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KLEBSIELLA PNEUMONIAE ENTEROTOXIN

I. EFFECT OF AERATION ON PRODUCTION AND TOXICITY ASSAY IN ANIMALS

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(Received May 26, 1980)

The toxin produced by aerobic stationary and anaerobic cultivation elicited a more marked gut dilatatory response than that obtained in aerobic shaken culture. For its assay, the rabbit ileal loop model proved satisfactory, whereas the rat ileal loop test yielded inconsistent results and the suckling mouse test was unsuitable.

Although klebsiellae were reported from diarrhoeal cases [1] these organisms were not thought to have an enterotoxic activity like enteropathogenic *Escherichia coli*. Later, klebsiellae were reported from cases of tropical sprue [2] and were the prominent species in some children and adults with acute diarrhoea [3, 4]. Now it is already known that *Klebsiella pneumoniae* possesses enterotoxigenic properties like *E. coli* and some elaborate toxin inducing fluid secretion in animals [5–7]. These, too, have been found to stimulate the conventional assay system for enterotoxins [8–10]. Others [11] showed that these strains induced water secretion or diarrhoea, when administered orally to the experimental animals.

These studies have made us to study the possibility of enterotoxin production by a laboratory strain of *K. pneumoniae* under different laboratory conditions.

Materials and methods

Source of organisms. A laboratory strain of *K. pneumoniae* (B-45-1), obtained from the Central Research Institute, Kasauli, India, was used to produce the enterotoxin. It was maintained on nutrient agar and stored at 4 °C for use.

Preparation of seed culture. For the production of enterotoxin, the medium of KLIPSTEIN and ENGERT [12] was used. A loopful of *K. pneumoniae* culture was added to 50 ml of growth medium, incubated at 37 °C for 6 h which constituted the seed culture for the production of enterotoxin.

Production of enterotoxin under different cultural conditions. To study the effect of aeration on the yield of enterotoxin, it was produced in aerobic shake, aerobic stationary and anaerobic culture. A seed culture of 0.5 ml was added to each of the 5 flasks of 1 litre capacity, containing 250 ml of growth medium. Two, 1 and 2 of these, respectively, were incubated under aerobic stationary, anaerobic and aerobic shaken conditions, respectively, at 37 °C for 18 h. Each broth culture was then centrifuged at 28 000 *g* for 45 min in a refrigerated centrifuge. The cell-free supernatant containing toxin was decanted, concentrated to 8–10 times of its volume in a rotating vacuum evaporator (Metrex Institute, India) in a water bath at 40 °C, filtered through Seitz filter and finally dispensed in sterile 10 ml vials and stored at −20 °C for subsequent use. The sterile growth medium treated similarly was used as a control of the toxin.

The sedimented cells, washed twice in phosphate buffer saline (PBS) pH 7.2, were used to prepare the cell lysate [13] by suspending these in 10 ml of PBS. The cells were disintegrated in an ultrasonic vibrator (Vibrionic Ltd., India) at 25 kilocycles/second for 30 min and then centrifuged at 7000 *g* for 15 min to remove the cell debris. The supernatant was the cell lysate used in the toxicity test. The total protein concentration of concentrated toxin and of the cell lysate was estimated [14], using crystalline bovine albumin as reference.

Determination of the number of organisms and the toxicity. The relation between the number of organisms and the toxin content of the culture was studied by the viable plate count method. The toxin produced under stationary aerobic conditions, and harvested at 0, 4, 8, 24, 36 and 48 h, was used for estimating its activity in the rabbit ileal loop model. The number of organisms at each of the above time intervals, was also determined by the viable plate count method.

Suitability of laboratory animal models for toxin assay. Infant mice, rats, rabbits and rats were used. (i) *Infant mice and rats.* Fifteen newborn suckling mice and 15 rats were used. Each animal received intragastrically [15] 0.1 ml of toxin, mixed with a drop of 1% pontamine sky blue dye, while the controls received the same amount of sterile growth medium mixed with the same dye. The animals were sacrificed after 1, 2, 4, 8 and 12 h. The weight of the gut and of the remaining body was recorded and toxicity was expressed as a ratio of gut weight per remaining body weight. Values above 0.09 were considered to mean a positive response.

(ii) *Rabbit ileal loop method.* The rabbit ileal loop method was used to study enterotoxicity [16]. Rabbits weighing 1.5 to 2.0 kg were fasted for 24 h before use. The small intestine exposed by laparotomy, was ligated into small loops measuring 10–12 cm. Ten loops were used in duplicate for each test and control. Each loop was injected with 2 ml of cell-free supernatant intraluminally and the control loop with the same amount of sterile growth medium. The animals were sacrificed after 4, 8, 12, 18 or 24 h. The volume of fluid accumulated in each test and control loop and its length were recorded. The gut dilatatory response was expressed as ml of fluid accumulated per cm of inoculated loop length and values of 1 or more were considered a positive response.

(iii) *Rat ileal loop model.* The above method was followed for testing enterotoxin in the rat ileal loop. Each loop received 1 ml of toxin and sterile growth medium as a control, respectively. The rats were then sacrificed at intervals of 4, 8, 12, 18, 24 and 36 h and the volume of accumulated fluid in the test and control loops and their length were recorded. The gut dilatatory response was expressed similarly as in the rabbit ileal loop method, and values beyond 0.3 were considered a positive response.

Results

The protein concentration of the concentrated toxin produced under three different cultural conditions was estimated and tested for its toxic activity in different laboratory animals. The protein concentration differed under different conditions. It was 20, 16.6 and 12.2 mg/ml in the stationary aerobic, anaerobic and aerobic shaken cultures, giving gut dilatatory values of 1.55, 1.21 and 0.67, respectively (Table I). The toxic activity appeared 8–10 h after inoculation and continued to grow up to 24 h in terms of gut dilatatory response of 1.486 (Fig. 1). Interestingly a direct correlation of the number of organisms and the production of toxin was noticed (Fig. 2); 7.8×10^5 organisms/ml gave maximum toxic values of 1.46 at 18 h of incubation. The number of organisms decreased with the decrease in toxin production. The effective dose of the toxin was found to be 19.6 mg/ml, giving a value of 1 and was considered to represent one effective dose.

Among the laboratory animals tested, the rabbit ileal loop model (Table II) gave encouraging positive results with a gut dilatatory value of 1.55, where-

Table I

Effect of aeration on the yield of enterotoxin and its toxicity in the rabbit ileal loop

Aeration condition	Protein mg/ml	Observation	Experiment-1			Experiment-2			Experiment-3			Mean ratio ± SD
			Fluid, ml	Length, mm	Fluid/length ratio	Fluid, ml	Length, mm	Fluid/length ratio	Fluid, ml	Length, mm	Fluid/length ratio	
Stationary aerobic	20	Test	15.2	10.1	1.5	16.4	10.6	1.55	15.8	9.9	1.60	1.55 ±0.07
		Control	1.6	7.9	0.2	2.1	8.1	0.25	1.6	6.9	0.23	0.226±0.033
Anaerobic culture	16.6	Test	12.2	10.1	1.20	12.6	10.1	1.25	11.5	9.5	1.20	1.21 ±0.158
		Control	1.6	7.9	0.2	2.1	8.1	0.25	1.6	6.9	0.23	0.226±0.033
Aerobic shaken culture	12.2	Test	5.4	7.7	0.70	5.1	8.2	0.63	6.5	9.5	0.68	0.67 ±0.038
		Control	1.6	7.9	0.20	2.1	8.1	0.25	1.6	6.9	0.23	0.226±0.033

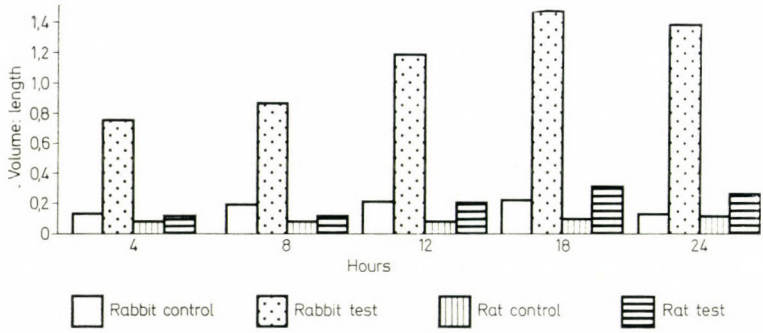

Fig. 1. Enterotoxigenic activity of *Klebsiella pneumoniae* (B-45-1) enterotoxin at different time intervals in rabbit and rat ileal loop

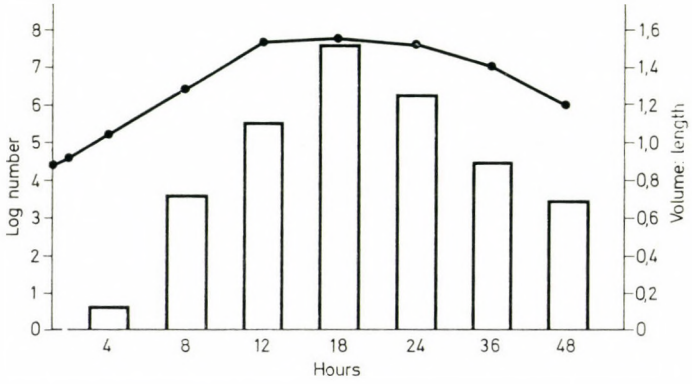

Fig. 2. Number of organisms producing enterotoxin and its enterotoxigenicity at different time intervals

as the rat ileal loop model was somewhat weaker and other models failed to give a positive response. The cell lysate failed to induce any gut dilatatory response in the rabbit ileal loop model.

Discussion

On the basis of toxin yield in terms of protein concentration and toxicity, the toxin produced aerobically under stationary conditions and anaerobically, gave a more marked gut dilatatory response than did the aerobic shaken culture. Its protein concentration was also higher under these conditions. Protein concentration of the toxic material was 20 and 16.6 mg/ml, respectively, under stationary aerobic and anaerobic conditions, and gave a gut dilatatory response of 1.55 and 1.25, respectively, which did not differ significantly. Therefore, the toxin was produced under stationary aerobic conditions.

Table II

Comparison of various animal models for the determination of their suitability for assay of K. pneumoniae (B-45-1) enterotoxin

Sample	Infant mouse gut			Infant rat gut			Rat ileal loop			Rabbit ileal loop		
	Gut wt, mg	Body wt, g	Mean gut/body wt ± SD	Gut wt, mg	Body wt, g	Mean gut/body wt ± SD	Fluid, ml	Length, cm	Vol./length ratio ± SD	Fluid, ml	Length, cm	Vol./length ratio ± SD
	133	2.093	0.062	361	6.103	0.056	3.6	8.1	0.406	15.2	10.1	1.55
Test	154	2.504	±	345	5.455	±	3.15	7.8	±	16.4	10.6	±
	125	1.99	0.002	349	6.980	0.012	3.50	9.2	0.093	15.8	9.9	0.07
	120	2.63	0.056	365	7.01	0.055	0.83	8.5	0.12	1.58	7.9	0.26
Control	122	2.73	±	368	6.70	±	0,95	7.9	±	2.05	8.1	±
	125	2.72	0.012	347	6.00	0.001	1,1	7.8	0.02	1,60	6.8	0.033

In previous studies, the toxin was found to be heat stable. These findings differed from those of other authors [24] who reported that *K. pneumoniae* produced heat stable (ST) enterotoxin anaerobically, whereas still others [25] reported that it produced both heat labile (LT) and ST under stationary anaerobic conditions but no toxin in shaken cultures. The latter investigators reported that only ST was produced under stationary aerobic conditions, which agrees with the present observations in that the toxin produced under stationary aerobic conditions was heat stable. On the other hand, ST might perhaps be produced under both aerobic and anaerobic conditions whereas the latter perhaps favoured the production of ST only.

The cell lysate failed to induce any gut dilatatory response, suggesting the absence of LT. It is not, however, unusual to come across such strains, producing either ST or LT alone or both, as KLIPSTEIN *et al.* [6] observed that out of 5 strains of *K. pneumoniae* only one strain produced both ST and LT whereas 3 produced ST and only one produced LT only. The strain under study produced only ST.

Infant mice were reported to be a suitable animal model for the assay of *E. coli* enterotoxin [15] but this model failed to work with the present enterotoxin. The same was the case with infant rats, but it needs repeated attempts to assess their suitability, but differences in the various species should be ruled out before labelling them completely unsuitable. Neither were observations with the rat ileal loop model encouraging as very high concentrations of toxin were needed and the results were inconsistent. On the other hand, the rabbit ileal loop model proved satisfactory and gave a satisfactory gut dilatatory response. Other authors [12] found it suitable for the assay of *K. pneumoniae* enterotoxin.

The LT of *E. coli* and the enterotoxin of *Vibrio cholerae* induce fluid accumulation in 8–10 h [16, 26] and the ST of *K. pneumoniae* and of *E. coli* also induced it in 2–18 h [27]. All the above observations presumably indicate its similarity with the LT of *E. coli* and the enterotoxin of *V. cholerae*.

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REFERENCES

1. OLARTE, J., FERGUSON, W. W., HENDERSON, N. S., MICH, L., TORREGROSA, L., MEXICO, D. F.: Amer. J. Dis. Child. **101,** 763 (1961).
2. KLIPSTEIN, F. A., HOLDEMAN, L. V., CORCINO, J. J., MOORE, W. E. C.: Ann. intern. Med. **79,** 632 (1973).
3. COELLO-RAMIREZ, P., LIFSHITZ, F., ZUNIGA, V.: Pediatrics **49,** 233 (1972).
4. HEYWORTH, B., BROWN, J.: Arch. Dis. Child. **50,** 27 (1975).
5. KLIPSTEIN, F. A., HOROWITZ, I. R., ENGERT, R. F., SCHENK, E. A.: J. clin. Invest. **56,** 789 (1975).

6. KLIPSTEIN, F. A., ENGERT, R. F., SHORT, H. B.: J. infect. Dis. **136,** 205 (1977).
7. KLIPSTEIN, F. A., ENGERT, R. F., SHORT, H. B.: Lancet **2,** 342 (1978).
8. GUERRANT, R. L., DICKENS, M. D., WENZEL, R. P., KAPIKIAN, A. Z.: J. Pediat. **89,** 885 (1976).
9. GUERRANT, R. L., MOORE, R. A., KIRSCHENFELD, P. M., SANDE, M. A.: New Engl. J. Med. **293,** 567 (1975).
10. WADSTROM, I., AUS. KETTIS, A., HABTE, D., HOMGREN, J., MEEUWISSE, G., MOLLBY, R.,
11. KLIPSTEIN, F. A., GOETSCH, C. A., ENGERT, R. F., SHORT, H. B., SCHENK, E. A.: Gastroenterology **76,** 341 (1979).
12. KLIPSTEIN, F. A., ENGERT, R. T.: J. infect. Dis. **123,** 200 (1975).
13. SMITH, H. W., GLYES, C. L.: J. med. Microbiol. **3,** 387 (1970).
14. LOWRY, O. H., ROSENBUROUGH, A. L., FARR, A. L., RANDELL, R. J.: J. Biochem. **193,** 265 (1951).
15. DEAN, A. G., CHING, Y. C., WILLIAMS, R. G., HARDEN, L. B.: J. infect. Dis. **125,** 407 (1972).
16. DE, S. N., CHATTERJEE, D. N.: J. Path. Bact. **66,** 559 (1953).
17. CAMPBELL, D. H., GARVEY, S. J., CREMER-SUSSDORF, D. H.: Methods in Immunology. A Laboratory Text for Instruction and Research. 2nd ed. Benjamin Inc., New York 1972.
18. BURROWS, W., MUSTEIKIS, G. M.: J. infect. Dis. **116,** 183 (1966).
19. KASAI, G. J., BURROWS, W.: J. infect. Dis. **116,** 606 (1966).
20. JACK, T. M., WU, B. J., BRAEMER, A. C., BIDLACK, D. E.: Infect. Immunol. **7,** 178 (1973).
21. JACK, T. M., WU, B. J.: Infect. Immunol. **6,** 342 (1971).
22. CRAIG, J. P.: Nature (Lond.) **207,** 614 (1965).
23. EVANS, D. J. JR., EVANS, D. G., GORBACH, S. L.: Infect. Immunol. **8,** 725 (1973).
24. KLIPSTEIN, F. A., ENGERT, R. F.: Infect. Immunol. **13,** 373 (1976).
25. KLIPSTEIN, F. A., LEE, C. S., ENGERT, R. F.: Infect. Immunol. **14,** 1004 (1976).
26. KLIPSTEIN, F. A., HOROWITZ, I. R., ENGERT, R. F., SCHENK, E.: J. clin. Invest. **56,** 799 (1975).
27. EVANS, D. G., EVANS, D., PIERCE, N. F.: Infect. Immunol. **7,** 873 (1973).

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IMMUNE REGULATORY FACTORS IN SHEEP FETUSES

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After an antigenic stimulus *in vitro*, a fraction was found in the supernatant fluids of spleen (S-1) and thymus (T-1) cell cultures of 70 to 80-day-old sheep fetuses infected with ovine adenovirus *in utero*. The fraction agreed with alpha-1-fetoprotein in mobility and its molecular weight was 67 000. Injected into adult sheep, S-1 and T-1 had a suppressive effect on the humoral immune response to both adenovirus and *Clostridium perfringens* D vaccine. Tests with peripheral lymphocytes suggested that fetal cell supernatants do not affect blastogenesis but markedly inhibit Ig synthesis as it could be demonstrated by immunofluorescence tests.

In recent years several data have shown that the immune regulatory factors are chemical messengers. Their main function is to stimulate or suppress the immune response, i.e. to evoke a quantitative or qualitative modification of the immune response [1–4].

Knowledge on the regulation of the fetal immune system is incomplete. It has been shown previously that at a definite stage of development the lymphoid system of the mammalian fetus gains in immunological competence [5–11] and all conditions of the immune response appear. Nevertheless, several data suggest that the immune responsiveness of the fetus is limited and is influenced by suppressive materials [12].

Fetal alpha-fetoprotein (AFP) has been shown to suppress both the primary and the secondary immune response under experimental circumstances [13, 14] and to inhibit the blast transformation of lymphocytes induced by mitogens [15].

Since fetal AFP is detectable in the newborn's serum for a certain period [16, 17], it probably plays a role in the reduced immune responsiveness of not only the fetus but of the newborn as well [18].

Earlier, we have already studied the changes of fetoprotein and lipoids in the serum, amnion and allantois of sheep fetuses at different stages of gestation and their immune response to adenoviruses [19]. The present investigation aimed at studying the function and ontogenesis of the ovine immune system, the substances secreted *in vitro* by fetal spleen and thymus cells and their effect on the development of the immune response of adult vaccinated animals.

Materials and methods

Intrauterine infection of fetuses. Experiments were performed on fetuses carried by 7 merino ewes. At 70–80 days of gestation after laparotomy the fetuses of 5 ewes were infected intramuscularly with the virus strain ORT/111, isolated from sheep. This virus strain is related to the bovine adenovirus type 2 [20]. A 10^5 $TCID_{50}$/ml virus suspension was used for the infection. The fetuses of 2 ewes served as controls and were inoculated with the virus-free tissue culture maintenance fluid. The ewes were killed by bleeding on the 19th day after infection and then thymus and spleen specimens were taken from their fetuses.

Production of cell supernatant fluids from spleen and thymus. The specimens were homogenized in PBS in a glass homogenizer. The coarse sediment was discarded then the cells were separated with Ficoll-Paque and suspended in PBS at a cell density of 5×10^6/ml. The cell suspensions of adenovirus infected or control fetuses were pooled separately. The cell suspensions were treated with heat-inactivated (80 °C) 10^5 $TCID_{50}$/ml suspension of the ovine adenovirus strain ORT/111 at 100 : 1, then incubated at 37 °C for 24 h. The spleen and thymus cell supernatants of the infected fetuses (S-1 and T-1) and of the non-infected fetuses (S-0 and T-0) were purified by centrifugation and filtrated through Seitz filter, then supplemented with 1% 0.1 M ε-aminocapronic acid, lyophilized and stored.

Gel filtration. The lyophilized supernatant fluids were diluted 1 : 10 and fractionated on a Sephadex G-200 pH 6.0 and on a K 25/30 Pharmacia column, with 2 M phosphate buffer. Two ml fractions were measured photometrically at 280 nm.

Preparation of immune sera, and of the anti-ovine-Ig conjugate, agar gel precipitation, and polyacrylamide gel electrophoresis were performed as described before [19].

Gradient gel electrophoresis was carried out on PAA 4/30 slab gel. The eluent was tris–borate–Na_2 EDTA buffer pH 8.35.

Virus and toxin neutralization tests were performed as described before [21, 22].

Autoradiography. The white blood cells from the vaccinated animals were separated by Ficoll-Paque (Pharmacia) and suspended at 10^6/ml density in Hanks' solution containing 10% fetal serum and antibiotics. Leukocyte suspension samples of 1.5 ml from each experimental animal were transferred to Leighton tubes. The control tubes were left untreated, the cell suspensions in the other tubes were stimulated with homologous antigen (0.1 ml inactivated virus suspension or 0.1 ml 0.05 IN anatoxin of *Clostridium perfringens* D) and incubated at 37 °C. In the 57th h of incubation 10 μCi [^{3}H] thymidine was added to each tube and 16 h later the slides were fixed in methanol and washed in PBS. Subsequently, the slides were immersed in Ilford-Nuclear K_2 emulsion, dried, and stored in the dark-room at 4 °C for 6 days. The slides were developed in A49-ORWO solution, fixed, and stained according to May–Grünwald [23].

Immunofluorescence tests. After 76 h incubation the antigen-stimulated and the control Leighton slides were fixed in acetone at 4 °C for 10 min, covered with anti-ovine Ig conjugate and incubated at 37 °C for 30 min [24], then washed in PBS 3 times for 10 min and counterstained with Evans blue (1 : 40 000), coated with 1 : 3 glycerol PBS and covered with a coverslip. The slides were evaluated by a Zeiss NU_2 type microscope using a BG_{12} and a BG_3 objective filter, and an OG_1 ocular filter.

Results

Supernatants of spleen and thymus cell cultures. On gel filtration of Sephadex G-100 column the S-1 and T-1 concentrates showed the absorption profile demonstrated in Fig. 1. Fraction II, indicated in Fig. 1, was not observed in the gel filtrates S-0 and T-0.

Polyacrylamide gel electrophoresis of the S-1 and T-1 concentrates showed only one fraction with the electrophoretic mobility of alpha-1-fetoprotein, while the S-0 and T-0 concentrates yielded negative results.

The molecular weight of this fraction of S-1 and T-1 concentrates approximated 67 000, as determined with gradient gel electrophoresis on a PAA 4/30 slab gel.

Table I

Effect of spleen and thymus cell supernatants on the immune response of vaccinated animals

Group	Vaccine	No. of animals	Supernatant	Virus or toxin neutralizing titre
a	Adenovirus	1, 2, 3, 4	S-1	1 : 4–8
		5, 6	S-0	1 : 64–128
		7, 8	—	
b	Adenovirus	1, 2, 3, 4	T-1	1 : 8
		5, 6	T-0	1 : 128
		7, 8	—	
c	*C. perfringens* D	1, 2, 3, 4	S-1	0.1–1.5 IU/ml
		5, 6	S-0	9.2–16.2 IU/ml
		7, 8	—	
d	*C. perfringens* D	1, 2, 3, 4	T-1	0.6– 2.1 IU/ml
		5, 6	T-0	9.1–15.6 IU/ml
		7, 8	—	

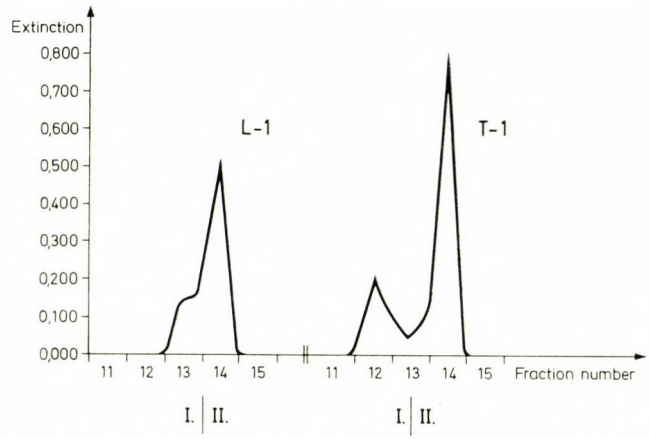

Fig. 1. Gel filtrate of spleen and thymus cell supernatant fluids

The S-1 and T-1 supernatants did not precipitate the anti-ovine immunoglobulin serum or the anti-ovine amniotic serum in an agar gel medium.

Effect of spleen and thymus cell supernatants on the immune response of adult sheep. One-year-old merino sheep were distributed in four groups each consisting of 8 animals. Groups *a* and *b* were inoculated with 2 ml of adenovirus vaccine intramuscularly [20] while groups *c* and *d* received 5 ml of *Clostridium perfringens* D vaccine (Phylaxia) intramuscularly. The experimental animals received 1 ml of the fetal spleen or thymus supernatant fluids (S-1, S-0, T-1, T-0) together with the vaccine and this was repeated 3, 6, 9 and 12 days later. Animals Nos 7 and 8 were the controls in each group, i.e., they received only vaccine (Table I). Blood samples were taken 1, 2, and 3 weeks after vaccination.

The effect of the supernatant fluids of the fetal spleen (S-1, S-0) and thymus (T-1, T-0) cells on the immune response of the animals injected with adenovirus or *C. perfringens* D vaccines is demonstrated in Table I, based on blood samples taken on the 21st day after vaccination.

Immune response of the animals vaccinated with adenovirus vaccine (groups a and b). The neutralizing antibody titres of the experimental animals which received vaccine and 5 times 1 ml of the S-1 or T-1 supernatant fluid were in the range of 1 : 4–1 : 8. S-0 or T-0 did not influence the immunogenic effect of the vaccine; the neutralizing antibody titres of their sera were, similarly as in the control animals, in the 1 : 64–1 : 128 range.

Immune response of animals vaccinated with C. perfringens D vaccine (groups c and d). In the experimental animals vaccinated with *C. perfringens* D, the toxin neutralizing titre amounted only to 0.1–1.5 IU/ml due to the 5 times 1 ml dose of S-1 supernatant. On the other hand, in the animals injected with S-0 and in the controls the titre was 9.2–16.2 IU/ml.

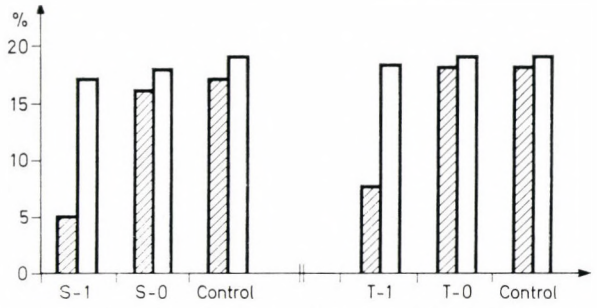

Fig. 2. Effect of the fetal spleen (S-1, S-0) and thymus cell (T-1, T-0) supernatants on the *in vitro* reaction of lymphocytes from animals vaccinated with adenovirus

In animals treated with T-1, the toxin neutralizing titres also decreased but at a lesser rate (0.6–2.1 IU/ml), while T-0 did not affect the immune response, i.e., the neutralizing antibody titre.

The blastogenic capacity of peripheral lymphocytes to homologous antigenic stimuli *in vitro* was then studied and the rate of Ig positive cells detectable by immunofluorescence. Figure 2 indicates the values in samples taken on the 21st day.

In the animals vaccinated with adenovirus and treated with S-1, S-0 (group *a*), [^{3}H]TdR-incorporation of antigen stimulated lymphocytes was 17–18%, which value was practically the same as in the untreated control animals. Nevertheless, the rate of Ig-positive cells determined by immunofluorescence was significantly lower in the animals treated with S-1 than in the controls; only 5% of the cells showed fluorescence, while the rate of Ig positive cells was 16–17% in both the control and the S-0 treated animals.

The rate of blastogenesis was similar in the animals inoculated with T-1 or T-0 (group *b*) and in the controls. The mean value of anti-ovine Ig-positive cells was 8% in animals treated with T-1, while it reached 18% in the untreated control or T-0 treated animals.

In the animals vaccinated with *C. perfringens* D (groups *c* and *d*) the reactions of lymphocytes *in vitro* were essentially similar as the reactions of animals vaccinated with adenovirus (groups *a* and *b*). The rate of blastogenesis in the lymphocytes following the antigenic stimulus *in vitro* reached the control values in the animals injected with the spleen and thymus supernatants, while the rate of Ig positive cells was significantly lower in the animals treated with S-1 or T-1.

Discussion

The functioning of fetal immune regulatory factors brings nearer to the understanding of the ontogenesis of the immune system. These factors presumably play a decisive role in the development of fetal immune tolerance as well as in the regulation of the weak immune response of newborns.

In the fetuses of various species, differentiation of the immune competent cells goes through similar stages but at different times [6, 7, 11, 25–27]. In ovine fetuses thymus and lymph nodes are detectable on the 35th to 50th day and lymphocytes appear in the blood and thymus between the 35th and 40th day. They appear in the spleen somewhat later, on the 58th to 60th day, and in the Peyer plaques on the 80th to 90th day. The immune responsiveness of the sheep fetus can be observed from the 41st day on, when it is able to produce antibodies neutralizing the phage $\varphi \times 174$ [27]. According to SILVERSTEIN *et al.* [9] a 66–70-day-old fetus can produce antibodies to ferritin only at low titre, it hardly reacts to ovalbumin and remains inactive to *Salmonella typhi-murium* or BCG. Other authors [22] demonstrated virus neutralizing antibodies in adenovirus-infected 70–80-day-old ovine fetuses. HUSBAND and MCDOWELL [28] could provoke a local immune response to *S. typhi-murium* and *Escherichia coli* administered on the 30th to 17th day prepartum. These data unambiguously prove the immunological responsiveness of the fetal immune system, but, at the same time, they point to its weak functioning.

The reduced or suppressed immune responsiveness persists for a certain period after birth [10]. SPEAR *et al.* [18] showed that although all the conditions of immune responsiveness were present in newborn mice, their response to antigens appeared only after the second week. DIXON and WEIGLE [29] transferred lymphoid cells of newborn mice into irradiated ones and found a normal immune reactivity. When, however, the sensitive lmyphocytes of adult mice with normal immune responsiveness were transferred into newborn mice, their reactivity was extremely low [30].

These observations point to the activity of some regulatory system in fetuses and, for a certain period, in newborns, which limits the immune response by its suppressive effect.

The present study was undertaken to establish whether the synthesis of mediators affecting the immune response takes place in a lymphoid system artificially induced to immune reaction and, if so, whether or not they are antigen specific.

Following an antigenic stimulus *in vitro*, a substance was detected in the supernatants of spleen and thymus cells (S-1, T-1) of 70–80-day-old ovine fetuses infected with adenovirus. The substance, separated by polyacrylamide gel electrophoresis, agreed in electrophoretic mobility with α1-fetoprotein and had a molecular weight of 67 000 as determined with gradient gel electrophoresis.

AFP in human [31, 32] and in various animal fetuses [16] is known to play an immunosuppressive role. According to MURGITA and TOMASI [14], AFP suppresses the immune response and also the mitogen-induced lymphocyte transformation [15].

From previous studies it is known that AFP is an embryospecific protein dominant in the fetal fluids from the beginning of embryonal life [33]. Chemi-

cally it is a glycoprotein with a simple polypeptide chain, resembling albumin in its aminoacid sequence. Its molecular weight is 68–72 000 [33–35].

According to our results, the substances S-1 and T-1 have an immunosuppressive effect on adenovirus-vaccinated adult sheep. This was indicated by their virus neutralizing titres which were significantly lower than those of the control animals. S-1 had a more expressed suppressive effect than T-1.

S-1 and T-1 suppressed the immune response of adult animals vaccinated with *C. perfringens* D as well, since the toxin neutralizing titres were very low in these animals.

Thymus and spleen cells were found capable of synthesizing the suppressive substance. The mediator showed no antigen specificity: its suppressive effect was manifest in both adenovirus and *C. perfringens* D vaccinated animals.

The results indicate that the immunosuppressive substances found in the supernatants of spleen and thymus cell cultures are produced by the activated lymphoid cells of the fetus. This is supported by the fact that such substances were not detectable in the supernatants of non-infected fetuses and that the suppressive supernatants did not react to anti-ovine Ig and anti-ovine amnion serum.

The reactions *in vitro* of the peripheral lymphocytes from groups *a*, *b*, *c*, and *d* might present a basis for the functioning of fetal mediators. The rate of blastogenesis determined with [^{3}H]TdR incorporation in samples obtained from animals inoculated with S-1 or T-1 was similar to the blastogenesis rate in the control animals, while the Ig synthesis, as determined by immunfluorescence, was clearly inhibited. Thus, the effect of the fetal mediators might be similar to the "antibody initiation suppressor factor" (AISF) of T cell origin, described by Douglas and Rubin [36].

Earlier it was shown [37] that a certain fraction of the amniotic fluid had an immunosuppressive effect. The fraction agreed in mobility with α-fetoprotein and its molecular weight was 67 000 as determined by gradient gel eletrophoresis. A comparison of the characteristics of the fraction isolated from amniotic fluid with that found in the S-1 or T-1 supernatants makes it evident that their electrophoretic mobility and molecular weight are similar.

The immunosuppressive effect of the immune regulatory α-globulin in adults (IRA) [13, 38–40] was proved to originate from its inherent AFP [41]. The synthesis of IRA has not been localized yet, but since it is extractable from bovine thymus [42] it is probably synthesised by T cells.

Further studies are required for clarifying the identity of the previously isolated amniotic fraction and of the fraction(s) in S-1 and T-1. Still, it can be assumed that the substance in S-1 and T-1 is comprised in the immunosuppressive AFP in the serum or other biological fluids of fetuses and newborn animals.

Certain experiments on the immunsuppressive effect of AFP yielded results contradicting the present ones [43]. This is probably due to the complicated isolation methods, which might affect the biological activity.

REFERENCES

1. Miller, J. F. A. P.: Progress in Immunology III. North Holland Pub. Comp., Amsterdam–New York–Oxford 1977. p. 353.
2. Rich, R. R., Rich, S. S.: In Lucas, D. O. (ed.): Regulatory mechanism in lymphocyte activation. Academic Press, New York 1977. p. 251.
3. Rubin, A. S.: Immunol. **26,** 629 (1979).
4. Rubin, A. S., McDonald, A. B., Conns, A. H.: J. Immunol. **111,** 1314 (1973).
5. Caldwell, J. L., Severson, C. D., Thompson, J. S.: Fed. Proc. **32,** 979 (1973).
6. Fennestag, K. L., Peterson, B. C.: J. infect, Dis. **110,** 63 (1962).
7. Osoba, D., Miller, J. P.: Nature (Lond.) **199,** 653 (1963).
8. Schnickel, P. G., Freguson, K. A.: Aust. J. exp. Biol. Med. Sci. **6,** 553 (1953).
9. Silverstein, A. M., Uhr, W. J., Kraner, K. L.: J. exp. Med. **117,** 799 (1963).
10. Symons, D. B. A., Binns, R. M.: Int. Arch. Allergy Appl. Immunol. **49,** 658 (1975).
11. Uhr, J. W.: Nature (Lond.) **187,** 957 (1961).
12. Sterzl, J., Silverstein, A. M.: Adv. Immuncl. **6,** 337 (1967).
13. Kamrin, B. B.: Proc. Soc. exp. Biol. (N. Y.) **100,** 58 (1959).
14. Murgita, R. A., Tomasi, T. B. Jr.: J. exp. Med. **141,** 269 (1975).
15. Murgita, R. A., Tomasi, T. B. Jr.: J. exp. Med. **141,** 440 (1975).
16. Tomasi, T. B.: In Lucas, O. D. (ed.): Regulatory mechanism in lymphocyte activation. Academic Press, New York 1977. p. 219.
17. Marti, J. S., Aliau, S., Moretti, J.: cit. Roushlati, E.: J. Immunol. **121,** 1687 (1978).
18. Spear, P. G., Wang, A. L., Rutishauer, U., Edelmen, G. E.: J. exp. Med. **138,** 557 (1973).
19. Tuboly, S., Szent-Iványi, T., Bauer, K.: Zbl. Vet. Med. **26,** 20 (1979).
20. Belák, S., Pálfi, V., Palya, V.: Zbl. Vet. Med. **23,** 320 (1976).
21. Bernáth, S.: Thesis. Budapest 1979.
22. Tuboly, S., Belák, S.: Magy. Állatorv. Lapja **34,** 772 (1979).
23. Varga, L.: Thesis. Budapest 1973.
24. Rose, N. R., Bigazzi, P. E.: Methods in immunodiagnosis. John Wiley, New York 1973. p. 110.
25. Dent, P. B., Perey, D. Y., Good, R. A.: J. Immunol. **101,** 799 (1968).
26. Globerson, A., Auerbach, R.: J. exp. Med. **126,** 223 (1967).
27. Tizard, I. N.: Veterinary Immunology. W. B. Saunders Company, Philadelphia–London–Toronto 1977.
28. Husband, A. J., McDowell, G. H.: Res. Vet. Sci. **25,** 343 (1978).
29. Dixon, F. J., Weigle, W. O.: J. exp. Med. **110,** 139 (1959).
30. Dixon, F. J., Weigle, W. O.: J. exp. Med. **105,** 75 (1957).
31. Goecken, N. E., Thompson, J. S.: J. Immunol. **119,** 139 (1977).
32. Yachin, S., Lester, E.: Clin. exp. Immunol. **26,** 484 (1976).
33. Gitlin, D., Boesman, M.: J. clin. Invest. **45,** 1826 (1966).
34. Abelev, G. I.: Adv. Cancer Res. **14,** 295 (1977).
35. Waldman, T. A., McIntire, K. R.: Cancer **34,** 1510 (1974).
36. Doeglas, G. N., Rubin, A. S.: Immunol. **32,** 669 (1977).
37. Tuboly, S.: Magy. Állatorv. Lapja **35,** 535 (1980).
38. Ashikawa, K., Inoue, K., Shimizu, T.: Jap. J. exp. Med. **41,** 339 (1971).
39. Mowbray, J. F.: Immunol. **6,** 217 (1963).
40. Trubowitz, S., Masek, B., Rosatio, A.: Cancer **19,** 2019 (1966).
41. Rouslahti, E., Terry, W. D.: Nature (Lond.) **260,** 804 (1960).
42. Carpenter, C. B., Boylston, A. W., Merril, S. P.: Cell. Immunol. **2,** 425 (1971).
43. Tyan, M. L.: Proc. Soc. exp. Biol. (N. Y.) **151,** 343 (1976).

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DRUG-RECEPTOR INTERACTION ON PLASMID ELIMINATION BY PHENOTHIAZINES AND IMIPRAMINE IN *ESCHERICHIA COLI*

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Plasmid elimination in *Escherichia coli* by a quaternary amine of chlorpromazine was demonstrated on different incompatibility groups of plasmid. The biological effect of the drug depends partly on the host bacteria and partly on the plasmid itself. Various receptor substrates such as adenosine, dopamine, histamine and norepinephrine do not alter the plasmid elimination by promethazine and imipramine. None of the known drug-receptors studied are involved in drug binding of the bacteria. The direct membrane action of imipramine and promethazine was demonstrated in electron microscopic studies and alterations in the bacterial membrane such as discontinuities, phase separation or rarely extensive lytic alterations were observed. Magnesium ions prevent the ultrastructural membrane alterations caused by imipramine and promethazine. There is some evidence that the drugs bind to two different receptor sites simultaneously on the plasmid replication site. The first and strongest binding has to be ionic through the side chain amino group, displacing the bivalent cations. In turn, the two aromatic rings of the fixed (ionically bound) drug molecules bind weakly through pi-electrons, hydrophobically or by a charge transfer complex. This weaker binding together with the ionic one are essential for biologic action and lead to the inhibition of plasmid replication. A schematic model of the effect of tricyclic psychotropic drugs on the bacterial membrane is proposed.

The quaternary amines of chlorpromazine and imipramine were effective as plasmid curing agents [1] despite the fact that the quaternary amines are not able to penetrate into cells [2]. These results suggest that the membrane effect of the drugs may play an important role in curing. In synaptosomes, the secondary amine of imipramine inhibits noradrenaline uptake, while the tertiary amine binds strongly to the serotonin carrier [3]. Both these drugs cause plasmid elimination [1]. In mouse neuroblastoma cells, both tertiary and secondary amines of imipramine block the histamine H_1 [4] and H_2 receptors [5]. Otherwise, imipramine and desipramine inhibit the neuronal uptake into the cerebral cortex of norepinephrine, serotonin and dopamine. The reduction of the aromatic rings, particularly of desipramine, abolished, however, the selectivity of the drug for the inhibition of norepinephrine uptake. On the other hand, saturation of the aromatic rings did not alter the inhibition of dopamine and 5-hydroxytryptamine uptake [6]. It has been shown that phenothiazines are antagonists of the central dopamine and norepinephrine sensitive adenylate cyclases [7, 8] which may represent the primary therapeutic site of action of neuroleptics. On the other hand, an antagonism of tricyclic drugs to the histamine-induced accumulation of cyclic AMP in tissue slices

has also been shown [9, 10]. It was remarkable that the different classes of drugs like antidepressants, neuroleptics and antihistamines exert a plasmid curing action on the same bacteria even though their affinity to various receptors is different (e.g. dopamine, norepinephrine, histamine and adenosine) in the central nervous system [10, 11]. We believe that one or possibly more of these receptor sites exist in the bacterial membrane which may influence plasmid replication.

The aim of the present paper is to correlate the plasmid elimination with some of the known receptors. To this purpose we have investigated the competition between drugs and biogenic amines or other substrates corresponding to the dopamine, norepinephrine, histamine, adenosine and calcium receptor sites.

Materials and methods

Chemicals. Imipramine (Melipramine®), promethazine (Pipolphen®), were obtained from EGYT Pharmaceuticals, Budapest. Histamine dihydrochloride, adenosine (Reanal, Budapest), l-noradrenaline-l tartrate (Merck–Schuchardt), dopamine (Sigma) were used. Chlorpromazine-phenacylammonium bromide was synthesized by us [1].

Bacterial strains. Escherichia coli K12 LE140 *tsx, str, Δ lac, su, λ*$^{-r}$, *mal,* (F-prime *lac*$^+$ [12]), *E. coli* K12 G. I. 65/R 144 drd$^+$ was obtained from Dr. B. WILKINS, University of Leicester which was col I$^+$, Kana^r. *E. coli* K12 W1 Azi/R 144 drd$^+$ (a transconjugant), *E. coli* JE 2571 *thr, leu, fla, pil*/Rm 98, (Rm 98 = Ap^r, Sm^r, Sp^r, Te^r), *E. coli* K12 JB 495 *pro, his, trp, lac, Nalidix A*/RP4 (RP4 = Kana^r, Carbeni^r, Te^r) were kindly provided by Dr. Á. KONDOROSI, Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged.

Culture media. MTY broth and MTY agar were prepared according to ALFÖLDI *et al.* [13]. Eosin–methylene blue agar (EMB) was used for detection of lac$^-$ colonies [14].

Elimination of F'lac and R-plasmids. An overnight preculture of *E. coli* strains was diluted 10^4 fold and distributed in 0.05 ml amounts (about 5×10^3 bacteria) into tubes with 5.0 ml MTY broth. Cultures were supplemented with different concentrations of curing agents. The samples were then incubated without shaking at 37 °C for 24 h in case of F'lac curing and for 48 h in the case of R-plasmid elimination. From tubes showing growth, different dilutions were prepared and 0.1 ml of samples plated on EMB agar in case of Lac-plasmid elimination or on MTY plates in case of R-plasmid carrying strains. After incubation overnight, the isolated colonies were replicated into MTY plates containing kanamycin (50 μg/ml) except for *E. coli* JE 2571 which latter was replicated on tetracycline (50 μg/ml) containing MTY plates. After incubation at 37 °C for 24 h, the number of colonies was compared with the number of colonies growing on the master plate. The R$^-$ colonies were checked for auxotrophy of *E. coli* JE 2571 and JB 495 and for resistance to streptomycin in case of *E. coli* LE 140. The R$^-$ colonies derived from *E. coli* K12 W1 Azi/drd$^+$ were identified on the basis of azide resistance.

Electron microscopy. E. coli K12 LE140 was cultivated in MTY broth (OD_{260} = 0.6) and 2.0 ml aliquots were distributed into centrifuge tubes. The bacteria were incubated in the presence of 1.0×10^{-3} M promethazine and 1.0×10^{-3} M promethazine plus 1.0×10^{-2} M $MgCl_2$ or 1.0×10^{-3} M imipramine and imipramine plus 1.0×10^{-2} M $MgCl_2$. Control bacteria were incubated similarly without the drugs, at 37 °C for 60 min. The samples were then cooled, prefixed, prepared for electron microscopy as described earlier [15], and the sections were studied under a JEOL 100 B electron microscope.

Results

The various plasmids belonging to different incompatibility groups were eliminated in the presence of the quaternary amine of chlorpromazine with different frequencies (Table I). One the other hand, one plasmid, R 144 drd$^+$

from two *E. coli* strains, could be cured at varying frequency (Table I). The results suggest that there are membrane receptors in the sensitive cells. In the case of strains resistant to plasmid elimination there are, however, no drug binding receptors or if there are, they have no connection with the plasmid replication sites. The phenothiazines and related compounds have at least four different well-known receptors in central postsynaptic nerve membranes. In some experiments the competition between plasmid curing compounds (promethazine and imipramine) and the substrates of the receptors (dopamine, norepinephrine, histamine, adenosine and calcium ions) were tested on an *E. coli* strain carrying F'lac plasmid. The plasmid curing effect of the drugs was investigated on bacteria which were pretreated with dopamine, norepinephrine, histamine, adenosine and $CaCl_2$, (from 1×10^{-4} to 1.0×10^{-3} M final concentrations). Table II shows that the various receptor substrates exerted neither a synergistic nor an antagonistic effect on the plasmid curing action of promethazine and imipramine. The efficiency of curing decreased somewhat in the presence of calcium ions. In control experiments, none of the receptor substrates showed plasmid elimination in the applied concentrations. The results indicate that binding of substrates and curing by these compounds must be different in *E. coli*.

Table I

Plasmid curing activity of chlorpromazine-phenacylammonium Br^- on E. coli strains carrying plasmids of different compatibility groups

Plasmid compatibility groups	Bacterial strains	Plasmid elimination, %	Effective concentration, M
F-like	*E. coli* K12 Le140/F'lac	20—29	$2.0-3.1\times10^{-5}$
I-like	*E. coli* K12 G.I. 65/R144 drd^+	0—0.2	$3.1-4.1\times10^{-5}$
I-like	*E. coli* K12 WI *Azi*/R144 drd^+	1—2.5	$1.0-2.0\times10^{-5}$
N	*E. coli* JE2571 *thr, leu, fla, pil*/Rm98	1—49	$1.0-2.0\times10^{-5}$
P-1	*E. coli* K12 JB495, *pro, his, trp, lac, Nalidix* A(RP4)	1—5.0	$2.0-3.1\times10^{-5}$

In a series of experiments the ultrastructural alterations of the bacteria exposed to promethazine and imipramine were studied (Fig. 1). Fig. 1/a shows the ultrastructure of normal untreated cells. The discontinuities of the bacterial membrane caused by promethazine can be seen in Fig. 1/b and the same effect of imipramine is shown in Fig. 1/c and 1/d. The ultrastructural changes of the

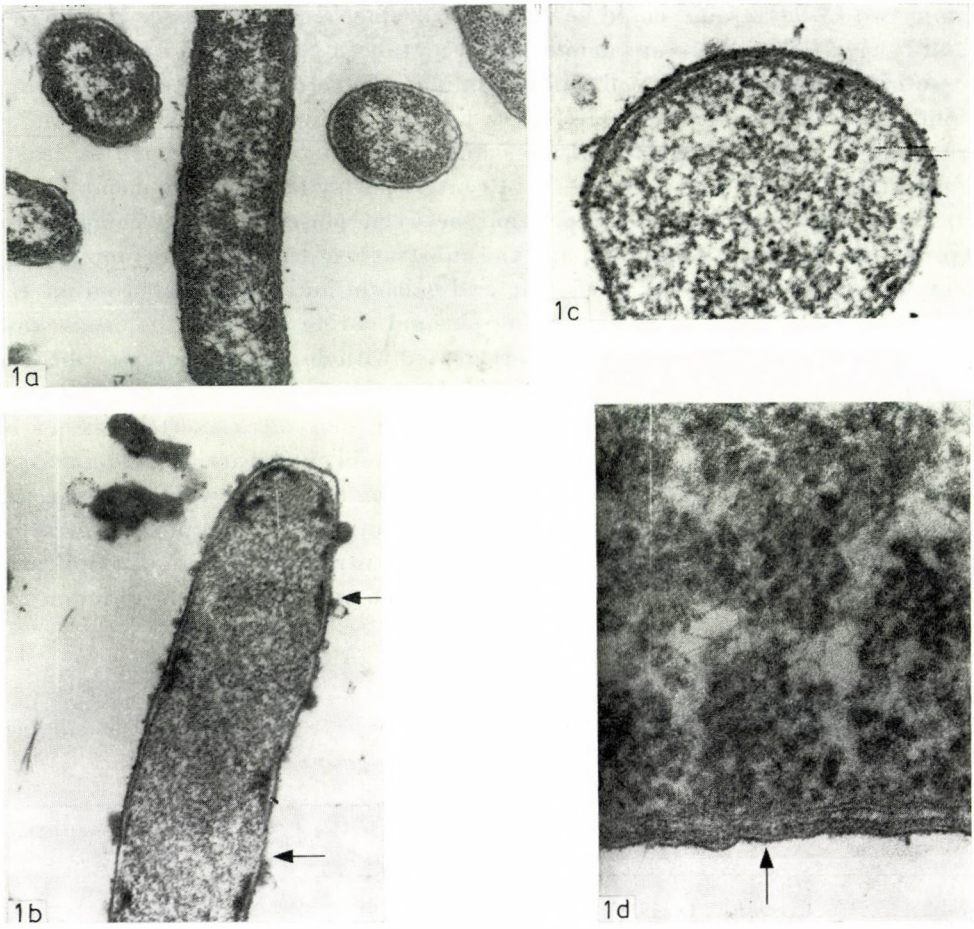

Fig. 1. Ultrastructural changes on bacterial membrane caused by imipramine and promethazine. *a)* Ultrastructure of control cells (30 000×2). *b)* Effect of 1.0×10^{-3} M promethazine (30 000×2). *c)* Effect of 1.0×10^{-3} M imipramine (80 000). *d)* Effect of 1.0×10^{-3} M imipramine (200 000×2) on the ultrastructure of *E. coli* membrane

bacterial membrane appeared as discontinuities in the membrane bilayers or membrane solubilization. Some of the promethazine-treated cells exhibited extensive lytic alterations while this effect was less pronounced in the presence of imipramine.

Incubation of the bacteria in the presence of the drugs and magnesium ions for 60 min did not produce any detectable change in the ultrastructure of the bacterial membrane and cell as compared to the control.

Table II

Plasmid curing effect of promethazine and imipramine in the presence of various compounds with different receptor affinity

Compounds	Concentration, M	Viable cells after 24 h	Lac⁻ colonies, %	No. of colonies tested
Promethazine	2×10^{-4}	5.6×10^{7}	25.0	1208
+ $CaCl_2$	5×10^{-4}	7.4×10^{7}	18.0	1700
+ Histamine	1×10^{-3}	8.0×10^{7}	23.0	1660
+ Norepinephrine	6×10^{-4}	4.2×10^{7}	17.0	1800
+ Adenosine	1×10^{-3}	3.7×10^{7}	26.5	1740
+ Dopamine	5×10^{-4}	5.8×10^{7}	12.0	3000
Imipramine	5×10^{-4}	5.0×10^{7}	29.0	1400
+ $CaCl_2$	5×10^{-4}	8.9×10^{7}	23.0	1020
+ Histamine	1×10^{-3}	4.6×10^{7}	20.0	890
+ Norepinephrine	6×10^{-4}	2.7×10^{7}	18.0	2300
+ Adenosine	1×10^{-3}	6.0×10^{7}	32.0	960
+ Dopamine	5×10^{-4}	2.4×10^{7}	30.0	2700
Control		3.5×10^{8}	0.0	1100
Dopamine	5×10^{-4}	1.5×10^{8}	0.0	2000
Histamine	1×10^{-3}	5.0×10^{8}	0.0	1583
Norepinephrine	6×10^{-4}	4.6×10^{8}	0.0	1012
Adenosine	5×10^{-3}	2.0×10^{8}	0.0	1500
$CaCl_2$	5×10^{-4}	2.0×10^{8}	0.0	927

Discussion

Studies on the replication of plasmid DNA in bacteria have shown that there are many plasmid specific events, e.g. appropriate promoters in the plasmid DNA, control of autonomous plasmid replication [16] and protein synthesis for several generations [17]. There are also many steps in transfer process for the mating signal to the phenotypic expression in the recipients, namely formation of open circular plasmid intermediate, donor conjugal-DNA synthesis, transfer of the nicked strand, recipient conjugal-DNA synthesis, recircularization in the donor cells, recircularization in the recipient cells [18] and establishment of plasmid molecules in recipients which requires more than five generations [19]. Excluding the SDS-like selection mechanism [20], plasmid replication can theoretically be affected in three different ways: (i) The DNA-complexing compounds like acridines and other tricyclic drugs, e.g., phenothiazi-

nes, show exterior and interior (intercalation) binding to the DNA [21, 22] and some of them may act as template blockers [23] on the DNA. (ii) The second possibility in an inhibition of one or more enzymes. DNA- or plasmid--primase and DNA-polymerase [24] are essential for the synthesis of plasmid DNA. These enzymes, however, require ribonucleotides as substrates among other important factors. It is known that chlorpromazine and its semiquinone--free radical inhibited the activity of both Mg^{++}- and Na^{+}, K^{+}-ATPases [25, 26]. (iii) In the third form of plasmid elimination, the bacterial membrane is involved. If there are special receptors on which the plasmid multiplies, an alteration of the physiological or structural conformation of the membrane may lead to a cessation or inhibition of plasmid replication. It has been shown that the phenothiazines and imipramine interact with certain phosphorylated components of the membrane [27] and alter the morphology of a wide variety of biological membranes, e.g. by producing discontinuities in the nuclear membrane of barley [28, 29].

Both promethazine and imipramine induce many well-defined alterations in the *E. coli* cells whereby the cell membrane is directly affected by the drugs. The characteristic membrane alterations suggest that the drugs increase permeability as a consequence of the disordered membrane structure, which cannot maintain the appropriate electrochemical proton gradient, may play an essential role in the membrane's active transport. At any rate, extensive lytic alterations have been observed in *Bacillus anthracis* [30] and *Mycobacterium tuberculosis* [31] in the presence of chlorpromazine and promethazine. Similar micromorphological alterations were found and membrane respiratory activity was inhibited in *Bacillus cereus* and *Bacillus subtilis* in the presence of chlorpromazine, tetracaine, or procaine [32]. After chlorpromazine treatment, mesosome-like structures appeared and the staining pattern changed from asymmetric to symmetric in *B. cereus* [32]. The phenothiazines and related compounds have stereospecific interactions with appropriate receptors, depending upon the molecule's configuration [33].

Further, our results show that there is no simple competition between receptor substrates and receptors, at least in *E. coli*. Dopamine, histamine, norepinephrine, adenosine and calcium all failed to prevent the plasmid elimination by promethazine and imipramine. On the basis of these results we suppose that none of the receptors known to be present in the postsynaptic nerve membrane can occur in the membrane of *E. coli* or, more exactly, the inhibition of plasmid replication has no relation to the known postsynaptic receptor conformations or receptor-like structures. It is possible that there are special membrane conformations or compartments in the bacterial membrane to which the drugs may bind.

Beside the receptors, the conformation or the electron stability of the drug molecules may also be important. The phenothiazine by itself may have

an antioxidant property which is related to the formation of semiquinone type radicals [34].

Chlorpromazine has been shown to be capable of depressing the excited state of certain molecules such as riboflavin, by bringing back an unpaired electron from a peripheral orbit toward a more central one [35]. It is possible that one molecule of the particular psychopharmacon has to bind to two different receptor sites simultaneously.

The first and strongest binding can be as a cation through the amino group of the chain, displacing the bivalent cations from the membrane. This binding is a reversible process, which depends on the number of bivalent cations and the number of drug molecules [36].

The ionically bound molecule can bind to the "second receptor site" hydrophobically through the two aromatic rings, or form a charge transfer complex with the "second receptor site" but distretches the layers of the bacterial membrane. This interaction is weaker than the first one, but much more stereospecific. Since substitution in the ring system prevents the plasmid elimination by chlorpromazine derivatives [36, 37], substitution on the side chain amino group does not alter the plasmid curing activity of the drugs

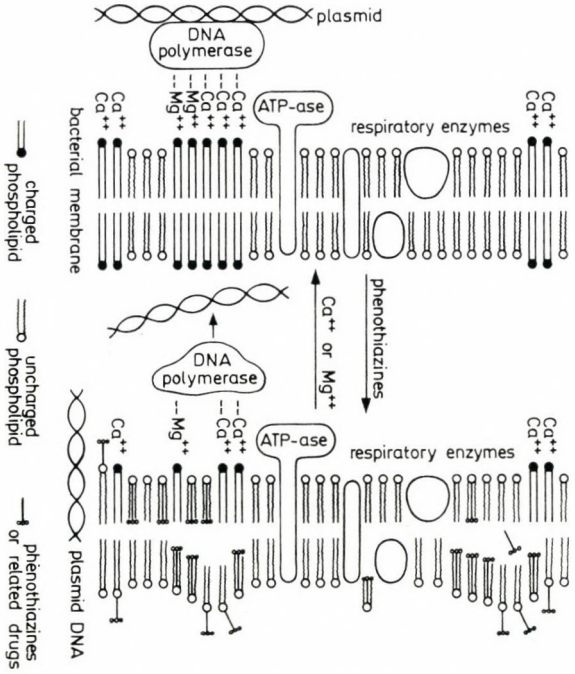

Fig. 2. A proposed model of the effects of tricyclic psychotropic drugs on the bacterial membrane (focussing on the phospholipid bilayer and plasmid replication site)

[1, 38]. It seems that the double-bound molecule can only inhibit plasmid replication.

On the basis of these results we propose the following mechanism to explain the plasmid elimination by the tricyclic drugs. The binding of tricyclic drugs preferentially displaces the bivalent cations, resulting in uncharged membrane lipids and a crystalline–liquid phase transition with increased fluidity in the membrane compartments (Fig. 2). On this altered membrane structure the plasmid DNA cannot bind to the replication site and/or some plasmid specific enzyme such as DNA-gyrase or DNA-polymerase can be inhibited. The hypothesis is made plausible by the fact that plasmid elimination occurs at subinhibitory drug concentrations in which case the membrane is nearly saturated with the drug molecules.

It seems that the drug tested can affect the bivalent cation binding of phospholipids and lipid fluidity, since bivalent cations prevent the antibacterial, plasmid curing [36, 37] and direct membrane effects. It is therefore concluded that tricyclic psychotropic drugs and bivalent cations may be competing for membrane receptor plasmid-sites on bacteria. It has been shown that by two chlorpromazine molecules one Ca^{++} is displaced from the human erythrocyte membrane ghosts [39].

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REFERENCES

1. MOLNÁR, J., DOMONKOS, K., MÁNDI, Y., FÖLDEÁK, S., HOLLAND, I. B.: Phenothiazines and Structurally Related Drugs. In USDIN, E. ECKERT, H. FORREST, I. (eds): Basic and Clinical Studies. Elsevier–North Holland, Amsterdam 1980. pp. 115–118.
2. GRUENER, R., NARASHI, T.: J. Pharmacol. exp. Ther. **181,** 161 (1973).
3. RAITERI, M., CARMINE, R., CERVONI, A. M., LEVI, G.: Eur. J. Pharmacol. **57,** 407 (1979).
4. RICHELSON, E.: Nature (Lond.) **274,** 176 (1978).
5. MAAYANI, S., GREEN, J. P., WEINSTEIN, H.: Fed. Proc. **37,** 612 (1978).
6. GRÜNEWALD, G. L., REITZ, T. J., RUTH, J. A., VOLLMER, S., EIDEN, L. E., RUTLEDGE, C. O.: Biochem. Pharmacol. **28,** 417 (1979).
7. CARLSSON, A., LINDQUIST, M.: Acta pharmacol. (Kbh.) **20,** 140 (1963).
8. NATHANSON, J. A.: Physiol. Rev. **57,** 157 (1977).
9. KAKIUCHI, S., RALL, T. W.: Molec. Pharmacol. **4,** 367 (1968).
10. PALMER, G. C., MANIAN, A. A.: In PALMER, G. C. (ed.): Neuropharmacology of Cyclic Nucleotides. Urban and Schwarzenberg, Munich 1979. pp. 53–111.
11. GREESE, I., BURT, D. R., SNYDER, S. H.: Life Sci. **17,** 993 (1975).
12. MÁNDI, Y., MOLNÁR, J., HOLLAND, I. B., BÉLÁDI, I.: Genet. Res. **26,** 109 (1976).
13. ALFÖLDI, L., RASKÓ, I., KEREKES, E.: J. Bact. **96,** 1512 (1968).
14. CLOWES, R. C., HAYES, W.: Experiments in Microbial Genetics. Blackwell Scientific Publications, Oxford 1968.
15. MOLNÁR, J., SZEPESSY, G. L.: Acta microbiol. Acad. Sci hung. **19,** 7 (1972).
16. SLOCOMBE, P. M., ELY, S., TIMMIS, K. N.: Nucl. Acids Res. **7,** 1469 (1979).
17. WOMBLE, D. D., ROWND, R. H.: Plasmid **2,** 79 (1979).

18. KINGSMAN, A., WILLETTS, N.: J. molec. Biol. **122,** 287 (1978).
19. CULLUM, J., COLLINS, J. F., BRODA, P.: Plasmid **1,** 536 (1978).
20. SALISBURY, V., HEDGES, R. W., DATTA, N.: J. gen. Microbiol. **70,** 443 (1972).
21. BLACKE, A., PEACOCKE, A. R.: Biopolymers **6,** 1225 (1968).
22. BARABÁS, K., MOLNÁR, J.: Acta microbiol. Acad. Sci. hung. **27,** 55 (1980).
23. OHNISHI, S., MCCONNELL, H. M.: J. Amer. chem. Soc. **87,** 2293 (1965).
24. LANKA, E., SCHERZINGER, E., GÜNTHER, E., SCHUSTER, H.; Proc. nat. Acad. Sci. (Wash.) **76,** 3632 (1979).
25. AKERA, T., BASKIN, S. I., TOBIN, T., BRODY, T. M., MANIAN, A. A.: In FORREST, I. S., CARR, C. J., USDIN, E. (eds): The Phenothiazines and Structurally Related Drugs. Raven Press, New Y rk 1974. p. 633.
26. SAMULES, A. M., CAREY, M. C.: Gastroenterology **74,** 1183 (1978).
27. HELLE, P.: Biochim. biophys. Acta (Amst.) **76,** 647 (1963).
28. ADAMS, J. D.: Pharmacology **13,** 137 (1975).
29. POLSON, C. D. A., ADAMS, J. D.: Pharmacology **16,** 83 (1978).
30. MOLNÁR, J., MÁNDI, Y., KIRÁLY, J.: Acta microbiol. Acad. Sci. hung. **23,** 45 (1976).
31. MOLNÁR, J., BÉLÁDI, I., FÖLDES, J.: Zbl. Bakt. I. Abt. Orig. A. **239,** 521 (1977).
32. SILVA, M. T., SOUSA, J. C. F., POLONIA, J. J., MACEDO, P. M.: J. Bact. **137,** 461 (1979).
33. BARBE, J.: In LE COFFIC, F., REY, A., CRESCIONI, C. (eds): Current Topics in Molecular Pharmacology and Drugs of the Future. Edition de Santé, Paris 1978. pp. 151–165.
34. LABORIT, H., DROUET, J., GERARD, J., JOUANY, J. M., NARVAES, C., NIAUSSAT, P., WEBER, B.: Int. Record Med. **173,** 351 (1960).
35. SZENT-GYÖRGYI, A.: Science **124,** 873 (1956).
36. MOLNÁR, J., SCHNEIDER, B.: Acta microbiol. Acad. Sci. hung. **25,** 291 (1978).
37. MOLNÁR, J., MÁNDI, Y., HOLLAND, I. B., SCHNEIDER, GY.: Acta microbiol. Acad. Sci. hung. **24,** 1 (1977).
38. FARKAS, S., MOLNÁR, J.: Acta microbiol. Acad. Sci. hung. **26,** 351 (1979).
39. KWANT, W. O., SEEMAN, P.: Biochim. biophys. Acta (Amst.) **193,** 338 (1969).

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CYTOTOXIC MATERIAL RELEASED FROM *STAPHYLOCOCCUS EPIDERMIDIS*

I. EFFECTS ON [^{3}H]THYMIDINE INCORPORATION OF HUMAN LYMPHOCYTES*

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Incorporation of [^{3}H]thymidine ([^{3}H]TdR) into tonsil lymphocytes was inhibited by native *Staphylococcus epidermidis* while *Staphylococcus aureus* Cowan I caused stimulation. The inhibitory effect of *S. epidermidis* was abolished by formalin treatment but not by heat killing. A toxic agent was released from *S. epidermidis* on gentle water extraction without lysing the bacteria. The extract contained protein and other UV-absorbing material, but did not exhibit haemolytic, lysozyme, catalase or protease activity. The heat-resistant, formalin-sensitive inhibitor present in the aqueous extract of *S. epidermidis* inhibited [^{3}H]TdR incorporation of lymphocytes in a dose-dependent manner and decreased the viability of lymphocytes.

Staphylococci are known to produce a series of agents affecting the metabolism of mammalian cells. These include substances of the cell wall (teichoic acids [1] and peptidoglycans [2]), or attached to the surface (protein A [3], polysaccharide A [4]) as well as numerous extracellular enzymes and toxins, esterases, haemolysins, etc. [5, 6]. Their biological effects are cytolysis (haemolysin [7, 8], epidermolytic toxin [8]) and metabolic inhibition [9] but also stimulation of lymphocytes (protein A [10], enterotoxins [11] and other mitogens [12], etc.). Such diverse biological effects render the pathogenesis of staphylococcal infections extremely complex. The relationship between pathogenicity and the agents of coccal origin is well established [13, 14] and a subject of intensive research.

Previously we have demonstrated that human lymphocytes bind several strains of bacteria [15]. The cytotoxic effect of one of these strains, *S. epidermidis*, is reported here. Cytotoxity was monitored by measuring [^{3}H]TdR incorporation of human tonsil lymphocytes. The toxic agent was released by *S. epidermidis*.

Materials and methods

Bacterial products. S. epidermidis was isolated from human tonsils. *S. aureus* was a gift of Dr. R. Varró (Human Institute for Serobacteriological Production and Research, Gödöllő). Cocci were grown on nutrient broth at 37 °C for 16 h. Viable cell count was determined by plating on brain heart infusion (BHI) agar.

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The released bacterial products were prepared as follows. The cocci were harvested by centrifugation then washed 3 times with 0.066 M phosphate buffered saline (PBS) pH 7.2. After suspending the cocci in PBS they were incubated at 4 °C for 24 h, then centrifuged. The supernatant was filtered through a Sartorius membrane (0.45 μm) and a G-5 glass-filter under sterile conditions, yielding the crude supernatant (SN).

Lymphocytes were prepared from freshly removed tonsils as described earlier [15]. Cells (10^6/ml) were suspended in 4 ml Eagle's MEM supplemented with 20% human AB serum, L-glutamine, and antibiotics. Incubation was performed at 37 °C in the presence 5% CO_2 gas. The additions were whole bacteria, SN or phytohaemagglutinin (PHA, 10 μg/ml, Difco). Cultures were pulse labelled with [^{3}H]TdR (3 μCi/ml; specific activity, 22 Ci/mM) for 4 h before harvest. After incubation, lymphocytes were collected by differential centrifugation. Incorporation of [^{3}H]TdR was determined in the fraction obtained by cold TCA precipitation and hydrolysis in PCA at 90 °C. Radioactivity was measured by liquid scintillation in a Beckman LS-350 spectrometer.

Control experiments showed that the bacteria did not incorporate [^{3}H]TdR under these experimental conditions.

Haemolytic assay. Suspensions of washed erythrocytes (sheep, rabbit, human) were mixed with SN (1 : 1 to 1 : 4, vol/vol) and incubated at 37 °C for one hour, then at 4 °C for 18 h.

Enzyme assays. Lysozyme activity was investigated using *Micrococcus lysodeicticus* as substrate in agar gel [16]. Catalase and protease activity was measured by standard techniques [17]. Esterase activity was demonstrated [18] by electrophoresis in 7.5% polyacrylamide gel and staining. The SN was analyzed for protein by the method of LOWRY *et al.* [19].

Results and discussion

Tonsil lymphocytes (1×10^6/ml) were cultured for six days in the presence of PHA, native *S. epidermidis* or *S. aureus* Cowan I (3×10^7/ml). Pulse labelling showed intensive DNA synthesis at the beginning (Fig. 1, day 0), which declined later. Rapidly decreasing spontaneous DNA synthesis in cultures of tonsil lymphocytes is well known [20]. Stimulation by PHA and by Cowan I (carrying protein A) was evident on the 3rd day. Thereafter the stimulatory effect of Cowan I increased further, in agreement with the results of others [21] who described maximum stimulation on the 7th day, although FORSGREN *et al.* [22] SAKANE and GREEN [23] found the peak of incorporation in lymphocytes around the 3rd day.

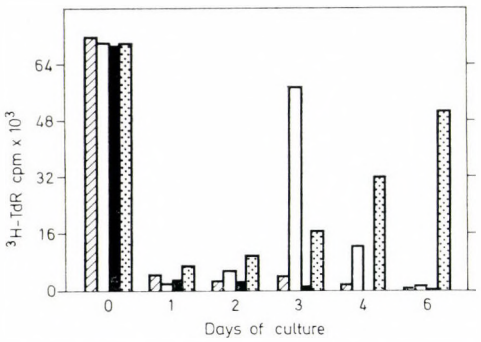

Fig. 1. [^{3}H]TdR incorporation of lymphocytes cultured in the presence of *S. epidermidis* (solid columns), *S. aureus* Cowan I (dotted column); PHA (open columns); control (shaded columns)

Inhibition of [^{3}H]TdR incorporation by *S. epidermidis* appeared on the 3rd day as compared to the control culture (Fig. 1). This was accompanied by a decrease in the number of viable lymphocytes (demonstrated by trypan-blue exclusion) from 72% (control) to 46% in the presence of *S. epidermidis*. Complete inhibition of [^{3}H]TdR incorporation by *S. epidermidis* was seen on the 4th day.

In the following experiment, different concentrations of bacteria (10^6–-3.10^7–10^8 colony-forming units per ml) were incubated for 24 h with lymphocytes, which previously had been cultured for 72 h in the absence (Fig. 2A) or presence (Fig. 2B) of PHA. *S. epidermidis* inhibited [^{3}H]TdR incorporation of unstimulated lymphocytes at concentrations of 3×10^7 and 10^8 c.f.u./ml. At the same c.f.u. concentrations Cowan I increased the incorporation of [^{3}H]TdR.

[^{3}H]TdR incorporation into lymphocytes pretreated with PHA was inhibited by Cowan I at each concentration (Fig. 2B), in agreement with the observation of WILLIAMS and KRONWALL [24] protein A inhibited the mitogenic effect of lectins. The inhibitory effect of *S. epidermidis* appeared only when high concentrations of these bacteria were applied. Stimulation of lymphocytes (DNA or antibody synthesis) by staphylococci has been demonstrated with whole bacteria [22, 25] but also with isolated protein A [23], enterotoxins [26] or staphylococcal phage lysate [27].

Generally, whole bacteria are applied after heat inactivation (80 °C, 5–10 min) and/or formalin (0.1–0.5%) treatment after which they may or may not retain their stimulatory effect but no toxic properties appear (Fig. 3). Since short heat-treatment did not decrease the viability of *S. epidermidis*, the bacteria were killed at 100 °C for 4 h. Heat inactivation did not abolish the stimulatory activity of Cowan I, probably due to the denaturation of protein A. However, when short heat inactivation of *S. epidermidis* was followed

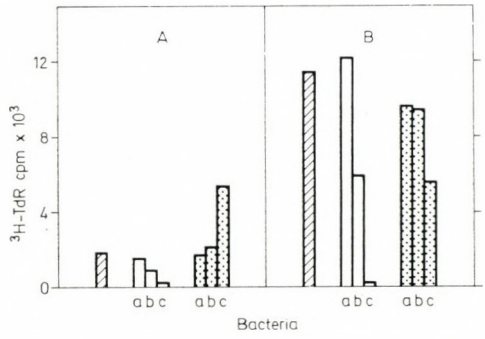

Fig. 2. Effect of *S. epidermidis* (open columns) and *S. aureus* Cowan I (dotted columns) on [^{3}H]TdR incorporation in lymphocytes which had been cultured for 72 h in the absence (A) or presence (B) of PHA. Thereafter they were incubated with different concentrations of bacteria a: 10^6 c.f.u./ml; b: 3×10^7 c.f.u./ml; c: 10^8 c.f.u./ml; control (shaded columns)

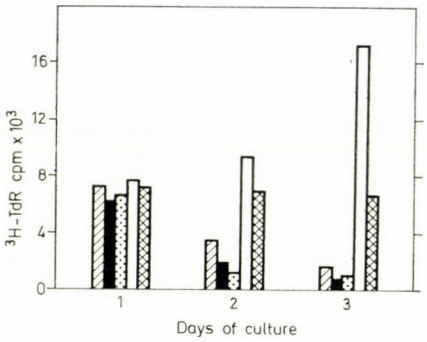

Fig. 3. [^{3}H]TdR incorporation of lymphocytes in the presence of *S. epidermidis* viable (solid columns) and heat-killed (dotted columns) or *S. aureus* Cowan I viable (open columns) and heat-killed (striped columns); control (shaded columns)

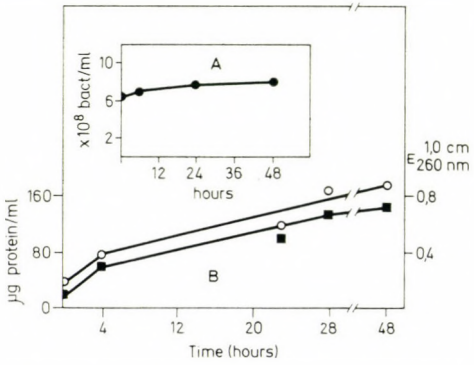

Fig. 4. Release of protein and other UV absorbing material by *S. epidermidis* into the PBS medium at 4 °C. Insert shows the viable cell count during incubation. (○——○) UV absorbance; (△——△) protein

by treatment with 0.5% formalin at 0 °C for 24 h, the bacteria lost their toxicity (data not shown). According to the above experiments, *S. epidermidis* contains a heat-stable, formalin-sensitive toxic material.

The inhibitory effect of whole, native cocci described above could be elicited by the aqueous extract of the bacteria as well. Release of toxic material from *S. epidermidis* was demonstrated as follows. *S. epidermidis* was suspended in PBS and incubated at 4 °C in order to avoid autolysis of the walls [28]. After 24 h the supernatant (SN) of the bacteria was prepared as described in Methods. The viable cell count of cocci did not change during 48 h of incubation at 4 °C (Fig. 4, insert) thus a lysis of bacteria could be excluded. Therefore the increase of protein and other UV (260 nm) absorbing material (to be characterized in another paper) in the medium was not due to cell death.

As shown in Fig. 5, [^{3}H]TdR incorporation was inhibited by SN of *S. epidermidis* while SN of Cowan I caused stimulation. Similar results were

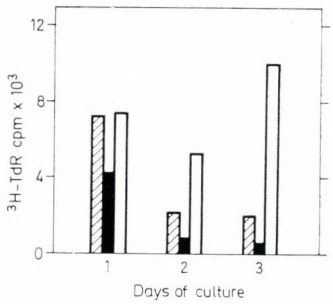

Fig. 5. [^{3}H]TdR incorporation of lymphocytes in the presence of supernatants of *S. epidermidis* (16.8 μg protein/ml, solid columns), and *S. aureus* Cowan I (5.3 μg/ml, open columns); control (shaded columns)

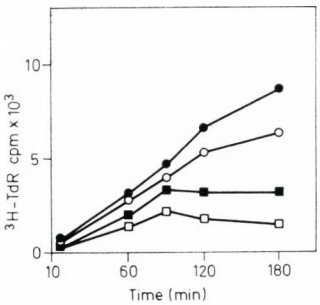

Fig. 6. Kinetics of [^{3}H]TdR incorporation of lymphocytes in the presence of various concentrations of supernatant of *S. epidermidis:* ○——○ 30 μg protein/ml; ▲——▲ 90 μg/ml; △——△ 190 μg/ml; ●——● control

obtained with SN prepared by longer (48 or 72 h) incubation of bacteria at 4 °C. The stimulatory effect of SN of Cowan I was most probably due to protein A released into PBS at 4 °C.

In order to examine the dose-response relationship of the toxic material released from *S. epidermidis*, SN was lyophilized and used in 3 hour experiments with lymphocytes (Fig. 6). Since human serum did not protect the lymphocytes against the inhibitory effect of SN, it was seen after a lag period of about 80 min; complete inhibition was caused by a dose of 90 μg SN protein/ml. The slow development of the inhibition renders a direct damage to DNA synthesis improbable.

We investigated the relationship of the SN inhibitor to known staphyloccal agents. The SN of *S. epidermidis* did not exhibit any haemolytic activity. Lysozyme, catalase and esterase are known to be secreted by cocci into the medium. Under our experimental conditions neither lysozyme nor catalase activities appeared in SN. A weak esterase activity was found in SN which disappeared after heat treatment, unlike the inhibitory effect of SN.

It was concluded that a new, water soluble, heat stable, formalin sensitive, cytotoxic material was prepared from *S. epidermidis* which inhibits the [^{3}H]TdR incorporation of tonsil lymphocytes. Purification and further properties of the toxic material will be described separately.

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REFERENCES

1. KNOX, K. W., WICKEN, A. J.: Bact. Rev. **37,** 215 (1973).
2. GROW, A., HELGELAND, S., ENDERSEN, C.: Zbl. Bakt. I. Abt. Suppl. **5,** 389 (1976).
3. GROV, A.: In Schlessinger, D. (ed.): Microbiology — 1977. American Society for Microbiology, Washington D.C. 1977. p. 350.
4. HAUKENES, G.: Acta path. microbiol. scand. **58,** 450 (1963).
5. WADSTRÖM, T.: Ann. N. Y. Acad. Sci. **236,** 343 (1974).
6. MCCARTNEY, C. A., ARBUTHNOTT, J. P.: In WADSTRÖM, T., JELJASZEWICZ, J. (eds): Bacterial Toxins and Cell Membranes. Academic Press Inc., London 1978. p. 89.
7. WISEMAN, G. M.: Bact. Rev. **39,** 317 (1975).
8. ROGOLSKY, M.: Microbiol. Rev. **43,** 320 (1979).
9. LOMINSKI, I., GEMMEL, C. G., ARBUTHNOTT, J. P.: J. gen. Microbiol. **52,** 107 (1968).
10. FORSGREN, A.: In Schlessinger, D. (ed.): Microbiology — 1977. American Society for Microbiology, Washington D.C. 1977. p. 353
11. LING, N. P., SPICER, E., JAMES, K., WILLIAMSON, N.: Brit. J. Haemat. **11,** 421 (1965).
12. KREGAER, A. S., CUPPARI, G., TARANTA, A.: Infect. Immun. **5,** 723 (1972).
13. VAN HEYNINGEN, W. E., ARSECULERATNE, S. N.: Ann. Rev. Microbiol. **18,** 195 (1964).
14. HOLT, R. J.: Brit. J. Derm. **86,** Suppl. 8, 42 (1972).
15. NAGY, ZS., ANTONI, F., SOLYMOSSY, M.: Acta microbiol. Acad. Sci. hung. **27,** 79 (1980).
16. PUSKÁS, M., STAUB, M., ANTONI, F.: Biochem. Med. **13,** 359 (1975).
17. AEBI, H.: In BERGMEYER, H. U. (ed.): Methods of Enzymatic Analysis. Academic Press, New York 1974. p. 673.
18. PAUL, J., FOTTREL, P.: Biochem. J. **78,** 418 (1961).
19. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., RANDALL, R. J.: J. biol. Chem. **193,** 265 (1951).
20. STAUB, M., SASVÁRI-SZÉKELY, M., SPASOKUKOTSKAYA, T., ANTONI, F., MERÉTEY, K.: Biochem. Med. **19,** 218 (1978).
21. RODEY, G. E., DAVIS, T., QUIE, P. G.: J. Immunol. **108,** 178 (1972).
22. FORSGREN, A., SVEDJELUND, A., WIGZELL, H.: Eur. J. Immunol. **6,** 207 (1976).
23. SAKANE, T., GREEN, I.: J. Immunol. **120,** 302 (1978).
24. WILLIAMS, R. C., KRONWALL, G.: Proc. Soc. exp. Biol. (N. Y.) **139,** 480 (1972).
25. MOLLER, G., LANDWALL, P.: Scand. J. Immunol. **6,** 357 (1977).
26. PEAVY, D. L., ADLER, W. H., SMITH, R. T.: J. Immunol. **105,** 1453 (1970).
27. DEAN, J. H., SILVA, J. S., MCCOY, J. L., CHANS, S. P., BAKER, J. J., LEONARD, C., HERBERMAN, R. B.: J. Immunol. **115,** 1060 (1975).
28. HAUKENES, G.: Acta path. microbiol. scand. **55,** 463 (1962).

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HIGHER RESISTANCE OF GERMFREE MICE TO DIANHYDRODULCITOL, A LYMPHOTROPIC CYTOSTATIC AGENT

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(Received December 19, 1980)

The same dose of dianhydrodulcitol (DAD) produced a lower mortality rate among germfree mice than among SPF or conventional C3H mice. On the other hand, it caused graver lymphoid atrophy in germfree mice. Their higher resistance, as evidenced by the mortality rate, can be explained on the basis of a histological study of the ileum. It showed milder alterations of the intestinal wall in germfree than in SPF mice. The lymphotropic cytostatic agent had a less direct toxic effect in germfree mice, due to the lacking damaging effect of endotoxin from the normal intestinal flora.

Several authors have reported on a higher resistance of germfree mice to certain antitumour agents like cyclophosphamide, nitrogen mustard or gamma rays [1–3]. Germfree mice have an underdeveloped lmyphoid system on account of the scarcity of antigenic stimuli from the endogenous and exogenous environment.

The present study was undertaken to examine the response of germfree mice to dianhydrodulcitol, a lymphotropic cytostatic agent.

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 for Laboratory Animals (LATI, Gödöllő, Hungary). The germfree state was maintained with VELAZ P–OI (Czechoslovakia) plastic isolators. Microbiological control of the GF state was done as described in literature [4]. The GF mice were fed with gamma ray sterilized pellets and autoclaved water *ad libitum.*

Treatment with dianhydrodulcitol (NSC-132313). Dianhydrodulcitol (DAD) is the epoxide of DBD, a lymphotropic cytostatic agent of the alkylating group [5]. The substance (Chinoin Chemical and Pharmaceutical Works Ltd., Budapest) was dissolved in distilled water and used within 30 min. The control mice received sterilized physiological saline administered in the same way and quantity as the DAD treatment.

Histological examination. The small intestine was prepared as described by SHIRAI *et al.*, then fixed and stained with haematoxylin–eosin [6].

Examination of the lymphoid system. The relative spleen and thymus weights and the spleen and thymus indices of mice died or sacrificed during the experiment were determined as follows;

$$\text{Relative lymphoid organ weight} = \frac{\text{lymphoid organ weight (mg)}}{\text{body weight (g)}}$$

$$\text{Lymphoid organ index} = \frac{\text{mean relative lymphoid organ weight in the DAD treated group}}{\text{mean relative lymphoid organ weight in the control group.}}$$

Statistical evaluation was done by Student's two sample t test. The accepted level of significance was $p = 0.05$.

Results

Three series of experiments were done. In Experiment I, GF, SPF, and CV mice were treated with a 15 mg/kg DAD dose. In Experiment II, GF and SPF mice were treated with DAD in doses of 12, 15 or 18 mg/kg, and in Experiment III, GF and SPF mice received DAD in a dose of 15 mg/kg. The mouse groups, their number and treatment are demonstrated in Table I.

Table I

Mouse groups and their treatments

Experiment	Group	No. of mice	Treatment (intraperitoneal)
I	GF–DAD	12	15 mg/kg DAD
	SPF–DAD	12	15 mg/kg DAD
	CV–DAD	12	15 mg/kg DAD
	GF	12	phys.NaCl
	SPF	12	phys.NaCl
	CV	12	phys.NaCl
II	GF–DAD	16	12 mg/kg DAD
		16	15 mg/kg DAD
		16	18 mg/kg DAD
	SPF–DAD	16	12 mg/kg DAD
		16	15 mg/kg DAD
		16	18 mg/kg DAD
	GF	12	phys.NaCl
	SPF	12	phys.NaCl
III	GF–DAD	20	15 mg/kg DAD
	SPF–DAD	20	15 mg/kg DAD

In Experiments I and II, the death rates caused by DAD were registered, and the relative lymphoid organ weights and indices of the died or sacrificed mice were determined. In Experiment III, the ileum of mice died or sacrificed at different times during the experiment was examined histologically.

