sera, conjugate alone, and serum without antigen were set up in every plate as controls. All sera were tested in duplicate, samples from cases and controls were tested on the same plate.

Immunofluorescence. Preparation of cells with EBV–EA: Raji cells were washed twice in RPMI-1640 medium and aliquots containing 10^6 cells per 0.2 ml were mixed with 0.45 μl of millipore-filtered culture medium of P3HR-1 cells. Mixtures were incubated for 30 min at 30 °C. Then to each aliquot 1.8 ml of complete medium (RPMI-1640 with 15% fetal calf serum) were added and cultures were incubated at 37 °C in the presence of 5% CO_2 for 72 h. For IF study, smears were prepared according to Neubauer et al. [12]. IF test on human and baboon sera were carried out as described for detecting herpes simplex virus IF antibodies [13]. Human and baboon sera were tested in 1 : 10 dilution. Drops were added to each smear fixed on slides. After a 30 min incubation at 37 °C, slides were washed with PBS for 15 min and covered with FITC-conjugated anti-human IgG (Wellcome Laboratories) diluted 1 : 15. Slides were incubated again and washed three times, counterstained with 0.06% Evans blue for 10 min, mounted with PBS glycerol (1 : 1) and examined by fluorescent microscopy. Reference positive and negative sera and conjugate were included into each test. Coded samples were examined by two observers.

Results

Standardisation of ELISA. Plates were sensitized with the three antigen preparations having the following components: P3 with VCA, EA and EBNA, R with EBNA and R + P3 with EA and EBNA. In ELISA these EBV anti-

gens were tested with the three reference human sera: (1) RK-1 with antibodies to capsid, early and nuclear antigens; (2) Jaboulin with antibodies to capsid and nuclear antigens; and (3) RK-3 without antibodies. These three sera were tested in 1 : 100 dilution. Results of these experiments are summarized in Table I. The reference negative serum RK-3 showed very low extinction values (< 0.10) with all the three antigens while the two reference positive sera reacted with the antigens in accordance with their IF activity. The extinction value of RK-1 serum was the highest with P3 antigen, and the lowest with EBNA positive antigen preparation (R), though the absorbance value was still in the positive range. In case of the Jaboulin serum there was no significant difference between its reactivity with R (EBNA) and R + P3 (EBNA and EA) fractions. Extinction values indicated a comparatively low antibody activity with these antigens. However, this serum reacted with the P3 antigen strongly showing that ELISA measure specifically the antibody activity to EBN antigens.

Table I

Antibody activity of reference human and baboon sera to EBV antigens

EBV Antigens	ELISA extinction values of sera						Blank without serum
	Human RK-1	Baboon 12342	Human Jaboulin	Baboon 13912	Human RK-3	Baboon 17062	
	by test positive with						
	VCA EA EBNA	VCA EA HUPNA	VCA EBNA	VCA	negatives		
P3 VCA EA EBNA	>2.00	1.40	0.78	0.35	0.05	0.03	0.04
R EBNA	0.58	0.38	0.37	0.07	0.07	0.06	0.03
R + P3 EA EBNA	0.92	>2.00	0.42	0.06	0.07	0.05	0.05
Blank without antigen	0.04	0.05	0.08	0.07	0.06	0.04	—

Reactivity of baboon sera with EBV antigens. Three reference baboon sera were tested with the same EBV antigens in ELISA. In IF test they reacted with HVP antigens as reference human sera did with the EBV antigens. Namely, serum No. 12343 was positive for VCA, EA and HUPNA, serum No. 13912 for VCA while serum No.17602 was the negative reference one. According to the results (shown also in Table I) baboon serum (No.13912) positive for VCA reacted only with the P3 antigen and the other (No.12343) with high IF titre to VCA, EA and HUPNA reacted strongly with both P3 and R + P3 antigens. In addition, it reacted also with the R (EBNA) antigen though less than did EBNA positive human serum. HVP negative serum (No.17602) was completely non-reactive with EBV antigens. Results indicated that EBV antigens can be used for testing anti-HVP activity of baboon sera.

Detection of antibodies to EBV–EA in baboon sera by ELISA and IF test. For the presence of antibodies to EA, 42 baboon sera were tested in parallel by the two methods. Taking an extinction value ≥ 0.15 as indicative for specific antibody activity, 8 sera were positive in ELISA. Of these, 6 were positive in the IF test. Two IF negative samples showed rather low extinction values (0.18 and 0.20), just above the cut-off level. Therefore ELISA measures anti-EA activity of baboon sera specifically and more sensitively than the IF test.

Abtibody activity of baboons with and without lymphoma. Sera of 23 baboon with lymphoma and of 24 age and sex matched healthy baboons were tested in ELISA by the EBV antigens. No significant difference was revealed between the two groups. In both groups antibody activity was comparatively low and it remained in the negative range with EA an EBNA. Regarding P3 (VC) antigens, the mean extiction value was slightly above the cut-off level with the sera taken from the 23 sick baboons (Table II).

Table II

Antibody activity in sera of lymphomatous and healthy baboons to EBV antigens in ELISA

Group	No. of sera	EBV antigens		
		P3 (VCA EA EBNA)	R(EBNA)	R + P3 (EA EBNA)
		Mean extinction value ± S. E.		
Baboons with lymphoma	23	0.16±0.03	0.12±0.02	0.12±0.04
Healthy baboons	24	0.10±0.01	0.10±0.02	0.06±0.01

Discussion

ELISA was originally described for detecting soluble EBV–EBNA [14] and we have extended its use for measuring serum antibody activity to EBV capsid, early and nuclear antigens. A comparison with the IF test showed that ELISA detects antibodies in human as well as baboon sera sensitively and specifically.

Activity of reference human and baboon sera with EBV antigens showed a close correlation; the highest r value (0.93) was found between reactivities to R + P3 and P3. This is explained by the fact that P3 has the highest amount of the early antigen component. Though those data are not shown here, it should be mentioned that no correlation was found when the same sera were tested with EBV and retrovirus (baboon endogenous type C, and Mason–Pfizer type D) antigens. Also, all sera tested in ELISA were completely non-reactive with fetal calf serum and human embryonic cell antigen.

All these facts indicate that ELISA is a reliable serological method for assessing antibody activity to EBV antigens. For laboratory confirmation of the clinical diagnosis, EBV-EA seems to be the most important [4, 6, 15]. In this respect ELISA may be a method of choice because it measures anti-EA antibody activity more sensitively than the IF test. Moreover, with the use of EBV antigens, antibody activity of baboon sera to HVP antigens can also be assessed. In agreement with the data published by Falk et al. [9], Kokosha et al. [16] and Neubauer et al. [12], we found that in ELISA baboon sera cross-reacted strongly with EBV capsid and early antigens. The fact that the HUPNA positive baboon serum reacted with EBNA suggests a close antigenic similarity between EBNA and HUPNA. This, however, still awaits confirmation as in other serological tests HUPNA positive baboon sera did not react with EBNA [12, 17].

Our results provided further data that ELISA can effectively be utilized in the field of EBV serology.

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INCIDENCE OF *PSEUDOMONAS AERUGINOSA* SEROGROUPS IN DRINKING WATER: USE OF SEROTYPING IN THE CONTROL OF WATER SUPPLIES

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From 26 568 drinking water samples collected in the years 1977–1981 from deep well supplies in Csongrád county, Hungary, 1269 *Pseudomonas aeruginosa* strains were isolated. The most frequent serogroups (Lányi–Bergan scheme) were O6 (9.5–69.2%), O1 (5.5–35.5%) and O9 (2.2–36.2%). Predominance of a given serogroup varied with, and was characteristic of, geographic areas of the county, water plants and distribution systems. Bacteriological monitoring has shown that serotyping of *P. aeruginosa* may be useful for evaluating the mechanical condition of the water supply.

Potential health hazards associated with *Pseudomonas aeruginosa* in drinking water have been extensively investigated [1]. The agent has been described in association with water-borne outbreaks of enteritis mainly among infants [2–6]. It is generally accepted that the presence of *P. aeruginosa* in drinking water should not be ignored [1]. The Hungarian National Standard for the Bacteriological Examination of Water [7] prescribes that any drinking water that contains this organism is of unsatisfactory quality.

For detecting in hospital infections the source of *P. aeruginosa*, serological, pyocine and phage typing methods are used all over the world. For tracing *P. aeruginosa* as a water-polluting agent, however, only few reports have been published [8–10].

The purpose of the present work was to survey the incidence of *P. aeruginosa* serogroups in drinking water obtained from deep wells.

Materials and methods

A total of 1269 *P. aeruginosa* strains was collected in the years 1977–1981 from drinking water in Csongrád county, Hungary. The organism was cultured as described in reference [7]. After biochemical identification, each strain was grown on ox-blood agar at 37 °C for 18–20 h and examined for O antigens by Lányi's slide agglutination method [11]. Serogroups were designated as in the scheme of Lányi and Bergan [12], where the numbering of O groups corresponds to Habs' symbols [13], except O2 (= Habs 2 + Habs 5) and O7 (= Habs 7 + Habs 8).

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Results

The source of *P. aeruginosa* strains is shown in Table I. In water plant and in distribution system samples the organism occurred with about the same frequency. The highest degree of pollution was detected in conduits connected freshly to the distribution system.

Out of the 1269 isolates, 1266 were serogrouped. In a village (Ambrózfalva) water plant, 107 samples were positive for *P. aeruginosa* serogroup O9; to avoid biassing of data, these strains were omitted from the following tables. The frequency of the remaining 1159 strains according to sources is presented in Table II. On the average, serogroup O6 strains occurred most frequently in plant and peripheral samples; strains O9 and O1 were next in order. In new extensions of the network serogroup O1 was the most frequent. The predominant serogroup varied with individual plants, distribution systems and sampling points.

Table I

Incidence of P. aeruginosa in drinking water samples collected in Csongrád county in the years 1977—1981

Source	No. of samples	Positive for *P. aeruginosa*	
		No.	%
Municipal water supply	2720	124	4.6
Municipal distribution system	15087	739	4.9
Other supplies	6630	201	3.0
New connections	2131	205	9.6
Total	26568	1269	4.8

Table III shows the incidence of *P. aeruginosa* in different geographic areas of the county. The highest number of polluted samples were encountered in village water supplies of the Makó district. Among large municipal supplies, samples taken from the Szeged distribution system and from the Szentes plant contained *P. aeruginosa* the most frequently.

Table IV shows the incidence of *P. aeruginosa* serogroups according to village and town supplies. In the Szentes and Makó district village plants serogroup O6 predominated. This serogroup was infrequent in the Szeged district, where the predominance of strains O9 was characteristic of most supplies. As *P. aeruginosa* occurred relatively infrequently in waters sampled in Csongrád, Makó, Hódmezővásárhely and Szentes, data for these towns have been summarized.

Table II

Incidence of P. aeruginosa serogroups in water supplies in Csongrád county (Percentage of serogroups without O9 strains isolated from samples collected in Ambrózfalva)

Serogroup (Lányi–Bergan)	Municipal supplies		Other supplies (199)	New connections (205)	Total (1159)
	plant (100)*	distribution system (655)			
1	13.0	18.3	11.1	41.1	20.6
2	10.0	6.0	12.1	2.9	6.8
3	7.0	4.9	2.5	11.7	5.9
4	1.0	0.5	1.0	—	0.5
6	22.0	29.6	33.7	14.6	27.0
7	17.0	9.4	7.5	2.9	8.6
9	15.0	18.8	19.6	18.0	18.5
10	6.0	4.7	2.5	0.5	3.7
11	3.0	4.1	1.0	2.4	3.2
nt	6.0	3.7	9.0	5.9	5.2

* No. of strains

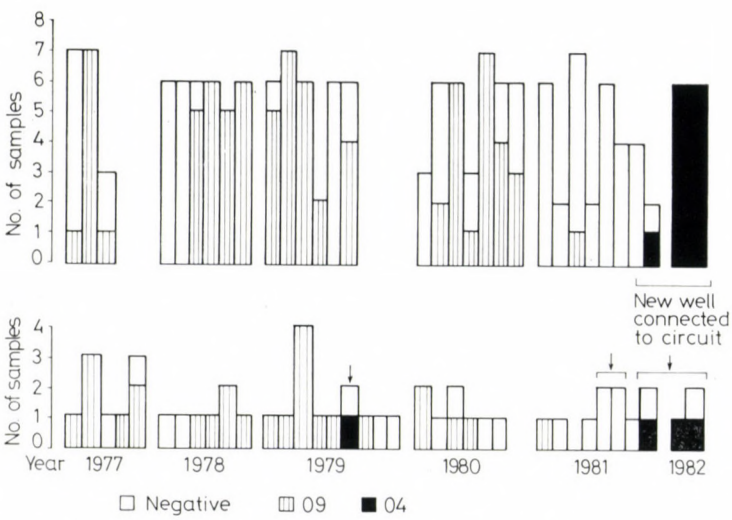

Fig. 1. P. aeruginosa serogroups isolated from the village water supply at Ambrózfalva. Upper columns: distribution system (of 167 samples, 92 positive); lower columns: plant (of 50 samples, 30 positive). Columns marked with arrow show samples taken from the new well established in 1979

Results obtained in two villages of the Makó district are worth detailing, since they prove the usefulness of *P. aeruginosa* serotyping. Figure 1 shows findings for samples taken from the wells, the engine compartment tap and the plant yard taps at Ambrózfalva. Serogroup O9 was demonstrated for years up to 1981 in well and tap samples. In 1979 a new well was drilled, the first examination of which yielded *P. aeruginosa* serogroup O4, but subsequent cultures failed to show this organism. To remove the obvious source of *P. aeruginosa* O9 contamination, in 1981 the old well was disconnected, and after a very carefully performed disinfection of the system, the new well was put into

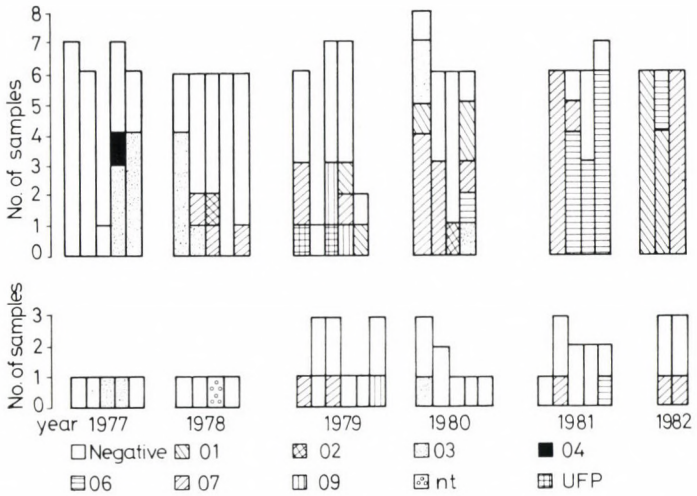

Fig. 2. P. aeruginosa serogroups isolated from the village water supply at Nagyér. Upper columns: distribution system (of 149 samples, 80 positive); lower columns: plant (of 45 samples, 11 positive)

operation. For a while it appeared that these measures had been effective: *P. aeruginosa* O9 disappeared from the system. At the end of 1981, however, *P. aeruginosa* O4 — which was isolated in 1979 from the freshly drilled well — was shown in a sample from the plant yard tap. By the year 1982, this serogroup had become characteristic as a single contaminant of the entire Ambrózfalva system.

The water supply at Nagyér is also heavily contaminated with *P. aeruginosa;* as shown in Fig. 2, 7 different serogroups occurred. In addition, nontypable (nt) *P. aeruginosa* and UFP (unclassified fluorescent pseudomonas [1, 14]) have also been isolated. The incidence of these bacteria varied irregularly in the 6-year period of monitoring.

Table III

Incidence of P. aeruginosa in drinking water samples collected from different supplies in Csongrád county
(Without *P. aeruginosa* strains isolated from Ambrózfalva)

Source	No. of samples	Positive for *P. aeruginosa*	
		No.	%
Village supplies			
Makó district (14)*			
Plant	581	26	4.5
Distribution system	2916	220	7.5
Szeged district (25)			
Plant	766	14	1.8
Distribution system	4794	101	2.1
Szentes district (8)			
Plant	304	9	3.0
Distribution system	1235	82	6.6
Town supplies			
Csongrád			
Plant	152	—	—
Distribution system	842	16	1.9
Hódmezővásárhely			
Plant	169	4	2.4
Distribution system	1090	14	1.3
Makó			
Plant	193	11	5.7
Distribution system	952	39	4.1
Szeged			
Plant	394	21	5.3
Distribution system	2233	175	7.8
Szentes			
Plant	114	12	10.5
Distribution system	870	12	1.4

* No. of supplies

Discussion

In studies reported previously [15, 16] we dealt with the association between the chemical and bacteriological quality of water derived from deep bore wells. We have shown that waters with higher than usual oxygen consumption (KOI_p), ammoniumtion concentration and earth gas content are characterized

Table IV

Incidence of P. aeruginosa serogroups in drinking water samples collected in different districts of Csongrád county
(Percentage of serogroups without O9 strains isolated from samples collected Ambrózfalva)

Serogroup (Lányi–Bergan)	Village supplies in district			Town supplies	
	Makó (248)*	Szeged (116)	Szentes (91)	Szeged (195)	Csongrád, Makó, Hódmező-vásárhely and Szentes (105)
1	13.3	11.2	5.5	35.5	12.4
2	3.2	8.6	20.9	1.0	9.5
3	7.3	6.0	—	5.1	3.8
4	1.2	—	—	—	1.0
6	35.1	9.5	69.2	13.3	27.6
7	23.0	7.8	2.2	2.6	5.7
9	6.8	36.2	2.2	25.1	26.7
10	0.8	9.5	—	8.2	7.6
11	6.5	3.4	—	4.1	1.9
nt	2.8	7.8	—	5.1	3.8

* No. of strains

by an increased total bacterial count. As the chemical parameters usually vary with the geographic area of the wells [17], we have assumed that there may be an association between the regional incidence of *P. aeruginosa* pollution and the chemical quality of the water. The present results have shown that *P. aeruginosa* occurs most frequently in the Makó district. This finding is in agreement with the above assumption, since waters in this region contain organic substances in significantly higher amounts than waters in any other areas of the county. The frequent occurrence of *P. aeruginosa* in such waters is associated with a high content of short-chain aliphatic hydrocarbons readily metabolized by the organism.

There are few literary data concerning the incidence of *P. aeruginosa* serogroups in water. The first report was published by Lányi et al. [8] on 195 strains isolated from drinking water and other samples in the area surrounding Budapest. Némedi and Lányi [9] described the serogrouping of 933 *P. aeruginosa* strains; 143 of these were isolated from drinking water, the rest from industrial water, swimming pools, surface waters and sewage.

The present results are comparable with those of the early reports, if Lányi's original antigen symbols [11] are expressed in terms of the Lányi–

Bergan system [12]. The incidence of serogroup O6 in Csongrád county (27%) was almost the same as in the material of Lányi et al. (26.7%) and of Némedi and Lányi (27.3%). Serogroup O1 occurred with the same frequency in Csongrád county and in the area surrounding Budapest (20.6 and 20.0%, respectively), but in the Budapest material it was less frequent (6.3%). Serogroup O9 was relatively frequent in Csongrád county even if strains isolated from the Ambrózfalva samples are omitted (18.5%); Lányi et al. [8] and Némedi and Lányi [9] found O9 strains only in 7.7 and 4.2%, respectively.

Bacteriological monitoring of the Ambrózfalva and Nagyér village water supplies indicates that serotyping of *P. aeruginosa* may be helpful in checking the mechanical condition of plants. Despite of the frequent isolation of *P. aeruginosa*, the condition of the Ambrózfalva plant and distribution network may be considered satisfactory. In this system, only a single contaminant *(P. aeruginosa* O9) occurred for years. After a thourough disinfection and installation of a new well, this organism disappeared. The fact that later the plant became a reservoir of another serogroup of *P. aeruginosa* (O4), was associated with an accidental contamination at drilling of the new well and the latent survival of the pollutant.

Without serotyping it could have never been revealed that pollutions of the Ambrózfalva and Nagyér supplies were sharply different in character. The large scale of serogroups isolated from the Nagyér samples is suggestive of unsatisfactory mechanical conditions, in consequence of which pseudomonads may gain access to the water at a variety of sites.

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RESPIRATORY EFFECT OF *HAEMOPHILUS INFLUENZAE* ENDOTOXIN IN MICE

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The effect of the endotoxin of a noncapsulated *Haemophilus influenzae* strain isolated from bronchitis was studied on the respiration of mice. Inhalation of the *H. influenzae* endotoxin resulted in heavy attacks of dyspnoea with non-specific histological changes in the lung. The endotoxin elicited no direct response in isolated organs, but produced cytotoxic changes in HEp-2 tissue cultures. The experiments led to the conclusion that the clinical signs and the histological changes may be explained by the direct damaging effect of endotoxin on the cells and by the released mediator substances.

Several endogenous and exogenous factors are involved in the development and progress of chronic aspecific respiratory diseases, among which bacteria and viruses are of decisive importance [1, 2]. Bacteriological studies all over the world have confirmed the frequent occurrence and outstanding role of haemophilus strains in chronic respiratory diseases [1, 3–16]. In lung infections caused by haemophilus strains, the enzymes, toxins, and cellular constituents of the bacteria produce tissue damage and stimulate the immune system in different degrees [17]. In our previous studies mice were subjected to the inhalation of a nutrient broth containing dead, partly autolysed haemophilus cells. Attacks of dyspnoea were observed and subsequent histological studies showed the characteristic features of interstitial pneumonia [18].

It seemed then worthwhile to look for the substance responsible for the attacks of dyspnoea among the cellular constituents of haemophilus. We extracted a lipopolysaccharide-type endotoxin from *H. influenzae*, determined its toxicity in rats and studied its pathogenic effect on the respiration of CFLP white mice, and on the lung tissues, on trachea and smooth muscle, and on HEp-2 tissue culture. The results are presented below.

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Materials and methods

Bacterial strain. Noncapsulated *H. influenzae* strain (designated T_2) was isolated from the pharynx of a patient with chronic bronchitis and maintained in Levinthal broth. The nutrient broth used in the fermentor was enriched with 10 μg/ml haematin (National Biochemical Corporation, Cleveland), 10 μg/ml DPN (Reanal, Budapest), 0.5% glucose, and 10% yeast extract. The yeast extract was prepared by Alexander's method using baker's yeast [19].

The endotoxin was prepared from the fermentor culture of the above-mentioned *H. influenzae* strain by the hot phenol water method of Westphal et al. [20]. The extracted endotoxin was dissolved in physiological saline and applied in the following experiments. 1. The toxicity of the preparation was determined in female R/AxLE/HF_1 rats (LATI, Gödöllő) weighing 150 g and previously hypersensitized to endotoxin with lead acetate. The single groups of 5 rats received 1, 2, or 5 μg/kg dose [21]. 2. Inhalation of endotoxin in groups of 5 mice each was performed under light ether anaesthesia once daily for 10 days. The inhaled amount was 1 μg or 10 μg in a 0.05 ml volume. The controls received the same amounts of physiological saline. 3. Quantitative determination of the increase in respiratory rate due to the inhalation of 1 μg endotoxin was done under pentobarbital anaesthesia. The intraperitoneal administration of 50 mg/kg pentobarbital was followed by a 10-min excitement and then quiet sleep for 60 min. In this interval 5 mice were made to inhale 0.05 ml of physiological saline and 5 mice 1 μg endotoxin. This procedure was repeated 10 times. 4. The effect of 1 μg endotoxin in Krebs's solution on the isolated trachea of guinea pigs was studied in vitro. 5. The effect of 1 μg endotoxin on homogeneous smooth muscle-nerve preparation from the guinea pig ileum was studied in Krebs's solution. 6. The effect of 1, 0.1 and 0.01 μg endotoxin was studied on the cells of permanent HEp-2 epithelial tissue cultures. The endotoxin was added to 20 tissue culture tubes and controlled microscopically daily for a week, to follow the development of toxic signs. The maintenance fluid was not changed during the observation period.

Results

1. A 1 μg dose of intravenously administered endotoxin killed every rat within 24 h, proving the toxicity of the lipopolysaccharide extract.

2. The inhalation of endotoxin in a dose of 1 μg or 10 μg produced attacks of dyspnoea of apprixomately the same intensity in mice. The symptoms were similar to those observed in animals subjected to inhalation of nonviable and autolysed bacteria in previous experiments [18]. Two to three minutes after inhalation the already awake animals started running about but stopped at intervals of about 30 sec, showing dyspnoea and tachypnoea. The first attacks lasted 5 min but later they became longer and graver. After the 7th or 8th inhalation the animals nearly suffocated, one mouse died 5 min after the 10th inhalation of 10 μg endotoxin. None of the animals inhalating physiological saline had an attack. The course of the respiratory attacks is demonstrated in Table I.

The animals were sacrificed after the 10th inhalation. Their lung, liver and spleen were examined macroscopically and histologically. The gross examination showed haemorrhagic foci. Histological examination revealed non-specific pathological changes only in the lungs of the suffocating animals that had inhaled 1 or 10 μg of endotoxin.

Peribronchial and perivascular round-cell infiltration with lymphocytes, macrophages and in some places a few granulocytes were found. Macrophage

Table I

Intensity of dyspnoeic attacks following endotoxin inhalation

No. of inhalation	Inhaled substance		
	1 μg endotoxin	10 μg endotoxin	physiological saline
1	++	++	—
2	+++	+++	—
3	+++	+++	—
4	++++	++++	—
5	++++	++++	—
6	++++	++++	—
7	++++	++++	—
8	++++	++++	—
9	++++	++++	—
10	++++	++++	—

++ The attack lasted 2 min
+++ The attack lasted 5 min
++++ The attack lasted 7–8 min

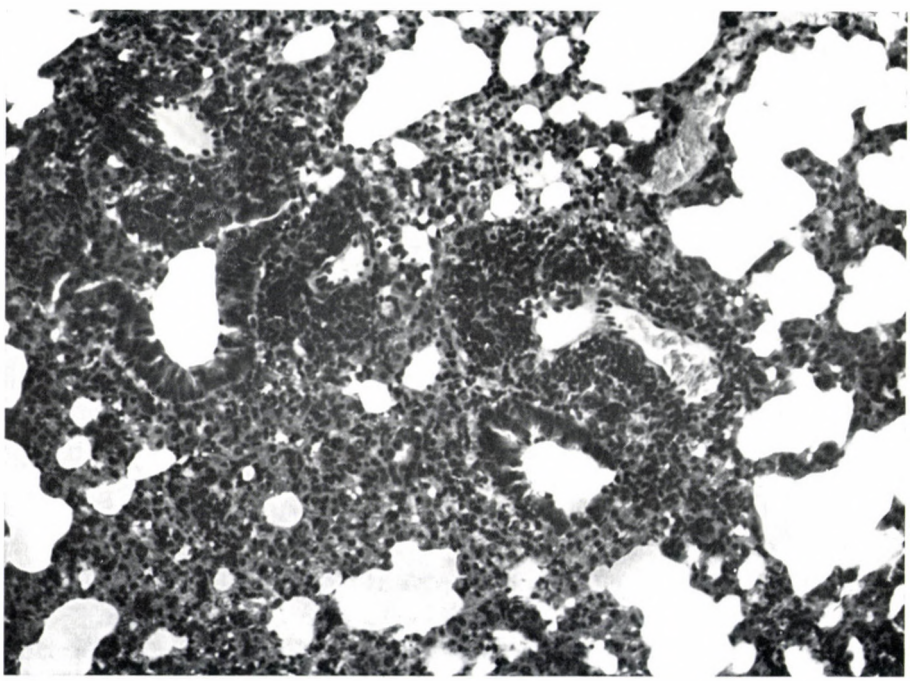

Fig. 1. Lung after the inhalation of 1 μg *H. influenzae* endotoxin 10 times. Haematoxylin–eosin staining, × 63. Perivascular and peribronchial round-cell infiltration

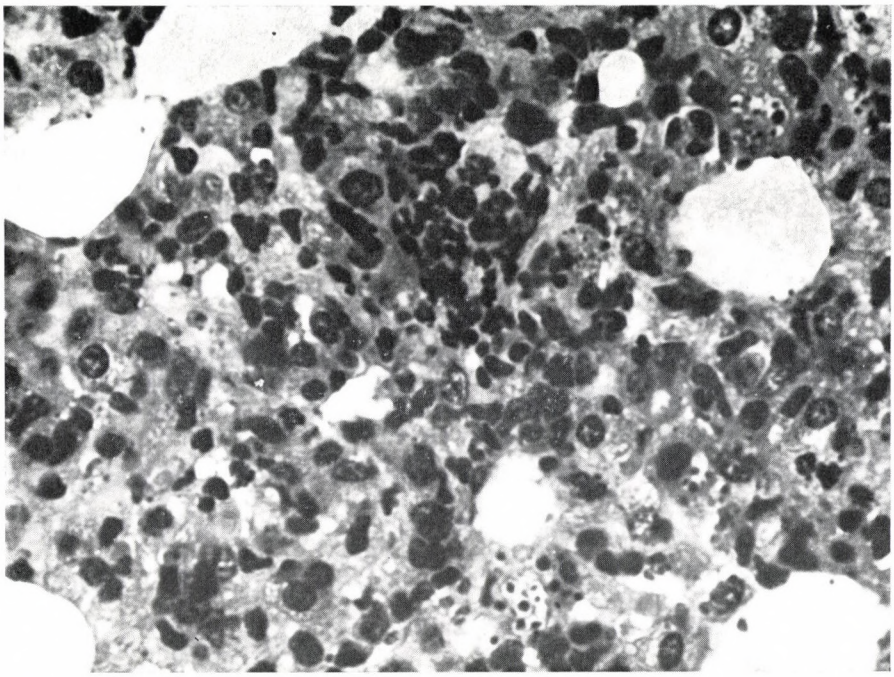

Fig. 2. Lung after the inhalation of 1 μg *H. influenzae* endotoxin 10 times. Macrophage collections around the alveoli. Haematoxylin–eosin staining, × 400

collections in the alveoli and along the alveolar septa could be seen in islets, of varying size. In some places the walls of alveoli were thickened by macrophages and lymphocytes, while in other places they were destroyed (Figs 1, 2).

3. The attack of dyspnoea following the inhalation of endotoxin was graver under deep than under superficial anaesthesia. The experiments done under deep anaesthesia were devised to quantify the response: following the inhalation of 1 μg endotoxin, hyperpnoeic and apnoeic periods alternated for 4–5 min. The increase in respiratory rate was 20–30% after the 2nd or 3rd endotoxin inhalation, while it reached 40% after the 8th or 9th inhalation. Inhalation of physiological saline resulted in a 10% increase in respiratory rate which was over within 1 or 2 min.

4. A 1 μg dose of the endotoxin failed to elicit any response from the isolated guinea pig trachea.

5. No contraction could be observed in the homogeneous smooth muscle-nerve preparation from guinea pig ileum exposed to 1 μg endotoxin.

6. The haemophilus endotoxin produced cytotoxic changes in HEp-2 tissue cultures. Their intensity and velocity depended on the concentration, as demonstrated in Table II.

Table II

Effect of haemophilus endotoxin on HEp-2 tissue culture

Amount of toxin	Days					
	1	2	3	4	5	6
1 μ/ml	+	++	detached			
0.1 μg/ml	–	–	+	++	++++	detached
0.01 μg/ml	–	–	–	(+)	+	++
Control	–	–	–	–	–	–

\+ 25% degenerated cells
++ 50% degenerated cells
+++ 75% degenerated cells
++++ 100% degenerated cells

Cellular changes are presented in Fig. 3, with cell degeneration, syncytial and round cells on the right, and the intact control tissue culture on the left.

