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METAL-ION ASSISTED DETERGENT ACTION ON BACTERIAL GROWTH: DETERGENT SPECIFICITY AND MECHANISM OF INHIBITION

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(Received September 26, 1983)

Detergents in general inhibit bacterial growth. The anionic detergent, sodium lauryl sulphate (NaLS), seems to have a more specific inhibitory effect on growth of the Gram-positive *Bacillus subtilis* than on growth of the Gram-negative *Escherichia coli*. The specificity of anionic detergent towards *B. subtilis* has been traced to the difference in the nature of the cell wall. The inhibitory action of the detergent is markedly increased by the presence of metal ions like Cd^{++} or Mg^{++}, due probably to the increase in the micellar phase, facilitating the solubilisation of bacterial species in the inner core of micelles. While metal ions like Cu^{++} do not influence the activity of NaLS, Na^{+}, Ca^{++} or Mn^{++} ions seem to act against the detergent action on bacterial growth, probably by altering the structure of micelles.

The history of surface active agents as bacteriocides and the detailed aspects of their chemistry, germicidal properties and applications have been reviewed by Glassman [1], Lawrence [2] and Davis [3, 4]. The mode of action of detergents on microorganisms is not precisely understood. Various modes of antimicrobial action have been proposed, including enzyme inhibition, protein denaturation, disruption of the cell membrane and causing leakage of vital constituents. It is, however, likely that a combination of actions is the cause of inhibition or of death of the cells. Certain organic antibacterial and antifungal agents exert their effect through the formation of toxic chelates of metal ions [5]. Also, metal ions have been shown to influence the antimicrobial properties of tetracycline due to the formation of insoluble complex [6].

In this paper we shall discuss some data on the influence of anionic and cationic surfactants on the growth of a Gram-positive *(Bacillus subtilis)* and a Gram-negative *(Escherichia coli)* bacterium in the presence of several metal ions.

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Materials and method

The microorganisms employed in the present study, viz. *Bacillus subtilis* and *Escherichia coli*, were obtained from the Indian Institute of Science, Bangalore. The bacteria were grown in nutrient broth (pH 7.0), or Czapek's Dox agar medium. The detergents employed, viz. cetyltrimethyl ammonium bromide (CTAB), cetyldimethyl benzylammonium chloride (CDBAC) and sodium laurylsulphate (NaLS) were of pure variety (BDH/Aldrich) and were further purified by the procedure of Duynstee and Grunwald [7].

The microorganisms were grown in nutrient medium of neutral pH and incubated at 37 °C for 16 h. The resultant growth was inoculated on Czapek's agar medium (pH 7.0) in Petri dishes. The antibacterial activity of detergents and the influence of metals on this activity were tested by the filter paper disk technique. The disks 20 mm in diameter were soaked in detergent solution of required concentration and placed on the agar medium seeded with the test bacterium. The dishes were incubated at 37 °C for 24 h and the inhibition zone in each dish was recorded. In the study of the influence of various metal ions on the growth of bacteria in the presence of detergent, 0.010 ml a solution of particular concentration was placed over the disks along with 0.010 ml of NaLS and inhibition was recorded as in the previous case. In the case of each metal ion, both in the presence and absence of detergents, the minimum concentration needed for inhibition has been determined. Results are shown in Table III in columns C and D. In all the cases, a control plate contained all the ingredients except the one whose influence was to be evaluated. The results reported are means of at least three replicas and each experiment was repeated three times.

Results and discussion

Choice of detergent

Surfactants generally affect the growth of bacteria, but activity of the surfactant varies with the microorganism. Therefore a preliminary study was made of the effect of CTAB, CDBAC and NaLS on the growth of *B. subtilis* and *E. coli*. Table I shows the results of this preliminary study. It is evident

Table I

Influence of detergents on bacterial growth

Detergent*	Inhibition zone, mm	
	B. subtilis	E. coli
Control	0.0	0.0
CTAB	23	0.0
CDBAC	0.0	0.0
NaLS	26	22

* Concentration employed $= 5.0 \times 10^{-3}$ M

that the anionic micelle-forming NaLS was more effective against the growth of *B. subtilis*. This specific influence on *B. subtilis* and *E. coli* [8] has been rationalised qualitatively.