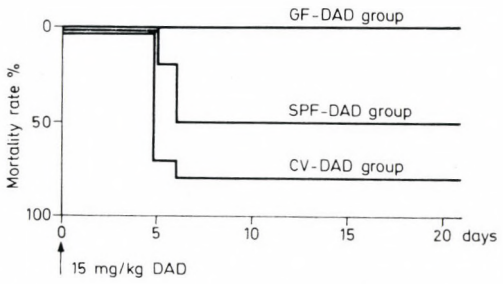

Fig. 1. Rate and time curve of mortality following DAD treatment in Experiment I

The GF state was checked microbiologically till the end of the experiments on the 21st day after treatment.

In Experiment I, 15 mg/kg DAD caused no death in the GF mice, while the mortality rate was 50% in SPF mice and 80% in CV mice, as registered on the 5th–6th day following treatment (Fig. 1).

There was no death in the mouse groups that had not received DAD.

The data of the lymphoid organs of mice dying or sacrificed on the 6th to 21st days are seen in Table II.

Table II

The lymphoid organs of mice died or sacrificed in Experiment I

Date	Group	No. of mice	Mean relative spleen weight	Spleen index	Mean relative thymus weight	Thymus index
5th and 6th day(died)	GF–DAD	0	—	—	—	—
	SPF–DAD	5	1.7	0.5	0.1	0.1
	CV–DAD	9	1.5	0.4	0.1	0.07
6th day (sacrificed)	GF	6	2.9	1.0	1.1	1.0
	SPF	6	3.3	1.0	1.0	1.0
	CV	6	3.6	1.0	1.3	1.0
21st day (sacrificed)	GF–DAD	12	4.4	1.4	1.1	1.0
	SPF–DAD	7	4.4	1.3	0.8	0.6
	CV–DAD	3	5.2	1.3	1.0	0.7
	GF	6	3.1	1.0	1.1	1.0
	SPF	6	3.2	1.0	1.2	1.0
	CV	6	3.8	1.0	1.4	1.0

Table II shows that grave lymphoid atrophy was observed in mice dying from DAD on the 5–6th day as compared to the GF, SPF, and CV mice sacrificed on the 6th day. The mice surviving DAD treatment displayed lymphoid

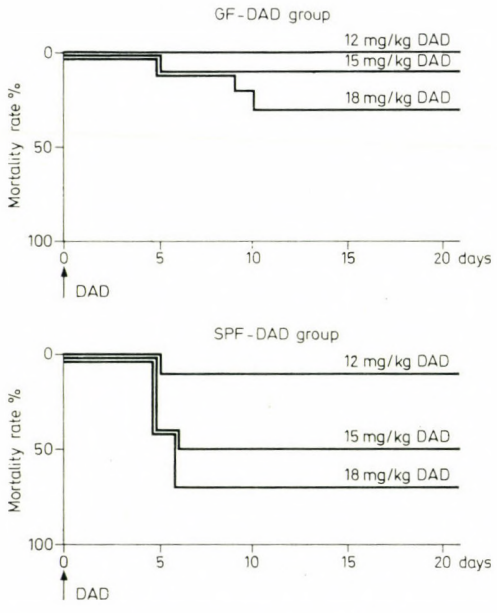

Fig. 2. Rate and time curve of mortality following DAD treatment in Experiment II

regeneration by the 21st day. The regeneration was almost equal in the GF–DAD, SPF–DAD and CV–DAD groups, as shown by the relative spleen and thymus weights. The spleen index pointed to hyperregeneration.

Figure 2 shows the time curve of mortality following DAD treatment with doses of 12, 15 and 18 mg/kg in Experiment II.

In GF mice a 12 mg/kg dose of DAD caused no death; the mortality rate was 10% after a 15 mg/kg, and 30% after a 18 mg/kg dose. In SPF mice the same doses caused death in 10, 50 and 70%, respectively. Thus, among SPF mice the LD_{50} was 15 mg/kg, while the same dose resulted in a death rate of 10% among GF mice.

The time curve of mortality revealed that the mice in the SPF–DAD group died on the 5th to 6th day, while those in the GF–DAD group died somewhat later, on the 5th to 10th day.

No death occurred among untreated GF and SPF mice during the test period.

Data for the lymphoid organs of mice dying from DAD treatment or sacrificed afterwards are demonstrated in Table III.

There were no significant differences between the data for the lymphoid organs of the GF–DAD and SPF–DAD groups with respect to the DAD dose. Hence, the data for the lymphoid organs were pooled for each group.

Spleen and thymus weight in the GF group was essentially the same whether the mice had been killed on the 6th or on the 21st day. Similarly,

Table III

The lymphoid organs of mice died or sacrificed in Experiment II

Date	Group	No. of mice	Mean relative spleen weight	Spleen index	Mean relative thymus weight	Thymus index
5th to 10th day (died)	GF–DAD	8	1.1 ± 0.1^a	0.2	0.2 ± 0.1	0.1
	SPF–DAD	28	2.1 ± 0.4^a	0.4	0.5 ± 0.3	0.2
6th day (sacrificed)	GF	6	3.8 ± 1.1	1.0	1.2 ± 0.3^b	1.0
	SPF	6	4.5 ± 0.9	1.0	2.1 ± 0.8^b	1.0
21st day (sacrificed)	GF–DAD	52	5.1 ± 2.2	1.3	1.2 ± 0.5	1.0
	SPF–DAD	32	5.3 ± 0.9	1.1	1.6 ± 0.5	0.7
	GF	6	3.8 ± 1.1	1.0	1.2 ± 0.3^c	1.0
	SPF	6	4.5 ± 0.9	1.0	2.1 ± 0.8^c	1.0

[a] $p < 0.001$
[b] $0.01 < p < 0.05$
[c] $0.01 < p < 0.05$

there was no difference between the 6 and 21 day values for the SPF mice. The relative spleen weight of the GF and SPF mice showed no significant differences on the 6th or 21st days, while relative thymus weight in the GF group was significantly lower than in the SPF group at any period.

Grave spleen and thymus atrophy was observed in both GF and SPF mice that had died from DAD treatment, but the spleen atrophy of GF mice was significantly graver than of SPF mice. This is indicated by the significantly lower mean relative spleen weight of died GF mice (GF–DAD) as compared to the died SPF mice (SPF–DAD), while there was no significant difference in relative spleen weight between the GF and SPF mice sacrificed on the 6th day.

A complete regeneration of the lymphoid organs was observed in mice surviving DAD treatment and sacrificed at the end of the experiment. Their relative spleen and thymus weights did not diverge in any significant degree from the corresponding values for the simultaneously sacrificed GF and SPF mice.

Experiment III was undertaken to examine the effect of DAD on the gastrointestinal tract [5] in GF and SPF mice. Twenty GF and 20 SPF mice were injected with a 15 mg/kg dose of DAD. A histological examination was undertaken on the ileum of mice dying from DAD treatment or sacrificed on the day of treatment (zero day) or on the 3rd, 12th or 21st days following treat-

ment. The histological specimens were examined as to the integrity of the intestinal mucosa expressed in percentage of total intestine length, and to changes indicating the effect of endotoxin, like inflammatory infiltrations or oedema. The extent is marked by crosses in Table IV, which summarizes the results.

Table IV

Intestinal changes in the mice of groups GF–DAD and SPF–DAD following DAD treatment

Day after treatment	No. of mice		Intestinal change: mucosal necrosis, %		muscle necrosis, %		inflammation		oedema	
	GF–DAD	SPF–DAD	GF–DAD	SPF–DAD	GF–DAD	SPF–DAD	GF–DAD	SPF–DAD	GF–DAD	SPF–DAD
0 day (sacrificed)	4	4	5	10	—	—	—	—	—	—
3rd day (sacrificed)	4	4	10	10	—	—	++	+++	—	+++
4th to 6th day (died)	2	6	60	90	30	90	—	±	—	±
12th day (sacrificed)	4	4	10	30	—	—	±	+	—	—
21st day (sacrificed)	6	2	10	10	—	—	±	+	—	—

No toxic signs attributable to DAD were observed in the GF or SPF mice sacrificed immediately after treatment (zero day). There were significant differences in the changes between the two groups of mice sacrificed or died later. Necrosis of the mucosa and inflammatory infiltration were much graver in SPF than in GF mice sacrificed on the 3rd day. No oedema was observed in GF mice. The dominant change in mice dying from treatment was a necrosis of the mucosa and muscle, but while these amounted to 90 and 90%, respectively, in the SPF–DAD group, in dead mice of the GF–DAD group the necroses amounted to 60 and 30% of the intestinal length. Pathologic changes in the mucosa were hardly detectable in GF–DAD mice sacrificed on the 12th day, while in mice of the SPF–DAD group mucosal necrosis was seen in 30%.

Discussion

The results of Experiments I and II show that the same dose of DAD caused lower death rates and later death in GF mice than in SPF or CV mice. Grave spleen and thymus atrophy developed in both SPF and GF mice dying

from DAD treatment, but spleen atrophy was significantly graver in the GF than in the SPF mice (Experiment II).

On the other hand, the changes of the intestinal tract were milder in GF than in SPF mice (Experiment III). By the end of the experiments a complete regeneration of the lymphoid organs (Experiments I and II) and of the intestinal mucosa (Experiment III) could be observed in mice surviving DAD treatment.

Since the death rate was lower among GF than among SPF mice, the GF mice, in spite of their underdeveloped lymphoid system, proved to be more resistant to the cytostatic agent than were the SPF or CV mice. The increased resistance of GF mice to DAD is explained by their GF state. In this state the direct toxic effect of DAD proved less grave since it had not been amplified by the acute damaging effect of the endotoxin from the normal intestinal flora. The characteristic signs of endotoxin effects like oedematous inflammatory changes and necrosis of the intestinal mucosa, were essentially milder in GF than in SPF mice. The mild endotoxin effect observable in GF mice might be explained by the fact that the GF condition does not imply an antigen-free state, and it is the endotoxin in food that might be responsible for the observed changes.

Our previous experiments showed an increased sensitivity to DAD of conventional mice that had been thymectomized neonatally [7] or suffering a GVH reaction [8], or treated with pertussis vaccine [9], or infected with LCM virus [10]. Similarly, an increased sensitivity could be observed in conventional newborn and suckling mice with underdeveloped lymphoid system [11] or in aged mice with thymus involution [12].

As compared to the normal DAD sensitivity of CV mice with developed and intact immune system, the sensitivity of CV mice with insufficient immune function changed in a direction opposite to that of GF mice with their equally insufficient immune function. The sensitivity of the latter diminished, while that of the former increased (Table V).

In previous experiments we attributed the increased sensitivity to DAD of conventional mice to a functional damage of the lymphoid system. The present results for GF mice, however, indicate that, apart from the actual functional state of the lymphoid system, the presence or absence of a normal intestinal flora is of decisive importance in respect of the sensitivity to DAD.

Our results are in accordance with those that emphasize the role of the normal intestinal flora in the development of gastrointestinal symptoms of the irradiation syndrome elicited by gamma rays [3].

Attention is called to the influence of a normal microbial flora on drug sensitivity during lymphotropic cytostatic treatment; they stress the importance of a germfree environment and the expediency of a so-called life island, an eventual reverse isolation [13].

Table V

Connection between gnotobiological condition, immunological state, and sensitivity to DAD in mice

Mice	State of the immune system	Function	DAD sensitivity
Germfree (adult)	under-developed	insufficient	diminished
Conventional (adult)	developed, intact	normal	normal
Conventional newborn, suckling	undeveloped		
neonatally thymectomized (adult)	impaired	insufficient	increased
suffering from GVH reaction (adult)			
LCM virus carrier (adult)			
treated with pertussis vaccine (adult)			
old	involuted		

REFERENCES

1. White, L. P., Caflin, E. E.: Science **140,** 1400 (1963).
2. Chakava, O. V., Ruban, S. Z.: Fol. microbiol. (Praha) **24,** 81 (1979).
3. Miller, I., Mandel, L., Moravek, F., Kruml, J., Klepalova, J.: Fol. microbiol. (Praha) **17,** 291 (1972).
4. Talafantova, M., Mandel, L.: Fol. microbiol. (Praha) **24,** 17 (1979).
5. Németh, L., Institoris, L., Somfai, S., Gál, F., Pályi, I., Csuka, O., Szentirmay, Z., Keller, B.: Cancer Chemother. Rep. **56,** 593 (1979).
6. Shirai, Y., Sunakawa, K., Ichihashi, Y., Yamaguchi, H.: Exp. Path. **17,** 158 (1979).
7. Anderlik, P., Szeri, I., Bános, Zs.: Kísérl. Orvostud. **26,** 393 (1974).
8. Anderlik, P., Szeri, I., Bános, Zs.: Ann. Immunol. hung. **18,** 53 (1975).
9. Anderlik, P., Szeri, I.: Acta microbiol. Acad. Sci. hung. **24,** 49 (1977).
10. Szeri, I., Alföldy, Z., Anderlik, P., Bános, Zs., Radnai, B.: Ann. Immunol. hung. **16,** 445 (1972).
11. Szeri, I., Anderlik, P., Bános, Zs.: Ann. Immunol. hung. **18,** 37 (1975).
12. Anderlik, P., Szeri, I., Bános, Zs., Wessely, M.: Kísérl. Orvostud. **31,** 167 (1979).
13. Blaha, M., Vanasek, J., Shiroky, O.: Fol. microbiol. (Praha) **24,** 96 (1979).

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A PROVISIONAL CHROMOSOME MAP OF *SHIGELLA* AND THE REGIONS RELATED TO PATHOGENICITY

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Based on the literature and the authors' studies, the genetic markers of *Shigella* and the chromosome regions connected with pathogenicity are listed. A provisional map of *Shigella* chromosome and another provisional map indicating the chromosomal regions related to *Shigella* virulence have been constructed. Both maps could be useful for further genetic investigations of *Shigella*.

The available genetic data obtained on the members of the genus *Shigella* permit to construct a provisional chromosome map of these bacteria which includes more than 40 markers. Most of these studies refer to *Shigella flexneri*, partly to *Shigella dysenteriae* 1 and some studies are concerned with *Shigella sonnei*.

Mapping of Shigella chromosomal genes

LURIA and BURROWS [1] were the first to accomplish recombination between *S. flexneri* recipients and Hfr strains of *Escherichia coli* K12. They showed the possibility of obtaining relatively stable hybrids and a similar localization of a number of the investigated markers with the localization of their alleles on *E. coli* K12 chromosome.

Later, similar intergeneric crosses using Hfr *E. coli* K12 with different orientation, not only demonstrated a similar localization of the investigated *S. flexneri* chromosome markers *(lac, ara, rha, xyl, mal, fuc, nad)* and their localization on *E. coli* but also revealed the circular structure of the *S. flexneri* chromosome [2]. Under natural conditions *S. flexneri* strains which utilize arabinose, rhamnose, xylose and maltose are found frequently [3] in contrast with the strains used in the mentioned experiments. Then it was shown that the strains of *S. dysenteriae* 1 which are phenotypical Mal$^-$, Mtl$^-$ after phage treatment or in their R state, begin to utilize maltose and mannitol [4]. These data indicate that among the *Shigella* strains which do not ferment certain carbohydrates some carry corresponding genes and that different strains differ from each other in the structure of the genes. This was experimentally shown for the *lac* operon of *S. dysenteriae* 1 [5]. After having obtained *S. flexneri*

donor strains it was possible to carry out comparative studies regarding the localization of several markers *(leu, ile, lys, his)* by kinetics of their transfer in the conjugations *E. coli* × *E. coli*, *E. coli* × *S. flexneri* and intraspecies *S. flexneri* × *S. flexneri*. The similarity of *S. flexneri* and *E. coli* K12 chromosome was demonstrated in spite of differences between the rates of transfer markers [6, 7].

A similar order of loci *(met A, thr, trp)* on the *E. coli* and *Shigella* chromosomes was shown in intraspecies crosses. High integration of markers in *E. coli* K12 Hfr × *S. flexneri* crosses was observed in contrast with the integration of markers in crosses of *E. coli* K12 Hfr with *Salmonella* or other representatives of *Enterobacteriaceae* [8]. The decrease in the rate of transfer in the *E. coli* × *Shigella* intergeneric crosses is known to be related to the incomplete homology of their DNA structure and host-cell specific modification and restriction [9].

Recent data, however, indicate an essential relationship of the structure of their genomes. It was even proposed to include *Escherichia* and *Shigella* into one genus [10].

A certain number of examples demonstrates that intergeneric crosses with *E. coli* K12 may be used for the preliminary determination of gene localization on the *Shigella* chromosome. The localization of *rfb* gene in *S. flexneri* 2a which is responsible for antigen 3,4 synthesis (primary S-specific chains: rhamnosyl-rhamnosyl-*N*-acetylglucosamine) near the *his* operon was first shown in intergeneric crosses with *E. coli* K12 by the loss of the marker [11], by its transfer to *E. coli* K12 [12] and later on intraspecies P1 transduction (cotransduction *his–rfb* 3,4–28.5%) [11].

Localization of the "Tp" locus responsible for some type- (serovar) specific antigens in the *lac–pro* region on the *S. flexneri* chromosome was demonstrated in intergeneric crosses with *E. coli* K12 Hfr by the loss of the corresponding antigenic factors in wild recipient strains [1, 13, 14]. Thereafter the transfer of antigen II from *S. flexneri* to *E. coli* K12 with *pro*$^+$ marker was carried out [12].

At the same time it was proved by immunochemical analysis that *S. flexneri lac*$^+$ hybrids lacking the corresponding serovar-specific antigens lose α-glucose in their LPS structure and are transformed into "Y"-like variants (—: 3,4) [12].

In some experiments with several converting phages [11, 16–18] conversion was carried out with the revealing serovar-specific antigens I, II, IV, V and group-specific factors 7,8 and 6. Comparative studies of the sensitivity of wild strains and *lac*$^+$ hybrids which were lacking serovar-specific antigens to the corresponding converting phages revealed that the *lac–pro* region of the *S. flexneri* chromosome is the so-called Tp locus the region of the attachment sites of converting prophages [11].

The attachment site of prophage 7,8 is in the same region [18]. Basing upon genetic findings it was suggested that phage 7,8 is characterized by a different kind of linkage (tandem?) with the chromosome, in contrast with the prophages I, II, IV, V [11]. In conversion, prophages I, II, IV and V behave as alleles. The possibility of subsequent lysogenization by phage II and 7,8 with the formation of bilysogenic serovar 2b (II; 7,8) as well as conversion of a variant X (—: 7,8) into V:7,8 was demonstrated [17].

The localization of some prophages in the "Tp" locus was confirmed by conjugation with the converted "Y" variants [19]. The possibility to reproduce conversion on *E. coli* K12 hybrids with previously acquired antigen 3,4 has been shown [16, 20].

Finally, the mapping of *kcp A* (keratoconjunctival provocation) locus was performed in the following way. At first it was demonstrated that 100% of the hybrids with substituted *lac–gal* region in intergeneric conjugation of *E. coli* K12 Hfr and virulent, keratoconjunctival positive (KK^+) strain of *S. flexneri* had lost their virulence. Thereafter in intraspecies transduction with the help of phage P1 cotransduction *kcpA* gene (68%) with *purE* marker (situated between *lac* and *gal)* was shown [21].

The localization of more than 15 markers of *Shigella* was determined by transduction (Table I).

The data obtained up to now permit to draw a provisional map of the *Shigella* chromosome which will be useful in further genetic analyses of *Shigella*. Figure 1 shows the provisional map of the *Shigella* chromosome which includes more than 40 markers. Due to the fact that the homology of *S. flexneri* and *E. coli* K12 chromosome was demonstrated in a number of papers [1, 2, 6, 8, 10], we have placed the investigated genes on the *Shigella* map according to the order of their allelic genes on the 100 min map of *E. coli* K12 [22]. We established that from 40 min the *Shigella* genes investigated to date only the localization of *glp K* gene failed to coincide with the localization of the allelic gene on the *E. coli* map [22, 23]. For some other genes, however, we only know the order of their arrangement but not their exact localization.

In Table I the list of the investigated *Shigella* markers is presented according to the nomenclature of BACHMANN *et al.* [22]. Column 3 shows the closely situated genes (determined in conjugation) or the linked genes (with indication of linkage per cent in transduction or conjugation).

Column 4 shows allelic genes on the *E. coli* K12 map (in minutes) which will allow a better orientation in the determination of the localization of the investigated *Shigella* genes. So it is known that the *arg* genes of *E. coli* K12 are localized at different chromosomal sites (at 6, 62, 68, 73, 88 min) [22]. The fact that the investigated *arg* gene of *Shigella* was found to be close to the *thr–leu* locus, allows to suggest that this gene may be an allele of the *argC*, *argE* or

Table I

List of genetic markers of Shigella

Gene symbol	Mnemonic	Relatively closely localized or linked genes	Map position*	Comments; alternative gene symbols	References
1	2	3	4	5	6
ara	Arabinose	*lac*, *leu*, *pil*	1	Utilization of arabinose	1, 2, 9
arg	Arginine	*thr–leu*	88	Synthesis of arginine	7
aroE	Aromatic	*rpsL*, *rpsE* (80% cotransduction)	71	Biosynthesis of aromatic aminoacids Transduction *E. coli* × *S. dysenteriae*	43
att I *II* *IV* *V*	Attachment	*lac–pro* (90–97% in conjugation)		Integration sites of *S. flexneri* corresponding prophages. Latter carry out modification (glycosylation) of the main structure of LPS (antigen 3,4); confers serovar (type) specificity (factors I, II, IV, V); Tp locus	1, 2, 12, 7, 11, 13 14, 15, 20
att 7,8	Attachment	*lac–pro*		Integration site of *S. flexneri* prophage 7,8. Glycosylation of the main structure of LPS (group-specific factor 7,8).	11, 18
att 6	Attachment	*lac–pro* (32% intraspecies conjugation)		Possible integration site of *S. flexneri* prophage 6. Acetylation of the main structure of LPS. (Group-specific factor 6).	11, 16, 44
crp		*rpsL* (28–33% intraspecies cotransduction)	73	Cyclic AMP receptor protein	45
cysB	Cysteine	*trpABCDEOP*	28	Synthesis of cysteine: transduction *S. dysenteriae* 1 × *E. coli* K 12	46, 47
fuc	Fucose	*nadB*	60	Utilization of fucose	2, 48
gal	Galactose	*nadA*	17	Utilization of galactose	48
glpK	Glycerolphosphate	? (60–65 min in intraspecies conjugation of *S. flexneri*)	87	Glycerol kinase; corresponds to *glpK* gene of *E. coli* K12, but is not cotransducted with *rha*	23
his	Histidine		44	Synthesis of histidine	7, 11, 12
hsd	Host specificity	*thr*, *pil*	98	Host restriction activity; *hsS*	9

ile	Isoleucine	*leu*	0	Synthesis of isoleucine	7
ilv	Isoleucine-valine	between *rha* and *xyl*	83		33
lac	Lactose	*pro*	8	Utilization of lactose; The following characteristics of *lac* operon of Lac$^-$ *S. dysenteriae* 1 strains was shown in transduction: $i^+o^+z^+y$ $i^+o^+z^-y$	5
leu	Leucine	*ara*, *ile*	2	Synthesis of leucine	7, 9
lon	Long form	*proC* (59–94% cotransduction)	10	Filamentous growth, radiation sensitivity, capsular polysaccharide synthesis. (Transduction *S. dysenteriae* 1 × *E. coli* K12)	49
mal	Maltose	between *xyl* and *rpsL*	74	Utilization of maltose	2, 50
metA	Methionine		89	Synthesis of methionine; map position was similar to that of *metA* gene of *E. coli* K12 (intraspecies conjugation)	51
mtl	Mannitol	*xyl*, *rfa*	80	Utilization of mannitol	52, 53
nadA	Nicotinamide adenine dinucleotide	*gal*	16	*S. flexneri* phenotype as a rule is Nad$^-$; point mutations in both genes	2, 48
nadB	Ditto	*fuc*	55	Ditto	2, 48
neaB	Neamine	*rpsL* (95.4% intraspecies cotransduction)	73	Resistance to neamine	25
pil	Pili	*hsd*, *thr*, *ara*	98	Presence or absence of pili (fimbriae)	2, 9
proAB	Proline	*thr–leu*, *arg*	6	Synthesis of proline; the order of genetic markers in *S. flexneri* is similar to that of *E. coli* K12: *pro–thr–leu–arg*; in *S. dysenteriae* 1 *proAB* is situated separately from *proC* as in *E. coli* K12	7, 49
proC	Proline	*lon* (59–94% cotransduction in *S. dysenteriae*)	9	Synthesis of proline; order of genetic markers in *S. flexneri* was *lac–proC*	49, 54
ptsH	Phosphotransferase system	*purC* (45% cotransductions)	52	Phosphotransferase system (transduction *S. flexneri* × *E. coli*)	55
ptsI	Ditto	Ditto	52	Phosphotransferase system: enzyme I	55

* Map position of allelic gene on *E. coli* map (min)

Gene symbol	Mnemonic	Relatively closely localized or linked genes	Map position*	Comments; alternative gene symbols	References
1	2	3	4	5	6
purC	Purine	*ptsHI* (45% cotransduction)	53	Synthesis of purine; transduction *S. flexneri* × *E. coli*	55
purE	Purine	Between *lac* and *gal* (in *S. flexneri*)	12	Synthesis of purine (conjugation and intraspecies transduction)	21
rfa	Rough	*mtl* (cotransduction: in *S. flexneri*, 20%; in *S. sonnei*, 16.3%)	80	Lipopolysaccharide core biosynthesis	52, 53, 56
rfb a3,4	Rough	*his* (28.5% intraspecies cotransduction)	45	Synthesis of main S-specific chains of LPS (*S. flexneri* group specific factor 3,4)	11, 12, 20
rfb aVI	Rough	*his* (45.3% interspecies conjugation)	45	Synthesis of *S. flexneri 6* serovar specific antigen VI	11, 57
rha	Rhamnose	*tna*	86	Utilization of rhamnose	2, 50, 57
rpsE	Ribosomal protein, small	*rpsQ* (98.1% intraspecies cotransduction).	72	30S ribosomal subunit protein S5; resistance to spectinomycin; *spc*	25, 43, see Fig. 3
rpsL	Ribosomal protein, small	*neaB* (95,4% intraspecies cotransduction)	72	30S ribosomal subunit protein S12; *strA*	25, 43, see Fig. 3
rpsQ	Ribosomal protein, small	*rpsE* (98.1% intraspecies cotransduction).	72	30S ribosomal subunit protein S 17: resistance to neamine; *neaA*.	25, 58, see Fig. 3
serB	Serine	*trpR*	100	Synthesis of serine	46, 47
thr	Threonine	*serB*, *trpR*, *hsd*	0	Synthesis of threonine	7, 9, 46, 47, 51
tna		between *rha* and *xyl*	82	Tryptophanase; (indole production); *ind*	2, 50, 57
trpAB CDEOP	Tryptophan	*cysB*	27	Synthesis of tryptophan; the order of structural genes in *S. dysenteriae* was identical to that of *E. coli* (transduction *S. dysenteriae* × *E. coli* K12)	46, 47, 51
trpR	Tryptophan	*serB*, *thr*	100	Regulatory gene for the *trp* operon in *S. dysenteriae*	46, 47
xyl	Xylose	*rfa* (70% linkage in conjugation)	79	Utilization of xylose	2, 50, 53

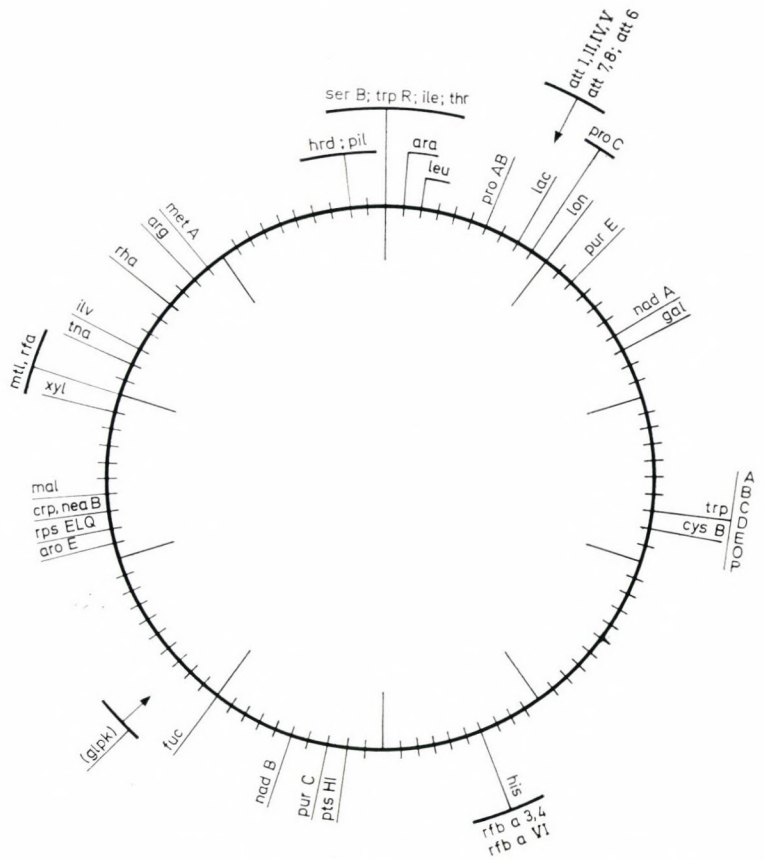

Fig. 1. Provisional linkage map of *Shigella* chromosome

argH genes of *E. coli* K12 (88 min). This suggestion can be controlled by investigation of corresponding enzymes.

Column 5 presents characteristics of the phenotypic expression of markers, with explanations and alternative gene symbols used in the works of different authors.

Regions of the Shigella chromosome related to pathogenicity

Since *Shigella* are pathogenic bacteria (aetiological factors of dysentery) not only the localization of different genes but also their pathogenetic importance were investigated in a number of studies. That is why we present in Fig. 2 another preliminary map of *Shigella* chromosome which indicates the regions related to the pathogenicity of the bacteria.

Investigation of the genetic control of bacterial pathogenicity is a difficult task because pathogenicity is multifactorial; its expression depends on

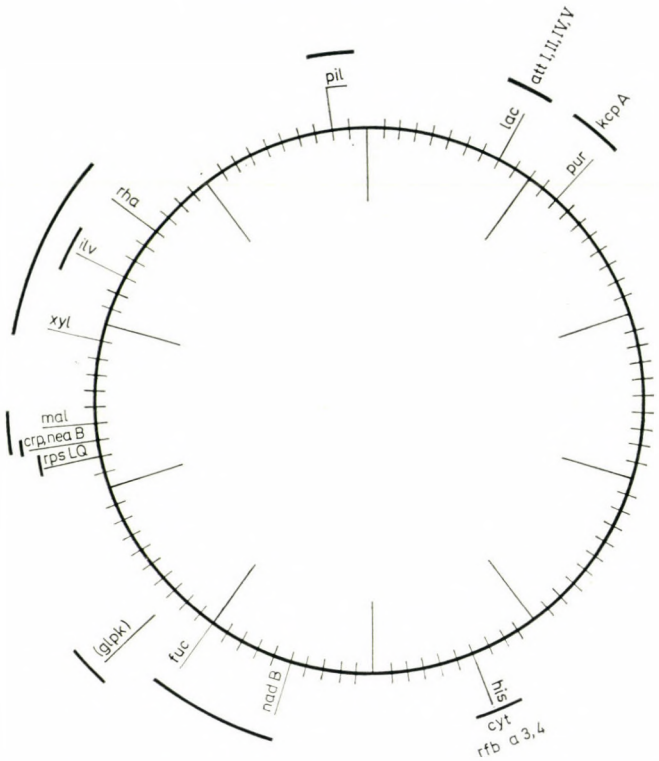

Fig. 2. Regions of the *Shigella* chromosome connected with pathogenicity

the activity of different and independent properties. Factors associated with pathogenicity cannot be selected by conventional methods used in bacterial genetics.

There are two main approaches in the genetic investigation of pathogenicity, viz.

(1) Investigation of the genetic control and regulation of known pathogenic factors (capsules, surface antigens, toxins, etc.);

(2) mapping on the chromosome of mutations or regions whose substitution in cross with non-pathogenic bacteria lead to a loss of virulence [24].

Both approaches were used with regard to *Shigella*. For instance in *S. flexneri* the genetic control of a known LPS O-antigen (the main structure-antigen 3,4 and serovar specific factors I, II, IV, V) was studied and *kcpA* gene was revealed by intergeneric crosses with *E. coli*. In each case the significance of the corresponding genotype variation for *Shigella* pathogenicity on different models of the infectious process was elucidated. The effect of different mutations *(glpK, rpsL, rspQ* and others) on the pathogenicity was also examined (Fig. 3) [23, 25]. Regarding such investigations it is important to determine not only the influence of the variations in the genome upon the bacterial patho-

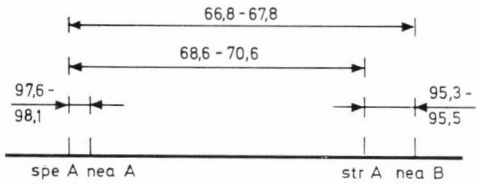

Fig. 3. Linkage map of *S. flexneri str–spc* region [25]. Symbols: *spcA–rpsE; neaA–rpsQ; strA–rpsL*

genicity but also to reveal which stages of the infectious process were influenced by these variations.

In view of the complex character of the infectious process, we divided the pathogenic factors into 3 main groups according to their functional importance:

(1) Factors which contribute to the entry of bacteria into the host and entertain interactions with mucosal membranes of definite ecological niches.

(2) Factors with resistance to humoral and cellular factors of the macroorganism defence and determine the rate of multiplication *in vivo*.

(3) Toxins and toxic products [26, 27].

A genetic control of neuro- (entero-) toxins of *Shigella* has not yet been investigated.

Shigellae are known to be facultative intracellular parasites. They are able to penetrate into, and multiply in, epithelial cells of the colonic mucosa [28, 29.]. This capacity correlates with the ability to cause keratoconjunctivitis (KK) in the guinea-pig. Negative strains as determined by the KK test are unable to cause dysentery in humans.

Interaction with mucosal membranes involves 3 main steps: adsorption, penetration and intraepithelial multiplication. In the experiments with ^{3}H-labelled *S. flexneri* virulent (KK^{+}) and R-mutants of different LPS chemotypes, it was shown that the main structure of *S. flexneri* LPS (antigen 3,4) is involved in the initial stage of attachment of bacteria ot the membrane of epithelial cells [30]. This structure probably is involved in the recognition of the specific receptor on the membrane: the replacement of antigen 3,4 by antigen O8 *E. coli* (with mannose instead of rhamnose in the LPS immunodeterminant group) in recombination leads to the loss of virulence [31]. In addition, the glucolipid which stimulates penetration isolated from the supernatant of the virulent strain of *S. flexneri* is only active with the homologous strain of *S. flexneri*, but not with *S. sonnei* or heated *S. flexneri* [32].

The LPS O-antigen alone does not, however, allow the *Shigella* to penetrate into the epithelial cells.

Two regions on the *Shigella* chromosome, *kcpA* and *glpK*, are significant for penetration [21, 23]. The products of these genes are unknown, but a biologically active factor was demonstrated in filtrates of virulent strains of *S. flexneri*,

in contrast with those of smooth mutants and hybrids lacking the penetration ability. A hypothetic penetration factor has therefore been assumed to be involved in the process of adhesion of *Shigella* onto the epithelium, also because the maximum radioactive label was observed with smooth KK^+ *Shigella* strains. Nonvirulent mutants of the smooth type (KK^-) are characterized by a low radioactive label. The problem is under discussion [30].

The LPS O-antigen, too, might play a role in intracellular survival and multiplication of the bacteria, because it was shown that *rha–xyl* hybrids of *Shigella* were able to penetrate into epithelial cells, but their intracellular multiplication was blocked [2]. The disturbance of LPS-synthesis in *rha–xyl* hybrids was demonstrated with the help of S- and R-specific phages [33].

As to the factors conferring resistance to humoral and cellular mechanisms of the host defence, the role of LPS O-antigen is well known: R-mutants of *Shigella* are avirulent and easily phagocyted [34]. In *S. flexneri* the main structure of LPS (antigen 3,4) plays an important role in this process. Secondary side chains (serovar-specific factors) confer additional resistance to humoral and cellular factors of the defence of the macroorganism [35, 36]. These observations correspond to the concept of main and secondary (additional) factors of bacterial pathogenicity [37].

Cases of dysentery induced by "Y" variants of *S. flexneri* lacking serovar-specific antigens have also been reported.

In *S. flexneri* factors inhibiting engulfment by phagocytes are absent. Virulent and avirulent strains are phagocyted with the same frequency, but in some virulent *S. flexneri* strains a factor killing macrophage (cytotoxin) was detected [38, 39]. Nevertheless the macrophages which survive 72 h following the infection digest not only Cyt^-, but also Cyt^+ *Shigella*. In contrast with *Salmonella*, *Shigella* are unable to multiply in macrophages, and therefore cannot induce a generalized infection. The gene (genes) responsible for cytotoxin synthesis is mapped near the *his* marker (28.8% of intraspecies cotransduction) [40].

It has been shown that induction of resistance against some antibiotics which influence the translation process leads to the loss of bacterial virulence, Such strains are not able to induce keratoconjunctivitis. The biological mechanisms of the loss of this property in $kcpA^-$ hybrids and streptomycin-resistant strains are different. In the latter case a blockage of intracellular multiplication takes place, but the capacity to penetrate into cells in maintained [41, 42].

At present it is difficult to say if mutations in ribosomal genes influence the synthesis of essential virulence factors or have more "unspecific" significance.

We hope that this short review of the important data on the problem of *Shigella* pathogenicity will facilitate understanding of the information presented in Table II.

Table II

List of the Shigella chromosome regions connected with pathogenicity

Region of the chromosome or gene symbol	Method of revealing	Characteristics	References
1	2	3	4
att I, II, IV, V	Conjugation with *E. coli* K12; conversion of var. Y	In absence of serovar-specific antigens I, II, IV, V resistance to humoral (immune sera), cell (macrophages) factors and surviving in KK sack is lowered	35, 36, 59
crp	Intraspecies transduction	Loss of the ability to induce keratoconjunctivitis in Crp strains by blocking penetration (HeLa cells)	45
cyt	Intraspecies transduction	Cytotoxin: hypothetic factor killing macrophages, taking part in cytopathic action on epithelial cells	37, 38, 39, 40
fuc–nadB	Conjugation with *E. coli* K12 Hfr	Virulence is lowered (model of starved guinea-pigs: 30–38% death in comparison with 76% in wild-type control strain). Mechanism is unknown	2
glpK	*glpK* mutation; transduction	5/9 *glpK* mutants of *S. flexneri* lose the ability to penetrate HeLa cells; 5/10 revertants and 7/18 *glpK*$^+$ transductants restore virulence	23, 30
kcpA	Conjugation with *E. coli* K12, intraspecies transduction *(kcpA–purE,* −68%); transfer F' plasmids of different length	*kcpA* hybrids lose the ability to induce keratoconjunctivitis; blocking penetration (HeLa cells); gene is recessive	21, 30, 42, 54
malA	Conjugation with *E. coli* K12 HfrC, selection for Mal$^+$	Restoration of the ability to induce keratoconjunctivitis in 1 of 3 spontaneous avirulent *Shigella* mutants. Reason for restoration of virulence not determined. It is possible that the mutant carried mutation for one of close *rps* genes	60
neaB	Intraspecies transduction	Loss of the ability to induce keratoconjunctivitis in Nea® strains	25
ilv	Intraspecies transduction of *ilv* marker from R mutants to virulent *Shigella* strain	Defect in synthesis of LPS with help of R-specific phages is shown. The ability to induce keratoconjunctivitis is preserved; rate of multiplication *in vivo* (lung model) is lowered; (*rfe* locus?)	33
pil	Conjugational transfer from *E. coli* K12	Lowering virulence of pil$^+$ hybrids (model of starved guinea-pigs: 29% death rate in comparison with 76% in control: wild strain). Mechanism is not clear; it is probably connected with the disturbance of specific attachment	2, 30

Region of the chromosome or gene symbol	Method of revealing	Characteristics	References
1	2	3	4
rfb	R mutants. Conjugational substitution of *rfb a3,4* for a O8 of *E. coli*	R mutants are easily phagocyted; role of antigen 3,4 in epithelial adhesion (HeLa cells) is shown; infectious process (model of starved guinea-pigs) is blocked	31, 30, 34
rha–xyl	Conjugation with *E. coli* K12	In *rha–xyl* hybrids: penetration ability is preserved; rate of multiplication in epithelium is lowered (infection of monkeys). It is probably connected with the disturbance of LPS core synthesis (*rfa* locus)	2, 33, 61
rpsL	Intraspecies transduction	Loss of the ability to induce keratoconjunctivitis in *rpsL* (Str®) transductants.	25, 41
rpsQ	Intraspecies transduction	Loss of the ability to induce keratoconjunctivitis in *rpsQ* (Nea®) transductants	25

Figure 2 shows the regions related to the pathogenic properties of *Shigella* and Table II characterizes their functional significance for the accomplishment of different stages of the infectious process.

REFERENCES

1. Luria, S. E., Burrows, J. W.: J. Bact. **74,** 461 (1957).
2. Falkow, S., Schneider, H., Baron, L. S., Formal, S. B.: J. Bact. **86,** 1251 (1963)
3. Ewing, W.: Differentiation of enterobacteriaceae by biochemical reactions revised. U. S. Department of Health, Education and Welfare. Atlanta 1974.
4. Li, K., Barksdale, L., Garmise, L.: J. gen. Microbiol. **24,** 355 (1961).
5. Franklin, N. O., Luria, S. E.: Virology **15,** 299 (1961).
6. Schneider, H., Falkow, S., Baron, L. S.: Genetics **48,** 907 (1963).
7. Schneider, H., Falkow, S.: J. Bact. **88,** 682 (1964).
8. Sanderson, K. E.: Advances in Genetics **16,** 35 (1971).
9. Falkow, S., Formal, S. B.: J. Bact. **100,** 540 (1969).
10. Colwell, R.: Int. J. syst. Bact. **24,** 422 (1974).
11. Petrovskaya, V. G., Bondarenko, V. M.: Int. J. syst. Bact. **27,** 171 (1977).
12. Formal, S. B., Gemski, P., Baron, L. S., LaBrec, E. H.: Infect. Immun. **1,** 279 (1970).
13. Timakov, V. D., Petrovskaya, V. G., Bondarenko, V. M.: Ann. Inst. Pasteur **118,** 3 (1970).
14. Timakov, V. D., Petrovskaya, V. G., Bondarenko, V. M., Kchomenko, N. A.: Int. J. syst. Bact. **22,** 149 (1972).
15. Manson, R., Simmons, D., Petrovskaya, V. G.: Europ. J. Biochem. **17,** 472 (1970).
16. Gemski, P. Jr., Koeltzow, D. E., Formal, S. B.: Infect. Immun. **11,** 685 (1975).
17. Giammanco, G.: Ann. Inst. Pasteur **114,** 63 (1968).
18. ПЕТРОВСКАЯ, В. Г., БОНДАРЕНКО, В. М.: Вестн. Акад. мед. наук. **12,** 52 (1969).
19. ПЕТРОВСКАЯ, В. Г., НЕВСКАЯ, Н. А., Генетика **10,** 61 (1975).
20. ПЕТРОВСКАЯ, В. Г., НЕВСКАЯ, Н. А.: Генетика **9,** 113 (1972).
21. Formal, S. B., Gemski, P. Jr., Baron, L. S., LaBrec, E. H.: Infect. Immun **3,** 73 (1971).
22. Bachmann, B. J., Low, K. B., Taylor, A. L.: Bact. Rev. **40,** 116 (1976).
23. Kim, R., Corwin, L. M.: Infect. Immun. **9,** 916 (1974).
24. Petrovskaya, V. G.: Genetical basis of the virulence of pathogenic and conditional-pathogenic enterobacteria. VII. Int. Congress of Infectious and Parasitic Diseases. Reports II. Varna 1978. pp. 32—35.

25. МАРАКУЩА, В. И., ПЕТРОВСКАЯ, В. Г.: Ж. микробиол. **12,** 25 (1980).
26. Smith, H.: Bact. Rev. **41,** 475 (1977).
27. ТИМАКОВ, В. Д., ПЕТРОВСКАЯ, В. Г.: Ж. микробиол. **9,** 3 (1977).
28. LaBrec, E. H., Schneider, H., Magnani, T. J., Formal, S. B.: J. Bact. **88,** 1503 (1964).
29. Takeuchi, A., Formal, S. B., Sprinz, H.: Amer. J. Path. **52,** 503 (1968).
30. Petrovskaya, V. G., Bondarenko, V. M., Mirolubova, L. V.: Zbl. Bakt. I. Abt. Orig. A. **243,** 57 (1979).
31. Gemski, P. Jr., Sheanan, D. G., Washington, O., Formal, S. B.: Infect. Immun. **6,** 104 (1972).
32. Osada, Y., Ogawa, H.: Microbiol. Immunol. **21,** 405 (1977).
33. ЛИХЕВА, Т. А.: Вестн. Акад. мед. наук. **1,** 34 (1976).
34. Slopek, S., Skurski, A., Michalska, E., Dabrowski, L.: Nature (Lond.) **182,** 1243 (1958).
35. ЛИХЕВА, Т. А.: Вестн. Акад. мед. наук. **1,** 34 (1976).
36. БЛИНОВА, Н. И.: Вестн. Акад. мед. наук. **12,** 44 (1973).
37. ПЕТРОВСКАЯ, В. Г.: Вестн. Акад. мед. наук. **12,** 6 (1973).
38. БЕЛАЯ, Ю. А., БРАНДЕ, А. И., ЩЕРБАКОВА, Е. Т., СОЛОВЕВ, Н. Х.: Ж. микробиол. **7,** 26 (1968).
39. Yee, R., Buffenmyer, C.: Infect. Immun. **1,** 459 (1970).
40. БОНДАРЕНКО, В. М.: Вестн. Акад. мед. наук. **1,** 14 (1976).
41. БОНДАРЕНКО, В. М., МАРАКУЩА, Б. И.: Ж. микробиол. **8,** 35 (1978).
42. БОНДАРЕНКО, В. М., ПЕТРОВСКАЯ, В. Г., КОРН, М. Я., ГАДОЩЕВИН, В. Н.: Ж. микробиол. **11,** 87 (1973).
43. Dekio, S.: Molec. gen. Genetics **113,** 20, (1971).
44. БОНДАРЕНКО, В. М., ПЕТРОВСКАЯ, В. Г.: Ж. микробиол. **4,** 79 (1977).
45. Manson, M. D., Yanofsky, C.: J. Bact. **126,** 668 (1976).
46. Manson, M. D., Yanofsky, C.: J. Bact. **126,** 679 (1976).
47. Gemski, P. Jr., Formal, S. B., Baron, L. S.: Infect. Immun. **3,** 500 (1971).
48. Markovitz, A., Rosenbaum, N., Baker, B.: J. Bact. **96,** 221 (1968).
49. Petrovskaya, V. G., Licheva, T. A.: Ann. Inst. Pasteur **118,** 761 (1970).
50. Kétyi, I.: Acta microbiol. Acad. Sci. hung. **17,** 167 (1970).
51. Godard, C., Hannecart-Pokorni, E.: Arch. int. Physiol. Biochim. **83,** 967 (1975).
52. Godard, C., Hannecart-Pokorni, E.: Ann. Inst. Pasteur **128A,** 19 (1977).
53. ЛИХЕВА, Т. А.: Ж. микробиол. **3,** 60 (1977).
54. ГЕРЩАНОВИЧ, В. И., УМЯРОВ, А. М., БОУРД, Г. И., БОЛЩАКОВА, ЛИХЕВА, Т. А., ПЕТРОВСКАЯ, В. Г.: Ж. микробиол. **11,** 83 (1980).
55. НАСТИХИН, И. А., ЛИХЕВА, Т. А.: Ж. микробиол. **9,** 68 (1979).
56. МАКЛЕЧОВА, Й. И., ПЕТРОВСКАЯ, В. Г.: Ж. микробиол. **1,** 44 (1976).
57. Bollen, A., Cabezon, T., de Wilde, M., Villarroel, R., Herzog, A.: J. molec. Biol. **92,** 795 (1975).
58. Kétyi, I.: Infect. Immun. **9,** 931 (1974).
59. Formal, S. B., LaBrec, E. H., Schneider, H., Falkow, S.: J. Bact. **89,** 835 (1965).
60. Formal, S. B., LaBrec, E. H., Kent, T. H., Falkow, S.: J. Bact. **89,** 1374 (1965).

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Note added in proof

In Fig. 2 *mtl* and *rfa* should be included at position 80 (see pp. 45 and 46, respectively).

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SOME CHARACTERISTICS OF NYSTATIN-RESISTANT STEROL MUTANTS OF *CANDIDA ALBICANS*

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Various stable, auxotrophic and nystatin-resistant sterol mutants of *Candida albicans* were isolated after nitrosoguanidine treatment. Sterol mutants were divided into groups on the basis of the ultraviolet spectra and thin-layer chromatographic patterns of their nonsaponifiable sterol extracts. They were further characterized by their conductometrically measured nystatin-induced ion release. These sterol mutants displayed a decreased growth yield and an increased cell volume. On media containing 0.01% of the carbon sources, most of them could assimilate glycerol, α-methyl-D-glucoside, DL-lactic acid, L-sorbose, L-arabinose and ribitol only to a significantly reduced extent, or not at all. It is presumed that these properties result from the altered sterol composition of the plasma membrane.

Since the discovery of the polyene macrolide antifungal antibiotics produced by various *Streptomyces* spp., these compounds, especially nystatin and amphotericin B, have widely been used to treat systemic mycoses. Polyene antibiotics interact with the sterol-containing membranes of sensitive organisms, causing permeability alterations leading to the loss of essential cytoplasmic constituents and culminating in cell death [1].

There are two basic approaches to the study of this interaction and the mechanisms of action of these agents. First, investigation of the effect of various polyene antibiotics different in chemical structure on ergosterol-producing, sensitive strains of fungi. Results concerning this subject have been reviewed in some excellent articles [1–3]. Second, examination of the effects of a given polyene antibiotic on different sterol mutants containing various intermediates of ergosterol biosynthesis in their plasma membrane [4]. This latter system allows to study beside the polyene antibiotic–sterol interaction, also the significance and function of ergosterol and its precursors in the plasma membrane and cell metabolism [5].

In the present work, the effect of nystatin on various types of nystatin-resistant sterol mutants of *Candida albicans* have been investigated.

Materials and methods

Strains. The prototrophic, ergosterol-producing, nystatin-sensitive, wild-type strain of *Candida albicans* (85/1975), designated *erg*$^+$, originating from the Department of Mycology, National Institute of Hygiene, Budapest. It was isolated from clinical material and used for the preparation of auxotrophic and nystatin-resistant mutants.

Culture media and conditions. Complete medium (YPG): 0.3% yeast extract, 0.5% peptone (Difco), 1% glucose. Minimal medium (MM): 1% glucose, 0.5% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4 and vitamins at pH 6, supplemented when required with 15 μg/ml adenine and 50 μg/ml of each of the required amino acids. Sabouraud liquid medium: 4% glucose, 1% peptone supplemented with vitamins, amino acids or adenine as described above. For solidification of media, 2% Bacto-agar (Difco) was used. Three-day-old stationary-phase cultures of cells grown in liquid YPG or MM in the manner mentioned below were used for mutagen treatment and sterol analysis. In all other experiments, overnight, exponential-phase cultures grown at 30 °C in YPG or Sabouraud liquid medium on a rotary shaker (Gallenkamp) at 200 rpm were used. The cells were washed four times in distilled water immediately before use.

Mycological studies. Both the wild-type strain and the mutants were identified by classical zymological techniques. These involved investigations on corn meal agar and colony morphology, including colour formation on Pagano–Levin–Trejo's TTC medium [6]. The well-known responses on these two media were considered the basic confirmation of the identity of the studied *C. albicans* strains. The results of further identification tests were regarded as variables which could be affected by mutagenesis. Carbohydrate assimilation tests were carried out on glucose-free solid MM containing 0.01% of each carbon source listed by VAN UDEN and BUCKLEY [7]. The media contained 5 μg/ml adenine and 15 μg/ml of each of the required amino acids. About 10^5 cells from overnight cultures were placed on a 1 cm^2 area of each medium and incubated at 30 °C. Results were evaluated every second day. Fermentation experiments on the recommended sugars were performed in Wassermann tubes using glucose-free liquid MM containing 0.5% of the required sugar and bromcresol purple indicator, and supplemented as for assimilation. After inoculation of 5 or 10 parallel tubes, they were covered with 2 ml Vaspar seal and incubated for one month at 30 °C. A test was classified as positive when a gas bubble was detected under the Vaspar seal.

Isolation of mutants. Mutagen treatments were carried out for 30–35 min at 37 °C with 1 mg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), buffered with 0.1 M Tris–HCl pH 8.2 [8] in a suspension containing 4×10^7 cell/ml of stationary phase. Two ergosterol-producing auxotrophs, an adenine-requiring (33 *ade*$^-$) and a threonine- and methionine-requiring (35 *thr*$^-$, *met*$^-$) strain, were used for the isolation of nystatin-resistant mutants of different origins [4]. These auxotrophs were designated 33 *erg*$^+$ and 35 *erg*$^+$, respectively. Nystatin-resistant mutants were maintained on YPG slants containing 50 U/ml nystatin.

Determination of minimum inhibitory concentration (MIC). MICs were determined by a two-fold serial dilution test on YPG solid media: 10^4 cells/cm^2 were plated on the medium containing the drug at appropriate concentration. MICs were recorded after 48 h incubation at 30 °C. The MIC was defined as the lowest concentration of antibiotic that inhibited growth completely.

Sterol analysis. Three-day-old cultures of cells were extracted as described by BREIVIK and OWADES [9], and the absorption spectra of the nonsaponifiable sterol extracts were recorded on a Specord UV–VIS spectrophotometer between 310 and 210 nm. Twenty mg dry sample were used for quantitative analysis of ergosterol. For thin-layer chromatography (TLC), samples of sterol extracts were applied in a volume of 25 μl to activated plates of silica gel G and developed with benzene : ethyl acetate (5 : 1, v/v) [10]. After drying of the plates, spots were detected under ultraviolet light or visualized with phosphoric acid : water (1 : 1, v/v) spray and heating of the plates under an infra lamp.

Determination of dry weight and cell volume. For determination of the dry weight yields of mutants grown in liquid MM, 10 ml culture was filtered through a Millipore membrane (pore size 0.45 μm), washed three times with 10 ml ice-cold distilled water, and dried for 4 h at 105 °C. The cell volumes of stationary-phase cells grown in YPG liquid medium were calculated by the following equation, after measurement of the short and long axes of 50 individual cells:

$$V = \frac{4\pi}{3} \cdot \frac{a}{2} \cdot \left(\frac{b}{2}\right)^2$$

where b and a are the average lengths of the short and long axes, respectively, in μm.

Ponceau red staining. To 0.9 ml 20% (wet weight/volume) cell suspension, 0.1 ml nystatin solution (1200 U/ml in 30% v/v dimethylformamide) was added. After 30 min incubation at 26 °C, 0.2 ml sample was withdrawn and added to 4 ml ice-cold water. The mixture was then centrifuged and resuspended in 1 ml ice-cold distilled water. After 10 min incubation at 4 °C, the suspension was again centrifuged and the pellet resuspended in 0.4 ml staining solution [11]. The proportion of red-stained cells was counted under a light-microscope after 5 min incubation at 26 °C.

Conductometry. The membrane-damaging effect of nystatin was characterized by measuring the nystatin-induced ion leakage of the cells [12]. Into six special home-made cuvettes for Philips electrodes, 4.5 ml 10% (wet w/v) cell suspensions were added and stirred magnetically. Conductometric electrodes (Philips, PW 9512/01) were immersed in each suspension. The six electrodes were connected to the channel-switch of a six-channel point-recorder of compensator type (Motorkompensator, type MK 3301–8.00, VEB Messgerätewerk Erich Winert, Magdeburg). The outlet of the switch was connected not to the built-in recorder, but to the inlet of the conductometer (type OK-102, Radelkis, Budapest), and the recorder outlet of the conductometer was in turn connected to the recording part of the compensator. Thus, the changes in conductivity measured by the six electrodes were registered automatically.

Spontaneous ion leakage in distilled water was followed for 30 min and thereafter 0.5 ml nystatin and control solutions were added to the parallel suspensions. The recordings were continued for at least a further 30 min. At the end of the experiments, the suspensions were boiled for 15 min, so that their ion content liberated by boiling could be determined. The relative ion liberation was calculated as described earlier [13]. For further evaluation, the dose-response curves demonstrating the initial rates of relative ion leakage were constructed. The initial rates were computed as the tangent to a hyberbola, fitted by the least squares method to the measured values.

Chemicals. Nystatin (5777 U/ml) was obtained from Chinoin, Budapest. Ergosterol, lanosterol, squalene and cholesterol were purchased from Sigma Chemical Co. Ltd., Saint Louis, U.S.A. Stigmasterol and β-sitosterol were purchased from Nutritional Biochemicals Co., Cleveland, U.S.A. Glycerol and α-methyl-D-glucoside were products of BDH Chemicals Ltd., England, while DL-lactic acid, L-sorbose, NTG and L-arabinose were from Fluka AG, Switzerland. All other chemicals were of the highest purity available commercially.

Results

1. *Description of mutants.* From the *erg*$^+$, wild-type strain of *C. albicans*, 39 different auxotrophic mutants were obtained by NTG treatment, with a frequency up to 0.26% if the proportion of survival was 9%. Two of them (27 *his*$^-$, 39 *met*$^-$) were used to produce double auxotrophic mutants. In these cases the frequency of mutation increased significantly if the proportion of survival was lower than 9%, but more than 90% of them had the same second auxotropic marker (Table I). Of the 33 different auxotrophs examined, 11 proved to be stable and no back mutation occurred. In some mutants, however, the frequency of revertants was between 1.74×10^{-3} and 3.2×10^{-4}. Auxotrophy was used as a marker for somatic hybridization experiments via protoplast fusion [14].

Since nystatin has been found to be the best agent for the isolation of different types of sterol variants [4, 15], it was applied for the selection of 20 nystatin-resistant, one-step mutants of different origin from two of the stable

Table I

Frequency of auxotrophic mutants of C. albicans obtained by NTG treatment

Strains	Survival %	No. of mutants	No. of types of (new) auxotrophy	Frequency of mutants, %
erg$^+$	9.0	39	39 different	0.26
27 his$^-$	4.2	33	31 ade$^-$, 2 ura$^-$	1.60
39 met$^-$	0.9	5	5 his$^-$	6.25

auxotrophic strains, designated *33 erg*$^+$, *ade*$^-$ and *35 erg*$^+$, *thr*$^-$ *met*$^-$, obtained from the above-mentioned 33 different auxotrophs. The *erg*-2, *erg*-16, *erg*-34 and *erg*-37 sterol mutants originated from the adenine-requiring *33 erg*$^+$ strain. All the others were descendants of the *35 erg*$^+$ strain. The MICs of these mutants are shown in Table II. Nystatin-resistant mutants were recultured 50 times on nystatin-free YPG, and only 3 of them lost their resistance to nystatin.

2. *Sterol analysis.* The UV absorption spectra of the nonsaponifiable sterol extracts of nystatin-sensitive and resistant mutants (Fig. 1) made it possible to divide all the 23 mutants into groups (I–VI). The wild-type strain and its auxotrophic mutants (type I) had absorption peaks at 271, 281 and 293 nm and a shoulder at 261 nm, corresponding to the peaks of the ergosterol standard. The type V sterol mutant produced 17.5% less ergosterol than did the parental strain, but a plateau appeared between 240 and 223 nm in its spectrum, suggesting the accumulation of a new sterol intermediate. Type II mutants seemed to be leaky as regards ergosterol production, with an ergosterol yield about 5% that of the parental strain. The spectra revealed a significant increase at the peak of squalene (216 nm) [15]. The absorption spectrum of the type IV mutant contained three peaks between 269 and 262 nm, with an increased one at 214 nm. Spectra of type III and VI mutants exhibited no absorbance between 310 and 250 nm, suggesting the lack of any $\Delta^{5,7}$-diene sterols in these mutants. However, a significant difference was found between them concerning the height of the peak at 213 nm. Most of the mutants belonged to these last two groups (Table II). All of the mutants were further studied by TLC analysis.

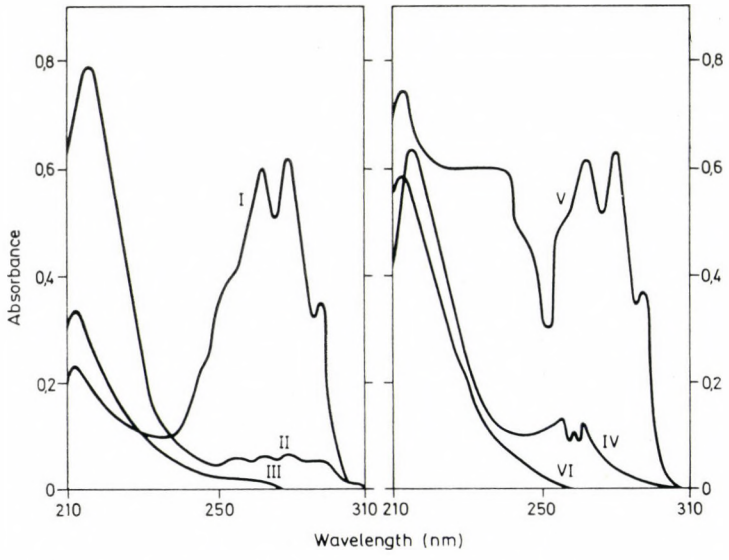

Fig. 1. UV absorption spectra of nonsaponifiable sterols extracted from nystatin-sensitive and resistant strains of *C. albicans*. I–VI represent the typical spectral groups

Table II

Minimum inhibitory concentration (MIC) of nystatin-sensitive and resistant strains of C. albicans, classified into different groups on the basis of ultraviolet spectra (UV) and thin-layer chromatographic (TLC) patterns

Strains	Nystatin MIC (U/ml)	UV spectrum	TLC pattern
erg$^+$	25	I	A
33 *erg*$^+$	25	I	A
erg-2	400	IV	F
erg-16	400	VI	B
erg-34	100	II	C
erg-37	200	VI	C
35 *erg*$^+$	25	I	A
erg-1	100	II	D
erg-12	100	V	D
erg-20	400	VI	B
erg-26	200	III	B
erg-27	200	VI	B
erg-28	400	III	E
erg-29	400	VI	E
erg-34	400	VI	E
erg-39	400	VI	E
erg-40	400	III	E
erg-41	400	III	E
erg-43	400	III	E
erg-45	400	VI	E
erg-48	400	VI	B
erg-50	400	VI	B
erg-51	400	II	D

TLC analysis of the nonsaponifiable sterol extracts supported the UV spectral results, and permitted further classification of the UV type III and VI mutants (Tables II and III). On the basis of these data, representative mutants were selected from the different groups and used in the following experiments (Table IV).

Sterol analysis has already been carried out by gas-liquid chromatography; this supports the results of UV and TLC analyses, showing the differences in the representative mutants. Results relating to fatty acid and phospholipid composition revealed significant changes in the sterol mutants as compared to

Table III

R_F values of major sterol classes of C. albicans nystatin-sensitive and nystatin-resistant strains and sterol standards on thin-layer chromatography

Standards	R_F	Strains	TLC pattern	R_F				
Ergosterol	0.25	*erg*$^+$	A	0.26	0.59	0.63	0.74	
Cholesterol	0.56	*erg-16, 20*	B	0.51	0.57			
Sitosterol	0.59	*erg-27*	C	0.51		0.57	0.68	
Stigmasterol	0.61	*erg-12*	D	0.25	0.55	0.74		
Lanosterol	0.70	*erg-40, 41*	E	0.52	0.58	0.71	0.77	
		erg-2	F	0.45	0.52	0.57	0.68	0.74

Table IV

Sterol contents, growth yields and cell volumes of nystatin-sensitive and resistant C. albicans strains

Strains	Ergosterol content[a] (quantitative UV data) (μg/mg dry wt.)*	Growth yield[a] (dry wt. mg/10 ml)*	Cell volume[b] (μm^3)
erg$^+$	35.21	25.5	—
33 erg$^+$	34.60	25.0	59.54 ± 6.13
erg-2	—	13.3	99.63 ± 4.66
erg-16	—	24.9	145.32 ± 10.98
erg-37	—	25.2	111.94 ± 9.67
35 erg$^+$	34.52	19.5	55.25 ± 8.89
erg-12	35.27	18.2	61.25 ± 5.12
erg-20	—	10.8	87.62 ± 9.23
erg-40	—	11.2	97.92 ± 10.11
erg-41	—	12.8	98.80 ± 9.29

[a] Cultures grown in liquid MM for 4 h at 30 °C

[b] Cultures grown in liquid YPG for 20 h at 30°C

[c] Mean ± standard error

* The accuracy of determination was $<2.5\%$

their ergosterol-producing parents, as a consequence of the altered sterol composition (unpublished data).

3. *Characteristics of sterol mutants.* With the exception of the *erg*-12 mutant, all the representatives lost their ability for ergosterol production. This phenomenon is associated with a significantly decreased growth yield and an

increased cell volume of the ergosterol mutants as compared to the original ergosterol-producing strains (Table IV). Auxotrophic mutants and their nystatin-resistant descendants formed white, glistening, smooth-surfaced colonies on Pagano–Levin–Trejo's TTC agar, and chlamydospores on corn meal agar. The wild-type strain and the auxotrophic mutants produced both acid and gas (CO_2) from glucose, galactose, maltose and trehalose after 6 days' incubation, and after 14 and 21 days the nystatin-resistant mutants displayed the same results as regards their reduced fermentation ability. Of the 43 carbon sources examined, the following were assimilated by the *erg*$^+$, 33 *erg*$^+$, 35 *erg*$^+$ strains alike: glucose, galactose, L-sorbose, sucrose, maltose, trehalose, soluble starch, D-xylose, glycerol, D-mannitol, α-methyl-D-glucoside, DL-lactic acid, succinic acid, citric acid, L-arabinose, D-fructose, melezitose, ribitol, D-glucitol and D-mannose. The resistant strains, however, could assimilate certain carbon sources only to a decreased extent or not at all (Table V).

The membrane-damaging effect of nystatin at different concentrations on nystatin-sensitive and resistant mutants of *C. albicans* were studied by conductometry (Fig. 2). Incubation for 30 min with 100 U/ml nystatin led to a 39.4% net relative ion leakage in the case of the 33 *erg*$^+$ cells, while 190 U/ml caused only a 25.3% relative ion leakage from the *erg*-2 cells. It is interesting that the spontaneous ion leakage of the *erg*-2 strain was two and a half times higher than that of the wild-type parent. It is seen, however, in the dose-response curves of different strains (Fig. 3) that the auxotrophic mutation did not

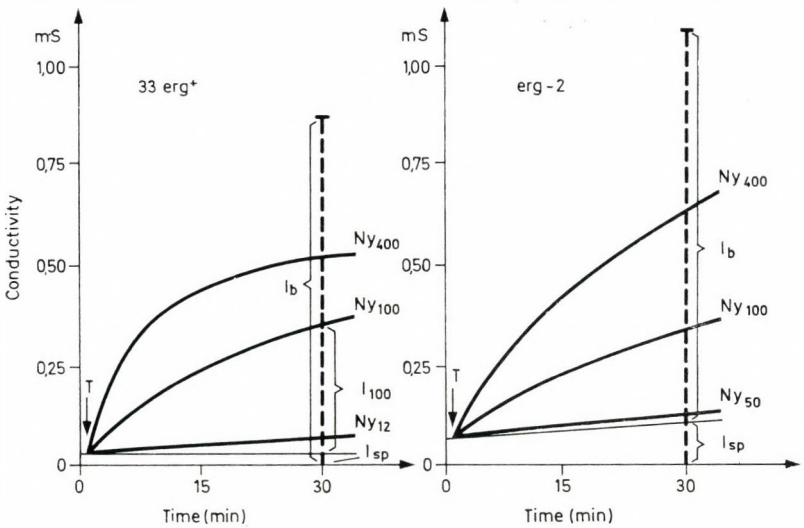

Fig. 2. Nystatin (Ny)-induced ion release in 33 *erg*$^+$ and *erg-2* strains of *C. albicans*. Index figures indicate the Ny concentration in U/ml. T = time of nystatin addition; Isp = spontaneous ion leakage; I_{100} = ion leakage induced by 100 U/ml nystatin; I_b = "total" ion content liberated by boiling

Table V

Assimilation patterns of nystatin-sensitive and resistant strains of C. albicans. Results after

Strains	Glucose		Glycerol		α-Methyl-D-glucoside	
	A	B	A	B	A	B
erg$^+$	++++	++++	+++	++	++++	++++
33 erg$^+$	++++	++++	+++	++	++++	++++
erg-2	++++	++++	−	−+	+	++
erg-16	++++	++++	−	++	+	+++
erg-37	++++	++++	−	−	+	++
35 erg$^+$	++++	++++	+++	+++	++++	++++
erg-12	++++	++++	−	+	++	++
erg-20	++++	++++	−	+	−	++
erg-40	++++	++++	−	−	−	+
erg-41	++++	++++	−	−	−	+

− no growth

+, ++, +++, ++++ weak, medium, good, excellent growth

± questionable growth

influence the barrier function of the plasma membrane of the 35 *erg*$^+$ strain in comparison with the *erg*$^+$ one. The 33 *erg*$^+$ mutant was, however, slightly more sensitive to nystatin, as indicated by the shift to the left of its curve. The mod-

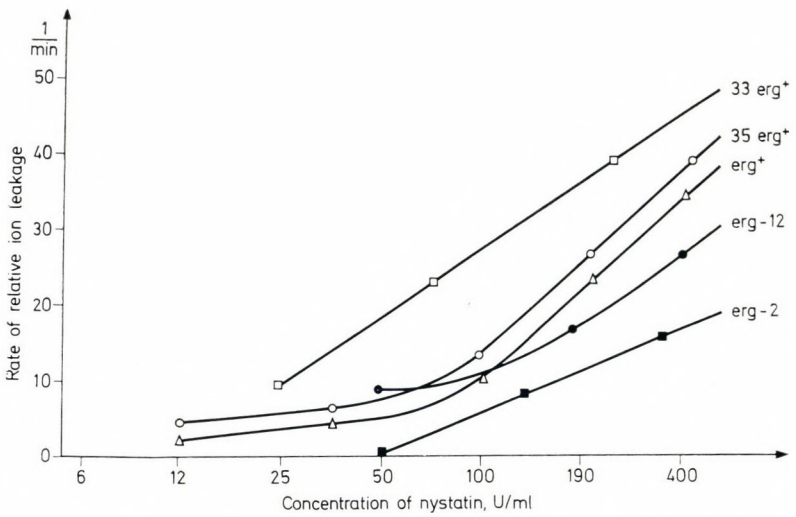

Fig. 3. Dose-response curves of ion leakage in nystatin-sensitive and resistant strains of *C. albicans*

3 days (A) and 14 days (B) incubation on MM containing 0.01% carbon sources

DL-lactic acid		L-sorbose		Ribitol		L-Arabinose	
A	B	A	B	A	B	A	B
+++	+++	++++	++++	++++	++++	+++	+++
+++	+++	++++	++++	++++	++++	+++	+++
+	+	+	++	+	+	+	+
−	±	++	++++	+	++	+	++
+	+	+	+	+	++	+	+
+++	+++	++++	++++	++++	++++	+++	+++
−	++	+++	++++	+++	++++	+	++
−	+	−	++	−	−	−	+
−	+	−	±	−	−	−	−
−	±	−	++	−	−	−	±

erately, 4-fold increased resistance of the *erg*-12 strain and the 16-fold increased resistance of the *erg*-2 strain are well characterized by their curves shifting to the right in consequence of their significantly decreased ion leakage. When nystatin-sensitive and resistant mutants were stained with Ponceau red following 120 U/ml nystatin treatment, no appreciable difference in staining (less then 5%) was observed between them.

Discussion

For the isolation of stable auxotrophic and nystatin-resistant mutants, NTG proved to be very effective. Further, as an alkylating agent, it rendered possible to treat stationary-phase cultures consisting of separated single cells and to reveal each of the mutants appearing in the surviving population. The 4–16-fold increased resistance of mutants was associated with the absence, or decreased yield, of ergosterol, in agreement with earlier findings [for reviews, see 1–3, 5]. Conductometric data support the results of MIC determinations on slants. The shift to the right of the dose-response curve of the *erg*-2 mutant indicates an increased resistance to nystatin as a consequence of its ergosterolless nature, while the different slopes of the curves suggest different mechanisms of action of nystatin on sensitive and resistant cells.

Ergosterol is of great importance as regards the plasma membrane functions [2, 5, 16], but its absence did not cause any demonstrable alteration of the plasma membrane ultrastructure of the *erg*-2 mutant, as revealed by freeze-

etch electron microscopy [17]. Significant changes were detected in the phospholipid fractions and fatty acid patterns of these resistant mutants (NOVÁK *et al.* [32]). These complex changes in membrane composition in response to the altered sterol composition tend to induce a reconstruction of the original membrane rigidity and function. The two and a half times higher spontaneous ion leakage of resistant mutants, however, may indicate incomplete restoration of the original membrane barrier. This phenomenon may offer an explanation for the decreased viability during storage of the nystatin-resistant mutants with altered permeability [18]. Preliminary [19] and the present results showed no penetration of Ponceau red into either the nystatin-treated sensitive or resistant cells. It is suggested that the membrane-modifying and the efflux barrier destroying effects of sterol blocking and nystatin treatment together could not result in the uptake of Ponceau red molecules.

The increased cell volume of the sterol mutants [20] may to a small extent also contribute to the increased resistance, as diploids having an increased surface–volume ratio proved to be partially resistant [21]. These facts together with the decreased growth yield pointed to certain physiological changes as a consequence of the altered lipid composition.

On the basis of the results we concluded that significant changes must be found in the uptake, and hence in the assimilation of carbon sources, the uptake processes being membrane-dependent. In fact, differences in assimilation between the nystatin-sensitive and resistant mutants have been found (Table V).

Although there are few data on the transport of these carbon sources in *C. albicans* [22], some comments may be made. Glycerol is transported by simple diffusion [23]. It has been suggested that L-sorbose, L-arabinose and glucose are taken up by a constitutive active system [24–27]. α-Methyl-D-glucoside is transported actively, but only after an induction period of 40 min. There are no data on the transport of ribitol in *C. albicans*. In *Saccharomyces cerevisiae* it is transported by a passive, simple diffusion process [28], while in *Rhodotorula gracilis* [29], *Torulopsis candida* [30] and *C. guillermondii* [31] an active carrier process has been reported. Results concerning the transport of lactic acid in yeast have not been published.

The non-growth or very slow growth of the resistant mutants on these substrates does not necessarily indicate a complete inability to transport or to assimilate these carbon sources. It may merely be due to slight changes in the properties of the plasma membrane of the nystatin-resistant sterol mutants, leading to a quantitative decrease in the permeation efficiency of one or other solute, possibly to below the maintenance level. It must be emphasized that the concentration of the carbon sources was as low as 0.01% (w/v). Thus, the alterations in the plasma membrane composition of the sterol mutants seems to be sufficient to reduce the net influx of some substrates into the cells, causing thereby their substrate fasting. Some results relating to the uptake of

different carbon sources in liposomes with different membrane composition have been reviewed by DEMEL and DE KRUYFF [16].

The general validity of the glycerol-negativity of nystatin-resistant sterol mutants should be checked on a broad basis. If it is true in general for sterol mutants, it could be used for their rapid isolation and identification.

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REFERENCES

1. KINSKY, S. C.: In GOTTLIEB, D., SHAW, P. D. (eds.): Antibiotics. Vol. 1. Mechanism of action. Springer-Verlag, Berlin–Heidelberg–New York 1967. p. 122.
2. NORMAN, A. W., SPIELVOGEL, A. M., WONG, R. G.: Advanc. Lipid Res. **14,** 127 (1967).
3. HAMILTON-MILLER, J. M. T.: Bact. Rev. **37,** 166 (1973).
4. MOLZAHN, S. W., WOODS, R. A.: J. gen. Microbiol. **72,** 339 (1972).
5. PARKS, L. W.: Crit. Rev. Microbiol. **1,** 301 (1978).
6. PAGANO, J., LEVIN, J. D., TREJO, W.: Antibiot. Ann. **1957–1958,** 137 (1958).
7. VAN UDEN, N., BUCKLEY, H.: In LODDER, J. (ed.): The yeast. North-Holland Publ. Co., Amsterdam–London 1970. p. 893.
8. HAMILTON-MILLER, J. M. T.: J. med. Microbiol. **5,** 166 (1972).
9. BREIVIK, O. N., OWADES, J. L.: J. Arg. Food Chem. **5,** 306 (1957).
10. WOODS, R. A., BARD, M., JACKSON, I. E., DRUTZ, D. J.: J. infect. Dis. **19,** 53 (1974).
11. MAAS, M., VAN STEVENINCK, J.: Experientia (Basel) **23,** 405 (1967).
12. BEREK, I., NOVÁK, E. K.: In LYR, H., RAWALD, W. (eds): Int. Symp. Wirkungsmechanismen von Fungiziden, Antibiotika und Cytostatika. Reinhardsbrunn 1969. Akademie-Verlag, Berlin 1970. p. 173.
13. DOBOLYI, Cs., EKÉS, M., NOVÁK, E. K.: In KOCKOVÁ-KRATOCHVILOVÁ, A., MINARIK, E. (eds): Proc. 1st Spec. Int. Symp. on Yeasts. Publishing House of the Slovak Academy of Sciences, Bratislava 1972. p. 95.
14. PESTI, M., NOVÁK, E. K., SVOBODA, A., FERENCZY, L.: Acta microbiol. Acad. Sci. hung. **27,** 249 (1980).
15. GRINDLE, M.: Molec. gen. Genet. **130,** 81 (1974).
16. DEMEL, R. A., DE KRUYF, B.: Biochim. biophys. Acta (Amst.) **457,** 109 (1976).
17. PESTI, M., NOVÁK, E. K., FERENCZY, L., SVOBODA, A.: Sabouraudia **19,** 17 (1981).
18. KARUNAKARAN, V., JOHNSTON, J. R.: J. gen. Microbiol. **81,** 255 (1974).
19. PESTI, M., SVOBODA, A., FERENCZY, L., KUSTÁN, L., NOVÁK, E. K.: Proc. 19th Hung. Meet. Biochem. Budapest 1979. p. 143.
20. HAMILTON-MILLER, J. M. T.: Microbios **8,** 209 (1973).
21. PATEL, P. V., JOHNSTON, J. R.: J. appl. Bact. **34,** 449 (1971).
22. KOTYK, A.: In SUOMALAINEN, H., WALLER, C. (eds): Proc. 3rd Int. Spec. Symp. on Yeasts (Otaniemi, Helsinki): Print Oy, Helsinki 1973. p. 103.
23. GANCEDO, C., GANCEDO, J. M., SOLS, A.: Eur. J. Biochem. **5,** 165 (1968).
24. DEÁK, T., NOVÁK, E. K.: Antonie van Leeuwenhoek **35,** Suppl. Yeast Symposium, Part II. 1969. p. 11.
26. NOVÁK, E. K., DEÁK, T.: In LYR, H., RAWALD, W. (eds): Proc. Int. Symp. on Mechanisms of Action of Fungicides, Antibiotics and Cytostatics. Reinhardsbrunn 1969. Akademie-Verlag, Berlin 1970. p. 201.
27. NOVÁK, E. K., DEÁK, T.: RGW-Symp. über Trägervermittelter Transport durch die Zellmembran. Rheimsberg 1975. Wiss. Z. Humboldt-Univ. Berlin, Math.-Naturw. Reihe **25,** 107 (1976).
28. CANH, D. S., HORÁK, J., KOTYK, A., RIHOVÁ, L.: Fol. microbiol. (Praha) **20,** 320 (1975).
29. KLÖPPEL, R., HÖFER, M.: Arch. Microbiol. **107,** 329 (1976).
30. HAŠKOVEC, C., KOTYK, A.: Fol. microbiol. (Praha) **18,** 118 (1973).
31. MIERSCH, J.: Fol. microbiol. (Praha) **22,** 363 (1977).
32. NOVÁK, E. K., PAKU, S., ZALA, J., HORVÁTH, I., VÍGH, L., PESTI, M.: Proc. 21st Ann. Meeting. Veszprém, 1981. p. 221.

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KURZMITTEILUNG

VERWENDUNG VON KANINCHENHODENZELLEN FÜR DIE PLAQUETECHNIK MIT *HERPESVIRUS HOMINIS* TYP 1 UND TYP 2

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(Eingegangen 30. Dezember, 1980)

K.-D. THIEL, P. WUTZLER and H. SCHWEIZER: **Rabbit Testicle Cell Plaque Technique for Herpesvirus hominis Type 1 and Type 2.** A reliable and simple test has been developed for the examination of *Herpesvirus hominis* type 1 and type 2, by the use of monolayer cultures of rabbit testicle cells overlayered with methyl cellulose.

Monolayerkulturen von Kaninchenhodenzellen eignen sich sehr gut für die Plaquetechnik mit dem *Herpesvirus hominis* Typ 1 und Typ 2. Die Vorteile dieser Zellen für die Plaquetechnik bei der Verwendung eines Overlays von Methylzellulose werden kurz beschrieben.

Bei der Suche nach einem optimalen Zell-Virus-System für einen schnellen und sicher reproduzierbaren Plaquetest prüften wir sowohl verschiedene permanente Zellinien als auch Primärzellkulturen (Tab. I) unter Methylzelluloseoverlay gegenüber *Herpesvirus hominis* Typ 1 und Typ 2. Bei der in-vitro Austestung antiviraler Substanzen [1, 2] konnten wesentliche Vorteile gegenüber anderen Zellinien nachgewiesen werden. Die Spendertiere für die Organgewinnung waren leicht zu beschaffen und konnten nach Hodenentnahme für anschließende Untersuchungen weiterverwendet werden [3]. Ein zunehmendes Alter der Kaninchen beeinflußte nicht die Sicherheit des Anzuchterfolges der Hodenzellen, denn von 3, 6, 9 und 12 Monate alten Versuchstieren konnten pro Gramm trypsinierten Organgewebes 50 bis 75 Millionen proliferierender Zellen gewonnen werden, wobei wegen der sicheren und raschen Vermehrung der Zellen Einsaatdichten von 2×10^5 Zellen pro ml Nährlösung genügten. Auch zeigten die Zellen über mindestens 15 bis 20 Subkulturen bei einer wöchentlichen Vermehrungsrate von 1 : 2 bis 1 : 4 ein gleichbleibend gutes, einheitlich fibroblastisches Zellbild. Vorteilhaft war die vergleichsweise zu anderen Zellinien schnellere und zahlreichere Plaquebildung bei gleichen Virusinokuli (Tab. I). Gegenüber Schwankungen in den Kulturbedingungen wie pH-Wert, Inkubationstemperatur, wechselnden Serumchargen oder Kultivierungsunterlagen waren Kaninchenhodenzellen wesentlich unempfindlicher als andere in Tab. I aufgeführte Zellinien.

Tabelle I

Dauer der Plaqueausbildung und Plaqueanzahl in verschiedenen Zellinien, beimpft mit je 2000 $TCID_{50}$/ml Herpesvirus hominis Typ 1 (Stamm KUPKA) und Herpesvirus hominis Typ 2 (Stamm US)

		VERO-Zellen	HEp-2-Zellen	Hühnerembryofibroblasten	Humane embryonale Lungenfibroblasten	Kälberhodenzellen	Kaninchennierenzellen	Kaninchenhodenzellen
Herpesvirus hominis Typ 1 (Stamm KUPKA)	Plaquebildung (std.p.i.)	48	72	—	72	72	72	48
	Plaqueanzahl	81	73	—	122	110	167	184
Herpesvirus hominis Typ 2 (Stamm US)	Plaquebildung (Std.p.i.)	60	72	48	72	60	72	48
	Plaqueanzahl	76	59	130	98	137	185	203

Aufgrund der von uns durchgeführten Untersuchungen sind wir der Meinung, daß Kaninchenhodenzellen für die Plaquebildung mit *Herpesvirus hominis* Typ 1 und Typ 2 wesentliche Vorteile gegenüber anderen Zellinien besitzen und wir aus diesem Grunde eine Anwendung dieses Zellsystems für sehr empfehlenswert halten.

LITERATUR

1. Thiel, K.-D., Klöcking, R., Helbig, B.: Zbl. Bakt. I. Orig. A **234,** 159 (1976).
2. Thiel, K.-D., Klöcking, R., Schweizer, H., Sprössig, M.: Zbl. Bakt. I. Orig. A **239,** 304 (1977).
3. Wutzler, P., Färber, I., Sprössig. M., Schweizer, H., Thiel, K.-D., Schneider, J.: Arch. Virol. **51,** 59 (1976).

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SHEEP-POX VACCINE PREPARED FROM FORMALDEHYDE INACTIVATED VIRUS ADSORBED TO ALUMINIUM HYDROXIDE GEL

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(Received August 5, 1980)

The aim of the study was to produce an efficacious formaldehyde inactivated, adsorbed vaccine from the Mongolian sheep-pox MH virus strain. Aluminium hydroxide gel prepared from $AlCl_3 \cdot 6\ H_2O$ proved to be the most efficacious adsorbent among the gels prepared from different substances. Above 1.8% Al_2O_3 content the unadsorbed virus quantity was less than 1% of the original one. Using gel prepared from $KAl(SO_4)_2$ and $AlCl_3 \cdot 6\ H_2O$, respectively, the quantity of adsorbed virus was the same during the adsorption period from 10 min to 24 h. Intradermal inoculation of sheep proved more advantageous for virus production than subcutaneous inoculation. Three vaccines containing different quantities of antigen were prepared from virus propagated in sheep. The vaccine containing 19 800 ID_{50} inactivated virus did not protect the sheep even against a virus challenge of 25 ID_{50}, while that of 67 000 ID_{50} content protected 50% of the animals infected with 125 to 287 ID_{50}, and that of 395 000 ID_{50} content protected 100% of the animals against challenge with more than 100 000 ID_{50}. More than 3 million sheep were inoculated in Mongolia with vaccines of 350 000 ID_{50} virus content in the last years. In the areas where vaccination has been introduced no sheep-pox epizootic has occurred.