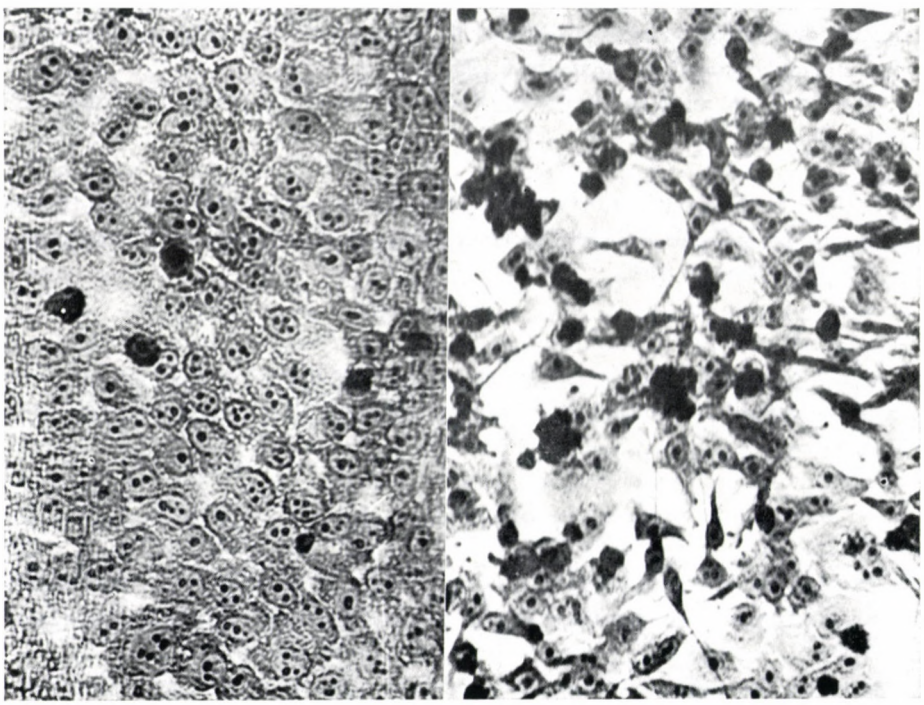

Fig. 3. HEp-2 tissue culture. Giemsa staining, × 400. Control (left); 1 μg/ml endotoxin caused damage within 3 days (right)

Discussion

Isolation of bacterial endotoxin and its study may lead to a better understanding of the pathogenesis of infectious diseases and to improvement of their therapy.

Raichvarg et al. [17] and Danny [22] considered the haemophilus endotoxin responsible for the blocking of ciliar movements and the destruction of epithelial cells. Dagre [23] found them to damage the function of the RES. Clarke [24] reported histamine release from non-sensitized human leukocytes and lung tissue under the effect of haemophilus endotoxin.

Earlier we have found that inhalation of nutrient broth containing non-viable, partly autolysed haemophilus cell was followed by dyspnoea [18]. Inhalation of 1 μg of the lipopolysaccharide extract of the bacteria prepared according to Westphal et al. [20] caused dyspnoea of a similar clinical course and pulmonary congestion which could be observed under general anaesthesia as well. The intensity of the attacks increased with every new dose of endotoxin, but even the first inhalation provoked dyspnoea.

Histological examination revealed features characteristic of interstitial pneumonia with perivascular and peribronchial lymphocytic infiltration without eosinophils. The alveolar walls infiltrated with macrophages were remarkably destroyed. Similar changes of pneumonitic character are caused by the inhalation of organic substances [25, 26].

Studying the mechanism of the acute attacks it was found that endotoxin failed to elicit any direct response from the smooth muscle-nerve or the isolated trachea preparations. Clarke [24], too, found that haemophilus antigens had no effect on the tracheal beta receptors. Thus we were led to the study of monolayer epithelial cell cultures, where the endotoxin proved cytotoxic in doses of 1 μg, 0.1 μg or even 0.01 μg.

Mastrobeanu et al. [27] have demonstrated that salmonella endotoxin penetrates the cell membrane and reaches the perinuclear zone and the cytoplasmatic vacuoles. Great significance is attributed to destruction by toxins of the membranes, the mitochondria, the nuclei and the lysosomes, and, to the released substances in respect of the tissue response and metabolism [28–31]. Electron microscopic studies were done in order to detect the cell damaging effect of haemophilus endotoxin and to gather further information on morphological changes.

During the attacks we could observe that the mucosa and the limbs of the mice were cyanosed. Endotoxins are characteristically toxic to vessels and they damage the capillary vessels by various mechanisms [32–36].

On the basis of the above facts it is thought that the acute attacks of dyspnoea and the interstitial pneumonia elicited by chronic endotoxin administration may be due to a direct damaging effect on the cells of endotoxin and the released mediators.

According to Hutás et al. [37, 38] and Miskovits and Szücs [39], chronic aspecific respiratory diseases (chronic bronchitis, bronchial asthma, obstructive emphysema) affect 20% of the Hungarian population. Postoperative complications were also more frequent among patients with respiratory disease [40].

The microorganisms growing in the lungs, the toxic substances released by them, and the tissue damage they cause play a decisive role in the development and gradual progression of these diseases. Bacterial destruction of the alveolar mucosa soon attains the lamina propria [41, 42]. According to the observations of Turk and May [43], haemophilus may be isolated from the lung even after an effective antibiotic treatment if the patient is symptom-free.

After antibiotic treatment and the administration of hyperimmune sera, a considerable quantity of endotoxin might be released following the sudden bacteriolysis. Spink et al. [44] have observed endotoxin shock in patients with brucellosis, pneumococcal pneumonia, and meningococcal meningitis following antibiotic therapy. When bacterial infections are treated with antibiotics during the exacerbation of chronic aspecific respiratory diseases, the endotoxin of the *in situ* disintegrate bacteria may produce cell damage and attacks of dyspnoea may be elicited by the released mediators. In order to avoid this, an attempt will be made to prevent endotoxin damage by a specific method using radiodetoxified endotoxin [45].

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STUDIES ON ENTEROTOXIN OF *SHIGELLA DYSENTERIAE* TYPE 1

I. EFFECT OF AERATION ON THE PRODUCTION OF ENTEROTOXIN AND SUITABILITY OF LABORATORY ANIMALS FOR ITS BIOLOGICAL ASSAY

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Shigella dysenteriae type 1 was used to study the influence of aeration on enterotoxin production and the suitability of laboratory animals for the toxin assay. The enterotoxin produced under aerobic shake sulture conditions gave a gut dilatatory response of 1.436 but that obtained in aerobic and anaerobic stationary cultures exerted no activity. The toxin fractionated on Sephadex G–150 column was more effective than that obtained by ammonium sulphate perecipitation. Only Sephadex column fraction II had a toxic activity. Among the three laboratory animal models tested, the rabbit ileal loop was suitable for the assay of toxin. The skin permeability test was unsatisfactory for enterotoxicity assay.

Shiga [1, 2] discovered that the bacillus described by him, produced a toxin causing hind limb paralysis in rabbits and labelled it a neurotoxin. The interest in *Shigella dysenteriae* greatly increased due to an epidemic in Central America in 1969 [3] and stimulated studies of its virulence. It was found that it produced an enterotoxin evoking functional and structural dearrangements in the bowel, resulting in clinical dysentery. Later the bacillus was reported [4] to produce a cell-free toxin causing transudation in the rabbit intestine. The role of the enterotoxin in the pathogenesis of bacillary dysentery is not clearly understood nor is it known whether it was a single moiety exhibiting neurotoxic and enterotoxic activities or two separate moieties were responsible for these effects. Now it is known that the toxin has three different activities, viz. neurotoxic, enterotoxic and cytotoxic ones [5].

In the present work the influence of aeration on toxin production and the suitability of different laboratory animals for its biological assay were studied.

Materials and methods

Organism. A strain of *S. dysenteriae* 1 was obtained from the Department of Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. It was used throughout the study to produce enterotoxin and was maintained on nutrient agar slant at 4 °C.

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Animals. Three types of laboratory animal viz. rabbits, rats and infantile mice were used for the assay of enterotoxin.

Production of enterotoxin. Production of seed culture. For the production of enterotoxin, a seed culture was prepared using sterile trypticase soy broth. A loopful of 24 h incubated *S. dysenteriae* 1 culture was inoculated in 150 ml of the medium and incubated at 37 °C for 6 h on a rotatory shaker. This constituted the seed culture to produce an enterotoxin.

Production of enterotoxin. To study the influence of aeration on the yield of enterotoxin, a medium of Casamino acid [6] was used to produce enterotoxin under three different conditions of aeration, namely aerobic shake culture, anaerobic culture and aerobic stationary culture. Four Erlenmeyer flasks, each of 500 ml capacity, containing 125 ml of growth medium were used. To each flask 0.5 ml of a 6 h seed culture was transferred. Two of these were incubated at 37 °C for 18 h and other two similarly aerobically under shaking. For anaerobic culture, 1 ml of seed culture was added to each of two 250 ml flasks filled with growth medium up to the neck of the flask, incubated without shaking under the same conditions as mentioned above. A flask containing the same amount of sterile growth medium incubated for the same time and temperature served as a control. The broth culture of each of two flasks of aerobical shake culture as well as aerobic stationary culture were pooled separately, centrifuged in a refrigerated centrifuge (K-24) at 14 000 r. p. m. for 30 min. The cell-free supernatant containing enterotoxin was decanted, concentrated to 7–8 times of its volume in a rotating vacuum evaporator (Metrex Instrument India) in a water bath at 42 °C, then filtered through Seitz filter. The bacterium-free filtrate was then dispensed into sterile vials and stored at −20 °C for subsequent study. Sterile growth medium was treated similarly and was used as a control.

Partial purification of enterotoxin. Ammonium sulphate was added slowly to the enterotoxin to 70% saturation under constant stirring at 4 °C, kept at the same temperature overnight and centrifuged at 15 000 rpm for 45 min. The supernatant was decanted and the pellet separated. This was then dissolved in a minimum amount of 0.05 M phosphate buffer (PB) solution pH 7.5 and dialysed three times against the same buffer at 4 °C for 24 h. The protein concentration of the precipitated toxin was estimated [7], dispensed in small vials and stored at −20 °C for subsequent use.

Gel filtration. Fractions of crude enterotoxin were subjected to gel filtration. Six ml of enterotoxin were applied on a Sephadex G-150 column. The Sephadex was equilibrated and eluted with 0.5 M PB of 7.5 pH. The flow rate was adjusted to 20 ml. The eluate was collected in 3 ml volumes in small test tubes and examined at 280 nm on a Beckman DU-2 spectrophotometer. The data were plotted and the samples of the same peak were pooled. Fractions pooled at each peak were concentrated against a saturated sucrose solution and tested for toxicity.

Suitability of animals for enterotoxin assay. For enterotoxin assay rabbits, rats and infantile mice were used.

Rabbit ileal loop method. White apparently healthy rabbits weighing 2 to 2.5 kg were used to test their suitability for biological assay of toxin [8]. The rabbits were fasted for 36 h with free excess to water. The intestine was exposed and ligated into loops measuring 9–10 cm each. Each loop was flushed with phosphate buffer saline pH 7.2 (PBS) before injecting toxin. Different protein concentrations of toxin were used to calculate the effective dose (ED) or one unit of toxin giving a gut dilatatory response of 1 or more [8, 9]. It was determined by locating the mid point of the dose response curve which exhibited a linear relationship between abscissa and ordinate. Two ml of enterotoxin were injected in each of two loops and the same amount of growth medium was injected in each of two further loops which then served as controls. The rabbits were sacrificed at 4, 8, 12, 18 and 24 h after inoculation. The volume of fluid accumulated in each loop and its length were recorded. Gut dilatatory response was expressed as a ratio of volume of fluid to length of the loop; a ratio of 1 or more was considered a positive response to toxicity.

Rat ileal loop [10]. Rats weighing 300–400 g were used after fasting for 36 h. The intestine was exposed and ligated as in the rabbits. In each test and control loop, 1 ml of enterotoxin (6 mg protein) and sterile medium was injected, respectively. The rats were observed and later sacrificed at 4, 8, 12, 18 and 24 h after inoculation. The fluid accumulated and the length of each loop of the test and control animals were recorded similarly as in the rabbits. The ratio of fluid to loop length was calculated and the dilatatory response was expressed as their ratio; 0.3 or more was considered a positive response to toxicity.

Infantile mouse gut method [11]. Mice 1–2 day-old were injected with 0.1 ml (600 μg protein) of enterotoxin, mixed with 1 drop of 5% pontamine sky blue intragastrically. The control mice received the same amount of sterile growth medium mixed with dye. The mice were killed at 2, 4, 8 and 12 h after inoculation. The intestine was separated and weighed. The remaining body weight was determined and the ratio gut weight by remaining body weight was determined. A ratio of 0.09 or more was considered a positive index of toxicity.

Skin permeability test [12, 13]. The skin on the back of healthy white rabbits was shaved and the area was divided into squares measuring 40–50 mm. In each square 0.1 ml (600 μg protein) of toxin and in the control squares the same amount of growth medium was injected intradermally. After 24 h 1.2 ml of 5% pontamine sky blue solution per kg body weight was injected intravenously. Two h later the diameter of each area stained blue was measured. A diameter of 7 mm or more was considered positive for the presence of permeability factor.

Results

Effect of aeration on the production of enterotoxin and its toxicity. This was studied under three different conditions. It was noticed (Table I) that enterotoxin produced by aerobic shake cultures caused a maximum gut dilatatory response of 1.436, whereas the toxin produced under stationary aerobic and anaerobic conditions failed to give a toxicity ratio of 1. The cell-free supernatant was precipitated with ammonium sulphate and dialysed three times and the precipitate dissolved in PB was tested for enterotoxicity. The ratio of fluid volume to loop length was 0.98 with 1.4 mg of enterotoxin protein concentration. The toxin partially purified on Sephadex G-150 column gave the elution pattern shown in Fig. 1. Three fractions collected were tested for toxic activity. A high molecular weight peak eluted as void volume was devoid of toxic activity. Fraction II consisting of eluent volume of 54–63 ml showed toxic activity, giving a ratio of 1.2 with 1.2 mg of enterotoxin protein concentration. Fraction III did not show any toxic activity.

The results of animal models for enterotoxin assay are presented in Table II. Of the three animal-models tested, the rabbit ileal loop method gave en-

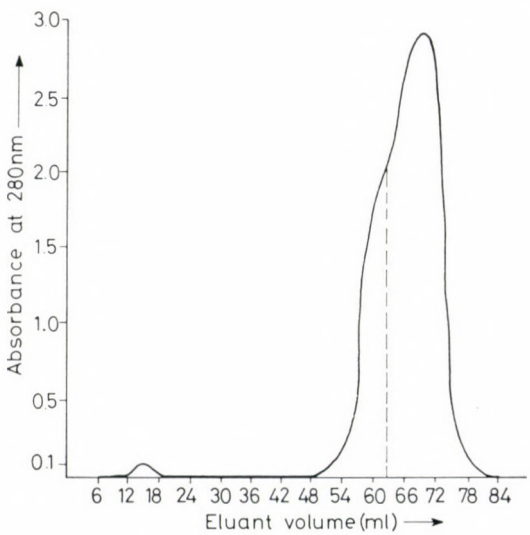

Fig. 1. Gel chromatographic pattern of crude *S. dysenteriae* 1 preparation on Sephadex G–150

Table I

Effect of aeration on enterotoxin production by Shigella dysenteriae 1 and its toxicity in rabbit ileal loop

Aeration condition	Sample	Experiment No. I			II			III			
		Fluid volume, ml	Loop length, cm	Volume per length	Fluid volume, ml	Loop length, cm	Volume per length	Fluid volume, ml	Loop length, cm	Volume per length	Mean ratio ± SD
Aerobic Shake culture	Test	16	11	1.45	11.4	8	1.42	14.7	10	1.47	1.436± 0.025
	Control	0.6	7	0.09	1.1	10	0.11	0.9	8	0.12	0.106± 0.012
Stationary culture	Test	3	10.5	0.30	3.6	11	0.33	3	8	0.37	0.333± 0.029
	Control	1.2	10	0.12	0.7	7	0.10	1	8.5	0.11	0.11± 0.008
Anaerobic culture	Test	1.0	11	0.10	0.8	9	0.09	16	12	0.13	0.11± 0.017
	Control	1.2	10	0.12	0.7	8	0.09	1	10	0.10	0.10± 0.013

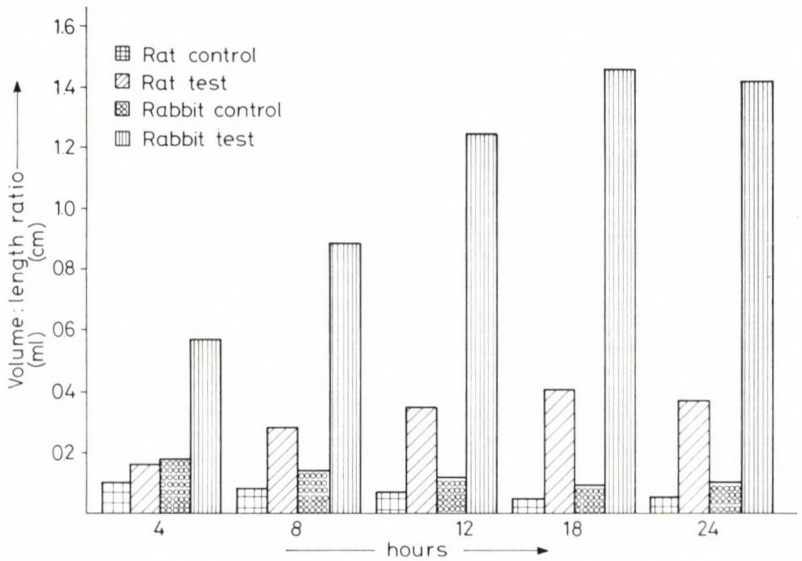

Fig. 2. Enterotoxicity of *S. dysenteriae* 1 enterotoxin at different time intervals in rat and rabbit ileum

couraging results with a toxicity ratio of 1.45. The rat ileal loop method gave also a positive result but the infantile mice failed to do so. Therefore the rabbit ileal loop method was used throughout the study.

The maximum of toxic activity was observed at 18 h after inoculation in both rabbits and rats (Fig. 2); the effect persisted till 24 h. The controls gave a negligible response in both cases.

As to the permeability to enterotoxin of the rabbit skin, no effect was found as the blue area was less than 7 mm in diameter in all cases (Table II).

Discussion

The toxin produced aerobically under shaking displayed its activity in the rabbit ileal loop (6, 14–16) by a dilatatory response of 1.436. Less toxin was produced under aerobic and anaerobic stationary conditions. The influence of shaking on the toxicity is not fully understood. Shaking provided more oxygen which might have influenced the production of toxin and its toxicity or release or both. The mechanical effect of shaking on the release of toxin cannot be ruled out, either. The influence of ingredients has not been studied.

The enterotoxin of *S. dysenteriae* 1 showed multiple bands of protein and the toxic moiety was only a minor component [16]. Similarly, shigella enterotoxin partially purified on Sephadex G-150 column gave three fractions viz. A, B and C and of these only fraction B was enterotoxic as well as neurotoxic

Table II

Comparison of animal models for the assay of enterotoxin of Shigella dysenteriae type 1

Sample	Infant mouse gut				Rat ileal loop				Rabbit ileal loop				Skin permeability test, diameter, mm
	Gut mg	Body g	Gut per body	Mean ratio ± SD	Fluid volume, ml	Loop length, cm	Volume per length	Mean ratio ± SD	Fluid volume, ml	Loop length, cm	Volume per length	Mean of ratio ± SD	
Test	209	2.712	0.07	0.06 ± 0.008	5	13.5	0.37	0·39 ± 0.016	14.7	10.0	1.47	1.45 ± 0.02	2.4
	97	1.984	0.05		4.5	11	0.41		12.8	9	1.43		
Control	80	2.114	0.04	0.045 ± 0.0077	0.6	8	0.07	0.06 ± 0.008	1.1	7	0.15	0.13 ± 0.02	2.0
	110	2.153	0.05		0.5	9	0.05		1.0	8.5	0.11		

whereas the others elicited neither of these activities [14]. By isoelectrofocussing in sucrose gradient, at pH 7.2 and 6.1, two fractions were separated by McIver et al. [16]. The former fraction showed neurotoxic as well as enterotoxic activities whereas the latter had a cytotoxic activity. The partially purifed toxin displayed a toxicity ratio of 0.98 with 1.4 mg protein concentration, i.e. below the ratio of toxicity. Fraction II gave a toxicity ratio of 1.2 whereas fractions I and III failed to show such activity, in agreement with the above findings but the method of fractionation was not the same as that used by McIver et al. [16].

Several animal models were tested in order to establish their suitability for assaying the enterotoxic activity. In these tests the rabbit ligated ileal loop model was found suitable as it gave a positive response. Rats were also useful but they had a lower ratio of accumulated fluid to loop length. The mouse gut model has been suggested to be the test of choice [17] for assaying *Escherichia coli* enterotoxin and being in addition less expensive and less time consuming than the others, failed to show encouraging results in the present study. Infantile mice were reported to respond to the heat stable extracellular enterotoxin of *E. coli* [18] but in the present study they did not respond to enterotoxin. It is assumed that either the heat stable moiety was lacking in the toxin used or else some other mechanism was responsible for the failing reaction.

The skin permeability reaction was reported to be specific for *Vibrio cholerae* enterotoxin [12] and for *Klebsiella pneumoniae* [19] and was considered suitable for assaying the toxin. The absence of skin permeability factor was reported in *S. dysenteriae* toxin [14] and the enterotoxin under investigation was also found to be lacking a permeability factor.

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URINARY TRACT INFECTIVITY OF R STRAINS OF *ESCHERICHIA COLI* CARRYING VARIOUS VIRULENCE FACTORS*

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The virulence factors of *Escherichia coli* supposed to act in urinary tract infections were studied on R strains in a suckling mouse model. The production of alpha-(diffusible-) haemolysin or the possession of antigen K1 enhanced the virulence significantly, while the type 1 (common) fimbriae failed to do so. An isogenic motile and non-motile pair of *E. coli* did not show any difference in infectivity in the model. The adhesins, the diffusible haemolysin, and the acidic polysaccharide K antigens (K1) are definitely additive virulence factors in the model. This is in good agreement with the experience of clinical bacteriology.

In a previous paper [1] an animal model was described for the study of urinary tract infections. By direct inoculation into the urinary bladder of suckling mice the virulence of strains of *Escherichia coli* or *Proteus mirabilis* (Vörös et al. to be published), and also their virulence factors were compared on the basis of the resulting chronic infection of the bladder and kidneys. In this respect the important role of F8 adhesin in the virulence of *E. coli* for urinary tract infections was observed [1]. In further experiments, comparing this suckling mouse model to the rapid, haematogenous mouse test of Van den Bosch et al. [2, 3] the alpha haemolysin of *E. coli* proved to be an additive virulence factor in urinary tract infections, demonstrating also the adhesive function of F7 [7].

The present paper is a summary of results of experiments on rough (R) strains of *E. coli* carrying various hypothetical virulence factors: alpha haemolysin, type 1 (common) fimbriae, and antigen K1. Some preliminary investigations were performed on the eventual role of motility (taxis), too.

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Materials and methods

Strains. R strains of *E. coli* were isolated from patients with urinary tract infection. They are listed in Table I indicating also their relevant factors of virulence.

The demonstration of antigen Kl was performed by using countercurrent immunoelectrophoresis [5], the diffusible (alpha) feature of the produced haemolysin was characterized by the overlayer method of Walton and Smith [6] and titres were determined from broth culture supernatant by the dilution technique. On the basis of the mannose sensitive haemagglutination of guinea pig erythrocytes, the presence of type 1 fimbriae was determined [7].

Table I

List of tested R strains of E. coli and their known virulence factors

Categories (groups)	Designation of strain	Virulence factors		
		antigen Kl	α-haemolysin (titre*)	type 1 fimbriae
	R120			
	R125			
	R215			
A	R256		none	
	R273			
	R319			
	R411			
	R138			
	R73	+		
	R135	+		
B	R365	+		
	R426	+		
	R378	+		
	R133		+ (16)	
C	R233		+ (32)	
	R251		+ (8)	
	R270		+ (32)	
	R6			+
	R104			+
D	R150			+
	R151			+
	R254			+
	R27	+	+ (8)	
E	R246	+	+ (8)	

* Reciprocal value of the supernatant dilution with haemolytic activity (end point)

A wild-type, motile strain of *E. coli* (O4 : K12 : H5) was mutagenized by ethylmethanesulphonate [8] and a H^- mutant was selected on soft agar (0.6%) plates. The defect in flagellar synthesis was tested further by the U-tube technique and by the lack of motility under the microscope.

Mice and the animal model. The bladder of three days old CFLP (LATI, Gödöllő, Hungary) outbred mice was inoculated by a volume of about 0.025 ml with a special fine (No. 22) needle. Unless otherwise stated, 4–10 mice per bacterial strains were used. The optimal dose used in the case of R strains was about 10^5 germs. For the technical control of inoculation Pontamine Sky Blue (6XB, Searle, England) in a final concentration of 0.05% was added to the inocula. Dissection of the animals was made under sterile conditions 14–24 days after inoculation. With the parallel checking of an eventual bacteriaemia (heart blood), samples of urine and both of the kidneys were plated. After the urinary bladder was removed, chopped and washed, it was treated in a Vortex-type apparatus (CSAV, 5001 H). This way the number of adhered bacteria was determined.

Statistical evaluation. Significant differences were calculated by the χ^2 test.

Results

Virulence factors of E. coli R strains causing urinary tract infections. The problem of reliable comparison was presented by the strains which were not isogenic derivatives with respect to virulence factors, being independent clinical isolates. A further difficulty of this kind was the dissection and bacteriological process making it impossible to carry out the experiments simultaneously. Standardization was attempted by the quasi identical dose of inoculation (10^5 germs), by the age of the animals (3 days), and by the arrangement of the experiments in which the strains carrying virulence factors and those without such character were tested in parallel. The results of model experiments are summarized in Table II.

Based on the presented data the additive role of alpha haemolysin as a virulence factor for urinary tract infection was confirmed also for strains lacking the somatic O antigen of *E. coli*. Comparison of the haemolysin producers to type 1 fimbriated strains seems to be more reliable than a comparison to totally avirulent strains. The latter group has no evaluable virulence: infection of the bladder occurred only in 10% of the animals without expressed adhesion ($< 10^2$ bacteria on the surface of the washed, vortexed bladder) and with only one case of ascending infection. Comparing these groups (C and D) the rate of infection proved to be significantly higher in the haemolytic group (bladder: $\chi^2 = 11.558$, $P < 0.001$; kidneys: $\chi^2 = 10.299$, $P < 0.01$). The mean number of bacteria on the bladder surface was 1.3×10^5 germs. A similar enhancement of virulence could be observed in the case of antigen $K1^+$ character compared with the type 1 fimbriated group (B and D) — (bladder: $\chi^2 = 8.963$, $P < 0.01$; kidneys: $\chi^2 = 4.249$, $0.05 < P < 0.02$) with a significantly higher rate of bladder and kidney infections, accompanied by a high surface germ count (mean value $= 1.1 \times 10^5$).

The combined presence of haemolysin production and K1 antigen was demonstrated only in two strains. Therefore a trend of additive effect can only be presumed.

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Table II

Urinary tract infectivity of groups of R strains of E. coli carrying various virulence factors in the suckling mouse model

Group of strains	Virulence factor	No. of strains	Bladder infectivity				Kidney infectivity		
			ratio[1]	germ count[2]	χ^2 value	P[3]	ratio[1]	χ^2 value	P[3]
A	none	8	0/52	.	.	.	0/52	.	.
B	antigen Kl	5	14/37	1.1×10^5	8.963[4]	<0.01	6/37	4.249[4]	0.05—0.02
C	α-haemolysin	4	14/32	1.3×10^5	11.558[5]	<0.001	9/32	10.299[5]	<0.01
D	type 1 fimbriae	5	4/12	$< 10^2$	4.529[6]	>0.02	1/42	1.107[6]	>0.2
E	Kl + α-haemolysin	2	11/20	6.3×10^6	.	.	6/20	.	.

[1] mice infected/inoculated, [2] mean number of bacteria from washed and vortexed bladder, [3] value of probability, [4] between groups B and D, [5] between groups C and D, [6] between groups A and D, . = not evaluated or not evaluable

The role of motility (taxis) in the virulence of urinary E. coli. An isogenic pair of *E. coli* (O4 : K12 : H5 wild-type and its H⁻ mutant) was tested in the model using graduated doses of inoculation. From the data summarized in Table III it is evident that there was no observable difference of the virulence with respect to motility.

Table III

Suckling mouse urinary tract virulence of a wild-type motile strain of E. coli (O4: K12:H5) and its EMS-induced non motile mutant derivative

Infecting agent	No. of mice	Doses of inoculation	Day after inoculation	Bladder infectivity			Kidney infectivity ratio[1]
				ratio[1]	germ count[2]	ID_{50} value[3]	
	10	1.7×10^4	21st	6/10	1.2×10^3		1/10
Wild-type	11	2.5×10^3	24th	2/11	5.4×10^3	6.0×10^3	0/11
$H5^+$	5	2.4×10^2	23rd	2/5	8.8×10^3		1/5
	10	2.6×10^4	21st	6/10	6.1×10^3		1/10
Mutant	10	6.5×10^3	24th	4/10	1.0×10^3	8.8×10^3	0/10
H^-	4	2.5×10^2	23rd	2/4	2.6×10^3		0/4

[1] mice infected/inoculated, [2] mean number of bacteria from washed and vortexed bladder, [3] endpoint of 50% of bladder infection by inoculation

Discussion

The presented data convincingly demonstrate the usefulness of the suckling mouse model in the evaluation of virulence factors of *E. coli.*

The important role of adhesins like the F8 [1], F7 [4] and others [9] has been proven earlier, as mentioned in the introduction. According to Ørskov et al. [10] the adhesins F7, F8 and F10 are important for the urinary tract pathogenicity of *E. coli.*

The presented results proved repeatedly the additive virulence function of alpha haemolysin, also in case of the missing somatic O-antigen. It should be emphasized that this is true only for the diffusible, so-called alpha haemolysin and not for the beta type, which is indistinguishable from the alpha type on blood agar plates. No pathogenic function of beta haemolysin was observed by us [11, 12]. Among the R strains investigated in the present experiments there was only a single beta haemolytic strain (No.R138) listed in group (A) carrying no known virulence factor.

The type 1 (common) fimbriae were the subject of a long discussion concerning their adhesive function. Ørskov et al. [13] were able to demonstrate that these do not adhere to the epithelial surface, but to the mucus and therefore they act as a "mucus trap" factor rather than a virulence enhancing

feature of the bacteria. Our results showed only an insignificant enhancing effect and the very low number of bacteria on the bladder surface is in good agreement with the conception of the Ørskovs.

The predominance of K1-antigen producing *E. coli* strains in cases of infantile meningitis or sepsis (14–16), as well as in urinary strains isolated mostly from children [5, 17, 18] was observed by many investigatoıs. The distinguished role of K1 in extraintestinal infections has not been clarified, but perhaps the most important is its marked antiphagocytic effect [19], at least of its O-acetylated form. Our results showed a virulence enhancing effect equivalent to the alpha haemolysin production.

The motility, i. e. chemotaxis, mediated by functioning flagellae has a role only in the enteropathogenic *Vibrio cholerae* [20–22]. The negative result in our preliminary experiments did not prove the lack of importance concerning the virulence of *E. coli* in urinary tract infections because our strain has other virulence factors (haemolysin and F8).

Our conclusions on the virulence factors of *E. coli* serving urinary pathogenicity are positive with respect to antigen K1, adhesins and alpha haemolysin, but negative for the type 1 (common) fimbriae and motility. This is in good agreement with the findings of clinical bacteriology. According to Evans et al. [23] strains of *E. coli* isolated from urinary tract infections (mostly pyelonephritis) are frequently carrying acidic polysaccharide K-antigens: predominant among them is K1 (29%) and so are K2, K12, K13. The rate of haemolysin production among them is even higher, 42%. Most of these strains belong to the type VI haemagglutination pattern, with their mannose resistant action on human erythrocytes (F8 belongs to this group), frequently in combination with the haemolytic and/or the $K1^+$ character (89%). It is easily acceptable that the maintenance of more than one virulence factor was the result of selection pressure. The simultaneous occurrence of the above mentioned three virulence factors was, however, observed in not more than 3%, so the presence of some contraselective force may be assumed. Checking of 408 urinary *E. coli* R strains for the presence of these three virulence factors resulted in not more than 3.5% positive strains [24].