According to Balozet [1], Bots [2], Manninger [3], Wynohradnik and Cristet [4], Pandey *et al.* [5] and Celiker and Arik [6] sheep can be immunized with living virus adsorbed to aluminium hydroxide gel without provoking serious postvaccinal reactions. The vaccination is, however, not completely harmless, because it induced epizootic in some cases.

Manninger in 1942 elaborated a sheep-pox vaccine inactivated with formalin and adsorbed to aluminium hydroxide gel. In 1948 [3] he could report that the virus completely inactivated with formalin possessed an excellent immunizing activity and this was fully corroborated by Delpy *et al.* [7].

The inactivated sheep-pox vaccine adsorbed to aluminium hydroxide was actually put on the market in Hungary, but as the country has been free from sheep-pox for two decades, production of the vaccine has ceased. In Mongolia, however, sheep-pox epizootics still occur and vaccination there is still of importance. As for this purpose the wish was to apply inactivated vaccine adsorbed to aluminium hydroxide gel, we have studied the possibility of production of an efficacious vaccine from the local sheep-pox virus strain. Thus, we have studied the virus adsorbing power of different aluminium hydroxide gels, the inoculation method affording the maximum virus yield,

the protective dose against the homologous artificial challenge, as well as the immunizing power of the vaccine against the challenge carried out with Hungarian sheep-pox virus.

Materials and methods

Aluminium hydroxide gel. Studies have been carried out with $Al(OH)_3$ gels prepared from Al_2O_3, $KAl(SO_4)_2$ and $AlCl_3 \cdot 6\ H_2O$, according to NAIL *et al.* [8]. The concentration of the gels has been determined by titration of the Al_2O_3 content.

Virus strains. The Mongolian MH and the Hungarian sheep-pox virus strains were used. The strains were propagated on sheep skin, harvesting was done on the 7th day following inoculation. The skin fragments displaying specific alterations were cut into small pieces, and with 9 parts of PBS solution were homogenized and centrifuged at 2000 *g* for 15 min. The supernatant to which 20% milk had been added, was freeze-dried and stored in ampoules at −20 °C. The virus content was determined by intradermal titration in sheep. The suspension was then used for inactivated vaccine production, as antigen in the complement fixation tests and as challenge virus in potency testing. The calculated virus doses applied in the course of potency testing were controlled in each case by repeated titration. The titre of both virus strains varied within $10^{5.0}$ to $10^{5.5}$.

Laboratory animals. Mongolian Merino, Hungarian Merino and Mongolian native sheep were used. The presence of antibody against sheep-pox was controlled in their sera with indirect complement fixation test [9]. Animals free from antibody were only used in the examinations. Before intradermal titration, the side of the animals was epilated with barium sulphide.

Complement fixation tests. Most complement fixation tests were carried out by a micromethod, only in determinations requiring great accuracy was a macro method used. In the micromethod, Takátsy's procedure was used, the results were recorded by 1 to 4 crosses depending on the degree of haemolysis. The 1 : 5, 1 : 10, 1 : 20 and 1 : 40 dilutions of the antigen were studied with standard hyperimmune serum dilutions of 1 : 2 to 1 : 64 with 5% complement and 2000-fold diluted haemolysin. To complete fixation 4 units were assigned and proportionally less with weakening of the reaction. The sum of the units gave the antigen content of the substances expressed in complement-fixing units. In the macromethod, Wassermann tubes were used and the results were read spectrophotometrically. In the course of both procedures the sheep red cell suspension was applied in such a dilution that the extinction amounted to 0.2 in the case of 100% haemolysis at 540 nm wavelength.

Freeze-dried complement and haemolysin (Phylaxia, Budapest) was used. The freeze-dried hyperimmune serum against sheep-pox was produced in sheep after hyperimmunization for 2 months. Regular control of the complement fixing system allowed the comparison of results of tests carried out in different periods.

Safety and potency testing. To control safety of the vaccine, 4 vaccinated susceptible and 2 untreated control sheep were kept together. In the course of the 21 day observation period no sheep-pox and rise in temperature above 1 °C did not occur. In potency testing, the sheep previously used in safety testing were applied. The potency of the vaccine was controlled by two methods. Each sheep was inoculated with 25 to 1000 ID_{50} virus following the vaccination. The controls contracted the disease, the vaccinated sheep were protected in 50%. In the other method the virus was titrated on the dorsal skin of the vaccinated and control animals, and the virus quantity neutralized by each animal was evaluated by the index method.

Results and discussion

Virus adsorption. Adsorbing ability of the aluminium hydroxide gels prepared from the three kinds of substance was studied together with the influence of the gel concentration on virus adsorption. MH Mongolian sheep-

pox suspension was mixed in equal volumes with different concentrations of the three kinds of aluminium hydroxide gel, and shaken for one hour. The mixture was stored at 4 °C for 7 days then it was centrifuged at 2000 *g* for 15 min and thereafter the virus content of the supernatant was estimated by complement-fixation test. Results are shown in Fig. 1.

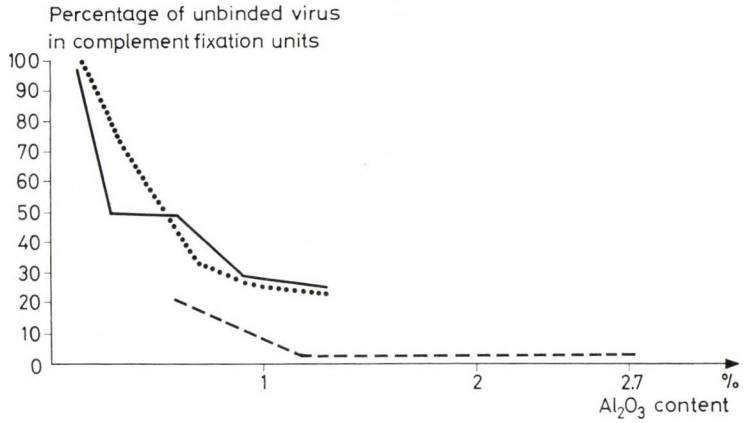

Fig. 1. Sheep-pox virus adsorbing capacity of three aluminium hydroxide gels prepared from different substances. $Al(OH)_3$ starting materials: – – – – $AlCl_3$; · · · · Al_2O_3: ——— $KAl(SO_4)_2$

The virus was best adsorbed by the gel prepared from $AlCl_3 \cdot 6\ H_2O$. This gel fixed 25 to 30% more virus than the others and above 1.8% Al_2O_3 less than 1% virus was found in the supernatant by means of complement fixation (Fig. 2).

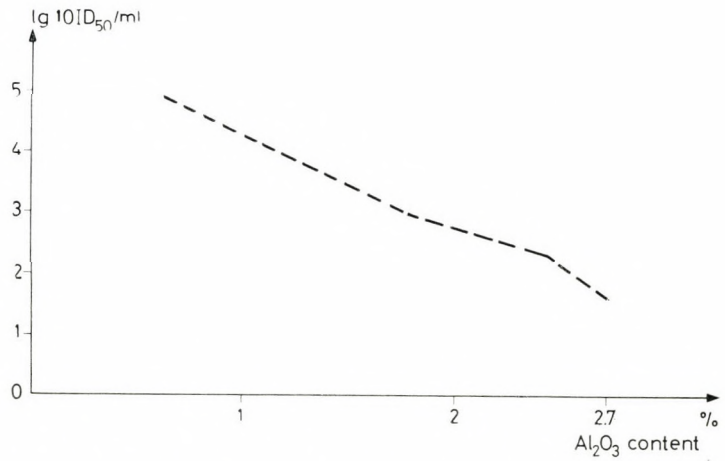

Fig. 2. Skin test in sheep for determination of sheep-pox virus adsorbing capacity of aluminium hydroxide gel prepared from $AlCl_3$; – – – – infective titre of unadsorbed virus

Intradermal titration proved to be more sensitive than the complement fixation test. At a 1.2% Al_2O_3 content, it detected an amount of 6309 ID_{50}, 5.58% of the original $10^{5.06}$ ID_{50}/ml virus content in the supernatant, while above 1.8% Al_2O_3 the non-adsorbed virus content was less than 1% of the original.

Virus growth. For vaccine production, the MH virus strain was propagated according to two methods. In the course of the first (Mongolian) method, the virus was inoculated subcutaneously and 6 to 8 days later, the virus content of the harvested epithelium and subcutaneous tissue was examined. A 1 : 10 (w/v) suspension was then prepared from both tissues and dilutions of 1 : 20, 1 : 40 and 1 : 80 were examined for antigen content by complement fixation test, using different dilutions of standard serum. The degree of haemolysis was determined spectrophotometrically at 540 nm.

Results are shown in Fig. 3.

The epithelial antigen gave a similar complement fixation at 1 : 80 dilution as the subepithelial one in 1 : 20 dilution. Thus, the epithelium contained four-times more complement-binding antigen than the subcutaneous tissue. Intradermal titration in sheep revealed $10^{4.5}$ ID_{50}/ml virus in the epithelium while merely $10^{3.5}$ ID_{50}/ml in the subcutaneous tissue.

According to the other method, the virus was inoculated intradermally. This elicited a considerable skin thickening with profound subcutaneous

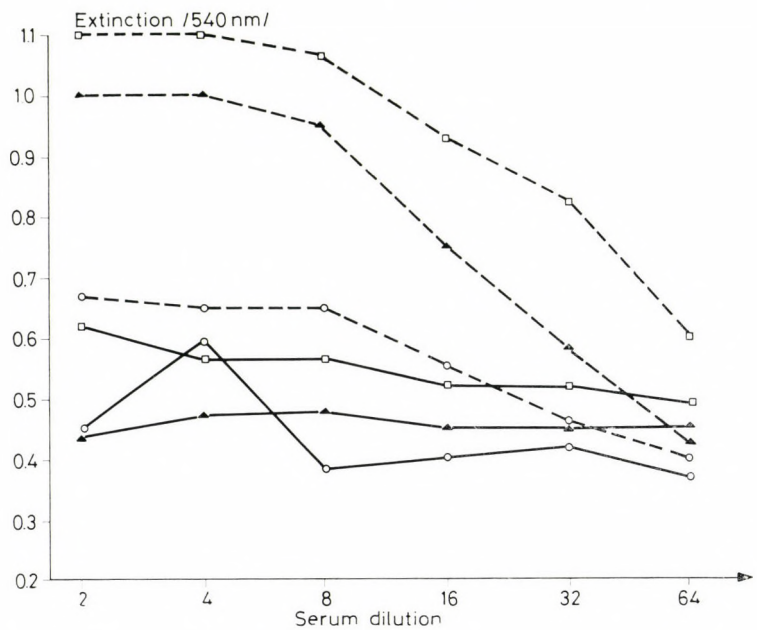

Fig. 3. Antigen content of epithelium and subcutaneous tissue of sheep inoculated with the MH Mongolian sheep-pox virus strain. Epithelium antigen □- - - -□ 1 : 20, △- - - -△ 1 : 40, ○- - - -○ 1 : 80; subcutaneous tissue antigen □——□ 1 : 20, △——△ 1 : 40, ○——○ 1 : 80

oedema. The virus content of the suspension prepared from the epithelium and subcutaneous tissue proved to be similar both in the complement fixation reaction and by intradermal titration. These examinations undoubtedly verified that intradermal inoculation is more advantageous for the purpose of virus propagation, because in such a way both the epithelium and subcutaneous tissue can be used for vaccine production.

Safety and potency testing of the inactivated vaccine. Three vaccines of different MH virus antigen content were prepared. The first vaccine contained aluminium hydroxide gel corresponding to 1.35% Al_2O_3 and 0.05% formalin as preservative. The vaccine was inactivated with formalin at 4 °C for 14 days, and contained per dose (5 ml) 19 800 ID_{50} inactivated virus. The vaccine proved to be safe in the course of testing. Potency testing was carried out in such a way that 4 sheep vaccinated by the subcutaneous route were challenged 3 weeks later together with 2 untreated controls, by intradermal inoculation on the lateral surface of the thigh of 0.5 ml 25 and 50 ID_{50} virus suspension. Specific lesions of sheep-pox developed in both the vaccinated and the control animals at the site of the injection in 4 to 6 days. Thus, the vaccine did not afford appropriate protection even against 25 infective units. The second vaccine was produced similarly with the difference that it contained 67 000 ID_{50} inactivated virus per 5.0 ml.

Four animals were vaccinated by the subcutaneous route in animals previously subjected to safety testing. In the course of the 17 days observation period, a slight subfebrility (maximum 0.8 °C) was observed on the 3rd and 4th days which disappeared by the 5th day. On the 21st day the 4 vaccinated animals and 2 untreated controls were challenged and 0.2 ml solution of the Mongolian MH strain was inoculated in five sites. The titres obtained after 10 days in the vaccinated animals were subtracted from those obtained in the controls; the results are summarized in Table I. It is seen that 2 animals possessed a certain protection while the two other were unprotected. The

Table I

Potency testing of vaccine produced from MH Mongolian sheep-pox virus strain in Mongolian Merino sheep (67 000 ID_{50} inactivated virus/dose)

Designation of sheep	Vaccine		Challenge strain	Protection index
	dose, ml	route of inoculation		prevented infective unit
1.	5.0	s.c.	MH	297
2.	5.0	s.c.	MH	39
3.	5.0	s.c.	MH	125
4.	5.0	s.c.	MH	39

third vaccine contained 395 000 ID_{50} inactivated virus per dose. Four Mongolian Merino and four Mongolian native sheep were vaccinated and kept together with 2 untreated controls each. The vaccinated animals had no symptoms except a maximum 0.7 °C rise of temperature on the 4th or 5th day. The control sheep had no symptoms whatever. The vaccinated and control sheep were tested on the 21st day using the MH Mongolian virus strain. In the course of the first method, the vaccinated and control sheep were inoculated intradermally with 3750 ID_{50} virus. None of the vaccinated animals showed a positive reaction while in the control ones specific nodules appeared on the skin. In the course of the test carried out by the other method, the same vaccine offered protection against more than 100 000 ID_{50} of virus (Table II). The same vaccine was inoculated into 4 Hungarian Merino sheep kept together with 2 untreated control ones. Slight subfebrility (maximum 0.8 °C) was observed in the vaccinated animals on the 3rd to 5th day. Apart from this no symptom was observed in any sheep in the course of 21 days. Then the sheep were challenged with the Hungarian sheep-pox strain titrated by intradermal inoculation in vaccinated and control sheep. Results are shown in Table III. The vaccine proved to be extremely efficacious, the virus titre in the control animals was $10^{5.5}$ ID_{50}/ml while in the vaccinated ones the concentrated virus suspension did not evoke a positive reaction. Thus, the vaccinees were protected against more than 310 000 ID_{50}.

The results proved that efficacious vaccine inactivated with formalin and adsorbed to aluminium hydroxide gel may be produced from the Mon-

Table II

Potency testing of vaccine produced from MH Mongolian sheep-pox virus strain (395 000 ID_{50} inactivated virus/dose)

Sheep		Vaccine		Challenge strain	Protection index
designation	species	dose, ml	route of inoculation		prevented infective unit
1	Mongolian	5.0	s.c.	MH	>3 750
2	Mongolian	5.0	s.c.	MH	>3 750
3	Merino	5.0	s.c.	MH	>3 750
4	Merino	5.0	s.c.	MH	>3 750
5	Mongolian	5.0	s.c.	MH	>100 000
6	Mongolian	5.0	s.c.	MH	>100 000
7	Merino	5.0	s.c.	MH	>100 000
8	Merino	5.0	s.c.	MH	>100 000

Sheep No 1 to 4 were inoculated into a single point, in sheep No. 5 to 8 virus titration was carried out

Table III

Potency testing of vaccine produced from MH Mongolian sheep-pox virus strain in Hungarian Merino sheep (395 000 ID_{50} inactivated virus/dose)

Designation of sheep	Vaccine		Challenge strain	Protection index
	dose, ml	route of inoculation		prevented infective unit
1	5.0	s.c.	Hungarian	310 000
2	5.0	s.c.	Hungarian	310 000
3	5.0	s.c.	Hungarian	310 000
4	5.0	s.c.	Hungarian	310 000

golian sheep-pox virus strain. The requirements are an adsorbent of good quality, and the amount of inactivated virus should be equivalent to 350 000 to 400 000 ID_{50} per dose.

The vaccine was not only efficacious but safe, too. Apart from a slight subfebrility, the vaccinated animals had no symptoms and were protected against the Mongolian MH strain and also against the Hungarian sheep-pox strain, indicating that the two strains are either identical or in close relationship in respect of antigenicity.

The experiments carried out in Mongolia and in Hungary also showed that the three sheep breeds under test (Mongolian, Mongolian Merino and Hungarian Merino sheep) did not differ in respect of immunization against sheep-pox.

The reliability of the experimental data has been verified in Mongolia [9] where after more than 3 million sheep had been vaccinated, no sheep-pox epizootic has occurred.

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REFERENCES

1. Balozet, L.: C. R. Acad. Sci. (Paris) **207,** 349 (1938).
2. Bots, J. J.: Vet. Bull. **18,** 457 (1942).
3. Manninger, R.: Bull. Off. int. Epiz. **29,** 237 (1948).
4. Wynochradnyk, V., Cristet, I.: Vet. Bull. **27,** 73 (1956).
5. Pandey, A. K., Malik, B. S., Bansal, M. P.: Indian. vet. J. **46,** 1017 (1961).
6. Celiker, A., Arik, F.: Brit. vet. J. **118,** 159 (1962).
7. Delpy, L. P., Rafyi, A., Mirchamsy, H.: Bull. Acad. vet. franc. **24,** 56 (1951).
8. Nail, S. L., White, J. L., Hem, S.: J. pharm. Sci. **65,** 1188 (1976).
9. Sodnomdosh, S.: Personal communication (1979).

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STIMULATORY EFFECT OF HERBICIDE 2,4-D ON THE HETEROTROPHIC MICROBIAL COMMUNITY IN THE WATER OF THREE FISH PONDS

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Herbicide 2,4-D at a dosage of 500 mg l^{-1} stimulated the proliferation of the heterotrophic bacterial community present in the water of three fish ponds over a period of one year. 2,4-D due to its toxic action might kill the planktonic algal population occurring in the pond water, which on degradation generated more nutrients conductive to bacterial proliferation. The microbial density in different hours were closely correlated ($p < 0.01 < 0.05$) with the variations of each of NH_4–N, NO_2–N and specific conductivity of water, while the relationship betwen the former and PO_4–P was reciprocal ($P < 0.001$).

Application of 2,4-dichlorophenoxy acetic acid (2,4-D) in fish culturing ponds has become a common practice for controlling aquatic weeds and algal blooms. The efficacy of the chemical had be en tested several times for killing either the submerged plants and emergent weeds [1, 2], or water hyacinth [3]. Applied at a rate of 260 mg l^{-1}, 2,4-D was found to be non-toxic to carp [4] but other studies, e.g. [5] indicate that the agent has definite deleterious effects on fish populations. Nonetheless, the effects of the compound on the heterotrophic microbial community in aquatic ecosystem have seldom been measured. The heterotrophic microorganisms play an important role in the nutrient cycle of a fish pond and are therefore closely associated with the growth of fish population. An attempt has been made to determine the action of 2,4-D at a recommended dose used in the field, on heterotrophic microorganisms occurring in the waters of three fish ponds.

Materials and methods

Three fish ponds belonging to each of the polyculture, traditional and monoculture system of fish farming, located at Kalyani fish farm, were selected for the study. The fish ponds were shallow with depth of water varying between 0.8 and 3.0 m. The polyculture fish pond was stocked with three species of Indian major carps *(Catla catla, Labeo rohita* and *Cirrhinus mrigala)* and the three exotic carps *(Hypophthalmichthys molitrix, Ctenopharyngodon idella* and *Cyprinus carpio)* in the ratio of 2 : 6 : 3 : 4 : 2 : 3, respectively, with a total stocking density of 6000 ha^{-1}. *Cirrhinus mrigala* was introduced in the monoculture fish pond maintaining the same stocking rate. The traditional fish pond had been stocked largely with the three species of Indian major carps. These fish ponds with the exception of the traditional one were fertilized regularly with inorganic and organic fertilizers. Surface water samples from each pond were collected in sterile glass bottles (250 ml capacity) in quadruplicate. On these, herbicide 2,4-D (commercial product) was added into two, at a dosage of 500 mg l^{-1}, while the remaining two served as controls. The study was carried out twice monthly from

December, 1978, until November, 1979. The bottles were kept at the water temperature of the pond for 24 h when the changes in heterotrophic bacterial numbers with respect to initial count were estimated by spread plate technique in triplicate using nutrient agar (Difco, Bacto) medium. Since the water temperature of these tropical ponds (22–32 °C) was higher throughout the year, incubation of plates for the growth of bacteria was thus made at a temperature of 37 $\pm$ 0.5 °C for 72 h. The number of developed colonies per plate were counted and the means of triplicate plates were obtained. The monthly mean values of bacterial populations are presented in Results. The population-size so obtained for each pond throughout the year was statistically compared between the treated and non-treated samples by using the paired t test.

Results

As can be seen in Table I, the average value in the treated water samples (4.93×10^5 cells ml^{-1}) exceeded nearly one and half-times the control (3.66×10^5 cells ml^{-1}) in the traditional pond ($t_d = 4.89$; $P < 0.001$). Even though the numerical superiority in the treated samples was relatively less in the ponds with mono and polyculture, the differences were significant ($t_d \geq 2.06$; $P < < 0.001 < 0.05$). Similarly, when the bacterial density occurring in the three ponds was considered together for comparison between treated and control series, the result ($t_{142} = 1.808$) was also highly significant ($P < 0.001$). The traditional and monoculture pond showed the greatest increase of bacteria in spring (239.53%) and autumn (669.23%), respectively, while the polyculture pond had two maxima occurring in spring (171.43%) and autumn (429.03%).

Table I

Heterotrophic bacterial population in different ponds ($\times 10^5$ ml^{-1})

Months	Polyculture		Traditional		Monoculture	
	with herbicide	control	with herbicide	control	with herbicide	control
December, 1978	12.6	15.9	6.9	4.25	9.75	5.25
January, 1979	7.7	6.25	5.9	5.05	4.55	3.85
February	3.8	2.2	3.6	2.60	4.7	3.5
March	9.0	4.4	14.3	11.05	11.8	8.8
April	6.15	4.55	5.1	3.15	6.55	5.8
May	4.91	3.25	4.85	5.3	7.35	5.3
June	15.05	13.2	8.1	5.7	11.05	9.6
July	14.4	16.3	5.7	3.45	8.4	4.3
August	4.8	4.1	1.45	1.05	3.38	2.5
September	1.54	1.3	1.13	0.55	1.88	0.9
October	2.54	0.9	1.45	1.25	1.66	0.4
November	1.03	0.69	0.60	0.48	1.02	0.64
Mean	6.96	6.087	4.92	3.66	5.96	4.24

The microbial density occurring in the water samples treated with and without herbicide has been measured after different time intervals of the short term experiment (4, 8, 16, 24, 36, 72, 168 and 240 h). The results are presented in Fig. 1. The bacterial numbers obtained in the experimental and control sets were compared statistically by using the paired t test. The results

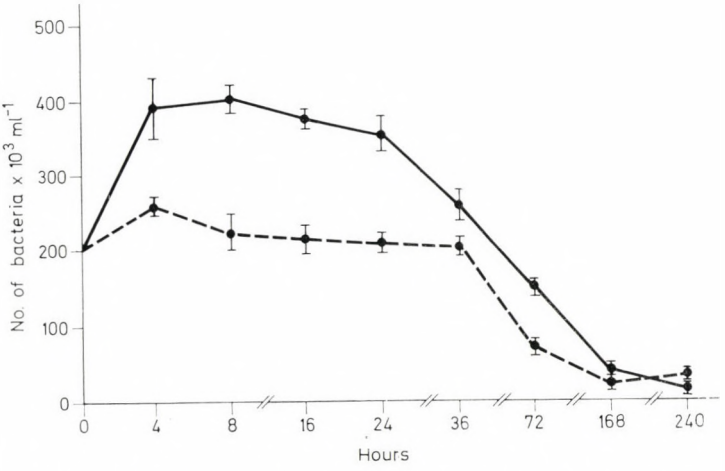

Fig. 1. Heterotrophic bacterial density in water in short term analysis

showed that the bacterial density in the treated bottles was distinctly higher than in the control in all the periods of the short term experiment ($t_d \geq 4.32$; $P < 0.001 < 0.05$). Although the highest count occurred at 8 h (Fig. 1), the size of the population was not markedly altered ($t_d \geq 3.38$; $P > 0.05$) between any two successive intervals for 24 h. The mean count obtained at 24 h differed greatly from the means found during the rest of the period ($t_d \geq 6.43$; $P < 0.001$). Changes in the physicochemical factors of water during different hours in the treated bottles were not apparent (Table II).

Discussion

It is evident that herbicide 2,4-D at the test dose had a definite stimulatory effect of the heterotrophic bacterial community in the water bodies, contrary to the detrimental impact often reported on most aquatic weeds and algae [1–3, 6]. 2,4-D due to its toxic action might kill the planktonic algal population occurring in the water, which on degradation, generated more N_i conductive to bacterial proliferation. The changes of bacterial numbers occurring in different hours in the bottles treated with herbicides (Fig. 1) were thus positively correlated with NH_4–N and NO_2–N of sample water ($P < 0.01 <$

Table II

Physicochemical factors (mean ± SD) in water samples in short term analysis

	0 h	24 h		48 h		72 h		168 h		240 h	
		treated	control	treated	control	treated	control	treated	control	treated	control
pH	7.9 ±1	8.1 ±0.1	8.07 ±0.08	7.6 ±0.1	7.1 ±1.0	7.5 ±0	7.1 ±1	7.6 ±1.5	7.5 ±1	7.7 ±0.1	7.7 ±0.1
CO_3^{-2} (mg l^{-1})	15 ±1	25.5 ±1.65	10.7 ±1.6	11 ±1.5	7 ±1.2	9 ±1	7 ±1.2	10.7 ±0.83	10 ±1	12 ±1.6	10.7 ±0.8
HCO_3^- (mg l^{-1})	141 ±0.8	135 ±3.6	132 ±8.6	131.5 ±1.65	114 ±2.4	135.5 ±0.86	116.5 ±4.7	124.7 ±2.76	124 ±2.8	130.2 ±0.94	125.5 ±1.6
NO_2–N (mg l^{-1})	0.252 ±0.008	0.190 ±0.07	0.192 ±0.014	0.205 ±0.005	0.175 ±0.004	0.121 ±0.001	0.167 ±0.003	0.047 ±0.005	0.030 ±0.012	0.129 ±0.001	0.051 ±0.002
NO_3–N (mg l^{-1})	0.051 ±0.001	0.036 ±0.007	0.048 ±0.001	0.034 ±0.001	0.042 ±0.002	0.035 ±0.001	0.040 ±0	0.040 ±0.001	0.035 ±0.001	0.035 ±0.001	0.041 ±0.0008
NH_4–N (mg l^{-1})	0.320 ±0.014	0.230 ±0.012	0.275 ±0.016	0.197 ±0.005	0.176 ±0.015	0.190 ±0.001	0.153 ±0.013	0.210 ±0.009	0.170 ±0.010	0.058 ±0.0007	0.140 ±0.001
PO_4–P (mg l^{-1})	0.00151 ±0.005	0.0016 ±0.002	0.00173 ±0.002	0.0015 ±0.001	0.00151 ±0.002	0.0028 ±0.001	0.00162 ±0.004	0.0050 ±0.003	0.00154 ±0.007	0.0053 ±0.002	0.00350 ±0.006
Specific conductivity (mhos $\times 10^{-3}$ cm^{-1})	0.238 ±0.002	0.377 ±0.004	0.245 ±0.001	0.346 ±0.009	0.241 ±0.002	0.206 ±0.004	0.171 ±0.001	0.251 ±0.001	0.180 ±0	0.241 ±0.002	0.190 ±0.003

< 0.05). As it can be seen in Fig. 2, the changes of population size of bacteria and that of specific conductivity of water were highly correlated ($P < 0.01$). It is possible that 2,4-D caused the death of planktonic organisms occurring in the bottles, which on degradation increased the availability of nutrients

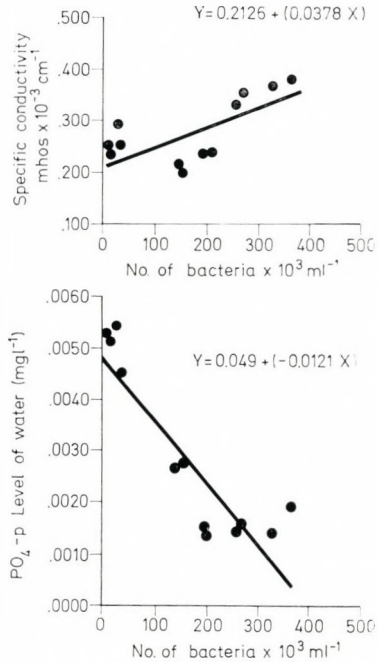

Fig. 2. Relationships between heterotrophic bacteria and specific conductivity, and PO_4–P level of water

conductive to microbial growth. Further, the inverse relationship between the heterotrophic bacterial population and the PO_4–P level of water was perhaps due to a mass decay of plankton in the early stage and of bacterial population in the late hours of herbicide treatment. It is known that many genera of heterotrophs used pesticides as substrates, either co-metabolizing the molecules or using them as nutrients [7]; low levels of herbicides often induced chlorophyll synthesis [8] and other toxic compounds were stimulatory to microbial community [9]. It has been observed that most microbial populations were either not reduced in numbers or were not too greatly altered by low concentrations of many herbicides, pesticides and insecticides [10].

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REFERENCES

1. Srinivasan, R., Chacko, P. I.: J. Bombay nat. Hist. Soc. **51,** 164 (1952).
2. Ramachandran, V., Ramaprabhu, T.: FAO Fish Rep. **44** (5), 92 (1968).
3. Jhingran, V. G.: Fish and Fisheries of India. Hindustan Publishing House, New Delhi 1975. p. 954.
4. Marini, E.: Notiz. Malattie Piante **19,** 17 (1952).
5. Smith, C. L.: The effects of water hyacinth mats and their control by 2,4-D on fish population. Thesis, Tulane University, 1952. p. 85.
6. Cullimore, D. R.: Weed Res. **15,** 401 (1975).
7. Alexander, M.: Introduction to Soil Microbiology. Second ed. Wiley Eastern Limited, New Delhi 1978. p. 467.
8. El-Nawawy, A. S., Hamdi, Y. A.: In: Stewart, W. D. P. (ed): Nitrogen Fixation by Free Living Microorganisms. Cambridge University Press, London 1975. p. 219.
9. Jensen, H. L., Schroder, M.: Arch. Microbiol. **58,** 127 (1967).
10. Van Schreven, D. A., Lindenbergh, D. H., Koridon, A.: Plant Soil **33,** 513 (1970).

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INHIBITION OF NITRIFICATION IN SOIL UNDER *JUNIPERUS PROCERA* WOODS IN ASIR REGION, SAUDI ARABIA

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Two plots representing an area dominated by *Juniperus procera* and another area lacking this species at two localities near to Abha, in the south-west Saudi Arabia, were studied. Soil samples from the 0–15 cm and 35–50 cm level were analysed for ammonium and nitrate nitrogen every other month for one year. The counts of *Nitrosomonas* and *Nitrobacter* were determined in the 0–15 cm level. The counts of *Nitrosomonas* and *Nitrobacter* were lower in soil samples collected from *J. procera* dominated areas than from those lacking this plant. The amount of nitrate nitrogen was lowest in plots with *J. procera* and highest in plots lacking *J. procera*. The amount of ammonium nitrogen, on the contrary, was highest in plots with *J. procera* and lowest in those lacking it. This trend was consistent throughout all sampling periods in both sampling levels at both investigated localities. The depletion in *Nitrobacter* counts and in the amount of nitrate nitrogen in plots with *J. procera* indicates an inhibitory effect exerted by this plant on nitrifiers.

Nitrification is one of the most important processes occurring in certain bacteria. In the present work the term nitrification is used for the biological process forming nitrite and nitrate from ammonium salts.

Several careful investigations in grass land [1–7] have indicated that the level of ammonium nitrogen was several times greater than the level of nitrate nitrogen. In these investigations it was pointed out that a grassy vegetation seems to exert a direct inhibiting action since after clearing, the nitrifying activity increases greatly. The same observation of ammonium nitrogen accumulation in forest-covered soils was noticed by some authors [5, 7–9]. The depletion of nitrate nitrogen was attributed to an inhibition of the nitrification process.

The above findings have made us to study the phenomenon in an area not investigated before regarding the nitrification process. The area is near to Abha (Asir region) in southwest Saudi Arabia; *Juniperus procera* dominates this area. Another area lacking this plant served as control. The present paper reports on the role of *J. procera* in inhibiting the process of nitrification in the investigated area.

Materials and methods

The investigated area. Two localities *viz.* AsSudah (2700 m a.s.l.) and Al-Jurrah (2300 m a.s.l.) west north and south east of Abha, respectively, were investigated. Al-Jurrah locality was covered with *Juniperus procera* associated with *Euryops arabica*, *Euphorbia cyparissioides*,

Asparagus sp., *Otostegia fruticosa*, *Gymnocarpus siniaca* and *Solanum* sp., AsSudah locality was covered with *J. procera*, *E. arabica*, *Euph. cyparissioides*, *Asparagus* sp., *Otostegia fruticosa* and *Dodonae viscosa*. Plants were kindly identified by Professor Dr. A. M. MIGAHID to whom the author expresses his appreciation and indebtedness. The associated species were of low frequency and distributed throughout the *J. procera* climax. The control site near to each of the above localities was completely free from *J. procera* but the above mentioned associated species were all represented with approximately the same frequency.

Sampling. Sampling was carried out aseptically over an area of 100×100 m with apparently homogeneous soil characteristics mainly loamy in the A and B horizons of AsSudah and Al-Jurrah localities and harbouring *J. procera*. The control area was sampled similarly. Starting in August, 1978, ten evenly distributed soil samples were collected in polyethylene bags from the 0–15 cm and ten from the 35–50 cm level in each site every other month for one year.

Soil analyses. Mechanical analysis, pH, total soluble salts and calcium carbonate were processed for each soil sample according to the techniques devised by BLACK [10].

Counting of Nitrosomonas and Nitrobacter. Soil samples were analysed for *Nitrosomonas* and *Nitrobacter* by the most probable number (M.P.N.) method of ALEXANDER and CLARK [11].

Media. The medium used for counting *Nitrosomonas* contained $(NH_4)_2SO_4$, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7\ H_2O$, 0.5; NaCl, 0.5 g/l, and traces of $Fe_2(SO_4)_3$ pH 8.5.

The medium used for counting *Nitrobacter* contained $NaNO_2$, 1; Na_2CO_3, 1; K_2HPO_4, 0.5; NaCl, 0.5; $MgSO_4 \cdot 7\ H_2O$, 0.3 g/l and traces of $FeSO_4 \cdot 7\ H_2O$.

Results

The characteristics of the soils are given in Table I. Soils of AsSudah and Al-Jurrah had a sandy clay loam and clay loam texture, respectively. The pH was practically the same in the investigated soil samples. The amount

Table I

Characteristics of soils 0–15 and 35–50 cm levels at AsSudah and Al-Jurrah with and without J. procera cover (control)

Locality	Sand	Silt	Clay	Organic carbon	$CaCO_3$	Total soluble salts	pH
	%						
AsSudah with *J. procera*							
0–15 cm	54.17	17.62	28.21	0.584	3.84	0.421	7.35
35–50 cm	58.64	13.87	27.49	0.537	2.16	0.467	7.35
without *J. procera* (control)							
0–15 cm	56.64	15.74	27.62	0.562	3.94	0.472	7.40
35–50 cm	59.34	13.58	27.08	0.521	2.53	0.489	7.35
Al-Jurrah with *J. procera*							
0–15 cm	43.16	24.55	32.29	0.520	4.14	0.349	7.40
35–50 cm	48.13	25.32	26.55	0.468	3.08	0.421	7.35
without *J. procera* (control)							
0–15	42.34	28.23	29.43	0.482	3.67	0.432	7.35
35–50 cm	46.17	25.39	25.44	0.446	2.32	0.456	7.35

of organic carbon did not vary considerably and ranged between 0.446 and 0.585%. The total soluble salts ranged between 0.349 and 0.489% whereas the amount of calcium carbonate varied considerably.

The amounts of ammonium and nitrate nitrogen are given in Table II. The amount of ammonium nitrogen was greater in plots with *J. procera* in either AsSudah or Al-Jurrah at both the 0–15 cm and 35–50 cm levels whereas in plots without *J. procera*, significantly lower values were recorded. On the other hand, the amounts of nitrate nitrogen behaved in the opposite way versus ammonium nitrogen where lower values were recorded in plots with *J. procera* than those recorded in plots without *J. procera*.

With regard to seasonal variations in the values of ammonium and nitrate nitrogen, the highest values were recorded in April and the lowest ones in December.

The counts of nitrifiers are represented in Fig. 1. The number of *Nitrosomonas* per g dry soil was higher in plots with *J. procera* than in those lacking this plant in both AsSudah and Al-Jurrah. The number of *Nitrobacter* was considerably higher in plots lacking *J. procera* than in plots dominated by

Table II

Amounts of ammonium and nitrate nitrogen (in oven dry soil) in 0–15 cm and 35–50 cm levels of selected sites at AsSudah and Al-Jurrah with and without J. procera cover (control)

Date	With *J. procera*				Without *J. procera* cover (control)			
	0–15 cm		35–50 cm		0–15 cm		35–50 cm	
	NH_4^+	NO_3^-	NH_4^+N	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
				AsSudah				
Aug.	8.86	1.24	7.63	1.21	3.12	6.32	4.36	7.42
Oct.	7.68	1.38	6.31	1.46	3.44	6.04	3.85	7.02
Dec.	7.24	1.03	6.14	1.16	2.93	5.62	3.21	6.14
Feb.	8.27	1.46	8.22	1.42	3.32	5.88	4.47	6.62
Apr.	9.41	1.62	8.66	1.56	3.56	6.48	4.68	7.46
June	7.04	1.42	7.23	1.30	3.21	5.61	4.21	6.26
				Al-Jurrah				
Aug.	9.24	1.32	7.86	1.18	3.16	6.32	3.68	6.66
Oct.	8.27	1.45	8.12	1.24	3.72	5.41	4.26	6.10
Dec.	7.48	1.32	6.84	1.16	3.04	5.11	3.34	5.82
Feb.	8.92	1.44	7.26	1.24	3.26	5.23	3.67	5.76
Apr.	10.16	1.52	9.12	1.36	3.84	6.28	4.63	7.41
June	8.36	1.26	7.64	1.12	3.21	6.16	3.82	6.61

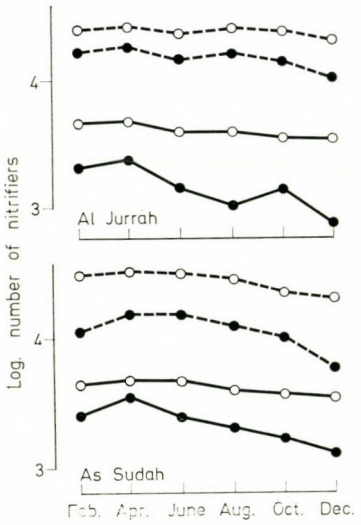

Fig. 1. Number of nitrifiers in 0–15 cm level of selected sites at Al-Jurrah and AsSudah with and without *J. procera* cover; ——— with *J. procera*, – – – – without *J. procera*, ○ *Nitrosomonas*, • *Nitrobacter*

this plant. The highest counts of both *Nitrosomonas* and *Nitrobacter* were recorded in April whereas the lowest values were recorded in December and ranged between 800 and 33 800/g dry soil.

Discussion

With the exception of the investigation of EL-HUSSEINY *et al.* [12] in the Riyadh region which is some 800 km far from the Asir region the present investigation was the first to be carried out in this region that differs from Riyadh region in vegetation, climatic and edaphic characteristics [13, 14] and own observations.

Results showed an inverse correlation between the amount of ammonium and the amount of nitrate nitrogen in all samples from soils covered with *J. procera* and those lacking it. This finding has been ascribed to an inhibition of nitrification by *J. procera*, since the amount of ammonium nitrogen was higher in plots with *J. procera* than in those lacking this plant, whereas the amount of nitrate nitrogen showed an opposite behaviour. On taking in consideration the amounts of nitrifiers, results indicate that the counts were higher in the soil samples taken from plots lacking *J. procera* than in those dominated by this plant. The obvious inference is that the nitrifiers are inhibited in some way in the plots dominated by *J. procera* so that the ammo-

nium nitrogen is not oxidized to nitrate as readily as in plots lacking *J. procera*. The associated species in plots with *J. procera* and those lacking it (control) at both Al-Jurrah and AsSudah showed approximately the same frequency, so *J. procera* appears to have a potential role in inhibiting nitrification. The lack of thoroughly penetrating fibrous roots due to the absence of grass or herbaceous ground cover in plots with *J. procera* might indicate that the low level of nitrate nitrogen was not due to an increased uptake of nitrates. The role of the microflora could not be excluded and as long as there is microbial activity nitrogen assimilation will take place. The microglora of *J. procera* rhizosphere and that of the soil apart showed no obvious qualitative differences [15]. The role of the microflora, however, has not been explored and needs further investigation. It is also obvious from the soil characteristics that the inhibition of nitrification in areas with *J. procera* was not due to the pH, organic carbon, $CaCO_3$ content or textural differences. The observed inhibition of nitrification is in accordance with the previous findings [5, 7–9, 16, 17].

The inhibition of nitrification was attributed to the influence of the plants exerted on the mineralization of nitrogen in the soil [2, 7]. The plant-produced toxins were expected to inhibit nitrification [18–20]. Plant tannins also were found to inhibit nitrification when added to soil [21].

Inhibition of nitrification is necessary to minimize nitrate pollution manifest by its role in eutrophication [22], infant and animal methaemoglobinemia and the formation of nitrosamines. In natural vegetation succession proceeds in the direction of inhibition of nitrification [7].

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REFERENCES

1. RICHARDSON, H. L.: J. agric. Sci. **28,** 73 (1938).
2. THERON, J. J.: J. agric. Sci. **41,** 289 (1951).
3. EDEN, T.: J. Soil Sci. **2,** 43 (1951).
4. MILLS, W. R.: East Afr. Agric. J. **19,** 53 (1953).
5. BERLIER, Y., DABIN, B., LENEUF, N.: Comparison physique, chimique et microbiologique entre les sols de forêt et de savane sur les sables tertiaires de la Basse Côte d'Ivoire. Trans. 6th International Congress of Soil Sciences E: 1956. pp. 499–502.
6. GREENLAND, D. J.: J. agric. Sci. **50,** 28 (1958).
7. RICE, E. L., PANCHOLY, S. K.: Amer. J. Bot. **59** (10), 1033 (1972).
8. JACOUEMIN, H., BERLIER, Y.: Evolution du pouvoir nitrifiant d'un sol de basse côte d'Ivoire sous l'action du climat et de la vegetation. Trans. 6th International Congress of Soil Sciences C: 1956. pp. 343–347.
9. NYE, R. H., GREENLAND, D. J.: The soil under shifting cultivation. Tech. Commun. 51. Commonwealth Bureau of Soils, Harpenden, England 1960.
10. BLACK, C. A.: Methods of Soil Analysis. American Society of Agronomy Inc., Madison, Wis. 1965.

11. ALEXANDER, M., CLARK, F. C.: Nitrifying bacteria. In: Black, C. A. (ed.): Methods of Soil Analysis. American Society of Agronomy Inc., Madison, Wis. 1965.
12. EL-HUSSEINY, T. M., ABU ZINADA, A. H., GHANNAM, M. A.: Bull. Fac. Sci. Riyad Univ. **8,** 93 (1977).
13. MANDAVILLE, J. P.: A contribution to the flora of Asir, South-western Arabia. Coconut grove, Miami, Field Research Publications IV, 1965, p. 13.
14. MIGAHID, A. M., HAMMOUDA, M. A.: Flora of Saudi Arabia. Riyad University Publication. 1974. p. 574.
15. EL-NAGGAR, M. R., EMARA, H. A.: In press.
16. RUSSELL, J., RUSSELL, E. W.: Soil Conditions and Plant Growth. 9th ed. John Wiley and Sons, New York 1961.
17. SMITH, W., BORMANN, F. H., LIKENS, G. E.: Soil Sci. **106,** 471 (1968).
18. MUNRO, P. E.: J. appl. Ecol. **3,** 231 (1966).
19. RICE, E. L.: Physiol. Plant. (1965).
20. RICE, E. L.: Physiol. Plant. **22,** 1175 (1969).
21. BASARABA, J.: Plant and Soil **21,** 8 (1964).
22. ALEXANDER, M.: Introduction to Soil Microbiology. 2nd ed. John Wiley and Sons, New York 1977.

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ANTHRAX-SPECIFIC "AP 50-LIKE" PHAGES ISOLATED FROM *BACILLUS CEREUS* STRAINS

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Phages exerting a specific action on *Bacillus anthracis* were isolated from mitomycin C-induced concentrated lysates of 5 *Bacillus cereus* strains producing megacin A (phospholipase A). In electron micrographs the phages closely resembled the anthrax-specific, lipid-containing phage AP 50 isolated earlier from soil sample. The phages were similar to AP 50 also in their antigenic and chemical structure, host range and sensitivity to organic solvents, detergents and caesium chloride. The DNA character of AP 50 nucleic acid was shown by agarose gel electrophoresis. AP 50 and related phages seem to represent a separate group of phages acting on *Bacillus* strains.

GAÁL and IVÁNOVICS [1] have shown a bacteriocin in the lysates of *Bacillus cereus* strain W induced with mitomycin C. Under similar conditions IVÁNOVICS *et al.* [2] obtained the same bacteriocin from five wild-type *B. cereus* strains isolated from soil samples. The bacteriocin was identified as megacin A [1, 2] corresponding to phospholipase A [3]. The lysates of two wild-type *B. cereus* strains yielded a specific phage identical in antigenic structure with phage *wx* of *B. cereus* strain W, which had been shown converting to phospholipase A production [2, 4]. Phages isolated from the remaining three *B. cereus* strains were assumed to be defective in converting capacity, but they carried a gene segment giving information for phospholipase A production. *B. cereus* strain W is polylysogenic, i.e. in addition to converting phage *wx* it carries an anthrax-specific β phage [5]. Five of the phospholipase A producing wild-type *B. cereus* strains isolated from soil samples were also polylysogenic: an anthrax-specific phage was shown in their concentrated lysate induced with mitomycin C; this phage was in several respects similar to the lipid-containing phage AP 50 isolated earlier from soil [6, 7]. The present study deals with the morphological and biological properties of AP 50 and related phages.

Materials and methods

Bacterial strains. B. cereus strains 04, 11, 23, 26 and 27 were isolated earlier from soil samples. Upon mitomycin C induction (0.5 μg/ml) their lysates contained 400–3200 U megacin A per ml [2]. Apathogenic, acapsulogenic Sterne strains CN 18-74 and CN 35-18 were used as indicators [8]. Host range examinations were carried out with 34 *B. anthracis* strains and with 52 strains representing other species of the genus *Bacillus* [7].

Phages. For comparison, phage AP 50 isolated from soil sample and its clear mutant AP 50 C were employed [6]. Phages isolated from *B. cereus* were designated as the corresponding strains, using the prefix AP 50 in view of their close relationship to that phage (e.g. AP 50-04, AP 50-11).

Culture medium and buffer. Liquid YP [9] and solid YP medium containing 1.5% agar were used. Phages were diluted with a mixture of 4 parts physiological saline and 1 part YP medium. For phage concentration the buffer described by RABUSSAY *et al.* [10] was used.

Isolation and concentration of phages. To exponentially growing broth cultures of *B. cereus* strains, 04, 11, 23, 26 and 27 1 μg mitomycin C was added per ml. The cultures lysed after residual growth were centrifuged at 5000 *g*, then concentrated with polyethyleneglycol (PEG, mol wt 6000) as described by YAMAMOTO *et al.* [11]. The concentrated lysate was drop-inoculated onto a layer of indicator strain Sterne CN 18-74. The target-like plaques produced were scraped off and propagated in a broth culture of CN 18-74. The propagated phage had a titre of 10^7–10^9/ml. Mass production of AP 50-like phages, their concentration and purification were performed as described for reference phage AP 50 [7]. Phage density was determined by CsCl gradient centrifugation.

Host range determination. The phage material was membrane filtered (pore size 0.45 μm) and spotted at 10-fold serial dilutions onto soft agar layer inoculated with the indicator strain.

One step growth of phages was determined in YP medium at 37 °C as described by ADAMS [12]. The m.o.i. was 0.1.

Antigenic structure. The serum of rabbits immunized intravenously with phage AP 50 was used as antibody. The K value was determined as described by ADAMS [12]. The serum was used at 1 : 100 dilution.

Sensitivity to organic solvents and detergents. The phage materials purified by cyclic ultracentrifugation were left to stand with 25% (v/v) ether, 5% (v/v) chloroform, 0.1% sodium laurylsulphate, 0.5% Triton X, 0.1% Brij 58 and 50% (w/v) CsCl. The number of survivors was determined by phage titration after 30 min, in the case of CsCl after 180 min exposure.

Nucleic acid determination. The phage materials purified by cyclic ultracentrifugation were treated with proteinase K (Merck; 0.1 mg/ml) at 37 °C for 60 min then incubated with sodium laurylsulphate at 37 °C for 5 min. Proteins were removed by phenol treatment and the phage material was dialysed against a buffer (10 mM Tris, 10 mM NaCl and 1 mM EDTA, pH 8). Nucleic acid samples were treated at 37 °C with RN-ase (Worthington; 0.05 mg/ml), DN-ase (Worthington; 0.05 mg/ml) for 3 min, with NaCl (0.1 M) for 3 h, with restriction endonucleases (Asu I and Bam H I; 50 U/ml) for 1 h. Untreated and treated nucleic acid samples were analysed by agarose gel (Sigma; 1%) electrophoresis at 4 mA per tube using a buffer containing 0.05 M Tris, 0.02 M NaOH, 2 mM EDTA and 0.02 M NaCl, pH 8.

Electron microscopic examination. The phage materials were purified by sucrose gradient centrifugation, treated with 50% CsCl for 180 min and mounted on LKB grids covered with Formvar film. After adsorption and washing, the preparations were stained with 2% uranyl acetate (w/v). For electron microscopy, a JEOL 100B apparatus operating at 80 kV was used.

Results

Isolation and plaque morphology of AP 50-like phages. The lysates of certain *B. cereus* strains producing phospholipase A after mitomycin C induction contained small amounts of a phage acting on *B. anthracis* strain Sterne 18-74 [2]. The plaque morphology of anthrax-specific phages obtained in this manner from *B. cereus* strains 04, 11, 23, 26 and 27 closely resembled the plaques of phage AP 50. On strains Sterne CN 18-74 and CN 35-18 the latter produced very turbid plaques 1–2 mm in diameter (Fig. 1a). A mutant of this phage, AP 50c produced target-like plaques 3–5 mm in diameter with a pin head size clear centre in the middle of the turbid area (Fig. 1b). Each AP 50-like phage of the five *B. cereus* strains exhibited this target-like morphology. Phage AP 50-04 produced on propagation both turbid and target-like plaques (Fig. 1c).

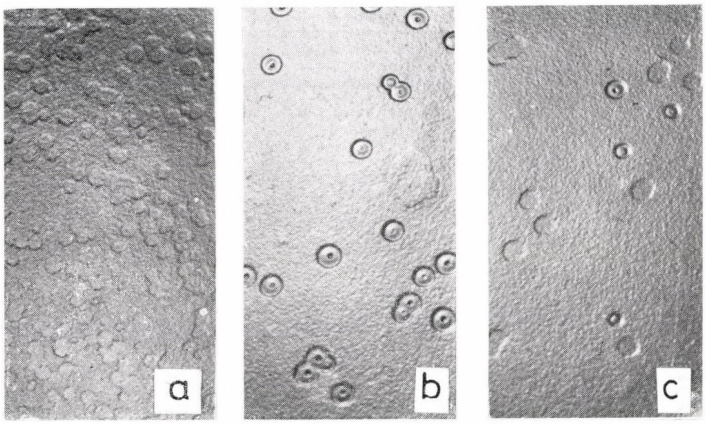

Fig. 1. Plaques of phage AP 50 (a), AP 50 c (b) and AP 50-04 (c). Original size

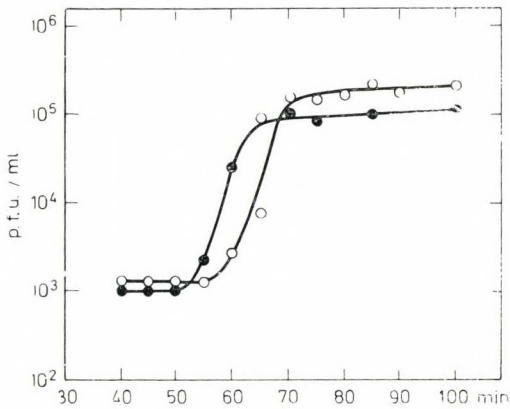

Fig. 2. One step growth curve of phage AP 50 (○———○) and phage AP 50-11 (●———●) on the Sterne strain CN 18-74

Host range. Twelve out of 34 *B. anthracis* strain were sensitive to the five AP 50-like phages. None of 52 strains belonging to 6 different species of the genus *Bacillus* was sensitive to phage AP 50 and to any of the AP 50-like phages.

One step growth. Figure 2 shows one step growth curves for phages AP 50 and AP 50-11. For AP 50-11 the latent period was somewhat shorter than for the reference phage (50 *vs.* 55 min). The average burst size for AP 50-11 was 240 p.f.u./cell. Other AP 50-like phages yielded closely similar results. The adsorption rate constant for AP 50-like phages against strain Sterne CN 18-74 ranged between 1.1×10^{-9} ml/min and 2×10^{-9} ml/min.

Antigenic structure of AP 50-like phages. Neutralization of AP 50-like phages by immune serum for reference phage AP 50 is shown in Table I. The K values were similar for each of the five AP 50-like phages.

Table I

K values for AP 50-like phages

Phages	K values*
AP 50	98
AP 50-04	70
AP 50-11	69
AP 50-23	86
AP 50-26	76
AP 50-27	81

* Determined with AP 50 immune serum diluted 1 : 100, average of four titrations

Resistance of AP 50-like phages. Various chemical substances exerted a fairly similar effect on all AP 50-like phages (Table II). Static exposure to 5% chloroform and 25% ether reduced the survivors to 0.2 and <0.01%, respectively. The titre was even lower if the phage suspensions were shaken during exposure. Sodium laurylsulphate exerted a similar degree of reduction in titre, but Triton X and Brij 58 were practically ineffective even after 24 h exposure. CsCl was less effective than the three other substances.

Nucleic acid determination. The nucleic acid fraction was isolated by cyclic ultracentrifugation from phage AP 50 material containing 10^{12} infective particles per ml. The nucleic acid fraction and its aliquots treated with DNase, RNase, NaOH and restriction endonucleases Asu I and Bam H I were examined by agarose gel electrophoresis. Figure 3 shows that the nucleic acid band is absent for the DNase treated, but is present for the untreated and RNase treated samples. This finding means that phage AP 50 contains DNA. Bam H I treatment resulted in bands identical with those for the untreated and RNase treated samples.

Table II

Sensitivity of AP 50-like phages to chemical substances

Treatment	Concentration, %	Per cent of survivors					
		AP50	AP50-04	AP50-11	AP50-23	AP50-26	AP50-27
Chloroform, 30 min	5.0	<0.01	<0.01	<0.01	0.05	<0.01	0.1
Ethyl ether, 30 min	25.0	0.1	<0.01	0.1	0.2	<0.01	0.1
Sodium laurylsulphate, 30 min	0.1	0.1	<0.01	0.05	0.1	0.1	0.05
Triton X, 30 min	0.5	100.0	98.0	100.0	100.0	88.0	92.0
Brij 58, 30 min	0.1	100.0	100.0	85.0	100.0	87.0	100.0
CsCl, 180 min	50.0	1.5	7.0	2.0	1.2	1.0	1.5

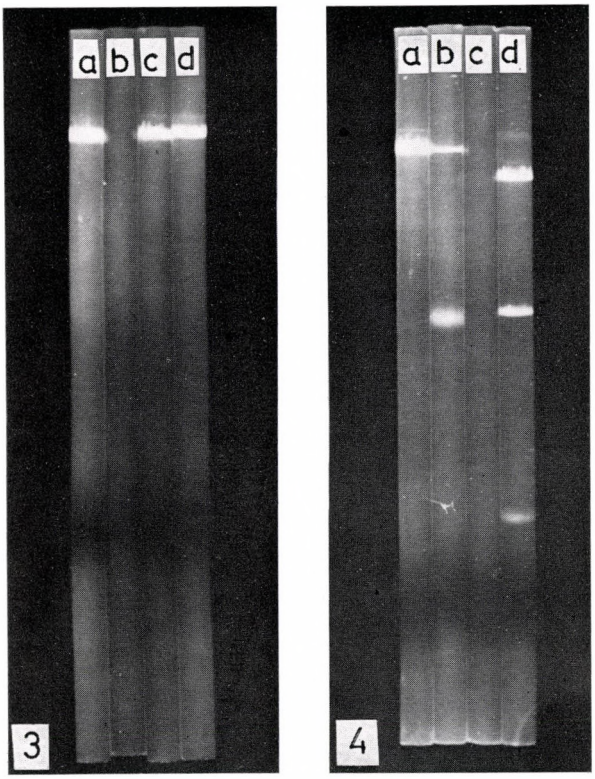

Fig. 3. Agarose gel electrophoresis of (a) untreated, (b) DNase 0.05 mg/ml, (c) RNase 0.05 mg/ml, (d) Bam H I 50 U/ml restriction endonuclease treated AP50 nucleic acid
Fig. 4. Agarose gel electrophoresis of (a) untreated, (b) NaOH 0.1 M, (c) DNase 0.05 mg/ml, (d) Asu I 50 U/ml restriction endonuclease treated AP 50 nucleic acid

Figure 4 shows that the nucleic acid is alkali-stable, which is another proof of its DNA character. Asu I, a less specific restriction endonuclease, split the phage DNA into three fractions differing in molecular weight (column 4 in Fig. 4).

Preformed CsCl gradient centrifugation showed that the density of AP 50-like phages ranged between 1.295 and 1.317 g/ml.

Electron microscopic examination of AP 50-like phages. The lysates of *B. cereus* strains isolated from soil samples and selected by megacin A (phospholipase A) production, were examined to show the presence of phages converting to the formation of this enzyme. In earlier experiments [2] the corresponding phages were demonstrated in *B. cereus* strains 23 and 26. These strains were, however, polylysogenic, as they released small numbers of hexagonal, tailless, nucleic acid deficient double membrane covered particles, in addition to *wx* phages (Bradley's group B). Phages similar in morphology were observed in *B. cereus* strains 04, 11 and 17 which produced phospholipase A in lower

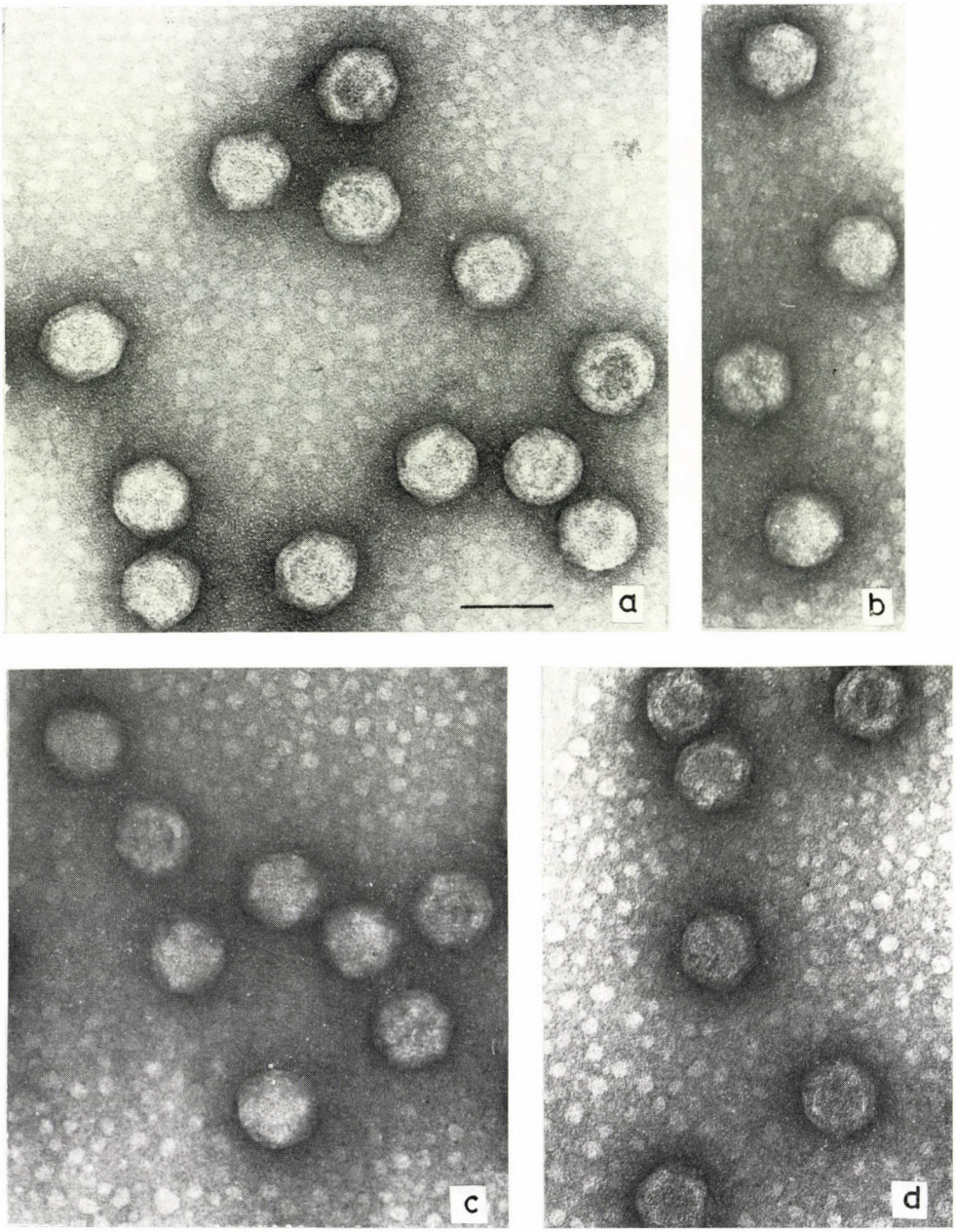

Fig. 5. Electron microscopic pictures of sucrose density gradient centrifuged AP 50 (a), AP 50-04 (b), AP 50-11 (c) and AP 50-23 (d). The bar is equivalent to 100 nm

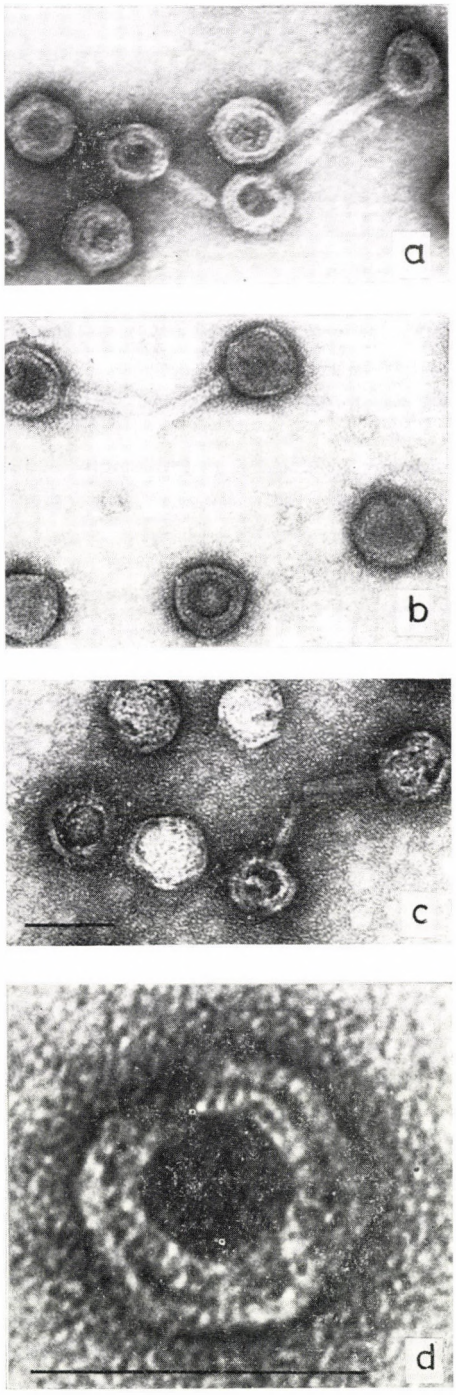

Fig. 6. Electron microscopic pictures of CsCl treated phages AP50-04 (a), AP50-11 (b), AP 50-23 (c), AP 50 (d). The bar is equivalent to 100 nm

titres. This structure was closely similar to that of phage AP 50 examined in detail earlier [6, 7].

Figure 5 shows AP 50-like phage particles propagated on strain Sterne CN 18-74, concentrated and purified by sucrose density gradient centrifugation. The particle population obtained in this manner was homogeneous. The phages were hexagonal, 80 nm in diameter, had no tail and were coated by a more or less visible double external membrane. Phages AP 50-11 and AP 50-27 were morphologically identical. The five AP 50-like phages were closely similar to reference phage AP 50 in being fairly sensitive to CsCl. Their electron microscopic picture changed considerably after exposure to 50% CsCl of the purified phage material: parallel with the decrease in titre (Table II), a large number of nucleic acid deficient phages with tail-like appendages appeared (Fig. 6). The double layer coat characteristic of AP 50-like phages was especially well-defined in nucleic acid deficient particles, the thickness of the two layers being approximately identical (8 nm). The effect of CsCl was similar to that described by SAKAKI and OSHIMA [13] for phage ΦNS11 and by OLSEN *et al.* [14] for phage PRD_1. The degrading effect of CsCl on PRD_1 can be prevented by the addition of 0.5% tryptone (Difco) [14]. In the case of AP 50-like phages tryptone was ineffective.

An attempt was made at eliciting the production of tail-like structures by exposure to effects other than that of hypertonic CsCl. Purified reference phage AP 50 and AP 50-like phages were vigorously shaken for 10 min with 2 mm glass beads. The resulting two-exponent fall in titre was not accompanied by the development of tail-like appendages. An osmotic shock with 4 M NaCl caused a three-exponent titre decrease with the appearance of nucleic acid deficient particles having no tail-like structures.

Discussion

The majority of *B. cereus* strains isolated from natural sources can be induced to lysis by UV irradiation [15] and by mitomycin C treatment. In lack of appropriate indicator strains, infective phages can infrequently be detected in the lysates [16, 17]. In the concentrated lysates of the induced strains, however, electron microscopy usually reveals intact phage particles [16].

Some *B. cereus* strains are double lysogenic, that is, they carry two phages different in morphological, biological and chemical properties. MCCLOY [5, 18] and MEYNELL [19] showed that *B. cereus* strain W carried an anthrax-specific phage not acting on other *Bacillus* species ($w\beta$). IVÁNOVICS *et al.* [3] described that *B. cereus* strain W had, in addition to the anthrax-specific phage, another phage (wx), which was responsible for phospholipase A production.

B. cereus strains $5P_3$ and $5P_{15}$ of M. Pollock's collection were also double lysogenic [16]. One of their phages acting on *B. anthracis* strain Davis, was characterized by hexagonal headed, non-contractile tailed particles similar in plaque morphology and electron microscopic picture in both strains. The other phage was morphologically identical (elongated head and non-contractile tail) but differed in size in the two strains. None of the two kinds of phage could be propagated in lack of a suitable indicator strain [16].

Accordingly, the double lysogeny of *B. cereus* strains examined in the present study is not surprising. Phages *wx*23 and *wx*26 of *B. cereus* strains 23 and 26 were characterized earlier [2]. These particles, although electron microscopically intact, could be propagated only on the homologous *cin*$^-$ strain. The anthrax-specific phages of these cultures and those of the other three *B. cereus* strains were identical in host range with the phage of the original AP 50 isolate [7, 20]. One step growth curves, adsorption rate constants and K values were also closely similar to earlier findings for phage AP 50 [7]. In sensitivity to chemicals and electron microscopic morphology, AP 50-like phages were practically identical with one another and with phage AP 50. It may be assumed that in the soil this group of phages is carried by *B. cereus* as a prophage.

Part of lipid containing phages characterized by cubic symmetry double capside (PR_3, PR_4, PR_5 and PRD_1) act on strains carrying a plasmid of the Gram-negative P_1, N and W incompability group [14, 21, 22]. Only three phages acting on *Bacillus* strains similar to the above group of phages have been described: Bam 35 [23] lyses part of *B. megaterium*, *B. cereus* and *B. thueringiensis* strains, ΦNS11 [13] is specific for *B. acidocaldaris* and AP 50 for *B. anthracis* strains that carry certain inducible and electron microscopically defective phage structures [20]. These phages are characterized by a tail-like structure appearing as a result of nucleic acid ejection [7, 24].

Nucleic acid studies on phage AP 50 have been revised in the present study. In previous examinations DNA determination by the method of BURTON [25] and RNA determination according to SCHNEIDER [26] showed that the phage material had no DNA, but contained 65 μg RNA per ml [7]. BURTON's diphenylamine method is relatively insensitive and requires a great amount of sample. SCHNEIDER's method has the disadvantage that the amount of RNA can be calculated only after a distraction of the DNA amount determined by other methods. In the present study the DNA character of AP 50 nucleic acid has been demonstrated by agarose gel electrophoresis.

The classification of phages Bam 35 and ΦNS11 into the *Tectiviridae* group has been accepted by the International Committee on the Taxonomy of Viruses. From the present data it may be concluded that, on the basis of morphology, physicochemical characteristics and DNA content, AP 50 and AP 50-like phages also belong to the *Tectiviridae*.

REFERENCES

1. GAÁL, V., IVÁNOVICS, G.: Z. allg. Mikrobiol. **12,** 393 (1972).
2. IVÁNOVICS, G., GAÁL, V., NAGY, E., PRÁGAI, B., SIMON, M.: Acta microbiol. Acad. Sci. hung. **23,** 283 (1976).
3. IVÁNOVICS, G., GAÁL, V., PRÁGAI, B.: J. gen. Virol. **24,** 349 (1974).
4. GAÁL, V., IVÁNOVICS, G.: Acta microbiol. Acad. Sci. hung. **20,** 209 (1973).
5. MCCLOY, E. W.: J. Hyg. (Lond.) **49,** 114 (1951).
6. NAGY, E.: Acta microbiol. Acad. Sci. hung. **21,** 257 (1974).
7. NAGY, E., PRÁGAI, B., IVÁNOVICS, G.: J. gen. Virol. **32,** 129 (1976).
8. STERNE, M.: Onderstepoort J. vet. Res. **9,** 49 (1937).
9. CSISZÁR, K., IVÁNOVICS, G.: Acta microbiol. Acad. Sci. hung. **12,** 73 (1965).
10. RABUSSAY, D., ZILLING, W., HERRLICH, P.: Virology **41,** 91 (1970).
11. YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWHORNE, L., TREIBER, G.: Virology **40,** 734 (1970).
12. ADAMS, M. H.: Bacteriophages. Interscience Publishers, New York 1959.
13. SAKAKI, Y., OSHIMA, T.: Virology **75,** 256 (1976).
14. OLSEN, R. H., SIAK, J. S., GRAY, R. H.: J. Virol. **14,** 689 (1974).
15. ALTENBERN, R. A.: Biochem. biophys. Res. Commun. **9,** 109 (1967).
16. NAGY, E., GAÁL, V.: Acta microbiol. Acad. Sci. hung. **21,** 229 (1974).
17. ALTENBERN, R. A., STULL, H. B.: J. gen. Microbiol. **39,** 53 (1965).
18. MCCLOY, E. W.: J. gen. Microbiol. **18,** 198 (1958).
19. MEYNELL, E. W.: J. gen. Microbiol. **28,** 103 (1962).
20. NAGY, E., IVÁNOVICS, G.: J. gen. Microbiol. **102,** 215 (1977).
21. BRADLEY, D. E.: Biochem. biophys. Res. Comm. **57,** 893 (1974).
22. WONG, F. H., BRYAN, L. E.: Canad. J. Microbiol. **24,** 875 (1978).
23. ACKERMANN, H. W., ROY, R., MARTIN, M., MURTHY, M. R. V., SMIRNOFF, W. A.: Canad. J. Microbiol. **24,** 986 (1978).
24. PALVA, T., BAMFORD, D.: In L. L. Randall and L. Philipson (eds): Virus Receptors (Receptors and Recognition) Series B, Vol. 7, Chapman and Hall, London 1980. p. 97.
25. BURTON, K.: Biochem. J. **62,** 315 (1956).
26. SCHNEIDER, W. C.: In S. P. Colowick and N. O. Kaplan (eds): Methods in Enzymology, Vol. III, Academic Press Inc., New York 1957. p. 680.

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EFFECT OF ENDOTOXIN PREPARATIONS ON THE COURSE OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN SUCKLING MICE

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Death occurred earlier and its rate was higher in suckling mice treated with parent or radio-detoxified endotoxin and subsequently inoculated intracerebrally with lymphocytic choriomeningitis (LCM) virus than in their virus infected but untreated control littermates. Thus, in suckling mice both the parent and the radio-detoxified endotoxin pretreatment contributed to the outcome of LCM virus infection in the form of lethal meningitis indicating its increasing effect on the cellular immunological reaction to the virus infection.

Lipopolysaccharide (LPS) endotoxins originating from Gram-negative bacteria are known to influence immune responsiveness. There have been reports on both the increase and the decrease of the immune response to heterologous antigens [1].

It is also known that the fatal outcome of lymphocytic choriomeningitis following intracerebral LCM virus infection is the consequence of a cell-mediated immune reaction to cells on the leptomeninx displaying viral (changed) antigens due to virus reproduction, and that cytotoxic T lymphocytes play a role in development of the disease [2–4]. Thus, the course of the virus infection in mice is highly dependent on the cellular immune responsiveness of the animals, and hence on their age.

LCM develops in adult mice with intact lymphoid system and the animals die on the 6th to 8th day following virus infection. In mice with insufficient T lymphocyte function, like in newborn mice with an undeveloped lymphoid system or adult mice with an impaired lymphoid system, do not develop meningitis following the infection but survive and become virus carriers [5, 6]. In the case of suckling mice the mortality rate and the time of death depend on their age at the time of inoculation. The rate of mice surviving the infection in a virus carrier state decreases with age, and old mice die sooner than young ones [7–9].

Substances with adjuvant activity contribute to, while immunosuppressive substances inhibit, the development of meningitis following intracerebral LCM virus infection in suckling mice [10–12].

In the present experiment the effect of native or radiodetoxified endotoxin was studied on the course of LCM virus infection in suckling mice.

Materials and methods

Endotoxin (LPS) preparation. The LPS was isolated by the hot phenol–water method of WESTPHAL *et al.* [13] from a fermentor culture of *Escherichia coli* O89. The preparation was purified by repeated ultracentrifugation at 100 000 × *g*.

Production of radio-detoxified LPS preparation. The 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 [14, 15].

Experimental animals. Outbred (LATI, Gödöllő, Hungary) 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 the neurological symptoms characteristic of LCM (tremor, convulsions) and death were controlled twice daily.

Experiments and results

To study the effect of endotoxin preparation on the course of LCM virus infection, two parallel experiments were undertaken on 12–13 day-old mice belonging to 17 litters. In each litter, the number of animals was reduced to 10. Five to 6 animals of each litter were treated with parent LPS (Experiment I) or radio-detoxified LPS (Experiment II). The animals received a single intraperitoneal injection of the parent LPS or radio-detoxified LPS preparation in a dose of 1 mg/kg body weight. In each litter the rest of the animals received PBS in the same way. On the following day the mice were infected intracerebrally, using 100 LD_{50} of the LCM virus, 6 litters in Experiment I and 5 litters in Experiment II.

Simultaneously with the LCM infection 6 other litters were inoculated intracerebrally with virus-free mouse brain suspension. Of these mice, those

Table I

Mouse groups and their treatment

Experiment	Groups	Number of mice	Intraperitoneal treatment	Intracerebral inoculation
I	LPS–LCM	30	LPS	LCM virus
	LCM	30	*	LCM virus
	LPS	15	LPS	**
	C	15	*	**
II	rdLPS–LCM	28	rdLPS	LCM virus
	LCM	22	*	LCM virus
	rdLPS	15	rdLPS	*
	C	15	*	**

* = PBS
** = Virus free mouse brain suspension

receiving LPS formed the group LPS, those receiving radio-detoxified LPS the group radio-detoxified LPS (rdLPS) and those treated with phosphate-buffered saline (PBS) the C group.

LPS pretreated, infected mice belonged to the LPS–LCM group, the radio-detoxified LPS pretreated infected ones to the rdLPS–LCM group.

The experiment was terminated on the 21st day after virus infection. The neurological symptoms characteristic of LCM and deaths were registered in the virus infected groups. The groups and treatments are presented in Table I.

Rate and time curve of death in the virus infected groups during the experiments are presented in Fig. 1.

In Experiment I 66% of the animals infected with virus but not treated with LPS died between the 8th and 12th day, in Experiment II 54% of only virus infected mice died between the 9th and 14th day. Ninety per cent of the virus infected and parent LPS treated mice (LPS–LCM group) died, thus the mortality rate was higher here than among the untreated littermates. Death

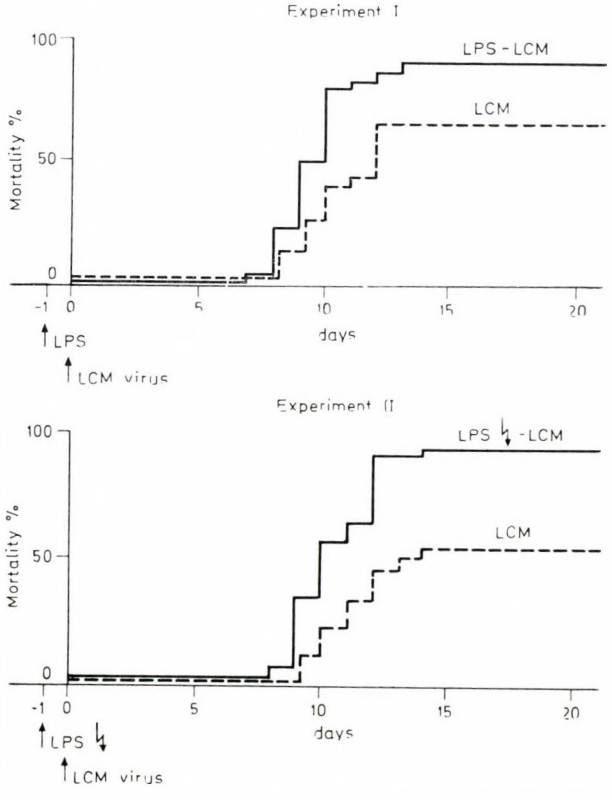

Fig. 1. Rate and time of death after LCM virus infection. LPS with arrow = radio-detoxified LPS

occurred from the 7th day after virus infection, i.e., earlier than in the untreated LCM group.

Eighty-five per cent of the virus infected and radio-detoxified LPS treated mice (group rdLPS–LCM) died between the 8th and 14th day after virus infection. Thus, the mortality rate was also higher and deaths occurred one day earlier than among the untreated control littermates.

Neither disease nor death occurred in the uninfected, i.e. in the parent or radio-detoxified LPS treated or control groups.

Discussion

The results showed that death occurred earlier and its rate was higher in mice treated with parent or radio-detoxified LPS and subsequently infected with LCM virus than in their virus infected but untreated control littermates. Thus, in suckling mice both the parent and radio-detoxified LPS treatment contribute to the outcome of LCM infection in the form of lethal meningitis, i.e. they enhance the cellular immune reaction to virus infection.

The results obtained are in good agreement with data in the literature on LPS treatment: stimulation of cellular immune responses, its contribution to the development of delayed type hypersensitivity or GVH reaction, and growing MIF production or proliferative response in mixed lymphocyte cultures [16–23].

It is remarkable that radio-detoxified LPS should produce an effect similar to that of parent LPS, i.e. gamma-irradiation did not influence the endotoxin's stimulating effect on the cellular immune reaction. This is in good agreement with our previous results showing that adjuvant activity of LPS on the humoral immune response is not influenced by radio-detoxification [24, 25].

REFERENCES

1. Morrison, D. C., Ryan, J. L.: Advanc. Immunol. **28,** 293 (1979).
2. Cole, G. A., Nathanson, N., Prendergast, R. A.: Nature (Lond.) **238,** 335 (1972).
3. Doherty, P. D., Zinkernagel, R. M.: Transplant Rev. **19,** 81 (1974).
4. Volkert, M., Marker, O., Bro-Jörgensen, K. J.: J. exp. Med. **139,** 1329 (1974).
5. Hotchin, J.: In Melnick, J. L. (ed.): Monographs in Virology. Vol. **3,** S. Karger, Basel 1971. p. 2.
6. Lehman-Grube, F.: In Gard, S., Hallauer, G., Meyer, K. F. (eds): Virology Monographs. Vol. **10,** Springer Verlag, Wien 1971.
7. Hotchin, J. E., Cinits, M.: Canad. J. Microbiol. **4,** 149 (1958).
8. Hotchin, J. E., Weigand, H.: N. Y. State Dept. Health, Ann. Rep. Div. Lab. Res. 1979. p. 21.
9. Hotchin, J., Weigand, H.: J. Immunol. **86,** 392 (1961).
10. Bános, Zs., Szeri, I., Anderlik, P., Radnai, B.: Acta microbiol. Acad. Sci. hung. **26,** 127 (1979).

11. Bános, Zs., Anderlik, P., Szeri, I., Radnai, B.: Acta microbiol. Acad. Sci. hung. **26,** 277 (1978).
12. Bános, Zs., Szeri, I., Anderlik, P.: Acta microbiol. Acad. Sci. hung. **26,** 29 (1979).
13. Westphal, O., Lüderitz, O., Bister F.: Z. Naturforsch. **7b,** 148 (1952).
14. Bertók, L., Kocsár, K., Várterész, V., Bereznai, T., Antoni, F.: Patent No. 162 973, Budapest 1973.
15. Füst, Gy., Bertók, L., Juhász-Nagy, S.: Infect. Immun. **16,** 26 (1977).
16. Lagrange, P. H., Mackaness, G. B.: J. Immunol. **114,** 447 (1975).
17. Lagrange, P. H., Mackaness, G. B., Miller, T. R., Padron, P.: J. Immunol. **114,** 442 (1975).
18. Skopinska, E.: Transplantation **26,** 420 (1978).
19. Rose, W. C., Rodey, G. E., Rimm, A. A., Truitt, R. L., Bortin, M. M.: Exp. Hemat. **4,** 90 (1976).
20. Forbes, J. T., Nakao, Y., Smith, R. T.: J. Immunol. **114,** 1004 (1975).
21. Ritter, J., Lohmann-Matthes, M. L., Sonntag, H. G., Fischer, H.: Cell. Immunol. **16,** 153 (1975).
22. Narayanan, P. R., Sundharadas, G.: J. exp. Med. **147,** 1355 (1978).
23. Narayanan, P. R., Kloehn, D. B., Sundharadas, G.: J. Immunol. **121,** 2502 (1978).
24. Elekes, E., Bertók, L., Merétey, K.: Acta microbiol. Acad. Sci. hung. **25,** 17 (1978).
25. Bertók, L.: Perspect. Biol. Med. **24,** 61 (1980).

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PROTECTIVE ROLE OF O ANTIGEN IN *SALMONELLA TYPHI-MURIUM* INFECTION

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Mice were actively immunized with preparations produced from different *Salmonella* strains. They were challenged with 2 LD_{50} of a virulent *S. typhi-murium* strain and examined for viable cell counts in the liver 4, 7 and 11 days postinfection. Whole cell vaccines, ribosomal extracts and endotoxin preparations of the O antigen-deficient variant of *S. typhi-murium* strain LT2-MI failed to protect the mice or did so in a much lower degree than preparations of the corresponding O antigen-bearing variant. Preparations from other salmonellae exerted a protective action only if the strain had an O-antigen identical with that of *S. typhi-murium*. The results pointed to a considerable protective role of the O antigen.

The classical bacterial cell vaccines are far from being ideal for the preventation of enteric infections as regards both efficiency and reactivity [1]. Some live vaccines prepared from attenuated cells [2, 3] have proved to be much more efficient than are the killed vaccines. Furthermore, efforts have been made to prepare vaccines which contain solely the purified protective antigen. The assumed protective effect of the O antigen raised the idea of producing lipopolysaccharide (LPS) vaccine from the cell wall of *Salmonella typhi* [4]. The protective action of the LPS vaccine was, however, unsatisfactory [5].