According to present knowledge a competition between the phagocytosis promoter adhesins and inhibitor K antigens stands to reason.

The results clearly demonstrate that *E. coli* R strains — generally believed to be avirulent — are able to induce kidney infections if they possess certain virulence factors. The fact that they are capable of maintaining urinary tract infections has already been reported [25].

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ANTIBODIES TO CORONAVIRUS OC 43 STRAIN IN THE POPULATION OF NORTH-EASTERN HUNGARY

(A NOTE)

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(Received July 21, 1982)

Monthly 120 serum samples were collected from healthy and clinically ill residents of North-Eastern Hungary in the years 1980 and 1981. Haemagglutination-inhibiting antibody to the strain Coronavirus OC 43 was detected in 58% of the 2881 samples. The rate of positive sera was the lowest, 23%, between 7 and 11 months of age and reached the peak of 70% between 15 and 19 years. It did not rise further with age. Paired sera were tested from 405 patients suffering from various diseases. A titre rise was shown in 23 patients; 12 of these were suffering from acute respiratory disease, another 7 showed simultaneous exacerbation of bronchial asthma or spastic bronchitis.

Our knowledge of the coronavirus aetiology of human respiratory and other diseases, and of the epidemiology of coronavirus diseases, has mainly been based on seroepidemiological studies [1–3]. Isolation of coronaviruses is too difficult for wide-scale investigations.

Serum samples were collected from healthy and ill residents of Northern Hungary over a period of two years to demonstrate antibodies to the strain Coronavirus OC 43. The results are published in the present report.

Materials and methods

Monthly about 120 serum samples, 2881 samples altogether, were collected in the years 1980 and 1981. Nearly two-thirds of the donors were patients presenting with various symptoms in clinics or hospitals. The remainder were healthy. The serum samples were stored at −20 °C, and heated at 56 °C for 30 min, before being tested.

The antigen used in the tests was prepared as described by Kaye and Dowdle [4] from the brain of Balb/c suckling mice intracerebrally infected with the Coronavirus OC 43 strain. The virus strain and the reference murine hyperimmune ascites fluid were kindly supplied by the National Institutes of Health (Bethesda, Md., USA).

Haemagglutination-inhibition (HI) test was performed in Takátsy's [5] Microtitrator trays. Four haemagglutinating units and 0.5% mouse erythrocyte suspension were used in the reaction mixture.

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Results and discussion

Figure 1 shows the percentage incidence of positive HI reaction in different age groups and the distribution of the positive reactions by age and titre; 57.7% of the samples contained HI antibodies detectable in the 1 : 10 serum dilution. The geometric mean of the titres was 1 : 16.9.

Of the samples collected from infants under 7 months 51%, of those collected from infants between 7 and 11 months of age 23.5% contained detectable antibodies. Thus, the frequency of positive reactions increased with age, up to 71% in the 15–19 year age group. There was no further increase in frequency in adult age. The geometric means for reciprocal titres ranged between 12.9 and 15.7 in the age groups under 15 years and between 18.1 and 19.3 for the older age groups.

The frequency of 1 : 10 and 1 : 20 titres consistently grew between 7 months and 14 years; higher titres occurred more frequently over 15 years than in children.

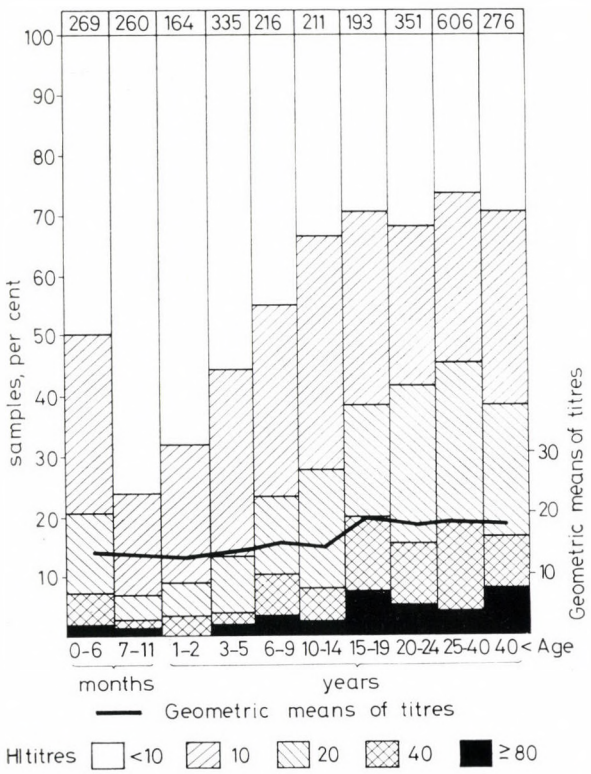

Fig. 1. Age distribution of the 2881 subjects tested for HI antibodies to the strain Coronavirus OC 43 and their distribution by serum titre

Table I

Changes in the HI antibody titre to strain Coronavirus OC 43. Serum pairs collected in 1980 and 1981

Age group, years	No. of serum pairs tested	Haemagglutination inhibition titres			
		< 10 in both samples	≥ 10 no change	Titre rise 4-fold or more	
				No.	per cent
0–1	67	44	18	5	7.5
2–14	86	38	43	5	5.8
15–24	109	31	70	8	7.3
25–40	105	29	73	3	2.9
40	38	11	25	2	5.3
Totals	405	153	229	23	5.6

Table I shows the titre changes for 405 pairs of serum samples. Four-fold or higher titre rise was demonstrated in 23 cases (5.6%).

In Fig. 2, the monthly distribution of the positive titre changes is shown. Fourteen of the positive patients suffered from acute or chronic respiratory disease. In the chronic cases, recrudescence of the disease occurred simultaneously with the titre rise. Some cumulation of titre rises was observed in the first quarter of both years.

The frequency and age distribution of positive sera in the present study were in good agreement with literary data based on similar serological surveys performed in European countries (6–9) and in America (10, 11).

It has been assumed [12] that the high titres of coronavirus antibodies in adult age may be due to secondary responses to re-infection. The high fre-

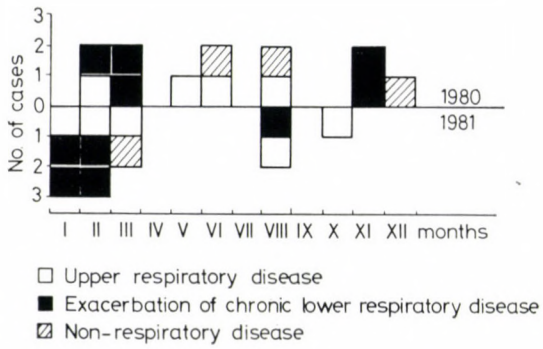

Fig. 2. Monthly incidence of serologically proved Coronavirus OC 43 infections in the years 1980 and 1981. The complement-fixing antibody titres to influenza A and B virus, to the common antigen of human adenoviruses and to respiratory syncytial virus were less than 1 : 4 in each case

quency of positive sera in the first 6 months of life may be attributed to maternal antibodies, though coronavirus infections may occur in young infants; according to Henigst [13] even intrauterine infection cannot be excluded.

Based on serological surveys performed in the years 1960–1967, Kaye et al. [12] assumed that the average annual incidence of infections caused by the Coronavirus OC 43 strain or closely related strains was about 7% in populations between 5 and 19 years of age, living in closed communities. Monto and Lim [14], who examined paired sera from subjects of various ages living elsewhere in the USA, found titre rises in 17.1%. Other authors [7], who had examined paired sera collected in Hamburg, reported titre increases in 6.6%. The not quite consistent results may be attributed partly to periodical fluctuations in the epidemiology of coronavirus infections [12, 14]. Outstanding peaks were observed in every third or second year, chiefly in the winter and early in spring in populations of given areas [10, 15]. The fact that 12 of our 23 cases with rising coronavirus antibody titres occurred in the first quarters of the calendar year agrees well with these observations.

We found a titre rise in seven patients who were suffering from bronchial asthma or spastic bronchitis. The serologically proved coronavirus infection co-incided with exacerbations in all the seven cases. This finding, too, is in accordance with the data in the literature [16, 17].

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CONTENTS

TO THE EIGHTIETH ANNIVERSARY OF PROFESSOR ZOLTÁN ALFÖLDY

The papers of this issue are dedicated to Professor Zoltán Alföldy, director emeritus of the Institute of Microbiology of Semmelweis University Medical School in Budapest, by his pupils on the occasion of his 80th anniversary.

Professor Zoltán Alföldy belongs to the generation of physicians that has kept besides the inevitable specialization its interest in the whole of medicine. Professor Alföldy was born in January 1904, and took his degree as medical doctor in Debrecen. He started here his career in 1928 at the Department of Medicine. The human medical attitude so characteristic of him was based on his practice. The severe epidemic of typhoid in the nineteen-thirties directed his attention to bacteriology. In 1937 he started working at the National Institute of Public Health (Budapest) where he joined the national research work against typhoid launched in 1938. From 1946 he was the head of the bacteriological department until 1950 when he became the director of the Institute of Microbiology of the Semmelweis University Medical School in Budapest. Since his retirement in 1974 he still works at the Institute as consultant.

Microbiology was taught by him for 25 years at the University and the lectures he delivered regularly were always remarkable. Most of the physicians of our days were his students and learned microbiology from his books.

Leptospirosis and the mechanism of action of disinfectants were the fields of his research. He published more than 70 papers, 10 books and many chapters of books. The first isolation of leptospira from a patient in Hungary

is linked with his name. Thereafter together with his collaborators he defined the regularities of the occurrence of leptospirosis in Hungary and proved the leptospiral origin of several epidemics of serous meningitis. Types of the strains occurring in Hungary were determined under his guidance.

Besides teaching and research, Professor Alföldy always took an active part in public life. Since the foundation of the Scientific Health Council he acted as secretary and then vice-president and is at present a member of the presidential board. From 1968 to 1970 he was the head of Scientific Research Department at the Ministry of Health. He took part in the work of editorial boards, committees and social organizations, keeping contact with many specialists in Hungary and abroad.

Professor Alföldy received many decorations and awards. He was decorated by the Kossuth Prize for his research on leptospirosis and on three occasions with the gold medal of the Order of Labour, among others.

Beside his activities he always devoted time and energy to the teaching and training of his pupils and successors, both in his close family and the large family of the Institute. All these characterize a man who has lived a full life and never losing anything from this completeness.

Dear Professor Alföldy,

In the name of all your pupils allow us, your two oldest collaborators, to wish you every happiness and best of health on the occassion of your 80th anniversary and please accept as a homage the papers written by your former and present collaborators.
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ARRANGEMENT OF HEXONS AND POLYPEPTIDE SUBUNITS IN THE ADENOVIRUS CAPSID

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With direct analysis of electron micrographs and with Markham's rotational integration technique we have demonstrated that in the adenovirus capsid the triangular profile of the hexon-end facing the virion surface is formed by three polypeptide subunits. The three vertices of the profile are formed by one polypeptide each and the sides by two neighbouring polypeptides. The mutual rotational and spatial orientation of the three hexon polypeptide subunits has been determined in relation to each other and to the pentons. Two polypeptides of the peripentonal hexons are oriented towards the penton. A "one-to-one" pattern was found in relation to two peripentonal hexons, "one-to-two" to two of the three neighbouring hexons and "two-to-two" to one hexon. "One-to-two" orientation is the general rule in the GONs. The mutual orientation between the hexons of the neighbouring GONs being in connection with each other and that of the polypeptide subunits of the peripentonal hexons is characterized by the regular alternation of the "one-to-two", "one-to-one" and "two-to-two" orientation. The mutual spatial orientation of hexons at different points of the capsid has been studied in model experiments and was characterized by the angle enclosed by the longitudinal axis of the given hexon and that of the other hexon in open position towards the virion surface, if the longitudinal axis of the hexons of the triangular faces are perpendicular to the plane of the face and those of the edge-hexons perpendicular to the plane of the edge and the longitudinal axis of the penton faces in radial direction the centre of the virion. In this way the longitudinal axes of the peripentonal hexons enclose with that of the penton an angle of $\sim 32°$ and with each other $\sim 36°$. The longitudinal axis of the edge-forming hexons shows a deviation of $\sim 21°$ in relation to the longitudinal axes of hexons situated on the neighbouring triangular faces. We present on a tentative virus model the mutual rotational and spatial orientation of all the polypeptide subunits of the adenovirus-building hexon capsomers. This corresponds to the two-, three- and fivefold rotational symmetry characteristic of the icosahedral capsid.

Adenovirus hexon capsomers consist of three polypeptide subunits arranged around an axis of threefold symmetry [1–5] and their ends facing the virion surface have a triangular profile [6, 7]. On electron micrographs the profile of the hexon-ends and the three polypeptide subunits are seldom distinguishable.

Previous experiments have shown that in the course of crystallization of adenovirus hexon proteins tightly packed two-dimensional crystalline arrays could arise besides the three-dimensional tetrahedral crystals [8]. Direct analysis of electron micrographs as well as optical diffraction procedure and Markham's rotational integration technique provided means for determination of both the form (profile) of the ends of the hexons constituting the

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crystalline array and of the mutual rotational orientation of the hexon polypeptide subunits [9]. In the two-dimensional hexon crystalline array one polypeptide subunit of a given hexon is situated nearest to two other polypeptide subunits of the neighbouring hexon. This means that the position of the polypeptide subunits follows the "one-to-two" pattern, accordingly the hexons and polypeptide subunits are in equivalent environment corresponding to the hexagonal packing and threefold symmetry.

On the basis of the mode of linkage between the hexons determined in the two-dimensional hexon crystalline array and of the electron microscopic examination of the virion the present report gives information on the mutual orientation of the hexons and polypeptide subunits in the virus capsid, taking into consideration the orientation within the GONs ("groups on nine hexons") which represent a significant part of the triangular faces of the icosahedral virion as well as the mutual orientation of the GONs.

Materials and methods

Virus propagation, separation and purification. Human adenovirus type 12 has been propagated on HEp-2 cell culture using Parker's 199 medium supplemented with 15% calf or bovine serum and antibiotics to prevent bacterial contamination. After the cell degeneration, cells were separated from the maintenance medium by centrifugation at 2000 rpm for 20 min. The infected cells were disintegrated by repeated freezing and thawing or by ultrasonic disintegrator. Virions were separated from the cells in an MSE Superspeed 50 ultracentrifuge on double CsCl cushion at 27 000 rpm for 1.5–4 h. For further purification the virion band was centrifuged in CsCl density gradient at 27 000 rpm for 24–48 h.

Virions of adenovirus strain isolated on primary rabbit kidney cell culture from the intestinal tract and different organs of 6–8 weeks old rabbits suffering from nonbacterial diarrhoea were also studied [10].

Electron microscopic examination. Virions separated and purified by ultracentrifugation were adsorbed to carbon-formvar-coated grid and negatively stained with 1% uranyl acetate. The preparations were examined in a JEM 100 B electron microscope at 60 kV. The micrographs were prepared with a basic magnification of 25 000–50 000 and the negatives were enlarged to a magnification of approximately 300 000.

The disturbing background granulation was eliminated by the technique of Yabe et al. [11]. Performing image analysis the apparent 10–12% increase of the virus particles caused by the uranyl acetate staining was taken into consideration [12]. Virions with capsids containing hexon capsomers of distinct profile and visible polypeptide subunits were chosen for serial examinations. These virions were rephotographed and their negatives were used in the following studies.

The technique of Markham et al. [13] was used for enhancing images and for examination of the hexon profiles and the rotational symmetry of the polypeptide subunits as well as other symmetry conditions recognizable in the virus particle. According to the technique after a shortened exposition time the photographic paper is appropriately rotated and this is followed by the next exposition. The number of rotations and expositions is defined by the number of periodically repeated image elements. Maximum enhancement is reached if the number of expositions is identical with the number of repeating elements, otherwise the image becomes blurred.

Results

Rotational orientation of peripentonal hexons in the plane of the electron micrograph. Direct examination of negatively stained preparations revealed several virions in the capsid of which the triangular profile of the hexons facing the virion surface as well as the three hexon-building polypeptide subunits and their orientation were well discernible. On the picture of human adenovirus type 12 (Fig. 1/a) the arrow indicates a peripentonally situated hexon of which not only the triangular profile but also the three polypeptide subunits and their mutual orientation as well as the Y-shaped slit at the centre are discernible. The virion was subjected to Markham's rotational procedure with an angle of rotation of 120° (three-part rotation) so that the middle of the triangular face was the rotational axis (Fig. 1/b). In this way the image of the profile of the peripentonal hexons improved on both ends of the three edges bordering the given triangular face. Their orientation is also clearly visible.

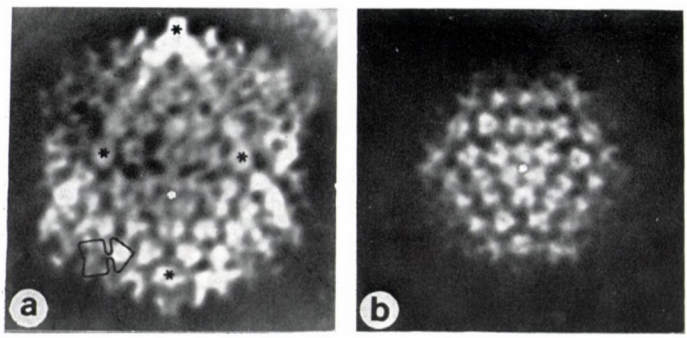

Fig. 1. (a) On the electron micrograph of human adenovirus type 12 the virion is visible approximately from the twofold symmetry axis (edge). Pentons on the vertices of two opposite triangular faces are marked by asterisk. Arrow points to the peripentonal hexon with clearly discernible triangular profile, Y-shaped slit among the three polypeptide subunits and visible orientation. Besides this hexon another one on the same edge with similar profile and orientation is also observable. This latter hexon belongs to the GON which constitutes one of the triangular face. The centre of one triangular face of the virion i.e. the centre of the area (small white point) among three hexons in the middle of the face (threefold symmetry axis) served as centre for Markham's rotational integration

(b) Picture of one triangular face of the virion processed by rotational technique. Angle of rotation: 120°. The image of the profile of the peripentonal hexon marked on the virion improved by the use of the technique. It is discernible that two polypeptide subunits facing the next lying penton and the third one are opposite in direction. It has become discernible that one polypeptide subunit of two neighbouring peripentonal hexons is near to each other. The triangular profile and orientation of the six hexons on one triangular face i.e all hexons among the three hexon rows on the edge is visible. The hexons are situated parallel with the edges and one vertex of their triangular profile (one polypeptide subunit) points anticlockwise, viewing it from above, from the threefold symmetry axis. The axis of the triangular profile running through the middle of the side and the opposite vertex deviate slightly from the longitudinal axis of the given hexon row towards the centre of the triangular face. On both sides of the edge-forming hexons the distance separating the edge- and face-building hexons are wider than those between the hexons on the edges and faces. Spaces separating the pentons and peripentonal hexons are also wider. Sings of interhexonal linking components are also visible at some sites [14, 15]

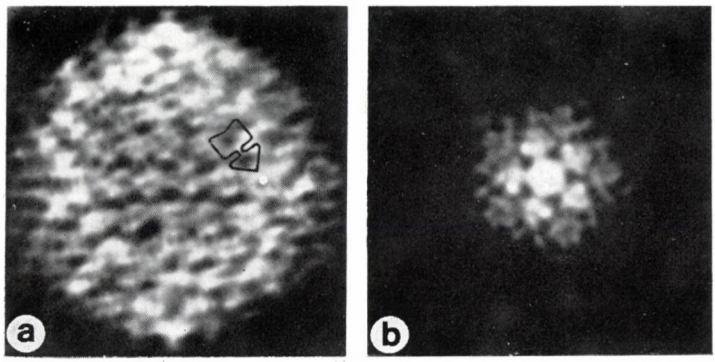

Fig. 2. (a) The penton serving as centre for Markham's rotational integration (fivefold symmetry axis) is marked by arrow on the electron micrograph of adenovirus isolated from rabbit
(b) Picture of the penton marked on the virion and of five peripentonal hexons processed by rotational technique. Angle of rotation: 72°. The triangular profile of the ends of the peripentonal hexons is well visible. The three polypeptide subunits building the complete hexon and the Y-shaped slit among them is discernible. It is also seen that two polypeptides of the peripentonal hexons are facing the penton. One polypeptide of each peripentonal hexons is near to each other and one is situated farther in radial direction from the penton. Linking components are also visible between the penton and the hexons [16]

Two of the three polypeptide subunits forming the triangular profile are situated near the penton and the third one on the edge of the capsid towards the penton situated farther. It is also clearly discernible that the three vertices of the triangular hexon profile are constituted by one polypeptide subunit each. The localization of the six peripentonal hexons on the three bordering edges shows a threefold symmetry also in respect of the polypeptide subunits.

Figure 2 presents an electron micrograph of an adenovirus isolated from rabbit. From the centre of the marked penton (Fig. 2/a) the micrograph was processed by Markham's rotational technique (Fig. 2/b) with a rotational angle of 72° (five-part rotation). The triangular profile of the five hexons surrounding the penton and the three polypeptide subunits around the Y-shaped slit are discernible. In can be seen that one side of each peripentonal hexon i.e. two polypeptide subunits, are facing the penton and one polypeptide is located on the edge in radial direction from the nearer penton towards the farther lying one. The five peripentonal hexons surrounding one penton display a position corresponding to fivefold symmetry in the respect of the polypeptide subunits too on the five edges starting from the penton.

According to the above observations the orientation of the peripentonal hexons was identical both by rotation from the threefold symmetry axis (centre of the triangular face) and from the fivefold symmetry axis (vertex). Consequently the mutual orientation of every peripentonal hexon i.e. of their polypeptide subunits to their six neighbours is as follows. Two polypeptide subunits i.e. one side of the triangular profile are oriented towards the penton,

and the same two polypeptide subunits are linked also with two neighbouring peripentonal hexons thus in the case of the neighbouring hexons the polypeptide orientation shows a "one-to-one" pattern. The third polypeptide subunit shows "one-to-two" orientation with a hexon of similar orientation belonging to the GON which takes part in the constitution of the edge of the capsid. Its fifth neighbour is a hexon which belongs to the same GON but is situated on the triangular face nearest to the given peripentonal hexon being in "one-to-one" linkage to it. The sixth neighbour is a hexon belonging to the other GON and linked with the given peripentonal hexon: it is situated on the triangular face of the capsid nearest to the peripentonal hexon displaying with it a "two-to-two" orientation. In this way each peripentonal hexon is in connection with two GONs.

Orientation of the GON-building hexons within the GON. On the micrograph of human adenovirus type 12 processed by Markham's rotational integration technique (Fig. 1/b) using for the centre of rotation the centre of the space among the three hexons in the middle of the triangular face i.e. the threefold symmetry axis, the profile and orientation of each of the six hexons building the triangular face are visible. Thus one define the position of the polypeptide subunits which make up the hexons as each vertex of the triangular profile is built of one polypeptide subunit whereas the sides are formed jointly by two polypeptide subunits. This is well observable in the case of the peripentonal hexons. Accordingly, the mutual orientation of six hexons belonging to the same GON and building one triangular face as well as that of their polypeptide subunits is always of the "one-to-two" type within the GON. As compared to three neighbouring edges they show a position in which a hexon row of three members runs parallel with the edge (which means six hexons as the hexons on the vertices participate in the formation of two edges) in such a way that every polypeptide subunit corresponding to one of the hexon vertices points in anti-clockwise direction, viewing it from above from the threefold symmetry axis.

Taking for axis of the triangular profile of the hexon-end the straight line which crosses the middle of one edge and the opposit vertex, this axis fails to appear to run parallel in certain cases with the longitudinal axis of the given hexon row, namely the vertices of the hexons constituting the triangular face are slightly turning towards the centre of the face.

Three further hexons which participate in the construction of the neighbouring edges also belong to the GON. Their profile became blurred in the course of the rotational procedure. Nevertheless, beside the peripentonal hexon marked on Fig. 1/a, this kind of hexon belonging to the GON is visible, its triangular profile and orientation are discernible. Within the GON this hexon is characterized also by "one-to-two" linkage. The orientation of the polypeptide subunits i.e. the direction of one polypeptide corresponding to the

vertex is identical with that of the GON-hexons constituting the triangular face.

Figure 3 gives a schematic representation of the uniform "one-to-two" orientation of the polypeptide subunits of nine hexons in one GON.

Orientation of the GON-hexons toward the neighbouring hexons. Each GON is situated on the triangular face of the capsid in such a way that two hexons of each of the three vertices (a total of six) are linked with two peripentonal hexons according to the orientation described earlier. The mutual orientation of the GON is that one hexon of each neighbouring GON participates in the formation of the common edge. These hexons are connected with each other as well as with two other hexons of the neighbouring GON situated near the common edge. Accordingly, three hexons of each of the two neighbouring GONs are in connection of five contact points. Two of them display "two-to-two" and three a "one-to-one" orientation (Fig. 4). One of the latter is situated in the middle of the longitudinal axis of the common edge built by two GONs, i.e. exactly on the twofold symmetry axis. From this point in two directions towards the pentons the orientation of the edge-building hexon polypeptides is a bilateral reflecting orientation, the hexons turn so-to-say toward each other in respect of the triangular profile and position of the polypeptide subunits. As each GON participates in the formation of three edges and has thus three neighbouring GONs, the above described observations apply to all the three sides of the GON. This orientation means that if the centre of a penton is taken for an imaginary axis of rotation, the orientation as one GON and that of the polypeptide subunits of hexons building the surrounding edges displays a rotation of 60° in relation to the neighbouring faces and surrounding edges. Thus in case of serial imaginary rotation with an angle of 60° not only the hexons but the polypeptide subunits of the hexons also reach an equivalent orientational position in the whole capsid, taking into consideration naturally the spatial deviations between the planes of the triangular faces (see later).

Fig. 3. Schematic drawing shows the "one-to-two" orientation of hexon polypeptides making up the group of nine hexons (GON) on one face and on the surrounding three edges of the virion, as well as the orientation within hexons and between them and the mutual orientation of the hexons within the GON. Polypeptide subunits of the hexons on the virion edges are shaded. Outlines of three polypeptide subunits around the Y-shaped slit represent the triangular profile of the hexon. The orientation corresponds to the threefold symmetry of the individual hexons and of the GON as well as to the hexagonal packing of the hexons in the virus capsid

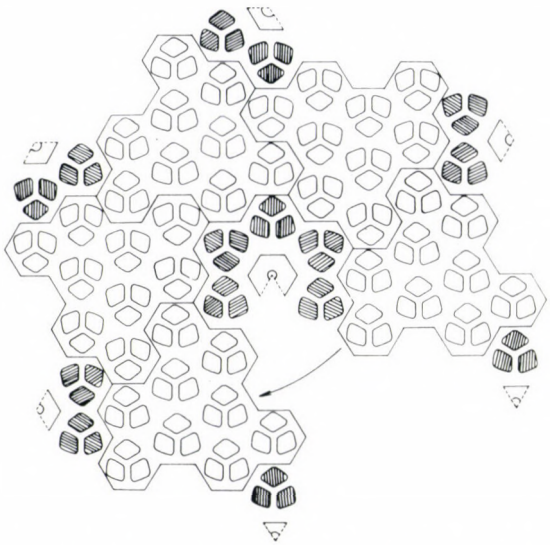

Fig. 4. Schematic drawing of the profile of hexons situated on five edges and five trianguiar faces around the vertex of the virion in flat projection. Orientation of their polypeptide subunits and mutual orientation of five GONs. The arrow points to the joining line of the "fifth" edge. Outlines of the three polypeptide subunits around the Y-shaped slits represent the triangular profile of the hexon. Peripentonal hexons are shaded. The border lines of the GONs are drawn. In the centre and on the other end of the edges proportional parts of the penton are indicated

Spatial orientation of hexons. In the course of the electron microscopic study of virions it was observed in several cases that the distances between the edges and triangular faces were wider than those among the hexons on the faces and on the edges. This phenomenon is visible on the adenovirus presented on Fig. 1/a and on the micrograph processed by rotational technique (Fig. 1/b). The distances separating the pentons and peripentonal hexons appear similarly wider. This phenomenon may be explained by the deviating spatial orientation of the hexons constituting the faces and edges as well as of the pentons on the vertices, which follows necessarily from the icosahedral structure of the capsid. The mutual spatial orientation of hexons situated on different parts of the capsid i.e. the deviation of their longitudinal axis was studied with the help of a virion model. To determine the mutual spatial orientation we set out from taking the longitudinal axes of the hexons on the triangular faces as perpendicular to the plane of the face, parallel with each other and with the threefold symmetry axis. The longitudinal axis of the hexons on the edges were considered to be perpendicular to the plane of the edges, parallel with each other and with the twofold symmetry axis. In the case of the pentons we assumed that their longitudinal axis faces in radial direction the centre of the virion and identical with the fivefold symmetry axis. The mutual spatial orientation was characterized by the angle enclosed by the

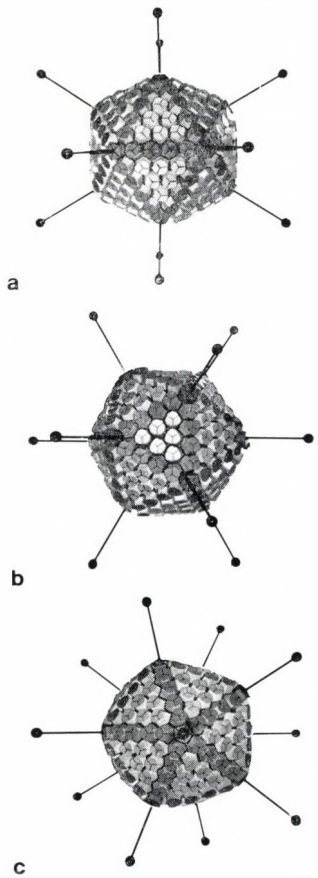

Fig. 5. Adenovirus model viewed from the twofold (a), threefold (b) and fivefold (c) symmetry axis. The model is built of hexons with hexagonal base and triangular outer-end profile. The polypeptide subunits around the Y-shaped slit and their mutual orientation are visible. The mutual spatial orientation of hexons situated on different parts of the capsid is different. The longitudinal axes perpendicular to the surface plane of the hexons on the edges and triangular faces enclose angles with each other (see text for further detail)

longitudinal axis of the hexon studied and that of the neighbouring capsomer used for comparison, in open position towards the virion surface (Fig. 5). Measurements performed on the model and the estimations made on the basis of the icosahedral structure suggested that the longitudinal axis of the penton and peripentonal hexons enclosed an angle of $\sim 32°$. The angle enclosed by the longitudinal axes of two neighbouring peripentonal hexons is $\sim 36°$. The longitudinal axes of the peripentonal hexon and of the neighbouring hexon on the edge show a parallel orientation. The longitudinal axis of the given peripentonal hexon encloses an angle of $\sim 21°$ on both sides with the longitudinal axes of the hexons on the triangular faces connected with it.

The longitudinal axes of six of the nine GON hexons constituting one triangular face of the virion run parallel and are perpendicular to the plane of the face. The other three hexons of the GON are components of the edges and their longitudinal axes are assumably perpendicular to the plane of the edges. On the basis of calculations and measurements — corresponding to the angle of deviation between the edges and faces — their longitudinal axis encloses an angle of $\sim 21°$ on both sides with that of the hexons of the same GON and with the longitudinal axis of the hexons of the GON on the neighbouring face, which are in connection with it. The direction of the longitudinal axes of the three edge-building hexons of the GON shows within the GON a deviation of $\sim 21°$, as compared to that of the six hexons building the triangular faces. The same applies to the longitudinal axes of hexons of three neighbouring GONs which build the common edges; they enclose an angle of $\sim 21°$ with the longitudinal axes of the hexons situated in the central GON. Three hexons of two neighbouring GONs each meet in five contact points of which two are on both sides of the hexon row of the edge. In the direction of their longitudinal axis there is a deviation of $\sim 21°$ on both sides of the hexons on the edge which corresponds to the deviation of $\sim 42°$ between the planes of the two triangular faces. Parallel orientation is displayed only by the longitudinal axis of the two neighbouring GON-hexons meeting on the fifth contact point in the middle of the longitudinal axis of the common edge. The concept that on rotation with an angle of 60° the polypeptide subunits of every triangular face and edge would get in identical orientational position in the space i.e. in the virion, is true only if the deviation of an angle of $\sim 42°$ between the planes of the triangular faces is also taken into consideration besides the rotation of 60°.

Discussion

The mutual rotational and spatial orientation of the polypeptide subunits of the peripentonal hexons was determined in relation to each other, to the penton and to the hexons of the neighbouring GONs in the virus capsid. Direct electron microscopic evidence was presented concerning the rotational orientation of GON-building hexons in the virus capsid which is in good accordance with the results of Nermut [2], Nermut and Perkins [7] and Burnett et al. [6] obtained with separated hexons and GONs. Determination of the spatial orientation of the GON-building hexons within the GON and that of the hexons of neighbouring GONs being in connection with each other provided means for the development of a tentative virus model which allowed to define theoretically the mutual rotational and spatial orientation of all the polypeptide subunits making up the whole capsid (Fig. 5) Our model expresses an interrelation of the threefold symmetry of the polypeptide subunits con-

stituting the complete hexon and the hexagonal packing of the hexons in the icosahedral capsid. The rotational and spatial orientation suggested on the model for the hexon polypeptide subunits corresponds in the whole capsid to the symmetry conditions determined by the two-, three- and fivefold symmetry axes characterizing the icosahedron, confirming that the suggested orientation reflects correctly the position of the hexon polypeptides in the capsid. The symmetry conditions characteristic of the icosahedron are true also in the sense of our proposal not only for the hexons but also for the hexon polypeptide subunits.

In the two-dimensional hexon crystalline array the "one-to-two" orientation appearing to be a general rule for the polypeptide subunits may be detected in the capsid uniformly only within the GON, although the spatial orientation of the edge-forming GON-hexons is necessarily different. The mutual orientation of the polypeptide subunits of the hexons of the neighbouring GONs being in connection with each other and that of the polypeptide subunits of the peripentonal hexons display besides the "one-to-two" pattern also "one-to-one" and "two-to-two" patterns with alternating regularity. The differences in the spatial orientation as well as the fact the mutual orientation of the polypeptide subunits pertains to the outer part of the hexons must, however, be taken into consideration. The hexagonal lower and middle parts of the hexons [6, 7] ensure a closed hexagonal packing for the capsomers in the inner layer of the virus capsid.

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POSSIBLE CAUSES OF THE LOSS OF SPECIFIC pBR322-Ad h 1 DNA RECOMBINANTS FOLLOWING TRANSFECTION

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Characteristics of pBR322/Ad h 1 DNA recombinants were studied which had been cloned using *Hin*dIII restriction endonuclease in a single "shot-gun" experiment. Both oxytetracycline and ampicillin resistance of the clones were found to be heterogeneous. Ad h 1 DNA fragments *Hin*dIII-A, and -C could be cloned only in combination with other fragments. Among the possible reasons of the loss of recombinants upon transfection the impairment of pBR322-specific gene functions by the inserts is discussed in addition to the increased tetracycline resistance, and the tertiary structure of recombinant DNA.

Cloned libraries of adenovirus DNA fragments have been prepared from the genomes of Ad h 2, Ad h 3, Ad h 5, Ad h 7, and Ad h 12 [1–6]. Internal *Hin*dIII-fragments of Ad h 1, and 6 DNA have been cloned recently in the pBR322–*Escherichia coli* system [7, 8]. The evaluation of "shot gun" experiments revealed that the cloning frequency of internal fragments correlated with the M_r values [7, 8]. The cloning frequency of a single fragment, *Hin*dIII-C, deviated from the approximately Gaussian distribution in cases of both Ad h 1, and 6 DNA, and both of the *Hin*dIII-C fragments have been shown to be located within the right region of sequence heterogeneity of the genomes [8–10]. In spite of the absence of sequence homology between these "type specific" restriction endonuclease fragments, both of them behave similarly in the pBR322–*E. coli* system.

In this work the successful cloning of the "type specific" *Hin*dIII-C fragment of Ad h 1 DNA is reported. The recombinant, which contains the *Hin*dIII-(C + H) insert was found to have the same regularity of tertiary structure as most of the recombinants. The tetracyclin resistance of the *Hin*dIII-(C + H) clones was found to be increased as compared to the majority of clones. No direct evidence could be obtained, however, which might explain its relatively low cloning efficiency. Indirect evidences indicate that reduced resistance to ampicillin, increased resistance to tetracycline, and possible changes in the tertiary structure of the recombinants may be involved in the phenomenon [12].

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Materials and methods

Cloning and clone selection. In all the presented experiments the same transfected *E. coli* HB101 population was used; it was obtained from a "shot-gun" experiment. The purification of DNA [7, 13–15], digestion with restriction endonucleases, [7, 13, 16, 17], ligation of pBR322 DNA with partially digested *Hin*dIII-fragments of Ad h 1 DNA, and the transfection have been performed according to standard protocols [18, 19, 20]. The transfected bacteria were regenerated at 37 °C overnight and samples were stored at −50 °C supplemented with 0.5 part glycerol.

Appropriate dilutions of the stock suspension were placed on YTB (yeast tryptone broth) plates containing 100 $\mu g/cm^3$ ampicillin. In order to reduce the passage level single colonies were picked up from this first ampicillin plate, and transferred in the form of radial lines beginning at a central hole in the agar (Fig. 1). One mg of dissolved oxytetracycline was placed into the central cavity, thus the clones could be tested for tetracycline resistance in the second passage, and the peripheral part of the linear colony was used for recombinant analysis. The distance of the colony from the central hole was used as arbitrary unit of the relative antibiotic sensitivity using mm units on a logarithmic scale. Relative sensitivity to ampicillin was measured by the same technique: in this case 10 mg of dissolved ampicillin was pipetted into the central hole.

Recombinants. Identification of the recombinants was performed by redigestion with *Hin*dIII alone, or with combinations of restriction endonucleases. In some case the presence of adenovirus specific nucleotide sequences was verified by blot-hybridization with nick-translated Ad h 1 DNA [7].

Eckhardt's technique [21] was used to screen for the superhelical structure of the recombinants directly from the colonies. In order to standardize the contact of bacteria with lysozyme and RNase, both enzymes were simultaneously mixed with the bacterial mass in Eppendorf vials. The electrophoretic mobility of the covalently closed recombinants was more reproducible if no agar cover-layer had been applied after placing the samples into the reservoirs.

Redigestion of the recombinants with restriction endonucleases was done by the modification of the procedure of Holmes and Quigley [22] using the material of colonies. The bacteria were suspended in 20 μl of 10 mM Tris-HCl pH 9.2 containing 500 $\mu g/cm^3$ lysozyme, 5 mg/cm^3 Triton X100, and 15 mM EDTA. The mixture was incubated at 100 °C for 60 s, followed by 15 min incubation at 0 °C. After a second incubation at 100 °C for 60 s in the presence of $MgCl_2$ (3 μl, 200 mM) the mixture was cooled quickly to 0 °C, and centrifuged in the Janetzki K24 centrifuge at 5000 rpm. The supernatant was suitable for digestion with restriction endonucleases after the addition of 3 μl of 350 mM Tris solution without counterions (P. Geck, unpublished results).

The restriction endonuclease fragments were separated in horizontal agarose slab gels (10 mg/cm^3; SeaKem HGTP) using the buffer system described by Helling et al. [23]. *Hin*dIII-fragments, and unit length DNA of Ad h 1 were included in all experiments as M_r standards. The electrophoretic mobility of superhelical recombinants could be standardized as equivalents of the migration distances of linear control molecules [24].

*Enzymes and biochemicals. Hin*dIII restriction endonuclease was purified according to the standard protocol obtained from the Cold Spring Harbor Laboratories [16, 17]. *Eco*RI was a kind gift from L. Jánossy. The polynucleotide ligase of T4 was obtained from C. Mulder. Lysozyme, pancreatic RNase, and most of the other chemical substances were purchased from Reanal (Budapest). HGTP and ME agarose preparations (SeaKem, Marine Colloids Inc, Rockwell, Maine) were used for preparation of the gels. Safety film DK5, 35 mm (ORWO, Wolfen, GDR) was used for the photography of fluorescent bands stained with ethidium bromide (Serva, Heidelberg, FRG).

Results

Cloning of type specific DNA fragment. In contrast to the clone selection technique [7, 18] based on tetracycline sensitivity, random selection was used as described in Materials and methods, and shown in Fig. 1. Thus the relative tetracycline sensitivity and the screening for recombinants could be done simultaneously from the same linear colonies. The inserts of recombinants obtained by conventional selection and random selection are shown in

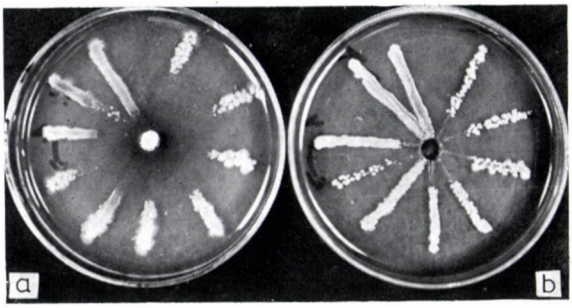

Fig. 1. Semiquantitative screening for antibiotic sensitivity of the clones. Holes were prepared in Petri dishes containing 30 cm^3 of YTB-agar medium. Hole "A" contained 100 μl oxytetracycline of 10 mg/cm^3; hole "B" contained 100 μl ampicillin of 100 mg/cm^3. Photograph 48 h after inoculation

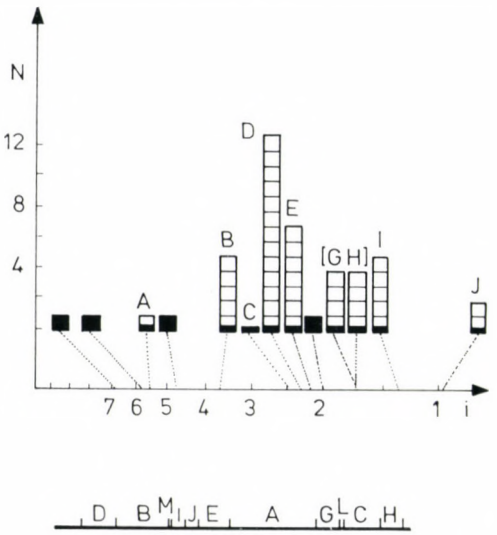

Fig. 2. Distribution of cloned Ad h 1 fragments as the function of M_r. Ordinate: number of clones (N); Abscissa: M_r of inserts (i; $\times 10^{-6}$) indicated by the dotted lines drawn to the columns. Solid squares indicate Ad h 1 inserts, which are neighbouring fragments within the physical map of the genome (insert: modified from ref. 24). TET-marker was used during selection of clones

Table I. The cloning frequency of *Hin*dIII-D was significantly higher in the case of conventional replica-plating. Nine of the 37 clones carried more than one *Hin*dIII-fragment. As shown by italics, 7 of these could not be the result of the cloning of partially digested, neighbouring DNA fragments present in the original reaction mixture used for transfection. Only 4 of the 37 clones harboured neighbouring *Hin*dIII-fragments of Ad h 1 DNA. The cloning frequency is also shown in Fig. 2. The number of clones carrying final *Hin*dIII-fragments is shown by open columns as a function of the M_r. Despite the

high number of *Hind*III-B, *Hind*III-D, and *Hind*III-E recombinants, no *Hind*III-C recombinant was identified. The physical map [24] included into Fig. 2 shows that the position of *Hind*III-B was identical with that of *Hind*III-C. Both the neighbouring fragments *Hind*III-H and -G occurred in recombinants. Nevertheless, only two of the potential *Hind*III-H framgents were identified exactly by double digestion with *Eco*RI, and *Hind*III. Since fragments of identical location within the physical map seemed to occur in identical number in the initial ligation-mixture, the absence of *Hind*III-C recombinants was not the result of the absence of *Hind*III-C fragment in the original DNA digest.

If the sensitivity to tetracycline had not been used as selective marker, the *Hind*III-C fragment was already recognized in one of the 8 isolated

Table I

Distribution of Ad h 1 DNA fragments cloned into the HindIII-site of pBR322

$M_r \times 10^{-6}$ of Ad h 1 inserts	Mode of selection			
	Without TET marker	No. of clones	Using TET marker	No. of clones
11.06			*A* + B + D	1
6.81	A + G	1	A + G	1
5.61			*B* + *E*	1
4.73			B + I	1
4.29	*D* + *E*	1	*D* + *E*	1
4.22			*B* + *J*	1
4.13			*D* + I + J	1
3.73	C + H	1		
3.50	B	1	B	3
3.41			*D* + *I*	1
3.34			*E* + *I*	1
2.18	D	3	D	10
2.11			E	4
			G	1
1.43*			H	2
	G or H	1	G or H	5
1.23			I	2
0.72			J	1
Sum of clones		8		37

* *Hind*III-G, and -H fragments of identical M_r have not been differentiated in 6 cases
Italics: fragments which have recombined during ligation

clones. All internal DNA fragments occurred in the 8 recombinants identified except *Hin*dIII-I, and -J (Table I). Three of the 8 recombinants were composed of more than one DNA fragment of Ad h 1. Results of the above experiments refer to some kind of correlation between tetracycline resistance and the cloning frequency of adenovirus fragments.

Tetracycline and ampicillin sensitivity of the clones. Stüber and Bujard [25] have shown that specific inserts might influence the expression of the tetracycline gene. Therefore, independently of the mode of selection, the second passages of the above characterized series of clones were inoculated onto YTB plates supplemented with 5, or 10 $\mu g/cm^3$ of tetracycline. All the clones were able to form colonies within 24 h. Five of the 8 clones which had been selected without tetracycline were able to grow even in the presence of 20 $\mu g/cm^3$ oxytetracycline. Nevertheless, 10 of the 36 clones which had been selected using the tetracycline marker were also able to grow in the presence of 20 $\mu g/cm^3$ oxytetracycline. These results showed that random selection had influenced only slightly the number of clones of elevated tetracycline gene expression.

The semiquantitative method described in Materials and methods was used to compare both the ampicillin and oxytetracycline sensitivity of the individual clones. The results are summarized in Fig. 3. The distance of the end points of linear colonies from the central antibiotic hole is given in log mm units. Filled circles indicate the degree of oxytetracycline sensitivity. The results are presented as the function of the increasing M_r values of the recombinants (abscissa). Colonies carrying religated and transfected pBR322 plasmids were included into each experiments as control. The upper margin of the parallelogram shows that all control colonies grew to 7 to 8 mm as measured from the central wells. All colonies except three with recombinants *Hin*dIII-(C + + H), -(G + H), and *Hin*dIII-(D + E) grew to 15 to 30 mm. Since the concentration of inoculated bacterial suspensions was different and the copy-number of the recombinants has not been determined, it may be concluded that the expression of tetracycline gene was reduced in most of the recombinants, but the differences observed in the case of individual clones are not conclusive.

In spite of the very high ampicillin concentration used in the tests (see Materials and methods) all control colonies grew to the central hole, thus the points were within the base line of the parallelogram. Comparable resistance to ampicillin was observed only in the case of 11 clones carrying recombinants. These are shown by vertical lines drawn to the abscissa in Fig. 3. Values indicating increased ampicillin sensitivity are shown by open circles connected with the corresponding points of tetracycline sensitivity. The reduction of the expression of beta-lactamase gene in the recombinants was surprising. The reduction seemed to be rather uniform, since in the majority of cases the

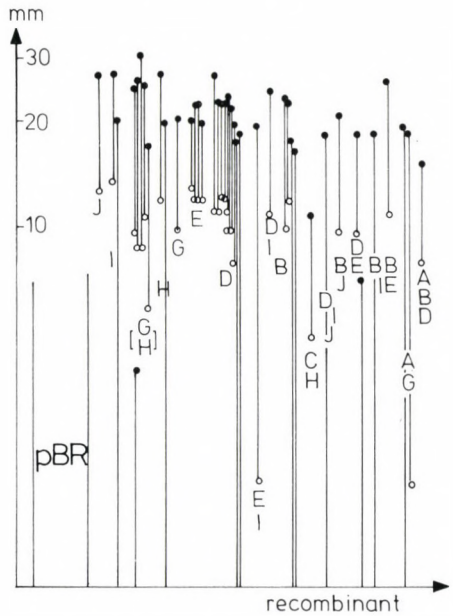

Fig. 3. Relative ampicillin (open circles), and tetracycline (solid circles) sensitivity of clones. Ordinate: Distance in mm of the end point of colonies from the central antibiotic-hole. Abscissa: Groups of clones as the function of increasing M_r of the recombinants. Capi al letters: Ad h 1 inserts. Upper and lower sides of paralellogram (pBR) show the values obtained with 16 control clones carrying recircularized and transfected pBR322 plasmid

degree of tetracycline and ampicillin resistance changed together (lengths of vertical lines between open and filled circles in Fig. 3). This may reflect differences in the copy-number of recombinants. As far as the recombinant *Hin*dIII-(C + H) is concerned, the relative resistance to tetracycline of this clone coincided with an increased resistance to ampicillin, which may be explained by the increased copy-number of this plasmid. The two other clones of high tetracycline resistance could not be examined in this way, since their resistance to ampicillin was found to be unchanged. Nevertheless, all the three clones of increased tetracycline resistance have been isolated without the application of oxytetracycline for selection.

Superhelical structure of the recombinants. According to previous data, gene expression may be influenced by the superhelical structure of DNA, and by the polarity of the inserts [12, 26]. The electrophoretic mobility of the superhelical DNA was examined as the function of the M_r of the recombinants. The results are plotted in Fig. 4, where continuous curves represent the results obtained in separate experiments. The distance of migration (d) is shown on the abscissa, and M_r values have been plotted according to a logarithmic scale on the ordinate. Absolute M_r values only may be read from the lowest continuous curve of superhelical recombinants, and from the dotted curve of

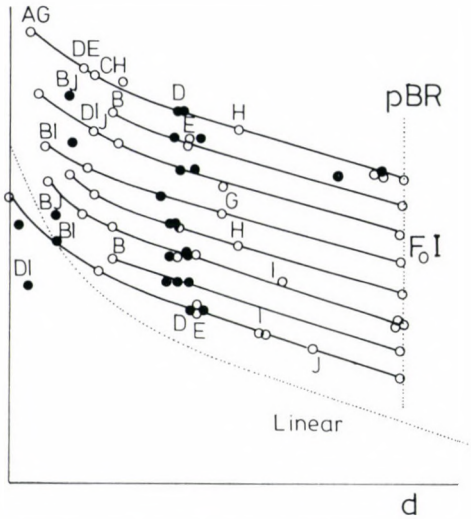

Fig. 4. Superhelical structure of recombinants. Ordinate: M_r of recombinants (continuous lines), and linear control fragments (dotted line). Abscissa: Migration distances standardized according to the linear controls, and superhelical pBR322 (vertical dotted line). Capital letters: Ad h 1 inserts of recombinants. Curves of superhelical DNA (FoI) shifted upward from experiment to experiment, therefore M_r values may be compared with those of linear controls when projected to the lowest curve. Open circles: recombinants following regularities of the curves. Solid circles: recombinants deviating from the curves

linear control DNA, since the other curves have shifted upwards. Migration distances measured in different experiments were standardized according to the pBR322 controls (vertical dotted line) and the M_r values of control *Hin*dIII fragments of Ad h 1 DNA. The uniformity of the curves showed that the electrophoretic mobility of recombinants followed the expected regularity. Some of the recombinants were deviating from this regularity (shown by filled circles in Fig. 4). The irregularities of *Hin*dIII-D recombinants (filled circles in the central region of the figure) could be verified experimentally by comparative experiments, but these were not followed further experimentally. Irregularities at the left termini of the curves ((B + I), (B + J), and (D + I) recombinants) were shown to have structural basis in all cases but one, (i.e. tandem recombination of complete pBR322 sequences with Ad h 1 DNA fragments). Ad h 1 *Hin*dIII-L, and -M fragments of 0.1, and 0.05×10^6 M_r could not be detected by the procedures used for plasmid analysis and it is therefore assumed that the four plasmids (unlabelled at the right upper part of Fig. 4) contain such minor inserts. The main result of these series of experiments is that *Hin*dIII-(C + H) recombinant follows the common regularity of the tertiary structure of superhelical molecules.

Discussion

*Hin*dIII-C (*Hin*dIII-C' in the case of Ad h 6) is the type specific DNA fragment of the genomes of both Ad h 1, and 6 which shows the most expressed sequence heterogeneity, and it is the most AT-rich region of both molecules [7–10]. The fact that these unique DNA fragments displayed identical behaviour if cloned into the pBR322-*E. coli* system seemed to be of interest for further studies.

Stüber and Bujard [25] observed that the expression of the tetracycline gene was only partially damaged by certain inserts at the *Hin*dIII site of pBR322. In other cases colinear integration of the inserts was shown to allow the appearence of tetracycline resistant phenotypes [26]. In order to prevent the loss of clones of unimpaired tetracycline resistance, random clone selection has been performed. As predicted by previous experiments [8] and personal communication of Garaev et al. *Hin*dIII-C recombinant has been isolated in case of both Ad h 1, and 6. The successful isolation of *Hin*dIII-C carrier clones would suggest that unimpaired tet-gene function had been the actual cause of the low cloning efficiency of this fragment. Comparative experiments, however, cast doubt on this assumption.

The first problem originates from the fact that true *Hin*dIII-C fragment as single insert has not been isolated. Both *Hin*dIII-A, and -C occur only in recombinants together with neighbouring or other Ad h 1 fragments. This may indicate that the viral sequences themselves do not interfere with the cloning, or the clone carrying *Hin*dIII-(C + H) is genetically altered. Genetic defects in *Hin*dIII-A which have been identified in 3 different clones in combination with other fragments seems improbable. If so, one of the junction points between pBR322 and the fragments *Hin*dIII-A or -C might be responsible for the absence of single-insert recombinants. Such a phenomenon might occur if a palindromic sequence is formed [27] or if the specific site of topoisomerase-like activity had been produced at the junction point, which would result in the formation of aberrant tertiary structure of the recombinants upon transfection. The possible role of topoisomerases in the reduction of cloning frequency is supported by the results indicating that all recombinants but one possess a tertiary structure following the same regularity. Minor deviations from this regularity, as observed in the case of *Hin*dIII-D recombinants, are currently examined.

The possibilities discussed above i.e. active promoters within the insert [25], polarity of the insert [26], and formation of palindromic structure [27] or topoisomerase site at one of the junction sites would only influence the cloning frequency or the expression of the tet-gene if the recombination had occurred in one specific direction. Two adenovirus promoters of opposite polarity could enhance tet-gene activity independently of the polarity of

integration, but only one promoter has been identified within the corresponding region of Ad h 2 [28]. One could argue that Ad h 1 and 6 genomes carry two promoters within the type specific DNA fragments. Nevertheless, the semiquantitative tests for the comparison of ampicillin and tetracycline resistance failed to prove unequivocally that *Hin*dIII-(C + H) clone was expressing tet-gene at a higher level than other clones. Both tetracycline and ampicillin resistance were found to be higher than those of the comparable clones of reduced ampicillin resistance, which might be the consequence of the higher copy-number of the recombinant.

It has been shown in this paper that the level of ampicillin resistance is impaired also of those clones, which harbour inserts at the *Hin*dIII-site of pBR322. Only 12 of the 42 clones tested retained the level of ampicillin resistance comparable to that of religated control pBR322 clones. We believe that this may be understood on the basis of the results of Stüber and Bujard [25] (Fig. 5).

On their modified drawing (Fig. 5) it is shown that the *Hin*dIII-insert interrupts the P2 promoter of the tet-gene, but the P1 promoter itself remains intact. Thus, initiation of transcription will occur independently of the presence or absence of an insert, and will produce mRNA for beta-lactamase together with the P3 promoter, located downstream from P1. Under natural conditions both mRNA populations may produce functional beta-lactamase enzymes. The three arrows represent transcripts due to the readthrough from the P1 promoter. Transcription will be interrupted by the insert if a termination signal is carried by it. In this case the downstream P3 promoter (not shown in Fig. 5) may increase the level of activity and produce sufficient enzyme resulting in a resistance to ampicillin comparable to that of the pBR322 without inserts.

When no termination signal is introduced by the insert, the transcription initiated at P1 will proceed to the structural gene of beta-lactamase, but no functional enzyme will be produced since stop codons within the insert, or

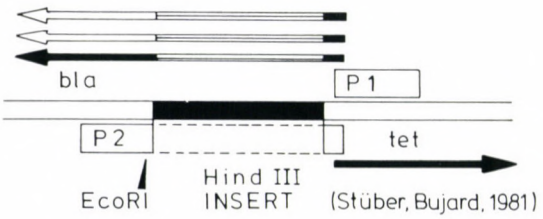

Fig. 5. Expression of P1 and P2 promoters of pBR322 modified from reference 25. Central lines with filled insert: DNA of pBR322. Arrow head: *Eco*RI restriction endonuclease site. Solid arrows: mRNA of beta-lactamase gene (bla), and tet-gene (tet) when the reading frame is unimpaired by the insert. Open horizontal arrows: mRNA produced from P1 promoter when the inserts are of 3n + 1, or 3n + 2 base pairs

the randomization of the reading frame by insert of 3n + 1 or 3n + 2 base pairs, will prevent correct translation. Thus, the level of ampicillin resistance may be impaired also at the level of translation.

In the experimental system used in this work ampicillin has been present during the regeneration of transfected bacteria. Therefore, only recombinants could be isolated where P3 promoter is sufficiently more active than P1. In this experimental system the influence of inserts at the *Hind* III-site of pBR322 to the rate of beta-lactamase initiation could not be examined for the same reason. In spite of this the main conclusion is that specific recombinants may be lost due to unimpaired tetracycline resistance and impaired resistance to ampicillin.

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POLYPEPTIDES AND IMMUNOREACTIVITY OF EMPTY ADENOVIRUS TYPE 1 PARTICLES

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Two distinct CsCl-stable empty particle populations were characterized in human adenovirus type 1 infected cells. Different sensitivity to proteases and polypeptide composition indicate that empty capsids *A* and *B* are degradation products of two different assembly intermediates. Both empty particle populations react with antihexon antibodies similarly to complete virions. Thus the arrangement of exposed type-specific epitopes is suggested to be unchanged during virus assembly. Experimental evidence is presented that both antifibre and antihexon antibodies trigger structural changes of complete virions, which are initiating the specific loss of core protein V from antibody-aggregated virions.

Conventional purification of adenovirus particles using CsCl buoyant density centrifugation regularly results in the separation of particle population(s) which are of 1.3 g/cm^3 density, do not contain nucleic acid and appear as disrupted core-less particles under the electron microscope [1–9]. More recent experiments have shown that cross-linking reagents may prevent the release of core structures from these particles and part of them remains floating in 1.315 g/cm^3 CsCl solution [9–11]. This observation is compatible with previous results on a precursor product relationship of some core-less and light particles and mature virions as measured by sedimentation through sucrose or Ficoll gradients [2, 6, 9, 11]. Only one band of empty particles has been observed in most experiments of Ishibashi and Maizel [4] and D'Halluin et al. [11]. In connection with our large scale purification of adenovirus type 1 (Ad h 1) particles, two distinct populations of empty particles have regularly been observed in CsCl gradients [12–14]. Examination of these two particle populations revealed that they have a characteristic polypeptide composition. The presence of unique scaffolding proteins allowed their distinction. One of them has been shown to be sensitive to proteases, which usually do not affect structural integrity of complete and "young" virus particles. In spite of biochemical differences, both of them could be agglutinated only with antisera

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directed against type specific hexon, thus their surface seems to be similar to that of complete virions [10]. It was an accidental finding of these experiments that the agglutination of complete virions with specific antisera directed to type-specific hexon epitopes may trigger the specific loss of the major core protein (V).

Materials and methods

Virus. Prototype strain of Ad h 1 was produced in suspension cultures of HEp-2 cells as described earlier [12–14]. Infected cells were collected by low-speed centrifugation and disrupted by one cycle of freezing and thawing after the addition of 5 mg/cm^3 Triton X100 and 0.4 M NaCl final concentrations [15]. The cell debris was removed by centrifugation at 5000 *g* at +4 °C for 20 min. Discontinuous CsCl gradients [3, 12] were run at 25 000 rpm at +4 °C in the 3 × 23 cm^3 rotor of the MSE Superspeed 50 type ultracentrifuge for 6 to 8 h, in order to separate particles of different buoyant density. Fractions collected through the bottom of the tubes were recentrifuged without dilution of the samples in discontinuous CsCl gradients. The samples were used as an additional layer at the corresponding density region of the gradient, thus quick pH and osmotic changes have been avoided. In some cases sucrose density centrifugation preceded further purification in CsCl equilibrium gradients [16].

SDS–polyacrylamide-gel electrophoresis. Samples were treated with 20 to 30 mg/cm^3 SDS (Reanal, Hungary) and 0.05 2-mercaptoethanol (Reanal, Hungary) at 70 °C for 15 min followed by immersion into a boiling water bath for 2 min. The mixtures were completed with 0.1 part glycerol containing 1 mg/cm^3 bromophenolblue before heating. Vertical slab gels at 40:1 acrylamide–bisacrylamide ratio of 0.1 to 0.15 concentration were used as separating gels [17]. The acrylamide component of the stacking gel has been omitted and the buffer system described by Laemmli [17] was used with 20 mg/cm^3 agarose (SeaKem HGTP, Marine Colloids Inc. Rockland, Maine, USA) in order to facilitate the migration of large polypeptides. Gels were run at 1 V/cm for 1 to 2 h at room temperature, then overnight at 4 V/cm. The presence of salt (1 M NaCl or 2 M CsCl) in the samples did not affect the quality of the electrophoretic patterns above 20 °C temperature. Gels were stained and photographed according to standard procedures [17].

Protease digestion and aggregation with antibodies. It was an essential point in the reported experiments that particles were not exposed to drastic osmotic changes and pH alterations. Therefore gradient fractions were either diluted stepwise to 1M concentration of CsCl or dialysed in 1M NaCl. Both manipulations were done using at least 20 mM Tris–HCl, pH 7.5. The system applied prevented aspecific aggregation of particles [18], and reduced protease activities possibly present as contaminants in the antisera and virus preparations.

Crystalline trypsin (Difco Laboratories. Detroit, Michigan, USA), chymotrypsin, papain and pepsin (Serva, Heidelberg, GFR) 0.1 mg/cm^3 were used at +4 °C for 10 to 16 h in the above buffer systems.

Reaction mixtures were stirred continuously with teflon coated magnetic stirrers. Complete and both populations of empty particles were simultaneously present in all reaction mixtures prepared with proteases. The samples were layered on the top of discontinuous CsCl gradients, and centrifuged to equilibrium in order to screen for resistant particle populations.

Antisera were prepared in rabbits against purified Ad h 1 hexon and fibre preparations as described earlier [19]. One of the antihexon sera (aH_3) and the antifibre serum (aF) were used in the form of purified IgG preparations obtained by DEAE Sephadex chromatography. Group specific antigens were tested with sheep serum directed against purified Ad *bos* 2 virions and rabbit IgG to simian adenovirus (SA7).

Undiluted serum samples were layered onto the top of virion particle suspensions in glass tubes 3 mm in diameter, in vertical position at room temperature. Following 1 to 3 h incubation large aggregates appeared and the 1M salt solutions allowed their spontaneous sedimentation to the bottom of the tube. In order to separate the aggregates the tubes were centrifuged at 1500 *g* at +4 °C for 5 min. The supernatant was carefully removed and the aggregates were directly processed to SDS–acrylamide gel electrophoresis or centrifuged to equilibrium in discontinuous CsCl gradients.

Results

Properties of empty particles. The densities of the two empty particle populations of Ad h 1 were found to be 1.302 to 1.305 g/cm^3 for particles *A* and 1.295 g/cm^3 for the upper *B* population in CsCl. Treatment with Na-desoxycholate and Triton X100 did not modify the densities nor their appearance in the electron microscope. When the extracts of the infected cells were centrifuged first in low salt sucrose gradient, only *A* particles could be recovered from the samples by recentrifugation in CsCl.