The unfavourable experience with vaccines of cell wall origin suggested that antigens present in the bacterial ribosome may account for the protective activity [6, 7]. VENNEMAN and BIGLEY [8] were the first to isolate from *S. typhi-murium* a ribosomal preparation which was free of cell wall products. They found the preparation as protective as are live attenuated vaccines. Since the ribosomal antigen (RA) is a heterogeneous substance, efforts have been made to identify its protective component. The protective effect was attributed to the RNA [9], an RNA–protein complex [10], a protein component [11], and to a high molecular weight glycoprotein [12]. In the last years, contamination of RA preparations by O antigen has attracted attention. EISENSTEIN [13], who demonstrated O antigen in both the RNA and the protein fraction of *S. typhi-murium* cells, has suggested that the O antigen was responsible for at least a part of the protective activity of RA preparations. LIN and BERRY [14] failed to demonstrate protection by an RA antigen that had been prepared from an O antigen-deficient (O^-) variant of *S. typhi-*

murium. A similar preparation obtained from the O^+ variant of the same strain was found to be protective.

In the light of the above findings, the views on the protective activity of the O antigen need further revision. Presumably, the controversies may be attributed to quantitative differences between the preparations used in the different experiments and to methodical differences in measuring protective activity.

The present work was aimed at examining the mouse-protective capacity of whole-cell bacterial vaccines, RA preparations and endotoxins prepared from O^+ and O^- strains of *S. typhi-murium* as well as of whole-cell vaccines that had been prepared from strains bearing or not bearing the O antigen common with *S. typhi-murium*. Immunized and non-immunized mice were challenged by artificial infection with virulent *S. typhi-murium*.

Materials and methods

Animals. CFLP albino mice of both sexes, 16–18 g in body weight, were used throughout. The mice were given mouse feed and water *ad libitum*.

Vaccines. All preparations, except the *Vibrio cholerae* vaccine, were prepared by us, with the following strains. *S. typhi-murium* No. 10040 (1,4,5,12 : i : —); *Salmonella java* No. 10014 (4,5,12 : b : —); *Salmonella cholerae-suis* No. 15011 (6,7 : — : 1,5); *S. typhi-murium* LT2-MI.

The strains except the Rc mutant LT2-MI, which had been kindly supplied by Dr. L. J. BERRY, were obtained from the strain collection of this Institute. Being defective in the uridine-diphosphate-D-galactose-4-epimerase enzyme, the LT2-MI strain does not synthesize galactose, hence it develops an O antigen-deficient culture in galactose-free medium. Except for the LT2-MI strain, which was grown in the fluid medium recommended by LIN and BERRY [14], all strains were cultivated in a medium containing 37 g/l Difco BHI agar. To support the growth of the variant producing the O antigen, 2 g of D-galactose per litre of medium were added. The medium used for cultivation of the O antigen-negative variant contained no galactose.

The 15 h cultures were washed in a phosphate buffer of pH 7.2 and suspensions of 35×10^9/ml density were prepared in the same buffer.

Heat-killed vaccines were prepared from *S. typhi-murium* strain No. 10040 and from the O^+ and O^- variants of the LT2-MI strain. The bacterial suspensions were inactivated for 30 min in boiling water.

Phenol-killed vaccines were prepared from *S. typhi-murium* strain No. 10040 and from the O^+ and O^- variants of the LT2-MI strain as well as from *S. java* and *S. cholerae-suis*. The bacterial suspensions were kept at 56 °C for 1 h, then, with 0.5% phenol added, at room temperature for 72 h.

The killed vaccines were stored in the lyophilized state at +4 °C.

Ribosomal antigens [8, 15] were prepared from washed suspensions of the virulent *S. typhi-murium* strain No. 10040 and from the O^+ and O^- variants of the LT2-MI strain. From the centrifuged sediment of the bacterial suspension, a suspension of $100–150 \times 10^9$/ml density was prepared in phosphate buffer pH 7.2 containing 3×10^{-4} M Mg^{++}, and the suspension was sonicated for 10 min at 150 W. The sonicated extract was centrifuged at +4 °C at 30 000 *g* for 10 min and the clear supernatant was centrifuged at 45 000 *g* for another 10 min. The second supernatant was incubated with 3 μg/ml DNase at room temperature for 30 min, then centrifuged at 100 000 *g* for 2 h. From the sediment, in phosphate buffer containing 10^{-4} M Mg^{++}, a suspension of 50 μg/ml was prepared, to which an equal volume of 1% SDS (sodium lauryl sulphate) was added. The mixture was kept at room temperature for 30 min and, subsequently, at +4 °C overnight. On the next day, the membrane debris were removed by centrifuging at 30 000 *g* for 15 min. The RA thus obtained was re-centrifuged at 100 000 *g*. The yield calculated for the initial bacterial mass was 1%. The RA was suspended

in phosphate buffer pH 7.2 to a concentration of 1 mg/ml, the suspension was sterilized by filtration through a G-5 glass filter, and the filtrate was stored at −20 °C.

Boivin extract [16] was prepared from the virulent strain No. 10040 and from the O^+ variant of the strain LT2-MI. One g of acetone dried bacterial mass was suspended in 20 ml distilled water and the suspension was allowed to stand at +4 °C with an equal volume of 0.5 N trichloracetic acid overnight. Then, the suspension was centrifuged and the process was repeated with the sediment. The supernatants were pooled, dialysed against tap water for 48 h and lyophilized.

Goebel extract was prepared from the O^- variant of the LT2-MI strain as recommended by MILLER and GOEBEL [17] for isolation of endotoxin from R-type bacteria. One g of the centrifuged culture was suspended in 10 ml distilled water and the suspension was incubated in a water bath at 65 °C for 1 h, then centrifuged. The sediment was resuspended and centrifuged again. The supernatants from the two centrifugations were united and lyophilised.

The V. cholerae vaccine was a commercial preparation of the Institute for Serobacterial Production and Research Human, Budapest. The vaccine contained 4×10^9/ml heat-inactivated cells of both the Ogawa and Inaba serotypes of *V. cholerae*.

Immune assay. Groups of mice were immunized subcutaneously with different doses of the vaccine under testing. The immunized animals, and the non-immunized ones kept together with them, were challenged 14 days after the immunization with 2–5 LD_{50} (i.e. about 50 viable cells) of the virulent strain No. 10040. In the 8 to 10-day period after the challenge, on three occasions three mice each were killed with ether in each group. The livers were ground with quartz sand and the pooled livers from each group were suspended in 7.5 ml of saline. Serial dilutions were made and 0.02 ml of each of the five dilutions were placed on each of two blood agar plates. The plates were incubated at 37 °C and at 24 h the colonies were counted to calculate the mean viable cell count/mouse liver. This count was higher than 10^5 for the control livers in at least one of the assays in each experiment. A preparation was considered protective if the mean viable cell count for the livers of the immunized mice remained below 10^5 throughout the observation period.

Demonstration of O antigen contamination. Two methods were used for this purpose.

(a) Determination of the specific antibody response of mice to the preparation under testing. Twenty mice of 16–18 g body weight were injected intraperitoneally at weekly intervals with rising doses (10, 20 and 50 μg dry material) of the preparation mixed to an equal volume of incomplete Freund's adjuvant. The mice were exsanguinated one week after the last injection. A proportional mixture of sera obtained from each group of mice was titrated for *S. typhi-murium* O agglutinin level by agglutination and by passive haemagglutination reaction in which sheep erythrocytes sensitized with an LPS preparation of *S. typhi-murium* origin were used. The LPS preparation was kindly supplied by Dr. MÁRIA ÁDÁM (National Institute of Hygiene, Budapest).

(b) Demonstration by passive haemagglutination of the O antigen contents of the preparation under testing. The reaction was performed as described by RAUSS and KÉTYI [18], using the passive haemagglutinating reagent described under (a) and 2 haemagglutinating units of *Salmonella* O serum 1,4,5,12.

Results

Mouse-protective activity of inactivated cell vaccines prepared from the O^+ and O^- variants of the LT2-MI strain (Figs 1, 2 and 3). The mouse-protective activities of phenol-killed vaccines are shown in Fig. 1. The viable counts of the challenge strain in the livers of the mice immunized with the O^+ vaccine was consistently under 10^5 and lower than the corresponding viable cell counts for the mice given the O^- vaccine. The viable counts were slightly lower for the latter group than for the non-immunized control group.

Similar results were obtained with the heat-killed vaccines (Fig. 2).

In Fig. 3, the viable counts for the livers of mice that had been immunized with different doses of phenol-killed vaccines are presented. The

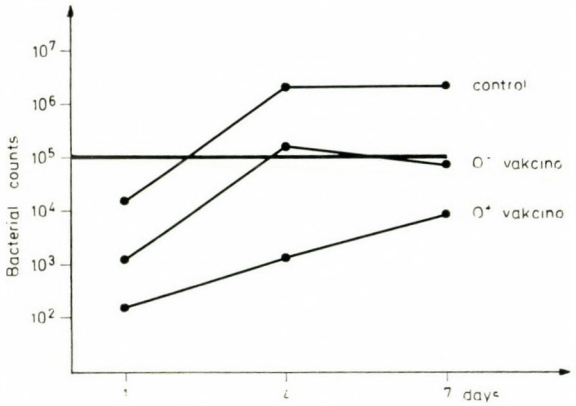

Fig. 1. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with phenol-killed O^+ and O^- vaccine. Bacterial counts in the liver, days after infection. Immunizing dose, 10^5 cells/mouse

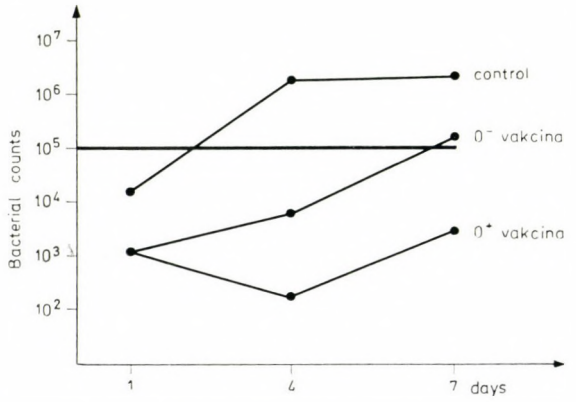

Fig. 2. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with heat-killed O^+ and O^- vaccine. Bacterial counts in the liver, days after infection. Immunizing dose, 10^5 cells/mouse

lowest immunizing dose was 10^3 for the O^+ vaccine and 10^8 cells for the O^- vaccine.

Mouse-protective activity of the RA preparations obtained from the O^+ and O^- variants of the LT2-MI strain (Figs 4 and 5). Mice were immunized with RA preparations obtained from the O^+ and the O^- strain, using an individual dose corresponding to 10 μg dry matter (Fig. 4). The results were similar to those shown in Figs 1 and 2. The mean viable count for the livers was strikingly lower in the O^+-immunized group than in the O^--immunized one. The O^- RA preparation showed no appreciable protective effect, though multiplica-

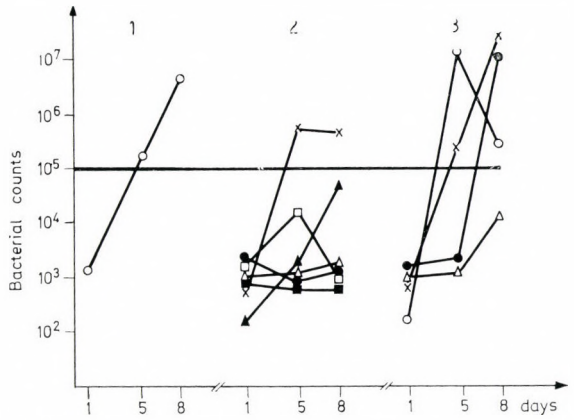

Fig. 3. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with phenol-killed O^+ and O^- vaccine. Bacterial counts in the liver, days after infection. 1 = Control; 2 = O^+ vaccine; 3 = O^- vaccine. Immunizing dose, cells/mouse △——△ 10^8; ○——○ 10^7; □——□ 10^6; •——• 10^5; ■——■ 10^4; ▲——▲ 10^3; ×——× 10^2

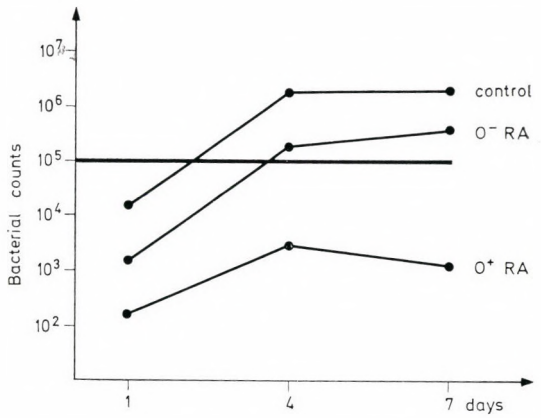

Fig. 4. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with ribosomal preparations of O^+ and O^- origin. Bacterial counts in the liver, days after infection. Immunizing dose, 10 μg/mouse

tion of the challenge cells was slightly inhibited at least up to the 7th day postinfection. The experiment illustrated in Fig. 5 shows the protective effect of different doses of O^+ and O^- RA preparations. The minimum protective dose of the former corresponded to 5 μg, whereas the O^- RA was ineffective even in a 250 μg dose.

O antigen content of the preparations. Table I shows the agglutination titres and the passive haemagglutination titres of mice that had been immunized with O^+ or O^- preparations. The preparations obtained from the O^+ variant of the LT2-MI strain induced a well-defined O antibody response

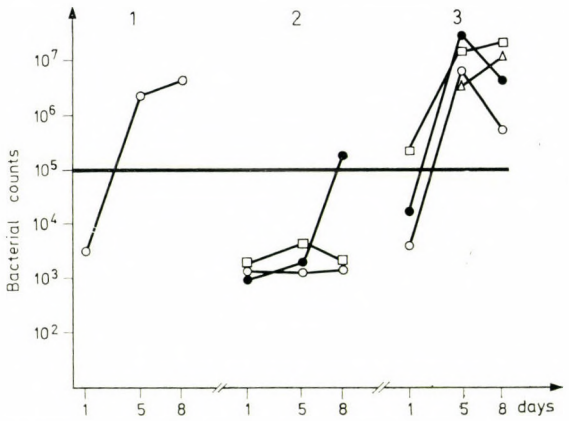

Fig. 5. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with ribosomal preparations of O^+ and O^- origin. Bacterial counts in the liver, days after infection. 1 = Control; 2 = O^+ RA; 3 = O^- RA. Immunizing dose, μg/mouse △——△ 250; ○——○ 50; □——□ 5; •——• 0.5

while those obtained from the O^- variant did not. Using the passive haemagglutination-inhibition method, we failed to demonstrate O antigen in the preparations, with the exception of the O^+-variant killed vaccine.

Mouse-protective effect against virulent S. typhi-murium of inactivated vaccines prepared from enteric bacteria bearing various O antigens (Fig. 6). Mouse groups were immunized with inactivated vaccines prepared from *S. java*, *S. cholerae suis* and *V. cholerae*. The immunizing dose contained 10^5 or 10^8 cells. The 10^8 dose of the *S. java* vaccine proved to be effective: the viable cell

Table I

O-antigen content of killed whole-cell vaccines and of ribosomal preparations

Preparation	Strain	Agglutination titres of pooled sera	Passive HA* titres of immunized mice	Passive HI+ titres
Phenol-killed vaccines	LT2-MI O^+	1/128	1/128	1/512
	LT2-MI O^-	0	0	0
Heat-killed vaccines	LT2-MI O^+	1/512	1/256	n.t.
	LT2-MI O^-	1/4	0	0
Ribosomal antigens	LT2-MI O^+	1/16	1/8	0
	LT2-MI O^-	0	0	0

* Haemagglutination
\+ Haemagglutination-inhibition
n.t. = Not tested

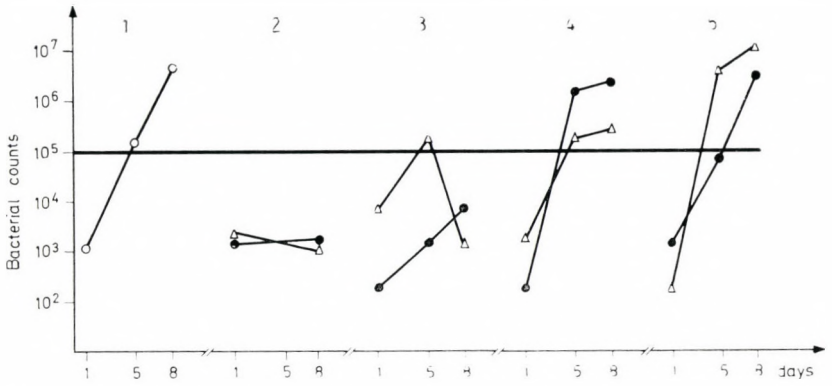

Fig. 6. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with various vaccines. Bacterial counts in the liver, days after infection. 1 = Control; 2 = *S. typhi-murium*; 3 = *S. java*; 4 = *S. cholerae-suis*; 5 = *V. cholerae*. Immunizing dose, cells/mouse • ——— • 10^8; △ ——— △ 10^5

count for livers did not exceed 10^4 in the 8-day period after challenge. The protection by the 10^5 cell/dose of the same vaccine was equivocal. In the same experiment, the *S. cholerae suis* and the *V. cholerae* vaccines showed no protective effect. It should be noted that the O antigen of *S. java* is identical with, whereas the O antigens of the other two strains are different from, the O antigen of *S. typhi-murium*.

Mouse-protective effect of endotoxin preparations (Fig. 7). The endotoxins used in these experiments were prepared from the virulent strain *S. typhi-murium* No. 10040 and from the O^+ and O^- variants of the LT2-MI strain. The immunizing dose uniformly corresponded to 5 μg dry matter. The liver

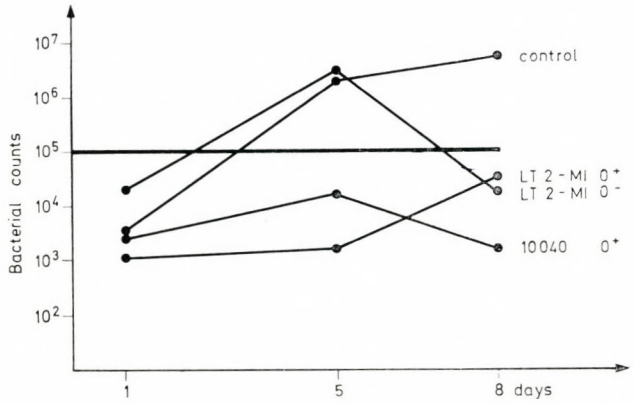

Fig. 7. Multiplication of virulent *S. typhi-murium* in non-immunized mice and in mice immunized with endotoxins from various *S. typhi-murium* strains. Bacterial counts in the liver, days after infection. Immunizing dose, 5 μg/mouse

viable cell count of the challenge strain remained below 10^5 during the whole observation period in the mice that had been immunized with the Boivin extract prepared from the O^+ strains, whereas the mice that had been pretreated with the endotoxin prepared from the O^- strain showed no appreciable protection, compared to the non-immunized control mice.

Discussion

The mouse-protective effect of preparations of *S. typhi-murium* origin is usually examined by challenging immunized and untreated animals with a virulent strain and comparing the survival rates after a 30-day observation period [8, 10, 11]. Some authors, however, examine the inhibition of the multiplication of the challenge bacterium in the organs of pretreated and non-immunized animals, usually 5 days after challenge [13, 19, 20]. ROBSON and VAS [21], using 10^6 viable *S. typhi-murium* cells for challenge, made quantitative re-isolation experiments after various intervals.

We, unlike the cited authors, used very low challenge doses (2–5 LD_{50}). Thus, non-immunized animals began to die 8–10 days after challenge. During this period, re-isolation can be attempted several times and the results may provide information on the immunogenicity of the preparation under study. Of course, the data thus obtained are valid only for the 10-day period after challenge unlike the survival data, which are related to the 30-day period.

Our observation suggest that the O antigen plays a decisive role in protecting mice against *S. typhi-murium*. The whole-cell vaccines, the RA extracts and the endotoxin preparations originating from an O^- strain of the bacterium failed to protect or did so in a much lower degree than did the corresponding O^+ preparations. Furthermore, of the *Salmonella* strains other than *S. typhi-murium*, only those bearing an O antigen identical with the *S. typhi-murium* O antigen proved to be protective.

Taking into account that very high doses of O^- preparations were found to be protective, it cannot be excluded that some bacterial components other than the O antigen may also protect animals.

REFERENCES

1. HORNICK, R. B., GREISMAN, S. E., WOODWARD, T. E., DUPONT, H. L., DAWKINS, A. T., SNYDER, M. J.: New Engl. J. Med. **283,** 686 (1970).
2. COLLINS, F. M., CARTER, P. B.: Infect. Immun. **6,** 451 (1972).
3. GERMANIER, R.: Infect. Immun. **5,** 792 (1972).
4. WESTPHAL, O., LÜDERITZ, O., BISTER, F.: Z. Naturforsch. **7b,** 148 (1952).
5. Polish Typhoid Committee: Bull. Wld. Hlth Org. **32,** 15 (1965).
6. YOUMANS, A. S., YOUMANS, G. P.: J. Bact. **91,** 2139 (1966).
7. WINSTON, S. H., BERRY, L. J.: J. RES **8,** 13 (1970).

8. VENNEMAN, M. R., BIGLEY, N. J.: J. Bact. **100,** 140 (1969).
9. VENNEMAN, M. R., BIGLEY, N. J., BERRY, L. J.: Infect. Immun. **1,** 574 (1970).
10. SMITH, R. A., BIGLEY, N. J.: Infect. Immun. **6,** 337 (1972).
11. JOHNSON, W.: Infect. Immun. **8,** 395 (1973).
12. HOUCHENS, D. P., WRIGHT, G. L.: Infect. Immun. **7,** 507 (1973).
13. EISENSTEIN, T. K.: Infect. Immun. **12,** 364 (1975).
14. LIN, J. H., BERRY, L. J.: J. RES **23,** 135 (1978).
15. YOUMANS, A. S., YOUMANS, G. P.: J. Bact. **99,** 42 (1969).
16. BOIVIN, A., MESROBEANU, I., MESROBEANU, L.: C. R. Soc. Biol. (Paris) **114,** 307 (1933).
17. MILLER, E. M., GOEBEL, W. F.: J. exp. Med. **90,** 255 (1949).
18. RAUSS, K., KÉTYI, I.: Schweiz. Z. Path. Bakt. **22,** 20 (1959).
19. VENNEMAN, M. R., BERRY, L. J.: Infect. Immun. **4,** 374 (1971).
20. VENNEMAN, M. R., BERRY, L. J.: Infect. Immun. **4,** 381 (1971).
21. ROBSON, H G., VAS, S I.: J. infect. Dis. **126,** 378 (1972).

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ISOLATION AND CHARACTERIZATION OF *MICROMONOSPORA HEVIZIENSIS* SP. NOV.

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Morphological, cultural and physiological description of a proposed new species, isolated from the bottom mud of the thermal lake Hévíz (West Hungary) and designated as *Micromonospora heviziensis* sp. nov., is given. *M. heviziensis* (type strain: No. II-176) is a characteristic and common member of the bacterial community in the curative mud of this famous watering place.

Studying the species composition of the bacterial population of the curative bottom mud of the thermal lake Hévíz in West Hungary, we have separated from among our isolated and selected representative *Micromonospora* strains five completely identical ones. The diagnostic properties of these clearly show that they belong to a new species, characteristically occurring in the curative mud of this well-known watering place. Description of the type strain and the taxonomic position of the new species, designated as *Micromonospora heviziensis* sp. nov., will be given.

Materials and methods

Isolation. The type strain II-176 and other representatives of *M. heviziensis* were isolated from bottom mud samples obtained from lake Hévíz about 20 cm under the mud surface where the water depth was about 3 m [1]. Dilution plates prepared with nutrient agar, starch nutrient agar and thiamine–sodium propionate agar [2] media were used for isolation. The plates were incubated at 28 °C.

Cultivation. The isolated strains were routinely cultured at 30 °C on the following medium: yeast extract, 1%; soluble starch, 2%; agar, 1.5% [3]. Cultures were transferred every two weeks.

Morphological–cultural properties. These were observed using the following media: yeast extract–malt extract agar (ISP Med. 2), oatmeal agar (ISP Med. 3), inorganic salts–starch agar (ISP Med. 4), peptone–yeast extract–iron agar (ISP Med. 6), tyrosine agar (ISP Med. 7) [4], all from Difco; Czapek agar and Emerson agar [5].

Micromorphology. Microscopic morphology of the mycelium was studied in yeast extract–starch liquid medium, after 10 and 21 days incubation. Samples were examined either without preparation under phase contrast illumination or fixed on slides and stained with carbolfuchsin. Electron microscopic observations were done at $\times 8000$.

Carbon source utilization. Growth with different carbon sources was determined using the basal medium proposed by LUEDEMANN [3]: yeast extract, 0.5%; $CaCO_3$, 0.1%; agar 1.5%. An aqueous solution of the carbon source, sterilized by filtration, was added to the basal medium to a final concentration of 1%, except for dextrin, inulin, salicin, dulcitol and i-inositol which were heat sterilized, and glycerol that was tested at 2% concentration.

Utilization of sodium salts of organic acids was tested in the following basal medium [6]: NaCl, 0.1%; $MgSO_4 \cdot 7\ H_2O$, 0.02%; $(NH_4)_2HPO_4$, 0.1%; K_2HPO_4, 0.05%; agar, 1.5%. As an indicator, 20 ml of a 0.04% solution of phenol red were added to one litre of the medium.

Salts of organic acids were added to a final concentration of 0.2%. The colour change in the medium and the intensity of growth were observed after 21–28 days incubation.

Cellulose decomposition was observed in tubes containing 5 ml of Luedemann's liquid basal medium, with a filter paper strip. After 1 month incubation at 30 °C, destruction of the paper was detected.

Growth on potato plugs. The method described by LUEDEMANN [3], with and without $CaCO_3$ on the potato plugs, was used.

Milk digestion. The zone of hydrolysis was measured after 21 days incubation on the following medium: yeast extract, 0.5%; glucose, 1.0%; $CaCO_3$, 0.2%; skim milk, 1.0%; agar, 2.0% [3].

Gelatin decomposition. Nutrient agar with 0.4% of gelatin was used and hydrolysis was tested with $HgCl_2$ one week after inoculation.

Hydrolysis of nucleic acids. The method proposed by JEFFRIES *et al.* [7] was employed.

Tributyrin utilization was detected in the following medium: glucose, 1%; yeast extract, 1%; glycerol tributyrate, 1%; agar, 1.5%, pH 7.0.

Aesculin hydrolysis. Aesculin medium (aesculin, 0.1%; ferric citrate, 0.05%; peptone, 1%; NaCl, 0.5%) was examined for blackening four weeks after inoculation [8].

Hippurate hydrolysis. Cultures were inoculated in hippurate broth [9]: tryptone, 1.0%; beef extract, 0.3%; yeast extract, 0.1%; glucose, 0.1%; Na_2HPO_4, 0.5%; sodium hippurate, 1.0%. After 6 weeks incubation, 1 ml of each culture was mixed with 1.5 ml of 50% (v/v) sulphuric acid. The appearance of finely divided crystals in the acid mixture after 4 h at room temperature indicated the presence of benzoic acid.

Decomposition of allantoin [10]. Allantoin was added to Rustigan and Stuart's broth (KH_2PO_4, 0.91%; Na_2HPO_4, 0.95%; yeast extract, 0.01%; phenol red, 0.001%) to a final concentration of 3.4% (w/v). The medium was then tubed, autoclaved, inoculated and incubated for 4 weeks. A shift to alkaline indicated the decomposition of allantoin.

Tellurite reduction. A method proposed by KURUP and SCHMITT [10] and modified by us was employed.

Nitrate reduction was tested in Difco nitrate broth and in glucose–nitrate broth [3] (yeast extract, 0.5%; glucose, 1.0%; KNO_3, 0.5%; $CaCO_3$, 0.1%) after 14 days incubation.

Utilization of amino acids as carbon and nitrogen sources. The following basal medium was used: yeast extract, 0.1%; $CaCO_3$, 0.1%; agar, 1.5%. All amino acids were added to a final concentration of 280 mg/l of nitrogen [11].

Utilization of amino acids and some inorganic N-compounds as sources of nitrogen. The basal medium employed was: glucose, 1.0%; yeast extract, 0.1%; $CaCO_3$, 0.1%; agar, 1.5%. In all cases the initial pH value of the medium was checked to be 7.0. N-compounds were added to a final concentration of 280 mg/l of nitrogen [11].

Temperature range of growth. Strains were cultivated on yeast extract–starch agar medium at 28, 30, 37 and 40 °C for 10 days.

Salt tolerance. NaCl was added to yeast extract–starch agar to final concentrations of 1.5%, 3%, 4.5% and 5%. Incubation lasted for two weeks [3].

Growth at different pH values. Liquid yeast extract–starch medium after heat sterilization was adjusted to pH 4.5, 5.0, 6.0, 7.0 and 8.0. Observations for growth were done two weeks after inoculation.

Antagonistic activity. Nutrient agar plates were inoculated with the *Micromonospora* strains to be tested for antibiotic activity in the form of a streak and after one week of incubation at 30 °C the sensitive bacteria (*Bacillus cereus* CCM 2010 and *Escherichia coli* DSM 30038) were inoculated perpendicularly to the line of actinomycete growth. For detecting antifungal activity, the one-week-old plate cultures of the antagonists were superinfected with a spore suspension of *Aspergillus flavus* F 108 and incubated again.

Sensitivity to antibiotics. Nutrient agar plates were seeded with 2 weeks old *Micromonospora* cultures, then sensitivity disks (Human, Budapest) were placed on the freshly inoculated plates. After 2 weeks incubation the radius of inhibition zones was measured.

Results and discussion

Description of the holotype strain of Micromonospora heviziensis sp. nov.

Name. Micromonospora heviziensis (Hévíz is a thermal lake and a health-resort in West Hungary; Hév = hot; víz = water; heviziensis = belonging to

Hévíz, Hungary; the source of the mud, from which the organism was isolated).

Type strain. No. II-176 (deposited in the Culture Collection of the Department of Microbiology of Eötvös L. University and in the ATCC).

Growth on different diagnostic media. Yeast extract–malt extract agar (ISP Med. 2): good growth, colonies usually strongly folded (with a lichenoid appearance), orange to orange-red. Weak production of a yellow soluble pigment. No brown superficial layer or any other coloured layer of spores may be observed.

Oatmeal agar (ISP Med. 3): poor growth, orange coloured colonies. No visible sporulation.

Inorganic salts-starch agar (ISP Med. 4): poor growth, orange to yellow-orange colonies. Absence of sporulation.

Peptone–yeast extract–iron agar (ISP Med. 6): poor growth, no sporulation. No production of melanoid pigment.

Tyrosine agar (ISP Med. 7): poor growth, no sporulation, no production of melanoid pigment.

Czapek agar: poor to fair growth, orange or pale orange colonies, no sporulation.

Emerson agar: good growth, lichenoid, orange to red coloured colonies.

Micromorphology. Spores randomly produced on the branching hyphal filaments. They are ellipsoidal to spherical, 0.8–1.0 μm in diameter and smooth walled. Spores occur singly, sessile or at the end of short branches. Sometimes chlamydospores or globose bodies may be found at the end of the filaments. A predominantly monopodial system of branching is characteristic of the sporulating hyphae.

Physiological–biochemical properties. Cellulose hydrolysis: fast growth on filter paper and rapid decomposition of cellulose. Carbon source utilization: good growth and positive utilization of D-glucose, L-arabinose, D-xylose, L-rhamnose, D-fructose, D-galactose, mannose, cellobiose, β-lactose, maltose, α-melibiose, sucrose, trehalose, L-melezitose, raffinose, dextrin, D-mannitol and i-inositol. Growth with sorbose, inulin, salicin, adonitol, dulcitol, glycerol and D-sorbitol was not better than on the negative control medium. Utilization of sodium salts of organic acids: good growth on acetate and pyruvate. No growth or only in traces on citrate, oxalate and benzoate. Growth on potato plugs: good growth on potato slices with and without calcium carbonate. Milk digestion: positive. The zone of hydrolysis is variable. Gelatin decomposition: good growth on nutrient agar plus gelatin. After 15 days incubation the radius of the zone of hydrolysis measures about 7 mm. No hydrolysis of nucleic acids. Tributyrin utilization: positive. Aesculin hydrolysis: strong. Hydrolysis of hippurate: negative. Decomposition of allantoin: negative. Tellurite reduction: positive. Nitrate reduction: negative. Utilization of amino acids as carbon

and nitrogen sources: DL-phenylalanine is utilized well. Growth with L-arginine is doubtful. No utilization of DL-alanine, L-glutamic acid, L-asparagine, L-histidine and L-cystine. Nitrogen source utilization: good growth with DL-alanine and L-asparagine. Doubtful or weak growth with L-lysine, L-arginine and L-cystine. No utilization of DL-phenylalanine, L-tryptophan, L-glutamic acid, L-histidine, ammonium sulphate, sodium nitrate and potassium nitrate. Good growth at 30 °C, no growth at 37 °C. Salt tolerance: good growth at 1.5% NaCl. No growth at 3% NaCl. No growth at pH 4.5, good growth at pH 5.0, 6.0, 7.0 and 8.0. Antagonistic activity: no antibiotic activity against *Bacillus cereus* CCM 2010, *Escherichia coli* DSM 30038 and *Aspergillus flavus* F 108. Sensitivity to antibiotics: resistant to penicillin (3 IU), oxacillin (10 μg) and oxytetracycline (30 μg). Moderately sensitive to oleandomycin (30 μg), tetracycline (30 μg), neomycin (100 μg), polymyxin-B (15 μg), chlortetracycline (30 μg), colistin (20 μg), lincomycin (10 μg), pristinamycin (10 μg), paromomycin (50 μg) and gentamicin (20 μg). Sensitive to methicillin (20 μg), chloramphenicol (30 μg), streptomycin (30 μg), erythromycin (10 μg), vancomycin (50 μg), kanamycin (30 μg), ampicillin (20 μg) and cephalosporin (10 μg).

Systematic position of the new species Micromonospora heviziensis

Strain No. II-176 is a typical member of the genus *Micromonospora*. To clarify its systematic position among the recognized species of this genus, we have compared it directly with authentic or type strains of 19 *Micromonospora* species obtained from the ATCC. Only strain RIA 472 of *M. fusca* was received from Prof. NAVASHIN (Research Institute of Antibiotics, Moscow).

The results clearly showed that strain No. II-176 represents a characteristic new species within the genus *Micromonospora*, differing in many properties from all of the authentic representatives of the known species. Strain II-176 is the only single organism which is capable of utilizing L-melezitose, a carbon source, which proved to be unsuitable for growth of all the other *Micromonospora* species. Besides it differs

(1) from *M. chalcea* strain ATCC 12452, because this is L-rhamnose, D-mannitol and i-inositol negative, and salicin positive;

(2) from *M. purpurea* strain ATCC 15835, because this is L-rhamnose, D-fructose, D-galactose, β-lactose, α-melibiose, raffinose, D-mannitol and i-inositol negative;

(3) from *M. echinospora* subsp. *ferruginea* strain ATCC 15836 and *M. echinospora* ssp. *echinospora* strain ATCC 15837, because these are D-fructose, D-galactose, β-lactose, α-melibiose, raffinose, D-mannitol and i-inositol negative;

(4) from *M. inositola* strain ATCC 21773, because this is L-rhamnose and D-mannitol negative, and inulin and adonitol positive;

(5) from *M. olivoasterospora* strain ATCC 21819, because this is L-arabinose, L-rhamnose, β-lactose, α-melibiose, raffinose, D-mannitol and i-inositol negative, and salicin positive;

(6) from *M. sagamiensis* strain ATCC 21826, because this is L-rhamnose, β-lactose, α-melibiose, raffinose, D-mannitol and i-inositol negative;

(7) from *M. purpureochromogenes* strain ATCC 27007, because this is L-arabinose, L-rhamnose, trehalose, D-mannitol and i-inositol negative, and inulin and glycerol positive;

(8) from *M. coerulea* strain ATCC 27008, because this is L-arabinose, L-rhamnose and i-inositol negative, and inulin positive;

(9) from *M. aurantiaca* strain ATCC 27029, because this is L-rhamnose, trehalose, D-mannitol and i-inositol negative;

(10) from *M. lilacina* strain ATCC 27030, because this is adonitol and glycerol positive;

(11) from *M. rubra* strain ATCC 27031, because this is sucrose negative, and inulin, salicin, adonitol and glycerol positive;

(12) from *M. carbonacea* strain ATCC 27114, because this is L-rhamnose, raffinose, D-mannitol and i-inositol negative;

(13) from *M. narashino* strain ATCC 27331, because this is L-rhamnose, D-mannitol and i-inositol negative, and salicin positive;

(14) from *M. brunnea* strain ATCC 27334, because this is L-arabinose, L-rhamnose, trehalose, D-mannitol and i-inositol negative, and inulin and glycerol positive;

(15) from *M. parva* strain ATCC 27358, because this is L-rhamnose, raffinose, D-mannitol and i-inositol negative;

(16) from *M. halophytica* subsp. *halophytica* strain ATCC 27596, because this is L-rhamnose, raffinose, D-mannitol and i-inositol negative, and salicin positive;

(17) from *M. megalomicea* subsp. *nigra* strain ATCC 27598, because this is L-rhamnose, D-galactose, α-melibiose, raffinose, D-mannitol and i-inositol negative;

(18) from *M. inyoensis* strain ATCC 27600, because this is L-arabinose, L-rhamnose, cellobiose, β-lactose, α-melibiose, raffinose, D-mannitol and i-inositol negative;

(19) from *M. fusca* strain RIA 472, because this is L-rhamnose, mannose and i-inositol negative, and salicin positive.

JOHNSTON [12] grouped the species of the genus *Micromonospora* on the basis of their macromorphological–cultural properties into four large groups.

(1) On the surface of the orange coloured colonies develops a distinct, brown to black spores containing layer.

(2) A separate superficial spore layer is lacking. Consequently the colour of colonies is identical with that of the vegetative mycelia: orange.

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Table I

Carbon source utilization by the type strain Micromonospora heviziensis (II-176) *and by closely related Micromonospora species*

	D-Glucose	L-Arabinose	D-Xylose	L-Rhamnose	D-Fructose	D-Galactose	Mannose	Sorbose	Cellobiose	β-Lactose	Maltose	α-Melibiose	Sucrose	Trehalose	L-Melezitose	Raffinose	Dextrin	Inulin	Salicin	Adonitol	Dulcitol	Glycerol	D-Mannitol	D-Sorbitol	i-Inositol
M. inositola (ATCC 21773)	+	+	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	+
M. parva (ATCC 27358)	+	+	+	−	+	+	+	−	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−
M. aurantiaca (ATCC 27029)	+	+	+	−	+	+	+	−	+	+	+	+	+	±	−	+	+	−	−	−	−	−	−	−	−
M. heviziensis (II-176)	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+

\+ positive
− negative
± weak or negative

(3) The colour of the colonies is lilaceous or red either with or without a distinct superficial blackish or brownish spore layer.

(4) The colonies covered with a superficial blackish or brownish spore layer are green or blue coloured.

Strain II-176 belongs to Group No. 2 of JOHNSTON, in which *M. inositola* [13], *M. parva* [14, 15] and *M. aurantiaca* [14] are the representative species.

Tables I, II and III give a comparison between strain II-176 and the type strains of the latter species. As can be seen, strain II-176 differs from

Table II

Nitrogen source utilization by the type strain Micromonospora heviziensis (II-176) *and by closely related Micromonospora species*

	As N-source												As C- and N-sources						
	DL-Alanine	DL-Phenylalanine	L-Tryptophan	L-Glutamic acid	L-Lysine	L-Asparagine	L-Arginine	L-Histidine	L-Cystine	$(NH_4)_2SO_4$	$NaNO_3$	KNO_3	DL-Alanine	DL-Phenylalanine	L-Glutamic acid	L-Asparagine	L-Arginine	L-Histidine	L-Cystine
M. inositola (ATCC 21773)	±	0	−	−	±	+	±	±	±	−	−	−	−	±	0	−	−	0	0
M. parva (ATCC 27358)	−	−	−	0	−	−	−	−	−	0	−	−	−	+	−	−	−	−	0
M. aurantiaca (ATCC 27029)	−	−	−	0	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−
M. heviziensis (II-176)	+	−	−	−	±	+	±	−	±	−	−	−	−	+	−	−	±	−	0

\+ positive
± weak or negative
− negative
0 total inhibition

M. inositola ATCC 21773, *M. parva* ATCC 27358 and *M. aurantiaca* ATCC 27029 not only in carbon utilization but also in the ability to grow with different N sources (DL-alanine, DL-phenylalanine, L-asparagine, L-lysine and L-histidine). Further differences are found in the antibiotic sensitivity spectra.

On the basis of the above data, strain II-176 is considered a representative of a new species of the genus *Micromonospora*. We designate and introduce it as the holotype strain of *M. heviziensis* sp. nov., which is a common inhabitant of the bottom mud of a low temperature (about 27 °C throughout winter) thermal lake. On the ecology of the new species and on the actinomycete community of the bottom mud of lake Hévíz we shall report elsewhere.

Table III

Antibiotic sensitivity of the type strain of Micromonospora heviziensis (II-176) *and of closely related Micromonospora species*

	Penicillin (3 IU)	Oxacillin (10 μg)	Methicillin (20 μg)	Chloramphenicol (30 μg)	Oleandomycin (30 μg)	Streptomycin (30 μg)	Tetracycline (30 μg)	Neomycin (100 μg)	Polymyxin-B (15 μg)	Erythromycin (10 μg)	Chlortetracycline (30 μg)	Oxytetracycline (30 μg)	Vancomycin (50 μg)	Kanamycin (30 μg)	Ampicillin (20 μg)	Colistin (20 μg)	Lincomycin (10 μg)	Cephalosporin (10 μg)	Pristinamycin (10 μg)	Paromomycin (50 μg)	Gentamicin (20 μg)
M. inositola (ATCC 21773)	37	17	—	29	39	17	39	23	11	ND	41	47	39	37	27	13	27	37	41	29	11
M. parva (ATCC 27358)	—	—	—	15	—	18	12	8	2	3	17	17	14	15	11	3	—	11	5	12	5
M. aurantiaca (ATCC 27029)	—	4	—	11	2	7	12	7	1	5	16	13	15	5	14	—	—	—	3	8	2
M. heviziensis (II-176)	—	—	10	18	8	14	2	2	1	14	3	—	15	15	19	2	3	13	5	7	3

Zones of inhibition in mm
ND = not determined
— = no inhibition

REFERENCES

1. FERNÁNDEZ, C.: Estudios en las Comunidades de Actinomicetos de Diferentes Ecosistemas Thesis. L. Eötvös University, Budapest 1980.
2. ROWBOTHAM, T. J., CROSS, T.: J. gen. Microbiol. **100,** 231 (1977).
3. LUEDEMANN, G. M.: Int. J. Syst. Bact. **21,** 240 (1971).
4. SHIRLING, E. B., GOTTLIEB, D.: Int. J. Syst. Bact. **16,** 313 (1966).
5. WAKSMAN, S. A.: The Actinomycetes. I–II. Williams and Wilkins Co., Baltimore 1961.
6. GORDON, R. E.: In GRAY, T. R. G., PARKINSON, D. (eds): The Ecology of Soil Bacteria. Univ. Press, Liverpool 1968. pp. 293–321.
7. JEFFRIES, C. D., HOLTMAN, D. F., GUSE, D. G.: J. Bact. **73,** 590 (1957).
8. COWAN, S. T., STEEL, K. J.: Manual for the Identification of Medical Bacteria. Univ. Press, Cambridge 1965.
9. GORDON, R. E., HORAN, A. C.: J. gen. Microbiol. **50,** 223 (1968).
10. KURUP, P. V., SCHMITT, J. A.: Canad. J. Microbiol. **19,** 1035 (1973).
11. SZABÓ, I. M.: Microbial Communities in a Forest-Rendzina Ecosystem. Akadémiai Kiadó, Budapest 1974.
12. JOHNSTON, D. W.: Actinomycetes in Aquatic Habitats. Thesis. Univ. Bradford 1972.
13. KAWAMOTO, I., OKACHI, R., KATO, H., YAMAMOTO, S., TAKAHASHI, I., TAKASAWA, S., NARA, T.: J. Antibiot. **27,** 493 (1974).
14. SVESHNIKOVA, M., MAXIMOVA, T., KUDRINA, E.: In PRAUSER, H. (ed.): The Actinomycetales. Gustav Fischer Verlag, Jena 1970. pp. 187–197.
15. Bergey's Manual of Determinative Bacteriology. 8th ed. Williams and Wilkins Co., Baltimore 1974.

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IMMUNOLOGICAL APPROACH TO THE PATHOGENETICAL ROLE OF *ESCHERICHIA COLI* ADHESIVE FACTOR "119" IN A SUCKLING MOUSE URINARY TRACT MODEL*

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In the course of urinary tract infections, suckling mice with maternal anti-pilus ("119") immunity showed a massive protection against a 119$^+$ strain of *Escherichia coli*. Animals could be protected against urinary tract infection by giving pilus antibody or pilus vaccine shortly after the infection. Results showed the importance of adhesive pili in initiating the urinary tract infection by *E. coli*.

A mannose resistant pilus with the capacity to agglutinate human erythrocytes provisionally designated "119" [1] was carried by about 40% of *Escherichia coli* strains isolated from human urinary tract infections in our material. The type strain No. 119 was analysed by ØRSKOV and ØRSKOV [2], who concluded that the strain carries two mannose resistant haemagglutinins: one is identical with F8, the other with the provisionally designated pseudotype 1. The strain No. 119 shows a mannose resistant adhesion to human uroepithelial cells [3]. For studying adhesion factor(s) and other virulence elements, an animal model was elaborated [4]. The translucent urinary bladder of 3-day-old mice is inoculated with 10^2–10^5 germs. With virulent *E. coli* strains, bladder and kidney infection is frequent and when the urinary tract infection lasts for 3 weeks, morphological changes of the kidneys and mainly their contraction can be seen.

Testing 119$^+$ and 119$^-$ pairs of strains [4] the piliated strain showed a higher rate of infection in bladder and kidney, with a higher number (3–4 log 10 exponent) of bacteria, than in the 119$^-$ ones.

A further approach to the role of adhesive pili in the infection process may be immunological. The results of such experiments are summarized in this paper.

Materials and methods

Strains. Pili were prepared from an *E. coli* strain O4 : K12 : H5 : 119. The identity of 119-complexes was proved by antigen adsorption. The O,H,K unrelated infecting agent was the type strain No. 119 (O18a,c : K5 : H— : 119) of *E. coli*.

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Serum. Anti-119 serum was prepared in adult mice immunized subcutaneously by the first dose and intraperitoneally by purified 119-pili 5 times in 4 day intervals. One week after the last dose, the animals were bled, their serum was collected, Seitz-filtered, and preserved by 0.01% merthiolate.

The serum titre was determined by haemagglutination inhibition. Serum dilutions were mixed with 119+ bacteria and thereafter with human red blood cells. The titre of the mouse serum was 1 : 512.

Pilus preparation was done according to STIRM and ØRSKOV [5] and EVANS *et al.* [6]. After cultivating the strain in Roux bottles at 37 °C for 24 h bacteria were harvested in PBS pH 7.2 containing NaN_3 (0.01%). After blendor homogenization at 0 °C for 30 min, the bacteria were removed by centrifugation (5000 *g*/min at 4 °C for 1 h). The supernatant was allowed to stand at 4 °C for 72 h. Repeated centrifugation was done at 20 000 *g*/min for 30 min at 4 °C and the supernatant was acidified with concentrated acetic acid to pH 3.5 and incubated overnight at 4 °C. The precipitate was collected, redissolved in PBS and the whole procedure was repeated three times. After the last precipitation the pellet was washed three times in 0.05 M acetate buffer pH 4.0, redissolved in 0.05 M Tris–HCl buffer pH 7.6, and stored at 4 °C.

Maternal immunity. Pregnant mice were immunized s.c. with purified pili. Females were given 0.5–0.5 ml of purified pili in 5 doses at 3 day intervals. The first 2–3 doses were inoculated before, the others after delivery.

Passive immunization. After bladder infection, suckling mice were given s.c. 0.05, 0.1, 0.1, 0.1 and 0.2 ml anti-pilus mouse serum at two day intervals.

Active immunization. Similarly to passive immunization, suckling mice were given purified undiluted pili using 0.05, 0.1, 0.3, 0.2 and 0.2 ml.

Bladder infection and bacteriological evaluation. As described earlier [4], 0.025 ml of about 10^3 germs was inoculated with a special fine cannula (No. 22) through the abdominal wall into the bladder of 3-day-old mice. Inoculation was checked by staining the inoculum with 0.05% Pontamine Sky Blue (6XB SEARLE, England).

Sections were prepared under sterile conditions. Infection of the removed bladder and the kidney was studied qualitatively and quantitatively using vortex-type treatment. The gross changes of kidneys were registered and all the animals were tested for bacteriaemia. Occasionally, bacteriaemic animals were excluded from the experiments.

Significance at 5% limit was estimated by the χ^2 test.

Results

Protective effect of maternal immunity. Females were massively immunized and their litter was infected in the bladder. Litters of the same age of non-immunized females served as controls. The protective effect of maternal immunity was evaluated at 14 and 23 days after urinary infection. In previous experiments [4] a slow spontaneous recovery from the urinary infection occurred, with protracted chronic pyelonephritis. Results are summarized in Table I.

It is seen in Table I that a significant protective effect was provided by maternal immunity. The incidence of bladder and kidney infection was lower and less germs (by 2–3 log 10 exponent) could be detected in the "immune" litters. The protection was more pronounced in the late period of infection: only one out of 8 mice showed infection of the lower part of the urinary tract; the germ count was only 10^3/ml. Control litters showed a high rate of bladder and kidney infection, with germ counts between 10^5 and 10^6/ml. In four cases, contracted kidneys were found (Table I).

Effect of passive anti-pilus (119) immunization. Three days old suckling mice were inoculated with *E. coli* No. 119. After infection, litters were pas-

Table I

*Effect of maternal anti-pilus (119) immunity on urinary tract infection in the suckling mouse model**

Organs investigated	Infection rate (mean log germ count)				χ^2 test between "immune" and control
	14 days after infection		23 days after infection		
	"immune" litters	control litters	"immune" litters	control litters	
Urinary bladder	2/13 (5.1)	10/14 (7.2)	1/8 (3.1)	12/18 (5.7)	11.452 $P < 0.001$
Kidneys	2/13 (4.2)	8/14 (7.2)	0/8 (.)	8/18 (5.8)	7.510 $0.01 > P > 0.001$
Contracted kidneys			0/8	4/18	

* Females were immunized before and after delivery with purified 119-pili prepared from an O, K, H unrelated strain of *E. coli* 119+. Challenge through the urinary bladder was carried out by about 10^3 germs of *E. coli* No. 119 in 3 days old mice

sively immunized with mouse anti-199 serum diluted 1 : 5, 1 : 25, and 1 : 125, with total serum quantities of 0.12, 0.024 and 0.0048 ml, respectively. Age matched non-immunized litters served as controls. The immune litters were divided into two groups. (1) Passive immunization was done early, 3–4 days after the infection. (2) Anti-pilus serum was given 7–14 days after bladder inoculation. Results are presented in Table II.

Table II

*Effect of passive immunization by anti-pilus (119) mouse serum on urinary tract infection in the suckling mouse model**

Interval between infection and immunization	Serum dose, ml (total)	Incidence of infection (mean log germ count)	
		Bladder	Kidneys
3–4 days	0.12	2/16 (4.1)	0/16 (.)
	0.024	2/12 (5.2)	0/12 (.)
	0.0048	4/10 (8.1)	0/10 (.)
Control	—	16/20 (8.1)	10/20 (7.6)
7–14 days	0.12	6/14 (7.2)	6/14 (3.1)
	0.024	8/10 (7.5)	8/10 (6.1)
	0.0048	8/12 (7.7)	7/12 (5.2)
Control	—	8/12 (7.8)	6/12 (4.1)

* The mouse anti-serum prepared from purified 119-pili of an O, K, H unrelated strain was given 5 times in dilutions 1 : 5, 1 : 25, and 1 : 125 s.c. into infected suckling mice. Bladder infection was performed on the 3rd day of life with *E. coli* No. 119 (about 10^3 germs). Sections were made 21–24 days after infection

It is seen that antiserum offerred protection only when it was given 3–4 days after infection. When it was administered later, during the course of the infection, it gave no protection (Table II).

Effect of purified 119-pilus vaccine. For active immunization, mice were divided into 3 groups. (1) Vaccination was done 2–4 days, (2) 4–12 days, and (3) 12–19 days, after bladder infection. Non-immunized suckling mice of the same age served as controls (Table III).

Table III

*Vaccination of suckling mice previously infected through their urinary bladder by purified 119-pili**

Organs investigated	Incidence of infection (mean log germ count)			
	Groups immunized on days after infection**			Control groups
	2–4	4–12	12–19	
Urinary bladder	4/18 (3.8)	5/9 (7.4)	8/10 (7.1)	17/27 (6.1)
Kidney	3/18 (4.8)	3/9 (6.1)	8/10 (4.8)	17/27 (5.7)
Contracted kidneys	0/18	0/9	2/10	4/27
$\varkappa^2$ analysis between vaccinated and control litters of mice	7.202 $P < 0.01$	0.155 $P > 0.5$	0.966 $P > 0.3$ bladder	
	9.375 $P < 0.01$	2.400 $P > 0.1$	0.966 > 0.3 kidney	

* Bladder infection was performed on the 3rd day of life, sections were made 14–19 days after infection and 4–8 days after the last dose of vaccine

** Purified 119-pilus vaccine given in different intervals after infection in 5 doses and 2 day intervals, s.c.

Vaccination gave results similar to those obtained after passive immunization. Early anti-pilus vaccination lowered the rate of bladder and kidney infection and to some extent the mean germ count of these infected organs. Vaccination carried out later, in the course of infection, was ineffective (Table III).

Discussion

Prevention of urinary infections by any kind of adhesive factor or other antigens and vaccines is of questionable value. Still, vaccines containing the adhesive factor K99 for the protection of calves, lambs [7, 8], or similar vaccines either with K99, or 987 adhesins [9] for suckling pigs, provide an effective level of maternal immunity. The inhibitory effect of K88 antigen given orally caused an early protection in the suckling mice model [10]. To assess the effectiveness of such prevention in the case of human urinary tract infections, e.g. in pregnant women at risk, more should be known about

adhesins taking part in this kind of infection and their exact role in the pathogenic process.

The present results have proved the importance of adhesin 119. It plays a role not only in lower urinary tract and bladder infection, but also in the development of pyelonephritis. Passive or active immunization performed in the course of infection is ineffective. This fact suggests — as expected — that adhesins are important virulence factors solely in initiating the pathological process, but subsequently the infection develops further.

The investigated pili, provisionally designated 119 are a mixture of two mannose resistant pili agglutinating only human red blood cells among human, bovine, guinea pig, and chicken cells. Separation of these pili has not been achieved, and so it is not known whether one or both of them are responsible for uroepithelial adhesion.

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REFERENCES

1. KUCH, B., PÁL, T., EMŐDY, L.: Acta microbiol. Acad. Sci. hung. **27,** 317 (1980).
2. ØRSKOV, I., ØRSKOV, F.: Personal communication.
3. KUCH, B., PÁL, T., EMŐDY, L.: Unpublished data.
4. KÉTYI, I.: Acta microbiol. Acad. Sci. hung. **28,** 393 (1981).
5. STIRM, S., ØRSKOV, F., ØRSKOV, I., MANSA, B.: J. Bact. **93,** 731 (1967).
6. EVANS, D. G., EVANS, D. J. Jr., TJOA, W. S., DUPONT, H. L.: Infect. Immun. **19,** 737 (1978).
7. ACRES, S. D., ISAACSON, R. E., BABIUK, L. A., KAPITANY, R. A.: Infect. Immun. **25,** 121 (1979).
8. SOJKA, W. J., WRAY, C., MORRIS, J. A.: J. med. Microbiol. **11,** 493 (1978).
9. MORGAN, R. L., ISAACSON, R. E., MOON, H. W., BRINTON, C. C., TO, C.-C.: Infect. Immun. **22,** 771 (1978).
10. DAVIDSON, J. N., HIRSH, D. C.: Infect. Immun. **12,** 134 (1975).

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PILUS ANTIGEN 987P PRODUCED BY STRAINS OF *ESCHERICHIA COLI* SEROTYPES O141 : K—, H- AND O8 : K85 : H—*

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Escherichia coli strains isolated from the alimentary tract of 68 weaned and 44 unweaned pigs with diarrhoea in various parts of Hungary, were tested for the presence of pilus antigens K88, K99 and 987P. K88 was detected in 30% of the strains from newborn pigs and in 12% of the strains isolated from weaned pigs. One strain carried K99. Based on agglutination test and immunoelectron microscopic studies with specific absorbed antisera, five non-haemolytic *E. coli* strains isolated from newborn pigs were found to produce so-called 987P pili. Three of these strains were designated serologically as O8 : K85 : H—,987P$^+$ and two as O141 : K— : H—,987P$^+$. The Y1 cell assay, the infant mouse assay, and the ligated intestinal loop assay in less than 3-week-old pigs indicated that none of the strains produced heat-labile enterotoxin but all produced a heat-stable enterotoxin detectable in infant mice and in pig loops (STa). All the strains induced diarrhoea in newborn, colostrum deprived pigs and colonized the lower small intestine by adhesion to the villous epithelium. The results have confirmed earlier findings about adhesive virulence attributes caused by 987P pili.

Pili (fimbriae) of certain enterotoxigenic *Escherichia coli* (ETEC) strains, defined for this paper as non-flagellar filamentous appendages of the bacterial surface ≤ 10 nm in diameter, are considered to be virulence factors [1–5] because they enable the bacteria to proliferate in the small intestine of man (CFA1, CFA2), pig (K88, 987P) and calf (K99).

The pilus antigen 987P has been described as a colonization factor of ETEC strains isolated from cases of diarrhoea in newborn pigs in the mid-western United States. These strains were nonhaemolytic and belonged to serogroups O9 and O20, that are not regarded as typical for porcine ETEC. Recently, MOON *et al.* [6] reported that several porcine ETEC strains belonging to serogroups O141, O101 and O149 produced the 987P antigen. O9 strains carrying this antigen were reported by SMITH and HUGGINS [7] from England and by GUINEE and JANSEN [8] from Holland. It therefore seemed worthwile to investigate the prevalence of the 987P antigen in another intensive pig rearing country like Hungary, where so far only the K88 antigen has been found on porcine ETEC strains [9].

* An essential part of the studies was performed in the Veterinary Institute, Szombathely, Hungary.

Materials and methods

Escherichia coli strains. In 1977, one hundred and twelve *E. coli* strains were isolated from the small intestine of 112 pigs with diarrhoea, 68 of which were weanlings and 44 were newborn (< 10 days old). In the same year they were sent in for further investigation from 6 different diagnostic laboratories representing all regions of Hungary. Subsequently all strains were tested for the presence of K88, K99 and 987P antigens by slide agglutination using absorbed antisera [10] and for haemolysin production on sheep blood agar. Strains carrying the 987P antigen were further tested for production of heat-stable [ST] enterotoxin using the infant mouse test, for heat-labile [LT] enterotoxin using the Y1 adrenal cell assay and for both toxins in ligated gut loop tests in pigs (< 3 weeks old) as described elsewhere [11–13]. *E. coli* 263 [O8 : K87,K88ab] was used as LT^+ control and *E. coli* 1261 [O138 : K81] as ST^+ control.

Intragastic inoculation of pigs. Strains found to be $987P^+$ were tested for colonizing ability by inoculating each strain orally into 6 newborn, caesarean derived colostrum deprived (CDCD) pigs as reported earlier [11, 14]. *E. coli* strains 987 (O9 : K103 : NM, $987P^+$ ST^+) and 124 (O8 : K50 : NM) were used as positive and negative control, respectively. Pigs were anaesthetized and killed 16 h after exposure. A 10 cm segment of the ileum was immediately removed for bacterial counts. Samples for frozen and paraffin embedded sections as well as for ultrathin sections were taken and processed as described previously [11, 14]. Frozen sections of the ileal segment of each pig were stained with fluorescein labelled monospecific anti 987P serum [10]. Weight loss during 16 h after exposure was also recorded.

Electron microscopy was performed on ultrathin sections from ileal segments of 8 pigs using a Philips 201c transmission electron microscope. Two pigs were inoculated with the negative control *(E. coli* 124) and two with the positive control strain (*E. coli* 987). Another four pigs were inoculated with two $987P^+$ isolates (Bp-S-68 and Sz-S-22). The pili were visualized by transmission electron microscopy using negative staining as described previously [15].

Immuno electron microscopy was performed on two $987P^+$ isolates as follows. Colonies grown on sheep blood agar [10] and agglutinable in anti 987P serum were suspended in 1 ml of phosphate buffered saline (PBS) and 0.05 ml of a 1 : 300 dilution of anti 987P serum was added. The mixture was incubated at 37 °C for 1 h and kept at 4 °C overnight. The same procedure was carried out with another 1 ml of the suspension, but with a 1 : 300 dilution of normal rabbit serum (NRS) instead of antiserum. Bacteria of both suspensions were sedimented at 100 *g* for 10 min, washed twice in distilled water and resuspended in 0.5 ml distilled water. Electron microscopy was performed as described earlier [10].

Serotyping. The O antigens were examined by routine procedures [16]. K antigen determination was carried out by agar electrophoresis [17] and by counter-current immunoelectrophoresis [18].

Results

Antigen K88 was detected in 13 of the 44 *E. coli* strains from newborn pigs (30%) and in 8 strains from 68 older pigs (12%). One strain carried K99. Five non-haemolytic strains isolated from newborn pigs carried 987P (11%). Results of serotyping and enterotoxin tests on these five strains are presented in Table I. Agglutination in anti 987P serum occurred when the strains were grown on Blood Agar Base (Difco) containing sheep red blood cells as described [10].

By electron microscopy, pili were seen on the bacteria of all the 987P agglutinable colonies. The pili were morphologically similar to those of the 987 strain [10], being approximately 7 nm in diameter. These agglutinating colonies were small and translucent. Larger, less translucent colonies were not agglutinable in anti 987P serum and no pili were seen by electron microscopy.

Table I

Serotype and enterotoxin production of 987P$^+$ ETEC strains and of a control LT producing E. coli strain

E. coli strains	Serotype	Enterotoxin production			
		pig-loop, netto/total	mouse, gut/body	Y-1 cell	character-istic toxin
Bp-S-68	O8 : K85 : H—, 987P$^+$	0.669	0.113	—	ST
Sz-S-20	O8 : K85 : H—, 987P$^+$	0.623	0.121	—	ST
Sz-S-22	O8 : K85 : H—, 987P$^+$	0.662	0.130	—	ST
Bp-S-56	O141 : K—, 987P$^+$	0.588	0.121	—	ST
Sz-S-24	O141 : K—, H—, 987P$^+$	0.540	0.112	—	ST
263	O8 : K87; K88 ab	0.604	0.060	+	LT

Immuno-electron microscopy revealed an electron dense layer on the pili of the bacteria to which the anti 987P serum had been added whereas no such layer was detected on the pili of the same bacterial population treated with

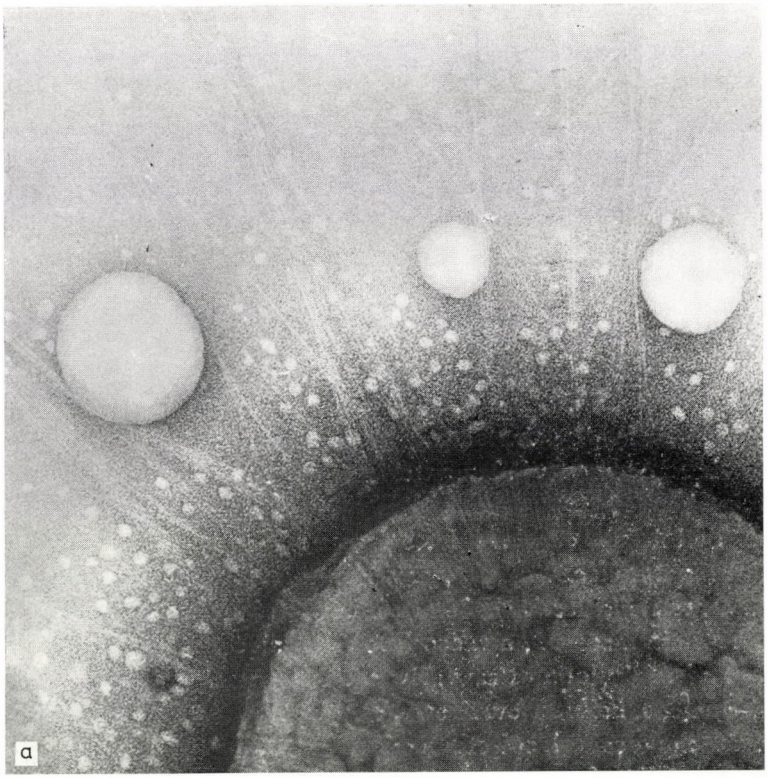

Fig. 1a. Pili of the *E. coli* strain Bp-S-68 (O141 : K— : H— : 987P$^+$) reacted with normal rabbit serum diluted 1 : 300. Approx. × 100 000

NRS, indicating a specific reaction of the 987P antibodies with the pili of the Hungarian 987P$^+$ bacteria (Fig. 1a and 1b).

All the 987P$^+$ strains colonized the small intestine of the CDCD pigs and induced mild to severe diarrhoea, very similarly to strain 987, while neither colonization nor diarrhoea was observed in pigs inoculated with the negative control strain. In fluorescein antibody stained frozen sections of the ileal segments from pigs inoculated with the 987P$^+$ strains the bacteria were seen in close association with the microvilli. These sections were therefore given high or moderate association indices (Table II). Electron microscopy of ultrathin sections from the ileum of the pigs inoculated with *E. coli* strains 987, Bp-S-68 and Sz-S-22 indicated that the bacteria formed a layer on the mucosal surface and that they were separated from the microvilli and from each other by an electron translucent region. In this region, numerous filamentous appendages were occasionally seen to connect the bacteria with the microvilli (Fig. 2).

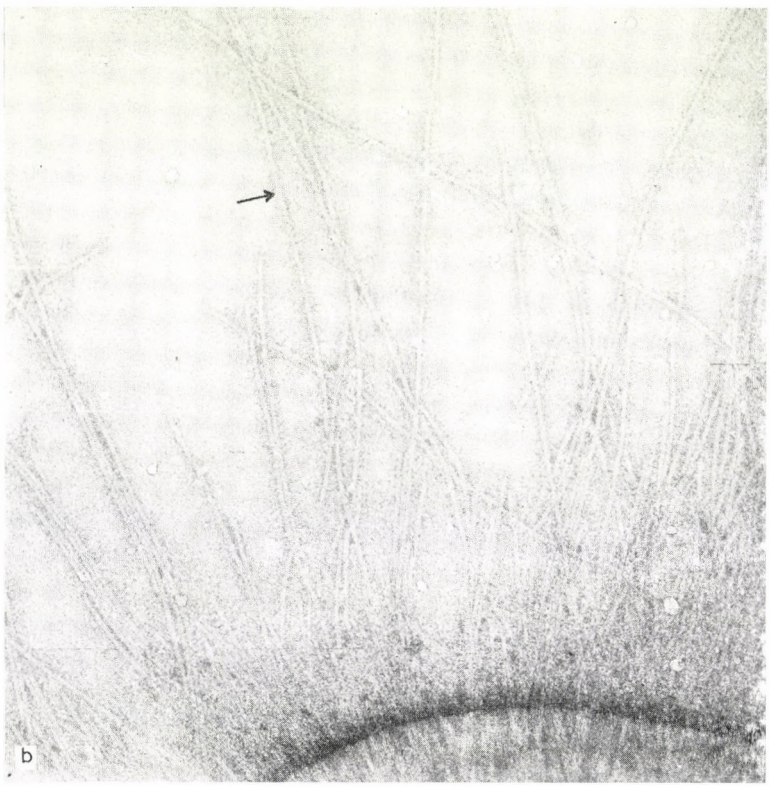

Fig. 1b. Pili of the *E. coli* strain Bp-S-68, reacted with anti 987P serum prepared in rabbits, diluted 1 : 300. A fine electron dense layer (arrow) can be detected on the surface of the pili, in contrast to Fig. 1a. Same magnification as with Fig. 1a

Table II

Results of intragastric inoculation of newborn pigs with 987P+ ETEC and with control strains

E. coli strains	No. of pigs. Diarrhoeal/ inoculated	Wt loss (per cent of initial body wt.)	Log_{10} of total viable *E. coli* of 10-cm ileal segments	Association index
Sz-S-20	5/6	12.4	9.85	3.3
Sz-S-22	6/6	19.3	9.24	5.0
Sz-S-24	2/6	7.2	9.20	4.5
Bp-S-56	6/6	15.1	9.67	3.0
Bp-S-68	4/6	19.7	9.57	3.7
987	5/5	16.3	9.94	3.8
124	0/6	3.3	6.50	1.1

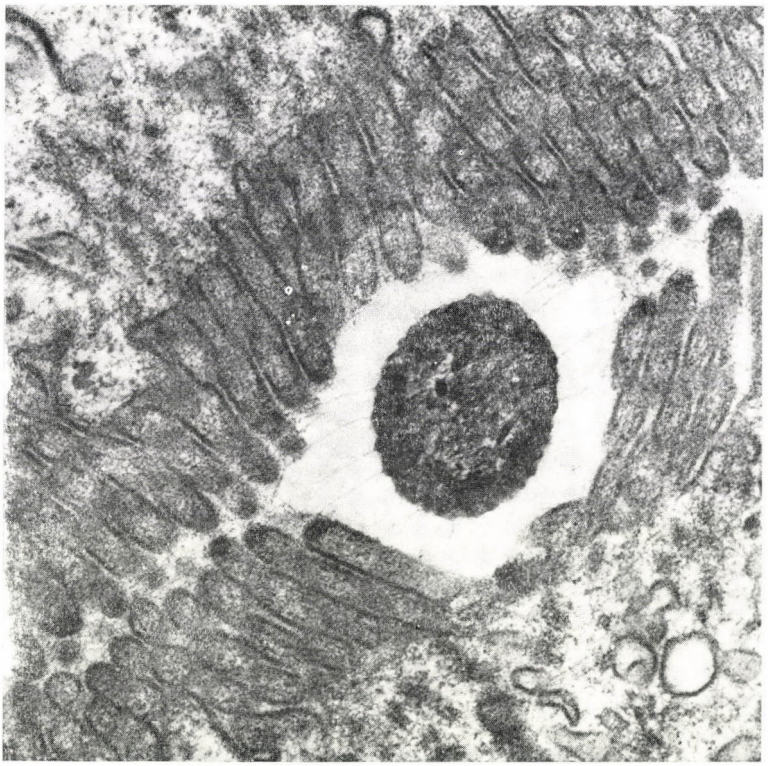

Fig. 2. A cell of the *E. coli* strain Bp-S-56 (O8 : K85 : 987P+) in the ileum of an orally infected CDCD pig 16 h post exposure, showing numerous filamentous appendages that connect the bacteria with the microvilli. Approx. × 43 000

Discussion

By means of electron microscopy, bacteria of non-haemolytic ETEC strains isolated from newborn pigs in Hungary and agglutinable in anti 987P serum were shown to be piliated. Immuno electron microscopy demonstrated that the anti 987P serum reacted specifically with the pili of the 987P$^+$ bacteria, and that these pili were morphologically similar to those of strain 987 [10]. The Hungarian 987P$^+$ strains were similar to the prototype strain 987 (O9 : K103, 987P$^+$ST$^+$) as regards type of enterotoxin produced and the absence of haemolytic activity. It is interesting to note that, so far, all 987P$^+$ strains have produced an ST toxin which is active not only in pigs but also in baby mice and therefore can be designated as STa [19] producers. Two of the 987P$^+$ Hungarian isolates (Table I) did not possess acidic polysaccharide K antigen. They were, however, potent STa producers possessing the adhesive pili 987P and were thus able to colonize the lower small intestine of the CDCD pigs and to produce diarrhoea in some of the pigs tested. One of these K-minus strains induced diarrhoea in only 2 out of 6 pigs. There was, however, a large amount of clear yellow fluid characteristic of ETEC diarrhoea in the small intestine of the 4 pigs that did not develop diarrhoea. Therefore our impression was that the strain produced another toxic substance and/or a low amount of STa. This hypothetical toxic substance possibly inhibited peristaltic movements of the small intestine and thus inhibited removal of the accumulated intestinal fluid and the development of diarrhoea. This hypothesis is supported by similar observations of METZ and OHGKE [20].

Immunofluorescent studies using absorbed anti 987P conjugate not only revealed adhesive abilities of these 987P$^+$ strains but also demonstrated — by specific fluorescence on the surface of the bacteria — the presence of 987P antigen in the small intestine of the CDCD pigs *in vivo*. This observation was further supported by electron microscopic studies that revealed fine structures between bacteria and the microvilli resembling those described by MOON *et al.* [21] for *E. coli* 987 and assumed to be pili or an aggregated form of pili.

Earlier studies [10] on 987P reported the presence of this pilus antigen on O9 and O20 strains isolated from newborn pigs. The present studies have demonstrated the 987P antigen on O8 and O141 strains which were also isolated from newborn pigs with diarrhoea. Porcine *E. coli* strains of serogroups O8 and O141 usually produce haemolysin, LT enterotoxin and the K88 antigen [22, 23]. The five strains studied here were found to be Hly$^-$, LT$^-$, K88$^-$ but STa$^+$ and 987P$^+$. Taking into account that antigen O141 is chemically the same as K85 [24], our O8 : K85 : 987P$^+$ STa$^+$ strains were probably similar to our O141 strains and to those reported by MOON *et al.* [6] to be O141 : 987P$^+$, STa$^+$STb$^-$LT$^-$.

Our results have confirmed and extended earlier findings about the pilus antigen 987P as an adhesive virulence factor; in this case in some O8 : K85 and O141 : K— strains. Our results also support earlier findings [14] by demonstrating that the acidic polysaccharide K antigen is not always essential for adhesion and colonization of 987P$^+$ strains. The K-minus strains, however, possessed antigen O141 which is an acidic lipopolysaccharide [24]. The studies also indicate that the number of serogroups incriminated in porcine neonatal diarrhoea is still limited and it seems that the association of certain serotypes and virulence attributes has once more been confirmed by this study.

Acknowledgements. *E. coli* strains 263, 1261, 124, 987 were kindly provided by Dr. H. W. Moon. Our thanks are due to Dr. I. Timar for providing the CDCD pigs, Dr. F. Dorner for the Y1 cells and LT standard, to Dr. E. Kudron for tissue culture work, to the colleagues in the veterinary institutes in Hungary for the field isolates of *E. coli*, and to Mrs Maria Bertha, Mrs Anna Varga and Mrs. Zsuzsa Szabó for valuable technical assistance.

REFERENCES

1. Evans, D. G., Silver, R. P., Evans, J. D., Chase, D. G., Gorbach, S. L.: Infect. Immun. **12,** 656 (1975).
2. Evans, D. G., Evans, D. J. Jr.: Infect. Immun. **21,** 638 (1978).
3. Moon, H. W., Nagy, B., Isaacson, R. E., Ørskov, I.: Infect. Immun. **15,** 614 (1977).
4. Ørskov, F., Ørskov, I., Smith, H. W., Sojka, W. J.: Acta path. microbiol. scand. Sect. B. **83,** 595 (1975).
5. Smith, H. W., Lingood, M. A.: J. med. Microbiol. **4,** 467 (1971).
6. Moon, H. W., Kohler, E. M., Schneider, R. A., Whipp, S. C.: Infect. Immun. **27,** 222 (1980).
7. Smith, H. W., Huggins, M. B.: J. med. Microbiol. **11,** 471 (1978).
8. Guinee, P. A. M., Jansen, W. H.: Zbl. Bakt. I. Abt. Orig. A. **243,** 245 (1979).
9. Semjén, G., Pesti, L.: Acta vet. Acad. Sci. hung. **23,** 219 (1973).
10. Nagy, B., Moon, H. W., Isaacson, R. E.: Infect. Immun. **16,** 344 (1977).
11. Bertschinger, H. U., Moon, H. W., Whipp, S. C.: Infect. Immun. **5,** 595 (1972).
12. Dean, A. G., Ching, Y. C., Williams, R. G., Harden, L. B.: J. infect. Dis. **125,** 407 (1972).
13. Donta, S. T., Moon, H. W., Whipp, S. C.: Science **183,** 334 (1974).
14. Nagy, B., Moon, H. W., Isaacson, R. E.: Infect. Immun. **13,** 1212 (1976).
15. Isaacson, R. E., Nagy, B., Moon, H. W.: J. infect. Dis. **135,** 531 (1977).
16. Ørskov, F., Ørskov, I.: Acta path. microbiol. scand. Sect. B. **83,** 595 (1975).
17. Ørskov, F.: Acta path. microbiol. scand. Sect. B. **84,** 319 (1976).
18. Semjén, G., Ørskov, I., Ørskov, F.: Acta path. microbiol. scand. Sect. B. **85,** 103 (1977).
19. Burgress, M. N., Bywater, R. J., Cowley, C. M., Mullan, N. A., Newsome, P. M.: Infect. Immun. **21,** 526 (1978).
20. Metz, H., Ohgke, H.: Zbl. Bakt. I. Abt. Orig. A. **235,** 53 (1976).
21. Moon, H. W., Nagy, B., Isaacson, R. E.: J. infect. Dis. **136** S 124 (1977).
22. Ørskov, F., Ørskov, I.: Fortschr. Vet. S **29,** 7 (1979).
23. Söderlind, O.: Zbl. vet. Med. B. **18,** 569 (1971).
24. Ørskov, I., Ørskov, F., Jann, B., Jann, K.: Bact. Rev. **41,** 667 (1977).

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Acta microbiol. Acad. Sci. hung. 29, 137—138, 1982

NOTE

AMIKACIN IN EXPERIMENTAL TUBERCULOSIS OF GUINEA PIGS

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(Received August 14, 1981)

Development of experimental tuberculosis of guinea pigs is inhibited by amikacin administered intramuscularly in daily doses of 7.5 mg/kg.

Amikacin (Biklin®, Bristol Myers), a new aminoglycoside derivative active mainly against *Pseudomonas aeruginosa* strains and *Enterobacteriaceae* was found to inhibit the growh of *Mycobacterium tuberculosis* strains in Šula medium [1]. As compared to the size of inoculum (10^5–10^6), the number of resistant mutants proved to be low (0–9) [2]. On the basis of the marked inhibitory action *in vitro*, the sensitivity of mycobacterial strains resistant to other antituberculotic drugs [1] and of the small number of resistant mutants in the population, it is suggested that amikacin might be an effective antituberculous agent. Thus, animal experiments seemed to be justified in this respect, especially in view of the fact that amikacin is less toxic than kanamycin

Materials and methods. Thirty male guinea pigs weighing 350–400 g each were inoculated subcutaneously over the sternum with 0.01 mg (moist weight) of the H37 RV strain of *Mycobacterium tuberculosis* cultured on Löwenstein–Jensen medium for two weeks. The animals were divided in three groups of 10 each as shown in Fig. 1. Guinea pigs of Group 1 had no treatment and served as control. In Group 2 and 3 treatment with amikacin was begun on the third day after infection. In Groups 2, amikacin was given in a single daily dose of 7.5 mg/kg in 0.5 ml solution into the inguinal region by the intramuscular route. Animals in Group 3, were given the same dose by the same route but twice daily at an interval of 6 h. The antibiotic was administered for six days in the first week and for five days in the following ones. Thus, the guinea pigs in Groups 2 and 3 had 37 days of treatment altogether. Fifty days after infection all animals were killed and a record was made of the extent of tuberculous disease seen at necropsy.

Results and discussion. Figure 1 shows the extent of tuberculous lesions observed in each animal at necropsy. The rectangle represents the liver and the oval represents the spleen. Dots indicate miliary or nodular lesions, and complete blackening a diffuse involvement of the organ. Arrows represent the site of infection [3]. In the untreated control animals, extensive tuberculous lesions were observed in the lungs, liver, at the site of infection and in the axillary lymph nodes. In the amikacin-treated animals in Groups 2 and 3, tuberculous changes proved to be much slighter. The spleen of five control guinea pigs and of five in Group 2 markedly was enlarged. In Group 3, the spleen was enlarged in two guinea pigs which had no tuberculous lesion.

Fig. 1. Schematic representation of the extent of disease observed in each guinea pig at necropsy on the 50th day. (a) Controls; (b) amikacin 7.5 mg/kg once daily; (c) amikacin 7.5 mg/kg twice daily

It was concluded that amikacin treatment favourably influenced the course of experimental guinea pig tuberculosis, proving thus the efficacy of the antibiotic *in vivo*.

REFERENCES

1. Fodor, T.: Pneumon. hung. **34,** 77 (1981).
2. Fodor, T.: Acta microbiol. Acad. Sci. hung. **28,** 419 (1981).
3. Feldman, W. H., Hinshaw, C. H.: Amer. Rev. Tuberc. **51,** 582 (1945).

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KLEBSIELLA PNEUMONIAE ENTEROTOXIN

II. PHYSICOCHEMICAL PROPERTIES OF ENTEROTOXIN

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(Received May 20, 1981)

Physicochemical properties and systemic effects of the enterotoxin of *Klebsiella pneumoniae* has been studied. The enterotoxin had a molecular weight between 10 000 to 50 000. It was protein in nature, and heat and acid stable, inducing a dilatatory response in the gut. It haemolyzed the erythrocytes of various animals including man. It had a capillary permeability activity. In addition, when administered parenterally it increased the level of blood glucose, serum cholesterol, serum alkaline phosphatase and serum acid phosphatase.

Non-invasive enterotoxin-producing strains of *Escherichia coli* in tropics have been reported as a common cause of acute diarrhoeal disorders [1–4]. Bacteriological examination of clinical specimens of acute diarrhoea, malnourished children, chronic diarrhoea, and tropical sprue, revealed *Klebsiella pneumoniae* [2, 5–9]. Although these organisms were isolated from the intestinal tract of sick persons, no pathogenic significance was attached to them. It has only recently been reported that they produce enterotoxin, inducing net secretion of water and electrolytes in rabbits [9, 10].

In a previous study we have reported on the influence of aeration factors on the production of enterotoxin by *K. pneumoniae* and the suitability of laboratory animals for its biological assay. In the present investigation, efforts were made to study the physicochemical properties of the enterotoxin and its systemic effects in experimental animals.

Materials and methods

Source of enterotoxin. K. pneumoniae strain (B-5-1) was used to produce an enterotoxin and to purify it [11].

Source of animals. Healthy rabbits weighing 2 to 2.5 kg were used for inoculation with enterotoxin using the ileal loop method [12]. The animals were fasted for 24 h before use, with free access to water. The time between inoculation and sacrifice was 18 h.

Molecular weight of enterotoxin. The molecular weight of enterotoxin was determined by the gel filtration technique [13]. Ten mg of toxin were placed on the Sephadex G-50 column and the eluates collected were monitored by a Beckman spectophotometer at 280 nm (DU-2). The pooled contents were dialysed against a saturated sucrose solution at 4 °C and each of the eluates was tested for toxicity.

Effect of heat on enterotoxin. The effect of heat on the activity of the toxin was studied in a water set at 45 $\pm$ 1, 56 $\pm$ 1, 70 $\pm$ 1 and 100 $\pm$ 1 °C for 30 and 60 min. Each sample was later tested for toxicity. Sterile growth medium treated similarly was used as control.

Effect of hydrogen ion concentration on enterotoxin. The toxin in 2 ml volume was placed in a small test tube, adjusted to pH 1, 1.5, 2.5, 3, 4, 4.5, 5, 6, 7, 7.5, 8.5, 9, 10, and 11, using 0.1 N hydochloric acid or sodium hydroxide and incubated at 37 °C for 4 h. Then the pH was readjusted to 7.0, and the sample was dialysed for 8–12 h and tested for toxicity. Sterile growth medium treated similarly was used as a control.

The nature of enterotoxin. In order to determine the nature of enterotoxin, 4 proteolytic enzymes namely trypsin, protease, lipase and pronase were used. The toxin was treated with each of these enzymes at protein concentration of 1 : 3, 1 : 5, 1 : 7 and 1 : 10 and tested for toxicity on rabbit skin. A ratio of 1 : 10 (enzyme : substrate) was tested for toxicity in rabbit ileal loop. The toxin was mixed with 10 mg/ml trypsin and pronase [4], incubated at 37 °C for 3 and 4 h, respectively, whereas protease and lipase were also separately mixed with toxin [15] and incubated at 37 °C for 80 min.

Systemic effects of enterotoxin. The effect of toxin was studied on blood glucose (BG), serum cholesterol (SC), serum alkaline phosphatase (SAP), and serum acid phosphatase (SACP), in adult white albino rabbits. Each rabbit received intravenously 1 ml of toxin containing 550 μg protein. The control rabbit received the same amount of sterile growth medium. Blood was collected from test and control animals at 0, 4, 12, 18, 24, 36, 48, 72, 120 and 144 h and BG [16], SC [17], SAP [18] and SACP [19] were determined. The results were recorded as mg/dl and K. A. Units/dl blood.

Haemolytic activity of enterotoxin. One per cent suspensions of erythrocytes of mice, rabbit, rat, sheep and man were tested for haemolytic activity of purified toxin [11]. Equal volumes of the erythrocyte suspension and enterotoxin were mixed and left to stand at room temperature for 4–8 h. The control contained equal volumes of erythrocyte suspension and growth medium, incubated similarly.

Effect of enterotoxin on capillary permeability. The enterotoxin was tested for its activity on capillary permeability in rabbit skin [20, 21]. A rapid type of capillar activity appeared in 2 h.