To find the reason of the phenomenon, model experiments were performed with different proteases. Results are shown in Fig. 1. The central tubes (C) show the distribution of prepurified *A* and *B* particles incubated at +4 °C for 16 h in the absence of enzymes. A faint band of complete virions (V) was released during incubation and recentrifugation. The material fractionated in the neighbouring tubes was stirred under identical conditions in the presence of papain (PA), pepsin (PE), chymotrypsin (CH) and trypsin (T). The protease treatment affected only the higher *(B)* population of particles, since this upper band disappeared upon chymotrypsin, trypsin and papain treatment. The unchanged band of *B* particles treated with pepsin did not indicate specific resistance against this protease since under the conditions applied the enzyme is completely inactive at pH 7.5. It shows that the protein itself does not influence the distribution of particles. Horizontal lines drawn to the control gradients indicate that complete virions and *A* particles remained

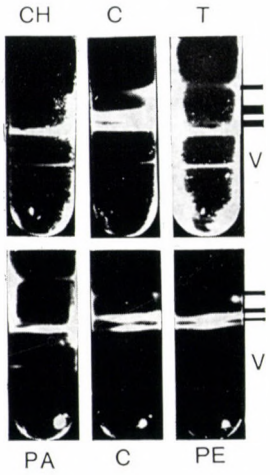

Fig. 1. Protease sensitivity of empty particles *B* of Ad h 1. Empty particles were purified by one cycle of CsCl centrifugation and 6 identical samples were stirred at +4 °C for 16 h after the addition of chymotrypsin, (CH), trypsin (T), papain (PA) and pepsin (PE) 100 μg/cm^3 each, or without enzymes, as controls (C). The samples were immediately placed onto preformed CsCl gradients and centrifuged at 26 000 rpm until equilibrium (8 h)

unchanged. Considerable density differences may be seen in the case of highest band of soluble components, nevertheless it has not been possible to determine the polypeptide composition of the materials concerned.

Polypeptide composition of empty particles. Empty particle populations *A* and *B* were purified by two cycles of equilibrium density centrifugation in CsCl. Unlabelled material was tested in order to detect the polypeptides present in large quantities within the particles. Characteristic differences as shown in Fig. 2, have repeatedly been observed. The insert in Fig. 2 was taken from an SDS–acrylamide slab gel stained with Coomassie brilliant blue. Different quantities of antibody-aggregated virus particles (lanes "v") were included as molecular weight control. The relationship of M_r values and migration distances are drawn below the control samples. Between the bands of penton (P) and fibre (F) the heavy chain of IgG molecules is visible (unlabelled bands in Fig. 2). Both empty particles proved to be devoid of core proteins (V and VII). The bands of hexon (H), penton (P) and IIIa, just above the dotted line indicating the position of fibre (F), were found to be of identical electrophoretic mobility in both particles *A* and *B*. Three unique bands are major structural components of the *A* particles. The apparent M_r values of the bands between structural components V and VI were estimated at 42×10^3, 34×10^3 and 27×10^3, respectively. Structural virion component VI and the band of M_r 34×10^3 were found to be absent from *B* particles.

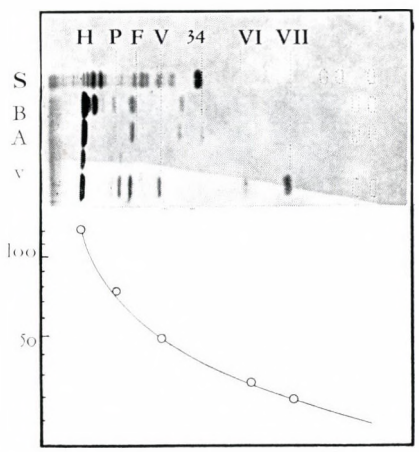

Fig. 2. SDS–polyacrylamide gel electrophoresis of polypeptides of salt-stable Ad h 1 particles. Particles purified by two cycles of equilibrium centrifugation were examined in 120 mg/cm^3 SDS–polyacrylamide slab gel as described in the text. Structural proteins of complete virions (V) were used as M_r controls. Hexon (H), penton (P), fibre (F), polypeptides V, VI, VI and VII are indicated accordingly by vertical dotted lines. Minor proteins visualized only in overexposed photographs are shown as dotted spots. The curve in the lower part of the Figure (ordinate: $M_r \times 10^{-3}$; abscissa: migration distance) was used to estimate M_r values of polypeptides of empty virion populations *A* and *B* and that resolved from the band of soluble proteins (S), which was taken from the bands of lowest buoyant density, shown in Fig. 1, tubes C

The apparent M_r value of the major unique band was also somewhat different (41×10^3) in comparison to that of *A* particles.

A completely new band of M_r 10^5 appeared in the lane of *B* particles between hexon and penton. In order to exlude the role of possible cross-contamination with neighbouring CsCl fractions, the material from the upper band (unlabelled in Fig. 1) was also included into the experiment as sample S. There are indications that very small amounts from the major bands of sample S contained the sample of *B* particles. Nevertheless, the appearance of the polypeptide of M_r 10^5 cannot be the result of cross-contamination, since its amount in sample S seems to be identical with the material of M_r 90×10^3, which is hardly visible in *B* particles. The M_r values of the polypeptides are summarized in Table I.

Table I

Polypeptides of complete and empty Ad h 1 particles

Designation or M_r value/10^3 of polypeptide	Particle		
	complete	empty *A*	empty *B*
hexon	+	+	+
100	—	—	+
penton	+	+	+
IIIa	+	+	+
fibre	+	+	+
V (core)	+	—	—
42	—	+	—
41	—	—	+
34	—	+	—
pVI	—	+	+
VI	+	+	—
VII (core)	+	—	—
VIII—XI*	+	+	+

* The amount of these materials was insufficient to detect and identify individual polypeptides. Two bands could be visualized in overexposed photographs (see dotted marking in Fig. 2) which seem to be identical in the case of all three particle populations

Immunoreactivity of empty particles. The new technique of immuno-aggregation of virus particles described in Materials and methods allowed the comparison of epitopes exposed at the external surface of virus particles. It has been shown that type-specific antigens are neutralized only at the surface of complete virions [20]. All three particle populations were examined with type-specific and heterospecific adenovirus antisera. Antifibre and antihexon sera directed to structural components of Ad h 1 were able to agglutinate all the

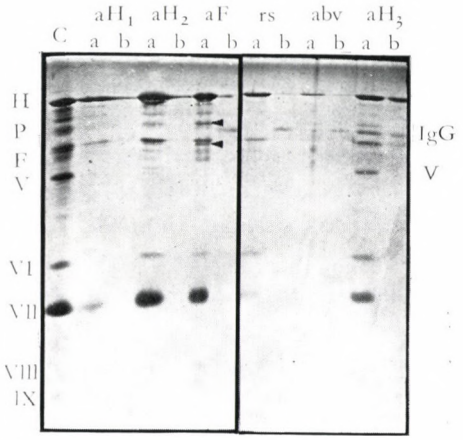

Fig. 3. SDS–polyacrylamide gel electrophoresis of the proteins from antibody-aggregated complete (a) and empty (b) particles. Virions were treated with hexon-specific antisera (aH_{1-2}), antihexon IgG (aH_3), antifibre serum (aF) and pooled normal sera (rs) of rabbit origin. Sheep antiserum to Ad bos 2 (abv) and untreated Ad h 1 virions (C) were included for control purposes. Visible bands formed by the heavy chain of IgG (IgG) and expressed bands of fibre and IIIa are indicated by arrowheads. Other details see in the legend to Fig. 2

three purified particle populations. The density of antibody-aggregated material was not modified to a measurable degree (experiments not shown). Figure 3 shows the SDS–acrylamide gel electrophoresis patterns of the polypeptides in the aggregated material. Polypeptides of untreated complete virions are shown in the control (C) lane. Lanes "a" show polypeptides of antibody-aggregated complete virions, lanes "b" that of the mixture of aggregated *A* and *B* empty particles. Pooled sera of slaughtery rabbits (rs) and antiserum against Ad bos 2 complete virions were included as controls. It can be seen in the lanes of abv-treated samples that some aggregation had occurred (hexon bands are visible) in contrast to the results of other authors [18]. Nevertheless, in lane "b" prepared with Ad bos 2 antiserum the band of IgG is more expressed than that of the hexon, indicating an extremely low degree of aggregation. In the material treated with pooled sera of healthy rabbits the presence of low levels of antibodies directed to rabbit adenovirus(es) [21] cannot be excluded. The visible band of IgG in lane "b" seems to be similar to that of the samples precipitated with Ad bos 2 antiserum.

The specificity of aggregation is demonstrated by sharp bands of penton and fibre (arrowheads in Fig. 3), which are only visualized following treatment with antifibre serum (aF). The comparison of the polypeptide patterns obtained with control sera and antihexon sera (aH_{1-2}) shows that specific aggregation occurred. The amount of aggregated empty particles allows only the visualization of hexon bands (b). These are more expressed than those in the control lanes (abv and rs). Hexon, IIIa, VI and VII and even two faint bands in the

region of minor polypeptides VIII and IX are clearly visible in the lanes of complete virions (a).

There is a significant difference between the polypeptide composition of virions aggregated with sera aH_1 and aH_2 and that of virions aggregated with aH_3. Core protein V is visible only in the latter. Results of CsCl centrifugation and those of control experiments (not shown) revealed that the absence of polypeptide V was not due to the different antibody titres of antihexon sera but to the time elapsed between antibody treatment and electrophoresis. When the aggregates had been boiled with SDS-2-mercaptoethanol on the day of centrifugation, polypeptide V was always present. The samples aH_3 have been prepared in this way. Samples aH_1 and aH_2 were stored at $+4$ °C in CsCl for 12 days. This incubation resulted in a specific loss of core polypeptide V even in the presence of CsCl, whereas core polypeptide VII was not affected.

Discussion

Two distinct populations of empty capsids have been characterized in CsCl equilibrium gradients used for the fraction of Ad h 1 particles. These empty capsids are artifacts, as shown by experiments with cross-linking chemicals [9–11], and assembly intermediates have been suggested to be the source of them [1, 6, 9–11]. One population of empty capsids of lower buoyant density were found to be sensitive to protease digestion, and these results seem to provide a possible explanation of their absence in virion preparations extracted under conventional conditions. Nevertheless the higher resistance to CsCl of particle *B* in the case of Ad h 1 cannot be excluded.

The polypeptide composition of the two empty particle populations seems to be specific. Previous papers have reported the presence of scaffolding proteins of cellular origin, precursors of structural proteins in both empty capsids and assembly intermediates [1–4, 6–9, 11, 22, 23]. Examination of unlabelled *A* and *B* particles revealed that both contain precursor of polypeptide VI (pVI) as shown in Table I and Fig. 2. The polypeptides of M_r 32–33 $\times 10^3$ and 40 $\times 10^3$ characterized by the above authors were estimated at M_r 34 $\times 10^3$ and 42 $\times 10^3$, respectively, and have been detected only in *A* particles. We could not decide whether the polypeptide of M_r 34 $\times 10^3$ was identical with pVIII or with the 33K protein mapped recently into the *Eco*RI-F fragment of the Ad h 2 genome [24]. In the latter case the 33K protein would be a structural protein of immature virions.

No mature polypeptide VI was found in the *B* particles; this would suggest that *B* particles had come from immature virions at an earlier step of virus assembly than *A* particles. Nevertheless the band indicating the presence of pVIII protein was completely absent. It is therefore suggested that pVIII is not a salt-stable component of empty particles. In contrast, the polypeptides

of M_r 42 $\times 10^3$ are stable but unequal components. Comparative experiments showed that every *A* an *B* particle preparation carried a component corresponding to "40K" protein, but of slightly different M_r value. Such minor differences of apparent M_r values have been described in connection with the phosphorylation and glycosylation of proteins [25, 26].

The 33K protein was also found to be phosphorylated [25]. The experiment shown in Fig. 2 and comparative studies indicated that the band corresponding to this protein among the soluble components (S) had a slightly higher apparent M_r than that of *A* particles. Based on the results of Axelrod [25] it is concluded that the soluble 33K protein is released from the particles due to structural changes upon phosphorylation. A direct experimental analysis is in progress.

A component of M_r 10^5 occurs in *B* particles. Previous data already suggested that a 100K protein was of essential significance in adenovirus assembly [6, 16, 27–30]. Earlier it had been specified as a component of immature virions [6, 16] but recently, by the use of monoclonal antibodies their function has been limited to the formation of native hexons within the cytoplasm [29, 30]. The contradiction might be resolved by supposing that 100K protein or its ternary complexes remain attached to the native hexons and transported into the nuclei, but structural alterations prevent their immunological detection by monoclonal antibodies directed against monomeric 100K.

We have no experimental evidence to prove that the protein of M_r 10^5 in *B* particles was identical with the 100K protein. One has to take into consideration that the band corresponds to the hexon precursor described by Boulanger et al. [31] which migrated slower in acrylamide gel than did the mature hexon, but the apparent M_r proved to be smaller in SDS–polyacrylamide gels.

In spite of the structural differences, virions and empty capsids reacted similarly with type-specific antihexon sera. No specific immunoaggregation was obtained with heterospecific antibodies. This indicates that hexons of every particle possess only type-specific epitopes at the external surface. The immunoaggregation technique does not allow a quantitative evaluation, since the possible highest concentration of antigen had to be used in order to obtain sufficient immunoaggregates for biochemical analysis.

Some of the immunoaggregates have been further purified by CsCl equilibrium centrifugation. Complete virions may be stored indefinitely at +4 °C in CsCl solution without any modification of the capsid proteins. When antibody-aggregates of complete virions (Fig. 3) had been stored for more than two days in CsCl at +4 °C, specific release of core protein V has been observed. This indicates that in addition to structural changes triggered by antibodies [32] neutralization with fibre- or hexon-specific antisera is associated with antibody triggered impairment of the capsid.

According to current knowledge concerning the adenovirus core [33–35] polypeptide V is first complexed with the genome during virus assembly. Polypeptides IVa_2, V, VI, VII, pVII and 40K have a DNA-binding property [36]. The presented results suggest that within the complete virion or during their disruption by antibodies, first the binding of polypeptide V is affected. The observed low rate of protein V disappearance raises the question whether adenovirus associated protease [37] or other mechanisms were also involved in the process.

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VIROLOGICAL STUDIES OF MALIGNANT TUMOURS OF THE UROGENITAL SYSTEM

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Tumours of the urogenital system were investigated to clarify an eventual virus–tumour connection. From 105 tumours virus antigens were found in 57 cases by immunofluorescent method, mainly with serum raised against the oncogenic type 12 adenovirus. Virus antigens were also present in peripheral lymphocytes of some patients. Occasionally electron microscopy revealed adeno-, herpes and C-type virus particles. In patient sera, antiviral antibodies were shown often and in high titre especially against adenovirus type 12 early non-virion antigens. Herpesvirus antigens were found in 6 cases among 40 prostate hypertrophy control patients, and in 2 among 15 non-tumorous specimens.

Several types of virus antigen or virus particles have been found in tumours of the urogenital system [1–13], but such data concerning adenoviruses are lacking. Earlier it was shown that women without clinical symptoms may carry latent adenovirus antigens in their genital organs. The frequency of virus antigens was higher in patients with carcinoma of the cervix [14]. As more types of human adenovirus proved to be oncogenic in experimental conditions, the question arose whether adenovirus antigens or particles were present in tumours of the urogenital system or antiviral antibodies in sera of patients. The same materials were studied for herpes simplex virus and antibodies.

Materials and methods

Specimens removed at operation or autosy of 160 persons were studied; 105 patients had renal carcinoma, malignant tumour of bladder, prostatic carcinoma, malignant disorders of testis and penis, as well as papilloma of the bladder. Specimens from 40 prostatic hypertrophy and 15 non-tumourous urogenital patients served as controls. All materials were prepared immediately after surgery or autopsy.

Virus antigens. Cytological smears were prepared on slides from the cells scraped off the fresh surface of specimens for immunofluorescent investigation. Smears were dried at room temperature and fixed with acetone, treated with immune sera raised in rabbits against adeno and herpes simplex viruses and conjugated with fluorescein-isothiocyanate or rhodamine.

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To search for virus antigens peripheral lymphocytes of some patients were washed three times in physiological salt solution and treated as mentioned above. Immunofluorescence was estimated at 600 × magnification under a Zeiss × Fluoval microscope [15].

Virus particles. Certain specimen cells were fixed in glutaraldehyde, subsequently in osmium tetroxide for electron microscopic examination. After dehydration in acetone the cells were embedded in Epon-812, ultrathin sections were cut and assayed by JEM 100 B electron microscope.

Antibodies. To demonstrate antiviral antibodies, all the sera were studied with complement-fixation test using Takátsy's microtitrator. In the tests adenovirus types 1 and 12 structure antigens, as well as their early non-virion antigens and herpes simplex virus served as antigens. The method of antigen preparation has been described earlier [16].

Results

Specific fluorescence indicated in the cells of 57 from 105 tumour patients the presence of virus antigens. They were herpesvirus antigen in 7 cases, type 1 adenovirus antigen (of latent character) in 15 cases, and type 12 adenovirus antigen (oncogenic type) in tumour cells of 35 patients. Division of tumours and occurrence of virus antigens are detailed in Table I.

Table I

Distribution of different tumours and virus antigens

Tumours		Virus antigens		
		Herpes-virus	Adenoviruses 12	1
Bladder tumour N = 41		3	13	3
Kidney carcinoma N = 23		—	6	6
Tumour of prostate N = 8		2	3	—
Tumour of testis N = 15		—	10	4
Tumour of penis N = 4		—	—	—
Bladder papilloma N = 14		2	3	2
Total	105	7	35	15
			57	

It seemed remarkable that the tumour cells of 4 patients contained both latent and oncogenic adenovirus antigens; in the cells of another tumour, adenovirus type 12 and herpes simplex virus antigens were present.

By the immunofluorescent technique in the peripheral lymphocytes of 14 cases from among 35 tumorous patients were different virus antigens detected, corresponding to the virus antigen positivity of tumours.

Different virus antigens in the same specimen were identified on the basis of fluorescent colours, thus antibodies against adenoviruses conjugated

to fluorescein-isothiocyanate gave yellow-green fluorescence while antibodies against herpesvirus conjugated to rhodamine displayed an orange-red fluorescence. Localization of virus antigens in the cells was also determined; adenovirus antigens could be seen mainly in the nuclei, while herpesvirus antigens were dispersed around the nuclear membrane or in the cytoplasm [17].

From the 40 control prostatic hypertrophy cases 6, and from the 15 non-tumourous urological patients 2 had herpesvirus antigens in their tissues.

Electron microscopy revealed virus particles in tumour cells in 6 cases; these proved to be adeno- or herpesvirus on the basis of morphology and size. The tumour cells carrying a large quantity of virus antigens contained virions, too. Besides, the two bladder-papilloma patients showed several agents resembling budding C-type oncogenic RNA viruses but neither adenovirus nor herpesvirus antigens by immunofluorescence.

Antibodies against herpes simplex virus in the sera of tumorous patients occurred less frequently than in the controls. Antibodies against adenovirus type 1 structure and non-virion antigens, as well as against adenovirus type 12 structure antigen showed a similar pattern. In contrast, antibodies against adenovirus type 12 early non-virion antigens were present in high titre in almost every patient with malignant tumour, except some in the terminal stage.

Discussion

In animals including primates it has been shown that viruses may play a role in the pathomechanism of tumours either directly or as co-carcinogens [18]. Such a connection has been assumed in humans but doubtless proof has not been known so far. The role of herpesviruses in certain tumours including urogenital malignancies appears to be very likely, mainly on the basis of the frequent presence of non-virion antigens or their antibodies, as well as of the results of molecular-virological studies [8, 13, 19–21].

To obtain more proof, we have investigated another oncogenic human adenovirus. We often found adenoviruses or their components, mainly the oncogenic type 12, besides several other earlier observed viruses, and their role has further been supported by the frequent presence of virus antigens in tumour-cells, and/or antibody against non-virion antigens in the patients' sera, and the occasional presence of virus particles. In certain tumour cells simultaneously with adenoviruses herpesvirus antigens, or particles, even agents resembling the RNA oncogenic viruses could be detected. It could not be decided whether the observed viruses had or had not a role in the genesis or maintenance of tumours, although several studies support the assumption.

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EFFECT OF ADENOVIRUS INFECTION ON HUMAN PERIPHERAL LYMPHOCYTES

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The effect of adenovirus infection on human peripheral lymphocytes has been studied in vitro. Virus antigens were found to multiply in the cell cultures but no infective virus was formed. On infecting the lymphocytes after stimulation with phytohaemagglutinin, infective virus was formed though in low titre. Adenovirus infection decreased the E-rosette forming activity and the response to phytohaemagglutinin of the lymphocytes. The immunomodulating drug levamisole failed to influence significantly the effect of the virus. Adenovirus thus causes several changes in the characteristics of peripheral lymphocytes which may lead to a disturbance of the immune function.

Several viruses are known to possess an immunomodulating effect which increases the sensitivity to other microbial infections [1–9]. Besides their protective function leucocytes may be permanent carriers of certain viruses. The phenomenon can be observed in several illnesses of unknown origin and occasionally in clinically healthy persons too [10–12]. Few data are available concerning the adenoviruses which are widespread, incline to exist in a latent manner and have considerable affinity to the immune system. Adenovirus has been isolated in infective form from the leucocytes of a child suffering from pneumonia, from a patient with recurring aphthae, and from an erythroleukaemic subject [13, 14]. Adenoviruses may multiply even in the stimulated lymphocytes of healthy persons [15]. The effect of permanent adenovirus carriership and the way of its influence on the lymphocytes is less known. The present study was undertaken to clarify this problem. Human lymphocytes were infected with adenovirus and/or the cells were stimulated with phytohaemagglutinin (PHA); the effect of the virus on the stimulation by PHA of the lymphocytes was studied, the immediate E-rosette forming activity of the leukocytes was measured and the appearance of virus antigens and infective virus as well as the effect of the immunomodulating levamisole on the control and infected cells were studied.

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Materials and methods

Lymphocytes. Blood taken with 2 mg/ml heparin from not virus-carrier persons without preservative was separated by filtration on fibreglass and sedimented at room temperature. The lymphocyte-rich supernatant was removed and diluted to contain 1.5×10^6 cells/ml. The cell suspension was placed into test tubes.

Virus. Human adenovirus type 5 (Ad5) was propagated and titrated on HEp-2 cell cultures [16]. Eagle's solution with 10% calf serum and the usual antibiotics were used as culture medium and with 1% rabbit serum for maintenance.

Virus infection. The first group of lymphocyte cultures was infected with 2×10^4 $TCID_{50}$ of Ad 5. The same virus quantity and PHA in 1 : 3000 final dilution was added to the second group. Only PHA in the mentioned quantity was given to the third group of cultures. Finally, lymphocytes without any treatment were cultured as control. Cultures treated with levamisole of 150 μg/ml final concentration were also examined [17]. Culturing was performed at 37 °C for 72 h. Viability of the cells was controlled with trypan blue staining at the beginning and at the end of the experiments.

Demonstration of virus antigens. Immunofluorescence studies were carried out to determine the rate of appearance and formation of virus antigens. Smears were prepared, dried at room temperature, fixed with acetone, treated with immune serum produced in the rabbit against Ad 5 and stained with the indirect method using fluorescein-isothiocyanate labelled anti-rabbit globulin [18]. Zeiss-Fluoval microscope was used for evaluation.

Recovery of virus. For the demonstration and titration of the infective virus, tenfold dilutions were prepared from the lymphocyte cultures after repeated freezing and thawing and placed on HEp-2 cell cultures. The cytopathic effect was observed daily. The virus yield was determined by the Reed–Munch formule [16].

Study of rosette-formation. The number of immediately rosette-forming cells in the lymphocyte cultures subjected to different treatments was determined by the method of Wybran and Fudenberg [19].

Cytological examination. After culturing smears were prepared from part of the cultures. The preparations were fixed and stained according to Giemsa and the number of lymphoblasts was determined by examining 300–400 cells and expressed in percents.

Isotope examinations. Other parts of the cultures were labelled with 5 μCi ^{3}H-thymidine 16 h before the end of culturing. Then the cells were washed in physiological saline and precipitated by cold trichloroacetic acid. After centrifugation the precipitate was dissolved in Triton X100 solution and the activity was determined in a scintillation fluid of PPO dissolved in toluol. The measurements were carried out in a Beckman IS 9000 fluid-scintillation instrument. Quenching of the samples was taken in correction automatically by ^{137}Cs outer standard on the basis of the shift-rate of the Compton-edge. ^{3}H-toluol standard and Triton solution were the samples of the previous quenching series. The quenching effect was produced by adding 0–0.9 ml of chloroform, and the rate of correlation was fed into the instrument. Accordingly the instrument measured the absolute activity of the samples (dpm). The stimulation index was calculated on the basis of the formula,

$$\text{SI} = \frac{\text{activity of treated lymphocytes}}{\text{activity of control lymphocytes}} .$$

Results were evaluated by Student's t test.

Results

Immunofluorescence examination revealed the appearance of adenovirus antigens in every infected lymphocyte culture, on the first day as membrane fluorescence and later in the characteristic form of nuclear fluorescence. Virus antigen was present on the second day in approximately 10% and on the third day in 60% of the lymphocytes. Blast cells showed intensive positivity in the stimulated lymphocyte cultures. Levamisole failed to influence the appearance of virus antigens.

Demonstration of infective virus was attempted on the third day. Reisolation was successful in stimulated cultures only and even then in very low titre (10^2–10^3 $TCID_{50}$/culture).

The E-rosette forming capacity of the lymphocytes was examined in the first 10 experiments daily, later every third day. Table I shows the results. The rate of rosette-forming lymphocytes varied between 30 and 50%. The value was not influenced by levamisole. Upon PHA treatment the number of rosette-forming cells rose, reaching the maximum on the third day. 25–30% of the lymphocytes formed giant rosettes, i.e. the lymphoid cell fixed on its surface 30 or more sheep erythrocytes. Upon treatment with PHA and levamisole the rate of rosette-forming cells rose significantly on the second and third days.

Virus infection reduced the number of rosette-forming lymphocytes and this decrease could be observed at an early phase of infection. Levamisole by itself failed to influence the effect of adenovirus. Fewer immediately rosette-forming cells were present also among the PHA-stimulated and adenovirus-infected lymphocytes. Results are summarized in Table II. The number of lymphoblasts was very low in the control cultures, their rate was always below 1%. More than 2% lymphoblasts were not found among the lymphocytes infected with adenovirus type 5 either. Upon PHA stimulation in non-infected cultures 54% of the cells were transformed into young cell forms i.e. lymphoblasts, whereas this value was 41% in stimulated and virus-infected cultures ($p < 0.01$). Results are presented in Fig. 1.

Levamisole treatment did not increase significantly the blast transformation of either the control or the infected lymphocytes.

Table I

Effect of phytohaemagglutinin and levamisole on active E-rosette forming capacity of lymphocytes, percent

Treatment	1st day	2nd day	3rd day	4th day	5th day
Control	32.25 ± 4.5	41.1 ± 7.3	44.3 ± 4.4	38.3 ± 4.7	50.5 ± 9.3
Levamisole	NS	NS	NS	NS	NS
	38.8 ± 6.8	45.8 ± 10.8	49.3 ± 3.9	43.8 ± 4.8	52.0 ± 9.5
PHA	NS	NS	$p < 0{,}01$	NS	NS
	46.1 ± 6.5	41.5 ± 4.0	60.0 ± 4.4	55.8 ± 9.2	—
Levamisole + PHA	NS	$p < 0.01$	$p < 0.05$	NS	—
	43.5 ± 8.3	48.7 ± 2.4	59.45 ± 3.6	61.0 ± 2.4	—

NS = Not significant

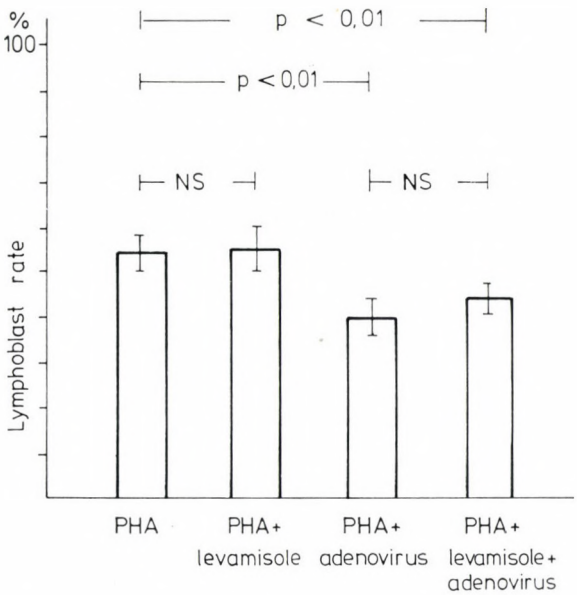

Fig. 1. Lymphoblast transformation under effect of phytohaemagglutinin, levamisole and adenovirus infection

Table II

Effect of phytohaemagglutinin and levamisole on the E-rosette forming activity of lymphocytes infected with adenovirus type 5 — compared to date of Table I as control

Treatment	1st day	2nd day	3rd day	4th day	5th day
Adenovirus type 5	17.1 ± 5.0	26.6 ± 6.5	29.0 ± 5.0	25.4 ± 4.1	40.0 ± 10.1
Levamisole + 5 AV	NS	NS	NS	NS	NS
	16.8 ± 4.6	29.5 ± 6.4	29.4 ± 4.5	22.1 ± 8.5	38.0 ± 6.7
PHA + 5 AV		NS	NS	$p < 0,05$	NS
	—	33.6 ± 8.0	29.7 ± 7.1	46.7 ± 4.5	36.6 ± 6.5
Levamisole + PHA +		$p = 0.05$	$p < 0.01$	$p < 0.01$	NS
5 AV	—	37.4 ± 4.6	40.8 ± 6.4	47.2 ± 5.0	38.5 ± 4.5

Isotope examinations confirmed the results obtained by light microscopic methods. Virus infection decreased the DNA synthesis of the cells very slightly, this was, however, below significance as compared to the control. Thymidine incorporation showed a manyfold increase upon PHA stimulation, its rate, however, varied with the individual cells. Data are presented in Fig. 2. Adeno-

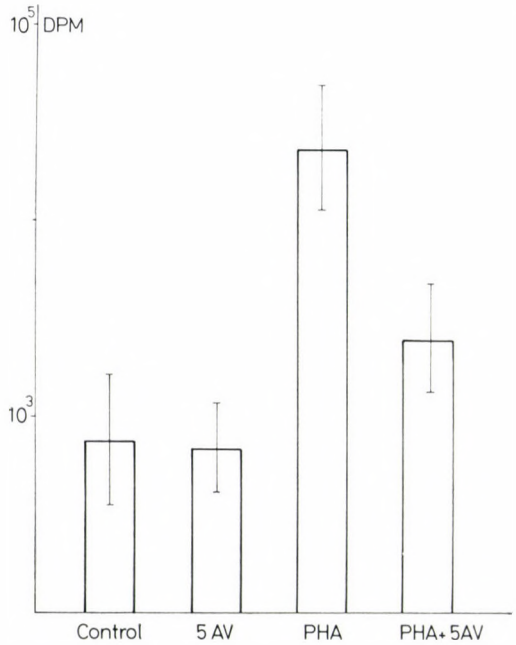

Fig. 2. Thymidine incorporation of lymphocytes under the effect of phytohaemagglutinin treatment and virus infection

virus infection decreased the DNA synthesis of PHA-stimulated cells to almost one tenth, this rate being much higher than the value obtained with the light microscopic method. Changes of the stimulation-index also supported the results presented in Table III.

Table III

Stimulation index under the effect phytohaemagglutinin treatment and adenovirus infection

Treatment	a; 5 AV	b; PHA	c; 5 AV + PHA
Stimulation index	0.8	10.95	1.9
	1.43	52.6	14.9
	0.87	33.1	0.88
	0.7	24.4	0.67
Average	0.95 ± 0.16	30.26 ± 8.73	4.59 ± 3.45
P	NS	$p < 0.01$ (3.5646)	NS to a $p < 0.01$ to b

Discussion

The results showed that human adenovirus type 5 infection changed several characteristics of the peripheral lymphocytes of healthy donors. The number of active T-cells i.e. the E-rosette forming capacity decreased and this could not be influenced significantly by levamisole. Chaturverdi et al. [20, 21] reported a similar decrease in patients with dengue haemorrhagic fever. A cytotoxic effect was observed by us in adenovirus transformed cells caused by adenovirus infected mononuclear leukocytes of healthy donors [22]. These cells released a mediator which decreased the phagocytic activity of polymorphonuclear cells [23].

Adenovirus infection causes a decreases of blast transformation of lymphocytes induced by PHA. The mechanism of the phenomenon is not clear but it has been assumed in the case of measles, rubella and lactate-dehydrogenase virus that by the virus-infected macrophages enzymes are released which impair the immunocompetent cells [24]. This possibility may be left out of consideration in our experiments as we have used separated mononuclear leukocytes and in the course of fibreglass filtration the majority of mononuclear macrophages adhered to the filter. According to our observations and on the basis of literary data the phenomenon might be in connection with the metabolic changes of the cell membrane and receptors [20].

Our results indicate that human adenovirus may modulate the characteristics of peripheral lymphocytes. Several virus infections are followed by bacterial infection and for this the immunomodulation caused by viruses may be responsible [8, 25–28]. One cannot exclude the possibility that the disturbance of the immune system brought about by virus infections may contribute to the development of immunopathological conditions and neoplastic changes [12, 29–31]. To obtain an answer to this question and to highlight its exact mechanisms, further examinations are needed.

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THE DIAGNOSTIC VALUE OF VARICELLA-ZOSTER VIRUS (VZV)-SPECIFIC ANTIBODIES IN THE EARLY PHASE OF VARICELLA IN IMMUNOCOMPROMIZED CHILDREN

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Varicella–zoster virus (VZV)-specific antibodies were tested by indirect membrane immunofluorescent technique in sera of 32 immunocompromized children during the first week after onset of varicella. VZV-specific IgM antibodies were found in sera of all but four patients. Three of the four IgM negative patients were followed up, and it was demonstrated that all of them gave a retarded antibody response. They had severe leukopenia and had several relapses of their VZV-infection. The results indicate (a) that the demonstration of VZV-specific IgM antibodies is of diagnostic value even in atypical varicella of immunocompromized patients; and (b) that in cases with retarded antibody response relapses can be expected.

Varicella (chickenpox) can easily be diagnosed by its characteristic simptoms in patients having normal immune systems. In immunocompromized patients, however, the disease often takes an atypical and sometimes a fatal course [1, 2]. Therefore in the latter cases varicella might be suspected only on epidemiological grounds i.e. in case of a known contact with varicella or zoster patients. The correct diagnosis is, however, important for the treatment of the patient as well as for the protection of other immunocompromized contact patients.

A straightforward diagnostic procedure would be the isolation of the VZV from the skin eruptions. This procedure, however, calls for special laboratory background, it is time consuming and a negative result does not exclude the VZV-infection. Furthermore, isolation of the virus is almost impossible after the 3rd day from the onset of the disease [3]. Williams et al. [4] elaborated an indirect membrane immunofluorescent technique for the demonstration of VZV-specific IgM and IgG antibodies and showed that the demonstration of IgM antibodies by this method in the early phase of varicella or the increase of IgG antibodies during the disease are of diagnostic value in patient with normal immune system.

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The aim of our study was to investigate whether the demonstration of VZV-specific antibodies in sera of immunocompromized patients had the same diagnostic value.

Patients and methods

Thirtytwo immunocompromized children hospitalized for varicella were included in the study. The age of the patients ranged from 1 to 15 years. All of them were under treatment with immunosuppressive drugs. Among them 19 had acute lymphoblastic leukaemia, 1 acute myeloblastic leukaemia, 1 undifferentiated cell leukaemia, 1 non-Hodgkin lymphoma, 2 Wilms tumour, 4 neuroblastoma, 1 mastocytosis, 1 mediastinal tumour and 2 had nephrosis. Twenty-four were in remission of their underlying disease and were still under maintenance therapy while the others were in the active phase of their disease and therefore under intensive immunosuppressive therapy. Symptoms of varicella were typical in 16 cases, while in the others signs of progressive varicella [2] could be observed. All patients were checked for VZV-antibodies immediately after admission i.e. within 7 days after onset of varicella. A few of them were, however, followed up serologically for a longer period.

The method used for the determination of VZV-specific antibodies was that of Williams et al. [4] with some modifications [5, 6].

Results

Table I demonstrates the VZV-specific IgM antibody titres of the patients. VZV-specific IgM antibodies could be demonstrated in the first week of varicella in all but 4 immunocompromized children. The proportion of IgM antibody positive patients tested either on the first to third or on the fourth to seventh day after the onset of the varicella was the same. As far as titres of VZV-specific IgG antibodies were concerned, these ranged from 1 : 5 to 1 : 405. There were only two children without detectable VZV-specific antibodies. The titres of the other patients were rather low especially during the first three days of the disease (Table II).

Table I

VZV-specific IgM antibody titres of immunosuppressed patients with varicella

Titres (reciprocals)	Number of patients		
	investigated on indicated days after onset of varicella		total
	1 to 3	4 to 7	
40	—	1	1
15	7	11	18
5	7	2	9
< 5	2	2	4
Positive/all patients	14/16	14/16	28/32

Table II

VZV-specific IgG antibody titres of immunosuppressed patients with varicella

Titres (reciprocals)	Number of patients		
	investigated on indicated days after onset of varicella		total
	1 to 3	4 to 7	
> 45	1	11	12
5 to 45	13	5	18
< 5	2	—	2

The patients without detectable VZV-specific IgM antibodies in the early phase of varicella deserve special attention. We could follow up three out of these four children and their case reports can be summarized as follows.

S. É. 3 yr. Underlying disease: acute lymphoblastic leukaemia in relapse. The child had typical varicella. All of the eruptions had already been crusted, when a zoster developed and became generalized. She was treated with cytosine arabinoside (Cytosar®, Upjohn) for five days whereupon she recovered. VZV-specific antibody titres on the 7th, 10th and 20th day were invariably IgM negative, IgG 1 : 10. Eight months later the child developed an atypical zoster in the S_2 dermatome with confluent vesicles and necrosis. At that time VZV could be isolated from the vesicles. Within a week varicella-like vesicles appeared all over the body. VZV-specific antibody titres on the 7th, 15th and 21st day were again invariably IgM negative, IgG 1 : 10. The child was treated by acyclovir (Zovirax®, Wellcome) for 7 days and recovered from the VZV-infection. Besides, the child was leukopenic (WBC 1.0 to 3.0 G/l) before and during both VZV-infections. Later she died from a cerebral haemorrhage.

B. O. 4 yr. Underlying disease: acute lymphoblastic leukaemia in remission. She developed typical varicella from which she recovered. During the following three months she had four relapses of VZV-infection: once in the form of varicella, three times in the form of severe generalized zoster. Each of the relapses were treated with antiviral drugs; three times with acyclovir and once with adenine arabinoside (Vidarabinphosphate 500®, Thilo) with success. The child had a severe leukopenia (WBC 0.8 to 2.0 G/l) during these three months with the exception of the last week when WBC count increased to 4.0 G/l. VZV could be isolated from the skin eruptions. She was checked weekly for VZV-specific antibodies but was negative until the 43rd day. The titres of the 43rd day were IgM 1 : 15, IgG 1 : 15; on the 48th day IgM 1 : 15, IgG 1 : 15; on the 58th day IgM 1 : 5, IgG 1 : 10; on the 86th day IgM 1 : 20, IgG 1 : 160. Since that time no relapse of the VZV infection has been observed.

K. Cs. 5 yr. Underlying disease: acute lymphoblastic leukaemia in remission. During a period of two months following a typical varicella the child had three relapses of VZV-infection in form of varicella successfully treated each time with antiviral drugs i.e. twice with acyclovir, once with adenine arabinoside. This child also had a severe leukopenia during the relapses (WBC 0.6 to 1.2 G/l). It was only by the end of the 2nd month that the WBC count increased to 4.0 G/l. Only specific IgG antibodies in low titre could be demonstrated in blood samples taken weekly up to the 58th day when an IgM titre of 1 : 20 and an IgG titre of 1 : 160 was observed. Further relapses of the VZV infection were not observed.

Discussion

It is generally accepted that the immune response of children with malignancy and/or under immunosuppressive treatment is impaired [7–9]. Most of them are, however, able to produce specific antibodies to both primary and secondary antigen stimuli, especially when they are in remission under maintenance therapy [7, 8, 10–16]. In this study we also found that most of our patients were able to produce both IgM and IgG specific antibodies after primary VZV-infection which were demonstrable as early as in the first week of varicella.

As mentioned in the introduction, in this early phase of varicella only VZV-specific IgM antibodies are of diagnostic value. With a few exceptions all of our patients were able to produce specific IgM antibodies irrespective of the activity of their underlying disease, the intensity of the immunosuppressive treatment or whether the course of varicella was typical or atypical. Thus, demonstartion of VZV-specific IgM antibodies seems to be a method useful for the diagnosis of varicella of immunocompromized children.

Although immunocompromized children are able to produce VZV-specific IgG antibodies as well, the demonstration of these antibodies is of little value for the early diagnosis of infection, since the increase in titre allows only a retrospective diagnosis.

The history of the patients who produced no IgM antibodies in the first week of varicella suggested that the absence of antibody production might be connected with their severe leukopenia; in two cases antibody production started only after the WBC count had increased. These case histories also showed that these children are prone to relapses until they have produced antibodies.

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QUANTITATION OF MACROPHAGE ACTIVATION

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Chemoluminescence is a sensitive and relatively easy method to quantitate macrophage activation. Cytotoxic assay as mesasured by ^{51}Cr release from tumour target cells is more involved and less sensitive. Our experiments indicate that, depending on the mode of activation, different effector mechanisms are operational. Thus, macrophages activated with double stranded yeast RNA show good chemoluminescence but no cytotoxic response while thioglycollate activated macrophages show the reverse response.

The macrophage plays a crucial role both in the induction of the immune response and as a powerful effector cell. In its latter capacity, the macrophage ingests and disposes of bacteria, tumour cells and participates in a number of immunopathological processes. Macrophages obtained from immune animals have altered morphology and metabolism [1, 2]. Such macrophages are said to be "activated".

Until recently, quantitative measurements of macrophage activation involved assays that required more than 5×10^6 macrophages and extended periods of time. We have evaluated two assay systems of macrophage-activated cytolysis of tumour cells and chemoluminescence, for their rapidity and sensitivity.

Materials and methods

Animals. White male Swiss Webster mice (18–20 g) were purchased from Canadian Breeding Farms, Quebec. Stock animals were maintained in temperature controlled rooms. Experimental animals were maintained in controlled rooms of similar temperature but separated from stock animals. All animals were kept in plastic cages and fed Purina mouse pellets and tap water ad libidum.

Bacterial cultures. Escherichia coli (O111, clinical isolate) was the bacterial culture used in the experiments. The organism was originally lyophilized. Cultures were maintained on blood agar plates at 4 °C and transferred monthly to ensure virulence.

Preparation of heat-killed organisms. Organisms were grown overnight in 100 ml of Tryptic Soy Broth. Four hours prior to harvesting, an additional 100 ml of Tryptic Soy Broth was added. Organisms were harvested by centrifugation at 2000 *g* for 20 min. The organisms were washed twice in 0.85% NaCl and resuspended in 0.85% NaCl. A sample was taken for bacterial enumeration using the Plate-Count Technique [3]. The remaining suspension of organisms was placed in a boiling water bath for 30 min. After 30 min, organisms were washed three

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times with 0.85% NaCl and resuspended in 0.85% NaCl. A 1 ml sample was taken to test sterility.

After the bacterial count was determined, cultures were diluted with 0.85% NaCl to 1×10^{10} organisms/ml. The suspensions were stored at 4 °C until needed.

Stimulation of animals. Animals were stimulated by one of two methods.

1. *Thioglycollate induced animals.* Animals were induced by injecting 1 ml of thioglycollate broth intraperitoneally (IP) four days prior to harvesting of peritoneal exudate cells (PEC). It has been shown [4] that the cell population in the peritoneal cavity, four days after injection, was primarily macrophages.

2. *RNA stimulation of animals.* Animals were stimulated according to the method of Medina [5]. Animals received a daily injection (IP) of 100 μg RNA (Yeast Core, Schwarz/Mann, Orangeburg, N. Y.) each day for three consecutive days. Five days after the last injection peritoneal exudate cells (PEC) were harvested.

Preparation of macrophage monolayers.

1. *Harvesting of peritoneal exudate cells.* Macrophage monolayers were prepared from peritoneal exudate cells (PEC).

Animals were sacrificed by cervical dislocation and washed down with 70% ethyl alcohol. Four ml of cold Hanks BSS containing 10 units/ml heparin was injected intraperitoneally (IP).

The abdominal cavity was gently massaged to increase PEC harvest. Peritoneal exudate cells were harvested by exposing the peritoneum, piercing it with siliconized Pasteur pipette, and aspirating off the fluid. The aspirated fluid was placed into siliconized 16 mm × 125 mm screw cap culture tubes and the tubes put into an ice bath.

Cell suspensions were centrifuged at 200 *g* for 10 min, washed once in Hanks BSS with heparin (10 units/ml) and resuspended in RPMI-1640 Complete Media containing 10% fetal bovine serum (FBS). Cell concentration was determined by haemocytometer. The cell suspensions were then adjusted to 4×10^6 cells/ml for "normal" PEC and 2×10^6 cells/ml for "induced" PEC. Since fewer "normal" PEC will adhere to glass than "induced" PEC, greater number of "normal" PEC were allowed for so that, after adherence, we would be comparing equal cell populations.

2. *Adherence to glass — macrophage purification technique.* Since macrophages adhere to glass or plastic, this can be used as a purification procedure.

(a) For ^{51}Cr-Cytotoxicity Test: 0.5 ml of the "normal" or "induced" cell suspension was added to a 16 mm tissue culture well (Tissue Culture Cluster, 16 mm dia., Costar, Cambridge, Mass.). After all wells were filled with cells, an additional 0.5 ml of RPMI-1640 Complete Media with 10% FBS was added, bringing the total volume per well to 1 ml. For the ^{51}Cr-Cytotoxicity Test, there should be a minimum of 6 "normal cell" (control) wells and 6 "induced cell" (test) wells per experiment. The tissue culture wells were then incubated for 2 h at 37 °C in a 5% CO_2 incubator.

(b) Chemoluminescence — Leighton Tube Coverslips. 0.5 ml of the cell suspension ("normal" or "induced" cells) was added to the Leighton tubes containing coverslips. An additional 0.5 ml of RPMI-1640 Complete Media with 10% FBS was added. The Leighton tubes were placed in a Leighton tube holder and incubated at 37 °C in a 5% CO_2 incubator for 2 h.

After 2 h incubation, tissue culture wells and Leighton tubes were removed from the incubator and the media aspirated off. The cells were washed 3 times with gentle agitation to remove nonadherent cells. One ml of RPMI-1640 Complete Media containing 10% FBS was then added to the macrophage monolayers. By morphological examination, the cell population was more than 90% macrophages.

Mithramycin assay for cell counting. In order to ensure that comparative cell numbers were being used, it was necessary to determine the number of macrophages present on the glass after nonadherent cells were remoed.

The mithramycin assay was performed as described by Hill and Whatley [6] and Kulisek [7]. Mithramycin was reconstituted with 0.3 M $MgCl_2$ to a final concentration of 500 μg/ml. The drug was further diluted to 100 μg/ml using 0.3 M $MgCl_2$ immediately prior to use.

Fluorescence was determined using a fluorometer (Turner Model 111, G. K. Turner Associates, Palo Alto, Ca.). Filter combinations Wratten #47 and #2A were used for an excitation wavelength of 436 nm and filters #55 and #2A-15 for an emmision wavelength of 540 nm. All readings were done at 30 × intensity for maximum sensitivity. A 10 × 75 mm glass tube was used as a cuvette.

Three Leighton tubes or tissue culture wells containing macrophage monolayers were used to determine the cell number present. The medium was removed and the monolayers gently washed three times with 0.01 M phosphate buffered saline (PBS) pH 7.2. The macrophage monolayers were frozen and thawed three times. The cells were scraped off the surface after

being frozen and thawed, in 0.5 ml of 0.01 M PBS with a flat-edge spatula. The cell lysate was brought to a final volume of 1.8 ml using 0.01 M PBS.

The cell lysate was sonicated (Sonifier Model W-350, Branson Instrumental In., Melville, N. Y.) for 10 s using a microtip head at 150 Watt power. A 0.9 ml sample was taken and 0.1 ml of mithramycin (100 μg/ml) was added. Mithramycin at 10 μg/ml in 0.01 M PBS served as a blank.

Fluorescence was determined in a Turner Fluorometer. Fluorescence units were referred back to Fluorescence-DNA standard curves and macrophage cell numbers derived from the DNA standards. The DNA content of macrophages was taken as 8.7×10^{-12} gms DNA/macrophage.

Some Leighton tube coverships were removed from the media and gently washed in 0.01 M PBS pH 6.4. Cells were stained with a 1 : 40 dilution of Giemsa stain (diluted with 01 M PBS, pH 6.4) for 25 min. Cell counts were then made by microscopic examination of the coverslips.

^{51}Cr-cytotoxicity test.

1. *B-16 Melanoma target cell.* The B-16 was originally a spontaneous skin tumour in C57Bl/6J mice. It is a pigmented spindle-shaped cell often producing haemorrhagic soft grey tumour masses. The B-16 tumour is syngeneic to C57Bl/6J mice and is passed as a subcutaneous implant. The tumour was received from Jackson Laboratory (Bar Harbor, Maine) as a subcutaneous implant.

2. *^{51}Cr-cytotoxicity test.* B-16 tumour cells grown as monolayers were harvested using 0.25% trypsin in Tris phosphate buffered saline (Tris–PBS). The cells were centrifuged at 200 *g* for 10 min, washed once in Tris–PBS and resuspended in Tris–PBS. Cell concentration and viability was determined by a haemocytometer and 0.2% trypan blue. The cell suspension was then diluted to 3×10^6 cells/ml with Tris–PBS.

To 1.0 ml of cell suspension, 100 μC of ^{51}Cr as sodium chromate (^{51}Cr-sodium chromate in saline, specific activity 300–500 μC/μg, 1 mC/ml, New England Nuclear, Boston, Mass.) was added and the cell suspension incubated at 37 °C in a 5% CO_2 incubator for 45 min with occasional shaking. The cells were then centrifuged at 200 *g* for 10 min, washed four times with RPMI-Complete Media containing 10% FBS, and resuspended in RPMI-Complete Media plus 10% FBS.

The cell concentration was determined by use of a haemocytometer and then diluted to give a final concentration of 4×10^4 cells/ml; 0.5 ml of the cell suspension was added to each tissue culture well of macrophage monolayers. To an additional 6 wells which did not contain macrophages, 0.5 ml of the labelled tumour cells were added and the final volume made up to 1 ml using RPMI-Complete Media with 10% FBS. The six wells were used to calculate the spontaneous release of ^{51}Cr from the tumour cells.

The macrophage monolayers containing ^{51}Cr labelled tumour cells were then incubated for 18 h at 37 °C in a 5% CO_2 incubator.

After 18 h, the medium was removed from the cells. The cells were washed twice with 0.01 M PBS and the supernatant and washings of each tissue culture well were combined. The supernatant was then centrifuged at 200 *g* for 10 min to remove any remaining cells. The supernatant was decanted into plastic microscintillation vials. A 1% solution of Triton X100 was added to the six wells "with tumour cells only" and to their six corresponding centrifuge tubes containing residual cells removed by the washings. The Triton X100 fluid was collected and placed into liquid scintillation vials. These vials represent "total ^{51}Cr label" left in the tumour cells after spontaneous release. Total ^{51}Cr label present was calculated by adding the cell ^{51}Cr label to the spontaneous released ^{51}Cr label. This activity represents 100% of releasable ^{51}Cr. The vials were then placed in a Gamma Scintillation Spectrometer (Packard Model 5375, Downers Grove, I11.): channel Setting E-F, 400–700; amplification 50%; time of counts 5 min).

The percent cytotoxicity can be calculated according to the formula of Cerottini and Brunner [4].

$$\% \text{ Cytotoxic effect} = \frac{IR - NR}{MR - NR} \times 100$$

IR: the immune release from activated macrophages and ^{51}Cr labelled tumour cells; NR: the normal release from nonimmune macrophages and ^{51}Cr labelled tumour cells; MR: the maximum release from tumour cells when they are lysed with 1% Triton X100.

Chemoluminescence

1. *Preparation of FBS-Luminol.* Luminol (5-amino 2, 3-dihydro 1, 4 phthalazinedione) (Eastman–Kodak, Rochester, N. Y.) was added to fetal bovine serum (FBS) that had been previously heat inactivated (56 °C waterbath for 30 min). Luminol was added to the FBS at a

concentration of 0.2 g per 10 ml of FBS; this is to obtain saturation of the FBS with luminol. The FBS–luminol solution was incubated for 1 h on a rocking platform mixer.

After one hour, the FBS-luminol solution was centrifuged at 700 g for 30 min, to remove any particulate matter. After centrifugation, the luminol saturated FBS was kept at room temperature (25 °C) until the chemoluminescence test could be performed. At room temperature the FBS–luminol solution will remain stable for 12 h.

2. *Chemoluminescence — Leighton tube coverslips.* Leighton tube coverslips containing macrophage monolayers were removed from the Leighton tubes and placed into a glass liquid scintillation vial containing 17 ml of Hanks BSS without heparin. The coverslip was placed into the scintillation vial in such a manner as to form a 45° angle with the bottom of the vial with the cells on the top side of the coverslip. Each coverslip for the chemoluminescence assay was added to a scintillation vial in the same manner. These vials were then placed in a 37 °C–5% CO_2 incubator until the chemoluminescence assay could be performed.

At the time of the chemoluminescence assay, a scintillation vial containing cells was removed from the incubator and 2.5 ml of FBS–luminol was added. The vial was placed in a Nuclear Chicago Scintillation Counter (Model Unilux II-A, Nuclear Chicago). The counter was placed out-of-coincidence by disconnecting one photomultiplier tube and adjusting the window openings to maximum range. The refrigeration was also disconnected; thus the inner temperature of the machine remained at 35 °C. The scaler was programmed for continuous 1 min counts. The point of addition of FBS–luminol is marked Time = —20 min.

The counting sequence was run for 20 min to establish a FBS–luminol plus cell base line of chemoluminescence. After 20 min (Time = 0 min) 100 μl of appropriate opsonizing antiserum (undiluted), 100 μl of bacteria (2×10^9 cells/ml), and 50 μl of guinea pig complement was added to the cells. Chemoluminescence counting was reinstated and continued for 45 min.

Results

Two procedures were used to measure macrophage activation. Pure monolayers (>95%) of macrophages were used in the ^{51}Cr cytotoxicity test and in the chemoluminescence assay. Different results were obtained using these methods.

Chemoluminescence. When macrophages become activated, one of the initial changes to occur is an increase in glucose oxidation through the hexose monophosphate shunt (HMS) [8]. Allen and Loose [9] showed that a chemoluminescence response occurs in actively phagocyting macrophages. This chemoluminescence response following phagocytosis correlates directly with the metabolic activation of the HMS. By measuring the chemoluminescence response, it is possible to measure quantitatively the degree of metabolic activity of the HMS through its generation of oxidizing radicals ($^1/_2$ O_2, HOOH, OH, O_2). The luminol will react with these oxidizing radicals by releasing a photon which can be detected in an out-of-coincidence liquid scintillation counter. The number of photons emitted will be a direct measure of the quantity of oxidizing radicals produced by the HMS and thus an indication of the cells activity.

A "normal" mouse macrophage chemoluminescence response curve is characterized by a rapid increase in photons per minute (ppm) starting at 2–3 min following the initiation of phagocytosis. The curve rises from a baseline of 2000 ppm $\pm$560 prior to the initiation of phagocytosis. The curve peaks in 8–12 min and has a magnitude of 49 $\times$ baseline ($\pm$3 $\times$ baseline) after peaking,

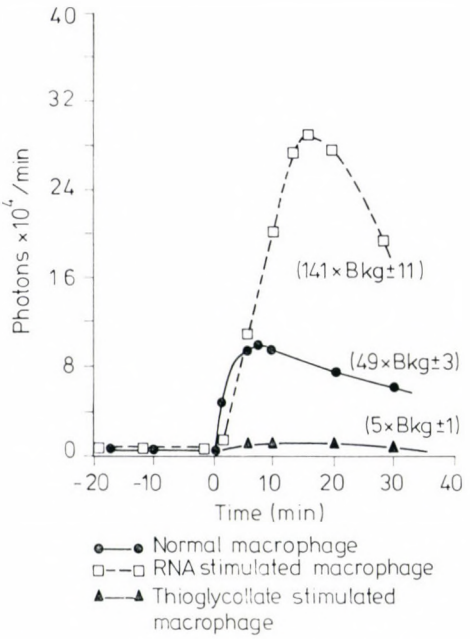

Fig. 1. Chemoluminescence of macrophages from thioglycollate and RNA stimulated mice

there is a gradual decrease in the chemoluminescence, as measured by the number of photons per minute (Fig. 1).

Controls consisting of opsonized bacteria in the absence of macrophage monolayers and macrophage monolayers without bacteria being present where performed. In no experiments with these controls did the chemoluminescence (CL) response peak at a magnitude greater than $5 \times$ baseline $\pm 1 \times$ baseline.

Time sequence of RNA stimulated mouse macrophages is shown in Fig. 2. Both normal and stimulated macrophages go through a time sequence, depending on how long the cells have been under tissue culture conditions.

Since Gram-negative organisms were used in this study, it was necessary to determine whether the lipopolysaccharide (LPS) contained on the bacterial cell wall was inducing the response. In place of bacteria 5.4×10^{-3} g *E. coli* LPS was added to the monolayers along with antisera and complement. A CL response occurred at 4 min with a $6.5 \times$ baseline magnitude. It was further shown that the CL response can be inhibited at any place in the curve with 200 mg Na azide and that a maximum CL response occurs when the bacteria are fully opsonized with specific antisera and C'. Our results indicate that for CL to occur, there has to be an intact functional macrophage actively in the process of phagocytosis.

Double stranded RNA [10] and thioglycollate were used to activate macrophages.

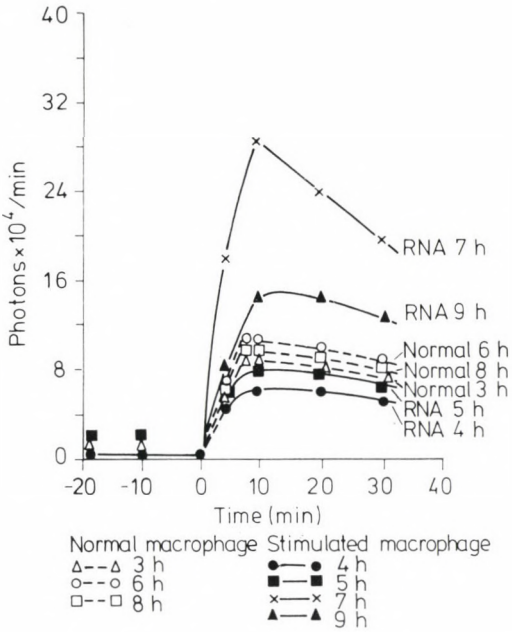

Fig. 2. Time sequence of chemoluminescence of RNA stimulated mouse macrophages compared to non-stimulated animals

RNA activated mouse macrophages expressed a CL response that peaked within 14 min at 141 × baseline (±11 × baseline) magnitude (Fig. 1). This represents a 187% increase above that received for the same number of normal mouse macrophages.

RNA activation of macrophages results in a nonspecific enhanced bactericidal state [10]. Our results indicate that RNA causes activation of macrophages. Macrophages obtained from RNA treated animals show an increase in their CL response. The CL corresponds to an increased phagocytic rate and metabolic activation of the HMS. The height of the CL response relates to the degree of metabolic activity of the HMS.

Thioglycollate, a chemotactic chemical, is used widely to induce peritoneal exudate cells. Thioglycollate induced Swiss mouse macrophages did not share the typical CL response. CL did not rise above 5 × (±1 ×) baseline (Fig. 1).

^{51}Cr-Tumouricidal assay. To show that thioglycollate-induced macrophages have not lost their activated state we decided to investigate their tumour killing ability. When macrophages become activated, they express a nonspecific cytotoxic effect on tumour cells [11]. The ^{51}Cr-release assay is based on the observation that when a cell membrane (tumour cell) is damaged or altered, ^{51}Cr is released into the surrounding media [12]. The amount of specific

release caused by activated macrophages will serve as a measure of their tumouricidal capacity and thus their degree of activation. The percent cytotoxicity caused by activated macrophages was calculated according to the formula of Cerottini and Brunner [13]. Results are expressed as percent cytotoxic effect [CE].

Our preliminary results indicated that the maximum sensitivity of the ^{51}Cr-release assay could be achieved when the macrophage to tumour cell ratio was 50 : 1 and a 18 h incubation period was used.

Thioglycollate-induced mouse macrophages were tested against a B-16 allogenic tumour. B-16 tumour cells were labelled and placed in contact with Swiss Webster mouse macrophages at a ratio of 50 : 1.

Thioglycollate-induced macrophages showed a cytotoxic effect of 27%. In addition to the cytotoxic effect, macrophage monolayers were characterized by extensively spreading cells with a highly vacuolated cytoplasm.

Our results indicate that thioglycollate will activate certain functions of macrophages but not others. We found that thioglycollate causes an increase in adherence, spreading on glass, degree of vacuolation and increased tumouricidal activity all morphological and physiological indicators of activation. In contrast, it suppressed the rate of phagocytosis and the metabolic activity of the HMS.

Macrophages were activated also by double stranded RNA and tested for their cytotoxic effect. RNA activated Swiss mouse macrophages showed no increase in their cytotoxicity (CE — 0) when tested against B-16 tumour cells. Our results indicate that double stranded RNA does not cause an increase in the tumouricidal activity of Swiss mouse macrophages when tested against B-16 tumour cells.

Although RNA does stimulate immunity to intracellular parasites [10] we failed to demonstrate activation of macrophages as measured by an increase in their tumouricidal effect. When RNA activated macrophages were tested for CL activity there was an enhanced metabolic activity of the HMS and the rate of phagocytosis. Microscopically, RNA activated macrophages appear morphologically similar to normal macrophages and there is no increase in percent adherence to glass as compared to their normal counterpart.

Discussion

The accumulation of research data over the years has provided a great deal of information on the role of the macrophage in host defense mechanisms. In response to an invading pathogen, macrophages will undergo certain metabolic and morphological changes resulting in a condition termed "activated" [11, 14, 15].

Since Metchnikof's [16] first mention of the macrophage, the measurement of activation has presented a special problem. Quantitative assay techniques of activation are based on the measurement of a particular enzyme or enzyme system. In order to perform these assay, a minimum number of cells must be present; usually $> 5 \times 10^6$. In many experiments on macrophage activation, mouse models are used. Since the harvest of peritoneal cells represents only $3–5 \times 10^6$ cells per mouse, activation experiments are limited by the number of animals needed.

In our results, we have presented two assay systems which meet the requirements of short duration (<24 h) and are quantitative and sensitive ($< 1 \times 10^6$ cells/assay). One assay system, chemoluminescence, measures the degree of metabolic activity of the HMS, while the other, cytolysis, measures the tumouricidal activity of activated macrophages.

The chemoluminescence assay is a rapid and sensitive assay that requires minimal preparation to be performed. Utilizing the fact that luminol will react with oxidizing radicals ($^1/_2\ O_2$, HOOH, OH, O_2) generated by the HMS, it is possible to measure the respiratory burst in macrophages.