Results

The filtration studies revealed two peaks (Fig. 1), a higher and a lower one; the former failed to show any enterotoxicity whereas the later proved toxic, giving a gut dilatatory value of 1.28. It was found to have a molecular

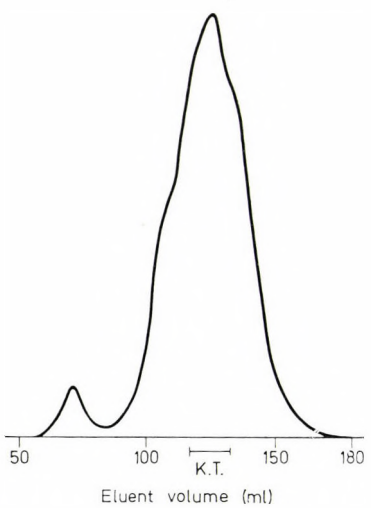

Fig. 1. Gel filtration of *K. pneumoniae* (B-5-1) enterotoxin in Sephadex G-50; K. T. = *Klebsiella* toxin

Table I

Effect of heat on enterotoxin

Temperature	Sample	Gut dilatatory value (volume: length)	
		30 min	60 min
45 °C	Test	1.34	1.31
	Control	0.21	0.205
56 °C	Test	1.36	1.275
	Control	0.18	0.14
70 °C	Test	1.175	1.11
	Control	0.205	0.145
100 °C	Test	1.165	0.835
	Control	0.21	0.20
Unheated toxin	Test	1.385	1.32
	Control	0.215	0.20

Mean of two experiments

Table II

Effect of enzymes on enterotoxin activity in rabbit skin

Enzyme used	Sample	Diameter of blueing (mm) at enzyme: substrate ratio			
		1 : 3	1 : 5	1 : 7	1 : 10
Pronase	Test	19.5	20.25	19.7	21.15
	Control	1.85	1.95	1.7	1.8
Trypsin	Test	19.5	20.15	20.15	21.25
	Control	1.375	1.625	1.40	1.7
Protease	Test	6.6	7.0	6.45	6.6
	Control	1.05	1.25	1.225	1.325
Lipase	Test	22.75	23.35	22.35	23.4
	Control	1.11	1.25	1.225	1.35
Untreated toxin	Positive control	22.5	22.75	23.9	24.1

Mean of two experiments

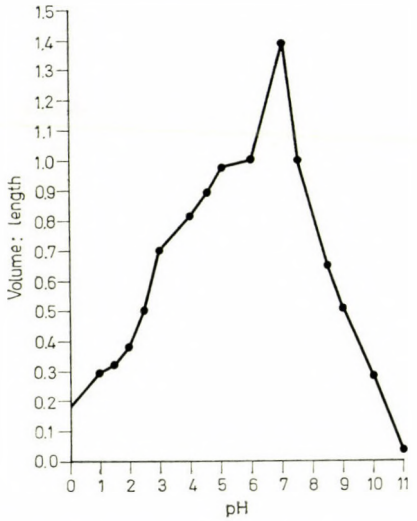

Fig. 2. Effect of hydrogen ion concentration on *K. pneumoniae* (B-5-1) enterotoxin

weight between 10 000 to 50 000 and was heat and acid stable. A slight loss in toxicity was noted when heating at 100 °C was continued for 60 min (Table I). A similar slight loss of activity was observed at alkaline pH (Fig. 2). The toxin was found to be protein in nature as it was inactivated by protease (Fig. 3) giving a gut dilatatory value of 0.85 against 1.4. g of untreated toxin.

While studying the effect of toxin on BG, SC, SAP and SACP, it was noticed that the level of all four increased. The BG level increased to 158 mg/dl from the 12th h and continued to increase till the 24th h and then decreased

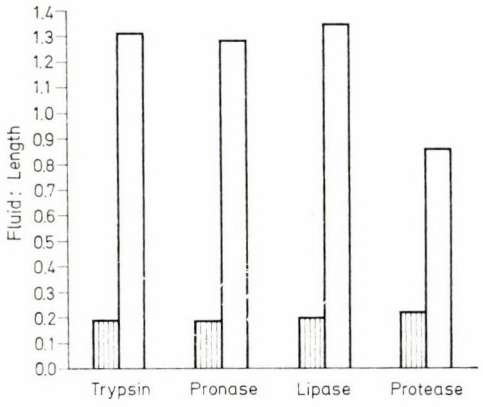

Fig. 3. Effect of enzymes on *K. pneumoniae* (B-5-1) enterotoxin in the ligated rabbit ileal loop; shaded columns: control; open columns: test

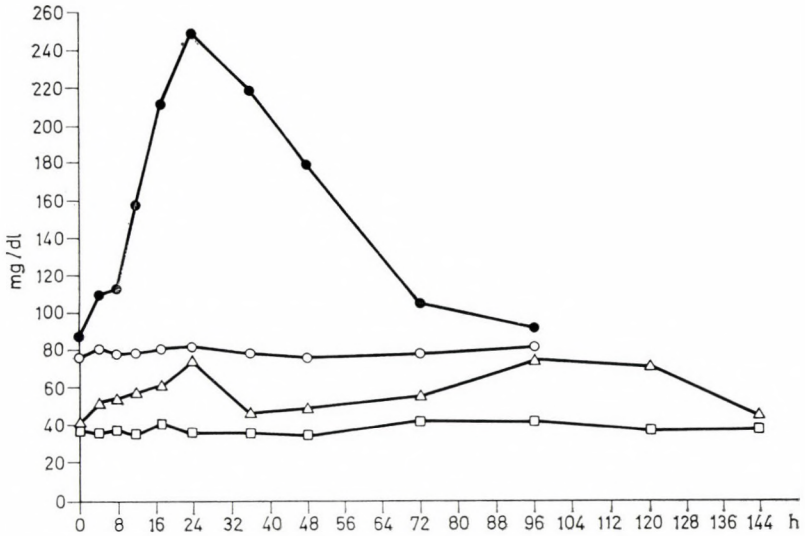

Fig. 4. Effect of parenteral administration of *K. pneumoniae* (B-5-1) enterotoxin on blood glucose and serum cholesterol in the rabbit; ○——○ glucose control; ●——● glucose test; □——□ cholesterol control; △——△ cholesterol test

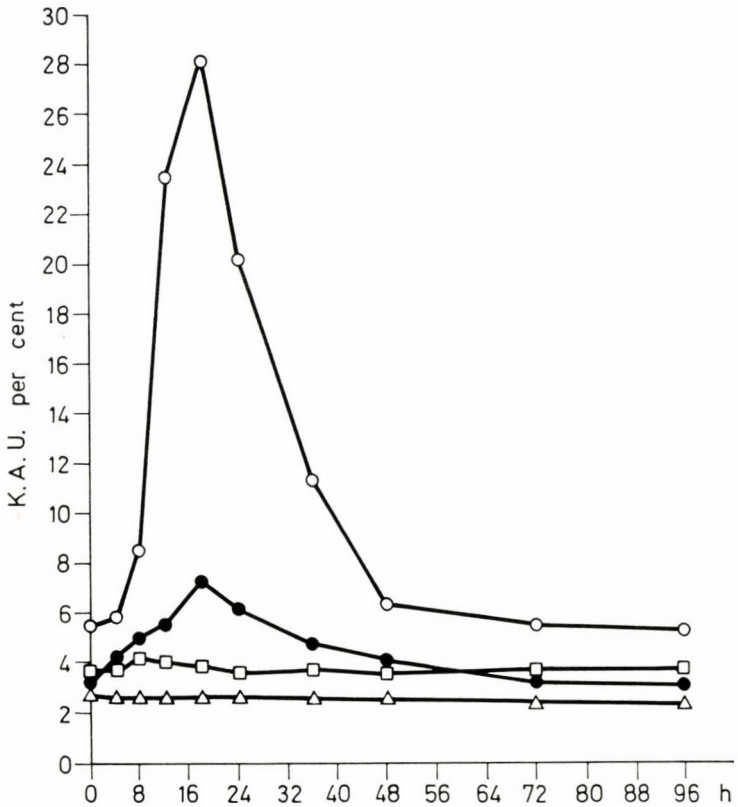

Fig. 5. Effect of parenteral administration of *K. pneumoniae* (B-5-1) enterotoxin on serum alkaline phosphatase and acid phosphatase; ○——○ alkaline phosphatase test; □——□ alkaline phosphatase control; ●——● acid phosphatase test; △——△ acid phosphatase control

gradually till 48 h, reaching the normal level in 72 h (Fig. 4). The maximum concentration was 248 mg/dl as compared to the control of 82 mg/dl. SAP also showed a rise from 8 h, reached its maximum (28.3 K. A. Units/dl) at 18 h and thereafter fell steeply to the normal after 36 h (Fig. 5). The enterotoxin haemolysed the erythrocytes of all the animals including man, and had a capillary permeability activity (Table II) giving a 22.5–24.1 mm blueing diameter as compared to the 1.05–1.95 mm diameter of the negative control.

Discussion

The physicochemical properties of *K. pneumoniae* enterotoxin were studied. It was observed that no factor could be pooled in void volume, using gel filtration, suggestive of the absence of an enterotoxin of smaller molecular weight like the heat labile toxin of *E. coli* [13, 14, 22]. The molecular weight of *K. pneumoniae* enterotoxin is between those of *E. coli* ST and LT [14, 15, 21, 23] and is probably not identical with any of them. The toxin was found to be heat stable confirming the similar observation of others [22], as it could not be inactivated at 100 °C for 30 min and it was unstable below pH 5. It was found to be protein in nature. Only protease inactivated it, causing a loss of toxicity.

The systemic effect of the enterotoxin caused an increase in BG, SC, SAP and SACP. While studying cholera toxin in mice, a rapid loss of liver glycogen *in vitro* studies was reported [24]. A sharp rise in plasma glucose level was observed on the parenteral administration of *E. coli* and *Vibrio cholerae* enterotoxins [25] which is suggestive of rapid glycogenolysis and the levels showed marked decrease within 48 h, due apparently to a loss of glycogen [26]. Similarly, a sharp rise in BG till 24 h was observed thereafter it decreased gradually. It may have been due to a slow depletion of glycogen. After 72 h it reached the normal level, confirming the similar observations for *E. coli* and *V. cholerae* [27, 28] enterotoxins. The SAP level, too, increased due perhaps to a hormone-like action of the enterotoxin on the cyclic AMP and adenyl cyclase effect, like it was reported for cholera enterotoxin [29]. SACP and SC may have increased due to stimulation by adenyl cyclase, as the presence of alkaline phosphatase and acid phosphatase was observed in intestinal exudates [10]. An increased level of serum cholesterol was observed by others and also by us in clinical sprue [31].

It may be inferred from the above that these systemic effects could be of some clinical importance. It seems that the enterotoxin may damage the liver, kidney and spleen, but to prove this, extensive histochemical studies will be necessary.

Like *V. cholerae* enterotoxin [32, 33] and LT of *E. coli* [21], the enterotoxin of *K. pneumoniae* affected the capillary permeability in the rabbit skin. These observations indicate that the enterotoxin of *K. pneumoniae* resembles

those of *E. coli* and *V. cholerae* as for as this activity is concerned, suggestive of the presence of a capillary permeability activating factor. The fact that this activity was lost on treatment with protease indicates that either the enterotoxin has a separate permeability factor or it exerts such an activity in addition to its enterotoxicity, similarly as *V. cholerae* enterotoxin.

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REFERENCES

1. Dupon, H. L., Formal, S. B., Hornick, R. B., Snyder, M. J., Libonate, J. P., Sheahan, D. J., Labrec, E. H., Kalas, J. S.: New Engl. J. Med. **1,** 285 (1971).
2. Gorbach, S. L., Banwell, J. G., Chatterjee, B. ., Jacobs, B., Sack, R. B.: J. clin. Invest. **50,** 881 (1971).
3. Gorbach, S. L., Kean, B. H., Evans, D. G., Bessudo, D.: New Engl. J. Med. **292,** 933 (1975)
4. Rowe, B., Taylor, J., Bettelheim, D. A.: Lancet **1,** 1 (1970).
5. Coello-Ramirez, P., Lifshitz, F., Zuniga, V.: Pediatrics **49,** 233 (1972).
6. Grocey, M., Stone, D. E.: Amer. J. clin. Nutr. **26,** 1170 (1973).
7. Heyworth, B., Brown, J.: Arch. Dis. Childh. **50,** 27 (1975).
8. Klipstein, F. A., Holdeman, L. V., Corcino, J. J., Moore, W. E. C.: Ann. intern. Med. **79,** 632 (1973).
9. Klipstein, F. A., Horowitz, I. R., Engert, R. F., Schenk, E. A.: J. clin. Invest. **56,** 799 (1975).
10. Klipstein, F. A., Schenk, E. A.: Gastroenterology **68,** 642 (1977).
11. Anil Jhanjee: M. Sc. Thesis, Panjab University, Chandigarh, India 1977.
12. De, S. N., Chatterjee, D. N.: J. path. Bact. **66,** 559 (1953).
13. Cambell, D. H. Garvey, J. S., Cremer, N. E., Sussdorf, D. F.: Methods in Immunology. 2nd ed. Benjamin inc., New York 1971.
14. Jack, T. M., Wu, B. J., Braemer, A. C., Bidlack, D. E.: Infect. Immun. **7,** 178 (1973).
15. Jack, T. M., Wu, B. J.: Infect. Immun. **5,** 342 (1971).
16. Folin, O., Wu, H.: In Varley, H. (ed.): Practical Clinical Biochemistry. 4th ed. Arnold Heinemann Pvt Ltd., India.
17. Saeckett, G. E.: In Varley, H. (ed.): Practical Clinical Biochemistry. 4th ed. Arnold Heinemann, Pvt Ltd., India.
18. King, P. R., King, E. J.: In Wootton, I. D. P. (ed.): Microanalysis in Medical Biochemistry. 5th ed. Churchill Livingstone, Edinburgh–London 1974.
19. King, E. J., Jegatheesan, K. A.: In Wootton, I. D. P. (ed.): Microanalysis in Medical Biochemistry. 5th ed. Churchill Livingstone, Edinburgh–London 1974.
20. Craig, J. P.: Nature (Lond.) **207,** 614 (1965).
21. Evans, D. G., Evans, D. J., Pierce, N. F.: Infect. Immun. **7,** 873 (1973).
22. Klipstein, F. A., Engert, R. F.: Infect. Immun. **13,** 373 (1976).
23. Bywater, J.: J. med. Microbiol. **5,** 337 (1971).
24. Zieve, P. D., Pierce, N. F., Greennough, W. B.: Clin. Res. **18,** 690 (1970).
25. Sigh, A., Gaind, S., Sethi, S. K., Vadehra, D. V.: Indian J. med. Res. **65,** 299 (1977).
26. Mohan, K.: M. Sc. Thesis, Panjab University, Chandigarh, India 1975.
27. Greybill, J. R., Kaplan, M. M., Pierce, N. F.: Clin. Res. **18,** 454 (1970).
28. Pierce, N. F., Wallace, C. K.: Gastroenterology **63,** 439 (1972).
29. Baker, A., Kaplan, M. M., Kimberg, D. V.: J. clin. Invest. **52,** 2928 (1973).
30. Evans, D. J., Chen, L. C., Curlin, G. T., Evans, D. G.: J. clin. Invest. **50,** 1218 (1972).
31. Klipstein, F. A., Short, H. B., Engert, R. F., Jean, L., Weaver, G. A.: Gasteroenterology **67,** 1035 (1976).
32. Ghosh, S. N., Mukherjee, S.: Ann. Biochem. **19,** 173 (1959).
33. Basumalik, K. C., Ganguli, N. C.: Indian J. med. Res. **52,** 894 (1964).

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KLEBSIELLA PNEUMONIAE ENTEROTOXIN

III. EFFECT OF *KLEBSIELLA PNEUMONIAE* ENTEROTOXIN ON THE INTESTINAL TRANSPORT AND HISTOPATHOLOGICAL CHANGES IN RABBIT ILEAL LOOP

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Klebsiella pneumoniae strain B-5-1 was used to study the effect of its enterotoxin on the intestinal transport and histopathological changes in the rabbit ileal loop. Enterotoxin increased the net flux of water and of electrolytes. Increased amounts of phospholipids and proteins were also detected together with structural and functional abnormalities in the ileum. All these suggest that *K. pneumoniae* enterotoxin not only induce a net flux of fluid and imbalance of electrolytes but also damages the intestinal structure.

It is beyond doubt that the intestinal tract of untreated tropical sprue patients is usullay colonized with coliform bacteria [1–5], which possess enterotoxigenic properties as their cell-free filtrate induces fluid secretion and structural abnormalities. Like the enteric pathogens, *K. pneumoniae* isolated from cases of tropical sprue also produces heat stable enterotoxin (ST), causing net secretion and electrolyte imbalance in the intestine [6]. The genesis of this toxin is uncertain as on antibiotic or chemotherapy rapid improvement of the intestinal structure and its function occurs [1, 2, 7–10].

It has therefore been attempted to establish whether the enterotoxin of *K. pneumoniae* had similar effect in the intestine, and so the effect of *K. pneumoniae* enterotoxin on intestinal transport and the intestinal structure was studied on the rabbit ileal loop model.

Materials and methods

Source of enterotoxin. A strain of *Klebsiella pneumoniae* (B-5-1), was used to produce the enterotoxin as described earlier [11]. A sterile Trypticase Soy broth of pH 7.2 served as the control. The total protein content of the enterotoxin was estimated [12] and adjusted to 19.6 mg/ml and it was stored at −20 °C for future use.

Animal inoculation. Healthy rabbits weighing 1.5 to 2.0 kg, fasted for 24 h with free excess to water were used throughout.

The rabbit ileal loop method has been described elsewhere [13]. Each of the test and control loops received 2 ml of enterotoxin and sterile medium, respectively. The rabbits were then sacrificed 18 h after inoculation. The volume of fluid accumulated in the test and control loops was collected and their length was recorded. Specimens of test and control loops were also collected for histological study.

Biochemical analysis of accumulated fluid. The accumulated fluid was analysed for sodium, potassium, chloride, bicarbonate, phospholipids and protein. Sodium and potassium were estimated [14] using an EEC flame photometer whereas bicarbonate and chloride were

determined according to SCHALES and SCHALES [15] and values were recorded as meq/litre of fluid. The proteins and phospholipids were determined by the method of LOWRY *et al.* [12, 16], and the values were recorded as mg/ml of fluid.

Histopathological study. Specimens obtained from the intestinal loops, were fixed in 10% formalin, sections of 7–8 μm thickness were cut, stained with haematoxylin–eosin and periodic acid Schiff stain, and examined under low and high power.

Results

The electrolyte, phospholipid and protein content of the accumulated fluid was considerably higher than in the controls (Fig. 1). The phospholipid and protein content was 2.7 mg and 70.2 mg/ml, respectively as compared to the control values of 24 μg and 8 mg/ml, respectively.

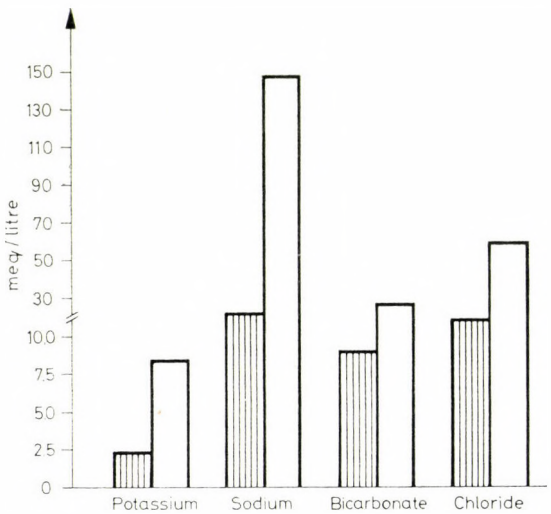

Fig. 1. Effect of *K. pneumoniae* (B-5-1) enterotoxin on electrolytes in the accumulated fluid of ligated rabbit ileal loop. Shaded column: control; open columns: test

The histological studies of the ileal loop revealed that the intestinal mucosa was damaged whereas no such changes were seen in the controls (Fig. 2). Mild blunting and broadening of villi (Fig. 3) showing variable size and shape were clearly observed. Necrosis of the upper portion of some villi and venous congestion with inflammatory cellular infiltration were also observed, followed by degeneration of the tip of the villi (Fig. 4). Figures 5 and 6 show slight venous congestion and infiltration with inflammatory cells. Atrophy with inflammatory exudate was observed on the tip of the villi (Fig. 7).

Discussion

The enterotoxin of *K. pneumoniae* was studied for its effect on intestinal transport and histological changes in the intestine, using the rabbit ileal loop model. It was observed that the enterotoxin induced net fluid secretion with an imbalance of the level of electrolytes, phospholipids and proteins and produced certain structural and caused functional abnormalities in the intestine.

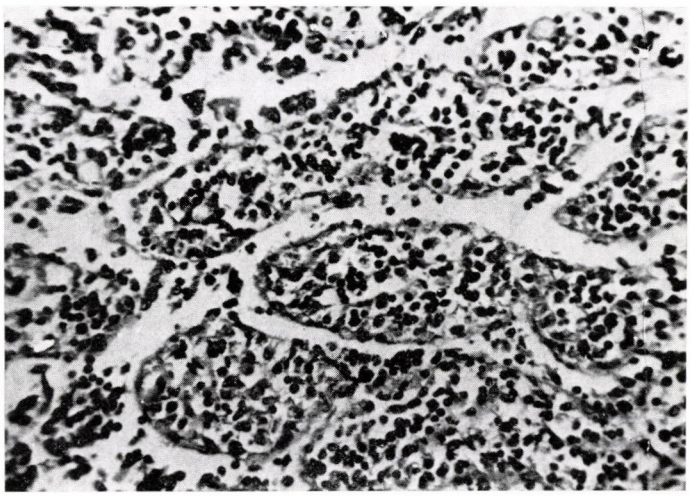

Fig. 2. Control ileal loop exposed to sterile Trypticase Soy broth shows normal mucosa and finger shaped villi. Haematoxylin–eosin, ×300

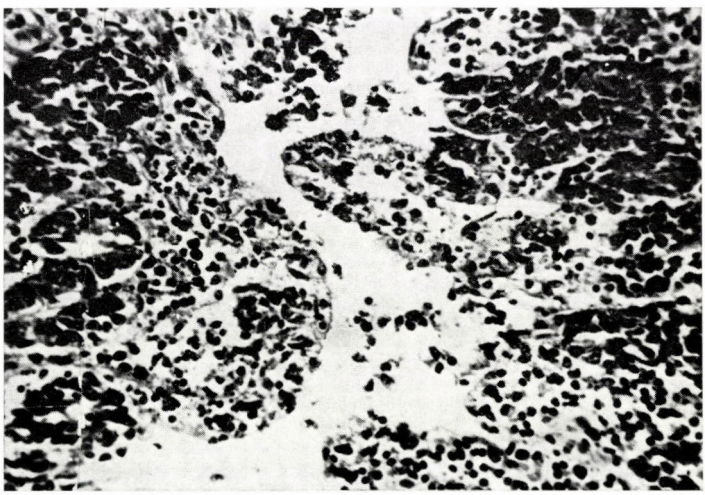

Fig. 3. Ileal loop exposed for 18 h to 20 mg of *K. pneumoniae* (B-5-1) enterotoxin, shows mild blunting, broadening, venous congestion and necrosis of the upper portion of villi. Haematoxylin–eosin, ×300

The enterotoxin of *Escherichia coli* and *Vibrio cholerae* evoke net fluid secretion and electrolyte imbalance in the rabbit ileal loop [17, 18]. BHOGAL (M. Sc. Thesis, Panjab University, India, 1973), and SAXENA (M. Sc. Thesis Panjab University, Chandigarh, India, 1978) reported similar observations with *E. coli* enterotoxin. Others [4, 19] reported that *K. pneumoniae* enterotoxin increased intestinal net secretion as compared to the controls.

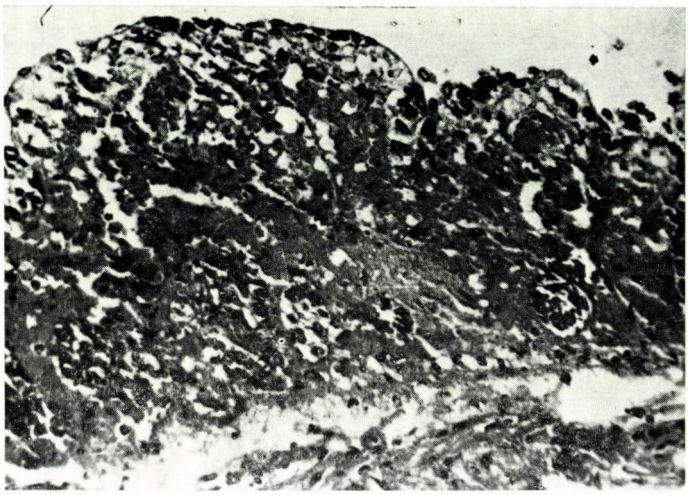

Fig. 4. Ileal loop exposed for 18 h to 40 mg *K. pneumoniae* enterotoxin, shows severe blunting, broadening and alterations in the size of villi. Haematoxylin–eosin, ×300

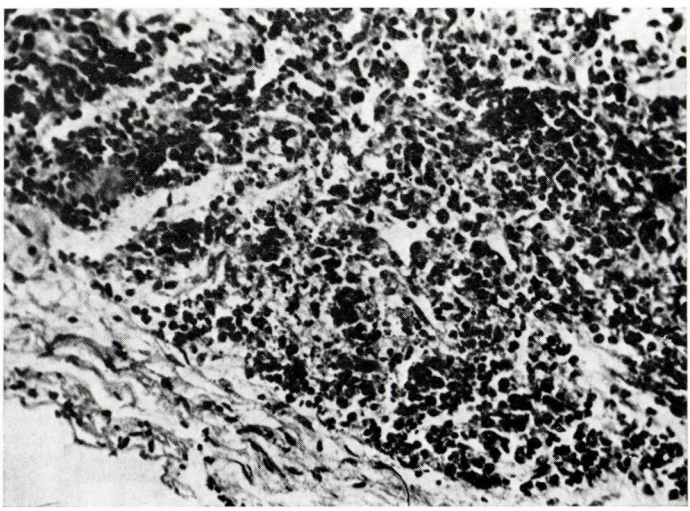

Fig. 5. Ileal loop exposed for 18 h to 20 mg of *K. pneumoniae* enterotoxin, shows slight congestion infiltration by inflammatory cells. Haematoxylin–eosin, ×300

It is uncertain whether *K. pneumoniae* enterotoxin is antigenically similar to the enterotoxins of *E. coli* and *V. cholerae* it resembles them in being stable in contrast to other enterotoxins [6], evoking net water secretion and electrolyte imbalence in rabbits [4, 5, 20]. Similarly, the enterotoxin of *E. coli* with its phospholipase-like activity causes leakage of phos-

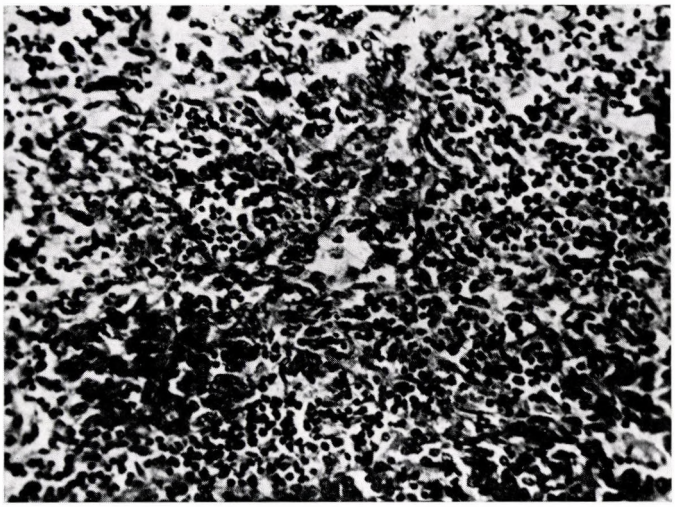

Fig. 6. Ileal loop exposed for 18 h to 40 mg of *K. pneumoniae* enterotoxin, shows severe congestion and increased inflammatory cell infiltration. Haematoxylin–eosin, ×300

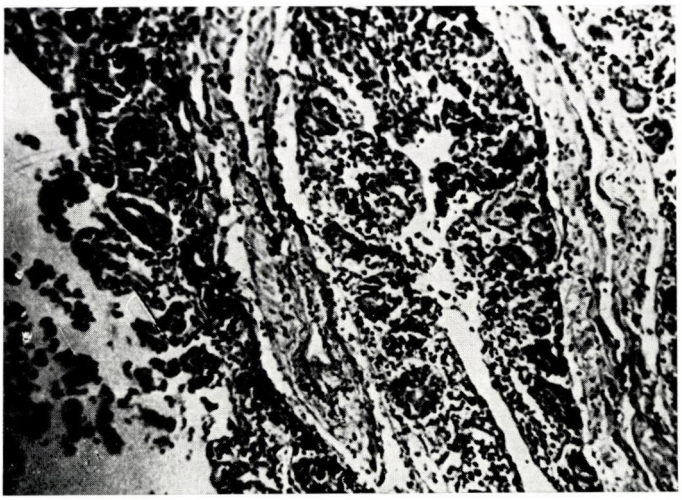

Fig. 7. Ileal loop exposed for 18 h to 40 mg of *K. pneumoniae* enterotoxin. Inflammatory exudate is clearly seen in the tip of villi. Haematoxylin–eosin, ×300

pholipids from the intestinal membrane and affects active transport, causes accumulation of excess fluid and electrolyte imbalance [21]. It is therefore presumed that a similar mechanism may act in the case of *K. pneumoniae* enterotoxin. The combined effect of several factors, viz. a phospholipase activity of enterotoxin through adenyl cyclase and histopathological changes in the bowel may explain the increased intestinal secretion and the disturbance of electrolytes. The exact mechanism of enterotoxin is not understood and for its explanation two possibilities have been suggested [22–25]. First a direct invasion of the intestinal mucosa by bacteria, producing the structural abnormalities associated with net secretion and electrolyte imbalance as in the case of *Salmonella typhi-murium* [26–28]. *Shigella dysenteriae* [29, 30] and *E. coli* [31, 32]. Second, some bacteria may elaborate toxin in the intestine, causing net fluid secretion. Non-pathogenic strains of *E. coli* and *V. cholerae* do not cause any structural changes [17, 33–36] but produce a secretory flux of chloride and water [18, 37] by a mechanism that appears to be related to the stimulation of mucosal adenyl cyclase activity [38, 39], whereas the enterotoxin of certain other bacteria produces both fluid accumulation and structural abnormalities [40–46]. A positive fluid response and structural abnormalities due to 4 coliform strains have also been recorded [47, 48]; they are presumably due to the toxic properties of enterotoxin resembling the heat stable enterotoxin of noninvasive *E. coli* strains evoking an unusually rapid fluid accumulation [20]. *E. coli* enterotoxin possesses phospholipase activity affecting the active transport through the intestinal membrane; this results in net secretion of fluid and electrolyte imbalance [49]. A similar mechanism may be responsible for the net secretory response in the case of *K. pneumoniae* enterotoxin.

The enterotoxin under study produced structural abnormalities in the rabbit ileal loop; their severity depended upon the amount of enterotoxin injected as in agreement with other observations [5]: we found mild and severe changes after injecting 20 and 40 mg/ml doses, respectively. The histological study revealed mild degenerative changes of the villi, or focal necrosis with venous congestion, and a shortening and blunting of the villi associated with cellular infiltration with inflammatory exudate.

The exact mechanism of action of enterotoxin is still open to discussion. Necrosis and avulsion of villi suggested that primarily the toxin may be acting on the vascular system, as it displayed an increased capillary permeability [50], followed by ischaemia leading to necrosis and other changes in the intestinal structure and its functions.

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REFERENCES

1. Gorbach, S. L., Banwell, J. G., Mitra, K., Chatterjee, R. B., Jacobs, B., Guhamazmuder, D. N.: Lancet **1,** 74 (1969).
2. Gorbach, S. L., Banwell, J. G., Jacobs, B., Chatterjee, B. D., Mitra, R., Sen, N. N., Guhamazmuder, D. N.: Amer. J. clin. Nutr. **23,** 1545 (1970).
3. Bhat, P. S., Shanta Kumari, S., Rajan, D., Mathan, V. I., Kapadia, C. R., Swarnbai, C., Baker, S. J.: Gastroenterology **62,** 11 (1970).
4. Klipstein, F. A., Holdeman, L. V., Corcino, J. J., Moore, W. E. C.: Ann. intern. Med. **69,** 632 (1973).
5. Klipstein, F. A., Schenk, E. A.: Gastroenterology **68,** 642 (1975).
6. Banwell, J. G., Sherr, H.: Gastroenterology **65,** 467 (1973).
7. Sheehy, T. W., Perez Santiago, E.: Gastroenterology **41,** 208 (1961).
8. Klipstein, F. A., Falaiye, J. M.: Medicine **48,** 475 (1969).
9. Mehta, S. K.: J. Ass. Phycus India **19,** 417 (1971).
10. Maldonado, N., Horta, E., Guerra, R., Perez Santiago, E.: Gastroenterology **57,** 559 (1969).
11. Klipstein, F. A., Engert, R. F.: J. infect. Dis. **132,** 200 (1975).
12. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J.: J. biol. Chem. **193,** 265 (1951).
13. De, S. N., Chatterjee, D. N.: J. Path. Bact. **66,** 559 (1953).
14. Hawk, P. B.: In Varley, H. (ed.): Practical Clinical Biochemistry. 4th Ed. Heinemann, London 1954. p. 491.
15. Schales, O., Schales, S. S.: J. biol. Chem. **140,** 879 (1941).
16. Youngburg, N. Youngburg, J.: J. Lab. clin. Med. **16,** 158 (1930).
17. Moon, H. W., Whipp, S. C., Bactz, A. L.: Lab. Invest. **25,** 133 (1971).
18. Pierce, N. F., Wallace, C. K.: Gastroenterology **63,** 439 (1972).
19. Klipstein, F. A., Horowitz, I. R. Engert, R. F., Schen, K. E. A.: J. clin. Invest. **56,** 799 (1975).
20. Evans, D. G., Evans, D. J., Pierce, N. F.: Infect. Immun. **7,** 873 (1973).
21. Smith, H. M., Halls, S.: J. Path. Bact. **93,** 531 (1967).
22. Babwekk, H. G. M., Girbacg, S. K., Mitra, R.: Amer. J. clin. Nutr. **23,** 1559 (1970).
23. Corcino, J. J., Maldomalom, M., Klipstein, F. A.: Gastroenterology **65,** 192 (1973).
24. Grady, G. F., Keusch, G. T.: New Engl. J. Med. **285,** 831 891 (1971).
25. Formal, S. B., DuPont, H. L., Hornick, R. B.: Ann. Rev. Med. **24,** 103 (1973).
26. Maenza, R. M., Powell, D. W., Plotkin, G. R., Formal, S. B., Jervis, H. R., Sprinz, H.: J. infect. Dis. **121,** 475 (1970).
27. Powell, D. W., Plotkin, G. R., Maenza, R. M.: Gastroenterology **60,** 1053 (1971).
28. Giannella, R. A., Formal, S. B., Damin, G. J., Collin, H.: J. clin. Invest. **52,** 441 (1973).
29. Gemski, P., Takenchi, A., Washington, O.: J. infect. Dis. **126,** 523 (1972).
30. Levine, M. M., DuPont, H. L., Formal, S. B., Hornick, R. B., Takeuchi, A., Gangarosa, E. J., Synder, M. J., Libonati, J. P.: J. infect. Dis. **127,** 261 (1973).
31. Taylor, J., Maltby, M. P., Payne, J. M.: J. Path. Bact. **76,** 491 (1958).
32. DuPont, H. L., Formal, S. B., Hornick, R. B., Synder, M. J., Libonati, J. P., Sheahani, D. G., Labrec, E., Kalas, J. P.: New Engl. J. Med. **285,** 1 (1971).
33. Patnaik, B. K., Ghosh, H. K.: Brit. J. exp. Pathol. **47,** 210 (1966).
34. Fresh, J. W., Versage, P. M., Reyes, V.: Arch. Path. **77,** 529 (1964).
35. Nerris, H. T., Majno, G.: Amer. J. Path. **53,** 263 (1968).
36. Staley, T. E., Jones, E. W., Smith-Staley, J. A.: Amer. J. Digest. Dis. **18,** 751 (1973).
37. Field, M. I., Fromm, D., Atawqati, Q., Greenough, W. B.: J. clin. Invest. **51,** 796 (1972).
38. Kimberg, D. V., Field, M., Johnson, J., Henderson, A., Gershon, E.: J. clin. Invest. **50,** 1218 (1971).
39. Evans, D. J., Chen, L. C., Curlin, G. T., Evans, D. G.: Nature (Lond.) **236**) 137 (1972).
40. Keusch, G. T., Grady, G. F., Mata, L. D., McIver, J.: J. clin. Invest. **51,** 1212 (1972).
41. Sullivan, R., Asano, T.: Amer. J. Physiol. **220,** 1793 (1971).
42. Prohaska, J. V., Jacobson, M. J., Drake, C. T.: Surg. Gynec. Obstet. **109,** 75 (1959).

43. PROHASKA, J. V.: Gastroenterology **51,** 913 (1966).
44. WARREN, S. E., JACOBSON, M., MIRANY, J.: J. exp. Med. **120,** 561 (1964).
45. KENT, T. H.: Amer. J. Path. **48,** 387 (1966).
46. KENUSCH, G. T., GRADY, G. F., TAKEUCHI, A., SPRINZ, H.: J. infect. Dis. **126,** 92 (1972).
47. SMITH, H. W., GYLES, C. L.: J. med. Microbiol. **3,** 387 (1970).
48. BYWATER, R. J.: J. med. Microbiol. **5,** 337 (1972).
49. TRIA, E. D., BARNALEI, O.: In: TRIA, E. D., SCANU, A. M. (eds): Structural and Functional Aspects of Lipoproteins in Living Systems. Academic Press, New York 1969.
50. JHANJEE, A.: M. Sc. Thesis. Panjab University, Chandigarh, India 1978.

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RADIODETOXIFIED ENDOTOXIN INDUCED LYSOSOMAL ENZYME LIBERATION AND TOLERANCE

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Beta-glucuronidase and cathepsin D activity was measured in rabbit sera. A significant increase in beta-glucuronidase activity was found following the intravenous application of a high dose of the parent endotoxin. The enzyme liberating effect of endotoxins irradiated by 50, 100, and 150 kGy was much weaker. Enzyme assays also confirmed that, independent of the irradiation, radiodetoxified endotoxins retain their endotoxin tolerance inducing effect.

In recent years several methods have been elaborated for the preparation of non-toxic endotoxins which retain their capacity to increase natural resistance, to induce endotoxin tolerance and to prevent shock. Promising are the chemically detoxified preparations [1–3] and the endotoxin detoxified by ^{60}Co gamma ray irradiation as described by Bertók *et al.* [4]. Earlier studies have demonstrated that the latter preparation had reduced lethal, haemodynamic, and complement activating effects [5]. It has much less haematologic effect and Shwartzman reactivity than the parent endotoxin [6].

Endotoxin is known to induce lysosomal enzyme liberation *in vivo* [7, 8] and *in vitro* [9] both in treated animals and in cell cultures. The release of lysosomal hydrolases correlates with the rate of the endotoxin effect. In the present experiments the effects of parent endotoxin and radiodetoxified endotoxin were tested by measuring the beta-glucuronidase and cathepsin D activity in the sera of treated animals.

Materials and methods

Endotoxin was extracted by the phenol-water method [10] from a fermentor culture of *Escherichia coli* and purified by repeated ultracentrifugation. The endotoxin was then suspended in distilled water (10 mg/ml) and irradiated by 50, 100, 150, or 200 kGy (^{60}Co Gamma Noratom 3500) according to the modified method of Previte *et al.* [11]. The biological effects of the parent and of the radiodetoxified endotoxins were measured in rabbits by determining the LD_{50} values and by haematologic tests as described earlier [6]. Irradiation resulted in a significant increase of the LD_{50} of the endotoxin.

The potency of the parent and the radiodetoxified endotoxin was tested by changing the serum lysosomal level.

Serum beta-glucuronidase (E. C. 3. 2. 1. 31) was determined by Fishman's method [12]. The substrate used was phenolphthalein-sodium-beta-d-glucuronate (Sigma).

For measuring cathepsin D (E. C. 3. 4. 4. 23), BARRETT's method [13] was applied. As a substrate a 4% solution of purified dialysed denatured bovine haemoglobin was used. Enzyme activity was measured in all experimental animals before application of the parent endotoxin or the radiodetoxified endotoxin, and the values obtained served later as controls. Enzyme activity was measured in blood samples taken from the ear vein in the 60th, 120th, or 180th min after treatment.

Experimental animals. New Zealand rabbits of both sexes were used, each weighing 2000–2500 g. The animals were kept under standardized conditions and fed on standard diet completed with green food. The schedule of treatment was as follows.

Group I. Rabbits in group I/a received intravenously LD_{30} of the parent endotoxin. Rabbits in groups I/b and I/c were treated with a single intravenous injection of 0.1 ml/kg endotoxin irradiated by 100 or 150 kGy, respectively.

Group II. In this group 4 subgroups of 5 animals each were treated. The animals in group II/a received, following WEISSMANN and THOMAS' method [7], the parent endotoxin for 20 days, then, after an interval of 48 h, LD_{30} of the parent endotoxin intravenously. The animals in groups II/b, c and d were treated with the endotoxin irradiated with 50, 100, or 150 kGy, respectively, for 20 days, then, after an interval of 48 h, LD_{30} of the parent endotoxin was given intravenously.

Group III. Rabbits were treated with endotoxin irradiated with 50, 100 or 150 kGy, then, after 48 h, each animal received a 100 μg/kg dose of that detoxified endotoxin intravenously, which had been used for the induction of tolerance.

Thus, in the first experimental group the effects on the lysosomal enzyme activity of the parent endotoxins were studied. In group II the tolerance-inducing effect of the parent and radiodetoxified endotoxins was compared, after a provoking dose of parent endotoxin. In group III, we measured the rate of enzyme liberation elicited by radiodetoxified endotoxin in animals previously made tolerant by the same radiodetoxified endotoxin.

Results

Enzyme activity in the rabbit sera was determined 60, 120 and 180 min after administration of the parent or the radiodetoxified endotoxin. The values obtained in the 60th min differed only slightly from the minute 0 control value. Peak activity was found at 120 min for both beta-glucuronidase and cathepsin D. Similar or somewhat decreased values were found at 180 min. For this reason only the values for the 120th min are indicated in the figures

The results for beta-glucuronidase are summarized in Fig. 1. It is seen that the parent endotoxin caused a great increase in activity (I/a), while the effect of endotoxin irradiated with 100 kGy was much weaker (I/b), and the endotoxin irradiated with 150 kGy produced only a slight increase (I/c). Group II served for studying the effect of parent endotoxin on beta-glucuronidase activity of rabbits in which tolerance had been induced by different endotoxins. The results showed that the parent endotoxin caused a smaller increase in activity in the animals whose tolerance had been induced by parent endotoxin (II/a) or irradiated endotoxin (II/b, c, d) than in animals without tolerance (I/a).

The experiments in group III showed that in rabbits made tolerant by irradiated endotoxin pretreatment, the same endotoxin failed to increase the beta-glucuronidase activity.

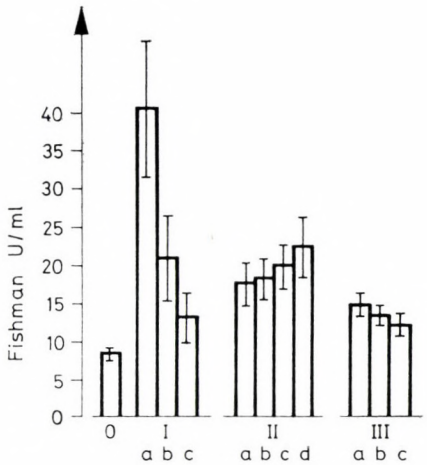

Fig. 1. Effect of endotoxins on serum beta-glucuronidase activity. New Zealand rabbits weighing 2000–2500 g each. The indicated values are the means of 5 experiments. 0 = serum beta-glucuronidase activity before treatment; all other values refer to the 120th min after treatment. I/a = single intravenous administration of parent endotoxin (LD_{30}) $p < 0.001$; I/b = effect of endotoxin irradiated with 100 kGy (100 μg/kg) $p < 0.01$; I/c = effect of endotoxin irradiated with 150 kGy (100 μg/kg) $p < 0.05$; II/a = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by parent endotoxin $p < 0.05$; II/b = effect of LD_{30} of the parent endotoxin in rabbits by 50 kGy $p < 0.05$; II/c = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by endotoxin irradiated by 100 kGy $p < 0.05$; II/d = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by endotoxin detoxified by 150 kGy $p < 0.02$; III/a = effect of endotoxin detoxified by 50 kGy (100 μg/kg) in rabbits previously made tolerant by the same endotoxin $p < 0.05$; III/b = effect of endotoxin detoxified by 100 kGy (100 μg/kg) in rabbits previously made tolerant by the same endotoxin $p < 0.05$; III/c = effect of endotoxin detoxified by 150 kGy (150 μg/kg) in rabbits previously made tolerant by the same endotoxin $p < 0.5$.

The results of studies of cathepsin D are summarized in Fig. 2. It shows that cathepsin D activity was raised to a lesser degree by irradiated endotoxin (I/b, c) than by the parent endoxin (I/a), as compared to the control values (0). The data in Fig. 2 demonstrate that in rabbits previously made tolerant by parent endotoxin, neither the parent endotoxin (II/a) nor the detoxified endotoxins (II/b, c, d) affected the cathepsin D activity. The results obtained in group III show that in rabbits previously made tolerant by radiodetoxified endotoxin the same endotoxin failed to cause an increase in cathepsin D activity.

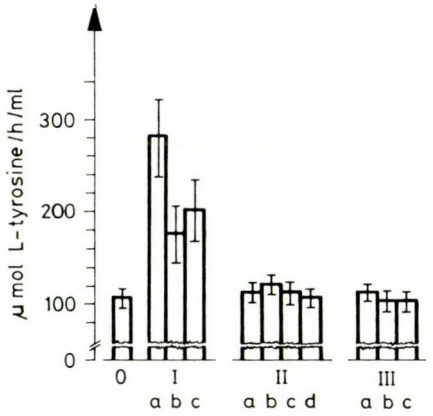

Fig. 2. Effect of endotoxin on serum cathepsin D activity. New Zealand rabbits weighing 2000–2500 g each. The indicated values are the means of five experiments. 0 = serum cathepsin D activity before treatment; all other values refer to the 120th min after treatment. I/a = single intravenous administration of parent endotoxin (LD_{30}) $p < 0.001$; I/b = effect of endotoxin irradiated by 100 kGy (100 μg/kg) $p < 0.01$; I/c = effect of endotoxin irradiated by 150 kGy (100 μg/kg) $p < 0.05$; II/a = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by the parent endotoxin (not significant); II/b = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by endotoxin irradiated with 50 kGy (not significant); II/c = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by endotoxin irradiated with 100 kGy (not significant); II/d = effect of LD_{30} of parent endotoxin in rabbits previously made tolerant by endotoxin irradiated with 150 kGy (not significant); III/a = effect of endotoxin detoxified by 50 kGy (100 μg/kg) in rabbits previously made tolerant by the same endotoxin (not significant); III/b = effect of endotoxin irradiated with 100 kGy (100 μg/kg) in rabbits previously made tolerant by the same endotoxin (not significant); III/c = effect of endotoxin irradiated with 150 kGy (100 μg/kg) in rabbits previously made tolerant by the same endotoxin (not significant)

Discussion

In an earlier paper [8] it has been shown that endotoxin increased the beta-glucuronidase and acid phosphatase activities in rabbit sera. At the same time the level of gamma globulin was observed to decrease significantly due to the increase in protease activity. It was also shown that these phenomena might be prevented by hypothermia, since the endotoxin hardly affects lysosomal enzyme release in hypothermic animals. The results presented confirm that the endotoxin produces a significant lysosomal enzyme mobilization increasing the activity of cathepsin D which plays an essential role in eliciting local and generalized Shwartzman reactions [8, 14]. Radiodetoxified endotoxins characteristically lack or hardly have the damaging effect of the parent endotoxin but retain its adjuvant, shock preventing and non-specific resistance increasing effects [15]. Radiodetoxified endotoxin affects lysosomal enzyme liberation to a much lesser degree than does the parent endotoxin and this is especially true concerning beta-glucuronidase release. It is not fully understood why the endotoxin irradiated by a higher dose of gamma rays

elicits a greater increase in cathepsin D activity than the endotoxin irradiated by a smaller dose.

Thus, radiodetoxified endotoxin has the same or nearly the same capacity to induce tolerance as has the parent endotoxin, and this capacity is not affected by irradiation. It might be concluded that by measuring the activity of the enzymes at issue will offer information concerning endotoxin tolerance.

These results have again confirmed that the increase in beta-glucuronidase activity is a sensitive marker of lysosomal enzyme liberation.

REFERENCES

1. Nowotny, A.: Nature (Lond.) **197,** 721 (1963).
2. Nowotny, A.: Ann. immunol. hung. **12,** 15 (1968).
3. McIntire, F. C., Hargis, M. P., Schenk, J. R.: J. Immunol. **117,** 674 (1976).
4. Bertók, L., Kocsár, L., Bereznai, T., Várterész, V., Antoni, F.: Hungarian Patent No. 62973. 1973.
5. Füst, Gy., Bertók, L., Nemes, A., Juhász-Nagy, S.: Infect. Immun. **6,** 26 (1977).
6. Szilágyi, T., Csernyánszky, H., Gazdy, E., Bertók, L.: Acta microbiol. Acad. Sci. hung. **25,** 159 (1978).
7. Weissmann, G., Thomas, L.: J. exp. Med. **116,** 433 (1962).
8. Szilágyi, T., Tóth, S., Csernyánszky, H., Sümegi, J.: Agents Actions **6,** 721 (1976).
9. McGivney, A., Bradley, S. G.: Proc. Soc. exp. Biol. (N. Y.) **155,** 390 (1977).
10. Westphal, O., Lüderitz, O., Bister, F. Z.: Naturforschung **7**/b, 148 (1952).
11. Previte, J., Chang, Y. E., Bisi, H.: J. Bact. **93,** 1607 (1967).
12. Fishman, W. H., Springer, B., Brunnett, R.: J. biol. Chem. **173,** 449 (1948).
13. Barrett, A. J.: Biochem. J. **104,** 601 (1967).
14. Szilágyi, T., Tóth, S., Lévai, G.: Acta microbiol. Acad. Sci. hung. **24,** 247 (1977).
15. Bertók, L.: Perspect. Biol. Med. (Chicago) **24,** 61 (1980).

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ESCHERICHIA COLI STRAINS ISOLATED FROM SURFACE WATERS. DISTRIBUTION BY RESISTANCE TO ANTIBIOTICS AND R-PLASMID TRANSFER

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Samples were taken from surface waters in Csongrád county in the months March through December, 1980. *Escherichia coli* isolates selected at random were tested for resistance to 5 antibiotics and for R-plasmid carriership. Of the strains isolated from Tisza river at each of 8 sampling sites, 50–60% were sensitive to all the 5 antibiotics. The percentage of sensitivity was much lower if only the strains isolated during the summer months, when the water level was high, or those isolated from affluents and backwaters were taken into account. The frequency of resistance was the highest for tetracycline, followed in order by ampicillin, streptomycin, kanamycin and chloramphenicol. R-plasmid was carried by 43% of the resistant isolates tested, mainly by multiresistant ones.

The actual state of surface waters depends on numerous continuously changing factors, viz. the temperature, chemical and biological contaminants including toxic factors, and the rate of flow. Waters are reached by contaminants from the air, from the soil and with sewages. Thus, characterization of a surface water on the basis of sampling at a given place usually cannot be reproduced.

It follows from the foregoing that the actual biological state of a surface water cannot be determined exactly because the actual values of some parameters are always unknown. Nevertheless, bacterial contaminations of surface waters have attracted much interest, and this is the reason why standards have been elaborated for both the Comecon countries and the European Common Market.

Recently, R-plasmids prevalent in bacteria, first of all in enteric bacteria, have rapidly been spreading all over the world. These biological contaminants reach the environment and from there the surface waters, with hospital sewages [1, 2] as well as garbages and sewages of animal farms and slaughterhouses [3, 4]. The occurrence of resistant and R-plasmid carrier bacteria has been examined by many authors in river water [5–8], waters of seaside resorts [9] as well as in untreated and sedimented sewages [10, 11]. Bacteria resistant to antibiotics and those carrying R-plasmid were detected in 43–90% and 34–70% of the samples, respectively. In Hungary, MILCH *et al.* [12, 13] examined in this respect *E. coli* strains isolated from the rivers Danube

and Drave in the years 1975 and 1976. Fifty-five per cent of the isolates proved to be resistant to antibiotic (s) and 70% of the resistant strains carried R plasmid.

In the present work, *E. coli* isolated from the surface waters of Csongrád county were examined for resistance to antibiotics and for R-plasmid carriership.

Materials and methods

Sampling. Samples were obtained from surface waters of Csongrád county by the staff of the Laboratory of the Lower Tisza Region Directorial Board of Water Affairs from the water current at sites indicated by us in the months March through December, 1980. In addition, samples were collected in the summer months from places marked out for bathing. The samples were transferred to our laboratory in sterile bottles and examined according to Hungarian Standards [14–16], with reasonable modifications.

Culture media. 1. Lactose broth. 2. Broth for R-plasmid transfer; Bacto peptone (Difco) 10 g; Lac Lemco meat extract (Oxoid) 10 g; NaCl 5 g; distilled water 1000 ml; pH 7.4; 121 °C 30 min. Resistant strains were isolated on eosin–methylene blue agar medium containing one of the following antibiotics: tetracycline, streptomycin, kanamycin (30 μg/ml of each), ampicillin and chloramphenicol (20 μg/ml of each). To select transconjugants, nalidixic acid (50 μg/ml) was added to the media.

Strains. From each culture, 8–10 colonies chosen at random were purified by plating on eosin–methylene blue agar medium. *E. coli* K12 Hfr *lac*$^-$ *nal*^{r} was used as the recipient strain.

Resistance was determined by Resistest disks (Human, Budapest).

R-plasmid transfer. Nalidixic acid sensitive *E. coli* strains resistant to one or more of the above antibiotics were used as donors. From fresh broth cultures of the donor and the recipient strain, 0.1 and 0.2 ml, respectively, were added to 4.5 ml nutrient broth. These cultures and separate control cultures of both strains were incubated at 37 °C for 24 h. The transconjugates thus obtained were dropped on eosin–methylene blue agar plates containing the respective antibiotic and nalidixic acid. After an incubation at 38 °C for 48 h, we used the *lac*$^-$ colonies to determine the antibiogram by the disk method. Phage typing and colicin typing were performed and *fi* specificity was examined, using the RNA phages f_2 and MS2. The transfer rate was not calculated.

Results

Two rivers, viz. the Körös and the Maros, flow into the river Tisza in Csongrád county. The drained sewages of some settlements between the rivers Danube and Tisza and of riverside towns mostly arrive unpurified, the undrained ones originating from holiday resorts densely built along the river and its backwaters and channels are washed into river Tisza by floods.

Figure 1 shows a schematic hydrographic chart of Csongrád county with the sites of sampling. According to hygienic scoring (I—IV) 90% of the samples collected during the 10-month period under study were contaminated (III) or highly-contaminated (IV), just like in the previous years. From March to December, 1980, 1360 isolates were tested and 52.2% proved to be sensitive to all the antibiotics included in the study; 20.1% were resistant to one, 11.8% to two and 15.9% to three to five drugs (Fig. 2). From March to the end of August the majority, from September to December only about 40%, of the isolates were resistant.

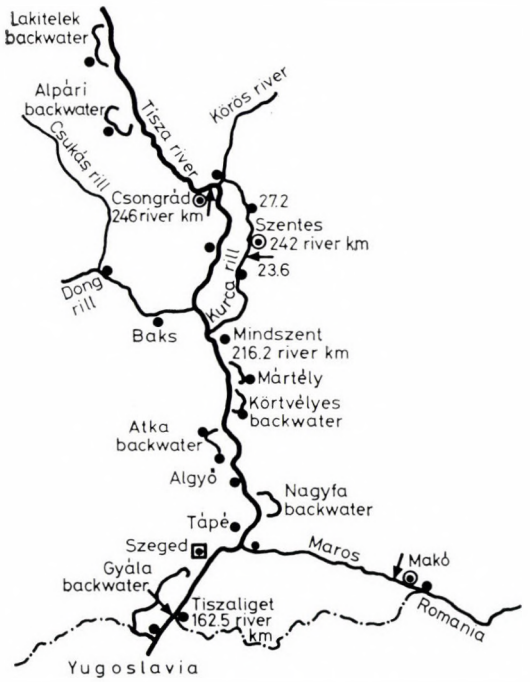

Fig. 1. Hydrographic chart of Csongrád county. Major towns Szeged (170 000 inhabitants), Csongrád, Szentes and Makó (20 000–30 000 inhabitants). Arrow: sewage inflow

A flood passed down in river Tisza late in February and a smaller one in April. The water level was high again from June to early September. Then the river subsided until November, when it tended to rise again. The affluents and the backwaters connected with the Tisza followed the fluctuations of the river.

The column diagrams in Fig. 3a show the average distribution by sensitivity to antibiotics of the *E. coli* strains isolated from the Tisza at 8 sampling sites during the whole period of sampling. In the samples collected at Csongrád Szentes, Mindszent, Algyő and Tápé, the average rate of sensitive strains was approximately 50%, whereas it was 57% at Szeged and Tiszasziget and 52% at the Yugoslav border. Taking into account that the sewage coming from Szeged reaches the Tisza between Tiszasziget and the border, the corresponding decrease in sensitivity seems to be moderate. If, however, only the values obtained in the three summer months (i.e. when the water level was high) are taken into account (Fig. 3b), the percentage sensitivity shows more pronounced differences between sampling sites.

In the column diagrams of Fig. 4, the resistant strains are analysed further. Figures on the top of the columns present the average number of antibiotics to which the strains isolated from the indicated place proved to be

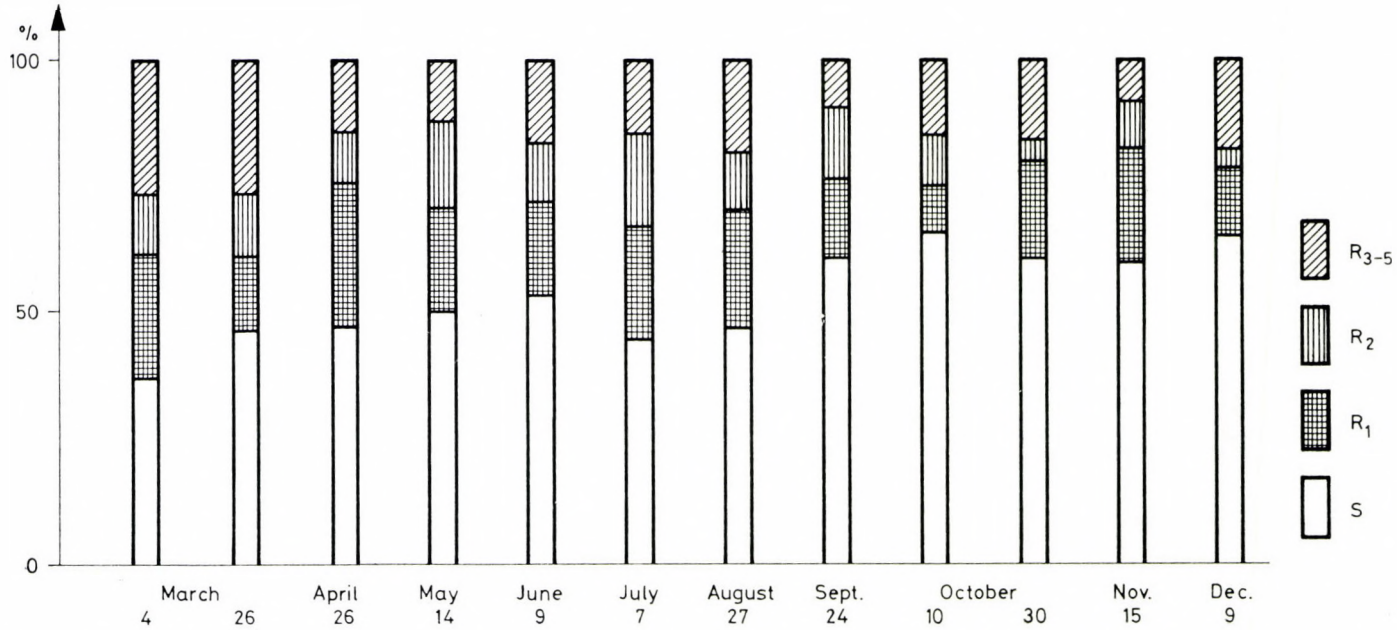

Fig. 2. E. coli strains isolated from surface waters of Csongrád county in the period March to December, 1980. Percentage distribution by resistance to antibiotics. S = strains sensitive to all the five drugs; R_1 = strains resistant to one drug; R_2 = strains resistant to two drugs; R_{3-5} = strains resistant to 3–5 drugs

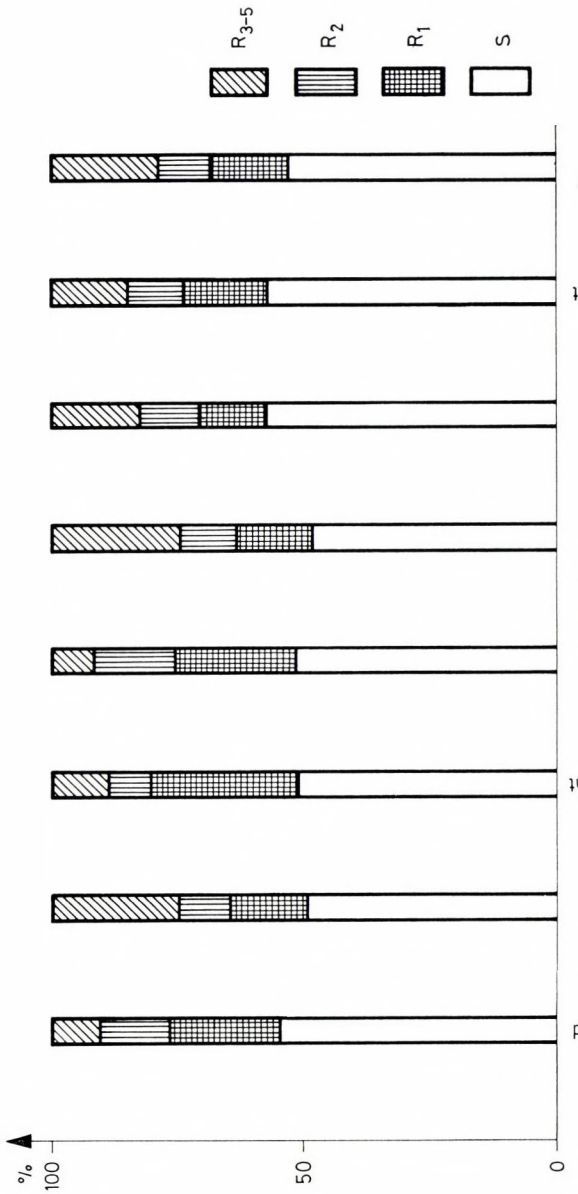

Fig. 3a. E. coli strains isolated from samples taken at 8 different sites of the river Tisza in the period March to December, 1980. Percentage distribution by resistance to antibiotics. For explanation, see Fig. 2

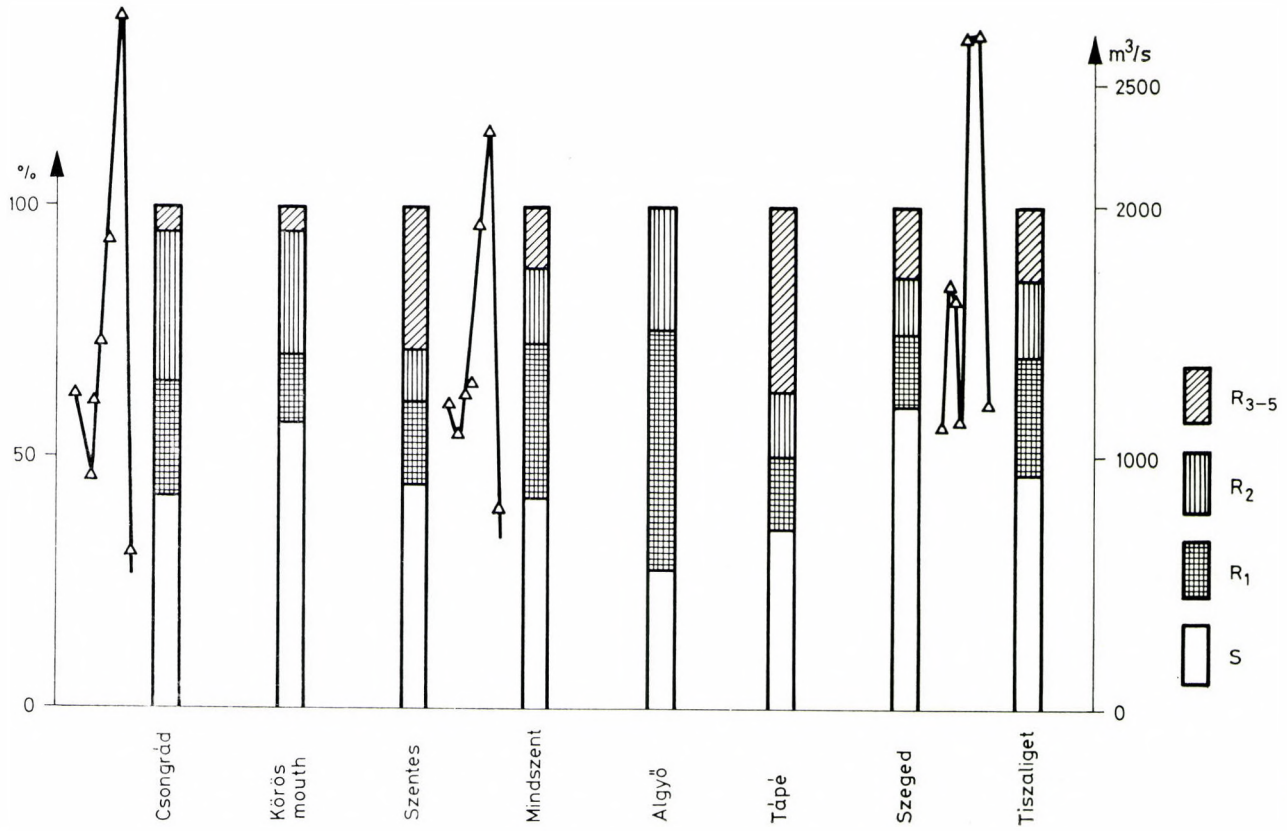

Fig. 3b. E. coli strains isolated in the summer months, 1980, from 8 sites of river Tisza. Percentage distribution by resistance to antibiotics. Mean values for samples taken every second week. △: flow rates m^3/s. For explanation, see Fig. 2

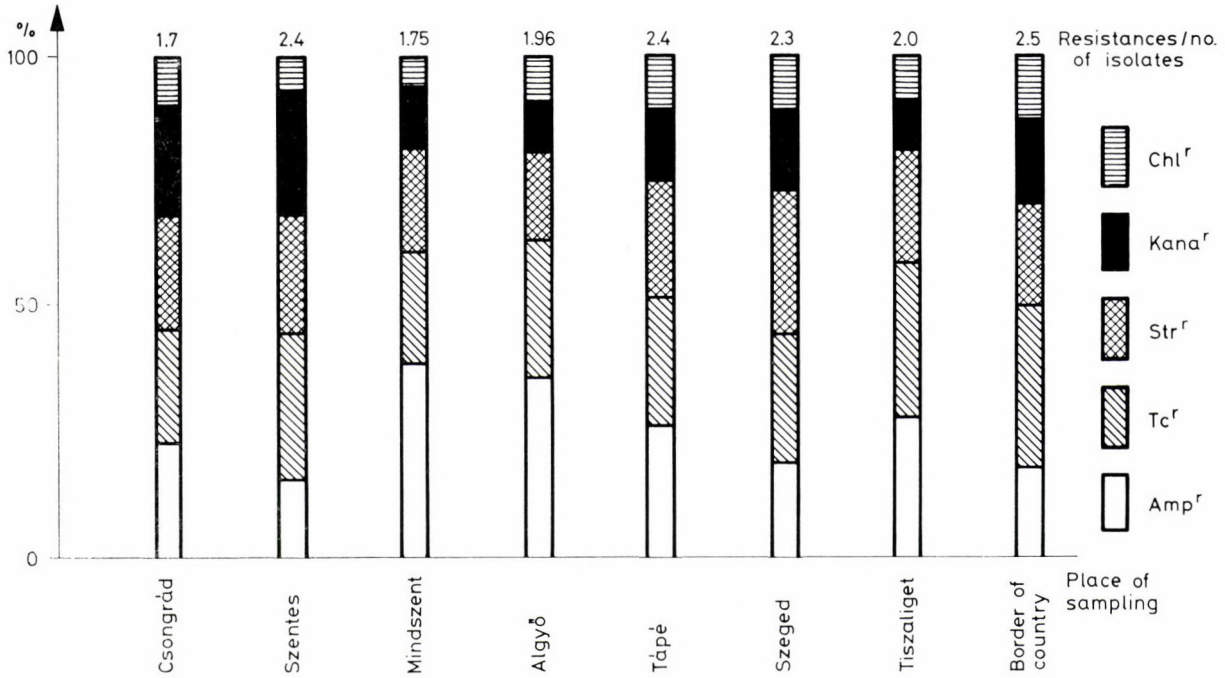

Fig. 4. Resistant *E. coli* strains isolated from river Tisza in the period March to December, 1980. Strains resistant to ampicillin (Amp^r), tetracycline (Tc^r), streptomycin (Str^r), kanamycin ($Kana^r$), chloramphenicol (Chl^r) in per cent

resistant. The frequency of multiresistant strains among those isolated from the Tisza at Szentes, Tápé and the Yugoslav border approximated, or even surpassed, 20% (see Fig. 3). Irrespective of the site of sampling, resistance was the commonenst to tetracycline (28%); to other antibiotics the following order was recorded: ampicillin (22%), streptomycin (22%), kanamycin (16%) and chloramphenicol (10%).

An analysis of the water samples collected from affluents and backwaters is shown in Fig. 5. The results obtained for both sampling sites of the Körös and for the vicinity of the river's mouth are consistent with the results obtained for the river Tisza, whereas both *E. coli* contamination and resistance to antibiotics were much higher in the samples taken from the Kurca, the Csukás rill the Dong rill and in those collected from the Maros near the Roumanian border.

The results for the backwaters are summarized in Table I. Among these, the backwater near Lakitelek and that near Alpár are in nature conservation areas outside Csongrád county. From the waters connected with the rivers there was no sampling in the summer months when the open-air baths were under water. Thus, the corresponding values are poorly informative. The rate of resistant strains was the highest, 75%, for isolates obtained from the backwater near town Csongrád, a surface water, not connected with the river Tisza.

Table I

E. coli strains isolated from backwaters

Backwater	Month of sampling	No. of colonies tested	No. of colonies resistant to antibiotics	Resistances per resistant strains
Alpár	March to December	28	21	1.33
Lakitelek	March to December	24	8	1.1
Csongrád	June, July	16	12	1.9
Mártély	June to October	18	4	1.0
Körtvélyes	September, October	14	4	3.75
Atka	June to October	22	12	1.33

The results of resistance transfer experiments are presented in Table II. Most of the R-plasmid carriers were multiresistant, and it was most common with these strains that the *fi* character of the recipient had changed due to the transfer.

R-plasmid transfer resulted in phage restriction in 29.5% of the R strains, and colicine was transferred together with the R plasmid in 10.5%.

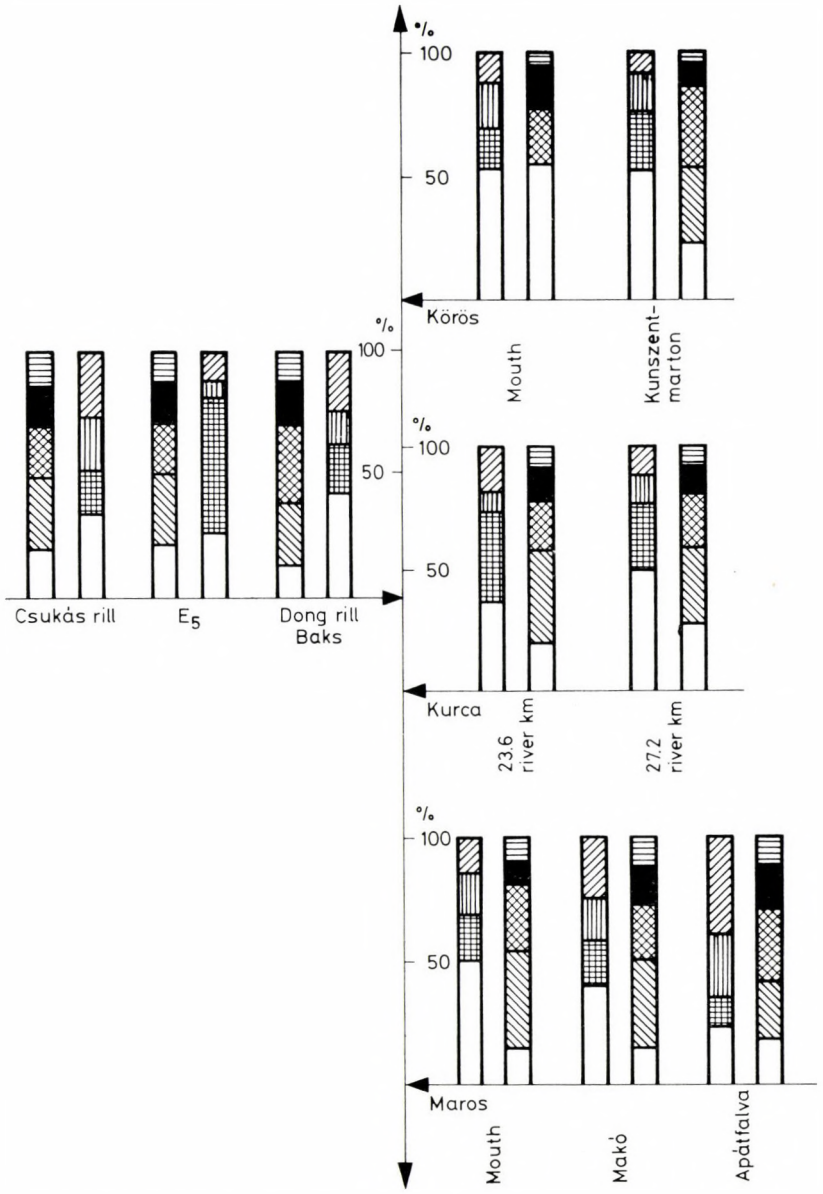

Fig. 5. E. coli strains isolated from the affluents of river Tisza. Resistance to antibiotics, per cent. For explanation, see Figs 2 and 4

Table II

Transfer of resistance

No. of strains tested	Resistances per strain	R+ strains		*fi*+	Phage restriction	Colicin transfer
		No.	per cent			
21	1	3	14.3	1	—	—
51	2	13	25.5	—	3	1
171	3–5	89	52.0	12	28	10
243	1–5	105	43.2	13	31	11

Discussion

The increasing prevalence of resistant and R-plasmid carrying bacteria has strikingly contributed to the recent deterioration of river waters. The regenerative capacity of river waters depends on the flux rate and the temperature of the water as well as on the quantity and degree of contamination of the sewage flowing into the river [19]. In conventional systems of sewage purification it was observed that the sedimentation system favoured the transfer of resistance from bacterium to bacterium. According to FONTAINE and HOADLEY [10], the faecal coliform count decreased considerably during sedimentation, but the ratio of resistant to sensitive strains did not change, only multiresistant strains showed some decrease in number. Experimental transfer of resistance to tetracycline has been shown even at 23 °C and 30 °C. BELL [20] reported a 95% decrease in coliform count during sedimentation of sewage; the coliforms resistant of five antibiotics disappeared and the number of those resistant to ampicillin dropped to one fourth. According to SHAW and CABELLI [21], the transfer frequency for *E. coli* is below 10^{-4} under optimum conditions, depending on the recipient as well as on the experimental conditions. Taking into account GRABOW's and their own calculations, SHAW and CABELLI concluded that a subject bathing in an objectionable water cannot swallow more than 1 to 5 *E. coli* or coliform bacteria carrying R-plasmid. Thus, a transfer of R-plasmid into a faecal *E. coli* strain living in the gut has little probability. According to ANDERSON [22] implantation in the bowel is unlikely even if 10^{10} bacterial cells are present there. This means that the risk of transferring resistance *in vivo* from an R-plasmid carrying strain to a faecal strain is very low.

Recently [23–25], however, it has been found that *Bacterioides fragilis*, an anaerobic microorganism present in the intestinal tract in thousandfold

the number of *E. coli*, may also serve as recipient for *E. coli* R-plasmids, and thus become a reservoir for the plasmid.

In the present report we wished to point to an environmental risk factor which is able to persist and spread and also to develop new resistances in the surface waters of Csongrád county. The persistence of this factor in rivers is ensured by the continuous inflow of unsatisfactorily purified sewages of human and animal origin and by the frequently occurring floods stirring up the rivers' sediment.

REFERENCES

1. Prikazsky, V., Koskava, I., Kremonova, Z., Krcméry, V.: Zbl. Bakt. Hyg. A **240,** 216 (1978).
2. Linton, K. H., Richmond, M. H., Bevan, R., Gillespie, W. A.: Med. Microbiol. **7,** 91 (1974).
3. Anderson, E. S.: Amer. Rev. Microbiol. **22,** 131 (1968).
4. DeZutter, L., Van Hoof, J.: Zbl. Bakt. Hyg. B **171,** 269 (1980).
5. Janouskova, J., Krcméry, V., Kadlecova, O.: Zbl. Bakt. Hyg. A **233,** 495 (1975).
6. Grabow, W. O. K., Prozesky, O. W., Burger, S. J.: Water Res. **9,** 777 (1975).
7. Kelch, W. J., Lee, J. S.: Appl. envir. Microbiol. **36,** 450 (1978).
8. Koditschek, L. K., Guyre, P.: Water Res. **8,** 747 (1974).
9. Smith, H. W.: Nature (Lond). **234,** 155 (1971).
10. Fontaine, T. D. III., Hoadley, A. W.: Hlth Lab. Sci. **13,** 238 (1976).
11. Cooke, M. D.: Antimicrob. Ag. Chemother. **9,** 879 (1976).
12. Milch, H., G. László, V., Hérmán, G.: Egészségtudomány **18,** 166 (1974).
13. Milch, H., Baron, F., Hoch, R., Pintér, E.: Egészségtudomány **19,** 72 (1975).
14. Hungarian Standard of the Bacteriological Control of the Drinking-Water No. 22901—71 (in Hungarian).
15. Methodological Guide (in Hungarian). National Institute of Hygiene, Budapest 1977.
16. Qualitative Classification of Surface Waters (in Hungarian) Eüsz-OVHSZ 141, Budapest.
17. Milch, H., Gyenes, M.: Acta microbiol. Acad. Sci. hung. **19,** 213 (1972).
18. Hegedűs, M., Fodré, Zs., Zsigó, M.: Tiscia (Szeged) **15,** 35 (1980).
19. Grabow, W. O. K., Prozesky, O. W., Smith, L. S.: Water Res. **8,** 1 (1974).
20. Bell, R. B.: Canad. J. Microbiol. **24,** 886 (1978).
21. Shaw, D. R., Cabelli, V. J.: Appl. envir. Microbiol. **40,** 756 (1980).
22. Anderson, J. D.: J. med. Microbiol. **7,** 85 (1974).
23. Guiney, D. G., Davis, C. E.: Nature (Lond.) **274,** 181 (1978).
24. Mancini, C., Behme, R. J.: J. infect. Dis. **36,** 579 (1977).
25. Privitera, G., Sebald, Fayolle, F.: Nature (Lond.) **278,** 657 (1979).

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HUMORAL IMMUNE FUNCTION AND EXPERIMENTAL *ESCHERICHIA COLI* INFECTION IN SPLENECTOMIZED DOGS

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Intravenous administration of sheep red blood cells resulted in a deficient initial antibody response and provoked a much lower concentration of plaque forming cells in the blood of splenectomized dogs than in the control group. The *in vitro* serum opsonizing activity on *Escherichia coli* M-S-15 remained normal after splenectomy, and no difference was found between the test and the control group in the intravascular clearance rate of bacteria injected intravenously.

Numerous animal and human studies have demonstrated that splenectomy leads to increased susceptibility to bacterial infections [1–5]. For this reason considerable interest has recently arisen in the role of the spleen in immunity.

There are, however, conflicting opinions concerning the importance of the spleen in host defense against bacterial invasion [3, 4, 6, 7].

Several immunological abnormalities have been demonstrated in the asplenic host, including a low serum IgM level [8, 9], decreased serum opsonizing activity [7, 10] and a failure to respond to intravenous particulate antigen challenge [11]. Although several studies have shown an increased mortality due to experimental bacterial infections in splenectomized rodents [1, 2], this could not be reproduced in higher mammals [12].

Thus, the mechanism leading to increased susceptibility after splenectomy remains enigmatic. In order to clarify the problem we have investigated the effects of splenectomy in the dog. This report describes variations of haemagglutinating antibodies and plaque forming cells, serum opsonizing activity and phagocytic function of splenectomized animals.

Materials and methods

Dogs. Twenty mongrel dogs of 10–17 kg body weight were used in the experiments. The animals were divided into two groups. The first group of 10 dogs underwent splenectomy, the remaining 10 animals served as controls.

Splenectomy. Dogs of the first group were anaesthetized by intravenous administration of hexobarbital-sodium (20 mg/kg) after injecting atropine (0.05 mg/kg), fentanyl (0.03 mg/kg) and droperidol (1 mg/kg) premedication intramuscularly. The dogs were ventilated by dinitrogen oxide–oxygen gas mixture (3 : 1) via intratracheal tube in a semi-closed system with the aid of an automatic respirator.

The immunological experiments were started 4 months after splenectomy. Neither splenectomy nor experimental infection caused mortality.

Isolation of leukocytes. Two types of cell preparations were obtained. Lymphocytes used in the Jerne single-cell assay (described below) were separated from heparinized venous blood using Ficoll (Pharmacia, Uppsala, Sweden)–Uromiro (Bracco, Milano, Italy) gradient centrifugation as described by BØYUM [13]. Cells were resuspended in Eagle's MEM (Grand Island Biological Company, Grand Island, New York, USA) supplemented with 20% pooled inactivated dog serum and 100 μg/ml streptomycin and 100 U/ml penicillin. Leukocytes used in the Maaløe assay (see below) were prepared from heparinized venous blood which was allowed to sediment for 30 min at room temperature. The leukocytes from the supernatant were washed twice in phosphate buffered saline (PBS), and resuspended in antibiotic-free Eagle's MEM. Cells were stained with crystal violet for counting. Trypan blue dye exclusion test showed a viability greater than 95%.

Immunization with sheep red blood cells (SRBC). SRBC in Alsever's solution were washed with three changes of MEM containing antibiotics before use for either immunization or the haemolytic plaque assay. Five ml of a 20% (vol/vol) cell suspension in MEM was administered to the dogs intravenously.

Preparation of bacteria. Overnight nutrient agar slope cultures of *E. coli* M-S-15 (oedema disease) bacteria were suspended in 0.9% NaCl. The amounts of bacteria were estimated by optical density at 690 nm in a Spekol UV photometer with the aid of a standard curve.

Haemagglutination assay for antibodies against SRBC. Titres of haemagglutinating antibodies were measured in dog serum with the standard microtitre method described by TAKÁTSY [14]. Results are expressed as the reciprocal of the highest dilution still producing agglutination.

Plaque assay for specific antibody forming cells. Direct (IgM) plaque formint cells (PFC) were detected by the method of JERNE and NORDIN [15] with the modifications described by KALTREIDER *et al.* [16]. Portions of the cell suspensions (5×10^6 lymphocytes per ml) ranging from 0.1 to 0.3 ml and 0.2 ml washed SRBCs (final concentration 15–20%) were added to glass tubes containing 2 ml of molten 0.7% agarose at 47 °C. The contents of the tubes were mixed by inversion and poured rapidly into Petri dishes 8 cm in diameter, previously coated with 5 ml of 1.4% agarose. The soft agarose mixture was allowed to gel at room temperature and the dishes were incubated for 45 min at 37 °C in humified air. Thereafter freshly restored guinea pig complement (Human, Budapest), was diluted 1 : 30 with MEM and 10 ml aliquots were added to each plate and the samples were incubated at 37 °C for additional 45 min. All assays were performed at least in duplicate. Plaques were counted under microscopic control. Results are expressed as PFC-s/10^6 viable lymphocytes.