The assay can be inhibited with sodium azide at any point during phagocytosis; adding credence to the fact that for a chemoluminescence response to occur, macrophages must possess a functional membrane and enzyme systems. When sodium azide was added at the same time as the initiation of phagocytosis, no chemoluminescence response appeared. If sodium azide was added at a point when the macrophages were actively phagocyting, the chemoluminescence response decreased drastically to backround levels. The effects of sodium azide on polymorphonuclear leukocytes indicates that chemoluminescence is dependent on functionally intact cells [17].

Our results along with those of Schleupner et al. [18] and Schadelin and Mandell [19] indicate that normal intact phagocyting macrophages exhibit a chemoluminescence response that is specific and can only be elicited from macrophages that are in the process of phagocytosis. This chemoluminescence response does not occur prior to or subsequent to phagocytosis [9].

The chemoluminescence response was tested on activated macrophages in order to establish if it could serve as a quantitative measure of macrophage activation. The chemoluminescence response for RNA activated Swiss mouse macrophages was 187% higher than that for normal macrophages. The general shape of the chemoluminescence response curve for activated macrophages was similar to that given by normal macrophages, but the magnitude at the peak chemoluminescence response was higher. When macrophages become activated there is a corresponding increase in the metabolic activity of the HMS. This results in increased production of oxidizing radicals. These radicals are responsible for the generation of photons from luminol. Thus with luminol, we are measuring the production of oxidizing radicals generated by the HMS

in response to phagocytosis. Schleupner et al. [18] have shown that *Corynebacterium parvum* activated macrophages show a greater chemoluminescence when phagocyting *Candida albicans* or zymosan as compared to normal macrophages in the process of phagocytosis. Our results and those of Schleupner et al. [18] indicate that chemoluminescence is a quantitative measure of macrophage activation. The peak of the chemoluminescence response curve is in proportion to the degree of activation.

The ^{51}Cr-release assay for measuring macrophage activation proved to be sensitive and of short duration. The assay, although not as sensitive as the chemoluminescence assay, has the added advantage of measuring a direct effect of macrophage activation, the ability to kill tumour cells. There were two disadvantages with the ^{51}Cr-release assay. Our initial problem with the assay was the high degree of ^{51}Cr- spontaneous release from the tumour cells. Under the best conditions it was not possible to reduce this release to less than 1.5% per hour; thus using a 18 h assay period, the spontaneous release accounted for 28% of the total incorporated label. Cerottini and Brunner [13] further showed that each tumour cell line will release the ^{51}Cr label at a rate dependent on how well the cells are adapted to tissue culture conditions. In addition, our results have shown that under maximum conditions there was only a 27% increase in release due to the cytotoxic effect of activated macrophages. Thus under experimental conditions, the spontaneous release was almost as high as the cytotoxic effect release. The ^{51}Cr-cytotoxic release assay did not show a cytotoxic effect for RNA activated macrophages, although there was an increase in cytotoxic release when thioglycollate-induced macrophages were used.

Our results have shown that the ^{51}Cr-release assay records a cytotoxic effect when thioglycollate-induced macrophage are used, but no cytotoxic effect when macrophages are activated by RNA. Using the chemoluminescence assay, RNA activated Swiss mouse macrophages express a 187% increase in the activity of the HMS as compared to their normal counterparts, whereas thioglycollate-induced macrophages were suppressed in their activity; the reverse situation when the ^{51}Cr-release assay was used. We propose, in addition to our two assay systems measuring different effects of activation, that thioglycollate and RNA do not activate macrophages by the same mechanism.

We conclude from our results that both methods, chemoluminescence and ^{51}Cr-release, quantitatively measure macrophage activation. The chemoluminescence assay was the more sensitive, less variable, simpler and more rapid of the two methods.

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BACTERIAL MODULATION OF THE CELLULAR IMMUNE RESPONSE IN MICE

I. THE COURSE OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN *BORDETELLA PERTUSSIS* VACCINE PRETREATED MICE WITH PHYSIOLOGICAL THYMUS INVOLUTION

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The cellular immune response to lymphocytic choriomeningitis virus infection was found to be normal in 6-month-old mice with physiological thymus involution, while it was reduced in 18-month-old mice. The *Bordetella pertussis* vaccine elicited immunosuppression in 6-month-old mice with normal immunological responsiveness, while it failed to affect the physiologically diminished cellular immune response in 18-month-old mice. The extent of immunosuppression elicited by the vaccine changed parallel to its concomitant spleen hypertrophy inducing effect.

A cellular immune reaction underlies the lethal lymphocytic choriomeningitis (LCM) where the T cells play an essential role in the development of the disease [1–3]. The course of the virus infection depends on the host's cellular immune responsiveness and, consequently, on the host's age. Adult mice with intact immune system develop acute LCM with fatal outcome, while suckling mice with their immature immune system survive the virus infection and become virus carriers [4].

Our previous studies of old mice with physiological thymus involution, i.e. lymphoid atrophy due to aging, have revealed a reduced cellular immune responsiveness to LCM virus; the characteristic neurological symptoms of LMC virus infection with its characteristic histological changes were present in few animals [5].

The *Bordetella pertussis* vaccine is known to have an immunomodulatory effect which may, however, work not only in one direction; it may cause both stimulation or suppression, depending on the circumstances [6–9].

According to our previous studies, the effect of pertussis vaccine on the course of LCM virus infection varies with the host's age; it restricts the cellular immune response to the virus infection in adult mice with intact immune system and amplifies it in suckling mice with immature immune system [10, 11].

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The present experiments were concerned with the influence of *B. pertussis* vaccine on the course of LCM virus infection in mice of various ages but all in the state of physiological thymus involution.

Materials and methods

Experimental animals. C57BL mice of different ages and both sexes were used.

The B. pertussis vaccine contained 30×10^9/ml killed bacteria suspended in physiologial saline(Human Institute for Serobacteriological Production and Research, Budapest). The mice received single intraperitoneal injection of 0.3 ml vaccine each, containing 9×10^9 bacteria. Control mice were treated with physiological saline pH 7.0 administered in the same way and quantity.

LCM virus infection. The applied W. E. strain was maintained in serial mouse brain passages. Physiological saline was used to prepare the brain suspensions and virus dilutions. The experimental animals were inoculated intracerebrally with 100 LD_{50} in 0.03 ml of pretitrated virus. Control animals received in the same way brain suspensions of uninfected mice. The neurological symptoms characteristic of LCM (tremor, convulsion) were checked twice daily and the incidence of death was registered.

Virus recovery. Brain suspension prepared from surviving and then sacrificed animals was inoculated intracerebrally in 1 : 10 dilution to several groups of mice. Characterstic neurological symptoms and death confirmed the recovery of LCM virus.

Examination of the lymphoid system. Mean relative lymphoid organ weights and spleen indices were determined:

$$\text{Relative lymphoid organ weight} = \frac{\text{mean lymphoid organ weight (mg)}}{\text{mean body weight (g)}}$$

$$\text{Spleen index} = \frac{\text{mean relative spleen weight in the vaccine treated group}}{\text{mean relative spleen weight in the control group}}$$

Statistical evaluation. For statistical evaluation Student's two sample t test was applied. The significance level was $p = 0.05$.

Results

Six-week-old (I), six-month-old (II) and 18-month-old (III) mice were treated with pertussis vaccine. On the next day some of them were infected with LCM virus. The experimental groups and treatments are presented in Table I.

The experiments were concluded on the 21st day after LCM virus infection. At the time of virus infection the untreated (unvaccinated) control mice were sacrificed and the relative thymus weight was determined in the three age groups. The data obtained are presented in Table II.

As it can be seen from Table II the relative thymus weight was significantly higher in the 6-week-old mice (group I) than in the groups II and III; while there was no significant difference in relative thymus weight between the 18-month-old and 6-month-old mice.

Table I

The experimental mouse groups and their treatments

Groups		Age (month)	No. of mice	Intraperitoneal inoculation	Intracerebral inoculation
	—I	1.5	20	*B. pertussis*	LCM
P-LCM	—II	6	20	vaccine	virus
	—III	18	20		
	—I	1.5	20	PBS	LCM
LCM	—II	6	20		virus
	—III	18	20		
	—I	1.5	20		
P	—II	6	20	*B. pertussis*	normal brain
	—III	18	20	vaccine	suspension
	—I	1.5	20		
C	—II	6	20	PBS	normal brain
	—III	18	20		suspension
Untreated	—I	1.5	10	—	—
	—II	6	10		
	—III	18	10		

Table II

Relative thymus weight in the age groups

Group		Mean relative thymus weight	Significance
Untraeted	—I	3.8 ± 1.3	$p < 0.001$
	—II	1.8 ± 1.0	
	—III	1.6 ± 0.5	$p > 0.05$

The rate and time curve of deaths in the LCM infected groups are presented in Fig. 1.

In groups LCM-I and LCM-II all the mice died between the 6th and 8th day after the infection. In agreement with our previous experiments, in group LCM-III 50% of the mice survived the infection and death occurred later between 8 and 18 days. In P-LCM-I and P-LCM-II groups of vaccinated mice death occurred between the 7th and 11th day with 15 and 20% of the mice surviving the virus infection, respectively. In group P-LCM-III the death

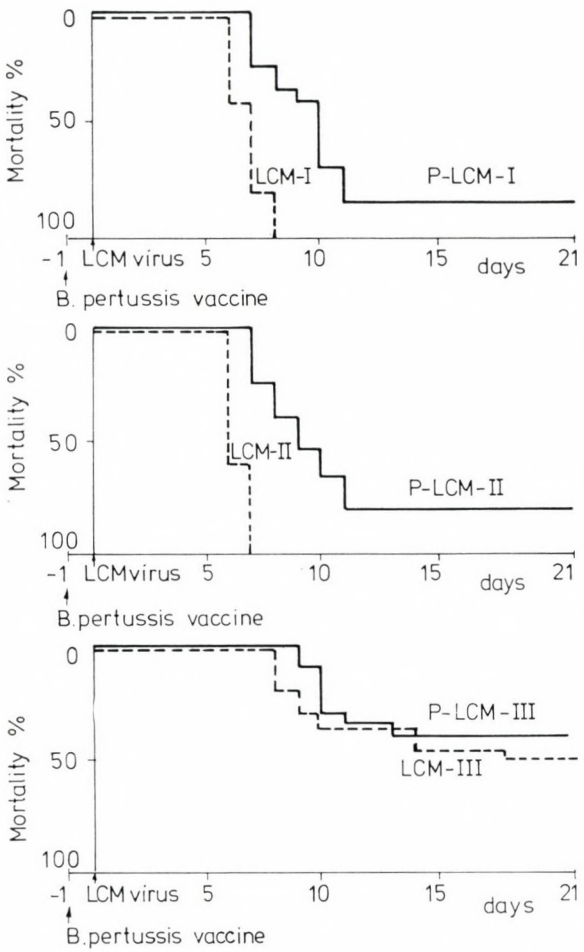

Fig. 1. Rate and time of death after LCM virus infection

occurred between the 9th and 13th day with a 60% survival rate. Neurological symptoms characteristic of LCM could be observed in all dying mice.

Half of the groups P and C were sacrificed on the 7th day of the experiment, the other half on the 21st day. No spontaneous death occurred in these groups. Relative spleen weight and indices of the sacrificed animals are presented in Fig. 2.

Expressed spleen hypertrophy was observed on the 7th day after vaccination in both the age-groups I and II, while the hypertrophy due to pertussis vaccine was significantly slighter in age-group III. By the 21st day spleen hypertrophy decreased as compared to its 7th day value; the rate of decrease was the lowest in group III.

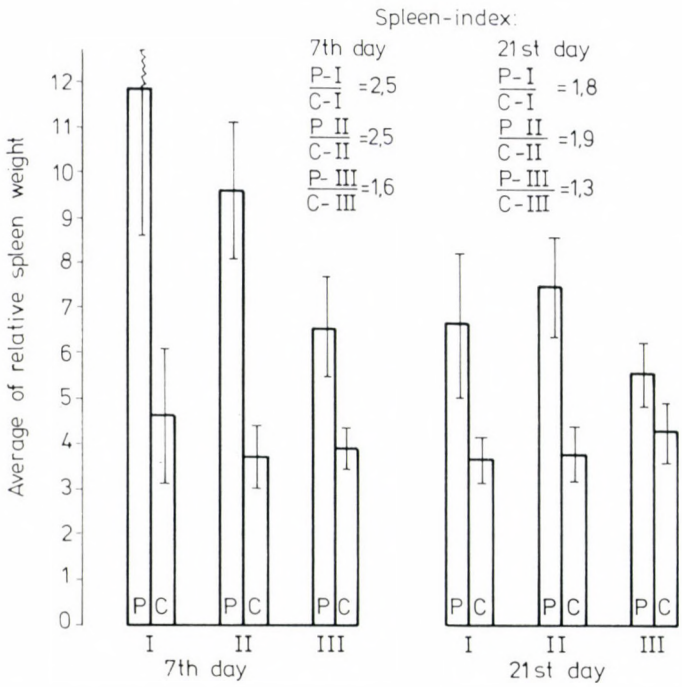

Fig. 2. Effect of *B. pertussis* vaccine on the spleen in mice of various ages

Discussion

Lymphocytes are known to leave the central lymphoid organs such as the thymus and migrate to the peripheral lymphoid organs, simultaneously with the beginning of thymus involution. In the present experiments the cellular immunological responsiveness to LCM virus was studied in mice of different ages in different thymic states as well as the effect of the *B. pertussis* vaccine on the course of LCM infection was observed in the different age groups. In spite of the fact that the peripheral lymphoid system of 6-month-old mice was already in the thymus involution state, they still had a complete cellular immunological capacity (100% death rate in group LMC-II). On the other hand, the cellular immune responsiveness of 18-month-old mice is already on the decline, in group LCM-III survival was 50%. The immunomodulatory effect of *B. pertussis* vaccine elicited immunosuppression in 6-week-old and 6-month-old mice, thus these vaccinated animals died later than their unvaccinated counterparts and part of them survived the virus infection as virus carrier. In 18-month-old mice the rate and time curve of death did not show any significant difference between vaccinated and un-

vaccinated animals, i.e. the vaccine failed to affect the physiologically declined cellular immunological capacity.

Evaluation of the relative spleen weight showed that *B. pertussis* vaccine caused slighter spleen hypertrophy in 18-month-old mice than in 6-month or 6-week-old ones. The immunosuppressive and spleen hypertrophy inducing effects of *B. pertussis* vaccine seem to run parallel. In the age groups I and II, namely, expressed spleen hypertrophy (spleen index 2.5) could be observed simultaneously with immunosuppression. On the other hand, the third age-group showed no change in cellular immunological capacity and spleen hypertrophy was also slight (spleen index 1.6).

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BACTERIAL MODULATION OF THE CELLULAR IMMUNE RESPONSE IN MICE

II. STIMULATION BY ENDOTOXIN OF THE REDUCED CELLULAR IMMUNE RESPONSE TO LCM VIRUS INFECTION IN AGED MICE

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Aging mice treated with radio-detoxified endotoxin and subsequently inoculated intracerebrally with lymphocytic choriomeningitis (LCM) virus died earlier and in a higher rate than the virus infected and otherwise untreated controls. Thus, in mice with insufficient T lymphocyte function due to aging, the radio-detoxified endotoxin pretreatment contributed to the outcome of LCM virus infection in the form of lethal meningitis. That indicates a stimulatory effect on the reduced cellular immunological reaction to the virus infection.

It is known that several microbes and microbial substances can modify immunological reactivity. They are important as natural stimulators of the developing immune system. The bacteria and their non-toxic purified products can be used as adjuvants and non-specific stimulators in infections, especially in virus infections, in immune deficiency states and in the case of tumours.

Lipopolysaccharide (LPS) endotoxins originating from Gram-negative bacteria are known to influence the immune responsiveness. There are data on increases and decreases of the immune response to heterologous antigens [1].

It is also known that the course of intracerebral lymphocytic choriomeningitis (LCM) virus infection depends on the cellular immune responsiveness of the host and hence on his age. In adult mice with intact immune system acute LCM has a fatal outcome. The disease and death are the consequences of the cytotoxic reaction of T-lymphocytes, specific to LCM virus antigen, to cells that express viral antigens on the leptomeninx [2–4].

In mice with insufficient T-lymphocyte function, like newborns or sucklings with immature immune system or aged mice or those having received immunosuppressive treatment, LCM fails to develop, and the mice surviving the infection become virus carriers [5–8]. Substances with adjuvant activity, like *Bordetella pertussis* vaccine or endotoxin preparations (both the parent

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and the radio-detoxified endotoxin) contribute to the development of meningitis following intracerebral LCM virus infection in suckling mice [9–11].

In the present experiment the effect of radio-detoxified endotoxin was studied on the course of LCM virus infection in aged mice with physiological thymus involution.

Materials and methods

Endotoxin (LPS) preparation. LPS was isolated by the hot phenol–water method of Westphal et al. [12] from a fermentor culture of *Escherichia coli* O89. The preparation was purified by repeated ultracentrifugation at 100 000 *g*.

Production of radio-detoxified endotoxin (rdLPS) preparation (Tolerin®, Humán, Budapest). LPS was "dissolved" in distilled water and irradiated (15 Mrad = 150 kGy) at a concentration of 10 mg/ml in a ^{60}Co source for radio-detoxification [13–15].

Experimental animals. Young (2 months old) and aged (18 months old) C57Bl inbred mice of both sexes were used.

LCM virus infection. The applied W. E. strain was maintained in serial mouse brain passages. Virus titration was performed by intracerebral inoculation of young adult mice. During the experiment the development of neurological symptoms characteristic of LCM (tremor, convulsions) and death were controlled twice daily.

Examination of the lymphoid system. The relative spleen and thymus weights and the spleen and thymus indices of mice were determined as follows:

$$\text{Relative lymphoid organ weight} = \frac{\text{lymphoid organ weight (mg)}}{\text{body weight (g)}}$$

$$\text{Lymphoid organ index} = \frac{\text{mean relative lymphoid organ weight in the experimental group}}{\text{mean relative lymphoid organ weight in the control group}}$$

Statistical evaluation was done by Student's two sample *t* test. The accepted level of significance was $p = 0.05$.

Results

In order to investigate the state of the thymus in mice, 10 young (2 months old) and 10 aged (18 months old) mice were sacrificed and their relative thymus weight and thymus index were determined. The mean relative thymus weight was significantly lower in the aged than in the young mice (thymus index = 0.4).

To study the effect of endotoxin on the course of LCM virus infection in aged mice, the animals received a single intraperitoneal injection of the rdLPS preparation in a dose of 10 mg/kg body weight. Control animals received PBS in the same way. On the following day the mice were infected intracerebrally with the pretitrated 100 LD_{50} of LCM virus.

Simultaneously with the LCM infection, mice were inoculated intracerebrally with virus-free mouse brain suspension. Of these mice, those receiving radio-detoxified endotoxin formed the rdLPS group and those treated with phosphate-buffered saline (PBS) the C group.

The infected mice pretreated with radio-detoxified LPS belonged to the rdLPS-LCM group.

The experiment was terminated on the 21st day after virus infection. The neurological symptoms characteristic of LCM as well as deaths were registered in the virus infected groups. The groups and treatments are presented in Table I.

Table I

Mouse groups and their treatment

Groups	No. of mice	Intraperitoneal treatment	Intracerebral inoculation
rdLPS-LCM	20	rdLPS	LCM virus
LCM	20	×	LCM virus
rdLPS	20	rdLPS	××
C	20	×	××

× = PBS
×× = Virus-free mouse brain suspension

Rate and time curve of death in the virus infected groups are presented in Fig. 1.

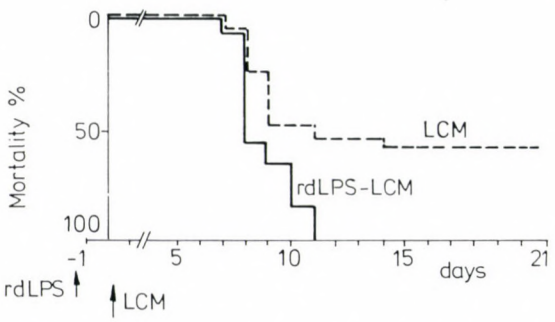

Fig. 1. Rate and time of death after LCM virus infection

Typical neurological symptoms of intracerebral LCM virus infection were apparent before death in each animal succumbing during the experiment.

Sixty per cent of the mice infected with virus but not treated with rdLPS, died between the 7th and 15th day after inoculation. Hundred per cent of the virus infected and rdLPS treated mice died between the 7th and 11th day following virus infection. Thus, the mortality rate was higher and death occurred earlier than in the untreated LCM group.

Simultaneously with infection of aged mice, parallel titration was performed on young animals. Hundred per cent of the mice infected with 100 LD_{50} of LCM virus died on the 7th to 9th day following infection.

Neither disease nor death occurred in the uninfected i.e. in the rdLPS treated or control groups.

To study the effect of treatment on the lymphoid system, 10 animals of the groups not subjected to virus infection were sacrificed on the 7th day after inoculation and the relative spleen weight and spleen index were determined. As compared to the untreated controls, rdLPS treated animals displayed increased spleen weight (spleen index = 1.8).

Discussion

The results showed that death occurred earlier and its rate was higher in aged mice treated with rdLPS and subsequently infected with LCM virus than in the virus infected but untreated control mice. Thus, in mice with thymus involution and insufficient T lymphocyte function due to aging, the rdLPS treatment contributed to the outcome of LCM infection in the form of lethal meningitis, i.e. it enhanced the reduced cellular immune reaction to virus infection.

The results obtained are in agreement with our earlier observation that rdLPS contributes to the development of the still insufficient cellular immune response of suckling mice [11]. Our earlier studies have also shown that *B. pertussis* vaccine has no enhancing effect on the reduced cellular immune response to LCM infection [8]. Both rdLPS and *B. pertussis* vaccine caused, however, spleen hypertrophy in aged mice.

The immunomodulatory effect of LPS preparations and *B. pertussis* on the immune response to LCM virus in mice is summarized in Table II.

Table II

Immunomodulatory effect of B. pertussis vaccine and LPS preparations on the immune response to LCM virus

Age of mice	The immune system		Effect of	
	State	Function	LPS	B. pertussis
Suckling	Undeveloped	Insufficient	Increased [11]	Increased [9, 10]
Young adult	Developed	Normal	No effect*	Diminished [8]
Old	Involuted	Insufficient	Increased	No effect [8]

* Unpublished data

LPS preparations enhanced the insufficient immune response to LCM virus in both aged and suckling mice. The immunomodulatory effect of *B. pertussis* vaccine depended on the age of the animals.

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EFFECT OF AN IRRADIATED *ESCHERICHIA COLI* ENDOTOXIN PREPARATION ON THE SENSITIVITY TO A LYMPHOTROPIC CYTOSTATIC AGENT IN GERMFREE AND CONVENTIONAL MICE

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A 18 mg/kg dose of dianhydrodulcitol, a lymphotropic cytostatic agent produced the same death rate among germfree as a 12 mg/kg dose did in conventional mice. Pretreatment with the same dose of an irradiated immunomodulatory endotoxin preparation had increased the sensitivity to these dianhydrodulcitol doses in the same degree in germfree as in conventiona mice. A study of the lymphoid organs and the intestinal wall indicate that both in germfree and conventional mice the dianhydrodulcitol sensitivity increasing effect of the endotoxin preparation was due to its stimulation of the lymphoid system. The higher resistance of germfree mice to dianhydrodulcitol is ascribed to their lack of a normal intestinal flora.

Several physiological and artificial conditions may influence the drug sensitivity of the organism. According to our earlier studies the presence or absence of the microbial flora plays a decisive role in the drug resistance of young adult mice. The sensitivity of germfree mice to dianhydrodulcitol (DAD), a lymphotropic cytostatic agent, was namely found to be reduced as compared to that of conventional mice. On the basis of the histological picture of their intestinal wall, the higher resistance to the drug of germfree mice was assumed to be due to the lack of endotoxin from their intestine [1].

The present study was undertaken to examine the effect of endotoxin on the sensitivity to DAD of germfree and conventional mice.

Materials and methods

Experimental animals. The experiments were carried out on 5–6-week-old germfree (Gf) and conventional (Cv) mice of both sexes weighing 20–25 g, purchased from the Zootechnical Institute for Laboratory Animals (LATI, Gödöllő, Hungary). The germfree state was maintained as described earlier [1].

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Treatment with dianhydrodulcitol (NSC-132313). Dianhydrodulcitol (DAD) is the epoxide of DBD, a lymphotropic cytostatic agent of the alkylating group [2]. The substance (Chinoin, Budapest) was dissolved in distilled water and used within 30 min.

Endotoxin preparation. The endotoxin was isolated by the hot phenol–water method of Westphal et al. [3] from a fermentor culture of *Escherichia coli* O89. The preparation was purified by repeated ultracentrifugation at 100 000 *g*. This native (parent) endotoxin was dissolved in distilled water and irradiated (15 Mrad = 150 kGy) at a concentration of 10 mg/ml in a ^{60}Co gamma source for radiodetoxification [4–5]. Preiliminary experiments showed that the parent endotoxin in a 10 mg/kg dose was highly toxic for mice with 50% of the mice dying on the 3rd day after treatment. The radiodetoxified endotoxin (rdLPS) used in a 10 mg/kg dose had no toxic effect whatever. The latter was used in the present work.

$$\text{Mortality index} = \frac{\text{mortality rate in the rdLPS-DAD group}}{\text{mortality rate in the DAD group}} .$$

Study of the lymphoid system. (a) The absolute lymphocyte count was determined in blood obtained from the caudal vein under standardized conditions. (b) Relative spleen and thymus weights and the spleen and thymus indices of mice died or sacrificed during the experiment were determined as follows:

$$\text{Relative lymphoid organ weight} = \frac{\text{lymphoid organ weight (mg)}}{\text{body weight (g)}} .$$

$$\text{Lymphoid organ index} = \frac{\text{mean relative lymphoid organ weight in the experimental group}}{\text{mean relative lymphoid organ weight in the control group}} .$$

Histological examination. The small intestine was prepared as described by Shirai et al. [6] then fixed and stained with haematoxylin-eosin.

Statistical evaluation was done by Student's two sample *t* test. The accepted level of significance was $p = 0.05$.

Experiments and results

Germfree (Gf) and conventional (Cv) mice were treated intraperitoneally with 10 mg/kg rdLPS. On the next day some of the Gf mice received 18 mg/kg, while some of the Cv mice received 12 mg/kg of DAD intraperitoneally. The control mice received physiological saline in the same way and quantity.

Table I

Mouse groups and treatments

Group	Treatment	
	1st day	2nd day
Gf —rdLPS-DAD	rdLPS	DAD
Gf —DAD	phys. NaCl	DAD
Gf —rdLPS	rdLPS	phys. NaCl
Gf —C	phys. NaCl	phys. NaCl
Cv —rdLPS-DAD	rdLPS	DAD
Cv —DAD	phys. NaCl	DAD
Cv —rdLPS	rdLPS	phys. NaCl
Cv —C	phys. NaCl	phys. NaCl

Each group consisted of 25 mice. The mouse groups and their treatments are demonstrated in Table I.

Four days after DAD treatment, thus 5 days after rdLPS treatment, 5 mice from each group were sacrificed and their ileum was processed for histology. In the DAD treated groups deaths were registered, and the relative spleen and thymus weights and indices of the died mice were determined. The experiment was terminated on the 21st day after DAD treatment.

On the 8th day after rdLPS treatment, when deaths occurred in the DAD treated groups, 10 mice each were sacrificed from the rdLPS and C groups. Before sacrifice, blood was taken for lymphocyte count determination. Afterwards the relative spleen and thymus weights and indices were determined. There was no death in the mouse-groups rdLPS and C during the 21 days of the experiment.

The rate and time curve of mortality in the DAD treated groups are presented in Fig. 1, showing an approximately similar death rate among Gf and Cv mice in spite of the differences in the dosage of DAD (groups Gf-DAD and Cv-DAD).

The mortality rate of Gf mice pretreated with rdLPS, was 100% while it was 90% in Cv mice (groups Gf-rdLPS-DAD and Cv-rdLPS-DAD). There was no significant difference in the time curves of death in the respective groups.

The data for relative spleen weight and the indices of the died and sacrificed mice are listed in Table II, showing that rdLPS treatment induced a

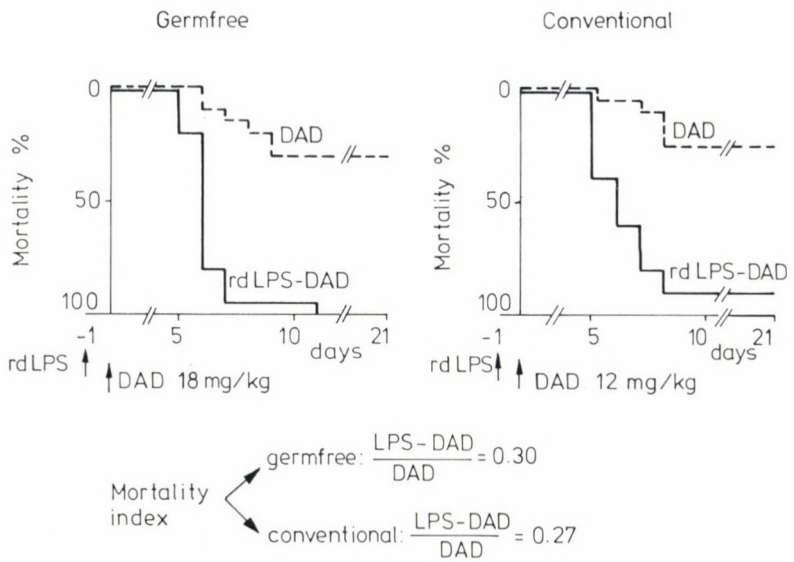

Fig. 1. Death rate and time curve in the DAD treated groups

significant hypertrophy in both Cv and Gf mice (groups rdLPS and C). DAD treatment caused severe spleen atrophy in both the rdLPS treated and untreated mice (groups Gf-DAD, Cv-DAD, Gf-rdLPS-DAD and Cv-rdLPS--DAD).

Table II

Relative spleen weight and spleen-indices in various mouse groups

Groups		Relative spleen weight		Spleen-index		
				DAD / C	rdLPS / C	rdLPS-DAD / rdLPS
Gf	—rdLPS-DAD	1.9 ± 0.6	$p < 0.001$	0.4	1.7	0.3
	—DAD	1.6 ± 0.4				
	—rdLPS	6.2 ± 1.4				
	—C	3.6 ± 0.9				
Cv	—rdLPS-DAD	2.4 ± 0.5	$p < 0.001$	0.5	1.6	0.4
	—DAD	2.1 ± 0.6				
	—rdLPS	6.1 ± 1.1				
	—C	3.8 ± 1.0				

The rdLPS treatment failed to cause a significant change in the absolute lymphocyte count and the relative thymus weight in the groups Gf-rdLPS and Cv-rdLPS as compared to the values obtained in the groups Gf-C and Cv-C.

Grave thymus atrophy was observed in the mice that had died of DAD treatment (thymus indices were about 0.3) as compared to the untreated groups. The rdLPS treatment failed to affect the degree of thymus atrophy.

On the 4th day after DAD treatment the ileum was examined in 5 mice each from the respective groups. The findings concerning the integrity of the intestinal mucosa and the eventual changes indicating the effect of endotoxin, like inflammatory infiltrations or oedema, were marked by crosses. The obtained results are summarized in Table III. It is seen that rdLPS treatment caused inflammation and oedema in the Gf mice while in the Cv mice no changes could be observed that would have been attributable to rdLPS (groups Cv-rdLPS and Cf-rdLPS). Presumably the Gf mice were sensitive to rdLPS since these animals met the endotoxin effect for the first time. DAD treatment caused inflammation, oedema, and necrosis both in Cv and Gf mice (groups Cv-DAD and Gf-DAD). Though Gf mice had received a higher dose, their inflammatory changes were slighter than those observed in Cv mice. On the other hand, the DAD elicited necrosis in the Gf mice treated with a higher dose was one grade graver (+++) than in the Cv mice treated with a smaller dose (++). The more serious changes could be equally observed in the Gf and Cv mice

receiving a combined treatment (groups Cv-rdLPS-DAD and Gf-rdLPS-DAD). The measure of alterations was equal among the two types of mice as to inflammation and necrosis but oedema was graver in Gf mice than in Cv mice.

Table III

Intestinal changes in the various mouse groups

Groups	Inflammation	Oedema	Mucosal necrosis
Cv-C	±	−	−
Gf-C	−	−	−
Cv-rdLPS	±	−	−
Gf-rdLPS	+	++	−
Cv-DAD	+++	+	++
Gf-DAD	+	+	+++
Cv-rdLPS-DAD	+++	++	+++
Gf-rdLPS-DAD	+++	+++	+++

Discussion

In previous experiments [1] the same dose of DAD produced a lower mortality rate and later death among Gf than among Cv mice. Grave spleen and thymus atrophy developed in both Cv and Gf mice dying of DAD treatment. On the other hand, the changes of the intestinal tract were significantly slighter in Gf mice than in Cv mice. Necrosis of the ileum was less severe in wall of the Gf mice and there was no inflammation or oedema. Consequently, the higher resistance to DAD of Gf mice may be explained by their germfree condition, since in this state the acute toxic effect of DAD is not amplified by the acute damaging effect of endotoxin as it occurs in the normal intestine.

Previous experiments have shown that the sensitivity to DAD of Cv mice with spleen hypertrophy, due to the immunomodulatory *Bordetella pertussis* vaccine, will increase as compared to unvaccinated controls. The lymphoid atrophy inducing effect of DAD was more expressed on the pertussis vaccine stimulated lymphoid system, and their combined administration caused a very grave thymus atrophy which may have accounted for the higher death rate [7].

Previous studies of rdLPS have demonstrated its immunomodulatory effect. It stimulates the cellular immune response in vivo [8, 9], increases the humoral immune response [10, 11], and leads to lymphocyte proliferation [12].

With our previous results in mind, this time we used a higher DAD dose (18 mg/kg) in the treatment of Gf mice than in that of Cv mice (12 mg/kg). The death rates in the two mouse groups (Gf-DAD and Cv-DAD) were nearly equal with this treatment schedule, again confirming the higher resistance of Gf mice to the applied cytostatic agent.

The same dose of rdLPS increased the drug sensitivity of Gf and Cv mice in a similar degree. After different DAD doses, the mortality index was 0.30 in the Gf, and 0.27 in the Cv mice.

The fact that the immunomodulatory, lymphoid proliferation inducing rdLPS increases the sensitivity to DAD might be brought into connection with its spleen hypertrophy inducing effect that was similar in the Gf and the Cv mice in accordance with previous results with *B. pertussis* vaccine. The lymphoid atrophy inducing effect of DAD could be observed more often in animals with stimulated lymphoid system and spleen hypertrophy due to rdLPS treatment, than in the mice not treated with rdLPS. That would explain the higher mortality rate of combined DAD-rdLPS treatment in both Gf and Cv mice.

The histological studies showed that the treatment with rdLPS had only slightly affected the DAD elicited intestinal changes in conventional mice while it had expressedly increased them in germfree mice as evidenced by oedema and inflammation, characteristic of an endotoxin effect. That confirms our suggestion that the increased drug resistance of germfree mice, as compared to the conventionals, is due to the absence of the endotoxin effect from normal intestinal flora. This may than account for the DAD sensitivity increasing effect of rdLPS as well as for the differences in DAD sensitivity of Gf and Cv mice.

In agreement with the results of *B. pertussis* vaccine treatment [7], the DAD sensitivity increasing effect of rdLPS might be explained by its lymphoid system stimulating and spleen hypertrophy inducing effects in both Cv and Gf mice. In the animals with rdLPS stimulated lymphoid system an irreversible lethal lymphoid atrophy was more frequent than in those not treated with rdLPS. In addition, this preparation increased the sensitivity to DAD of Gf mice by producing an intestinal damage characteristic of an endotoxin effect. The differences in DAD sensitivity between Gf and Cv mice, and the higher resistance of Gf mice can both be explained by the lack of endotoxin in the intestine.

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EFFECT OF IRRADIATED HAEMOPHILUS INFLUENZAE ENDOTOXIN PREPARATIONS IN MICE

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A detoxified substance (rdLPS) was produced from *Haemophilus influenzae* endotoxin by ionizing radiation and its capacity to prevent attacks of dyspnoea elicited by endotoxin inhalation in mice has been studied. The rdLPS proved to be an effective stimulant of aspecific immune resistance of mice but it could only partly prevent attacks of dyspnoea.

Endotoxin was prepared from the fermentor culture of a *Haemophilus influenzae* strain according to the method of Westphal et al. [1]. Mice made to inhale it developed acute dyspnoeic attacks leading with time to interstitial pneumonia. The effect was attributed to the direct cell damaging effect of the endotoxin and to the released mediator substances [2, 3].

Balogh et al. [4–7] have shown the protecting effect of radiation-detoxified endotoxin preparation of *Escherichia coli* (Tolerin®) in several endotoxin-induced diseases as well as the concomitant activation of the aspecific protecting mechanisms of the organism.

This gave the idea of detoxifying the lipopolysaccharide-type *H. influenzae* endotoxin (rdLPS) for studying the endotoxin induced dyspnoeic attacks under Tolerin® and rdLPS protection and the effect of rdLPS on dyspnoea state. Changes in the aspecific resistance of the organism were also studied.

Materials and methods

Bacterial strain. A noncapsulated *H. influenzae* strain was isolated from the pharynx of a patient with chronic bronchitis. The endotoxin prepared from a fermentor culture of this strain, the rdLPS prepared from this parent endotoxin and Tolerin® prepared from *E. coli* O89 endotoxin were used.

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Methods. (A) The endotoxins were prepared from the fermentor culture of the strains with the hot phenol–water method of Westphal et al. [1]. (B) The respective Tolerin® and rdLPS were prepared from the various endotoxins by irradiation with 150 kGy Cobalt gamma source [8].

The combined effect of Tolerin®, rdLPS and endotoxin was studied in CFLP white mice (LATI, Gödöllő). Each experimental group consisted of 10 animals.

1. The *E. coli* Tolerin® was administered intravenously, intraperitoneally or by intratracheal inhalation in a dose of 50 μg in order to determine whether it was able to prevent the dyspnoeic attack induced by 1 μg *H. influenzae* endotoxin inhaled 48 h afterwards.

2.1. *H. influenzae rdLPS* was given intraperitoneally in doses of 1 μg, 10 μg, 25 μg and 50 μg, respectively, followed by *H. influenzae* endotoxin inhalation in doses of 1 μg under light ether anaesthesia. The inhalation of endotoxin began 24 or 48 h later and was repeated daily for 10 days.

2.2. rdLPS was given intraperitoneally in a dose of 25 μg once daily for 3 days followed 48 h later by the inhalation of 1 μg endotoxin daily for 10 days.

2.3. Animals showing severe dyspnoea due to three endotoxin inhalation pretreatments received 1, 10, 25, or 50 μg rdLPS intraperitoneally, followed by renewed endotoxin inhalation 24 or 48 h later.

3. Twenty-four or 48 h after the inhalation of 1 μg, 10 μg, 25 μg, or 50 μg doses of rdLPS, the mice were made to inhale daily 1 μg endotoxin for 10 days.

4. The mice were made to inhale rdLPS in 1, 10, and 25 μg doses 10 times in order to study its direct effect on their respiration.

Immunological status of mice treated intraperitoneally with 25 μg rdLPS for 3 days.

1. Determination of the total peripheral WBC and lymphocyte counts.

2. Determination of the peritoneal cell count. The abdominal cavity was washed with 2 ml Parker's medium. The cell count was determined in Bürker's chamber.

3. Determination of macrophage phagocytosis [9]. The overetherized mice received intraperitoneally 2 ml Parker's medium containing heparin, 10% calf serum, buffer, Na-pyruvate and L-glutamate. The peritoneal fluid was then aspirated and centrifuged twice at 800 *g* at +4 °C for 20 min in heparin-free medium. The pellet was suspended in 2 ml medium and, after cell count determination, cultured in plastic Petri dishes, then incubated in a CO_2 atmosphere. Non-adhering cells were washed out after 2 and 24 h, then a *Candida albicans* suspension was added. Two hours later the phagocyte index, i.e. the average number of phagocyted fungi per cell was determined under a phase contrast microscope.

4. Granulocyte phagocytosis [9]. Mice were given 2 ml of 6% dextran solution (molecular weight, 200 000) intraperitoneally and 3 h later the peritoneal fluid was aspirated into plastic tubes containing 0.1 ml heparin and 0.1 ml physiological saline and centrifuged at 800 *g* for 15 min. The pellet was washed with Hanks' solution three times (800 *g*, 10 min), then suspended in 1 ml Hanks' solution and the cell count was determined in a Bürker chamber. Subsequently a suspension was prepared from *C. albicans*, containing 10 times more yeast cell per ml than the granulocyte suspension. The yeast suspension was added to the granulocytes and incubated at 37 °C for 3 h. Finally, smears were prepared, stained with Giemsa and the number of phagocyted yeast cells per granulocyte was determined.

Results

Table I shows the combined effect of the endotoxin and Tolerin®, rdLPS on the respiration.

1. Tolerin® prepared from *E. coli* parent endotoxin applied in high doses and given to the mice by various routes failed to reduce the dyspnoeic attacks induced by endotoxin inhalation 24 or 48 h later.

2.1. Twenty five and 50 μg rdLPS prepared from *H. influenzae* endotoxin administered intraperitoneally, mitigated the dyspnoeic attacks due to endotoxin inhalation 24 or 48 h later. Protection was most expressed if 50 μg rdLPS was applied after a 48 h incubation period.

Table I

Effect of Tolerin® and rdLPS on the dyspnoeic attacks due to endotoxin inhalation in mice

	Pretreatment	Provocation	Dyspnoeic response after 10 endotoxin inhalations
1.	*E. coli* Tolerin®		
	50 μg i.v.		strong
	50 μg i.p.		strong
	inhalation of 50 μg		strong
2.1.	*H. influenzae* rdLPS		
	1 μg i.p.		strong
	10 μg i.p.		strong
	25 μg i.p.		slight
	50 μg i.p.	1 μg *H. influenzae* endotoxin inhalation	slight
2.2.	25 μg i.p. rdLPS on three subsequent days		slight
2.3.	Inhalation of 1 μg *H. influenzae* endotoxin on three subsequent days, then intraperitoneal rdLPS in doses of	24 or 48 h later	
	1 μg		strong
	10 μg		strong
	25 μg		strong
	50 μg		strong
3.	*H. influenzae* rdLPS inhalation		
	1 μg		strong
	25 μg		strong
	50 μg		strong

The lungs of the animals were examined histologically. The groups pretreated with 25 or 50 μg rdLPS showed a mild form of interstitial pneumonia which developed in control mice with severe dyspnoea due to 1 μg endotoxin inhalation. The perivascular and peribronchial round cell infiltration consisted of less cells and involved smaller areas. The round cell infiltration consisted of lymphocytes, macrophages and some leukocytes. There were macrophage and lymphocyte collections in the alveoli, in their walls as well as in the alveolar septa (Figs 1, 2).

2.2. Intraperitoneal rdLPS administration repeated three times failed to further mitigate the dyspnoeic attacks due to endotoxin inhalation. The histological changes were not less, either.

2.3. An attempt was made to treat the mice that were pretreated with endotoxin three times and showed severe dyspnoea, with various doses of rdLPS in order to influence the dyspnoeic condition. The attacks did not, however, improve on rdLPS.

3. Pretreatment with rdLPS inhalation followed inhalation of 1 μg endotoxin resulted in severe dyspnoeic attacks. The rdLPS inhalation was not reaction-free, either.

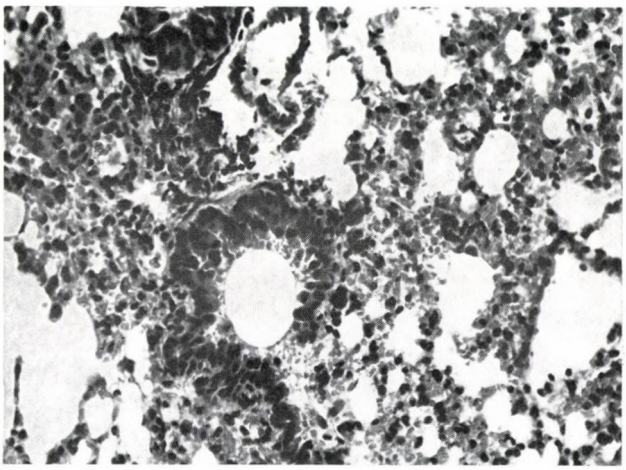

Fig. 1. Histological picture of mouse lung after 10 inhalations of 1 μg *H. influenzae* endotoxin. Haematoxylin–eosin staining, X63. Extended peribronchial and perivascular infiltration characteristic of interstitial pneumonia. Macrophage collections around the alveoli

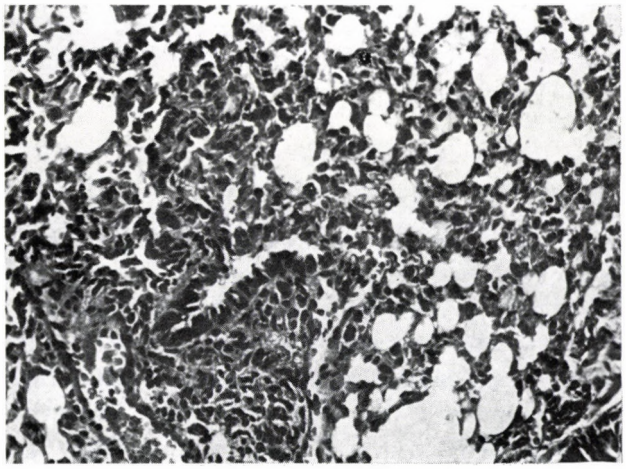

Fig. 2 .Histological picture of mouse lung pretreated with 50 μg rdLPS and 48 h later subjected to 10 inhalation of endotoxin. Haematoxylin–eosin staining, X63. The peribronchial and perivascular round cell infiltrations are slighter and less than those in Fig 1

4. The effect of serial rdLPS inhalation was then studied. After the tenth inhalation the same dyspnoeic attacks developed as in the controls under the effect of 1 μg endotoxin inhalation. Histology showed interstitial pneumonia but in somewhat milder form than after endotoxin inhalation (Fig. 3).

Table II summarizes the evaluation of the immunological state of 10 mice treated with rdLPS intraperitoneally 3 times. The 10 control mice received physiological saline in the same way and quantity.

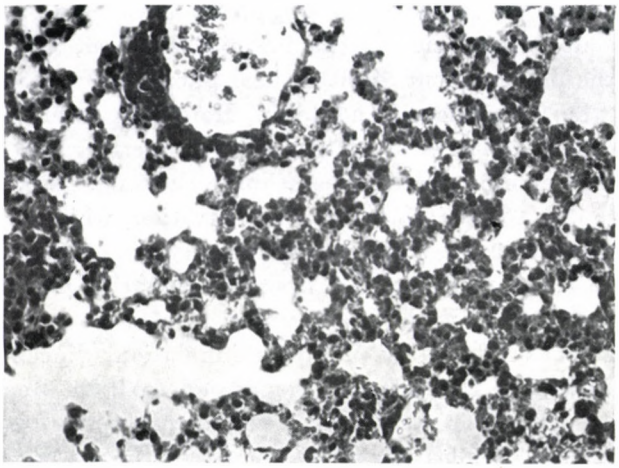

Fig. 3. Histological picture of mouse lung after 10 inhalations of 1 μg rdLPS. Haematoxylin–eosin staining, X63. Interstitial pneumonia of medium intensity

Table II

Immunological status of mice treated with rdLPS intraperitoneally 3 times (mean values)

		Test mice		Control mice
Peripherial blood	WBC count	7300 ± 500	$p < 0.001$	4500 ± 300
	absolute lymphocyte count	5000 ± 800	$p < 0.001$	3400 ± 400
Peritoneal cells	Total cell count	12.5×10^6 cell/mouse		6×10^6 cell/mouse
	Macrophage phagocytosis	28 *C. albicans*/cell		14 *C. albicans*/cell
	Granulocyte phagocytosis	15 *C. albicans*/cell		6 *C. albicans*/cell

1. The absolute WBC and lymphocyte count increased to double in peripheral blood after rdLPS treatment.

2. The total peritoneal cell count and the activity of peritoneal macrophages and granulocytes also increased to double.

Discussion

Tolerin® is the radiodetoxified *E. coli* O89 parent endotoxin developed by Bertók et al. [8]. It was shown to prevent successfully septic and haemorrhagic shock, liver damage in acute myocardial infarction, and endotoxin shock in animal experiments [4—7].

In previous studies it was observed that inhalation of *H. influenzae* endotoxin caused acute dyspnoeic attacks in mice and led, in case of chronic treatment, to the development of interstitial pneumonia [2, 3].

Studying the *H. influenzae* endotoxin, Raichvarg et al. [10, 11], Danny [12], and Dagre [13] reported on similar results asserting that the endotoxin impaired ciliar movements and RES function in the lung. Clarke [14] observed histamin release due to *H. influenzae* administration, while Snella et al. [15] registered an increase in the number of macrophages and granulocytes in the bronchial lavage fluid after bacterial lipopolysaccharide inhalation.

In the present study we have attempted to prevent or reduce by rdLPS the dyspnoeic attacks induced by endotoxin inhalation. Since *E. coli* Tolerin® had failed to influence the attacks, the radiodetoxified preparation of *H. influenzae* parent endotoxin was applied. In accordance with previous results [4–7], intraperitoneal application of 25 and 50 μg doses were found capable to mitigate the dyspnoeic attacks elicited by endotoxin after a 48 h incubation period in mice. Clinical signs and histological study showed that rdLPS was the most effective in a 50 μg dose. The effect of rdLPS pretreatment could be somewhat improved by repeating it three times, but still so it failed to offer protection against a fully developed dyspnoeic condition.

Since rdLPS by intratracheal administration had a direct effect, and had even provoked dyspnoeic attacks, serial rdLPS inhalation was attempted and as an experiment in vitro, the preparation was added to a HEp-2 tissue culture which displayed cytopathogenic changes due to *H. influenzae* endotoxin [3].

Serial rdLPS inhalation evoked increasing dyspnoeic attacks and histological changes took place in the lungs. In the HEp-2 tissue culture degenerative changes had appeared. It was concluded that the effect of endotoxin and that of rdLPS were identical on the lung tissue (alveolar epithelium, macrophages) and on the tissue culture.

As to the general immunological status of the animals after systemic rdLPS treatment, the preparation had increased not only the total WBC count and the absolute lymphocyte count but also the total peritoneal cell count and the phagocytic activity of peritoneal macrophages and granulocytes. Thus, in agreement with data in the literature we also found that rdLPS was capable of increasing the non-specific resistance of the organism [16–19]. This general stimulatory effect does not, however, mean a perfect protection against the dyspnoeic attacks induced by endotoxin.

For the stimulation of non-specific resistance only systemic administration is suited since the changes in the lung after rdLPS inhalation showed that irradiation of the parent endotoxin had not significantly modified the effect of the endotoxin on the HEp-2 tissue culture and on the alveolar epi-

thelial cells and macrophages. This means in other words that direct cell effects occur and the release of mediator substances might provoke dyspnoeic attacks.

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RESISTANCE TO BACTERIA OF PLASTICS USED IN DENTAL PRACTICE

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The resistance to bacteria of composition filling materials (Evicrol®, Isopast®, Micromix® and Superlux®), of acrylic base-plate used for removable dentures and of acrylic (Medident®) teeth has been examined. Out of the 6 samples tested with *Pseudomonas aeruginosa* in accordance with the Hungarian standard, Evicrol®, Superlux® and the base-plate proved to be resistant; Isopast® was attacked moderately and Medident® teeth and Micromix® showed intermediate results.

Microbiological corrosion of plastics which otherwise are resistant to physical and chemical influences has been observed in an increasing number of cases. Due to their organic composition and to materials used in the course of their production, plastics can serve as carbon source for microbes. Proliferating microbes may damage the plastics and thus cause hygienic and technical injuries and iatrogenic infections [1–6].

Plastics were adopted into dental practice about 40 years ago. The base material of dental prostheses is built up from acrylic resin macromolecules, composed of methacrylic acid methylester. Acrylic resin is used to make crownworks, bridges, aesthetic fillings, partial or total dentures; in these cases both the teeth and the base-plate consist of acrylic resin. Due to its numerous disadvantages, however, acrylic resin has been practically replaced by compositional filling materials. Their ground material consists of organic and inorganic components. The organic part is the product of a reaction between metacrylic acid and an epoxy resin; the bond between organic and anorganic compounds is guaranteed by silans which are organic silicon-compounds.

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Besides their advantage, disadvantages have also been reported. Thus, *Pseudomonas aeruginosa* can proliferate on plastic denture base-plates that remain soft [7] or on the tube of the turbine-spray [8–11].

In view of the above-mentioned observations, it seemed worthwile to examine the behaviour of plastics used in dental practice towards microbes.

Materials and methods

Tested samples. Acrylic base-plate (Spofa–Dental, Czechoslovakia) used for removable dentures; surface 45 cm^2. Medident® teeth (Industry for Dental Technology, Hungary) used for removable dentures, surface 5 cm^2. Composition filling materials: Evicrol® (Spofa, Czechoslovakia), which is organic–inorganic, Isopast® (Vivadent, Liechtenstein), Micromix® (Platzer Dental, Wien) Superlux® (Dental Material Ges., Hamburg) which contain organic–organic components.

The filling materials were smeared on both sides of a 2.5 × 1 cm coverslip and then printed flat. The surface was 5 cm^2. Each sample was examined in triplicate.

Examination of microbiological corrosion. We followed the MSz 8888/25–75 home standard developed according to international standards [12, 13]. Organic materials as carbon sources were tested with *P. aeruginosa* in carbon-free solution containing NH_4NO_3 1 g; KH_2PO_4, 1 g; Na_2HPO_4, 1 g; NaCl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; saturated $FeCl_3$ solution, 3 drops in 1000 ml of distilled water. The test strain was isolated from a patient.

If the tested sample contained an exploitable carbon source, the number of viable *P. aeruginosa* cells was rising from the initial 10^5/ml value. The materials were qualified on basis of the viable cell-maximum. The number of viable cells determined in carbon-free solution or in a solution containing dioctyl-sebaceic acid served as controls. In the latter solution the maximum germ count (N_{max}) exceeded 10^9/ml, while in the former it remained under 10^7/ml.

Thus the sample was considered resistant (R) if the germ count did not exceed 10^7/ml; moderately attackable (MA) where N_{max} was between 10^7–2×10^8/ml, and attackable (A) where N_{max} exceeded 2×10^8/ml. The quantity of the solution was 1 ml per 1 cm^2 sample. The examination was done at 37 °C, during a period of 28 days.

The behaviour of samples soaked in sunflower oil was also examined. The samples were weighed and kept in the oil for 14 days, then the rest of the oil was blotted up with paper and the samples were weighed again. They all weighed more than before treatment. After exposition to *P. aeruginosa* at 37 °C for 28 days, the excess weight disappeared and the germ count increased.

Results

Resistant were the base-plate, Evicrol® and Superlux®. Isopast® was attacked moderately and Medident® teeth and Micromix® showed intermediate behaviour (Table I).

Table II shows the mean weight of samples, the quantity of oil fixed to the samples and the results concerning their surface and weight. A significant quantity of oil was fixed to each sample and each sample was attacked by *P. aeruginosa*, the germ count exceeded 2×10^8/ml.

The quantity of fixed oil — in the case of Isopast® and Micromix® and except the base-plate — resulted in an increase of the germ count above 10^9/ml.

Table I

Qualification of samples according to MSz 8888/25–75 standard

Samples	Cells/ml	Qualification
Evicrol®	$<10^7$	R
Isopast®	3×10^7	MA
Micromix®	10^7	R/MA
Superlux®	$<10^7$	R
Medident® tooth	10^7	R/MA
Base-plate	$<10^7$	R
Controls		
Dioctyl—sebaceic acid	$>10^9$	
Sunflower oil	$>10^9$	
Carbon-free solution	$<10^7$	

Table II

Samples kept in sunflower oil

Samples	Weight of			Fixed oil/sample per cent	Cells/ml
	samples mg	oil fixed mg	oil fixed to samples mg/cm²		
Evicrol®	598	170	34	28.4	$<10^9$
Isopast®	406	174	34.8	43	$>10^9$
Micromix®	395	178	35.7	45	$>10^9$
Superlux®	336	178	35.7	53	$<10^9$
Medident® tooth	1304	174	34.8	13.3	$<10^9$
Base-plate	4800	26	0.57	0.0054	$<10^9$
Controls					
Dioctyl-sebaceic acid					$>10^9$
Sunflower oil					$>10^9$
Carbon-free solution					$<10^7$

During the testing period each sample came off the coverslip supposedly due to swelling caused by the fixed oil.

The following sequence could be established as regards the affinity to oil: filling material > Medident® teeth > base-plate.

Discussion

From the examined samples, Isopast® proved to be directly exploitable by microbes, therefore it may be a culture medium not only for *P. aeruginosa* but also for the *Candida* species occurring in the oral cavity [7, 14]. Depending on their affinity toward oil or liposoluble materials, the other samples may also serve as culture media. The oral flora can therefore be altered and as a consequence the incidence of these microbes may rise in the dysbacterioses noticed during antibiotic therapy and in respiratory infections. Due to the swelling of the filling-materials an increased formation of secondary caries can be observed. Discolouration may also originate, apart from pigmented foods, from microbes. One should not disregard the epithelial atypias due to the persistance of probable liposoluble carcinogens.

Nyárasdy et al. [15] examined 338 compositional fillings, 13% of which developed secondary caries. They found loss of material in 73% of the fillings; marginal sealing was insufficient in 77%; colour and transparency were perfect only in 13%.

It is supposed therefore that on the basis of these observations the attackability of filling-materials and microbiological corrosion might be brought into correlation.

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BACTERIAL COLONIZATION OF NEWBORN INFANTS IN AN INTENSIVE CARE UNIT

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Bacterial screening of external ear, umbilical and gastric fluid samples from infants at admission to the intensive care unit yielded opportunistic pathogens in 22% and 12% in the years 1980 and 1982, respectively. As a result of improved hygienic measures and more rational use of antibiotics, bacterial colonization of infants staying longer than 3 days decreased from 56% to 19%. Antibiotic treatment is not indicated if opportunistic pathogens except *Streptococcus agalactiae* (group B), are cultured from infants without clinical symptoms.

Establishment and functioning of intensive care units for infants have effectively contributed in the last years to decreasing the frequency of perinatal mortality. Intensive care, however, is closely associated with apparatuses and antibiotic treatment, which considerably enhances the chance of bacterial colonization and neonatal infections [1–9]. The causative agents of neonatal infections in a ward vary at different periods of time [10]. To obtain data for a more effective control of potential infection hazards, in the years 1980 and 1982 we conducted a surveillance in our intensive care unit.

Materials and methods

Bacteriological screening of external ear, umbilical and gastric fluid samples was performed on the day of admission and several times during hospitalization. Blood and CSF samples were screened when the obstetric history suggested a potential infection. Catheter tips were cultured after exchange transfusions. Plastic tubes, intravascular catheters and cannulae removed from the babies were also screened. Diagnostic bacteriological examinations were carried out whenever indicated by the clinical symptoms (sepsis, local lesions, etc).

Bacteriological methods were as described in Standard Methods (National Institute of Hygiene, Budapest). *Streptococcus* group B strains were serotyped in the National Institute of Hygiene.

Results

Screening cultures at admission yielded opportunistic pathogens in 22% in 1980 and 12% in 1982. Clinical signs of infection (sepsis, pneumonia) were recorded in 8 and 13 infants, respectively. Infants without symptoms were

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Table I

Opportunistic pathogens isolated at screening from gastric fluid, external auricular canal and umbilicus of newborn infants

	No. of infants positive at admission*		No. of infants colonized during hospitalization	
	1980	1982	1980	1982
Pseudomonas aeruginosa	20	14	51	15
Escherichia coli	18	3	30	9
Klebsiella	10	4	40	19
Staphylococcus aureus	9	1	7	6
Enterobacter	4	—	8	2
Acinetobacter	2	1	6	1
Haemophilus influenzae	1	—	—	—
Streptococcus agalactiae (group B)	1	6	—	—
Streptococcus pyogenes	—	1	—	—
Streptococcus faecalis	—	—	—	1
Two different bacteria	4	3	4	2
Total	69	33	146	55

* No. of infants older than 24 h: 10 in 1980 and 7 in 1982

Table II

Opportunistic pathogens isolated from the blood of newborn infants

	1980 No. of infants positive			1982 No. of infants positive		
	at admission	on further examination		at admission	on further examinations	
Klebsiella	1	10	(11)	1	6	(7)
Pseudomonas aeruginosa	1	3	(4)	1	3	(4)
Staphylococcus aureus	—	3	(3)	—	—	
Staphylococcus epidermidis	1	1	(2)	2	4	(6)
Enterobacter	1	—	(1)	—	1	(1)
Haemophilus influenzae	1	—	(1)	—	—	
Escherichia coli	1	—	(1)	—	2	(2)
Streptococcus alpha-haem.	1	—	(1)	1	—	(1)
Streptococcus agalactiae (group B)	—	—		4	—	(4)
Streptococcus pyogenes	—	—		1	—	(1)
Candida albicans	—	2	(2)	—	—	
Total	7	19	(26)	10	16	(25)

Figures in brackets show the total number of blood samples

regarded as colonized if one or more bacterial species could be cultured at least from two different sites or on two or more occasions from one site.

Infants staying in the intensive care unit for 3 or more days were colonized in 56% in 1980 and 19% in 1982. Table I shows bacteria cultured on screening. As compared to 1980, in 1982 the number of Gram-negative bacteria decreased whereas that of group B streptococci increased. Positive results were encountered most frequently in gastric fluid samples. With one exception, infants colonized by streptococci at different sites, harboured the agent also in their external auditory canal.

As shown in Table II, the incidence of bacteriologically confirmed septicaemia was 26 in each year, i.e. for the total number of patients, 8.2% in 1980 and 9.0% in 1982. The presence of group B streptococci in blood cultures in 1982 was in correlation with their increase in other samples. In 9 infants the organism cultured from blood was shown from other sites of the corresponding infant. B group streptococci belonged to serotypes I/a, I/a–I/b, I/c, II and III^{8}R.

Discussion

In intensive care units for newborn infants the number of nosocomial infections is comparatively high. Goldmann et al. [2] described a 15.3% frequency of infections associated with Gram-negative bacteria (70%) including klebsiellae (25%); staphylococci played a less prominent, although important role. Korányi and Vörös [11] reported that 24% of newborn infants were colonized by klebsiellae. Eisenach et al. [1] described that in a 12-month period 10% of the patients were infected with a kanamycin resistant R factor carrying enteric bacterium; the frequency of colonization was 90%.

Várkonyi [12] examined perinatal colonization in a 15-month period. At admission the frequency of colonization was 22%; Gram-positive bacteria were present in 61.2%, Gram-negative bacteria in 77.9% of the colonized infants. Infections accompanied by clinical symptoms were caused in 11.9% by Gram-positive and 17.9% by Gram-negative bacteria.

Borderon et al. [13] have shown that under normal conditions newborn infants are colonized by antibiotic sensitive *Escherichia coli*. As an effect of antibiotic treatment, resistant *E. coli*, *Klebsiella*, *Citrobacter* and *Pseudomonas* become predominant.

Screening at admission may contribute to the prevention of infections by opportunistic pathogens. The mere presence of these at different sites of infants displaying no clinical symptoms of infection does not justify antibiotic treatment. An exception is *S. agalactiae* (group B), which is more invasive than the others; accordingly, we prescribed antibiotics whenever it was isolated on screening. If sepsis is suspected, blood culture is essential, since bacterial

samples taken from different sites may give a negative result and even later the colonizing agent is not necessarily identical with the organism invading the blood stream.

In our ward the frequency of opportunistic pathogens decreased considerably in 1982 as compared to 1980. The change may be attributed mainly to a rigorous checking of hygienic measures including hand disinfection and to a more rational application of antibiotics.

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