Determination of serum opsonizing activity. Serum opsonizing activity was determined by the MAALØE method [17], with the modifications described by COHN and MORSE [18] and HIRSCH and STRAUSS [19]. Tests were done in siliconized glass or plastic tubes. Each tube contained 0.5 ml leukocyte suspension (2×10^7 polymorphonuclear leukocytes per ml, obtained from a third-party normal mongrel dog) +0.4 ml opsonin (sera stored frozen not longer than 14 days) + 0.1 ml *E. coli* suspension ($0.5–2 \times 10^6$ bacteria per ml). For the test the following controls were set up: tubes with leukocytes and bacteria without opsonin, tubes of leukocytes and bacteria with heat-inactivated sera, tubes with sera and bacteria without leukocytes. The samples were shaken at 37 °C for 60 min. For detecting extracellular inactivation of the bacteria, one set of tubes was incubated at 37 °C for 60 min. For the enumeration of total viable bacteria, aliquots were removed at 0 and 60 min and plated at suitable dilutions giving between 10 and 100 colonies on nutrient agar or blood agar plates. At the end of the incubation the tubes containing the phagocytic mixtures were centrifuged at 800 rpm for 10 min. Samples were removed from the supernatant for the determination of extracellular viable bacteria. Aliquots of the washed resuspended leukocyte pellets were plated for the determination of viable leukocyte-associated bacteria. All viable counts were performed at least in duplicate.

Clearance of bacteria after intravenous inoculation. Five ml of *E. coli* M-S-15 suspension ($0.5–1 \times 10^8$ bacteria per ml) were injected into the femoral vein of each animal. Aliquots of blood were then removed at 30 and 90 min, diluted in saline and plated on blood agar as described above. After an overnight incubation, the colony forming units per ml of blood were determined. The mean bacterial loading dose (0 min) was about 10^5 bacteria per ml blood, based on the assumption that the total blood volume amounts to 1/12 of the dog's total body weight. The geometric mean of colony forming units in each group of animals was plotted against time on a semilogarithmic graph.

Student's *t* test was used for statistical analysis.

Results

Titres of haemagglutinating antibodies in sera after immunization. Immunization by a single injection of SRBC resulted in a rapid increase in the serum haemagglutinin titre in the normal group ($0.001 < p < 0.01$ on the first day after immunization). In the splenectomized group until the 5th post-immunization day there was no significant increase in agglutinating antibody titres. After the 5th day there was a steady increase in the haemagglutinating titres of the test group but it still remained at a significantly lower level than that for the control group ($p < 0.001$); Fig. 1).

PFC response after intravenous SRBC immunization. As shown in Fig. 2, intravenous administration of SRBC resulted in significantly higher concentrations of plaque forming cells in the normal dogs than in the splenectomized group ($0.02 < p < 0.05$). In the splenectomized group, despite of immunization, in most of the cases the blood concentration of PFC-s did not exceed the background value (1–2 PFC per 10^6 lymphocytes) found in non-immunized normal dogs.

Serum opsonizing activity on E. coli M-S-15 bacteria. As can be seen in Fig. 3, there was no difference in serum opsonizing activity between control and splenectomized animals. The number of initial colony forming units was reduced to less than 10% on incubating phagocytic mixtures in both groups. Neither was there a considerable difference in the number of leukocyte-associated as well as free viable bacteria between the two groups. Heat inactivation

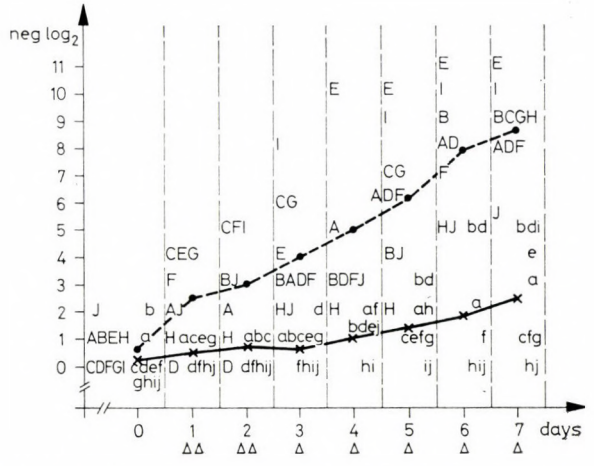

Fig. 1. Haemagglutination titres of dog sera against SRBC after immunization. One normal (capital letter) and one splenectomized (script letter) animal is matched. Each letter represents a value obtained from one animal on a given post-immunization day. Day 0 represents haemagglutinin titres before immunization. • – – – • means of the normal group; ×——× means of the splenectomized group. △ $p < 0.01$; △△ $0.001 < p < 0.01$

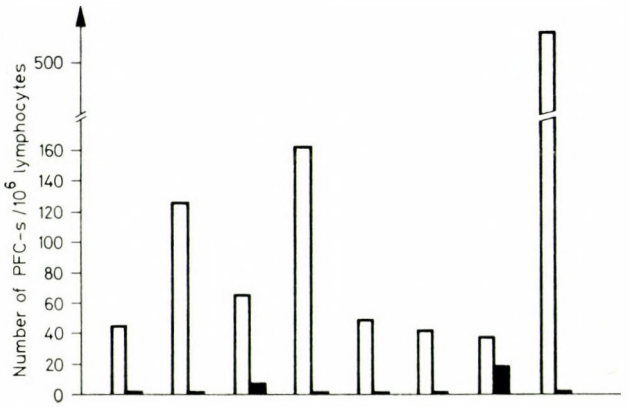

Fig. 2. Plaque forming response of blood lymphocytes. Each pair of columns represents the results of a single experiment in matched splenectomized (solid columns) and normal (blank columns) dogs. Testing plaque forming cells was done 7 days after immunization

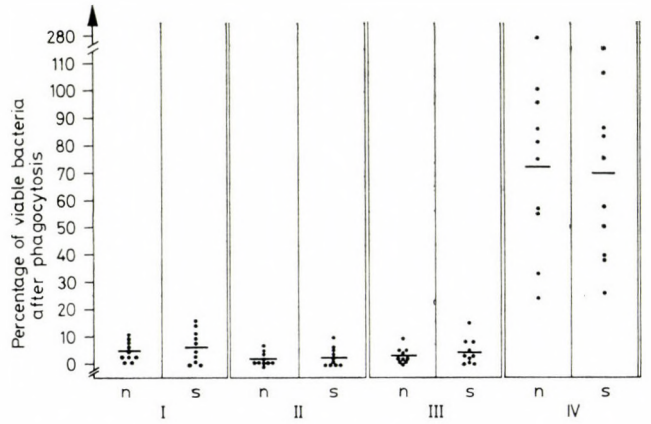

Fig. 3. Percentage of viable bacteria after phagocytosis. Each point represents the amount of viable bacteria after the phagocytic assay as a percentage of the initial bacteria per ml suspension value. n = incubation with serum of normal dogs; s = incubation with serum of splenectomized dogs; I = viable counts performed immediately after incubation from whole phagocytic mixtures (bacteria + serum + leukocytes); II = number of free viable bacteria remaining in the supernatant after centrifugation; III = number of leukocyte-associated bacteria; IV = result after incubation with heat-inactivated sera. Horizontal bars represent the means of each group

(56 °C, 30 min) resulted in a significant decrease of serum opsonizing activity in both groups ($0.001 < p < 0.01$ for normal and $p < 0.001$ for splenectomized dogs).

Clearance of bacteria injected intravenously. In both the test and the control animals there was a rapid exponential decrease in the number of colony

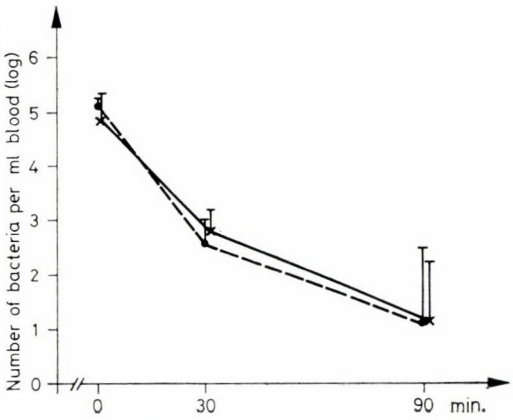

Fig. 4. Clearance of bacteria after intravenous inoculation. The log number of CFU-s per ml blood is represented as a function of time after intravenous inoculation. • – – – • normal dogs; ×——× splenectomized dogs

forming units. After 90 minutes the number of bacteria was less than 0.1% of the initial loading dose.

Discussion

The asplenic and/or splenectomized state carries an increased risk of fulminant bacterial sepsis (Overwhelming Postsplenectomy Infection — OPSI) [3–5], the immunological mechanism of which is not completely understood.

Splenectomized humans [8] and rats [9] show a low serum IgM level and a deficient antibody response to intravenous particulate antigen challenge.

Although several clinical [7] and laboratory [10] observations have shown a decreased serum opsonizing activity after splenectomy, there are data suggesting that splenectomy alone is not sufficient to cause such immunological aberration; e.g. WINKELSTEIN and LAMBERT [20] observed a normal serum opsonizing activity in splenectomized children who had had severe sepsis.

Most of the experimental studies performed in splenectomized rodents have shown a marked delay in the rate of intravascular clearance of intravenously injected pathogens [21–24].

In the present study striking similarities and marked discrepancies were found between the two groups of animals. In contrast to the normal group, the splenectomized animals had a low serum haemagglutinating antibody level after intravenous SRBC immunization. This finding is in agreement

with those described for other species including man [25], rabbit [26], rat [11] and mouse [27]. Thus, the spleen seems to be of primary importance in the initial antibody response to blood-borne particulate antigens.

Intravenous administration of SRBC-s provoked a much higher number of plaque forming cells in the normal group than in splenectomized animals. This finding is consistent with the data obtained in the mouse by LOZZIO and WARGON [28].

In contrast, there was no significant difference in serum opsonizing activity between the two groups. This observation is in contrast with the decreased postsplenectomy serum opsonizing activity found in rodents by LIKHITE [10].

Less than 0.1% of the bacteria injected intravenously could only be reisolated from the circulation. This finding does not confirm data reporting a delayed intravascular clearance rate of bacteria in the splenectomized rat [22], rabbit [21], guinea-pig [23], and mouse [24].

The *in vitro* opsonizing activity of sera was significantly decreased by heat-inactivation in both groups.

Thus our data show that splenectomy does not affect the phagocytic function either *in vitro* or *in vivo*. Similarly, it was reported that in splenectomized humans the intravascular clearance of bacteriophage ΦX 174 injected intravenously was at its normal level despite of decreased antibody production [29].

The present findings support the data published by AHLSTEDT [30] regarding the crucial role of the complement system in opsonization. Furthermore our results suggest that the function of the removed spleen may at least partly be taken over by the remaining part of the RES, thus lessening the risk of postsplenectomy complications.

Still, the decreased IgM production as a possible factor in the pathogenesis of OPSI should not be neglected. There is evidence indicating that bactericidal and opsonizing properties of IgM antibodies are more effective than those of IgG antibodies [31]. Furthermore, there is an increased incidence of sepsis in patients with IgM deficiency [32].

Since susceptibility to infections is influenced strongly by inherited factors [24], we cannot draw far-reaching conclusions for the relation between splenectomy and predisposition to infection. Our experiments performed on genetically heterogenous mongrel dogs nevertheless suggest that the deprival of an organ capable of recognizing and filtering blood-borne particulate antigens — under some circumstances — may account for increased incidence of severe infections.

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REFERENCES

1. Dickerman, J. D., Chalmer, B., Horner, S. R.: Pediat. Res. **14,** 1139 (1980).
2. van Wyck, D. B., Witte, M. H., Witte, C. L., Cole Thies, A.: J. surg. Res. **28,** 14 (1980).
3. Robinette, C. D., Fraumeni, J. F.: Lancet **2,** 127 (1977).
4. Krivit, W., Giebink, G. S., Leonard, A.: Surg. Clin. N. Amer. **59,** 223 (1979).
5. Leonard, A. S., Giebink, G. S., Basel, T. J., Krivit, W.: World J. Surg. **4,** 423 (1980).
6. Ly, B., Albrechtsen, D.: Acta med. scand. **209,** 21 (1981).
7. Passl, R., Eibl, M., Egkher, E., Frisee, H., Gaudernak, T., Neugebauer, G., Vécsei, W.: Wien. klin. Wschr. **88,** 585 (1976).
8. Krivit, W.: Amer. J. Hemat. **2,** 193 (1977).
9. Rozing, J., Brons, N. H. C., Benner, R.: Immunology **34,** 909 (1978).
10. Likhite, V. V.: Nature (Lond.) **253,** 742 (1975).
11. Schwartz, A. D., Dadash-Zadeh, M., Goldstein, R., Luck, S., Conway, J.: Blood **49,** 779 (1977).
12. Saslaw, S., Carlise, H. N.: Proc. Soc. exp. Biol. (N. Y.) **123,** 565 (1966).
13. Bøyum, A.: Scand. J. clin. Lab. Invest. **21,** Suppl. 97, 28 (1968).
14. Takátsy, G.: Acta microbiol. Acad. Sci. hung. **3,** 191 (1956).
15. Jerne, N. K., Nordin, A. A.: Science **140,** 405 (1963).
16. Kaltreider, H. B., Kyselka, L., Salmon, S. E.: J. clin. Invest. **54,** 263 (1974).
17. Maaløe, O.: On the Relation Between Opsonin and Alexin. Einar Munksgaard, Copenhagen 1946.
18. Cohn, Z. A., Morse, S. I.: J. exp. Med. **110,** 419 (1959).
19. Hirsch, J. G., Strauss, Bella: J. Immunol. **92,** 145 (1964).
20. Winkelstein, J. A., Lambert, G. H.: J. Pediat. **87,** 430 (1975).
21. Ellis, E. F., Smith, R. T.: Pediatrics **37,** 111 (1966).
22. Cooney, D. R., Swanson, S. E., Dearth, J. C., Dewanjee, M. K., Telander, R. L.: J. pediat. Surg. **14,** 336 (1979).
23. Hosea, S. W., Brown, E. J., Hamburger, M. I., Frank, M. M.: New Engl. J. Med. **304,** 245 (1981).
24. Shinefield, J. R., Steinberg, C. R., Kaye, D.: J. exp. Med. **123,** 777 (1966).
25. Rowley, O. A.: J. Immunol. **65,** 515 (1950).
26. Taliaferro, W. H., Taliaferro, L. G.: J. infect. Dis. **87,** 37 (1950).
27. Battisto, J. R., Cantor, L. C., Borek, F., Goldstein, A. L., Cabrerra, E.: Nature (Lond.) **222,** 1196 (1969).
28. Lozzio, B. B., Wargon, L. B.: Immunology **27,** 167 (1974).
29. Sullivan, J. L., Ochs, H. D., Schiffman, G., Hammerschlag, M. R., Miser, J., Vichinsky T., Wedgwood, R. J.: Lancet **2,** 178 (1978).
30. Ahlstedt, S.: Acta path. microbiol. scand. Sect. C. **89,** 23 (1981).
31. Michael, J. G., Rosen, F. S.: J. exp. Med. **118,** 619 (1963).
32. Hobbs, J. R., Millner, R. D. G., Watt, P. Y.: Brit. med. J. **4,** 583 (1967).

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METHYLATED NUCLEIC ACID BASES IN *MYCOBACTERIUM* AND MYCOBACTERIOPHAGE DNA

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Methylated bases of the DNA of two mycobacteria *(Mycobacterium phlei* and *Mycobacterium smegmatis* var. *butyricum)* and two mycobacteriophages (Phage phlei and Phage butyricum) have been studied. In both the bacterial and the phage DNAs 5-methyl-cytosine and 6-methyl-aminopurine could be detected. Using L-(methyl-H^3)-methionine as methyl donor not only the methylated bases of bacterium and phage DNA proved to be radioactive, but also the non-methylated purine residues and thymine. Possible pathways of this phenomenon are discussed.

Though the presence of methylated bases has been observed in DNA of viruses, bacteria, plant and animal cells, the biological meaning of methylation has been revealed only in the last decade; it plays a central role in modification and restriction phenomena [1, 2]. DNAs of mycobacteria and mycobacteriophages, however, have scarcely been investigated in this respect [3, 4].

In this paper we investigated the methylated bases in the DNA of two mycobacterium strains *(Mycobacterium phlei, Mycobacterium smegmatis* var. *butyricum)* and their phages (Phage phlei, Phage butyricum) as the first step to study their role in modification and restriction.

Materials and methods

Bacteria and phages. M. phlei and *M. smegmatis* var. *butyricum* and their phages (Phage butyricum and Phage phlei) of our culture collection were used.

Preparation of labelled DNA. Bacteria were grown by shaking at 37 °C in Sauton medium [5], supplemented with L-(methyl-H^3)-methionine (400 kBq/ml ~ 10 μCi/ml) and adjusted to pH 7.4. After 48 h cultivation glycine was added to achieve a concentration of 0.2 M [6]. Two hours later the cells were collected by centrifuging, resuspended in TNE buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM EDTA; pH 7.4) and lysozyme (Calbiochem) was added at a concentration of 1 mg/ml. The resuspended cells were incubated at 37 °C for 30 min, then for 2 h at room temperature with 0.1 volume of 10% sodium dodecylsulphate.

Nucleic acids were extracted with the phenol method [7]. For removing RNA the extract was dissolved in 0.3 N KOH and kept at 37 °C for 18 h. After adjusting the pH to 1.0 the resulting pellet was centrifuged, washed with 0.1 N HCl, then with 60, 70 and 80% ethanol and dried.

For preparing labelled phage DNA, bacteria grown in the presence of L-(methyl-H^3)-methionine were resuspended in fresh Sauton medium (containing 4 mM and 1 mM $CaCl_2$ for Phage phlei and butyricum, respectively), infected with the respective phages at a multiplicity of 1, and L-(methyl-H^3)-methionine was added in the concentration described above. After 18 h phage lysates were concentrated and purified as described earlier [5]. DNA of the purified phages was extracted according to MANDELL and HERSHEY [8].

DNA hydrolysis and chromatography. DNA was hydrolysed with 70% perchloric acid at 100 °C for 60 min.

Chromatography was carried out on Whatman I paper using a butanol–water–ammonia (60 : 10 : 0.1) solvent system [9]. Developed chromatograms were cut up in 2 cm strips and eluted with 0.1 N HCl. Radioactivity was measured in a Packard 3330 scintillation counter using Bray scintillation cocktail [10].

The eluted samples were occasionally concentrated and rechromatographed together with appropriate reference methyl bases either on MN 300 cellulose layers using methanol–HCl–water (65 : 17 : 18) and/or butanol–methanol–water–ammonia (60 : 20 : 20 : 0.1) solvent system [11] or on Fixion-50 × 8 chromatoplates (Chinoin, Nagytétény) precoated with a strong cation exchanger. In the latter case 2.8 N HCl was used for developing chromatograms [12]. The appropriate spots were eluted with 0.1 N HCl from the cellulose MN 300 layers and with 10% NaCl in 0.1 N HCl at 80 °C for 10 min from the Fixion-50 × 8 plates. Radioactivity was determined as described above.

Results

Table I shows the radioactivity of the bacterial and phage preparations. All the DNAs examined proved to be highly radioactive. Specific activity of bacterial DNAs was markedly higher than that of phage DNAs. DNAs of *M. butyricum* and Phage butyricum had a higher specific activity than that of *M. phlei* and Phage phlei.

Table I

Specific activity of [H^3]*-methyl labelled bacterium and phage DNA*

	dpm/10 μg DNA
M. butyricum	53.190
Phage butyricum	5.553
M. phlei	10.332
Phage phlei	1.446

Radioactivity of eluates of the paper strips obtained after chromatography is shown in Fig. 1. It can be seen that the pattern of radioactivity was different in the four kinds of DNAs examined. In the DNA of *M. butyricum* and Phage butyricum, activity prevailed in strips corresponding to purine bases (guanine and adenine) while the pattern of *M. phlei* and Phage phlei DNA proved to be more diffuse; practically all strips were more or less radioactive.

The results presented in Fig. 1 demonstrate the distribution of labelled DNA bases using L-(methyl-H^3)-methionine as methyl donor, but do not answer the question whether or not DNA of mycobacteria and their phages contains methylated bases.

To identify the presence of minor bases, corresponding areas of chromatograms were eluted, concentrated and rechromatographed as described in

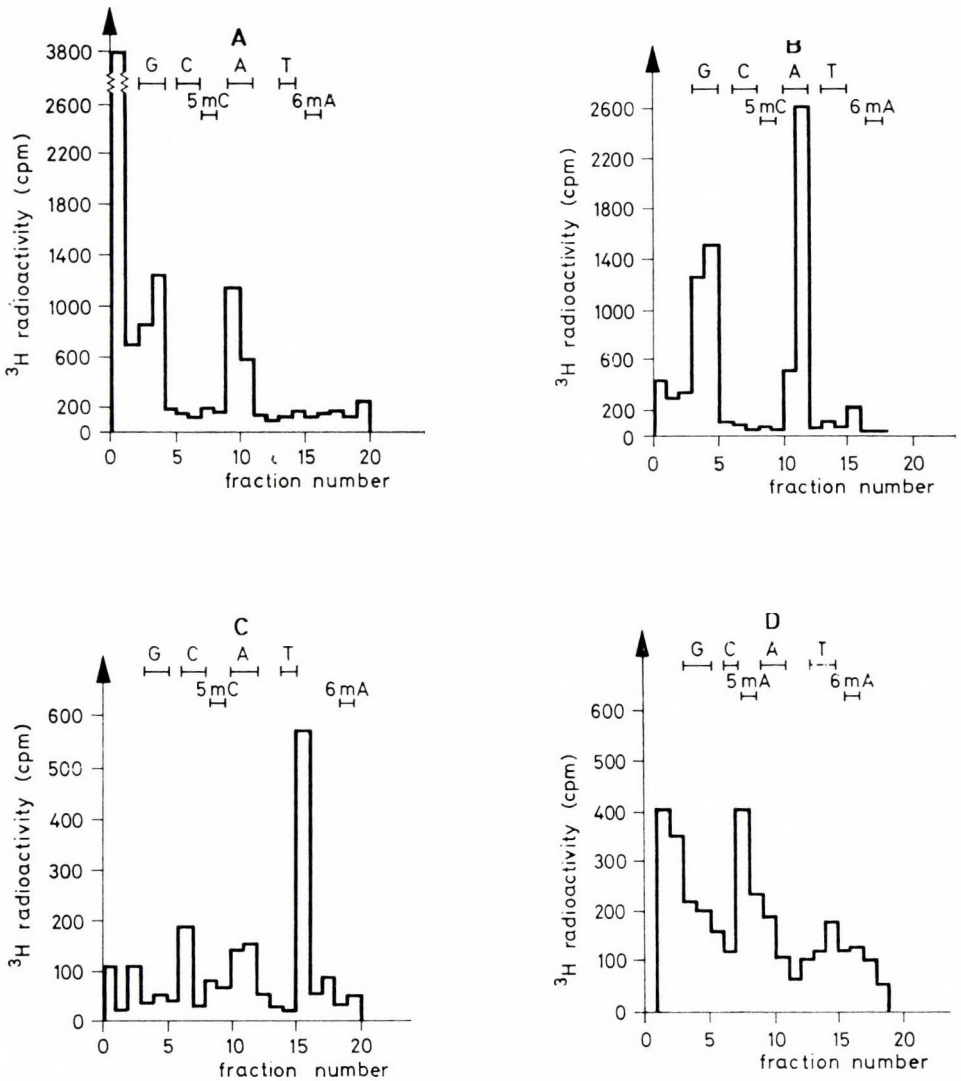

Fig. 1. Patterns of [H³]-methyl-labelled bacterium and phage DNA bases obtained by paper chromatography. Positions of nucleic acid bases are marked on the upper part of the figure. A = *M. butyricum* DNA; B = Phage butyricum DNA; C = *M. phlei* DNA; D = Phage phlei DNA. Abbreviations: G = guanine, A = adenine, 5mC = 5-methyl-cytosine, T = thymine, 6mA = 6-methyl-aminopurine

Materials and methods. Table II shows that in all DNA preparations studied radioactivity was found at positions of 5-methyl-cytosine and 6-methyl-aminopurine.

Table II

Rechromatography of fractions of [H^3]*-methyl labelled bacterium and phage DNA hydrolysates obtained by paper chromatography on Cellulose MN 300 thin-layers and on Fixion-50×8 chromatoplates for determination of methylated bases*

	Solvent system	[cpm] on area corresponding to	
		5-methyl-cytosine	6-methyl-aminopurine
M. butyricum	BMA	362	529
	MHW	284	1052
	Fixion	235	546
Phage butyricum	BMA	179	378
	MHW	55	220
	Fixion	—	—
M. phlei	BMA	183	256
	MHW	—	—
	Fixion	123	215
Phage phlei	BMA	1559	401
	MHW	77	150
	Fixion	—	—

BMA = butanol–methanol–ammonia solvent system
MHW = methanol–HCl–water solvent system
Fixion = Fixion-50×8 ion exchange chromatography

Discussion

A considerable incorporation of the labelled methyl group into the DNA of the two mycobacterial strains and mycobacteriophages investigated was observed. L-Methionine is the direct precursor of S-adenosyl-methionine, the methyl donor for the methylation reaction. Specifc DNA methylases transfer the methyl group from S-adenosyl-methionine to adenine and cytosine residues at specific sites of the DNA. Chromatographic analysis of DNA hydrolysates revealed that the DNAs examined contain the usual methylated bases 5-methyl-cytosine and 6-methyl-aminopurine [3].

The methyl group of L-methionine, however, could react with tetrahydrofolic acid forming N^5-methyl-tetrahydrofolate. Then two pathways are possible: (i) to participate in forming the purine ring, (ii) to methylate deoxyuridine-monophosphate forming thymidine-monophosphate. Thus, the radioactivity from the labelled methyl group of L-methionine might be present not only in the purine rings (adenine and guanine) but also in the methyl group

of thymine and this explains why we could detect radioactivity also in areas corresponding to thymine.

The presence of the same methyl bases in the DNA of mycobacteria and mycobacteriophages raises the question whether phage specific or host specific methylases are responsible for the methylation of phage DNA. Further studies are in progress in this regard.

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REFERENCES

1. ARBER, W.: Science **205,** 361 (1979).
2. WARREN, R. A. J.: Ann. Rev. Microbiol. **34,** 137 (1980).
3. HALL, R. M.: The Modified Nucleosides in Nucleic Acids. Columbia University Press, New York and London 1971. p. 281.
4. VANYUSHIN, B. I., BELOZERSKY, A. N., KOKURINA, N. A., KADIROVA, D. H.: Nature (Lond.) **218,** 1066 (1968).
5. SOMOGYI, P. A., PETROVSKY, G. V., GRIGOREV, V. B.: J. gen. Virol. **29,** 235 (1975).
6. MIZUGUCHI, Y., TOKUNAGA, T.: J. Bact. **104,** 1020 (1970).
7. SOMOGYI, P. A., PETROVSKY, G. V., FÖLDES, I.: Acta microbiol. Acad. Sci. hung. **25,** 37 (1978).
8. MANDELL, J. D., HERSHEY, A. D.: Anal. Biochem. **1,** 66 (1960).
9. NIKOLSKAYA, I. I., LOPATINA, N. G., DEBOV, S. S.: Biochim. biophys. Acta (Amst.) **435,** 206 (1976).
10. BRAY, G. A.: Anal. Biochem. **1,** 279 (1960).
11. RANDERATH, K., RANDERATH, E.: In GROSSMAN, L., MOLDAVE, K. (eds): Methods in Enzymology. Vol. 12/A. Academic Press, New York and London 1967. p. 323.
12. TOMASZ, J.: Anal. Biochem. **68,** 226 (1975).

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LOCALIZATION OF GENES CODING FOR MACROLIDE RESISTANCE ON THE PENICILLINASE PLASMID OF ISOLATES OF AN EPIDEMIC *STAPHYLOCOCCUS AUREUS*

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Genetic localization of gene(s) coding for erythromycin and oleandomycin resistance in five isolates of phage complex 52, 52A, 80, 81, multiple antibiotic resistant epidemic *Staphylococcus aureus* was studied. The 100% coelimination rate of erythromycin, oleandomycin, penicillin (penicillinase production) and certain heavy metal ion resistances from each of the clinical isolates and the 100% cotransduction rate of these resistance markers from two clinical isolates as well as changes in the partially purified extrachromosomal DNA patterns of the clinical wild types after elimination and the recipients after transduction indicated that erythromycin and oleandomycin resistance determining gene(s) resided on the penicillinase–heavy metal ion resistance plasmid in each of the isolates. The electrophoretic mobility of these macrolide–penicillinase–heavy metal ion resistance plasmids (MacPc plasmids) was the same in four strains and higher in one strain. These MacPc plasmids did not confer any resistance to spiramycin and lincomycin (even after induction) or to kanamycin, which features differentiate them from MacPc plasmids pI 258 and pTU 512 formely identified in *Staphylococcus aureus* in Japan.

A total of 654 *S. aureus* strains were isolated during an outbreak at three epidemiologically related day-nurseries in Pest county, Hungary, in the years 1977 to 1979. More than one half of the isolates (58.3%) belonged to one phage complex, the 52, 52A, 80, 81-complex, and proved to be multiple antibiotic resistant, mainly with a pattern of erythromycin, penicillin, chloramphenicol and tetracycline resistance. As many as 328 isolates out of the 381 belonging to the 52, 52A, 80, 81-complex were tested for their macrolide–lincosamide cross-resistance using erythromycin, oleandomycin, spiramycin and lincomycin disks. Four different cross-resistance patterns were found among the isolates; 239 strains proved to be resistant to erythromycin and moderately resistant to oleandomycin but sensitive to spiramycin and lincomycin (ery-r ole-r strains); 81 strains were resistant to erythromycin and oleandomycin as well as inducibly resistant to spiramycin and lincomycin induced by both erythromycin and oleandomycin (ery-r ole-r spi-ir lcm-ir strains); 7 strains showed erythromycin resistance as well as erythromycin induced oleandomycin, spiramycin and lincomycin resistance (ery-r ole-ir spi-ir lcm-ir strains); and a single strain proved to be resistant to each examined drug (ery-r ole-r spi-r lcm-r strain). The similarity in phage sensitivity and dissimilarity in

macrolide–lincosamide cross-resistance of the isolates can be explained either by a change in their genetic status involving macrolide and lincosamide resistance determinant(s) or by supposing the causative agents to have originated from different clones. To prove or exclude the one clone origin of the 52, 52A-80, 81-complex strains required the characterization of their macrolide–lincosamide resistance determinants in more detail. This paper deals with the genetic localization of gene(s) encoding the ery-r ole-r phenotype that was characteristic of the majority of 52, 52A, 80, 81-complex strains at the beginning of the outbreak. Results of the genetic analysis of *S. aureus* strains displaying the other macrolide–lincosamide cross-resistance phenotype will be described elsewhere.

Materials and methods

Bacterial strains. Five *S. aureus* clinical isolates were chosen for this study. Some characteristics of the clinical wild type strains as well as of *S. aureus* strains of laboratory origin are shown in Table I. Each of the wild type strains was isolated during the outbreak at nurseries in Pest county in 1977 and was identified at the National Institute of Hygiene, Budapest. *S. aureus* RN 981 was obtained from Dr. E. H. Asheshov. Strain BE 1222/2 was isolated in the same outbreak and cured of its macrolide-lincosamide resistance determinant in our aboratory.

Table I

S. aureus strains

Strain	Origin	Phage pattern at RTD × 100*	Antibiotic resistance phenotype**	Reference
PM 246	clinical wild type	80/81	pen$^+$ chm-r tet-r ery-r ole-r	this paper
PM 1669	ditto	80	ditto	ditto
PM 2104	ditto	80	ditto	ditto
PM 2017	ditto	80	ditto	ditto
PM 1424	ditto	52/52A/80	pen$^+$ chm-r ery-r ole-r	ditto
NCTC 8325	laboratory	47/53/75/77/84/85/ +	sensitive	[1]
RN 981	ditto	47/53/75/77/84/85/ +	sensitive	[2, 3]
NCTC 8360	ditto	strong reaction with each basic set phage except for 3A and 94	nov-r	[1]
BE 1222/2	ditto	6/47/53/ +	pen$^+$ chm-r tet-r	this paper

* + = further weak reactions.

** pen$^+$ = penicillinase production; chm-r = chloramphenicol resistance; tet-r = tetracycline resistance; ery-r = erythromycin resistance; ole-r = oleandomycin resistance; nov-r = novobiocin resistance.

Media. For the resistance elimination experiments and for the MIC determination of penicillin, erythromycin and oleandomycin, CY broth and agar [4] were used. Agar diffusion antibiotic sensitivity tests were carried out on Mueller–Hinton agar containing 2 ml per litre of Lissamine rhodamine B 200 from a 0.2% stock solution. The medium used for phage-typing,

for transduction experiments and for detection of penicillinase production consisted of yeast extract, 5 g; Witte peptone, 10 g; NaCl, 5 g per litre distilled water, pH 7.5, supplemented with 11 g of Oxoid No. 1 agar when used as a solid medium. For MIC determination of heavy metal ions, spiramycin and lincomycin, Oxoid Nutrient Broth No. 2 was used. Additives to media will be described later.

Antibiotic sensitivity tests. In agar diffusion sensitivity tests the following set of Resistest (Human, Budapest) disks was used: penicillin (3 IU), chloramphenicol (30 μg), tetracycline (30 μg), erythromycin (10 μg), oleandomycin (30 μg), spiramycin (30 μg), lincomycin (10 μg), oxacillin (10 μg), streptomycin (30 μg), neomycin (100 μg), kanamycin (30 μg), novobiocin (30 μg) and gentamicin (20 μg). Macrolide–lincosamide cross-resistance of the strains was tested by a modified method of WEISBLUM and DEMOHN [5] in which the soft agar overlayer was omitted.

In quantitative antibiotic sensitivity tests an agar dilution method [6] was used for the estimation of MIC values of erythromycin (Sigma), oleandomycin phosphate (Sigma) and benzylpenicillin (Biogal). Plates were prepared containing the drugs in the following concentration ranges: erythromycin from 0.39 to 200 μg/ml, oleandomycin from 2.4 to 1250 μg/ml, and penicillin from 0.39 to 25 μg/ml. MICs of spiramycin (Selectomycin, Chemie Grünenthal) and lincomycin hydrochloride (V/O Medexport) were determined by tube dilution test [6]. The tubes contained 0.7 to 12 μg/ml of both drugs. The same tube dilution method was used to determine MICs of mercury(II) chloride, cadmium sulphate, disodium hydrogen arsenate and lead acetate, using 6.25 to 100 μg/ml, 3.9 to 250 μg/ml, 25 to 400 μg/ml, and 6.25 to 100 μg/ml concentration ranges, respectively.

Detection of penicillinase production. The plate method of WORKMAN and FARRAR [7] was used.

Phyge-typing. Propagation, quantitative and qualitative control of *S. aureus* typing phages and interpretation of the results were carried out according to BLAIR and WILLIAMS [1]. The basic set of phages consisted of the recommended ones [8].

Elimination of antibiotic resistance markers. The procedure of curing and selection for penicillin or erythromycin has been described elsewhere [9]. Eliminant colonies were identified by phage typing, tested for antibiotic resistance pattern including macrolide–lincosamide cross-resistance, and for penicillinase production.

Propagation of transducing phage. S. aureus typing phage 80 (NCTC 9788) was used as transducing vector in all resistance transfer experiments. Broth propagation [1] of phage 80 on donor strains was carried out in three successive cycles. Phage suspensions were filtered through syntered glass filters, titrated on the donor strains and stored at 4 °C.

Preparation of antiphage serum. Preparation of antiphage serum against *S. aureus* typing phage 55 (NCTC 8429) and K-value determination were performed according to the method of ADAMS [10]. In transduction experiments as much serum was added to the phage–bacterium mixture which completely inhibited plaque formation of transducing phage in 15 min at 37 °C.

Transduction experiments. Transductions were carried out by a modified method of STIFFLER *et al.* [11]; instead of sodium citrate, antiphage serum was added to prevent further phage adsorption. Selection was carried out either on plates containing benzylpenicillin in a concentration of 0.1 μg/ml for recipients NCTC 8325 and RN 981, and 0.7 μg/ml for recipients NCTC 8360 and BE1222/2, or on plates containing 0.6 μg/ml erythromycin lactobionate (Antibiotics Plant, Jassy). Transductants were identified as described above for the eliminants.

Preparation of partially purified plasmid DNAs and electrophoresis. The lysostaphin–Brij-cleared lysate–phenol and chloroform extraction–alcoholic concentration method to obtain partially purified extrachromosomal DNAs and the technique of agarose-gel electrophoresis of the preparates were described elsewhere [12]; instead of Tris-acetate, Tris-borate buffer [13] was used to fill the tanks of the electrophoresis apparatus and to prepare 0.7% agarose gels.

Results

Characterization of macrolide–lincosamide cross-resistance of strains PM246, PM1424, PM1669, PM2017 and PM2104. Each clinical wild type strain grew near the edge of the erythromycin disk, showed an inhibition zone of approximately 12 to 14 mm around the oleandomycin disk and, a larger than 20 mm inhibition zone around the spiramycin and lincomycin disks

(Fig. 1). None of the inhibition zones was deformed in any direction. Comparing this macrolide–lincosamide cross-resistance pattern to that of the strain NCTC 8325 with inhibition zones larger than 20 mm around erythromycin, oleandomycin, spiramycin and lincomycin disks, the clinical wild type strains PM246, PM1424, PM1669, PM2017 and PM2104 were thought to be resistant to erythromycin, "moderately" resistant to oleandomycin, and sensitive to spiramycin and the lincosamide representative lincomycin. Neither erythromycin nor oleandomycin acted as an inducer in spiramycin or lincomycin resistance in these strains. Sensitivity of the wild type strains to spiramycin and lincomycin was confirmed by quantitative antibiotic sensitivity tests as shown in Table II.

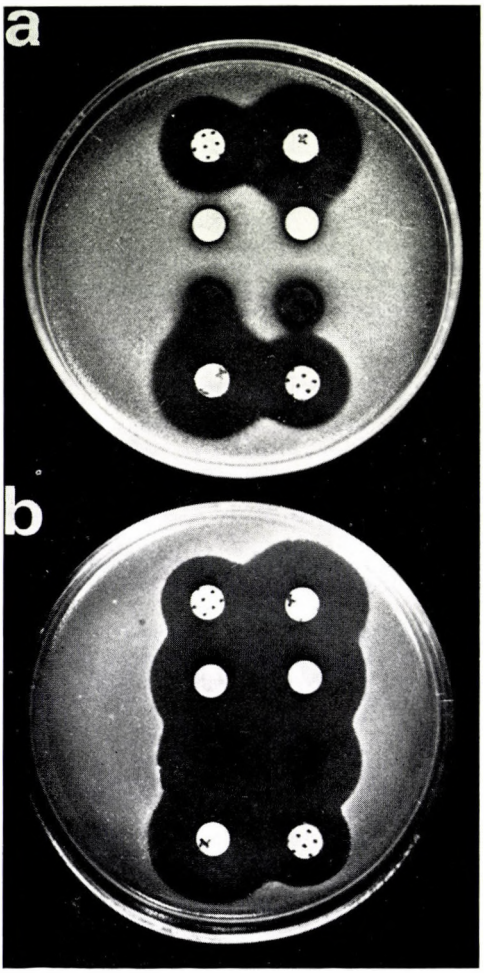

Fig. 1. Macrolide–lincosamide cross-resistance pattern of the clinical wild type strains. *S. aureus* PM 2104 (a); *S. aureus* NCTC 8325 (b). Antibiotic disks: spiramycin, erythromycin, oleandomycin and lincomycin (left, from top to bottom); lincomycin, erythromycin, oleandomycin and spiramycin (right, from top to bottom)

Table II

MIC values of spiramycin and lincomycin for S. aureus NCTC 8325 and the five clinical wild type strains

Strain	MIC values (μg/ml)	
	spiramycin	lincomycin
NCTC 8325	1.5	3.0
PM 246	3.0	1.5
PM 1424	3.0	1.5
PM 1669	6.0	1.5
PM 2017	3.0	< 0.7
PM 2104	6.0	3.0

Linkage of determinant(s) of erythromycin and oleandomycin resistance to determinants of penicillinase production and certain heavy metal ion resistances in strains PM246, PM1424, PM1669, PM2017 and PM2104. Data in Table III show that treatment of each of the clinical wild types with ethidium bromide and selection for eliminants on penicillin or erythromycin — containing agar plates resulted in detection of only one type of eliminant colonies; erythromycin and oleandomycin sensitive colonies with no penicillinase production. Neither a separate loss of macrolide resistance and penicillinase production nor a coelimination of other resistance markers was observed. Strains PM246 and PM2104 were used as donors in resistance transfer experiments. Data in Table IV show that two types of transductant colonies were observed depending on the recipient strain used. Transductant colonies derived from recipient strain BE1222/2 became resistant to erythromycin and oleandomycin, while colonies derived from the other recipients proved to be cotransductants of erythromycin and oleandomycin resistance as well as penicillinase production not depending on the selective drug used. No cotransduction of other antibiotic resistance traits was observed using benzylpenicillin or erythromycin selection. Data inculded in Table V show that both linked elimination and linked transduction of determinants of macrolide resistance and penicillinase production resulted in a change of the MIC value not only of erythromycin, oleandomycin and benzylpenicillin but of certain heavy metal ions as well. The determinant of cadmium resistance was coeliminated from each wild type strains, and also the mercury resistance of each strain but PM2017 originally sensitive to mercury ions, while the determinant of lead ion resistance only from strains PM246, PM1424 and PM2104. After transduction in one transductant derivative, *S. aureus* JL47/32, linkage of determinants of cadmium, mercury and lead ion resistances to determinants of macrolide resistance and penicillinase production could clearly be demonstrated.

Table III

Elimination of macrolide resistance from the five clinical wild type strains

Strain	Antibiotic resistance phenotype before elimination*	No. of colonies tested	Antibiotic resistance phenotype after elimination**	Frequency of elimination, %
PM 246	pen$^+$ chm-r tet-r ery-r ole-r	320	pen$^-$ chm-r tet-r ery-s ole-s	31.0
PM 1424	pen$^+$ chm-r ery-r ole-r	140	pen$^-$ chm-r ery-s ole-s	17.0
PM 1669	pen$^+$ chm-r tet-r ery-r ole-r	240	pen$^-$ chm-r tet-r ery-s ole-s	12.0
PM 2017	ditto	400	ditto	50.0
PM 2104	ditto	240	ditto	40.0

* Abbreviations used are the same as in Table I.

** pen$^-$ = no penicillinase production; ery-s = sensitive to erythromycin; ole-s = sensitive to oleandomycin

Table IV

Transduction of macrolide resistance from the two clinical wild type strains

Transductional cross*	No. of colonies tested	Phenotypic markers appeared in the recipients after transduction**	No. of colonies showing the phenotypic change
PM 246 $\xrightarrow[\text{Em}]{80}$ BE 1222/2	1	ery-r ole-r	1
PM 246 $\xrightarrow[\text{Em}]{80}$ NCTC 8360	20	pen$^+$ ery-r ole-r	20
PM 246 $\xrightarrow[\text{Pc}]{80}$ NCTC 8360	20	ditto	20
PM 2104 $\xrightarrow[\text{Em}]{80}$ BE 1222/2	20	ery-r ole-r	20
PM 2104 $\xrightarrow[\text{Em}]{80}$ NCTC 8360	20	pen$^+$ ery-r ole-r	20
PM 2104 $\xrightarrow[\text{Pc}]{80}$ NCTC 8360	20	ditto	20
PM 2104 $\xrightarrow[\text{Em}]{80}$ NCTC 8325	4	ditto	4
PM 2104 $\xrightarrow[\text{Em}]{80}$ RN 981	1	ditto	1

* Description of transductional crosses: donor $\xrightarrow[\text{selective drug}]{\text{vector}}$ recipient; Pc = penicillin; Em = erythromycin.

** Abbreviations used are the same as in Table I.

Table V

MIC values of penicillin, erythromycin, oleandomycin and heavy metal ions for the five clinical wild type strains, for their eliminants and transductants, and for the recipients used in the respective transductional crosses

Strain	Origin*	MIC values (μg/ml) of**						
		Pc	Em	Om	$[Hg^{2+}]$	$[Cd^{2+}]$	$[AsO_4^{2-}]$	$[Pb^{2+}]$
PM 246	clinical wild type	>25.0	50.0	19.0	100.0	250.0	200.0	>100.0
BE 246/1	eliminant of PM 246	<0.04	<0.39	<2.4	6.25	3.9	200.0	<12.5
NCTC 8360	laboratory, recipient	0.09	<0.39	<2.4	25.0	250.0	200.0	>100.0
JL 73/82	PM 246 $\xrightarrow[\text{Em}]{80}$ NCTC 8360, transductant	>25.0	25.0	19.0	>100.0	62.5	400.0	>100.0
PM 1424	clinical wild type	25.0	25.0	9.9	>100.0	250.0	>400.0	>100.0
BE 1424/1	eliminant of PM 1424	<0.04	<0.39	2.4	<6.25	<3.9	>400.0	<6.25
PM 1669	clinical wild type	>25.0	200.0	19.0	>100.0	250.0	400.0	>100.0
BE 1669/1	eliminant of PM 1669	<0.04	<0.39	<2.4	12.5	31.0	>400.0	>100.0
PM 2017	clinical wild type	>25.0	50.0	9.9	<6.25	250.0	>400.0	50.0
BE 2017/1	eliminant of PM 2017	<0.04	<0.39	<2.4	<6.25	15.0	400.0	>100.0
PM 2104	clinical wild type	>25.0	50.0	19.0	>100.0	250.0	400.0	>100.0
BE 2104/1	eliminant of PM 2104	<0.04	<0.39	<2.4	<6.25	3.9	200 0	<6.25
NCTC 8325	laboratory, recipient	<0.04	<0.39	<2.4	<6.25	<3.9	100.0	<6.25
JL 47/32	PM 2104 $\xrightarrow[\text{Em}]{80}$ NCTC 8325, transdnctant	25.0	25.0	9.9	>100.0	125.0	400.0	>100.0

* Description of transductional crosses is the same as in Table IV.

** Pc = penicillin; Em = erythromycin; Om = oleandomycin; (Hg^{2+}) = mercury ion; (Cd^{2+}) = cadium ion; (AsO_4^{2-}) = arsenate ion; Pb^{2+}] = lead ion.

Relation of linked determinants of macrolide resistance, penicillinase production and heavy metal ion resistances to extrachromosomal DNA fractions in strains PM246, PM1424, PM1669, PM2017 and PM2104. Figure 2 shows extrachromosomal DNA patterns of strains PM246 clinical wild type (lane 1), BE246/1, a coeliminant of PM246 lacking macrolide resistance, penicillinase production and heavy metal ion resistances (lane 2), *S. aureus* NCTC 8360

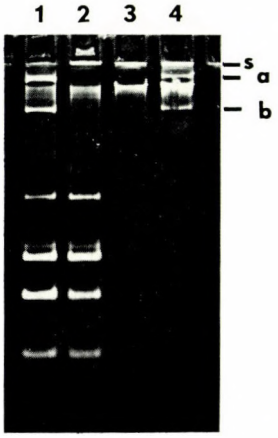

Fig. 2. Extrachromosomal DNA patterns of *S. aureus* PM 246 and its derivatives. *S. aureus* PM 246 clinical wild type strain (1); *S. aureus* BE 246/1 eliminant of PM 246 (2); *S. aureus* NCTC 8360 recipient (3); *S. aureus* JL 73/82 transductant (4). Electrophoretic separation was from top to bottom. An "s" mark indicates the start of electrophoretic separation. For further details, see the text

used as recipient in PM246 $\xrightarrow[\text{Em}]{80}$ NCTC 8360 transduction experiment (lane 3), and JL73/82, a transductant obtained in this transduction experiment (lane 4). In Fig. 2 an "s" mark indicates the start of electrophoretic separation. Comparing the pattern of PM246 to that of BE246/1, it can be seen that coelimination of the resistance markers was accompanied by a loss of two DNA bands from the cells of the clinical wild type strain in positions indicated by "a" and "b" in the picture. Strain 73/82 showed two additional DNA bands in its extrachromosomal DNA pattern in the same "a" and "b" positions as compared to the pattern of NCTC 8360. Figure 3 shows the extrachromosomal DNA patterns of strains PM2104 clinical wild type (lane 1), BE2104/1, an eliminant of PM2104 which has lost the capability to produce penicillinase and resistance to macrolide antibiotics and heavy metal ions (lane 2), *S. aureus* NCTC 8325 used as recipient in the PM2104 $\xrightarrow[\text{Em}]{80}$ transduction experiment (lane 3), and JL47/32, a transductant isolated in the same transduction experiment (lane 4). Marks "a" and "b" indicate the position of two DNA bands

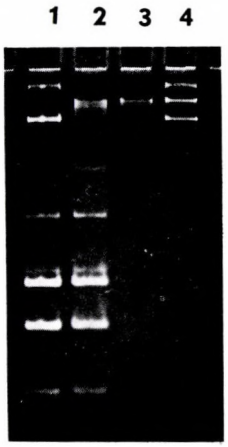

Fig. 3. Extrachromosomal DNA pattern of *S. aureus* PM 2104 and its derivatives. *S. aureus* PM 2104 clinical wild type strain (1); *S. aureus* BE 2104/1 eliminant of PM 2104 (2); *S. aureus* NCTC 8325 recipient (3); *S. aureus* JL 47/32 transductant (4). Electrophoretic separation was from top to bottom. An "s" mark indicates the start of electrophoretic separation. For further details, see the text

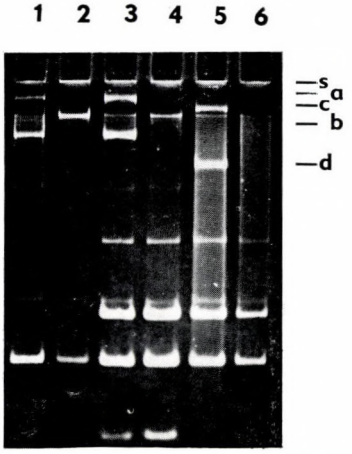

Fig. 4. Extrachromosomal DNA patterns of *S. aureus* PM 1424, PM 1669, PM 2017 and their derivatives. *S. aureus* PM 1424 clinical wild type strain (1); *S. aureus* BE 1424/1 eliminant of PM 1424 (2); *S. aureus* PM 1669 clinical wild type strain (3); *S. aureus* BE 1669/1 eliminant of PM 1669 (4); *S. aureus* PM 2017 clinical wild type strain (5); *S. aureus* BE 2017/1 eliminant of PM 2017 (6). Electrophoretic separation was from top to bottom. An "s" mark indicates the start of electrophoretic separation. For further details, see the text

present in PM2104 before elimination and in JL47/32 after transduction but absent in the BE2104/1 eliminant and *S. aureus* NCTC 8325 recipient. Figure 4 shows extrachromosomal DNA patterns of strains PM1424 (lane 1), PM1669 (lane 3), PM2017 (lane 5) clinical wild types as well as BE1424/1 (lane 2),

BE1669/1 (lane 4) and BE2017/1 (lane 6) eliminants for penicillinase production, macrolide and heavy metal ion resistances derived from PM1424, PM1669 and PM2017, respectively. DNA bands in positions "a" and "b" present in strains PM1424 and PM1669 were absent in their respective eliminants. Strain PM2017 had no DNA bands in "a" and "b" positions but after elimination the DNA bands in "c" and "d" positions were absent. Figure 5 shows extrachromosomal DNA patterns of the five clinical wild type strains in one electrophoretic run.

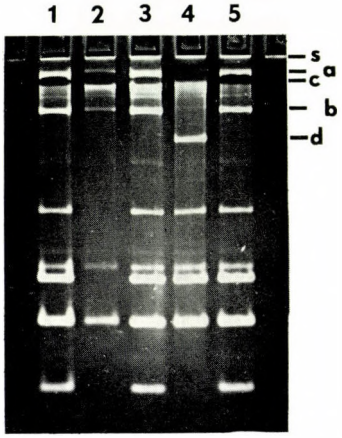

Fig. 5. Extrachromosomal DNA patterns of *S. aureus* clinical wild type strains. *S. aureus* PM 246 (1); *S. aureus* PM 1424 (2); *S. aureus* PM 1669 (3); *S. aureus* PM 2017 (4); *S. aureus* PM 2104 (5). Electrophoretic separation was from top to bottom. An "s" mark indicates the start of electrophoretic separation. For further details, see the text

DNA bands related to genetic determinants of penicillinase production as well as macrolide and heavy metal ion resistances in the strains run with approximately the same velocity in strains PM246 (lane 1), PM1424 (lane 2), PM1669 (lane 3) and PM2104 (lane 5), while that of strain PM2017 (lane 4) moved faster: bands present in "a" and "b" as well as in "c" and "d" positions, respectively.

Discussion

The present study was aimed at establishing the genetic location of macrolide resistance determinant(s) in five *S. aureus* strains chosen from among the 239 isolates of a multiple antibiotic resistant, 52, 52A, 80, 81-complex epidemic organism which exhibited erythromycin resistance and oleandomycin resistance but no spiramycin and lincomycin resistance. A series of data obtained in resistance elimination and transduction experiments as

well as in gel electrophoretic analysis of extrachromosomal DNA preparations suggest that each of the five clinical wild type strains examined carries a penicillinase and heavy metal ion resistance determining plasmid which also harbours the gene(s) governing erythromycin and oleandomycin resistance in the cells.

Most of the experimental data supporting this statement have been collected in the case of strains PM246 and PM2104. These data include a 100% coelimination rate of the genes governing macrolide resistance with those governing penicillinase production. This was accompanied in the BE246/1 and BE2104/1 eliminant derivatives tested by a loss of genes governing the mercury, cadmium and lead resistances in the cells. The elimination of this linkage group of genes from strains PM246 and PM2104 resulted in the loss of at least two well visible DNA bands from the extrachromosomal DNA pattern of both wild type strains indicating the relationship of linkage group of genes governing penicillinase production, macrolide and heavy metal ion resistances with extrachromosomal DNA fractions in strains PM246 and PM2104. It is worth mentioning that unless a special effort is made in purifying plasmid DNAs [e.g. 14–17], one plasmid species can be present in more than one molecular form in the preparations [18]. The different molecular forms of a plasmid separate in gel electrophoresis at different velocities [19]. This allows to interpret the results of elimination and gel electrophoretic analysis of DNAs of the wild type strains and their eliminant derivatives as elimination of one plasmid species coding for penicillinase production as well as macrolide and heavy metal ion resistances from strains PM246 and PM2104. In transductional crosses using PM246 and PM2104 as donors and NCTC 8360, NCTC 8325 as well as RN981 (recombination deficient) strains as recipients, all the 85 transductant derivatives isolated proved to be cotransductant for erythromycin and oleandomycin resistances, penicillinase production as well as heavy metal ion resistances. The latter was demonstrated in at least one transductant. Extrachromosomal DNA patterns of transductant derivatives JL73/82 and JL47/32 displayed two additional DNA bands as compared to the patterns of their respective recipients proving the relationship of penicillinase production, macrolide and heavy metal ion resistance governing linkage group of genes with extrachromosomal DNA fractions in the transductants. Moreover, the two additional DNA bands in the transductants appeared in the same position as those which disappeared from the wild types after elimination. These transductional as well as gel electrophoretic data can be interpreted as transduction of one plasmid species coding for penicillinase production as well as macrolide and heavy metal ion resistances from both clinical wild type strains PM246 and PM2104, and fully confirm the data obtained by elimination experiments. There were 21 transductant derivatives of BE1222/2 recipient in which cotransduction of macrolide resistance with penicillinase production was not detected. Although no effort was made to prove it, it is presumed that, as in the other three recip-

ients, cotransduction of genes of penicillinase production and macrolide resistance might occur in this recipient. The resident penicillinase production of the recipient, whether it was coded by chromosomal genes [20–23] or by a penicillinase plasmid of another incompatibility group [24], could mask this genetic event, or might have been replaced by the expression of the newly incorporated genes in the case of the presence of a penicillinase plasmid of the same incompatibility group [24]. In no case could the change be detected, at least by the method used in these experiments, in the penicillinase producing status of the transductant derivatives as compared to the recipient strain. These later transductional data of course do not confirm but do not exclude the validity of the transductional data obtained with recipients NCTC 8360 NCTC 8325 and RN981.

Data of elimination experiments as well as comparative gel-electrophoretic analysis of extrachromosomal DNA of wild types and eliminant derivatives are only available for evaluation in the case of strains PM1424, PM1669 and PM2017. All eliminant derivatives of these wild types proved to be coeliminants for erythromycin and oleandomycin resistance as well as for penicillinase production. One eliminant colony of each clinical wild type BE1424/1, BE1669/1 and BE2017/1, was tested further for heavy metal ion resistances and it was found that coelimination of the mercury resistance determinant from strains PM1424 and PM1669, the cadmium resistance determinant from all the three wild types, and the lead resistance determinant from strain PM-1424 had occurred together with those governing penicillinase production and macrolide resistance in the wild type strains. The relationship of penicillinase production, macrolide and heavy metal ion resistance determining linkage group of genes and extrachromosomal DNA fractions of these strains was proved by gel electrophoretic analysis of the DNA of the wild type strains and their eliminant derivatives; coelimination of penicillinase production, macrolide and heavy metal ion resistance from the cells resulted in concomitant loss of two DNA bands from the extrachromosomal DNA patterns of all the three clinical wild type strains. These experimental data are interpreted as an elimination of one plasmid species from the clinical wild type strains PM-1424, PM1669 and PM2017 coding for the penicillinase production and macrolide resistance as well as for certain heavy metal ion resistances of these strains.

Although all the five strains randomly chosen from the 239 erythromycin and oleandomycin resistant isolates of the multiple antibiotic resistant epidemic *S. aureus* were similar in that their gene(s) governing erythromycin and oleandomycin resistance resided on the penicillinase plasmid of the strains, some variations were found in the heavy metal ion resistances coded by these plasmids. Apart from the cadmium resistance determinant and, (except for the mercury sensitive strain PM21017) the mercury resistance determinant which could be localized on the same penicillinase plasmid of these strains,

the site of the lead resistance determinant on the same plasmid could only be demonstrated in strains PM246, PM1424 and PM2104. It needs further studies to clarify to what extent these variations in heavy metal ion resistance can be attributed to the host organisms [24] or to the carried penicillinase plasmids themselves. It also needs further studies to establish whether the penicillinase plasmid identified in the mercury sensitive PM2017 strain, which possesses a higher electrophoretic mobility than those in the other four strains, can be regarded as a naturally occurring deletion mutant of penicillinase plasmids found in strains PM246, PM1424, PM1669 and PM2104, where deletion could have involved some important sequences necessary for mercury resistance, or it is a different penicillinase plasmid carrying some common sequences involved in the erythromycin and oleandomycin resistance of the strains.

Macrolide resistance, penicillinase production and heavy metal ion resistance determining naturally occurring plasmids (MacPc plasmids) were identified in two series of *S. aureus* strains, both from Japan. The MacPc plasmids identified in *S. aureus* by MITSUHASHI *et al.* [25] in 1963 displayed a high level ($>$ 800 μg/ml) macrolide resistance including erythromycin, oleandomycin and spiramycin. Besides, these plasmids also coded for high level lincomycin resistance. The MacPc plasmids described by KONO *et al.* [26] in 1978 resembled in every respect the MacPc plasmids found in 1963 with regard to their high level macrolide–lincosamide resistance, but had the peculiar feature to code for kanamycin resistance. The molecular weight of MacPc plasmid pTU512, (a representative plasmid of the MacPc series described in 1978) was approximately the sum of the molecular weight of plasmid pI258, (a representative of the 1963 series of staphylococcal MacPc plasmids) and a kanamycin resistance plasmid described by STIFFLER *et al.* [27] in *S. aureus*. Accordingly, KONO *et al.* [26] concluded that the new variant of MacPc plasmids could evolve by recombination between a pI258-like penicillinase plasmid and the kanamycin resistance plasmid.

The MacPc plasmids described in this study differed from those found earlier in *S. aureus* in at least four respects: they did not code for kanamycin resistance, lincomycin resistance and, among from the tested macrolides, spiramycin resistance; furthermore, the coded oleandomycin resistance seemed to be slight ($\leq$19.0 μg/ml). Further physicochemical and genetic investigations are needed to explore whether this third variant of MacPc plasmids found in the five isolates of the epidemic *S. aureus* in some nurseries could evolve by certain genetic events from penicillinase plasmids like pI258 or pTU512 and spreading from Japan to our country, or a separate evolutionary pathway played a role in their appearance.

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REFERENCES

1. Blair, J. E., Williams, R. E. O.: Bull. Wld Hlth Org. **24,** 771 (1961).
2. Novick, R. P.: Virology **33,** 155 (1967).
3. Wyman, L., Goering, R. V., Novick, R. P.: Genetics **76,** 681 (1974).
4. Novick, R. P.: J. gen. Microbiol. **33,** 121 (1963).
5. Weisblum, B., Demohn, V.: J. Bact. **98,** 447 (1969).
6. Anderson, T. G.: In Blair, J. E., Lenette, E. H. (eds): Manual of Clinical Microbiology. American Society for Microbiology, Bethesda 1970. pp. 299–310.
7. Workman, R. G., Farrar, W. E., J. infect. Dis. **121,** 433 (1970).
8. Asheshov, E. H., Skalova, R.: Int. J. Syst. Bact. **25,** 233 (1975).
9. Bán, É., Milch, H., Jánosi, L.: Zbl. Bakt. Suppl. **5,** 325 (1976).
10. Adams, M. H.: Bacteriophages. Interscience Publishers, New York 1959. pp. 461–466.
11. Stiffler, P. V., Sweeney, H. M., Cohen, S.: J. Bact. **120,** 934 (1974).
12. Jánosi, L., Bán, É.: Zbl. Bakt. Suppl. **10,** 581 (1981).
13. Greene, P. J., Betlach, M. C., Gooman, H. M., Boyer, H. W.: In Wickner, R. B. (ed.): Methods in Molecular Biology, DNA Replication and Biosynthesis. Dekker, New York 1974. pp. 87–111.
14. Cohen, S. N., Miller, C. A.: Nature (Lond.) **224,** 1273 (1969).
15. Freifelder, D. R., Freifelder, D.: J. molec. Biol. **32,** 15 (1968).
16. Radloff, R., Bauer, W., Vinograd, J.: Proc. nat. Acad. Sci. (Wash.) **57,** 1514 (1967).
17. Zasloff, M., Ginder, G. D., Felsenfeld, G.: Nucleic Acids Res. **5,** 1139 (1978).
18. Clowes, R. D.: Bact. Rev. **36,** 361 (1972).
19. Aaij, C., Borst, P.: Biochim. biophys. Acta (Amst.) **269,** 192 (1972).
20. Asheshov, E. H.: Nature (Lond.) **210,** 804 (1966).
21. Harmon, S. A., Baldwin, J. N., Ten, W. C., Critz, D. B.: Canad. J. Microbiol. **12,** 973 (1966).
22. Poston, S. M.: Nature (Lond.) **210,** 802 (1966).
23. Sweeney, H. M., Cohen, S.: J. Bact. **96,** 920 (1968).
24. Peyru, G., Wexler, L., Novick, R. P.: J. Bact. **98,** 215 (1969).
25. Mitsuhashi, S., Morimura, M., Kono, K., Oshima, H.: J. Bact. **86,** 162 (1963).
26. Kono, M., Sasatsu, M., Ubukata, K., Konno, M., Fujii, R.: Antimicrob. Ag. Chemother. **13,** 691 (1978).
27. Stiffler, P. W., Sweeney, H. M., Schneider, M., Cohen, S.: Antimicrob. Ag. Chemother. **6,** 516 (1974).

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LYMPHOCYTIC CHORIOMENINGITIS (LCM) VIRUS CARRIER CELL CULTURES IN HUNGARIAN LABORATORIES

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One of the HEp-2 sublines maintained in the authors' laboratory was found to carry LCM virus. The virus proved to be identical with the prototype strain LCM-Am except that its multiplication rate in cell cultures and its mouse pathogenicity were limited. Forty-six cell cultures maintained in 10 Hungarian laboratories were examined for LCM carriership. Sixteen cultures including 11 HEp-2 sublines, all originating from a culture brought into Hungary in 1959, proved to carry the virus. Three FL sublines maintained in two laboratories and two sublines, viz. an RK-13 and a HeLa, maintained in a third one, were also contaminated by LCM virus. In these cases, the carrier HEp-2 subline was the probable source of infection and virus transmission is thought to have occurred in the course of manipulation with cell cultures. The necessity of introducing strict preventive measures in tissue culture laboratories is emphasized in the interest of the laboratory workers and for obtaining reliable laboratory results.

We were surprised to note in 1978 that the HEp–2 cell line (subline) maintained in our laboratory contained an antigen which reacted in the immunofluorescence (IF) test with human blood sera positive for antibodies to the LCM virus. The unexpected finding raised a number of questions, viz. (a) is the reaction specific; (b) if it is, i.e. the reaction is due to an LCM virus infection, is the infection latent or persistent; (c) is the carried virus identical with the prototype strain of the LCM virus; (d) where and at what time had become the subline infected; (e) are other cell lines (or sublines) maintained in our laboratories also infected by the LCM virus; (f) are there similarly infected cell lines among those maintained in laboratories collaborating with us in cell culturing?

The present work was undertaken to elucidate these questions.

Materials and methods

Cell lines and cell strains. In this paper all the cell cultures maintained through passage s or kept in deep-frozen state in a given laboratory are designated as cell sublines. All sublines being in continuous passage or kept in deep-frozen state in our laboratories were tested for LCM virus IF antigen. Further samples were sent us from nine laboratories in which, among other sublines, those received from us earlier had been used. Altogether forty-six samples were tested for carriership (see Table II).

Of the cell lines under study, eight (HeLa, HEp-2, HEp-2(C), FL, AV-3, L-41, MRC-5 and HD) had been established from human cells, four (III/1, Vero, BGM and CV-1) from simian cells, and the remaining four from pig (PKS), rabbit (RK-13), Syrian hamster (BHK-21) and mouse (L) cells. The histories of the sublines provided with the same designation were different.

Some sublines with the same designation had originated from different sources, while others had originated from the same source but were maintained in separate ways in the laboratories. We made attempts to find the possible common origin of the sublines on the ground of information kindly supplied by the maintaining institutes.

Virus strains. For comparative studies, the Armstrong E-350 strain of LCM virus (in the following, LCM-Am strain) maintained in mouse passages was used. Infection was initiated in Vero cell cultures with 10% suspension of infected mouse brains which contained 10^7 intracerebral (i.cer.) LD_{50} per 0.03 ml. In IF studies, Vero cells infected with this suspension were used. The strain isolated from our HEp-2 subline was designated as LCM-P (P = persistent).

Infectivity studies in cell cultures. Tube cultures of non-carrier Vero and L-41 sublines being in continuous passage in this laboratory were used in attempts to isolate virus and in the comparative studies. The growth medium contained 10%, the maintenance medium contained 2%, bovine serum in Eagle MEM solution. Ten cultures were inoculated with 0.1 ml of each of undiluted, 10-fold diluted and 100-fold diluted culture fluids. While incubated at 37 °C, one of the 10 cultures was removed for IF tests on each of days 5, 8, 12 and 14, the others were observed microscopically every day for 14 days. The same method was employed in the course of passages in Vero and L-41 cell cultures. Passage materials were titrated as follows. Serial tenfold dilutions were prepared, from 10^{-1} to 10^{-8}, and 4 cultures were inoculated with each dilution (0.1 ml/culture). The cultures were incubated at 37 °C up to the 14th day, when they were examined by IF technique for the presence of LCM antigen in the cells.

Mouse pathogenicity and cross protection tests. Mice of 12–14 g body weight were used in these tests throughout. The intraperitoneal and intracerebral inoculum was 0.3 and 0.03 ml, respectively. Each group of mice treated identically consisted of 10 animals. "Control" mice received 0.03 ml saline i.cer. Groups of mice were inoculated with HEp-2 culture fluids under testing or with virus suspensions from serial passages initiated with the same culture fluids in Vero or L-41 cell cultures. The virus suspensions contained 10^6 $TCID_{50}$/ml. The i.cer.-inoculated mice were observed for 14 days, those inoculated i.p. and the control mice were observed for 28 days. On the 28th day, the mice being still under observation were challenged with 100 LD_{50} of the LCM-Am strain i.cer., and the animals were observed for further 14 days.

Immunofluorescence tests. Details of the IF test have been published elsewhere [1]. The following reagents were used. In the direct method, anti-LCM rabbit immonoglobulin fluorescent conjugate; in the indirect method, rabbit, guinea-pig and mouse antisera, human serum samples from subjects with LCM history, and anti species-globulin fluorescent conjugates.

The *complement fixation (CF) and agar gel immunodiffusion (ID) tests* for the detection of LCM antigen were performed as described elsewhere [2].

Other tests. Ether sensitivity test, iododeoxy-uridine (IDU) inhibition and amantadine inhibition tests were performed as described by GOBARA *et al.* [3], PFAU *et al.* [4] and WELSH *et al.* [5], respectively. IDU was used at a concentration of 50 μg/ml and amantadine at a concentration of 25 μg/ml culture medium.

Electron microscopic studies. Cells of virus carrying HEp-2 cell cultures were removed from the glass wall mechanically and washed in PBS twice. After sedimentation, the cells were fixed in 2.5% glutaraldehyde, each time for an hour. Postfixation was carried out in 1% OsO_4 for 1 h, and the preparation was dehydrated in an alcohol series. Ultrathin sections embedded in Araldite were double-contrasted in 1% uranyl acetate and 1% lead citrate and examined in a JEM 100C electron microscope.

Results

Identification and characterization of the virus carried by our HEp-2 subline. The basic observation was that serum samples from a patient whose LCM ininfection had been proved serologically gave a positive IF reaction with "uninfected" cells of the HEp-2 subline which had been maintained in our laboratory since 1977. The reaction was repeated with human sera of known anti-LCM IgM and anti-LCM IgG titres. The same titres were obtained whether Vero cells infected with the LCM-Am strain or "uninfected" HEp-2 cells were used as antigen. Again, the same results were obtained with immune sera irrespective of the animal species (rabbit, guinea pig or mouse) in which they

had been raised. The antigen, showing cytoplasmic granular IF, was present in 60–70% of the cells in the HEp-2 cultures (Fig. 1).

The soluble antigens prepared by freezing and thawing either from non-inoculated HEp-2 cells, or from Vero cells inoculated with the LCM-Am strain proved to be identical both in CF and ID tests carried out with anti-LCM immune sera.

The electron micrographs prepared from HEp-2 cell cultures showed formations characteristic of arenaviruses, viz. polymorphous enveloped virions with electron-dense granules in them. Some virions were already released from the cells, others were in the state of budding off (Fig. 2).

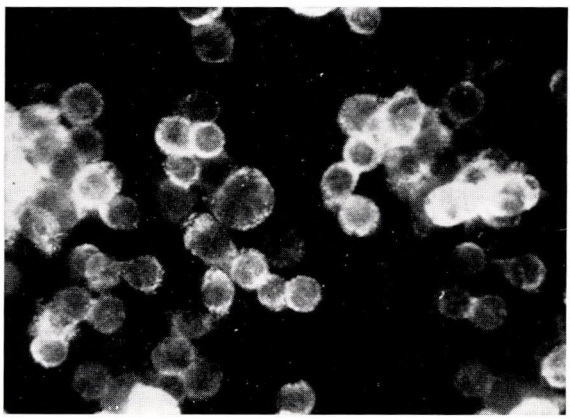

Fig. 1. LCM virus specific IF antigen in cells of the carrier HEp-2 cell line. Coarse granular cytoplasmic fluorescence of variable intensity in different cells

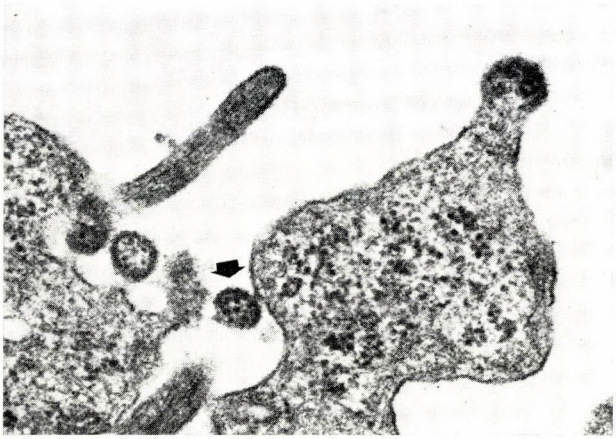

Fig. 2. LCM-P virus particle budding off on a process of a carrier HEp-2 cell. The cytoplasmic membrane is thickened. Note the ribosome-like electron-dense formation beneath the membrane. Arrow points to an extracellular mature virion

Thus the electron microscopic examinations suggested that there was a productive replication cycle advancing in the HEp-2 cells. Infectivity tests performed in Vero and L-41 cultures supported the occurrence of such a cycle. In cultures of these cells inoculated with the undiluted medium of HEp-2 cell cultures, a virus (LCM-P) could be revealed, which multiplied much slower than the LCM-Am strain without any cytopathogenic action but detectable by appearance of LCM specific antigens in the cells. If the virus suspension was titrated in the cell line from which it had originated, the virus reached considerable infective titres as high as 10^6 $TCID_{50}$/0.1 ml. In further passages, however, the titre tended to decline, especially if undiluted inocula were used.

The strain isolated in Vero and L-41 cultures lost its infectivity completely on ether treatment. Its replication in Vero and L-41 cells was not influenced by IDU at the concentration applied but it was significantly inhibited by amantadine.

None of the mice developed illness after i. p. or i. cer. inoculation with culture media obtained from virus carrying HEp-2 cells. The same was observed if mice were inoculated with relatively high-titre suspensions of the LCM-P strain produced in Vero or L-41 cell cultures. The 10 mice inoculated i. p. with the LCM-P strain isolated in Vero cell cultures and another 10 mice inoculated with the same virus isolated in L-41 cultures proved to be resistant to the challenge with the LCM-Am strain given 28 days later. All the control mice died within 7 days after the same challenge.

It may therefore be stated that our HEp-2 cell subline was carrying the virus persistently and that the LCM-P isolate was identical with the prototype strain in all of its properties except the multiplication rate and mouse pathogenicity (see Table I).

LCM virus carriership of sublines maintained in our laboratory and in other laboratories. Based on the IF tests, 16 of the 46 cell cultures proved to carry the LCM virus (Tables I and II). The 16 cultures included 11 HEp-2 sublines which had been maintained in 8 institutes. On the basis of information received from the corresponding institutes, all these had originated from a common source (Table III).

The original Hep-2^b culture was brought into Hungary by a member of Institute G. Samples from the subline maintained in Institute G were supplied to further two institutes in 1960 and 1962, and all the resulting sublines proved to be carriers in the present study. It was therefore assumed that either the culture imported in 1959 had already been carrier or the subline had become infected while it was passaged in Institute G. The incriminated subline was lost in Institute G in 1976 and was replaced there by a subline received from Institute C. The fact, however, that the HEp-2 subline passaged in Institute C proved to be carrier, whereas that passaged in Institute G was found free of the virus seems to be inconsistent with this history of the subline.

Table I

Comparison of strains LCM-Am and LCM-P

Property	Virus strain	
	LCM-Am	LCM-P
Virion morphology	Identical electron microscopic picture, characteristic of arenaviruses	
IF antigen	identical	
CF antigen	identical	
Precipitating ID antigen	identical	
Ether sensitivity	+	+
IDU (50 μg/ml) inhibition	−	−
Amantadine inhibition (25 μg/ml)	+	+
CP effect in cell cultures (Vero and L-41)	−	−
Rate of multiplication in cell cultures	fast	slow
Mouse pathogenicity	+	−
Mouse protection test	The mice inoculated i.p. with the LCM-P strain acquired resistance to the i. cer. lethal dose of the LCM-Am strain	

Table II

Cell cultures found positive for LCM carriership

Submitting institute	No. of cultures tested	Positive cultures		Negative cultures
		Designation	Number	Designation
OKI*	13	HEp-2[b], Fl[c]	2	HeLa, HeLa, HEp-2(C)[a], L-41, AV-3, III/1, Vero, BGM, CV-1, BHK-21, RK-13[d]
A	2	HEp-2[b], HEp-2[b]	2	—
B	3	HEp-2[b]	1	HeLa, HEp-2[e]
C	1	HEp-2[b]	1	—
D	5	HEp-2[b], HEp-2[b], FL[e], FL[c]	4	HeLa
E	3	—	0	HEp-2[e], HEp-2[e], Vero
F	2	HEp-2[b], HEp-2[b]	2	—
G	2	—	0	HEp-2, HEp-2
H	12	HEp-2[b], HeLa(S), RK-13[d]	3	HeLa, HEp-2(C)[a], AV-3, MRC-5, HD, Vero, PKS, L, II/D
I	3	HEp-2[b]	1	HeLa, AV-3
Totals	46		16	

* National Institute of Hygiene, Budapest.
[a–e] The sublines designated with the same letter originated from the same source

Table III

Origin of the virus carrier HEp-2 cell subline and its distribution in Hungarian institutes

Institute	Origin		Maintenance discontinued (date)
	Year	Institute	
G	1959	An institute abroad	1976
	1976	Institute C ?	—
OKI*	1960	Institute G	1976
	1977	Institute A	—
H	1962	Institute G	—
D	1964	Institute H	—
A	1965	Institute D	—
	1977	Institute H	
B	1975	Institute A	—
F	1975	OKI	—
C	1976	OKI	—
I	1976	Institute G	—

* National Institute of Hygiene, Budapest.

We received our FL^c subline from Institute D in 1975, and Institute D had received it in 1964 from Institute H. Meanwhile, the subline was maintained in Institute D. By the time of our studies, the passage of this subline had been discontinued in Institute H. Thus the subline could not be tested for carriership. Two other cell cultures (HeLa, RK-13) current in Institute H proved, however, to be infected. Presumably, the infection was transferred from the carrier HEp-2 subline that was also passaged in Institute H.

Discussion

Based on the present studies, there is little doubt that the LCM virus carrier HEp-2 subline had already been infected when it was received in this Institute. It remained, however, undetected when, where and how it had become infected. Most probably it was brought to Hungary in a carrier state. LCM virus is well-known to induce in its natural host, the mouse, persistent infection and that it is easy to establish virus carrier cell cultures artificially [5–11]. Spontaneous LCM virus carriership in murine cell culture has been reported [12—14], and in a study [15] LCM virus was found in harvests of cell cultures which were inoculated with mouse passage material of rabies virus. Our own results suggest that cross-contamination of tissue cultures may occur with

the LCM virus as a result of improper laboratory practice. This might have been the mechanism of the original contamination of HEp-2 cell line under study.

It is generally accepted that defective interfering (DI) particles play an essential role in development of persistent LCM and other arenavirus infections in tissue cultures [11]. In the course of 20–50 serial passages, an equilibrium may develop between infective virions and DI particles while infectivity remains low despite the presence of well-demonstrable LCM-specific antigens in the cultured cells. The carrier cultures grow as do the corresponding non-carrier ones and may be superinfected with other viruses. It is therefore not surprising that the carriership of the HEp-2^b line could have remained unrecognized for a long time.

It has repeatedly been reported [9, 10, 15] that in the course of persistent infection of cell cultures the mouse pathogenicity of the carried virus was declining. The same must have happened to the LCM-P strain. A correlation between mouse pathogenicity and human pathogenicity might explain why no illness attributable to accidental laboratory infections has been observed during the wide use of the carrier culture over many years. Though it cannot be excluded that inapparent infections may have occurred. The potential risk connected with carrier cell cultures is well-illustrated by the outbreak experienced in an oncological laboratory of the USA [14], where hamsters were inoculated with tumour cells which later proved to carry LCM virus. Of the laboratory workers who had been in contact with inoculated hamsters, 38 became infected and 21 of these developed typical LCM.

In addition to threatening the health of laboratory workers, cell cultures carrying LCM virus may falsify laboratory results. Virus strains passaged in virus carrier cell cultures become contaminated with the LCM virus, and if animals are immunized with antigens prepared from carrier cultures and/or laboratory tests are performed with the resulting antisera, the diagnostic use of any of these reagents will be misleading. Any kind of biological experiment may lead to false results if cell cultures carrying an unrecognized virus are used in it.

Spontaneous infection of cell cultures may occur with viruses other than LCM virus as well [16]. This is true not only for cultures of established cell lines, but also for primary cultures prepared from human cells or those of lower-animals. Accidental infection with some of the tissue-culture-carried viruses may be fatal in outcome, e.g. those with *Herpesvirus simiae* [17] or with the Marburg virus [18].

The present observations support the view that uninoculated tissue cultures must be regarded as potentially hazardous material. Accordingly, strict safety measures and adequate laboratory techniques similar to those applied in microbiological work should be instituted in all laboratories working with tissue cultures.

REFERENCES

1. Simon, M.: Acta microbiol. Acad. Sci. hung. **20,** 11 (1971).
2. Simon, M.: Acta virol. (Praha) **14,** 369 (1970).
3. Gobara, F., Itagaki, A., Ito, Y., Saito, K., Katsumoto, T., Kimura, G.: Microbiol. Immunol. **21,** 207 (1977).
4. Pfau, C. J., Pedersen, R., Volkert, M.: Acta path. microbiol. scand. **63,** 181 (1965).
5. Welsh, R. M., Trowbridge, R. S., Kovalski, J. B., O'Connel, C. M., Pfau, C. J.: Virology **45,** 679 (1971).
6. Lehmann-Grube, F., Slenczka, W., Tees, R.: J. gen. Virol. **5,** 63 (1969).
7. Lehmann, Grube, F.: Lymphocytic Choriomeningitis Virus. Virology Monographs. **10,** Springer, New York 1971. p. 24.
8. Staneck, L. D., Trowbridge, R. S., Welsh, R. M., Wright, E. A., Pfau, C. J.: Infection and Immunity **6,** 444 (1972).
9. Hotchin, J.: Progr. med. Virol. **18,** 81 (1974).
10. Hotchin, J., Sikora, E.: Infection and Immunity **7,** 825 (1973).
11. Pedersen, R.: Structural Components and Replication of Arenaviruses. Advances in Virus Research. **24,** Academic Press, New York 1979. p. 277.
12. Molomut, N., Padnos, M., Smith, L. W.: J. nat. Cancer Inst. **34,** 403 (1965).
13. Ofodile, A., Padnos, M., Molomut, N., Duffy, J. L.: Infection and Immunity **7,** 309 (1973).
14. Anonymous: Amer. J. med. Assoc. **228,** 815 (1974).
15. Wiktor, T. J., Fernandes, M. V., Koprowski, H.: J. Bact. **90,** 1494 (1965).
16. Hellman, A., Oxman, M. N., Pollack, R. (eds): Biohazards in Biological Research. Cold Spring Harbor Laboratory, 1973.
17. Hummeler, K., Davidson, W. L., Henle, W., LaBoccetta, A. C., Ruch, H. G.: New Engl. J. Med. **261,** 64 (1959).
18. Martini, G. A., Siegert, R. (eds): Marburg Virus Disease. Springer, Berlin–New York 1971.

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LEUKOCYTE MIGRATION INHIBITION (LMI) BY A SPECIFIC ANTIGEN IN HUMAN TOXOPLASMOSIS

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(Received December 11, 1981)

The diagnostic value of the leukocyte migration inhibition test was examined by comparing it to other specific laboratory methods i.e. complement fixation, indirect haemagglutination and skin test in 10 patients with acquired lymphoglandular toxoplasmosis. In the acute phase the serological tests gave more reliable results than did the LMI test. In subacute and chronic cases, however, there was a good correlation between dermal reactivity and LMI test. LMI is believed to represent a specific test for the detection of cellular hypersensitivity in human toxoplasmosis and so it can be used as a non-invasive method instead of dermal tests.

Acquired human toxoplasmosis is often difficult to diagnose. Epidemiological, clinical and serological data fail to give sufficient information on the occurrence of an active infection. In this study the diagnostic efficacy of the leukocyte migration inhibition test (LMI) was evaluated by comparison to the complement fixation (CF), indirect haemagglutination (IHA) and skin tests.

Materials and methods

Patients. Ten patients with acquired lymphoglandular toxoplasmosis were tested. The period between onset of the disease and time of testing varied from 2 weeks to 4 years. The disease was diagnosed on the basis of clinical symptoms, lymph node biopsy (microscopic examination: Piringer–Kuchinka lymphadenitis), epidemiological and serological data.

Controls. Ten healthy individuals who had never had toxoplasmosis nor any contact with toxoplasma infected animals served as controls. Toxoplasma skin test, CF and IHA tests of all control subjects were negative.

Serological tests. CF test and IHA test were carried out by the method of ENGELBRECHT, [1] and JACOBS and LUNDE [2], respectively.

Sensitivity tests. Skin tests and LMI tests were done by using the same antigen, with 0.25% phenol killed *Toxoplasma gondii* suspension (National Institute of Hygiene, Budapest). For the LMI test the suspension was washed three times in Hanks' BBS to remove traces of phenol.

Skin tests were performed by injecting intradermally 2500 *Toxoplasma gondii* in 0.1 ml saline solution. Local reactions were read after 72 h. Skin reactions of 10, 20, 30, 40 or $>$ 40 mm in diameter were recorded as $+$, $++$, $+++$, $++++$.

LMI tests were performed by the method of SØBORG and BENDIXEN [3]. Each migration experiment was carried out in triplicate with toxopasma antigen while 3 chambers without antigen served as controls. The migration index (MI) was calculated according to the formula

$$\text{MI} = \frac{\text{migration in antigen-containing culture}}{\text{migration in antigen-free culture}}$$

MI-s less than 0.8 and more than 1.2 proved significant when evaluated with Studient's *t* test.

The serological, skin and LMI tests were done on the same day. Blood was drawn before performing the skin test.

Controls were tested on a single occasion, the patients were tested twice at variou intervals.

Results

MI values are given in Fig. 1. The range of MI values in the patient group was 0.56 to 0.91 in the lst sample and 0.51 to 0.77 in the 2nd, sample, while in the control group the range was 0.78 to 0.98. The difference was statistically significant with Student's *t* test ($p < 0.001$).

Individual MI values and other findings are presented in Table I. The highest antibody levels were generally found when symptoms were still present. In contrast, though all the skin tests done gave positive results, the reactions were weaker when testing within 3 months after onset of the disease.

Table I

Clinical and laboratory data of patients with toxoplasmosis

No.	Patient	Time between onset of disease and testing	Clinical symptoms	Reaction to skin test	MI	IHA	CF
1.	K. A.	2 weeks	+	n. d.	0.91	1 : 512	1 : 20
		5 months	−	+++	0.74	1 : 2048	1 : 320
2.	K. E.	4 years	+	+++	0.57	1 : 1024	1 : 20
		4.5 years	−	+++	0.57	1 : 256	1 : 20
3.	W. E.	6 weeks	+	++	0.61	1 : 1024	1 : 80
		3 months	−	+++	0.58	1 : 4096	1 : 80
4.	F. Gy.	4 weeks	+	+	0.61	1 : 1024	1 : 80
		6 months	−	+++	0.54	1 : 256	1 : 20
5.	K. J.	6 weeks	+	++	0.61	1 : 256	1 : 20
		3 months	−	+++	0.60	1 : 64	1 : 10
6.	K. H.	4 weeks	+	++	0.89	1 : 1024	1 : 80
		5 months	−	++++	0.77	1 : 512	1 : 40
7.	S. S.	4 months	+	+++	0.60	1 : 256	1 : 10
		6 months	−	++++	0.51	1 : 64	1 : 5
8.	B. F.	5 months	+	+++	0.59	1 : 512	1 : 80
		7 months	−	+++	0.59	1 : 512	1 : 80
9.	M. L.	2 years	+	+++	0.60	1 : 512	1 : 40
		2.5 years	−	+++	0.60	1 : 64	1 : 20
10.	Cs. Zs.	6 months	+	++++	0.56	1 : 128	1 : 10
		10 months	−	+++	0.51	1 : 64	1 : 5

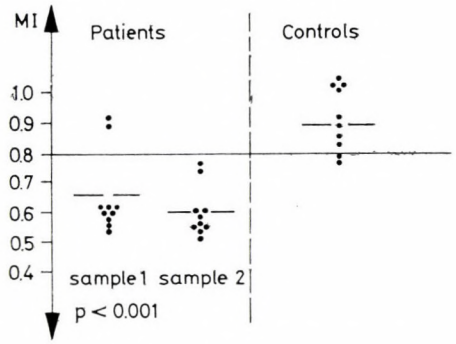

Fig. 1. Leukocyte migration indices of patient with toxoplasmosis and from control subjects

All but two LMI tests were positive. Both negative tests were performed in the acute phase i.e. 2 to 4 weeks after onset of the disease. Later the tests became positive in these cases too.

Discussion

We found that LMI with killed *T. gondii* is a highly specific test for the detection of cellular hypersensitivity in human toxoplasmosis. When comparing the diagnostic value of different tests it seems, however, that the serological methods are more reliable than skin and LMI tests in the acute phase of the disease. The LMI test seems to be of the same diagnostic value as the skin test, i.e. its positive result points to an earlier toxoplasma infection long after antibody titres had decreased, but gives no information as to the activity of the disease. It ought to be considered whether LMI test should be used as a non-invasive method instead of a skin test.

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REFERENCES

1. Engelbrecht, E.: WHO Scientifical Documents Series WHO/ZOON/ **70,** 112 (1970).
2. Jacobs, L., Lunde, M. N.: Science **125,** 1035 (1957).
3. Søborg, M., Bendixen, G.: Acta med. scand. **181,** 246 (1967).

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NOTE

CAMPYLOBACTER JEJUNI CONTAMINATION OF SLAUGHTERED CHICKENS

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(Received July 2, 1981)

Gut samples from 50 nonselected slaughtered chickens were obtained in two poultry processing plants and cultured for *Campylobacter jejuni* and *Salmonella*. Positive results were obtained in 84% and 4%, respectively. Viable *C. jejuni* and *Salmonella* were detected in every phase of processing, even during packaging for commercial purposes. Of surface samples taken from 118 slaughtered chickens prepared for delivery to consumers, 88 were contaminated by *C. jejuni* and 17 by *Salmonella*.

In the present work, the rate of contamination of slaughtered chickens by *Campylobacter jejuni* was examined in different phases of processing. For comparison, the samples were examined for *Salmonella*.

Materials and methods. Samples were taken from two poultry processing plants. Plant 1 belonged to a co-operative farm and had a capacity of 4000 chickens daily. Plant 2 belonged to a state farm and had a capacity of 20 000 chickens daily. The chickens processed in Plant 1 and Plant 2 had originated from three flocks and a single flock, respectively. Guts were placed each in a polyethylene bag. Samples were obtained from the external and internal surfaces of the chickens and the livers with sterile cotton swabs soaked in Todd–Hewitt's nutrient broth. The swabs were then placed each in a sterile test tube containing the same medium and sent to the laboratory within 3 h.

In the laboratory, the coecum was opened under aseptic conditions and samples from the intestinal contents as well as from the surface swabs were inoculated into SKIRROW's medium [1]. Isolation of *C. jejuni* was performed in SKIRROW's selective medium [1] containing 5% defibrinated and haemolysed bovine blood, vancomycin (5 mg/l), polymyxin B (2500 IU/l) and trimethoprin (5 mg/l). The inoculated media were incubated at 42 °C in a gas mixture containing oxygen, carbon dioxide, hydrogen and nitrogen at 5%, 10%, 10% and 75% partial tension, respectively. *C. jejuni* was identified as described in handbooks. To isolate *Salmonella*, Rappaport's enrichment medium was inoculated with the same inocula and incubated at 36 °C for 24 h. Subcultures on brilliant green and bismuth sulphite media were incubated at 36 °C for 24 h.

Results. C. jejuni was isolated from gut samples of 26 of the 30 chickens tested in Plant 1 and from 16 of the 20 chickens tested in Plant 2. From Plant 1, two *Salmonella* strains were isolated.

Of the 50 surface samples taken immediately after evisceration, 47 proved to be positive for *C. jejuni* and 3 for *Salmonella.*

Of the samples taken during commercial packaging, 45 were positive for *C. jejuni* and 29 for *Salmonella.* Of the 72 chickens sampled in the consumers' kitchen, 52 yielded *C. jejuni* and 1 yielded *Salmonella.*

The serotypes of *Salmonella* isolated were, *S. thompson*, *S. infantis*, *S. derby* and *S. typhi-murium.*

Discussion. The 84% contamination rate of non-selected chickens by *C. jejuni* is in good agreement with the results reported by GRANT *et al.* [2]. These authors isolated *C. jejuni* from 83% of 46 slaughtered chickens bought at the New York City market.

LUECHTEFELD and WANG [3] examined 600 turkeys over a one-year period. All the cloacal swabs and fresh faecal samples proved to be positive for *C. jejuni.*

RETTING [4] described *C. jejuni* as a member of the normal intestinal flora of chickens and turkeys, among others. According to this author, "vibrio" hepatitis of chickens and human enteritis were caused by *C. jejuni.*

We isolated *C. jejuni* from 82% of the chickens sampled in shipping rooms and kitchens, whereas LUECHTEFELD and WANG [3] isolated the same microorganism from 94% of turkeys before deep-freezing.

Of the 30 deep-frozen chickens sampled by us in the kitchen, 16 (53%) proved to be positive for *C. jejuni.* This percentage is lower than those found by us for the chickens tested immediately after killing. The difference might be attributed to a possible high sensitivity of the bacterium to environmental conditions or this group of chickens had not been infected immediately after being slaughtered.

The survival of *C. jejuni* on the surface of chickens was investigated by SMITH and MULDOON [5]. The bacterium survived at 3 °C for 5 days and at −23.5 °C for 20 days or more. ROBINSON *et al.* [6] succeeded in isolating the organism from chickens frozen over a period of 2 months or even longer.

We were surprised at the massive infection by *C. jejuni* of slaughtered chickens which were just to be delivered to consumers, for the final phase of the technology included floating in chlorinated water. Still, as we were informed later, the chlorinizing equipment in Plant 2 had been out of function at the time of sampling. Thus, the time and duration of chlorination was not controlled reliably. LUECHTEFELD and WANG [3] called attention the importance of adequate chlorination. They kept slaughtered turkeys in ice water containing 340 μg/ml chlorine overnight in a tank and found 34% of the samples positive for *C. jejuni.*

Recently, in Csongrád county in Southern Hungary through a period of nearly 6 months we have observed cumulations of *C. jejuni* enteritis mainly affecting young children. As source of infection slaughtered chickens were suspected in about 80% of the cases.

The cumulations followed, first of all, the periods in which foods enriched with chicken liver had been processed. A similar, though less pronounced relationship was noticed between processing of slaughtered poultry and subsequent cumulation of cases. (Personal communication, Dr. ILONA KISS, Public Health Station, Szeged).

More detailed studies are needed to elucidate the epidemiological relationships of *C. jejuni* in Hungary.

REFERENCES

1. SKIRROW, M. B.: Brit. med. J. **2,** 9 (1977).
2. GRANT, I. H., RICHARDSON, N. J., BOKKENHEUSER, V. D.: J. clin. Microbiol. **11,** 508 (1980).
3. LUECHTEFELD, N. W., WANG, W. L.: J. clin. Microbiol. **13,** 266 (1981).
4. RETTING, P. J.: J. Pediat. **94,** 855 (1979).
5. SMITH, M. V., MULDOON, P. J.: Appl. Microbiol. **27,** 995 (1974).
6. ROBINSON, D. A., GILBERT, R. J., SKIRROW, M. B.: Envir. Hlth **88,** 140 (1980).

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NOTE

CAMPYLOBACTER JEJUNI ENTERITIS: INCIDENCE, AGE DISTRIBUTION AND CLINICAL SYMPTOMS

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(Received November 16, 1981)

Campylobacter jejuni was isolated in Csongrád county, Hungary, during a 7-month period from 267 out of 2892 patients with enteritis and from 36 out of 708 symptomless contacts. Seventy-four per cent of the patients were under 6 years of age. Predominating clinical symptoms were mucous stools, malaise and pyrexia.

Reports from throughout the world indicate that *Campylobacter jejuni* is one of the most common causative agents of human enteritis [1, 2]. In Hungary, the organism was first isolated by ÁDÁM *et al.* [3]. In this paper we give an account of the incidence, age distribution and clinical symptoms of campylobacter enteritis observed in Csongrád county, Hungary.

Materials and methods. C. jejuni was cultured on Skirrow agar [2] modified in the National Institute of Hygiene, Budapest [4]. The inoculated plates were incubated in approximately 7% CO_2 + 7% O_2 atmosphere at 43 °C for 48 h. Isolates giving positive oxidase and catalase tests, showing characteristic morphology and motility under the microscope, growing well on Skirrow agar in the above atmosphere but not in air and exhibiting a characteristic antibiotic sensitivity spectrum, were regarded as *C. jejuni*. Antibiotic sensitivity of the isolates was tested on antibiotic-free Skirrow agar using disks (Human, Budapest). For the detection of other enteric pathogens, the faecal samples were seeded on desoxycholate citrate, bismuth sulphite, eosin methylene blue and 7.5% salt blood agar plates.

Results and discussion. Between January 1 and July 31, 1981, 3690 faecal samples of 2892 patients, and 737 faecal samples of 708 symptomless persons living in the family of the patients were examined. *C. jejuni* was isolated from 267 patients (9.2%) and from 36 contacts (5.1%). Accordingly, out of 3600 persons 303 (8.4%) were positive for *C. jejuni*. Taking into account the repeated isolations from the same persons, a total of 349 strains were cultured from the patients and 46 strains from the contacts. Environmental examinations were performed in 207 families; the 36 positive contacts belonged to 24 different families. Fifteen of the contacts were under 6 years, two were between 6 and

14 years, and 36 were older than 14 years. Four *C. jejuni* strains were isolated from the faeces of dogs and pigeons in the environment of the patients.

Age distribution and clinical symptoms of *C. jejuni* positive patients are presented in Table I. The frequent incidence of respiratory symptoms in children is noticeable. Bloody and mucous diarrhoea in the same patient was frequently observed (26 children and 7 adults). Pyrexia and malaise occurred frequently together in children.

Table I

Symptoms and age groups of patients with campylobacter enteritis
Total number of patients, 267

Symptoms	Patients		Distribution of symptoms according to age groups, per cent of patients			
	No.	%	under 1 year (87 patients)	1–6 years (110 patients)	6–14 years (18 patient)	above 14 years (52 patients)
Enteric						
bloody stools	68	25	26	26	17	25
mucous stools	162	61	66	72	44	35
watery stools	70	26	15	20	39	54
Upper respiratory	104	39	34	67	—	—
General						
pyrexia	146	55	61	65	28	29
malaise	168	63	61	75	61	40
abdominal pain	120	45	*	66	50	73

* Not evaluable.

The duration of the illness was usually 3–4 days. From the onset of the symptoms, the patients excreted *C. jejuni* for 5–14 days.

Every strain isolated at the first examination of the patients was tested for antibiotic sensitivity. The strains were uniformly sensitive to erythromycin, chloramphenicol, nalidixic acid and nitrofurantoin. To tetracyclines 74% of the strains were sensitive, 10% moderately sensitive and 16% resistant. To ampicillin 44% were sensitive, 31% moderately sensitive and 27% resistant. For the therapy of campylobacter enteritis, erythromycin is the preferred drug [5]; upon our recommendation, usually this antibiotic was prescribed by the physicians for the treatment of *C. jejuni* positive patients.

Enteric pathogens other than *C. jejuni* were shown in 141 out of the 2892 patients (4.9%) in the following distribution: *Salmonella* 85 (2.9%), *Shigella* 12 (0.4%), *Yersinia enterocolitica* 10 (0.3%), enteropathogenic *Escherichia coli*

22 (0.7%). *C. jejuni* and one of the above organisms occurred together in the faeces of 19 patients (4.8%).

Our examinations have shown that, in agreement with literary data, *C. jejuni* is a prominent causative agent of enteritis and occurs more frequently than other known enteropathogenic bacteria together.

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REFERENCES

1. Skirrow, M. B.: Brit. med. J. **2,** 9 (1977).
2. Butzler, J. P., Skirrow, M. B.: Clin. Gastroent. **3,** 737 (1979).
3. Ádám, M., Bartha, T., Pintér, M.: Annual Meeting of the Hungarian Society of Microbiology, Veszprém 1980.
4. Lányi, B., Ádám, M., Rédey, B., Konkoly Thege, M., Pechó, Z., Bartha, T.: Egészségtudomány **26,** 74 (1982).
5. Svedhem, A., Kajser, B., Sjögren, E.: J. Antimicrob. Chemother. **7,** 301 (1981).

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ENZYME-LINKED IMMUNOSORBENT ASSAY IN THE SERODIAGNOSIS OF SYPHILIS

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(Received September 28, 1981)

Enzyme-linked immunosorbent assay using ultrasonic lysate of *Treponema pallidum* and *Treponema reiteri* as antigens was used for the detection of antisyphilitic antibodies in various stages of syphilis. The conjugate was goat antiserum to human IgG labelled with horseradish peroxidase. A comparison with results of the *T. pallidum* immobilization test, Rapid Plasma Reagin test, Kolmer complement fixation reaction using cardiolipin and Reiter protein antigens showed that ELISA was more sensitive but less specific with *T. pallidum* antigen, whereas less sensitive but more specific with *T. reiteri* antigen. Absorption of group specific treponemal antibodies was needed to make the method reliable.

The enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of specific antibodies and thus for the diagnosis of many different diseases [1–3] and of infectious and non-infectious agents [4–6]. Still, few papers have dealt with its application in syphilis serology, even though the test seems to be useful for the purpose. The purpose of the present work was to investigate the possibility of using ELISA to detect a specific antibody in human syphilis and to evaluate its usefulness in serodiagnosis.

Materials and methods

Sera to be tested for antitreponemal antibodies were obtained from 160 normal persons, from 126 syphilitic patients and from 45 presumably false positive cases.

The sera were tested with the *Treponema pallidum* immobilization test (TPIT), the fluorescent treponemal antibody absorption (FTA—ABS) test, the Reiter protein complement-fixation (RPCF) test, the Rapid Plasma Reagin (RPR) slide test, the Kolmer complement-fixation test (CCFR) and by ELISA.

The RPR and FTA-ABS tests were performed according to the procedure recommended in the Manual of Tests for Syphilis, 1969 [7]. Complement fixation reactions were done with 5 U of complement [8]. The TPIT was performed with the Budapest strain of *T. pallidum* [9].

Antigens. Ultrasonic preparations of *T. pallidum* Budapest and *Treponema reiteri* were used as antigens. Treponemes were extracted from infected rabbit testes [9]. The medium with treponemes was centrifuged at 200 *g* for 20 min to remove gross tissue debris and particles. The organisms were then sedimented in the cold at 19 000 *g* for 90 min, washed three times with saline, diluted to 10^7 germs/ml with saline and homogenized with needle and syringe. The treponemal suspension was treated with the Lehfeld sonicator at 8 intensity (about 24 W/cm^2) at 0 °C temperature for 10 min. The sonicated material was controlled by dark field microscopy: all treponemes had to be minced.

The Reiter strain was grown in thioglycolate medium, to which 10% rabbit serum was added. After incubation at 35 °C for 5 days the treponemes were separated by centrifugation, washed and sonicated [10].

ELISA. Polystyrene flat bottomed plates with wells (Labor MIM, Hungary) were used to bind soluble antigens. Binding of antigen to the plastic was carried out at 4 °C overnight. Microtitre wells were coated with antigen in 0.1 M carbonated buffer at pH 9.6. Sera were diluted 1 : 200 with phosphate buffered saline (PBS) containing 0.05% Tween 20. After washing the plates, 100 µl of human serum in diluting buffer was added. The plates were incubated at 37 °C for 1 h. After washing the plates three times, 100 µl of a 1 : 20 000 dilution of enzyme conjugated antibody (peroxidase labelled goat antihuman IgG, Human Institute for Serobacteriological Production and Research, Hungary) in diluting buffer were added. The plates were incubated at 37 °C for 1 h. Excess conjugate was removed by washing three times. Finally, the amount of enzyme bound to the wells was determined by adding to each well 150 µl of 10 mM citric acid phosphate buffer containing orto-phenylene diamine and hydrogen peroxide (Reanal, Hungary). The reaction was stopped after 1 h incubation by the addition of 50 µl of 4 N H_2SO_4.

The results of the tests were evaluated when the control sera had reached the 0.8–1 extinction value, generally after 10–15 min incubation.

The developed colour was measured spectrophotometrically at 490 nm, by a Linson 3 photometer supplied with special microcuvettes.

Controls. Background optical density controls consisted of the highest tested concentration of all reagents in all combinations.

Negative control values were determined by estimating the values given by the normal uninfected population. The negative control serum used in all tests gave an extinction value of less then 0.3 in all experiments. All results of 0.3 lower extinction value were considered negative.

The positive control level was determined by estimating the extinction values given by TPIT positive cases. The extinction value indicative of infection was 0.8 or higher. The extinction range of the positive control serum used in all experiments was between 0.8–1.0.

The weakly reactive results were accepted between 0.3–0.7 extinction range, provided the same value was obtained in repeated tests.

Results

Comparison of the results of ELISA with those of other serological tests for syphilis yielded divergent data (Table I). Tests performed with pathogenic *T. pallidum* antigens (FTA-ABS, TPIT) produced the best correlation with ELISA though there were some disagreeing cases, especially among ELISA reactive ones and those reacting in other tests.

The sensitivity of ELISA is defined as the percentage of reactive or weakly reactive results obtained among specimens reacting in the different serological tests. The RPR reactive sera proved to be reactive in ELISA using Reiter antigen in 64%, and with *T. pallidum* antigen in 76%. The CCFR reactive sera were reactive in ELISA with the use of Reiter antigen in 57% and with *T. pallidum* antigen in 69%. The TPIT reactive sera were reactive in ELISA using Reiter antigen in 75% and using *T. pallidum* antigen in 84%. The FTA-ABS reactive sera reacted in ELISA with Reiter antigen in 57% and with *T. pallidum* antigen in 86%. The sensitivity of ELISA was higher with *T. pallidum* antigen in all groups than with Reiter antigen.

The specificity of ELISA was defined as the percentage of negative results obtained with specimens giving a negative result in the serological tests. The RPR non-reactive sera were negative in ELISA using Reiter antigen in

Table I

Evaluation of ELISA using Reiter and T. pallidum antigens, on the basis of the syphilis seroreactions

Test		ELISA (Reiter antigen)						ELISA (*T. pallidum* antigen)					
		R	WR	N	T	S	SP	R	WR	N	T	S	SP
RPR	R	51	15	37	103	64	—	51	23	23	97	76	—
	N	3	12	54	69	—	78	9	12	34	55	—	61
CCFR	R	54	18	54	126	57	—	50	19	31	100	69	—
	N	2	8	41	51	—	80	5	11	24	40	—	60
RPCFR	R	41	15	20	76	73	—	42	19	17	78	78	—
	N	12	11	64	87	—	73	13	11	37	61	—	60
TPIT	R	37	11	14	62			40	8	5	53		
	WR	—	1	2	3	75	—	2	11	4	17	84	—
	N	4	10	54	68	—	79.4	13	7	43	63	—	68
FTA-ABS	R	17	5	16	38	57	—	22	4	4	30	86	—
	N	3	1	30	34	—	88	6	3	18	27	—	66

R = reactive; WR = weakly reactive; N = non-reactive; T = total; S = sensitivity; Sp = Specificity

78%, and using *T. pallidum* antigen in 61%. The CCFR non-reactive sera were negative in ELISA with Reiter antigen in 80% and with *T. pallidum* antigen in 60%. The RPCFR non-reactive sera were non-reactive in ELISA with Reiter antigen in 73% and with *T. pallidum* antigen in 60%. The FTA-ABS non-reactive sera were non-reactive in ELISA using Reiter antigen in 88%, and using *T. pallidum* antigen in 66%. The specificity of ELISA with *T. pallidum* antigen was lower in all groups than with Reiter antigen.

Table II

Evaluation of ELISA using Reiter and T. pallidum antigens on syphilitic and non-syphilitic sera

	ELISA (Reiter antigen)						ELISA (*T. pallidum* antigen)					
	R	WR	N	T	S	SP	R	WR	N	T	S	SP
Normal	3	7	120	130	—	92	5	11	144	160	—	90
BFP	4	6	25	35	—	71	11	5	29	45	—	64
Untreated sy II	17	0	0	17	100	—	18	0	0	18	100	—
Treated sy II	21	0	0	21	100	—	21	0	0	21	100	—
Late latent sy	13	17	12	42	71	—	30	10	5	45	89	—
Cardiovascular sy	1	1	1	3	66	—	1	2	0	3	100	—
Neuro sy	9	4	4	17	76	—	12	5	3	20	86	—
Connatal sy	0	3	1	4	75	—	3	1	1	5	80	—
Treated sy years ago	7	1	5	13	?	?	2	8	4	14	?	?

R = reactive; WR = weakly reactive; N = non-reactive; T = total; S = sensitivity; SP = = specificity; BFP = presumed biological false positive; sy = syphilis

Results are shown in Table II. The investigations with syphilitic sera showed that the sensitivity differed according to the antigen used. In the group of the treated and untreated secondary syphilitic patients the sensitivity was 100%. In the group of patients with late latent syphilis the sensitivity of ELISA was with Reiter antigen 71%, and with *T. pallidum* antigen 88%. A few cardiovascular, neurological and connatal cases of syphilis were also examined and their sensitivity seemed always to be higher with *T. pallidum* antigen. The specificity of ELISA was examined with healthy normal and false positive sera; in these groups the higher specificity was obtained with Reiter antigen i.e. with normal persons the specificity of ELISA using Reiter antigen was 92%, while using *T. pallidum* antigen it was 90% and in the presumed false positive group the specificity with Reiter antigen was 71% and with *T. pallidum* antigen 64%.

Discussion

It is generally accepted that ELISA offers a useful alternative to the immunofluorescent tests [2]. The advantages are a high sensitivity, objective evaluation, possibility of automatization and a low cost, while the disadvantages as compared to immunofluorescence are a less reliable specificity and the difficulty of preparing the antigen. VELDKAMP and VISSER using *T. pallidum* antigen found the test to be extremely sensitive and specific [11]. The sensitivity of the test in all stages of syphilis was very high and equal to that of FTA-ABS. SACCHI *et al.* [12] obtained good results performing the test with purified protein antigen extracted from Reiter treponema and they reported that ELISA was more sensitive than any of the other tests. Because of its high sensitivity and specificity, the simple technique and the objective method of reading, the test was thought to be of value for the serodiagnosis of syphilis. From other experiments using enzyme-labelled anti-IgM conjugate it was also concluded that the test will be useful in syphilis serology [2].

Experiments with ELISA of *T. pallidum* smears and diluting the sera in FTA-sorbent [13, 14] showed the test to be more sensitive than the TPIT; ELISA proved as specific and at least as sensitive as immunofluorescence for detecting anti-treponemal antibodies.

Other authors [15], however, concluded that the reaction can be carried out only in highly specialized laboratories and therefore does not represent an important advance in the routine diagnosis of syphilis.

Our study has not confirmed the results of VELDKAMP *et al.* [11] and SACCHI *et al.* [12], who performed the test without sorbent material. The specificity of our tests, particularly in the presumably false positive group, was too low, even if not all of these reactions were non-specific. The criteria of false positivity in our material was a non-reactivity to TPIT. The high prevalence of

reactive ELISA tests among the false positive sera may be interpreted in two ways. A positive ELISA may indeed detect a history of treponemal disease. In this case, we must postulate that the prevalence of syphilis is quite high among the patients from whom our problem sera were collected. Taking into consideration the disadvantages of the TPIT, its clinical role is seriously questioned according to newer data [16]; with part of our sera this is likely to be the case. It also seems that lipid antigens, and so cardiolipin, cannot be found in the polystyrene well in the usual way [17].

There were, really, non-specific positive tests among our sera, particularly with normal sera. The lower level of ELISA specificity using *T. pallidum* antigen compared to Reiter antigen could have been due to cell debris which was sonicated with *T. pallidum*. The cause of the lower specificity in our material may be the difference in antigen quality. The above mentioned authors must have used more purified antigen than we did. SACCHI *et al.* [12] used a purified protein derivate of Reiter treponema; we used an antigen made of Reiter treponema by a more simple method. In other serological tests this antigen proved to be equal to the purified one [10].

In spite of the fact that the *T. pallidum* antigen was prepared according to the prescription of VELDKAMP *et al.* [11] we could not reproduce their extremely good results. In view of the problem of preparing a really purified *T. pallidum* antigen, it seems to be more convenient to carry out the test with an adequate sorbent medium, as in FTA and TPHA tests. Some of the above mentioned tests yielded good results on using FTA-sorbent. Our preliminary experiments using the sorbent medium of the TPHA test [18] gave promising results. Research will be continued in this direction.

In conclusion, using this kind of antigen without a sorbent medium, ELISA cannot substitute the immunofluorescence method. On the basis of theoretical considerations, of other authors' and of our own results, it seems that with a suitable sorbent and after improving the specificity, the test could be a reliable tool in the serodiagnosis of syphilis.

REFERENCES

1. ENGVALL, E., JONSSON, K., PERLMANN, P.: Biochim. biophys. Acta (Amst.) **251,** 427 (1971).
2. VOLLER, A., BIDWELL, D. E., BARTLETT, A.: Bull. Wld Hlth Org. **53,** 55 (1976).
3. VAN WEEMEN, E. K., SCHUURS, A. H. W. M.: FEBS Letters **15,** 232 (1971).
4. BÁNYAI, A., KÁVAI, M., ZSINDELY, A., PAPP, S., SZEGEDI, GY.: Orv. Hetil. **121,** 1371 (1980).
5. PÁCSA, S., PEJTSIK, B.: Orv. Hetil. **119,** 2071 (1978).
6. PÁCSA, S., PEJTSIK, B.: Orv. Hetil. **121,** 151 (1980).
7. Manual of Tests for Syphilis. National Communicable Diseases Center, Atlanta, Ga. 1969.
8. HORVÁTH, I.: Syphilis szerológiai reakciók. In: Sós, J. (ed.): Laboratóriumi diagnosztika. Medicina, Budapest 1974.
9. HORVÁTH, I., SIMON, E.: Euphoria et Cacophoria. **1,** 3 (1974).
10. KIRÁLY, K., PRERAU, H.: Z. Immun.-Forsch. **134,** 32 (1967).
11. VELDKAMP, J., VISSER, A. M.: Brit. J. vener. Dis. **51,** 227 (1975).
12. SACCHI, G., ALESSI, E., VALCURONE, G., BUZZETTI, C., TASSI, G. C.: Boll. Ist. sieroter. milan. **57,** 490 (1978).

13. PATRAMANIS, I., MARKETAKIS, J., KAKLAMANIS, E., TZAMOURANIS, N., PAVLATOS, M.: J. Immunol. Meth. **2,** 251 (1973).
14. PETTS, V., ROITT, I. M.: Clin. exp. Immunol. **9,** 407 (1971).
15. POSPISIL, L., KRATOCHVIL, F.: Dermatologica **149,** 78 (1974).
16. REIN, M. F., BANKS, G. W., LOGAN, L. C., LARSEN, S. A., FEELEY, J. C., KELLOGG, D. S., WIESNER, P. J.: Sex. transm. Dis. **7,** 101 (1980).
17. REGGIARDO, Z., VAZQUEZ, E., SCHNAPER, L.: J. Immunol. Meth. **34,** 55 (1980).
18. HORVÁTH, I., MARSCHALKÓ, M., RÁCZ, I.: Zbl. Bakt. I. Abt. Orib. A. **246,** 423 (1980).

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THE STABILITY OF ENTEROTOXIN PRODUCTION IN *YERSINIA ENTEROCOLITICA* AND THE METHANOL SOLUBILITY OF HEAT-STABLE ENTEROTOXIN

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Twenty five strains of *Yersinia enterocolitica* serogroup O3, were isolated from human enteritis and studied for heat-stable enterotoxin production. Enterotoxin production was found even in the crude supernatant fluid of cultures that had been stored in stock agar for a year. According to the suckling mice and rabbit gut loop tests, after 1 to 5 years storage the filtrate showed heat-stable enterotoxin activity only in a purified and concentrated form. Following more than 5 years storage positive results could be obtained only in rabbit gut loop test. After 9 years the freeze dried strains still showed a full capacity of heat-stable enterotoxin production. Studies with concentrated substances showed that even after more than 9 years, there was no spontaneous loss of heat-stable enterotoxin production, only quantitative changes occurred. The methanol solubility of the heat-stable enterotoxin of *Y. enterocolitica* is — as distinct from the heat-stable enterotoxin of *Escherichia coli* — homogeneous and only the methanol soluble fractions showed any activity. The activity of methanol soluble enterotoxin from several years old subcultures could be demonstrated in an isolated rabbit gut loop model even when it failed to show any activity in suckling mice.

In recent years several reports have been published on human infections caused by *Yersinia enterocolitica*. PAI and MORS [1] showed that numerous types of the bacteria produced heat-stable enterotoxin (ST) causing fluid accumulation in suckling mice and in the rabbit gut loop. Most experiments on *Y. enterocolitica* were performed in suckling mice; the rabbit gut loop test was used for the detection of enterotoxin production only in the case of a few strains [1, 2]. The test was not applied for the study of methanol soluble fractions, although it helps to separate the enterotoxin from the ST of *Escherichia coli*. Previously we described the enterotoxin production of some *Y. enterocolitica* strains isolated in Hungary and also some characteristics of the toxin [3]. The present study was undertaken to examine the stability of the enterotoxin production of *Y. enterocolitica* strains stored under various conditions and for various periods of time, and to determine the activity of the methanol soluble and insoluble fractions of the toxin by various methods.

Materials and methods

Y. enterocolitica strains. Thirteen strains isolated from human enteritis in the year of the study and 12 formerly isolated strains, serogroup O3, were studied. The *E. coli* P16 strain O9 : K9 used for comparing the methanol fractions was kindly supplied by Dr. M. N. BURGESS (Nutritional Research Centre, Tadworth, Surrey, England).

Culture media, growth. Cultivation took place in Syncase broth medium at 25 °C in a shaker thermostat (Psycroterm, New Brunswick) [1].

Filtrates. The cultures were centrifuged at 7000 *g* for 30 min and the supernatants were filtered through a membrane filter (Nr. 1121 Göttingen, pore size 0.45 μm).

Heat-stable enterotoxin (ST) was tested in 2- to 4-day-old mice following the method of DEAN *et al.* as modified by JACKS and WU [4]. Three mice in each group received 0.1 ml filtrate orally through a tube. Four hours later the animals were sacrificed and the gut: rest body weight ratios were determined. The reaction was regarded as positive if the index was over 0.083. The rabbit gut loop model was performed according to DE *et al.* [5]. Intestinal segments of about 5 cm were prepared. One ml filtrate was added to the isolated ileal loop and the index for fluid accumulation was determined by calculating the $\frac{\text{full loop} - \text{empty loop}}{\text{empty loop}}$ ratio 6 h later. The obtained value was positive if higher than 1.

Methanol precipitation of the filtrates was performed according to Mullan's method as modified by BOYCE *et al.* [6]. The filtrates were concentrated to 1 : 5 or 1 : 10 of the original volume, then dialysed against distilled water for 2 days. One material was ultrafiltered through an Amicon PM 10 membrane. The concentrated filtrates were dropped into absolute methanol of 15-fold volume, under intensive shaking, then the solution was stored in the refrigerator for 18 h, since in this way the precipitation was more efficient than at room temperature. Following centrifugation at 23 000 *g*, the methanol soluble and insoluble fractions were collected separately. The methanol was removed by distillation and the components were dissolved in distilled water to their original volumes and filtered through a membrane filter.

For the detection of heat-labile enterotoxin (LT), Chinese hamster ovary (CHO) cell culture was used as described by GUERRENT *et al.* [7].

Results

The filtrates of all the 12 strains, isolated in the year of the experiment and cultivated at 25 °C, proved to be toxic for suckling mice (Table I). The activity of the filtrate of one strain was checked by the rabbit gut loop test, too, 6 h after administration. The filtrate caused a significant fluid accumulation in this case as well. Heating the filtrate to 100 °C for 15 min did not influence the result. In order to determine how long toxic activity can be preserved, filtrates were prepared from the subcultures every half year and their activity was determined in the suckling mice test (Table II). The activity of the filtrates decreased after a year, after a year and a half the crude filtrates showed no toxic activity.

The filtrates of five-year-old subcultures were concentrated 10-fold. Three from the 5 so far negative strains [3] gave a seemingly positive reaction both in suckling mice and in the rabbit gut loop test (Table III). Strain No. 797 was isolated some months earlier than the others and strain OKI 134 was an old laboratory strain; the concentrate filtrates of these two strains were negative in suckling mice, though they showed some positive effect in the rabbit gut loop test.

The raw filtrates of the 9-year-old lyophilized strains were highly enterotoxic both in suckling mice and in the rabbit gut loop test (Table IV). The filtrates of two of these strains prepared after nine years storage on stock agar were negative.

The capacity of enterotoxin production did not change significantly in the half-year-old subcultures.

Table I

ST producing capacity in suckling mice test of Y. enterocolitica strains isolated in the year of the experiment

Strains	Mean indices
OKI 29002	0.150
OKI 6676	0.090
OKI 4472	0.138
OKI 31620	0.127
OKI 6998	0.121
OKI 52535	0.087
OKI 3812	0.101
OKI 48731	0.132
R 45320	0.155
R 62476	0.176
R 61907	0.162
R 40476	0.145
Controls	
E. coli crude ST	0.092
Culture media	0.049

The enterotoxin of 4 strains used for determining the stability of enterotoxigenicity and the enterotoxin of three earlier lyophilized and one freshly isolated strain were treated with methanol. The activity of the concentrated filtrates, of the methanol soluble and insoluble fractions was tested in the suckling mice and rabbit gut loop tests (Table V). The filtrate of the newly isolated strain No. 5319 was ultrafiltered through Amicon PM 10 membrane. In accordance with earlier results [6], the activity was observable in the reten-

Table II

Decrease in enterotoxigenic activity in 18 months (suckling mice test)

Strains	Mean index values after isolation for		
	6 MONTHS	12 MONTHS	18 MONTHS
OKI 4472	*0.138*	*0.119*	0.061
OKI 48731	*0.132*	*0.100*	0.052
R 45320	*0.155*	*0.128*	0.054

Italicized values are positive mean indices

Table III

ST production of 4-year-old Y. enterocolitica strains stored in stock-agar

Strains	Suckling mice test		Rabbit gut loop test (6th hour)	
	Crude, filtrate	Purified, concentrated, filtrate	Crude, filtrate	Purified, concentrated, filtrate
3064	0.054	*0.101*	0.48	*1.98*
OKI 134	0.069	0.061	0.88	*1.30*
797	0.063	0.065	0.92	*1.35*
1866	0.079	*0.088*	0.53	*2.00*
269	0.066	*0.148*	*1.2*	*2.36*
Controls				
E. Coli ST	0.092		*1.6*	
Culture media	0.049		0.32	

Italicized values are positive mean indices

tate fraction. The data in Table V show that the methanol soluble fraction of the 5- or 10-fold concentrated filtrates displayed enterotoxic activity in the suckling mice and rabbit gut loop tests. The methanol insoluble fractions were

Table IV

The stability of enterotoxin production of freeze-dried Y. enterocolitica

Strains	Storage in freeze-dried state 9 years		Storage in stock agar, 6 months	9 years
	Suckling mice index	Rabbit gut loop index	Suckling mice index	Suckling mice index
3637	*0.123*	*1.7*	.	.
3188	*0.125*	*1.8*	.	.
3186	*0.140*	*1.7*	*0.148*	*0.079*
3956	*0.137*	*1.8*	*0.100*	0.069
Controls				
E. coli ST	*0.096*	*1.6*		
Culture media	0.42	0.7		

Italicized mean indices are positive

inactive in both tests. The concentrated filtrates of the strains 797 and OKI 134 using both their methanol soluble and insoluble fractions, were enterotoxin negative in the suckling mice test, but in the rabbit gut loop test the the concentrated filtrates and the methanol soluble fractions were enterotoxin positive even if old strains were used. The methanol soluble fraction (ST a) of the enterotoxin of the *E. coli* P16 strain, which was used for comparison, showed a positive reaction in the suckling mice test but only a slight enterotoxic activity in the rabbit gut loop test. The methanol insoluble fraction was inactive in suckling mice but enterotoxin positive in the rabbit gut loop test. The CHO test performed with the filtrate of each strain indicated no LT effect.

Table V

Activity of the concentrated filtrates, and their methanol-soluble and insoluble fractions in suckling mice and the rabbit gut loop test

Strain	Concentrated	Concentrated filtrate in		Methanol soluble fraction in		Methanol insoluble fraction in	
		suckling mice	rabbit gut loop	suckling mice	rabbit gut loop	suckling mice	rabbit gut loop
R 5819	10×	*0.136**	*2.7*	*0.137*	*3.5*	0.060	0.50
R 7538	10×	*0.148*	*2.1*	*0.125*	*1.6*	0.061	0.40
R 3394	10×	*0.130*	*3.4*	*0.118*	*2.4*	0.058	0.20
R 3186	5×	*0.120*	*2.5*	*0.119*	*2.6*	0.075	0.50
R 3956	5×	*0.122*	*1.6*	*0.118*	*2.6*	0.072	0.40
5319	10×	*0.118*	*1.5*	*0.095*	*1.6*	0.049	0.70
797	10×	0.065	*1.8*	0.055	*2.0*	0.060	0.80
OKI 134	10×	0.060	*2.0*	0.055	*1.7*	0.055	1.00
E. coli P 16							
STa, STb	10×	*0.150*	*2.0*	*0.145*	1.1	0.072	*1.4*
Syncase	10×	0.042	0.4	.	.	.	.

* Index values: italicized mean indices are positive.

Discussion

Though the experiments were performed with relatively few strains, the enterotoxin-positive effect of every strain isolated in the year of the study was nevertheless striking. Boyce *et al.* [6] observed a stable ST production for 5 months and found that the strains stored in fluid cultures at —40 °C preserved

their capacity of enterotoxin production just as the strains did which were cultivated on agar media and passaged monthly. The latter, however, lost their enterotoxigenicity if not passaged for 3 months. The strains collected by PAI and MORS [1] for 3 years and stored in glycerol at −20 °C, preserved their enterotoxin positivity. Only a small part of the reference strains produced enterotoxin although their storage in stock agar might have played a role in this. Thus, forbearing storage (as deep-freeze, freeze-drying) seems considerably to contribute to the preservation of the enterotoxin producing capacity.

The enterotoxin of *Y. enterocolitica* behaves differently from the ST toxin of the used *E. coli* strain; only the methanol soluble fraction of the former is enterotoxin positive in both the rabbit gut loop and the suckling mice tests. The methanol soluble fraction (ST a) of *E. coli* ST is, on the other hand, negative in the rabbit gut loop test, while its methanol insoluble fraction is positive [8, 9]. BOYCE *et al.* [6] already described the activity of the methanol soluble fraction of *Y. enterocolitica* in the suckling mice test.

The genetic control of enterotoxin producing capacity is still uncertain. PAI and MORS [1] suppose that the enterotoxigenicity of *Y. enterocolitica* is of chromosomal origin. In an animal model experiment they found after 6 months of observation that the strains preserved their enterotoxigenic capacity [10]. In a previous report we suggested the possibility of plasmid information because the strains stored in subcultures seemed to lose their capacity of ST production [3]. In contrast, concentrated filtrates of these subcultures were now found enterotoxin positive in both tests or, at least in the rabbit gut loop test.

Hence, it is probably only a quantitative change in ST production that takes place and the loss of information can be excluded. Our present results support the chromosomal control of the enterotoxigenic character. The enterotoxigenic properties of *Y. enterocolitica* could not be connected to plasmid, as in the case of other pathogenetic factors.

Verification of the genetic control of enterotoxin production requires further direct experiments.

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REFERENCES

1. PAI, C. H., MORS, V.: Infect. Immun. **19,** 908 (1978).
2. ROBINS-BROWNE, R. M., STILL, C. S., MILIOTIS, M. D., KOORNHOF, H. J.: Infect. Immun. **25,** 680 (1979).
3. VELIN, D., EMŐDY, L., PÁCSA, S., KONTROHR, T.: Acta microbiol. Acad. Sci. hung. **27,** 299 (1980).
4. JACKS, T. M., WU, B. J.: Infect. Immun. **9,** 342 (1974).
5. DE, S. N., BNATTACHARYA, K., SARKAR, J. K.: J. Path. Bact. **71,** 201 (1956).
6. BOYCE, J. M., EVANS, D. J., EVANS, D. G., DU PONT, H. L.: Infect. Immun. **25,** 532 (1979)

7. GUERRANT, R. L., BRUTON, L. L., SCHNAITMAN, T. C., REBHUN, L. I., GILMAN, A. G.: Infect. Immun. **10,** 320 (1974).
8. MULLAN, N. A., BURGESS, M. N., NEWSOME, P. M.: Infect. Immun. **19,** 779 (1978).
9. BURGESS, M. N., BYWATER, R. J., COWLEY, C. M., MULLAN, N. A., NEWSOME, P. M.: Infect. Immun. **21,** 526 (1978).
10. PAI, C. H., MORS, V., SEEMAYER, T. A.: Infect. Immun. **28,** 238 (1980).

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EFFECT OF ENDOTOXIN AND RADIO-DETOXIFIED ENDOTOXIN ON CELL MEMBRANES *IN VITRO*

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The effect of parent lipopolysaccharide (LPS) and radio-detoxified endotoxin (rdLPS) on various human blood cell membranes proved to be different as detected by ^{3}H-concanavalin A-binding technique *in vitro*. The lectin-binding ability of erythrocyte membranes did not change upon treatment by either endotoxin, whereas that of lymphocytes was stimulated by LPS at 10–50 μg/ml concentration as well as by rdLPS at the lowest dose applied, i.e. 0.1 μg/ml. The LPS-treated platelets bound ^{3}H-concanavalin A less than did the untreated controls; on the other hand, the rdLPS did not change the lectin-binding surface of these cells. The affection by radiation of cell membranes could be prevented by pretreatment with endotoxins. This fact, however, could not be considered a radioprotective effect. The micromorphological investigations by scanning electron microscopy (SEM) support our data concerning the functional alterations of plasma membranes of platelets and lymphocytes after LPS and rdLPS treatment as well as after the combined effect of endotoxin pretreatment and X-irradiation, since a severe smoothening of the cell surface could be observed.

Experience shows that human beings are more sensitive to bacterial endotoxin than other mammals. Endotoxins identified as lipopolysaccharides (1, 2) of Gram-negative bacteria affect virtually every organ in the course of endotoxaemia. The latter occurs frequently in radiation illness and it manifests with intestinal syndrome and endotoxin shock. In endotoxin-resistent animals as well as in the case of endotoxin tolerance, however, certain favourable properties of lipopolysaccharides dominate. One of the most important actions of endotoxins is their capacity to stimulate the organism's natural defence. That is why numerous attempts have been made to obtain an endotoxin that does not exert toxic effects but possesses beneficial properties. Among various detoxification procedures we applied radiation treatment on endotoxin from *Escherichia coli* O89. In this way several noxious properties of LPS were diminished, whereas the endotoxin tolerance-inducing, immuno-adjuvant, shock--preventing, non-specific resistance-enhancing capacity and a certain radioprotective effect of the radiation-treated LPS were preserved [3–5].

Although the mechanisms by which LPS interacts with the cell are unknown, it was suggested that all endotoxin-mediated cell responses involve an interaction at the plasma membrane [6, 7]. In this context it is important to know whether the LPS influences certain well-defined membrane-bound functions, e.g. the binding of lectins. In the present work, therefore, the effects of the parent LPS and that of radio-detoxified LPS were studied upon concanavalin A receptors of human blood cells *in vitro*. Furthermore, the influence of

endotoxin pretreatment on radiation-induced functional alterations of cell membranes was also tested. The functional changes of cell surfaces were followed by scanning electron microscopy, among others.

Materials and methods

Preparation, detoxification and labelling of endotoxin. Extraction of endotoxin from a fermenter-grown culture of *E. coli* O89 was performed by the warm phenol–water method [8]. For detoxification, the material was irradiated with 150 kGy in a ^{60}Co source (Noratom-Gamma 350C). The LD_{50} values of the parent and irradiated preparations were determined in appropriate animal species [9]. Labelling of LPS with ^{51}Cr was carried out as described earlier [10].

LPS and rdLPS treatment of human blood samples. Human citrated blood samples (3–5 ml) from healthy donors were treated with LPS and rdLPS in final concentrations (v/v) of 0.1–50 μg/ml at room temperature for 1 h. After incubation with the endotoxins, separation of blood cells was carried out by centrifugation in Ficoll-Uromiro discontinuous gradient as described elsewhere [11]. The populations of platelets, lymphocytes and erythrocytes were isolated and washed with physiological saline.

^{3}H-concanavalin A (^{3}H-Con A) binding by human blood cells. Labelling of separated cell fractions with radioactive lectin was carried out in a volume of 0.2 ml; i.e. 0.1 ml 1 μCi/ml ^{3}H-ConA [^{3}H(G), NET-491, specific radioactivity 38.8 Ci/mmol, New England Nuclear, Boston, Mass., USA] was added to 0.1 ml cell suspension. ^{3}H-ConA labelling of erythrocytes (4×10^7 cells/0.1 ml) was performed at room temperature for 30 min, lymphocytes ($1–1.5\times10^6$ cells/0.1 ml) and platelets (1×10^6 cells/0.1 ml) were incubated with radioactive lectin at room temperature for 10 min. The unbound radioactivity of labelled cells was removed by repeated washings with cold physiological saline [12].

Irradiation technique. The blood samples (5 ml) pretreated with endotoxins for 1 h were X-irradiated in teflon tubes with 1 Gy for examinations of platelets and lymphocytes. Irradiation was carried out with a THX-250 X-ray apparatus under conditions as follows: 200 kV, 1.45 mm Cu, SSD 60 cm, dose-rate 0.317 Gy_{water} per min. Exposures were measured by a calibrated ionization chamber (Farmer dose meter Type 2502/3, 0.6 cm^3 ionization chamber). For calculation of the dose absorbed in water, conversion factors and the value of HVL were used.

Radioactivity measurements. For liquid scintillation counting the blood cells labelled with ^{3}H-ConA were dissolved in Bray's solution, and radioactivity was measured with a liquid scintillation spectrometer (Searle Analytic Inc., Mark III, Model 6880). For conversions of cpm to dpm the external standardization method was applied. The extent of binding was expressed in terms of dpm per 10^6 cells for each cell type.

Scanning electronmicroscopy (SEM). The cells were fixed with 2% glutaraldehyde at 4 °C for 1 h. After washing in buffer for 15 min, the materials were fixed in 2% OsO_4 for 1 h at 4 °C, then washing in buffer for 15 min was repeated. The cells were dehydrated in a series of ethanol and amylacetate, which was followed by critical point drying (Sorval critical point drying system) in CO_2 and gold coating. Specimens were photographed using JSM 50 A SEM at accelerating voltages of 20 kV at tilt angles of 45°.

Results

In the first series of experiments the effect of endotoxins in concentrations from 0.1 to 50 μg/ml was tested on human platelets, lymphocytes and erythrocytes. By means of concanavalin A-binding technique the specific functional alterations of plasma membranes could be determined since the lectin bound directly to glucose and mannose groups of oligosaccharides on glycoproteins.

The results summarized in Fig. 1 show that the reaction of different cell membranes against LPS varied. As compared to the controls, treatment with

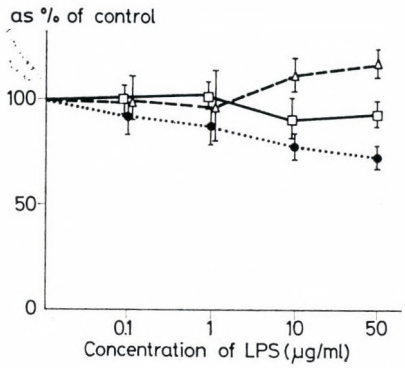

Fig. 1. Effect of LPS at various concentrations on the ^{3}H-ConA-binding to plasma membranes of human blood cells. Values are expressed in percents of the non-treated control. The points represent results of six measurements from two separate experiments. The SE values of the controls were $< \pm 10\%$. •.....• platelets, △------△ lymphocytes, □———□ erythrocytes

the parental endotoxin did not alter the ability of erythrocytes to bind ^{3}H--ConA. Binding by the platelet surfaces decreased as the concentration of LPS increased, whereas the ^{3}H-ConA-binding of lymphocytes showed an increasing tendency on high doses of LPS. Thus, the membranes of lymphocytes and particularly of platelets reacted against endotoxin, although in different manner and direction. The availability of ConA-binding sites on lymphocytes increased while on the platelets decreased.

SEM also revealed severe changes of the membranes. Figure 2 shows striking morphological changes after incubation with LPS, i.e. the characteristic discoid shape of cells (2b) was transformed, and the appearance of numerous long (2–10 μm) pseudopodia could be observed (2a).

The LPS detoxified with 150 kGy did not cause alterations in ConA-binding properties of either the platelets or red blood cells. The lymphocyte surfaces, however, bound the lectin at an increased level (Fig. 3), even at the lowest concentration of detoxified endotoxin, i.e. in the presence of 0.1 μg/ml.

In the second series of experiments the influences of endotoxins on radiation-induced membrane alterations were studied. It had already been demonstrated on blood cells of irradiated mice and various human cells irradiated *in vitro* that X-irradiation alone provoked an early and temporary increase of ^{3}H-ConA-binding (11–14). It was found that the pretreatment of blood samples with 10 μg/ml endotoxin at room temperature for 1 h inhibited the development of radiation effect as it was detected in various time-points of post-irradiation period after applying 1 Gy dose for the platelets (Fig. 4). Similar tendency was observed on the lymphocyte population, too (Fig. 5).

Although both types of endotoxins proved to prevent the radiation--induced increase in lectin-binding on the platelets and lymphocytes, the radio-

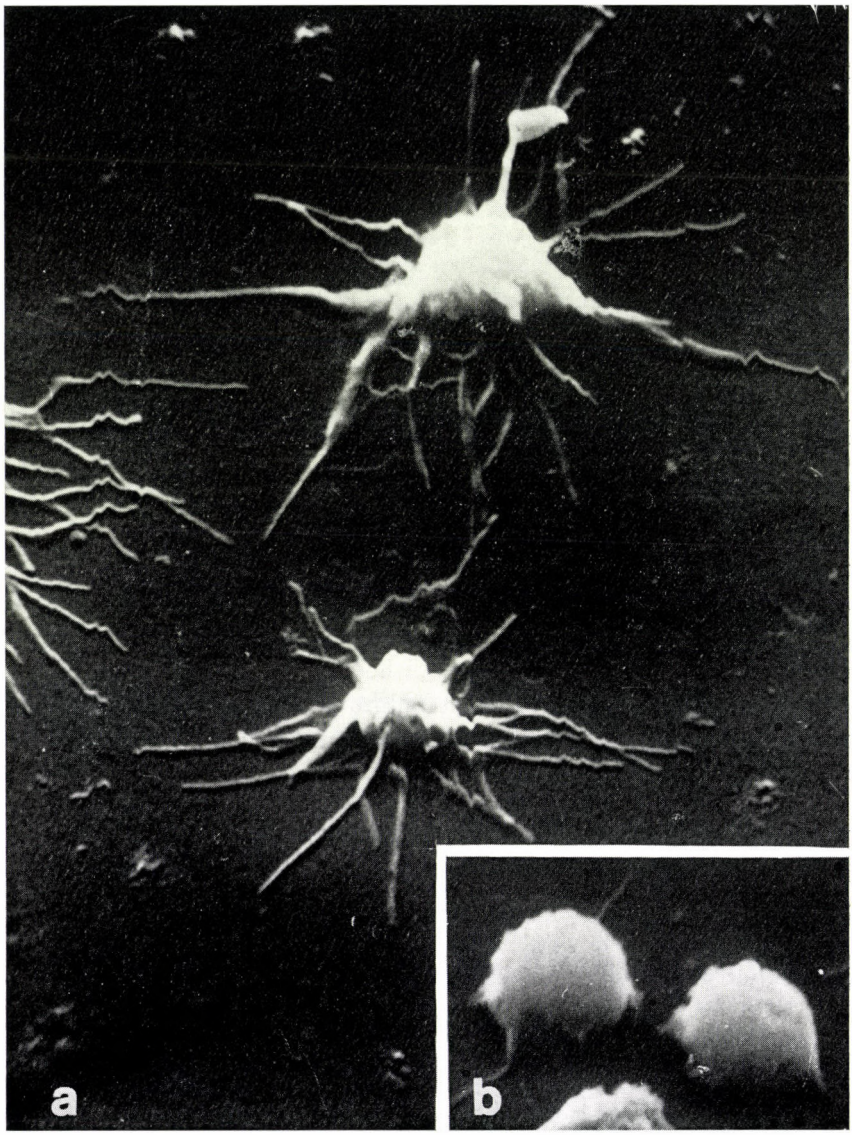

Fig. 2. Micromorphological (SEM) appearance of human platelets treated by 10 μg/ml LPS (a) as compared to the non-treated control (b) ×11 400

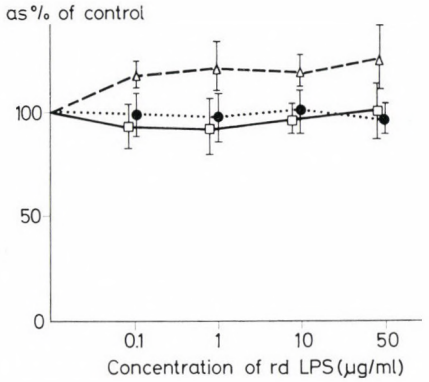

Fig. 3. Effect of rdLPS at various concentrations on the ^{3}H-ConA-binding to plasma membranes of human blood cells. Values are expressed in percents of the non-treated control. The points represent the results of six measurements from two separate experiments. SE values of the controls were $< \pm 10\%$. • . . . . • platelets, △ ------ △ lymphocytes, □ ——— □ erythrocytes

detoxified LPS depressed the levels of bound radioactivity in a greater extent than the parent endotoxin.

Micromorphological studies by SEM revealed severe alterations on the surface of human lymphocytes (Fig. 6). Treatment of the cells with the radiodetoxified LPS resulted in a reduction of the number of microvilli (6b) and the appearance of some bleb-like extensions as compared to the control (6a). Upon the combined effects of two factors, i.e. LPS-treatment and X-irradiation, the lymphocytes became smooth, almost "bald", and only some scattered microvilli and small spherical extensions could be seen (6c).

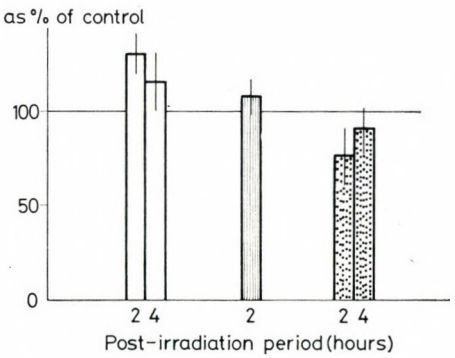

Fig. 4. Effect of various endotoxin preparations on the development of radiation-induced affection of plasma membranes of human platelets. ^{3}H-ConA-binding was measured at various points of time after X-irradiation with 1 Gy on the irradiated control (open), on cells pretreated with 10 μg/ml LPS for 1 h (shaded) and on cells pretreated with 10 μg/ml rdLPS for 1 h (dotted). Values are expressed in percents of ^{3}H-ConA-binding by the non-irradiated control. The points represent results of six measurements from two separate experiments. The SE values of the controls were $< \pm 10\%$

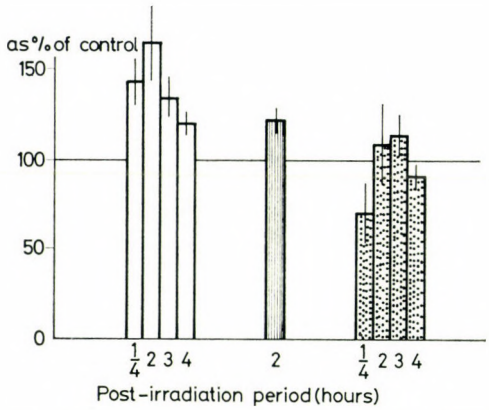

Fig. 5. Effect of various endotoxin preparations on radiation-induced affection of the plasma membranes of human lymphocytes. ^{3}H-ConA-binding was measured at various points of time after X-irradiation with 1 Gy on the irradiated control (open), on cells pretreated with 10 μg/ml LPS for 1 h (shaded) and on cells pretreated with 10 μg/ml rdLPS for 1 h (dotted). Values are expressed in percents of ^{3}H-ConA-binding by the non-irradiated control. The points represent results of six measurements from two separate experiments. The SE values of the controls were $< \pm 10\%$

Discussion

The fact that endotoxin interacts with the cell membranes, i.e. lysosome, mitochondria and plasma membranes, is already known from the literature (6, 7, 15–18). It has also been suggested that a generalized affection of a plasma membrane might be a sufficient triggering signal for all cell responses [6] leading to multiple reactions of the organism. The effect of LPS detoxified with 150 kGy γ-radiation has been mostly examined in animals and the experimental results showed that the endotoxin modified in this way could be considered a potent stimulator of non-specific resistance [19]. Besides, its radioprotective property has also been observed [4, 5]. From this point of view, do the plasma membranes play any role?

In the present work comparative studies of the effect of parent and radio-detoxified LPS were carried out *in vitro*. In the experiments it was attempted to approach natural conditions, therefore the endotoxins were added not to isolated cells but to whole blood. Under such circumstances both the toxic and the detoxified endotoxins may exert more than direct membrane action. The presence of various substances in blood plasma, the interaction between them and the LPS-preparations, the reaction of other cell types could modify the membrane changes observed after treatment of erythrocytes, lymphocytes and platelets. The reaction of isolated blood cells against the endotoxins would provide a model of the direct effect.

Methodological procedures, i.e. cell separation from the blood treated with LPS and rdLPS as well as cell washing did not influence significantly the

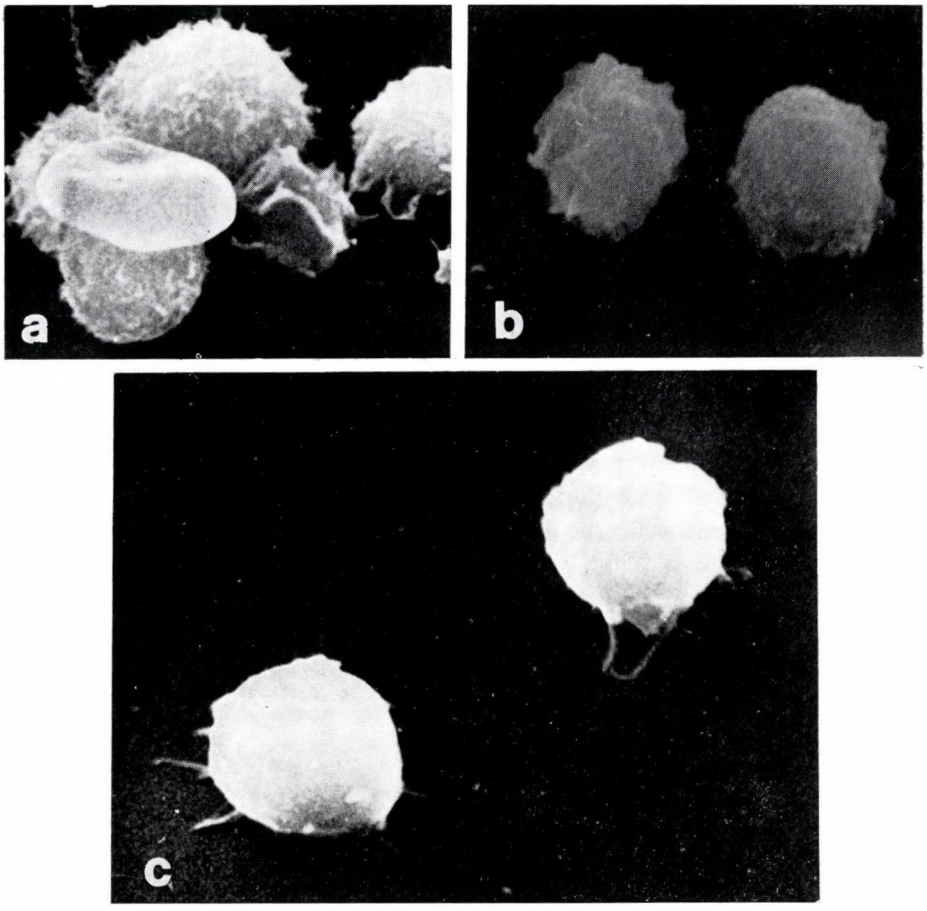

Fig. 6. Scanning electronmicroscopic appearance of human lymphocytes. (a) Control, ×5700; (b) cells treated with 10 μg/ml rdLPS for 1 h, ×3800; (c) cells pretreated with 10 μg/ml rdLPS then X-irradiated with 1 Gy, ×7900

amount of endotoxin bound to the cell surfaces. It was checked by measurements of radioactivity of the cells labelled with ^{51}Cr-endotoxin before and after manipulations (unpublished data). Our present results, however, showed that both endotoxins altered the surface of platelets and lymphocytes functionally and morphologically as detected by the lectin-binding technique and SEM. Lectin-binding by erythrocytes proved to remain unaltered. Our data show that the effect of LPS depends on the cell type. This phenomenon might depend on the composition of membranes. The role of phospholipids in the response of lymphocytes and platelets is very likely as it was suggested that in these cells the phospholipids bind the LPS [20], while in the erythrocytes LPS-binding seemed to occur mostly through glycoproteins [21]. By comparing the different effects of endotoxins on different cells, other authors, too, concluded

that the variation in the phospholipid compositions from one cell type to the other might account for the specificity on the biological reactions elicited by lipopolysaccharides [22]. No explanation is available of the direction of responses of lymphocytes and platelets. It is, however, evident from the data presented that LPS preparations affect the bindings of other ligands.

The effect of endotoxins on the plasma membrane resulted in an inhibition of a radiation-induced phenomenon [11–14], i.e. the increase in lectin-binding did not occur. As far as the mechanism of endotoxin effect is concerned, the question arises whether this inhibition means that the endotoxins stabilize the cell membranes against radiation and so protect the cell, or it is simply due to a more profound disturbance of plasma membrane by LPS on the basis of which the radiation-induced membrane affection as detected by lectin-binding cannot develop. On the basis of the surface alterations revealed by SEM, i.e. impoverishment in visible formations, the "bald" appearance, the possibilities that the cells suffer losses in ConA-binding sites or these sites become covered by LPS coating the cell, cannot be excluded.

In conclusion, the changes observed in the function and morphology of cell membranes upon the effect of parent and radio-detoxified endotoxin undoubtedly point to the influence of LPS preparations on the behaviour of cells in cell-to-cell or cell-to-bacteria interactions, e.g. phagocytosis [23] as an intensified cellular process or activity in the course of radiation-disease.

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REFERENCES

1. Rowley, D.: Brit. J. exp. Path. **37,** 223 (1956).
2. Landy, M.: Ann. N. Y. Acad. Sci. **66,** 292 (1956).
3. Bertók, L., Kocsár, L., Várterész, V., Bereznai, T., Antoni, F.: Hungarian Patent No. 162973, Budapest 1973.
4. Bertók, L., Kocsár, L.: Izotóptechnika **11,** 543 (1972).
5. Bertók, L., Kocsár, L.: Wiss. Z. Karl-Marx Univ. Leipzig **23,** 65 (1974).
6. Morrison, D. C., Oades, Z. G., di Pietro, D.: In Agarwall, M. K. (ed.): Bacterial endotoxin and host response. Proc. IVth Int. Congress of Immunology. July 1980, Paris, France. p. 311. Elsevier North Holland Biomedical Press, Amsterdam, New York, Oxford 1980.
7. Bradley, S. G.: Ann. Rev. Microbiol. **33,** 67 (1979).
8. Westphal, O., Lüderitz, O., Bister, F.: Naturforsch. **7b,** 148 (1952).
9. Füst, Gy., Bertók, L., Juhász-Nagy, S.: Infect. Immunol. **16,** 26 (1977).
10. Kocsár, L. T., Bertók, L., Varga, L.: Ann. immunol. hung. **17,** (supplementum) 71 (1973).
11. Kubasova, T., Varga, L. P., Köteles, G. J.: Int. J. radiat. Biol. **40,** 175 (1981).
12. Kubasova, T., Köteles, G. J., Varga, L. P.: Int. J. radiat. Biol. **40,** 187 (1981).
13. Köteles, G. J., Kubasova, T., Varga, L.: Nature (Lond.) **259,** 507 (1976).
14. Kubasova, T., Csáky, L., Köteles, G. J., Varga, L., Sztanyik, B. L.: Proc. IVth Int. Congress of IRPA, Paris, France, **4,** 1203 (1977).

15. Bona, C., Chedid, L., Lancensans, A.: Infect. Immunol. **4,** 532 (1971).
16. Esser, A. F., Russell, S. W.: Biochem. biophys. Res. Commun. **87,** 532 (1979).
17. Springer, G. F., Adye, J. C., Bezkorovainy, A., Murthy, J. R.: J. infect. Dis. **128,** 202 (1973).
18. Springer, G. F., Adye, J. C., Mergenhagen, S. E., Rosenstreich, D. L.: In Schlessinger, D. (ed.): Microbiology. American Society for Microbiology, Washington, D. C. 1977. p. 326.
19. Bertók, L.: Perspect. Biol. Med. (Chicago) 24, 61 (1980).
20. Springer, G. F., Adye, J. C.: Infect. Immunol. **12,** 978 (1975).
21. Springer, G. F., Adye, J. C., Bezkorovainy, A., Jirgensons, B.: Biochemistry **13,** 1379 (1974).
22. Kabir, S., Rosenstreich, D. L., Mergenhagen, S. E.: In Jeljaszewicz, J., Wadström, T. (eds): Bacterial toxins and cell membranes. Academic Press, New York 1978. p. 59.
23. Kisida, E., Döklen, A., Bertók, L.: Kísérl. Orvostud. **28,** 561 (1976).

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SENSITIZING ACTIVITY TO EGG PROTEIN OF AN $AlPO_4$-ADJUVATED FULL-VIRUS INFLUENZA VACCINE

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The sensitizing activity to egg protein of an $AlPO_4$-adjuvated purified and concentrated influenza-A vaccine was examined in animal experiments and in man. Intravenous injection of ovalbumin caused anaphylactic symptoms and/or fatal anaphylactic shock in prevaccinated guinea-pigs. Ovalbumin-specific antibodies detectable by the passive haemagglutination reaction (PHA) appeared in the blood serum of the vaccinated animals. Model experiments with purified ovalbumin suggested that 1 human dose of the vaccine contained egg protein in the range from 0.1 to 1ng, and that the antigenic effect of the vaccine grew to more than 10^3-fold by its adsorption to $AlPO_4$ gel. Adults who in previous years had been immunized with similarly prepared influenza vaccine several times responded with mild reactions; symptoms suggestive of hyperergy did not occur, irrespective of the vaccination history. In the prevaccination serum sample of some vaccinees, ovalbumin-specific PHA antibodies were found up to titres independent of the number of the previous immunizations. The concentration of the ovalbumin-specific antibodies of the IgE class was by several orders of magnitude lower in the postvaccination samples than in the serum of some patients hypersensitive to egg protein.

The full-virus influenza vaccines produced in embryonated eggs, even the purified ones, cannot be administered to egg-sensitive subjects owing to their residual egg-protein content.

Due to the short duration of the vaccination-induced immunity, it may happen that some subjects are immunized 5–10 times yearly, thus the idea has been raised that the vaccine itself might induce hypersensitivity. Based on animal experiments mainly aluminium-gel-adsorbed vaccines have been suspected, for adjuvants containing aluminium have been found considerably to raise the anaphylactogenic activity of ovalbumin [1] and of full-virus influenza vaccines [2]. In rabbits and other rodents, the production of globulins of the IgE class is stimulated by the same preparations [3]. In man, the sensitizing activity of non-adjuvated full-virus influenza and mumps vaccines purified by adsorption to, and elution from, erythrocytes was examined by RATNER *et al.* [4]. Threehundred and nineteen healthy children were involved in the studies. Each child was vaccinated repeatedly and subjected to egg protein skin tests (11–38 injections per child). Specific dermal sensitivity to egg protein, without any clinical symptom or sign, was seen in 1.6% of the vaccinees. To our best knowledge, the sensitizing activity of adjuvated influenza vaccines has not been examined in man so far.

In Hungary, an $AlPO_4$-gel-adsorbed purified and concentrated full-virus vaccine has been used since 1960. Although many persons have been vaccinated

several times yearly, major reactions have not been observed and minor reactions were infrequent. In spite of the favourable observations, we considered justified to examine the possible sensitizing effect of the vaccine to egg protein.

Materials and methods

Vaccine. The formalin-inactivated influenza-A vaccine used in the studies had been produced in the embryonated egg and purified and concentrated by TAKÁTSY's method (5) including adsorption to, and elution from, formalinized chicken erythrocytes, and precipitation by dialyzation against 0.4% saline. The inactivated virus suspension was adsorbed to $AlPO_4$ gel. One human dose of the vaccine contained 500 HAU of each of H1N1 and H3N2 influenza A virus adsorbed to 1.5 mg $AlPO_4$ gel.

Vaccinees. Adults 18–65 years of age working in the health service who during the previous few years had been immunized 0 to 10 times with a vaccine the production technology of which is outlined above. (Some details of the technology were changed during these years). The vaccinees were questioned of the reactions that had appeared during the 48 h period following vaccination and the reactions were registered in detail.

Serum samples examined for anti-ovalbumin. (i) Paired sera taken before vaccination and 4 weeks thereafter; (ii) serum samples from adults of unknown influenza vaccination history; (iii) serum samples from children never vaccinated against influenza before; (iv) serum samples from adults sensitive to egg protein and those suffering from skin diseases such as chronic urticaria, psoriasis vulgaris and atopic dermatitis; (v) serum samples from guinea-pigs injected with purified ovalbumin.

The serum samples were heated at 56 °C for 30 min and stored at —20 °C until used.

Examination of sera for ovalbumin specific antibodies. (1) *Passive haemagglutination (PHA) and PHA inhibition (PHAI) reaction.* Tanninized sheep erythrocytes (100 ml 2% suspension) were senzitized with 50 mg purified ovalbumin. Preparation of the reagent was the same as described elsewhere [6] for the sensitization of erythrocytes with bovine serum albumin.

The sera were titrated in Takátsy's Microtitrator system [7] using 25 μl loops and 25 μl drops. Saline of pH 7.4 containing 1% normal rabbit serum was used as diluent. One drop of reagent was added to each mixture. A reference serum, i.e. a pool of sera from guinea pigs immunized by ovalbumin was included in each tiration panel. The trays were allowed to stand at room temperature for 30 to 60 min before the results were read. The serum titre was the highest dilution giving a HA reaction of three crosses or stronger. The titre of the reference serum ranged between 1 : 6400 and 1 : 12 800 as examined on different occasions.

To check specificity, the serum samples whose PHA titre was 1 : 16 or less were titrated for PHAI in the Microtitrator system. One drop of 0.1% purified ovalbumin was added to each serum dilution. After incubation at room temperature for 1 h, one drop of reagent was added to each mixture and the results were read after an additional incubation of 30 to 60 min at room temperature.

A PHA titre not higher than 1 : 16 was regarded as positive if ovalbumin caused at least a four-fold drop of titre.

(2) *Radio-immunoassay (RIA) for measuring antibodies of the IgE class.* Purified ovalbumin was bound to CNBr-Sepharose 4B. Serum sample diluted with IgE-free horse serum, if necessary, was added to a mixture of equal volumes of gel bound to 0.2 ml albumin and 0.05 M phosphate buffer of pH 7.4. The mixtures were kept at 4 °C for 18 h, and washed 3 times with saline containing 0.5% Tween 80 then 0.05 ml of ^{125}I-labelled anti-human-IgE serum (Phadebast: 126 kBq) was added to the washed gel. The gel was incubated at 4 °C for 18 h and washed again with the Tween-containing saline. The radioactivity of the washed gel was measured in a Gamma NP-354 automatic Gamma counter. Various dilutions of a reference mixture composed of serum samples of patients sensitive to egg protein were tested in each panel. The anti-ovalbumin content of the sera was expressed in per cent of the reference value.

Systemic anaphylaxis test in guinea-pigs. Commercial guinea-pigs 300–350 g in weight were used throughout. Influenza vaccine or purified ovalbumin was administered subcutaneously. The doses are presented under Results. For provocation, 1 ml of a 10% ovalbumin solution was injected intravenously. Any typical anaphylactic symptom was considered a positive reaction.

Results

Sensitization of guinea pigs against ovalbumin with unadsorbed and $AlPO_4$-adsorbed influenza A virus vaccine. Two human doses of the vaccine showed a well-defined anaphylactogenic effect (Table I). The virus components of the vaccine were examined in the unbound state so that 500 HAU of the H1N1 or the H3N2 component was adsorbed to 3 mg of $AlPO_4$ gel. The nonadsorbed virus components failed to sensitize the animals while the adsorbed ones sensitized them.

Table I

Sensitizing activity of influenza vaccine to ovalbumin in guinea pigs

Product under testing	Preparatory dose		No. of animals	Result of ovalbumin provocation		
	Virus HAU	$AlPO_4$ mg		no symptom	anaphylactic symptoms	
					survived	died
Adsorbed influenza-A vaccine	1000 H1N1 + 1000 H3N2	3	4	1	1	2
Intermediate vaccine products	500 H1N1	—	4	4	—	—
	500 H1N1	3	4	—	2	2
	500 H3N2	—	4	4	—	—
	500 H3N2	3	4	—	2	2

Table II

Sensitizing activity of unadsorbed and adsorbed purified ovalbumin in guinea-pigs

Preparatory dose		Experiment 1				Experiment 2			
			Result of provocation by ovalbumin				Result of provocation by ovalbumin		
albumin μg	$AlPO_4$ mg	No. of animals	no symptom	anaphylactic symptoms		No. of animals	no symptom	anaphylactic symptoms	
				survived	died			survived	died
1	3	5	—	2	3	7	1	1	5
10^{-1}	3	5	—	2	3	7	3	3	1
10^{-2}	3	.	.	.	.	6	3	1	2
10^{-3}	3	.	.	.	.	6	5	—	1
10^{2}	—	4	3	—	1	7	—	5	2
10	—	5	3	2	—	7	6	1	—
1	—	5	4	1	—	7	6	1	—
10^{-1}	—	5	4	1	—	.	.	.	.

Sensitization of guinea-pigs with unbound and $AlPO_4$-adsorbed ovalbumin. Varying amounts of purified ovalbumin were adsorbed to $AlPO_4$ gel. Similarly as in the preceding experiment, there were appreciable differences in sensitizing activity between the same amounts of unadsorbed and adsorbed albumin (Table II). If the results of Experiment I and II are evaluated jointly, taking into account only the most severe symptom, the death of animals due to anaphylactic shock, it is clear that the sensitizing activity of ovalbumin was increased at least by three orders of magnitude by adsorption to $AlPO_4$ gel.

Anti-ovalbumin PHA titres of serum samples taken from guinea-pigs injected with unadsorbed and $AlPO_4$-adsorbed purified ovalbumin. Guinea-pigs were injected with various doses of unadsorbed ovalbumin or the same doses of ovalbumin in the adsorbed form (Table III). Serum samples taken by heart

Table III

Specific serum PHA titre of guinea-pigs inoculated with unadsorbed or adsorbed purified ovalbumin

Immunizing dose		No. of sera	Distribution of sera by anti-ovalbumin PHA titre 1:					
albumin μg	$AlPO_4$ mg		8	16	32	64	128	256
10^{-2}	3	9	2	5	1	1	—	—
10^{-3}	3	9	1	—	1	3	4	—
10^{-4}	3	6	—	—	1	2	3	—
10^{-5}	3	8	8	—	—	—	—	—
10^{2}	—	8	1	1	3	2	1	—
10	—	9	1	—	2	4	2	—
1	—	8	3	1	—	2	2	—
10^{-1}	—	9	8	—	—	—	—	—
—	—	10	10	—	—	—	—	—

puncture in the 4th postvaccination week were titrated against ovalbumin by the PHA test. Samples from 10 untreated guinea-pigs served as control. An appreciable antibody titre ($\leq$1 : 16) in 50% of the animals required at least 1 μg ovalbumin to be injected in the unadsorbed form or 10^{-4} μg to be injected in the adsorbed form.

Vaccination reactions in primovaccinated and in 1 to 10 times prevaccinated persons. Table IV clearly shows that the vaccination caused very weak, if any, side effects. Anaphylactic symptoms, e.g. urticaria, was observed in no case. There was no correlation between the number of prevaccinations and the frequency of vaccination reactions.

Anti-ovalbumin PHA titres in serum samples from persons differing in vaccination history. Paired sera from 33 vaccinees were tested for PHA. The

Table IV

Side reactions in vaccinees with different vaccination history

No. of previous influenza vaccination	No. of vaccinees	No reaction	Local pain	Slight malaise
0	12	9	3	0
1	12	8	3	1
2 or 3	11	10	1	0
4—10	13	10	2	1
Total	48	37	9	2

prevaccination titres are grouped according to vaccination history in Table V. Only four of the titres $\leq 1 : 16$ were found to be specific when checked by the PHAI reaction. The PHA activities of further 20 sera could not be absorbed with ovalbumin. In these cases, the PHA was certainly caused by antibodies to sheep erythrocytes. The remaining 9 serum samples had significant (1/32–1/512) PHA titres, but showed no relationship to the number of previous influenza vaccinations.

Table V

Anti-ovalbumin PHA titre in serum samples from subjects with different vaccination history

No. of previous influenza vaccinations	No. of persons	Distribution of the sera by PHA titre 1:						
		8	16	32	64	128	256	512
0	12	9	—	1	—	2	—	—
1–3	8	5	—	—	—	—	1	2
4–10	13	10	—	—	2	1	—	—

The prevaccination and postvaccination samples agreed well in PHA titre, i.e. the vaccination neither initiated specific PHA antibody production nor raised the prevalent titres.

In Table VI, the distribution of various groups of humans by anti-ovalbumin PHA titre is shown. Group A consists of the present vaccinees, the sera in group B were chosen from samples submitted from adults for syphilis serology. In the latter group, the vaccination history was unknown. Groups A and B were age and sex matched in composition. The donors of the serum samples in groups C, D and E had never been vaccinated against influenza. Those of group C were healthy infants 7 months of age sampled 4 weeks after the third injection of the basal immunization against diphtheria–pertussis–tetanus.

Table VI

Anti-ovalbumin PHA titre of subjects grouped by age and influenza vaccination history

Group	No. of sera tested	Distribution of the sera by PHA titre 1:									
		8	16	32	64	128	256	512	1024	2048	4096
A: adults 4 weeks after influenza vaccination	33	24	—	1	2	3	1	2	—	—	—
B: adults age- and sex-matched to group A*	33	23	1	—	4	4	1		—	—	—
C: 7-month-old infants**	25	20	—	1	1	2	1		—	—	—
D: 1- and 2-year-old children*	25	9	—	2	4	2	3	2	2		1
E: 5-7-year-old children*	24	13	5	5	1	—	—	—	—	—	—

* From screening tests.
** Serum samples taken 4 weeks after diphtheria–pertussis–tetanus immunization.

Groups D and E consisted of sera taken from 1 to 7 years old healthy children who had been sampled for mumps serology. The frequency of sera with PHA titres $>1:16$ was surprisingly high in the children 1 and 2 years of age. In this respect, there was a significant difference between the 7-month-old infants (group C) and the 5- to 7-year-old children (group E) ($\chi^2 = 9.959$; $P < 0.01$) and also between the former and the combined A + B group of adults ($\chi^2 = 12.091$; $P < 0.01$).

Table VII

Antibodies to ovalbumin in serum samples from healthy vaccinees and from dermatological patients

Healthy vaccinees	No. of previous influenza vaccinations	Anti-ovalbumin			
		PHA titre		IgE per cent	
		before vaccination	after vaccination	before vaccination	after vaccination
T.A. ♀	—	8	8	0.5	0.5
K.J. ♀	—	128	128	1.0	0.5
M.E. ♀	—	8	8	n.t.	1.3
N.S. ♂	—	8	8	n.t.	1.1
Sz.F. ♀	1	8	8	0.5	0.5
B.E. ♀	1	8	8	1.0	0.5
Sz.K. ♂	1	8	8	0.5	0.5
S.A. ♀	1	8	8	0.6	3.3
K.M. ♂	1	256	256	n.t.	1.1
S.K. ♂	1	512	512	n.t.	1.5
C.P. ♀	2	8	8	0.8	2.0
Zs.J. ♀	4	8	8	0.5	0.8
T.Zs. ♀	4	8	8	1.3	0.5
M.E. ♀	4	8	8	n.t.	1.4
M.A. ♀	5	64	64	1.5	0.9

Ovalbumin-specific antibodies determined by the PHA test and RIA in serum samples taken from influenza vaccinees and from dermatological patients. Paired sera or postvaccination serum samples from 15 vaccinees were tested for anti-ovalbumin by the PHA test and RIA. No relationship was found between the number of previous vaccinations against influenza and the serological results or the results of the two different tests. The ovalbumin-specific IgE contents were low; 2- or 3-fold differences occurred between the prevaccination and postvaccination titres in 4 of the 10 serum pairs tested. These differences could be attributed to the error of the method applied. In one case (SA ♀), the specific postvaccination IgE content was 5-fold of the prevaccination value. Unfortunately, we could not repeat the reaction. The PHA titres remained unchanged. In 18 dermatological patients, the oral sensitivity to egg albumin was examined. Generally, the PHA titres were low, showing correlation neither with the hypersensitivity to egg nor with the result of RIA. In two cases of chronic urticaria (TL ♀ and BM ♀), the anti-ovalbumin level of the IgE class was by two and three orders of magnitude, respectively, higher than the values measured in the sera of other patients or those of the vaccinees.

Patient	Clinical diagnosis	Result of oral provocation	Anti-ovalbumin	
			PHA titre before provocation	IgE per cent
O.K. ♂	urtic.chron.	—	8	0.5
T.F. ♀	urtic.chron.	—	8	0.5
H.L. ♂	urtic.chron.	—	8	n.t.
P.A. ♂	urtic.chron.	+	8	0.5
T.L. ♀	urtic.chron.	++	8	36.5
B.M. ♀	urtic.chron.	+++	32	280.0
F.M. ♂	urtic.chron.	—	8	n.t.
Zs.J. ♀	urtic.chron.	—	8	n.t.
J.L. ♂	urtic.chron.	+	8	2.3
O.J. ♀	atopic.derm.	—	16	n.t.
B.A. ♀	psor.vulg.	—	8	n.t.
B.I. ♂	psor.vulg.	—	8	n.t.
M.A. ♂	psor.vulg.	—	8	n.t.
M.B. ♂	psor.vulg.	—	8	5.6
V.J. ♂	psor.vulg.	—	8	n.t.
M.J. ♂	psor.vulg.	—	8	n.t.
S.A. ♀	psor.vulg.	—	128	0.5
H.L. ♀	psor.vulg.	—	8	0.5

n.t. = not tested

Discussion

The present anaphylaxis tests in guinea-pigs (Table I) obviously indicate that the influenza vaccine examined by us does contain some residual egg protein and that the anaphylactogenic activity of the latter is significantly supported by $AlPO_4$. The adsorbed bivalent vaccine or its adsorbed components proved to be anaphylactogenic, whereas the unadsorbed components had no such property. Still, the residual egg protein did not sensitize human vaccinees. The vaccine caused no hyperergic reaction even in the vaccinees who in the previous years had received 4 to 10 times influenza vaccine prepared by approximately identical technology. Neither PHA antibodies specific for egg protein were produced nor the concentration of pre-existent PHA antibodies was raised by the vaccination.

Ten serum pairs from vaccinees were examined by RIA for changes in the ovalbumin-specific IgE class antibodies. An appreciable rise was observed in a single case: the postvaccination sample contained 5 times as much of this antibody as did the prevaccination sample. It would be of interest to know the frequency and the possible clinical importance of such increases in antibody titre, for the postvaccination titres of patients non-sensitive to egg protein were far lower than the titres of those patients who proved to be severely hypersensitive in the case of oral provocation (Table VII).

The fact that we succeeded in sensitizing guinea-pigs but not human vaccinees may partly be attributed to the species difference. But we have to take into account that our guinea-pigs were overimmunized, their dose/body weight ratio being about 180 and 360 times as high as the corresponding ratio applied in man.

The animal experiments carried out with purified ovalbumin indicated that one human dose of the vaccine might contain 0.1–1 ng residual egg protein, since 0.1 ng $AlPO_4$-adsorbed ovalbumin, or even more, was necessary for inducing 50% of the experimental animals to produce a detectable amount of specific antibody, whereas the minimum anaphylactogenic dose was found to be about 1 ng (Table II).

The titres found in prevaccination serum samples called our attention to the occurrence of "natural" antibodies to ovalbumin. After we had observed that the titres were independent of the number of previous influenza vaccinations, we examined their frequency in different age groups. The data in Table VI suggest that the existence of antibodies to ovalbumin is a general phenomenon, and their presence may be related to the age of the individual and to his diet. Ovalbumin-specific antibodies occur at a surprisingly high frequency in children under 2 years of age, i.e. when egg and foods containing egg are given liberally. RATNER *et al.* [8], while examining the importance of the skin test with egg protein, arrived at a similar conclusion. Obviously, the specific

PHA antibodies to ovalbumin have no pathological importance, for none of the seropositive subjects suffered from manifest hypersensitivity. Furthermore, we failed to find any relationship between the PHA titre and the ovalbumin-specific IgE content of the sera (Table VII).

REFERENCES

1. KABAT, E. A., MAYER, M. M.: Experimental Immunochemistry. 2nd ed. Charles C. Thomas, Publisher, Springfield, Illinois, USA, 1961. pp. 269–316.
2. WEEKE-LÜTTMANN, M., SCHRAMM-THIEL, N.: Develop. biol. Standard. **39,** 219. S. Karger, Basel 1977.
3. WHO Scientific Group on Immunological Adjuvants. Geneva, 6–10 October 1975. WHO Techn. Rep. Ser. No. 595. 1976.
4. RATNER, B., UNTRACHT, S.: Amer. J. Dis. Children **83,** 309 (1952).
5. TAKÁTSY, GY.: Acta med. Acad. Sci. hung. **3,** 185 (1952).
6. ERDŐS, L., LÁNG, CS., JASZOVSZKY, I., NYERGES, GY.: J. biol. Standard. **3,** 77 (1975).
7. TAKÁTSY, GY.: Acta microbiol. Acad. Sci. hung. **3,** 191 (1955).
8. RATNER, B., UNTRACHT, S., HERTZMARK, F.: New Engl. J. Med. **246,** 533 (1952).

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EFFECT OF IONIZING RADIATION ON CHEMICAL AND BIOLOGICAL PROPERTIES OF *SALMONELLA MINNESOTA* R595 LIPOPOLYSACCHARIDE

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Lipopolysaccharide of the *Salmonella minnesota* Re mutant R595 was irradiated with ^{60}Co gamma doses of 50, 100, 150 and 200 kGy. The irradiated preparations were less toxic, less active in the Shwartzman reaction and as activators of the complement system, but they had retained the protection activity against the lethal action of endotoxin. The irradiation resulted in a dose-dependent decrease in the amounts of constituents (glucosamine, KDO, fatty acids) of the original lipopolysaccharide. With increasing irradiation doses increasing amounts of the irradiated material became dialysable (up to 21% in the 200 kGy sample). Only 50% of total fatty acids were present in the 200 kGy preparation compared to the parent lipopolysaccharide. The degradation products formed during irradiation have not been identified.

Various attempts have been made to modify endotoxins chemically or physicochemically in order to decrease their toxic (unwanted) effects without affecting their benificial (wanted) properties [1–6].

Thus, detoxified products have been obtained from *Escherichia coli* O89 endotoxin after gamma-irradiation under defined conditions [6]. Such preparations, depending on the dose of irradiation, were shown to exhibit low lethal toxicity and Shwartzman reactivity, and to induce only minor changes in blood pressure and white blood cell counts. On the other hand, they were still effective in preventing shock and conferring endotoxin tolerance [6–13].

Since the lipid A component of endotoxins represents the biologically active part of the endotoxin molecule [14], it could be expected that, as a result of irradiation, the lipid A structure would undergo chemical modifications leading to the observed changes in biological activity. The present paper describes comparative chemical and biological analyses of parent and irradiated endotoxin. The core-defective endotoxin of a *Salmonella minnesota* Re mutant has been selected for these studies, since it contains only KDO and lipid A [14]. Due to the high content of lipid A, even small chemical changes would be detectable.

Material and method

Endotoxin. S. minnesota Re mutant R595 was cultivated [15] and the Re lipopolysaccharide isolated by use of the phenol: chloroform: petroleum ether method [15]. It was subjected to electrodialysis and converted to the uniform triethylamine salt form [16]. In some cases, the lipopolysaccharide of *E. coli* O89 was used.

Irradiation of endotoxin. Samples (150 mg/5 ml) of the Re mutant lipopolysaccharide R595 in the form of its triethylamine salt were irradiated with ^{60}Co doses of 50, 100, 150 and 200 kGy [6]. A brown coloration developed which increased with the dose of irradiation. The samples were freeze-dried.

Chemical analyses. 2-Keto-3-deoxyoctonate (dOclA, KDO) was determined by the thiobarbituric acid reaction [17], glucosamine by the Morgan–Elson method [18], and phosphate according to LOWRY *et al.* [19]. 4-Amino-4-deoxyarabinose was estimated qualitatively after paper electrophoresis [20]. Fatty acids were liberated as methylesters by boron trifluoride--methanol and estimated by gas liquid chromatography as described earlier [21].

Biological tests. Lethal toxicity of the preparations was tested in female Wistar R/Ax Long Evans (hooded) F_1 hybrid rats (110 $\pm$ 5 g, LATI, Laboratory Animal Institute, Gödöllő, Hungary) which had been hypersensitized with lead acetate [22]. The LD_{50} values were calculated according to REED and MUENCH [23].

The local Shwartzman reaction was tested in male albino rabbits (3500 g), whose back was depilated. They received a preparative dose intracutaneously, and 24 h later, the provocative dose intravenously. The haemorrhagic and necrotic areas were recorded after 24 h.

The activity of the preparations to interact with the complement system was tested as described previously [6].

The capacity of the preparations to induce tolerance was tested in the same rat strain as used for the lethal toxicity test. The animals were injected intravenously with the minimal tolerance-dose (50 μg/rat) of parent endotoxin or same dose of irradiated preparations and challenged 24 h later by an intravenous injection of lead acetate (5 mg/rat) and a minute amount (0.5 μg/rat) of endotoxin.

Results

Chemical analyses. On dialysis of the 200 kGy sample, about 21% of the material was dialysable. This low molecular weight fraction contained about 24% of the phosphate present in the original preparation but only small amounts of glucosamine and KDO. All samples were dialysed and the high molecular weight fractions analysed for glucosamine, KDO, phosphate, 4-amino-arabinose and fatty acids. The results, which are summarized in Table I, show that irradiation resulted in a dose-dependent decrease in the amounts of constituents of the preparations. With the lowest dose (50 kGy) glucosamine decreased by 9% and with the highest dose (200 kGy) by 37% of the value determined in the non-irradiated original lipopolysaccharide. In the case of KDO, the above doses caused a decrease of 16 and 54%, while the corresponding losses of phosphate were 8 and 24%, respectively. 4-Amino-arabinose, detectable in the original lipopolysaccharide as a prominent spot, could just be identified in the 200 kGy sample.

The contents of individual fatty acids in the irradiated preparations also decreased with the increasing dose of irradiation. Only 50% of total fatty acids were present in the 200 kGy preparation compared to the original lipopolysaccharide.

The results of these analyses showed that in the non-irradiated lipopolysaccharides the determined constituents account for about 87% of the product. In the 200 kGy preparation this value is about 46%. Since the non--dialysable fractions of the irradiated samples have been analysed, it is concluded that part of the degradation products of original constituents are still

Table I

Chemical analysis of untreated and irradiated lipopolysaccharide R595. Analyses were performed after dialysis of the samples

Lipopolysaccharide preparation	Yield after dialysis	Glucos-amine	KDO	Phos-phate	4-Amino-arabino-se	Fatty Acids				Sum of	
						Cl2	Cl4	Cl6	$\frac{3-OH}{Cl4}$	fatty acids	consti-tuents
	per cent of analysed samples										
Untreated (parent)	100	10	16.6	7.5	4+	9.0	6.7	7.0	30.0	52.7	86.8
50 kGy	95	9.1	13.9	6.9	4+	8.5	6.4	5.3	24.0	44.2	74.0
100 kGy	91	7.6	11.0	6.6	3+	7.8	6.1	4.4	20.5	38.8	58.0
150 kGy	81	7.5	9.5	6.0	2+	6.2	5.1	4.0	19.4	34.7	54.7
200 kGy	79	6.3	7.7	5.7	1+	4.7	4.0	3.1	14.4	26.2	45.9
Maximal loss after irradiation	21	37	54	24		48	40	55	52	50	47.0

bound in the modified product. Another part of the degradation products is liberated and appears in the dialysates.

Biological analyses. Lethal toxicity of the preparations was tested in rats pretreated with lead acetate. The results of Table II show that the LD_{50} dose increased with the dose of irradiation applied to the endotoxin. About 120 times as much of the 200 kGy sample was needed for the LD_{50} as compared with the original endotoxin.

The local Shwartzman reaction was studied in rabbits prepared intracutaneously with either the parent or irradiated samples, and challenged 24 h later intravenously with the parent lipopolysaccharide. Tables III and IV show that the Shwartzman-inducing capacity of the irradiated preparations was significantly decreased.

The anti-complementary activity was tested with human serum. The results (Fig. 1) showed a dose-dependent inactivation of the samples, the 200 kGy preparation being hardly active.

Table II

Lethal toxicity of parent and irradiated lipopolysaccharide R595 in lead acetate sensitized rats (LD_{50} values)

Lipopolysaccharide preparations	LD_{50} μg/kg
Parent (untreated)	0.6
50 kGy-treated	9.8
100 kGy-treated	34.8
150 kGy-treated	56.6
200 kGy-treated	73.0

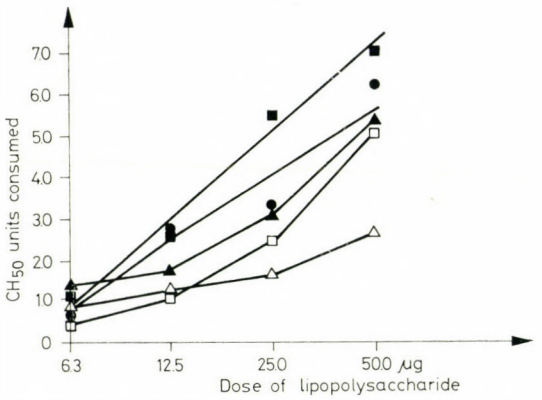

Fig. 1. Consumption of complement activity from normal human serum by untreated and irradiated lipopolysaccharide R595. Complement activity available: 7.0 CH_{50}. Untreated lipopolysaccharide: ■—■; irradiated lipopolysaccharide: 50 kGy ○—○, 100 kGy ▲—▲, 150 kGy□—□, 200 kGy △—△

Table III

Activity of parent and irradiated lipopolysaccharides R595 in the local Shwartzman reaction (rabbits)

Preparation	Provocation after 24h	Shwartzman reactivity maximal/minimal diameter in mm
Physiological NaCl i. c.		0/0
Parent–LPS 50 µg i. c.	LPS	27/24
50 kGy–LPS 50 µg i. c.	(E. *coli* O89)	17/16
100 kGy–LPS 50 µg i. c.	200 µg	15/15
150 kGy–LPS 50 µg i. c.	i. v.	14/12
200 kGy–LPS 50 µg i. c.		10/10

Table IV

Activity of parent and irradiated (200 kGy) lipopolysaccharides R595 in the local Shwartzman reaction (rabbits) (Dose dependence)

Preparation	Provocation after 24 h	Shwartzman reactivity maximal/minimal diameter in mm
Physiological NaCl i. c.		0/0
Parent–LPS 5 µg i. c.	Parent–LPS	12/12
Parent–LPS 10 µg i. c.	R595	16/12
Parent–LPS 20 µg i. c.	50 µg	20/20
200 kGy–LPS 5 µg i. c.	i. v.	6/4
200 kGy–LPS 10 µg i. c.		8/8
200 kGy–LPS 20 µg i. c.		10/8

Induction of tolerance towards an otherwise lethal dose of original endotoxin was tested in rats treated with lead acetate. As shown in Table V, all animals were protected to the challenge whether or not they had been pretreated with original or irradiated endotoxin.

Table V

Endotoxin tolerance-inducing capacity of parent and irradiated lipopolysaccharide R595 (in the rat)

Pretreatment	Challenge after 24 h	Mortality dead/total
Nil		10/10
Parent–LPS 50 μg i. v.	Lead acetate	0/5
50 kGy–LPS 50 μg i. v.	5 μg i. v.	0/5
100 kGy–LPS 50 μg i. v.	+	0/5
150 kGy–LPS 50 μg i. v.	Parent–LPS	0/5
200 kGy–LPS 50 μg i. v.	R595	0/5
	0.5 μg i. v.	

Discussion

Previous work on the ^{60}Co-irradiated *E. coli* O89 endotoxin showed that this treatment resulted in a characteristic change in biological activity of the endotoxin: some typical effects had been abolished while others had been retained [6, 13]. The purpose of the present investigation was to study the chemical changes in the endotoxin molecule brought about by irradiation and causing the altered biological properties. Since lipid A has been recognized as the endotoxically active centre of endotoxins [14], it was anticipated that irradiation would affect the lipid A component of the lipopolysaccharide. We therefore selected for the chemical analyses the core-defective lipopolysaccharide R595 of a *S. minnesota* Re mutant [14], which consists of about 70% lipid A and contains KDO as the only additional constituent.

Samples of this endotoxin were irradiated with 50, 100, 150 and 200 kGy. Biological tests performed with these preparations showed that the Re endotoxin was detoxified to a degree depending on the dose of irradiation in an analogous way as the previously investigated irradiated S form lipopolysaccharide of *E. coli* O89 [6, 13]. The irradiated preparations were less toxic, less active in the Shwartzman reaction and as activators of the complement system, but they had retained the protection activity against the lethal action of endotoxin.

The same samples that had been tested biologically were subjected to chemical analyses. It was found that with increasing doses of irradiation increasing amounts of the irradiated material became dialysable (up to 21%

in the 200 kGy sample). In these low molecular weight degradation products phosphate could be detected in appreciable amounts (about 24% in the 200 kGy sample), while other consituents of the original endotoxin were only present in trace amounts.

The non-dialysable, brownish materials contained the constituents known to be present in R595 lipopolysaccharide, i.e. KDO, glucosamine, phosphate, 4-aminoarabinose and fatty acids. Depending on the radiation dose applied their amounts were, however, significantly decreased. In the 200 kGy sample some of the constituents were present only to 50% as compared to the original lipopolysaccharide. As a consequence, the total amount of known constituents in the 200 kGy sample accounted for only 46%, as compared to 87% in the untreated lipopolysaccharide. Therefore, an appreciable fraction of the irradiated samples must consist of original constituents in chemically altered form which are not detected by the analytical method applied.

The results of this investigation demonstrate the complex composition of the irradiated preparations, and it is at present not possible to correlate between structure and presence or absence of endotoxic activities. We know that endotoxic activity is linked to lipid A. Therefore, the loss of KDO by itself would not alter the spectrum of activities. We have also shown that the active structure in the lipid A molecule is represented by the acylated and phosphorylated glucosamine disaccharide backbone of lipid A [24]. Furthermore, the analysis of a lipid A precursor molecule has provided evidence that the presence of four moles of β-hydroxymyristic acid on the backbone is sufficient to render the molecule an endotoxin [24]. In other words, the three non-hydroxylated fatty acids can be lacking without an appreciable loss of activity. We can speculate that such simple chemically modified structures occur in the irradiated samples, and endow the preparations with some of the original activities. The isolation and chemical characterization of such modified molecules will, however, be difficult as long as the degradation products formed during irradiation have not been identified.

REFERENCES

1. Freedman, H. H., Sultzer, B. M., Kleinberg, W.: Proc. Soc. exp. Biol. (N. Y.) **107,** 819 (1961).
2. Noll, H., Braude, A. I.: J. clin. Invest. **40,** 1935 (1961).
3. Nowotny, A.: Nature (Lond.) **197,** 721 (1963).
4. Previte, J. J.: J. Bact. 1907 (1967).
5. Nowotny, A.: Bact. Rev. **33,** 72 (1969).
6. Füst, Gy., Bertók, L., Juhász-Nagy, S.: Infect. Immun, **16,** 26 (1977).
7. Balogh, A., Bertók, L.: Acta chir. Acad. Sci. hung. **15,** 21 (1974).
8. Balogh, A., Bertók, L., Nemes, A., Juhász-Nagy, S.: Orv. Hetil. **115,** 2055 (1974).
9. Szilágyi, T., Csernyánszky, H., Gazdy, E., Bertók, L.: Acta microbiol. Acad. Sci. hung. **25,** 159 (1978).
10. Csordás, T., Bertók, L., Csapó, Zs.: Obst. Gynec. Invest. **141,** 159 (1978).

11. Orbán, I., Bertók, L., Regős, J., Gazsó, L.: Acta chir. Acad. Sci. hung. **19,** 339 (1978).
12. Bertók, L., Elekes, E., Merétey, K.: Acta microbiol. Acad. Sci. hung. **26,** 135 (1979).
13. Bertók, L.: Persp. Biol. Med. **24,** 61 (1980).
14. Galanos, C., Lüderitz, O., Rietschel, E. Th., Westphal, O.: Int. Rev. Biochem. Biochem. Lipids II, **14,** 239 (1977).
15. Galanos, C., Lüderitz, O., Westphal, O.: Eur. J. Biochem. **9,** 245 (1969).
16. Galanos, C., Lüderitz, O.: Eur. J. Biochem. **54,** 603 (1975).
17. Waravdekar, V. S., Saslaw, L. D.: J. biol. Chem. **234,** 1945 (1959).
18. Strominger, J. L., Park, J. T., Thompson, R. E.: J. biol. Chem. **234,** 3263 (1959).
19. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., Farr, A. L.: J. biol. Chem. **207,** 1 (1954).
20. Volk, W. A., Galanos, C., Lüderitz, O.: Eur. J. Biochem. **17,** 233 (1970).
21. Gmeiner, J., Martin, H. H.: Eur. J. Biochem. **67,** 487 (1976).
22. Selye, H., Tuchweber, B., Bertók, L.: J. Bact. **91,** 884 (1966).
23. Reed, L. J., Muench, H.: Amer. J. Hyg. **27,** 493 (1938).
24. Lüderitz, O., Galanos, C., Lehmann, V., Mayer, H., Rietschel, E. Th., Weckesser, J.: Naturwissenschaften **65,** 578 (1978).

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EPIDEMIC PULMONARY INFECTION ASSOCIATED WITH *MYCOBACTERIUM XENOPI* INDIGENOUS IN SEWAGE-SLUDGE

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Mycobacterium xenopi was isolated from the sputum of 21 patients with clinical signs of pulmonary disease and of 52 asymptomatic subjects living in the environment of a sludge pool. *M. xenopi* was cultured in high numbers from sludge samples. The infections were assumed to occur partly via dry sludge particles scattered by the wind in summer, and partly by sludge used as fertilizer.

Routine X-ray screening of the population of Pécs, Hungary, revealed pulmonary changes which, as a result of microscopic and cultural examination of sputum specimens, were assumed to be associated with non-tuberculous mycobacteria. From the year 1978 onwards, these microorganisms were isolated from 97 subjects living in one district of the town. This paper gives an account of clinical, bacteriological and epidemiological investigations in connection with this unusual observation.

Materials and methods

Bacteriological examinations. The samples (sputum, sewage inflow, fresh and old sedimented sludge, soil) were pretreated with 0.125% chlorhexidine gluconate for 16 h. The samples were then inoculated into Löwenstein–Jensen slants containing lincomycin, nystatin, nalidixic acid and clotrimazole (10 μg/ml each). The cultures were examined several times during 8 weeks incubation at 25, 37 and 45 °C. Identification of the isolates was performed as recommended by international standard methods. The following characteristics were determined: Ziehl–Neelsen staining, colony morphology, time and temperature of growth, photo- or scotochromogeny, niacin production, catalase activity (semiquantitative test at room temperature and 68 °C), amidases on Boenicke's series, nitrate reduction, Tween 80 hydrolysis, beta-galactosidase, arylsulphatase, 5% sodium chloride tolerance, iron uptake, resistance in Löwenstein–Jensen medium to isoniazid (10 μg/ml), thiophene-2-carboxylic acid hydrazide (1 μg/ml), hydroxylamine (250 μg/ml) and para-nitrobenzoate (500 μg/ml).

Skin tests were performed with preparations obtained from the State Serum Institute, Copenhagen (sensitin: *M. xenopi* RS 631; tuberculin: PPD RT 23).

In environmental examinations the following was considered: average weekly temperature, direction of prevailing wind, location of buildings, time of staying in the incriminated district and housing situation of the inhabitants, presence or absence of indoor plants and place of purchasing vegetables.

Clinical examinations were repeated several times especially of patients showing a progression or regression of the disease.

Results and discussion

Environmental investigation

Figure 1 shows the district of Pécs where most of the mycobacterial infections occurred. Considering the distribution of cases in the area, the sludge pool was suspected as the most probable source of the infections. This assumption was supported by the results of the following environmental investigations.

In the parks of the new building estate the excavated contents of the sludge pool were used as fertilizer. Some inhabitants carried home this material for manuring their indoor plants. Part of the inhabitants purchased vegetables from a local garden manured with the contents of the sludge pool. The prevailing wind blows to the direction of the building estate without any hindrance from the sludge pool, which is at a distance of 500–1000 metres from the buildings. In summer the sludge pool dries up and becomes dusty.

M. xenopi was cultured from the fresh sewage inflow. Samples taken from different sites and depths of the sludge pool yielded a number of mycobacterial isolates, namely 43 *M. xenopi*, 2 *M. fortuitum*, 7 *M. terrae*, 6 *M. chelonei*, 1 *M. phlei* and 4 *M. smegmatis*. In addition, 25 isolates remained unidentified, as they failed to grow on subcultures.

Bacteriological and clinical findings

Table I

Incidence of M. xenopi and M. avium infections in the town of Pécs, 1978—1981
Total number of strains isolated, *M. xenopi* 237, *M. avium* 41. All strains were cultured from sputum specimens

	Positive for *M. xenopi*			Positive for *M. avium*		
	on single isolations	on repeated isolations	total	on single isolations	on repeated isolations	total
No. of asymptomatic persons	36	16	52	16	5	21
No. of ill persons	5	16	21	2	1	3
Total no. of persons	41	32	73	18	6	24

The results of clinical and bacteriological examinations are summarized in Table I. A total of 97 subjects was positive for nontuberculous mycobacteria (73 for *M. xenopi* and 24 for *M. avium*). Clinical changes were present in 24 subjects (21 males and 3 females) between 32–72 years of age; 21 out of them were positive for *M. xenopi*. It was striking that except for two subjects they all lived south-east from the sludge pool. One patient was regularly working in the very neighbourhood of the sewage plant. Several of the patients were alcohol addicts.

The mycobacteria were shown on repeated examinations in many patients, especially in those with extensive X-ray changes. Some of the bacteriolo-

Fig. 1. District of Pécs with distribution of mycobacterial infections. Each dot represents one person positive for *M. xenopi*

gically positive subjects who had been asymptomatic at the time of the laboratory examination, developed clinical changes in 1–4 years. Illness was diagnosed on the basis of complaints in 12, and on routine screening in 15 persons.

In the history there were different lung diseases (10 bacteriologically controlled, healed tuberculosis, 1 aspergilloma, 1 relapsing pneumothorax, 1 bronchial asthma and 4 silicoses). As documented by previous X-ray screenings, 7 patients had had no pulmonary changes.

X-ray alterations in the lung varied in size. In one patient a small (1×1 cm) unilateral, in 13 patients somewhat larger unilateral, in 8 patients extensive unilateral changes were demonstrated. In 2 patients with gross bilateral changes the disease had a severe course; one of these patients died. Except in the patient with minimal change, the formation of cavities was characteristic. They differed in size and in the thickness of their wall; between them a normal lung tissue was only occasionally visible. The X-ray findings were characteristic.

Recovery has been recorded after a course of antituberculous treatment (6 patients), and after combined antituberculous + erythromycin + sulphonamide therapy (1 patient). Seven out of 8 patients who are still receiving antituberculous drugs, are improving. One patient shows no improvement after 8 months antituberculous treatment. At present only 4 patients can be considered to have recovered completely. A therapeutic response usual in tuberculosis was observed in 2 patients; the rest improved considerably slower.

Fifteen patients were skin-tested with *M. xenopi* sensitin (xenopin) and with PPD. Results were as follows: positive xenopin, negative PPD 4 patients; positive xenopin, weakly positive PPD 4 patients; positive xenopin, positive PPD 4 patients; negative xenopin, negative PPD 2 patients; negative xenopin, positive PPD 1 patient. Accordingly, the xenopin skin-test may be considered useful as an indicator of *M. xenopi* infection.

Few data are available on the incubation time, course and therapy of *M. xenopi* infections [1]. There are even less observations on human mycobacterial infections conveyed by water and sewage. An endemic occurrence of *M. kansasii* in water supply systems has been described [2].

It may be concluded that sewage-borne mycobacterial infections can be prevented by environmental sanitation and by heating of the sludge before use as fertilizer. The time and temperature of treatment is critical, since *M. xenopi* is resistant to moderate heating and survives in soil for several years.

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REFERENCES

1. KOIZUMI, J. H., SOMMERS, H. M.: Amer. J. clin. Path. **73,** 826 (1980).
2. KAUSTOVÁ, J., OLSOVSKI, M., KUBIN, O., ZATLUKAL, M., PELIKAN, V., HRADIL, N.: J. Hyg. Epid. (Praha) **25,** 24 (1981).

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ACTIVE AND PASSIVE MOUSE-PROTECTING CAPACITY OF *PSEUDOMONAS AERUGINOSA* PROTEIN VACCINES

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Six different vaccines were prepared, each containing the soluble, practically lipopolysaccharide-free protein extract of 2 or 3 *Pseudomonas aeruginosa* strains. In active mouse protection tests the vaccines were shown to give protection against both homologous and heterologous serotype strains, and against strain PA-103 producing exotoxin A. In rabbits the vaccines were found to stimulate the production of protective antibodies demonstrable in a passive mouse protection test. The immune serum had a protective effect against the exotoxin A-producing strain PA-103, too. Toxicity of the vaccines was studied in mice (mouse weight gain test) and in rabbits (intracutaneous skin test and pyrogenicity). The vaccines were not or only sligthy toxic.

In recent years the preparation of various *Pseudomonas aeruginosa* vaccines has been described (i) whole-cell vaccines [1–5], (ii) cell-free vaccines [6–13], and (iii) ribosomal vaccines [14–16]. All vaccines have been tested in animals and some in a few volunteers and patients with burns, but their effectiveness has not been properly proved. Some recommended vaccines have turned out to be strongly reactive [17].

The present experiments were undertaken to prepare a vaccine suitable for active immunization against *P. aeruginosa* infection. The preparation contained partially purified protective antigen(s) of cellular protein nature without contamination or with minimum lipopolysaccharide (LPS) content. Preliminary experiments have shown the soluble antigen(s) obtained from acetone-inactivated bacteria, to confer significant protection to animals, as indicated by the mouse protection test [18] and the rat-sepsis model [19].

In this paper the effectiveness of the bi- and trivalent vaccines in active and passive mouse protection and toxicity tests will be reported.

Materials and methods

Pseudomonas aeruginosa strains. (1) Strains 170004, 170008, 170011, 170014, 170015, 170016, 170017, 170019 and 170023 were obtained from the Hungarian National Collection of Medical Bacteria [20]. Table I shows their serotypes and those of the other strains according to Lányi's serogrouping system [21, 22]. (2) Strains Nos 8 and 868 were freshly isolated from patients (Vishnevski Institute of Surgery, Soviet Academy of Medical Sciences). (3) An exotoxin A producing strain, designated PA-103 [23].

Cultivation. The strains were grown on agar media containing trypsin-digested casein at 37 °C in a Shesterenko culture apparatus [18] for a period of 18 h.

Preparation of the cell-free extract vaccines. The cultures were washed off isotonic saline, the suspension was centrifuged, the supernatant discarded and the sediment was washed 3 times with acetone and then dried. The starting wet extract (SWE) was prepared as follows. A 10% suspension was made from the dried bacteria with distilled water and then homogenized in an LKB mechanical disintegrator at 8000 rpm for 5–8 min. The suspension was centrifuged, the supernatant was decanted, the sediment extracted 3 times as described above, and the supernatants were pooled. The mixture was centrifuged first at 1000 *g* for 60 min then the supernatant was ultracentrifuged (Spinco L ultracentrifuge) at 300 000 *g* twice for 8 h [18]. The supernatant fluid thus obtained was a partially purified aqueous extract (PPWE). The substances were filtered through Millipore filters (0.22 μm) and were used either in fluid or in lyophilized form.

Animals. Swiss mice and Chinchilla rabbits were used.

Active mouse protection test. Mice weighing 18–20 g were used. The animals were immunized intraperitoneally with different doses of the vaccines (cp. results) and then challenged intraperitoneally 7 days later. For challenge 16–18-hour-old agar cultures suspended in isotonic saline were used. The animals were under observation for 3 days. The LD_{50} and ED_{50} values were calculated from the rate of survival.

Passive mouse protection test. Rabbits weighing 2000–2500 g were repeatedly immunized intravenously and the sera obtained during and after the immunizations were tested. Mouse groups were immunized intraperitoneally with different amounts of the immune sera, then 2 h later were challenged intraperitoneally. The ED_{50} values of the sera and the LD_{50} values of the challenge strains were determined.

Determination of the LD_{50} of the vaccines. Mice weighing 18–22 g were injected intraperitoneally with different amounts of the vaccines. Observing them for 3 days, LD_{50} values were calculated from the survival rates.

Mouse weight gain test. Groups of 5–10 mice of 18–20 g weight were injected intraperitoneally with a vaccine containing either 250 or 25 μg protein. One group received isotonic saline. The animals were observed for 10 days and their body weight was measured daily before and after the vaccination.

Intracutaneous test in rabbits. Rabbits weighing 2000–2500 g were injected intracutaneously with a vaccine containing 1000, 100, 10 or 1 μg protein. Isotonic saline served as control. The skin reactions were evaluated 24 and 48 h later.

Pyrogen test in rabbits. Groups of 3 rabbits were injected intravenously with a vaccine containing 200, 100 and 40 μg protein, respectively. The rectal temperature of the animals was taken first before the injection and then 30 min, 1, 2, 6 and 24 h later.

Germ counts of the vaccine and challenge cultures were determined visually by comparing to the International Reference Preparation for Opacity. The number of viable bacteria was determined by the spread plate method, counting the colony forming units.

Chemical assays. Protein was determined by the method of LOWRY *et al* [24], carbohydrates (hexoses) by anthrone reagent [25]. Heptoses [26] and 2-keto-desoxy-octanates (KDO) were assayed as desribed in [27].

Statistical evaluation. The LD_{50} and ED_{50} values and fiducial limits were determined by VAN-DER-WARDEN's method [28].

Results

Composition fo the pseudomonas vaccines (PV). The vaccines (see TableII) were prepared by mixing the PPWE-s on the basis of their dry weight (PV-2, PV-3) or protein content (PV-4, PV-5 and PV-20). The chemical analyses showed the vaccines to consist mainly of proteins, presumably outer membrane proteins. Their molecular weight was between 10 000 and 100 000 daltons according to ultrafiltration on Diaflo membrane. There was only a small amount of heptoses in the vaccines; while the starting wet extract (SWE) contained 4.5–6% heptose, the purified preparation contained only 0.09–1.1% KDO, the characteristic constituent of LPS, was almost completely absent.

Table I

Strains used for the production of vaccines and for challenge

Strain	Serotype*
170004	O(3a),3c : H1
170005	O3a,3d : H1
170008	O4a,4b : H2a,2b
170011	O5a,5b,5c : H1
170014	O6 : H2a,2b
170015	O7a,7b : H2a,2c
170016	O7a,7c : H2a,2c
170017	O8 : H1
170019	O10a : H1
170023	O13 : H1
8	O3a,3d,3e : H?
868	O3a,3d,(3e) : H?
PA-103	O7

* LÁNYI's serogrouping system [21, 22]

Active mouse protecting capacity of the vaccines. Table III shows the summarized results of the active mouse protection test repeated 3 times with 4 vaccines. Challenge was performed with 6 different strains of various serotypes. In the case of PV-2, PV-3 and PV-4, the immunizing dose was 9–10 μg protein, and 25 μg in the case of PV-5. The obtained data show that every vaccine induced both homologous and cross protection. It is worth to consider that the vaccines induced protection against the exotoxin A producing strain PA-103, too. The vaccines did not differ considerably in efficiency.

The efficacy of vaccine PV-5 was studied in detail. Table IV summarizes the results. The ED_{50} differed only slightly after challenge with strains of homologous (170008 and 170015) or heterologous (170004, 170011) serotype. Adequate protection was observed also after challenge by strains 170014, 170017 and PA-103. On the other hand, less than 50% (15–45%) of the animals survived challenge with strains 8, 170019 and 170023. Though strains 8 and 170004 belong to serogroup O3, their ED_{50} values differed significantly.

The results of 3 mouse protection tests with vaccine PV-5 are summarized in Table V. The LD_{50} values were determined in both immunized and control animals. The immunizing dose was 100 μg protein. The vaccine induced protection against both homologous and heterologous challenge. Determination of the LD_{50} indicated a definite dose effect, while this was not always demonstrable in the case of ED_{50} values. A paradoxical effect could often be observed: the survival rate was higher after a smaller immunizing dose than after a higher one (cp. Table IV).

Table II

Composition and characteristics of P. aeruginosa vaccines

Vaccine	Strains used in the vaccines	Ratioprotein: carbohydrates (hexoses)	Heptoses, %	KDO, %
PV-2 (dried)	170008 170015 PA-103 (1 : 1 : 0.5)*	8.0	1	0.007
PV-2 (fluid)	170008 170015 PA-103 (1 : 1 : 0.15)	4.5	NT	NT
P-V3 (dried)	170008 170015 PA-103 (1 : 1 : 1)	5.3	1.1	Ø
PV-4 (dried)	170008 170015 (1 : 3)	7.0	0.7	Ø
PV-5 (fluid)	170008 170015 (1 : 3)	16.7	0.09	traces
PV-20 (dried)	8 868 170015 (1 : 1 : 1)	14.2	0.6	Ø

* In brackets: proportion of the strains in the vaccines
NT = not tested

Table VI shows the results of 3–4 active mouse protection tests performed with vaccine PV-20. Composition of the strains forming this vaccine was different from that in PV-5 (cp. Table I). Vaccine PV-20 induced a high level protection. The immunogenicity index (LD_{50} value of the immunized group/LD_{50} value of the control group) was 8.5–23.8 in the case of a 25 μg immunizing (protein) dose, 5.9–15 in the case of a 2.5 μg, and 7.7 in the case of 0.25 μg.

Passive mouse protecting capacity of the vaccines. Results are shown in Table VII. Pool I consisted of the sera of 5 rabbits collected after the second immunization. Pool II was collected after the fourth and pool III after the ninth immunizing inoculation. The sera collected after the fourth and ninth

Table III

Results of active mouse homologous and cross protection tests

Vaccine	Challenge strain					
	170015	PA-103	170004	170011	170014	170016
PV-2	220* (183–264)**	>200	346 (240–501)	525 (380–724)	282 (204–389)	288 (209–318)
PV-3	240 (200–289)	>200	NT	NT	NT	NT
PV-4	186 (162–214)	>200	200 (159–251)	288 (229–363)	NT	263 (191–363)
PV-5	239 (165–345)	>200	427 (295–617)	575 (417–794)	NT	372 (269–513)
Control (not immunized)	56 (44–70)	15 (11–20)	07 (77–148)	177 (129–246)	66 (52–83)	186 (135–257)

* LD_{50} values ($\times 10^6$ bacteria)
** Fiducial limits of the LD_{50} values
NT = not tested

Table IV

Results of active mouse protection tests performed with vaccine PV-5

Challenge strain	Challenge dose $\times 10^6$ bacteria	LD_{50} in challenge dose	Immunizing dose, μg					ED_{50} value, μg	Fiducial limits, μg
			250	25	2.5	0.25	0.025		
170015	200	6.9	19/20*	23/30	15/30	14/30	20/30	1.1	0.8— 1.6
180008	200	3.2	17/20	27/40	15/40	20/40	24/40	1.5	0.7— 3.4
8	100	5.1	8/20	14/40	12/40	7/40	17/40	>250!	
170004	200	3.2	16/20	24/40	18/40	19/40	16/40	2.7	1.2— 5.9
170011	250	3.2	20/20	11/20	9/20	5/20	11/20	2.3	1.1— 5.1
170014	400	3.9	28/40	10/40	7/40	8/40	11/40	25.1	12.6—50.1
170016	100	1.9	—	12/20	15/20	8/20	9/20	7.6	3.5—16.6
170017	200	1.5	40/40	37/40	35/40	37/40	36/40	<0.025	
170019	800	6.4	18/40	11/40	12/40	7/40	6/40	>250!	
170023	400	4.6	—	9/20	9/20	8/20	8/20	>250!	
PA–103	100	10.0	40/40	18/40	16/40	11/40	8/40	5.9	3.1—11.2

* Mice survived/total

Table V

Results of active mouse protection tests performed with vaccine PV-5; determination of the LD_{50} values

Challenge strain	Immunized*		Control	
	LD_{50}	Fiducial limits	LD_{50}	Fiducial limits
170015	229**	174–302	46.8	32–68
170008	302	240–380	61.7	46.8–81.3
8	97	76–120	15	11–20
170004	168	132–209	31	23–41
170011	219	166–288	96	79–115
170014	398	302–525	132	96–182
170016	162	135–195	58	44–76
170017	346	276–437	54	37–78
170019	473	376–596	186	135–257
170023	372	295–468	174	107–251

* Immunizing dose: 100 μg protein
** LD_{50} values ($\times 10^6$ bacteria)

Table VI

Results of active mouse protection tests performed with vaccine PV–20

Challenge strain	Challenge dose $\times 10^6$ bacteria	Immunizing dose, μg			
		25	2.5	0.25	Control
8	800	9/10*	10/10	10/10	—
	400	26/40	37/40	10/10	—
	200	10/40	15/40	9/10	20/20
	100	3/40	7/40	5/10	19/20
	50	0/40	1/40	1/10	20/20
	25	—	0/10	0/10	9/20
	12.5	—	—	—	1/20
LD_{50} ($\times 10^6$ bacteria)		309	195	100	13
Fiducial limits		251.2–371.5	162–234	87–115	10–17
170015	800	8/10	10/10	—	—
	400	18/30	21/30	—	—
	200	9/30	10/30	—	15/15
	100	1/30	5/30	—	13/15
	50	0/20	1/20	—	10/15
	25	—	0/20	—	4/15
	12.5	—	—	—	0/15
LD_{50} ($\times 10^6$ bacteria)		339	234		40
Fiducial limits		257–447	195–282		33–48

* Mice died/total

Table VII

Results of passive mouse protection tests performed with PV-2 immune sera

Immune serum	ED_{50}/ml	Fiducial limits	Challenge strain	Challenge dose ($\times 10^6$ bacteria)	LD_{50} in challenge dose
Pool I	0.04	0.03 –0.06	170008	200	16
	>0.1		170015	400	16
	>0.1		PA-103	200	8
Pool II	0.02	0.01–0.03	170008	200	16
	0.05	0.03–0.08	170015	400	16
	0.02	0.01–0.03	PA-103	200	8
Pool III	0.15	0.12–0.21	170008	200	16
	0.05	0.03–0.08	170015	400	16
	0.02	0.017–0.038	PA-103	200	8
	0.1	0.07–0.14	8	100	4

immunizing inoculation were definitely protecting. The protecting effect was demonstrable against the exotoxin A producing strain PA-103 and, though to a lesser extent, against the heterologous strain No. 8.

The LD_{50} value of the vaccines. The LD_{50} of the vaccines was found to exceed the 2 mg protein value.

Results of the mouse weight gain test. The PV-2, PV-5, and PV-20 vaccines were studied. One day after inoculation with 250 μg protein a weight loss could be observed, but after 48 h the animals regained their original weight; later, their weight increased and in this respect there was no difference between treated and control groups. There was no loss in weight among the animals inoculated with 25 μg protein; the dynamics of weight gain was essentially the same in the inoculated and the control animals.

Results of the intracutaneous test. The vaccines PV-2 and PV-5 were studied. None of the applied doses caused skin necrosis or induration in the rabbits. Erythema appeared 24 h after inoculation with a 1000 or 100 μg protein dose, but it vanished after 48 h.

Results of the pyrogen test. The vaccine PV-2 was studed. The increase in temperature did not exceed 0.5 °C after inoculation with any of the doses.

The above studies proved the preparations to be not, or only slightly, toxic.

Discussion

Water soluble, partially purified antigen(s) (PPWE) of 2–3 *P. aeruginosa* strains selected in preliminary experiments were isolated. Vaccines were produced from the extract on the basis of the dry weight or the protein content.

Determining the heptose and KDO contents, we found that the vaccines practically did not contain LPS. Active immunity against challenge with *P. aeruginosa* strains was induced in mice by immunization. Protection extended to almost every strain of a heterologous O antigenic structure. Immunization induced in rabbits the production of passive mouse protecting antibodies.

The experiments led to the conclusion that the bacterial cell (probably the outer cell membrane) contains common protective antigen(s) of protein nature (molecular weight 10 000–100 000 daltons). The isolated antigens may have been identical with the original endotoxin protein (OEP) described by HOMMA *et al.* [12].

Our previous experiments [29] showed that two protein antigens could be isolated from the aqueous extract: the protein *A* (molecular weight 12 000–14 000 daltons) and protein *B* (molecular weight 11 000–37 000 daltons), as determined by gel chromatography. Both antigens were effective in the active mouse protection test but protein *A* seemed to induce a stronger cross protection [29]. We suppose that the produced vaccines contain the protein *A* and *B* antigens of the strains. Earlier results led us to conclude [29] that the two protein antigens together induce protection. That has been confirmed by the present experiments.

We have attempted to produce a vaccine which contains no or only very little LPS because the heptavalent LPS vaccine [6, 7] is known to induce severe reactions [17].

OEP antigen as well as multicomponent vaccines containing protease and elastase toxoids have successfully been applied for preventing severe haemorrhagic pneumonia epidemics caused by *P. aeruginosa* in mink farms [13]. An effective protection required, however, repeated vaccinations. This vaccine has not been tried in humans. The polyvalent vaccine, produced from the surface antigens of 16 *P. aeruginosa* serotypes was effective in mice and in humans during clinical tests [9–11].

The use of 16 different serotypes of strains seems pointless if an effective vaccine can be produced from 2 or 3 suitably selected strains. Our water-extraction procedure allowed the isolation of the active cellular components of *P. aeruginosa*. The vaccines were highly purified by separating the cell wall LPS by repeated ultracentrifugation. Thus our vaccines were practically non-toxic for the experimental animals. Adsorption of PPWE to aluminium hydroxide gel might increase the efficiency.

Determination of the real efficacy of the vaccines requires further careful immunological and clinical studies.

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REFERENCES

1. Sato, H., Diena, B. B.: Rev. Canad. Biol. **33,** 93 (1974).
2. Akatova, N. S.: Zh. Mikrobiol. (Mosk.) **9,** 89 (1976).
3. Meitert, E., Meitert, T., Petrovici, M., Sima, Fl., Savulian, C.: Arch. roum. Path. exp. **37,** 251 (1978).
4. Moroz, A. F., Radkevitch, S. A., Antsiferova, N. G., Brodinova, N. S.: Zh. Mikrobiol. (Mosk.) **8,** 44 (1981).
5. Moroz, F., Antsiferova, N. G., Radkevitch, S. A., Mishina, V. S.: Zh. Mikrobiol. (Mosk.) **10,** 74 (1981).
6. Hanessian, S., Regan, W., Watson, D., Haskell, T. H.: Nature (Lond.) **229,** 209 (1971).
7. Fisher, M. W.: J. infect. Dis. **130,** S149 (1974).
8. Berche, P., Véron, M., Tinelli, R.: Ann. Microbiol. **127A,** 247 (1976).
9. Jones, R. J., Roe, E. A., Lowbury, E. J. L., Miler, J. J., Spilsbury, J. F.: J. Hyg. **76,** 429 (1976).
10. Miler, J. J., Spilsbury, J. F., Jones, R. J., Roe, E. A., Lowbury, E. J. L.: J. med. Microbiol. **10,** 19 (1977).
11. Jones, R. J., Roe, A. E., Gupta, J. L.: Lancet **2,** 977 (1979).
12. Abe, C., Shionoya, H., Hirao, Y., Okada, K., Homma, J. Y.: Japan J. exp. Med. **45,** 355 (1975).
13. Aoi, Y., Noda, H., Yanagawa, R., Homma, J. Y., Abe, C., Morihara, K., Goda, A., Takeuchi, S., Ishihara, R.: Japan J. exp. Med. **49,** 199 (1979).
14. Lieberman, M. M.: Infect. Immun. **21,** 76 (1978).
15. Gonggrijp, R., Mullers, W. J. H. A., Lemmens, P. J. M. R., Van Boven, C. P. A.: Infect. Immun. **27,** 204 (1980).
16. Gonggrijp, P., Mullers, W. J. H. A., Van Boven, C. P. A.: Infect. Immun. **33,** 178 (1981).
17. Pennington, J. E.: In Dogget, R. G. (ed.): Pseudomonas aeruginosa. Clinical Manifestations of Infection and Current Therapy. Academic Press, New York 1979. p. 192.
18. Stanislavsky, E. S., Zhvanetskaya, M. I., Mashilova, G. M., Gladus, M. A.: Zh. Mikrobiol. (Mosk.) **8,** 66 (1980).
19. Stanislavsky, E. S., Kolker, I. I., Grishina, I. A., Zhvanetskaya, M. I.: Zh. Mikrobiol. (Mosk.) **3,** 65 (1978).
20. Lányi, B., Konkoly, Thege, M.: Hungarian National Collection of Medical Bacteria. Catalogue of Strains. 2nd ed. National Institute of Hygiene, Budapest 1978.
21. Lányi, B.: Acta microbiol. Acad. Sci. hung. **17,** 35 (1970).
22. Lányi, B., Bergan, T.: In Bergan, T., Norris, J. R. (eds).: Methods in Microbiology. Vol. 10. Academic Press, New York 1978. p. 93.
23. Liu, P. V.: J. infect. Dis. **128,** 506 (1973).
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: J. biol. Chem. **193,** 265 (1951).
25. Roe, J. H.: J. biol. Chem. **212,** 335 (1955).
26. Osborn, M. J.: Proc. nat. Acad. Sci. (Wash.) **50,** 499 (1963).
27. Lüderitz, O., Westphal, O.: Eur. J. Biochem. **14,** 175 (1970).
28. Van-Der-Warden, B. L.: Mathematical Statistics (Russian). Inostr. Lit. Moscow 1960.
29. Stanislavsky, E. S., Palkina, N. A., Bulk, B. F., Zhvanetskaya, M. I., Tchekan, L. V., Mashilova, G. M.: Zh. Mikrobiol. (Mosk.) **8,** 37 (1981).

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CYTOTOXIC MATERIAL RELEASED FROM *STAPHYLOCOCCUS EPIDERMIDIS*

II. FRACTIONATION AND SOME EFFECTS OF THE FRACTIONS ON LYMPHOCYTES AND HEPATOCYTES*

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Cytotoxic substance(s) of about 4×10^3 molecular weight, containing 9.5% peptide and 73% carbohydrate was released from *Staphylococcus epidermidis* in phosphate buffered saline. The material was soluble in ethanol and was heat-resistant. It blocked amino acid uptake and E-rosette formation of human tonsillar and blood lymphocytes. In isolated mouse hepatocytes the toxin inhibited protein synthesis, but only in the presence of calcium ions. The results suggest that eukaryotic cell membranes are damaged by the coccal agent.

Staphylococci have been shown to produce about 30 different extracellular toxins and enzymes [1]. Beside secreted substances, surface components of bacteria are liberated and elicit diverse biological effects. Thus, peptidoglycans from the cell wall inhibit the migration of leukocytes [2]; lipoteichoic acid is mitogenic in lymphocyte cultures [3] and induces lysosomal enzyme release of macrophages [4].

Data reported in an earlier paper [5] showed that a cytotoxic agent is released from *S. epidermidis* in phosphate buffered saline (PBS) at 4 °C. The material inhibited [^{3}H]thymidine (TdR) incorporation into DNA of lymphocytes and decreased their viability as determined by trypan blue exclusion.

The present paper describes the partial purification and physicochemical characterization of the agent. Some features of the toxic effect on lymphocytes and isolated liver parenchymal cells (hepatocytes) are also reported.

Materials and methods

Preparation of lymphocytes and of the crude bacterial supernatant (SN), and [^{3}H]thymidine incorporation were performed as described earlier [5]. Amino acid (37 MBq/ml [^{14}C]valine, spec. activity: 7.4 GBq/mmol) incorporation into hot TCA insoluble material of lymphocytes and hepatocytes was measured as described [6].

Isolated hepatocytes were prepared from CFLP mice with the collagenase perfusion method [7, 8] and incubated as detailed previously [6]. In cases when calcium was omitted from the incubation mixture, 0.2 mM ethyleneglycol bis (2-aminoethyl ether)-N,N-tetraacetic acid (EGTA) was added [9].

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Uptake of labelled compounds. Lymphocytes (4×10^6 cells/ml) were suspended in Hanks medium supplemented with SN or its fractions and 37 MBq/ml [^{14}C]valine, [^{3}H]thymidine (9.9×10^5 MBq/mmol). At 1–5 min intervals aliquots were withdrawn and passed through Whatman GF/C glass fibre filter. After washing with PBS, radioactivity was measured in a Beckman liquid scintillation spectrometer.

E-rosette test was performed by the method of GUPTA *et al.* [10].

Other assays. Protein and carbohydrate were determined according to LOWRY *et al.* [11] and NELSON [12], respectively.

Ethanol extraction. One of the Sephadex gel filtration fractions of SN (f_2) was lyophilized and the residue was extracted with 80% ethanol. The ethanol-soluble and insoluble fractions were dried and re-suspended in PBS.

Results

Fractionation and characterization of the crude supernatant. Lyophilized SN powder consisted of bacterial products (16%) and salts from PBS. The bacterial products included 18% protein, 3% carbohydrate (hexose, pentose) and unidentified organic material. SN was fractionated and desalted by Sephadex G-25 gel filtration. Three main fractions (f_1, f_2, f_3) were obtained by measuring OD 280 nm (Fig. 1). The largest fraction (f_1) was excluded from the gel. The rest of the material appeared as two distinct peaks corresponding to molecular weights of 4×10^3 for f_2 and 2×10^3 for f_3. The light absorbtion of SN and of its fractions was investigated between 220 and 310 nm. We found a significant extinction only around 260 nm. Figure 2 shows the UV absorbtion spectrum of SN and its fractions.

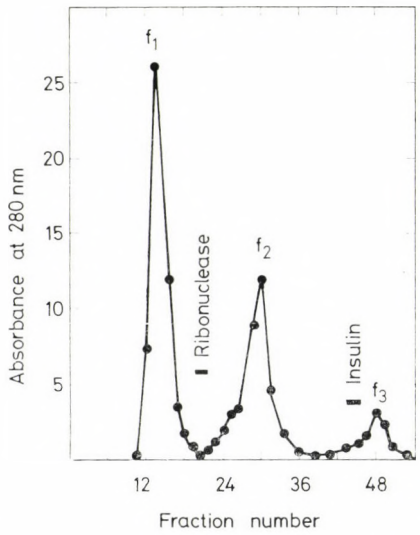

Fig. 1. Sephadex G-25 filtration of crude supernatant (SN) of *S. epidermidis.* Column, 1×45 cm; eluant, distilled water. Fractions of 3 ml were collected. Ribonuclease (mol wt 13 600) and insulin chain B (mol wt 3480) were used as markers

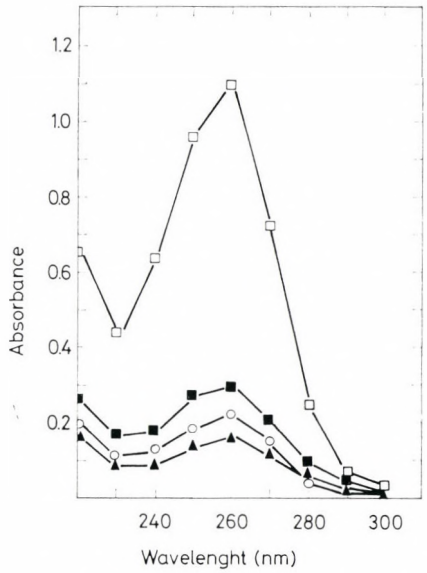

Fig. 2. Absorption spectra of SN from *S. epidermidis* and of gel filtration fractions. Concentration, 37.5 μg protein/ml each. SN ■ ——— ■; f, ▲ ——— ▲; f_2 ○ ——— ○; f_3 □ ——— □

The composition of the fractions differed significantly from that of SN. Table I shows the results of analysis of the SN fractions. Fraction 2 was relatively rich in carbohydrate.

Further purification of f_2 was performed by ethanol extraction; 84% of f_2 protein was recovered in the ethanol extract which contained most of the UV absorbing material.

Effect of SN and of its fractions on precursor uptake and incorporation of lymphocytes. We compared the effects of the SN fractions on [^{3}H]TdR incorporation of tonsillar lymphocytes with that of SN. Fraction 1 (f_1) and f_3 did

Table I

Analytical data of gel filtration fractions of SN

	Approx. molecular weight	OD 260/280	Per cent of organic material		
			peptide	pentose, hexose	other
SN	—	3.3	18	3	79
f_1	$>10^4$	1.7	12	15	73
f_2	4×10^3	3.2	9.5	73	19
f_3	2×10^3	4.7	1.4	ND	ND
ethanol-extract of f_2	ND	2.8	7.6	51	ND

ND = Not determined

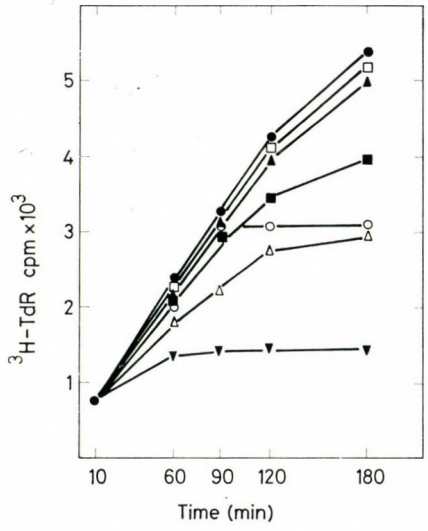

Fig. 3. Kinetics of [^{3}H] thymidine incorporation of tonsillar lymphocytes in the presence of SN and its fractions. Control • ——— •; f_3 85 μg protein/ml □ ——— □; f_1, 110 μg/ml ▲ ——— ▲; f_2, 20 μg/ml ■ ——— ■; f_2, 60 μg/ml △ ——— △; f_2, 105 μg/ml ▼ ——— ▼; SN, 80 μg/ml ○ ——— ○

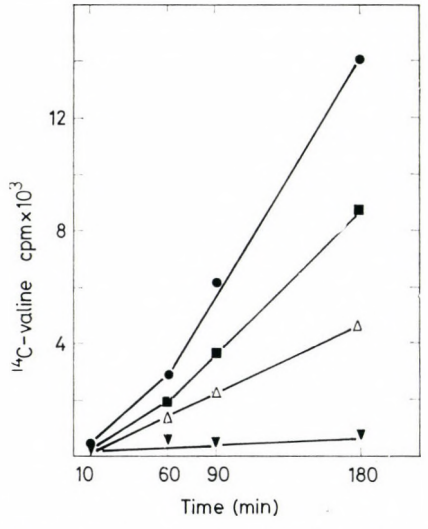

Fig. 4. Kinetics of [^{14}C]-valine incorporation of tonsillar lymphocytes in the presence of various concentrations of fraction 2 of SN. Control • ——— •; f_2, 20 μg protein/ml ■ ——— ■; f_2, 60 μg/ml △ ——— △; f_2, 105 gμ/ml ▼ ——— ▼

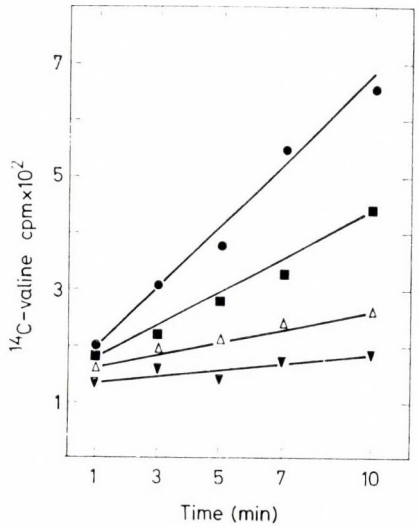

Fig. 5. Kinetics of [^{14}]-valine uptake of tonsillar lymphocytes in the presence of various concentrations of fraction 2 of SN. Control • ——— •; f_2, 20 μg protein/ml ■ ——— ■; f_2, 60 μg/ml △ ——— △; f_2, 105 μg/ml ▼ ——— ▼

not cause significant inhibition. The incorporation of [^{3}H]TdR into lymphocytes was decreased by f_2 to about the same extent as it was by SN (Fig. 3). The inhibitory effect of f_2 became manifest after a lag period of 45–90 min (depending on the dose), similarly as it was found with SN. Amino acid incorporation of tonsillar lymphocytes was also inhibited by f_2. However, [^{14}C]valine incorporation of lymphocytes was promptly inhibited by f_2 (Fig. 4).

In order to explain the difference between the onset of the effects of f_2, we examined the precursor uptake by tonsillar lymphocytes. The uptake of [^{3}H]TdR and also of [^{3}H]glucose remained undisturbed for 10 min (not shown), while a prompt, dose-dependent inhibition of [^{14}C]valine uptake of lymphocytes was caused by f_2 (Fig. 5). The uptake of [^{3}H]leucine and [^{3}H]glutamic acid was also inhibited immediately by f_2 (not shown). This finding was in good agreement with the prompt inhibition of amino acid incorporation (Fig. 4).

Heat treatment of f_2 (100 °C for 30 min) did not alter its inhibitory effect on uptake and incorporation of labelled precursors.

The biologically active components of f_2 were recovered in the ethanol-soluble fraction of f_2 (see above). Inhibition of precursor uptake and incorporation was demonstrated with the ethanol-soluble fraction of f_2 while the ethanol-insoluble part did not show any inhibitory effect.

These experiments were repeated using peripheral blood lymphocytes instead of tonsillar lymphocytes, with similar results.

Influence on amino acid incorporation of hepatocytes. The biological effects of f_2 were investigated in addition to lymphocytes in isolated mouse liver

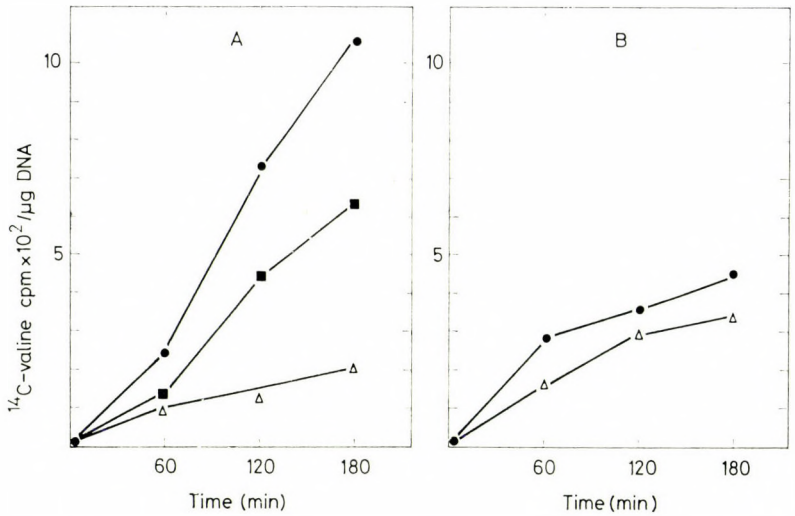

Fig. 6. Effect of fraction 2 of SN on [^{14}C]-valine incorporation into hepatocytes in the presence (A) and absence (B) of calcium ions. Control • ——— •; f_2, 20 µg protein/ml ■ ——— ■; f_2, 60 µg protein/ml △ ——— △

cells. Fraction 2 caused a marked, dose dependent inhibition of amino acid incorporation into the protein of hepatocytes (Fig. 6*A*). In the experiments shown in Fig. 6*A* and *B* the significance of Ca^{++} ions is demonstrated. When Ca^{++} was omitted from the incubation medium (Fig. 6*B*) the rate of incorporation was considerably reduced in the absence of inhibitor, too [9]. Addition f_2 caused a negligible decrease of [^{14}C]valine incorporation. Thus, the inhibitory effect of f_2 on protein synthesis was fully manifest only in the presence of Ca^{++} ions.

Table II

Inhibition of spontaneous rosette formation

Substance	µg/ml	E-binding lympho-cytes, per cent of total
None		13.5
f_2	34	5.9
f_2	68	3.3
f_2	135	1.7
Heat-treated f_2	79	0.6
SN	35	8.0
SN	70	5.5
SN	140	4.9
Heat-treated SN	80	0.5

Inhibition of rosette formation. The disturbance of precursor uptake of lymphocytes can be regarded as an indication of a surface effect of f_2. E-rosette formation of lymphocytes is known to be dependent on an intact cell surface. Generally, 10–15% of tonsillar lymphocytes are able to form E-rosettes. The number of rosettes strongly decreased in the presence of f_2. The inhibition of E-rosette formation was even more expressed in the presence of heat-treated f_2 (Table II).

Discussion

We have demonstrated a new, biologically active substance which is easily released from resting *S. epidermidis* [5]. The biological effects of this agent are an inhibition of DNA and of protein synthesis of eukaryotic cells (lymphocytes and hepatocytes), and inhibition of E-rosette formation.

Several characteristics distinguish our material from those described earlier. Losnegard and Oeding [13 a, b], Haukenes [14 a—f] and Grow [15] have reported on the release of polysaccharide A (a teichoic acid-mucopeptide complex) from *S. aureus* and *S. epidermidis*. It proved to be an insoluble, 8000 molecular weight fragment of the cell wall showing antigenic properties. Our material might be similar in some respects, although heat-treatment of cocci prevented the release of polysaccharide A but not of f_2 (it cannot be excluded that a heat-stable wall lytic enzyme was present in our strain). It should be noted that lipoteichoic acid (LTA) is one of the surface antigens [16], and it is continuously released in a soluble form from bacteria into the medium [17–20]. Ion exchange chromatography of polysaccharide A yielded an antigenically active substance which did not absorb UV light at 260 nm [21]. The biologically active agent in our material was soluble in ethanol and exhibited UV light absorbtion. This was probably due to the presence of nucleic acid. It is known that DNA is attached to a distinct site of bacterial membrane (mesosome) which at the same time is the exclusive site for the occurrence of LTA in Gram-positive bacteria [18].

Earlier reports on surface components of cocci described the mitogenic, stimulatory effect of protein A [22] LTA [3] and other unidentified materials [23]. In contrast, our agent was toxic in both lymphocyte and hepatocyte cultures.

The results showed that the agent causes disturbances in the structure and function of the eukaryotic cell membrane. This was indicated by the reduced rosetting of lymphocytes and by the inhibition of amino acid transport. The agent may bind to membrane proteins responsible for rosette formation and amino acid uptake. A complete desorganization of the membrane can, however, be excluded, since the uptake (transport) of glucose and thymidine was not inhibited. The decrease of DNA synthesis which developed later (Fig. 3) might be a secondary effect.

A series of membrane active agents (lysolecithin, melittin, Ca^{++} ionophore A23187, phalloidin, etc.) has been shown to act on isolated hepatocytes in the presence of calcium ion only. This has led SCHANNE *et al.* [24] to suggest that Ca^{++} influx is the final common pathway of cell death. Similarly, the marked toxic effect of f_2 on hepatocytes developed only in the presence of calcium ion. We therefore suggest that f_2 is a membrane active agent.

Our findings seem to be important with respect to coccal infections, where antibacterial therapy generally results in bacteriostasis. In such situations resting bacteria may release agents similar to the one described here which may cause tissue damage.

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REFERENCES

1. WADSTRÖM, T., THELESTAM, M., MÖLLBY, R.: Ann. N. Y. Acad. Sci. **236,** 343 (1974).
2. GROV, A., HELGELAND, S., ENDERSEN, C.: Zbl. Bakt. I. Abt. Suppl. 5, 389 (1976).
3. BEACHEY, E. H., DALE, J. B., GREBE, S., AHMED, A., SIMPSON, W. A., OFEK, I.: J. Immunol. **122,** 189 (1979).
4. PAGE, R. C., DAVIES, P., ALLISON, A. C.: Int. Rev. Cytol. **52,** 119 (1978).
5. SOLYMOSSY, M., NAGY, ZS., ANTONI, F.: Acta microbiol. Acad. Sci. hung. **29,** 27 (1982).
6. MANDL, J., GARZÓ, T., MÉSZÁROS, K., ANTONI, F.: Biochim. biophys. Acta (Amst.) **586,** 650 (1979).
7. BERRY, M. N., FRIEND, D. S.: J. Cell Biol. **43,** 506 (1969).
8. KREBS, H. A., CORNELL, N. W., LUND, P., HEMS, R.: In LUNDQUIST, F., TYGSTRUP, N. (eds): A. Benson Symposium VI. Munksgaand, Copenhagen 1974. p. 726.
9. MANDL, J., GARZÓ, T., ANTONI, F.: Biochem. Pharmacol. In press. —, (1982).
10. GUPTA, S., PAHWA, R., SIEGAL, F. P., GOOD, R. A.: Clin. exp. Immunol. **28,** 347 (1977).
11. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., RANDALL, R. J.: biol. Chem. **193,** 265 (1951).
12. NELSON, N.: J. biol. Chem. **153,** 375 (1944).
13. LOSNEGARD, N., OEDING, P.: Acta path. microbiol. scand. **58,** 482 (a), 493 (b) (1963).
14. HAUKENES, G.: Acta path. microbiol. scand. **55,** 110 (a), 117 (b), 289 (c), 299 (d), 308 (e), 450 (f) (1962).
15. GROW, A.: Acta path. microbiol. scand. **76,** 611 (1969).
16. VAN DRIEL, D., WICKEN, A. J., DICKSON, M. R., KNOX, K. W.: J. Ultrastruct. Res. **43,** 483 (1973).
17. MARKHAM, J. L., KNOX, K. W., WICKEN, A. J., HEWETT, M. J.: Infect. Immun. **12,** 378 (1975).
18. HUFF, E., COLE, M. R., THEODORE, T. S.: J. Bact. **120,** 273 (1974).
19. JACQUES, N. A., HARDY, L., KNOX, W. K., WICKEN, A. J.: Infect. Immun. **25,** 75 (1979).
20. WICKEN, A. J., KNOX, K. W.: Biochim. biophys. Acta (Amst.) **604,** 1 (1980).
21. HAUKENES, G.: Acta path. microbiol. scand. **55,** 463 (1962).
22. MÖLLER, G., LANDWALL, P.: Scand. J. Immunol. **6,** 357 (1977).
23. KREGER, A., CUPPARI, G., TARANTA, A.: Infect. Immun. **5,** 723 (1972).
24. SCHANNE, F. A. X., KANE, A. B., YOUNG, E. E., FARBER, J. L.: Science **206,** 700 (1979).

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THE MICROSCOPICAL PATTERN OF GROWTH OF *MYCOBACTERIUM LEPRAE-MURIUM* "DOUGLAS" IN MICROCOLONIES

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A thin section technique of Ogawa egg yolk culture medium inoculated with *Mycobacterium leprae-murium* was found to reveal microscopical growth of the strain which could not be demonstrated by macroscopical examination. A peculiar structure of the growth, characterized by many lytic spots different in size, was observed indicating the possible presence of a temperate phage which may interfere with the synthesis of nucleic acids needed for the active multiplication of *M. leprae-murium*.

In systematic studies of colony sections of different mycobacteria [1–3], profound differences were found in their micromorphological texture, in particular in the Argentinian bovine strains isolated from abbatoir specimens in Ramos Mejia Buenos Aires, in which phage microlysis inside the colonies was observed, which could never be found in human strains of mycobacteria [4].

In strains of *Mycobacterium africanum*, which are mainly characterized by dysgonic colonies, we found a subcolonial mesh-like growth which was not visible on gross inspection of the culture tubes. For this reason we applied the same technique of thin colony sections for culture media of *Mycobacterium leprae-murium* in which macroscopically visible growth was not observed.

Materials and methods

In the Douglas strain of *M. leprae-murim* maintained in white mice (ICR-Velaz SPF strain) for many years we could demonstrate by using a continuous culture technique in liquid medium, active fissions of the mycobacterial cells, without any production of macroscopic colonies [5]. The inoculum was prepared from a liver specimen taken from a mouse inoculated six months before. The suspension contained approximately 5.2×10^6 per ml of acid-fast bacilli and was inoculated in an amount of 0.1 ml following the technique of Pattyn and Portaels [6] on Ogawa yolk medium incubated in 5% CO_2 at 33 °C. After 5 months incubation the culture tubes were regularly checked for visible growth and some of those suspicious of growth were examined in thin sections by the following technique. The media with suspected microcolonies were first treated with 10% formol solution for 2 h. After pouring off the disinfectant, selected parts of the medium were cut out in the form of tiny blocks and transferred in Petri dishes containing a thin layer of melted 2% agar. A second layer of this agar covering the whole surface of culture medium was then added to fix any microcolonies. After dehydration in alcohol, acetone and benzene the blocks were embedded in paraffin and thin sections were cut with a Reichert horizontal microtome.

For staining, Ziehl–Neelsen and the PAS technique were used throughout and the sections were examined at 400–1000× magnification. Colour pictures were made on ORWO film and copies on Kodak colour paper.

Results

Among approximately two hundred sections prepared from several culture media eight were found to contain microcolonies. On Ziehl–Neelsen staining the colonies were mainly not acid-fast (Fig. 1, 2) but with PAS they proved almost entirely acid fast with the exception of small superficial and basal layers (Fig. 3) and small regions in the upper part of the colony (Fig. 4). In contrast to observations made in *M. africanum*, in deeper strata of the medium no growth strictly confined to the surface of the medium was encountered. In the *M. leprae-murium* "Douglas" strain the growth had the appearance of thin microscopical layers propagated on the surface of the medium which maintained its green colour in all the tubes examined, thus macroscopically there was no difference between positive and negative tubes. Only by chance could a few sections positive for microscopical growth be found.

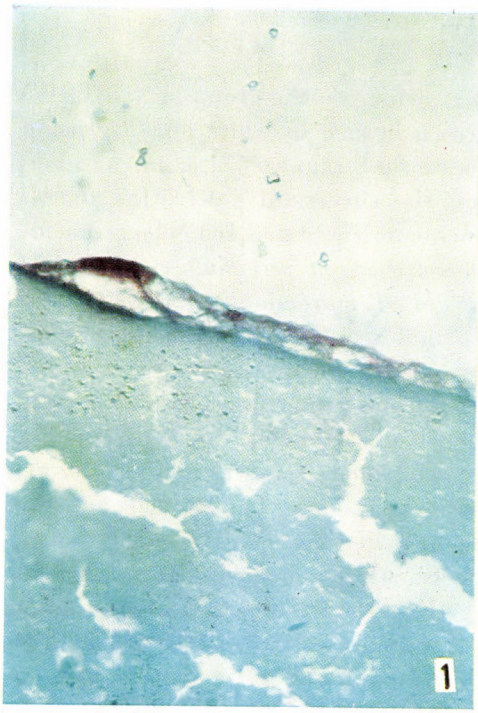
The inner structure of the growth was peculiar, displaying a net-like pattern with many small, medium-size and big holes of regular and irregular shape, resembling the phage lytic spots in the advanced stage of development leading in certain regions of the colony to its complete dissolution. The beginning of this lytic process is seen in Fig. 3, where on the left side the acid-fast structure and its density are well-preserved, while on the right side of the colony in this initial phase of lysis many tiny lytic spots are already visible surrounded by empty spaces; they are weakly acid-fast with tiny not acid--fast cords on the surface of the colony. Some other details of another colony are shown at 1000× magnification in Fig. 4. Even at this magnification no distinct acid-fast rods typical of mycobacteria were seen owing probably to the detrimental effect of the organic solvents used for dehydration on the morphological structure of the rods.

Fig. 1. Membraneous microcolony of *M. leprae-murium* Douglas strain covering partly the surface of Ogawa egg-yolk medium. The culture was incubated in 5% CO_2 at 33 °C for 5.5 months. On Ziehl–Neelsen staining the microcolony is not acid-fast except for a small region in the upper strata of the colony. Its microtexture is characterized by small and large lytic spots ×400

Fig. 2. Web-like not acid-fast microcolony of the same strain processed in the same way as in Fig. 1. Contrary to the colony depicted in Fig. 1 the growth of this colony penetrates partly into the surface strata of the egg-yolk medium. Note the larger size of this colony ×400

Fig. 3. Another colony of *M. leprae-murim* stained with PAS technique. Accumulation of many tiny lytic spots on the left side and protuberant not acid-fast cords on the right side of the colony ×400

Fig. 4. Details of another *M. leprae-murium* colony stained with PAS. The colony is permeated by many different lytic spots of irregular cavities destroying major parts of the whole body of the colony ×1000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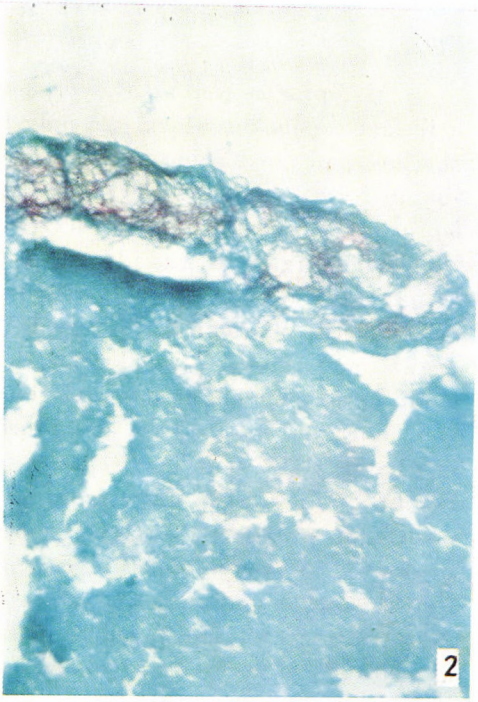
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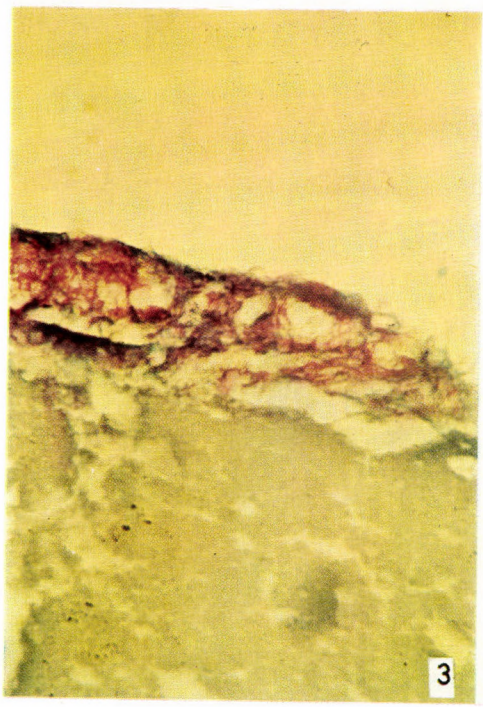
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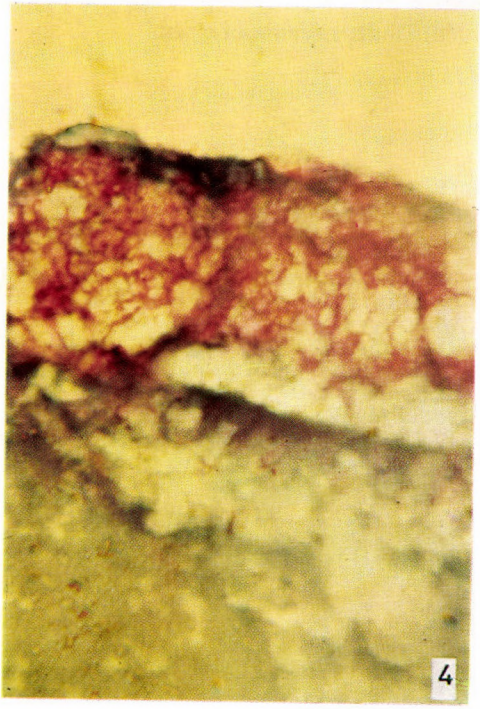
4

Discussion

M. leprae as well as *M. leprae-murium* belong to the group of "difficult" acid-fast organisms which can only be grown *in vivo* in white mice by using the foot-pad inoculation technique developed by SHEPARD [7] or in the nine-banded armadillo [8]. Recent reports on the successful cultivation of *M. leprae* in a special medium [9] have not been confirmed and the cultured acid-fast microorganism was identified as *Mycobacterium scrophulaceum*. Better results were achieved with *in vitro* cultures of *M. leprae-murium*. OGAWA and HIRAKI [10] obtained in 1% egg yolk medium a buff-coloured membraneous growth and single yellowish colonies approximately 4 mm in diameter which could be propagated on the same medium up to 8 generations. Similar results were obtained by PATTYN and PORTAELS [6] on the same egg yolk medium incubated at 33 °C in 5% CO_2 atmosphere. This technique was followed by us, but in 5.5 months no macroscopically visible colonies were observed, and the positive microscopic growth was confirmed only in a few sections of the Ogawa medium. The microscopical texture of this growth has never been investigated by any author and our observation along these lines is the first one reported in the literature. The peculiar picture of this growth characterized by the appearance of many tiny, medium-sized and confluent lytic spots, seems to indicate the presence of a temperate phage which could profoundly interfere with the multiplication of *M. leprae-murium* marking thus this strain as a "difficult mycobacterium".

REFERENCES

1. HENNEBERG, G.: Bildatlas der pathogenen Microorganismen. G. Fischer Verlag, Stuttgart 1969.
2. ŠULA, L.: Bull. Wld Hlth Org. **43,** 699 (1970).
3. ŠULA, L., CVEJNOVÁ, Z.: J. Hyg. Epidem. (Praha) **16,** 28 (1972).
4. ŠULA, L., ŠULOVÁ, J., SPURNÁ, M.: Zbl. Bakt. I. Abt. Orig. A, **223,** 520 (1973).
5. ŠULA, L., DUBINA, J.: Bull. Wld Hlth Org. **45,** 209 (1971).
6. PATTYN, S. R., PORTAELS, F.: Int. J. Leprosy **48,** 7 (1980).
7. SHEPARD, C. C.: J. exp. Med. —, 445 (1960).
8. KIRCHHEIMER, W. F., STORRS, E. E.: Int. J. Leprosy **39,** 693 (1971).
9. SKINSNES, O. K., MATSUO, E., CHANG, P. H. C., ANDERSON, B.: Int. J. Leprosy **43,** 193 (1975).
10. OGAWA, T., HIRAKI, M.: Kitasato Arch. exp. Med. **45,** 147 (1972).

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NOTE

A NEW *SALMONELLA* SEROTYPE *SALMONELLA ARIZONAE* (47 : *l*, v : z) WITH DULCITOL POSITIVE AND H_2S NEGATIVE VARIANTS

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A new *Salmonella* subgenus III serotype: *Salmonella arizonae* 47 : *l*, v : z and its dulcitol positive and H_2S negative variants isolated from two sampling points of a lake are described.

In the course of routine survey of the water of Lake Balaton in May and June, 1981, three isolates of a new serotype of *S. arizonae (Salmonella* subgenus III.) were found in two distant bathing resorts (Révfülöp and Keszthely).

Materials and methods. Isolation of cultures was performed by passing 1 litre of lake water through membrane filter [1].

The filter was then transferred to 100 ml buffered peptone-water [2] and incubated at 37 °C for 20 h. Ten ml of the peptone-water culture were then transferred to 100 ml Müller–Kauffmann [3] and Preuss [6, 7] enrichment broth and incubated for 20 h at 43° and 37 °C, respectively. Isolation was carried out on Wilson–Blair bismuth sulphite and on brilliant green agar. The Müller–Kauffmann enrichment broth was cultured for a further day at the same temperature and streaked out again. From the Preuss broth 1 ml was transferred to 5 ml of the fresh enrichment medium. Biochemical and serological methods were as described in KAUFFMANN [4] and LE MINOR [5].

Results. According to biochemical features the three isolates belonged to *Salmonella* subgenus III (see Table I). Strain No. 3041 isolated in May at the first sampling point (Révfülöp) did not split dulcitol. Strain No. 3155 found in May at the second sampling place (Keszthely) was biochemically typical but strain No. 3734 isolated there in June did not produce H_2S.

All three isolates belonged to serogroup X (47), they absorbed completely serum O of *S. bergen* (47_1, 47_2). Further examinations in the International Salmonella Centre (Paris) showed them to have 47_3, 47_4 antigens, too.

All three isolates possessed H antigens *l*, v and z. The cultures in the corresponding phases absorbed serum H *l*, v *(S. london* phase 1) and serum H z *(S. poona* phase 1).

Isolates of the same serotype could not be shown in further samplings. The three isolates of the new serotype have been included in the Hungarian Collection of Medical Bacteria, Budapest, under accession Nos 40044, 40045 and 40046.

Table I

Biochemical properties of S. arizonae 47:l, v: z

Reaction	4044	40045	40046
Adonitol	—	—	—
L-Arabinose	+	+	+
Cellobiose	—	—	—
Dulcitol	+	—	—
D-Fructose	+	+	+
D-Galactose	+	+	+
D-Glucose	+	+	+
Glycerol (gas)	—	—	—
m-Inositol	—	—	—
Lactose	+	+	+
D-Maltose	+	+	+
D-Mannitol	+	+	+
D-Mannose	+	+	+
Melibiose	+	+	+
Raffinose	—	—	—
L-Rhamnose	+	+	+
Salicin	—	—	—
D-Sorbitol	+	+	+
Sucrose	—	—	—
D-Trehalose	+	+	+
D-Xylose	+	+	+
ONPG	+	+	+
Methyl red 37 °C	+	+	+
Voges–Proskauer 37 °C	—	—	—
Urea	—	—	—
Indole	—	—	—
H_2S	+	+	—
Gelatin (film)	+[2]	+[2]	+[2]
Ammonium citrate	+	+	+
D-Tartrate	—	—	—
Malonate	+	+	+
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+[2]	+[2]	+[2]
Arginine dihyrolase	+[2]	+[2]	+
Tryptophan deaminase	—	—	—
KCN	—	—	—

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REFERENCES

1. Deák, Zs.: Egészségtudomány **14,** 324 (1970).
2. Edel, W., Kampelmacher, E. H.: Bull. WHO **48,** 167 (1973).
3. Kampelmacher, E. H.: Zbl. Bakt. I. Orig. **204,** 100 (1967).
4. Kauffmann, F.: The Bacteriology of Enterobacteriaceae. Munksgaard, Kopenhagen 1966. p. 21.
5. Le Minor, L.: Entérobactéries. La Tourelle, Saint-Mandé 1972. p. 17.
6. Preuss, H.: Z. hyg. **129,** 187 (1949).
7. The Hungarian Standard for the Examination of Drinking Water. MSz 22901–71.

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BOOKS RECEIVED

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SETLOW, J. K., HOLLAENDER, A. (eds): Genetic Engineering. Principles and Methods. Vol. 3. Plenum Press, New York–London 1981. pp. ix + 346. Price U. S. $ 39.50.

HOLLAENDER, A., RABSON, R., ROGERS, P., SAN PIETRO, A., VALENTINE, R., WOLFE, R. (eds): Trends in the Biology of Fermentations for Fuels and Chemicals. Vol. 18 in Basic Life Sciences (HOLLAENDER, A., General Editor). Plenum Press, New York–London 1981. pp. xii + + 591. Price U. S. $ 65.00.

SCHLESSINGER, D. (ed.): Microbiology — 1981. American Society for Microbiology, Washington, D. C. 1981. pp. xi + 424. Price U. S. $ 22.00.

LOEWUS, F. A., RYAN, C. S. (eds): Recent Advances in Phytochemistry. Vol. 15. The Phytochemistry of Cell Recognition and Cell Surface Interactions. Plenum Press, New York–London 1981. pp. ix + 277. Price U. S. $ 37.50.

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