The Gram-negative cell wall is chemically more complex than the Gram-positive cell wall [9] in the sense that the former contains more amino acids and

lipopolysaccharides. Hence, even if in Gram-negative bacteria the peptidoglycan is removed by detergents, other layers of the envelope may still adhere to the spheroplasts and may prevent the fragile cytoplasmic membrane from bursting. As the combination of NaLS and the Gram-positive *B. subtilis* yielded a better result, this particular set has been chosen for further studies.

Inhibition of growth of B. subtilis by NaLS

NaLS inhibited the growth of *B. subtilis* in concentrations ranging from 0.0010 to 0.10 M. The maximum inhibition occurred at 6.0×10^{-3} M which happens to be the critical micellar concentration of NaLS [10, 11] (Table II) — The anionic detergent probably disorganises the cell membrane and denatures the proteins essential for metabolism and growth. As the inhibition is increasing with the increase of detergent concentration below critical micellar concentration, the monomers seem to be effective growth-inhibitors for *B. subtilis*. The monomers easily penetrate through the cell membrane resulting in disruption of the cells. This suggestion gains further support from the observed order of inhibition zone with the used surfactants, viz. NaLS, CTAB, CDBAC, where the NaLS which has a lipophilic chain smaller than that of the others, was able to penetrate through the cell and inhibit the growth of *B. subtilis*.

Table II

Influence of NaLS on growth of B. subtilis

$\times 10^{-3}$ M NaLS per disk	Inhibition zone, mm
0.0	0.0
1.0	22
2.0	24
4.0	27
6.0	30
8.0	28
10.0	28
20	28
50	28
100	28

Effect of metal ions on the growth of B. subtilis in the presence and absence of NaLS

Table III summarizes the influence of sulphates of the metals like calcium, copper, magnesium, manganese, nickel, sodium and zinc on the growth of *B. subtilis* both in the presence and absence of NaLS. It is evident from

Table III

Combined effect of metal ions and NaLS on B. subtilis

Metal ion	Inhibition zone diameter, mm*		Minimum concentration of metal ion required for inhibition $\times 10^{-3}$	
	A	B	C	D
Control no metal ion	0.0	30	—	—
Na^+	20	30	4.0	6.0
Ca^{++}	20	20	3.0	8.0
Mn^{++}	20	20	4.0	7.0
Zn^{++}	28	26	0.70	3.0
Ni^{++}	20	34	0.90	1.0
Mg^{++}	20	36	3.0	8.0
Cu^{++}	40	40	0.50	0.90
Cd^{++}	24	48	0.70	2.0

A — 1.0×10^{-2} M metal ion in the absence of NaLS
B — 1.0×10^{-2} M metal ion in the presence of 6.0×10^{-3} M NaLS
C — in the presence of 6.0×10^{-3} M NaLS
D — in the absence of detergent
* Disk diameter, 20 mm

these results that Zn and Cu inhibited the organism in the absence of detergent while other metals by themselves did not affect its growth. Metal like Na, Ca and Mn block the inhibition due to NaLS whereas Mg and Cd reinforced its inhibitory ability of NaLS. The inhibitory capacity of Cu was unaffected by NaLS.

The increased activity of NaLS in the presence of Cd^{++} and Mg^{++} may be ascribed to the surfactant — metal complex binding with cellular proteins and denaturing them. Alternatively, these salts may increase the micellar surface area by altering the aggregation of surfactants [12], facilitating the solubilization of bacterial species in the inner core of micelles, where surface tension is low. This in turn slows down the growth rate of the organism. In the presence of Ca^{++} or Mn^{++} or Na^+ ion, NaLS lost all its activity, probably due to the change in the micellar structure so that it cannot interact with the membrane [13]. There seems to be a fairly linear relationship between the radii of the metal ions and their inhibitory capacity in the presence of NaLS, wherever the metal ion reinforces the inhibitory action of NaLS. Similar observations have been made by Gupta et al. during the study of metal ion action on the antimicrobial activity of tetracycline hydrochloride [6].

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COMPARISON OF *LISTERIA* SEROTYPES AND PHAGE TYPES ISOLATED FROM SHEEP, OTHER ANIMALS AND HUMANS

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A total of 814 different faecal, nasal, vaginal and milk samples of 50 healthy cotted sheep was investigated for *Listeria* during an epidemic-free summer season. Forty ewes and 5 tegs excreted *Listeria* strains; 18.1% of all samples were positive. A total of 151 *Listeria monocytogenes* strains belonging to serotypes 1/2, 4, 5, 6 and unknown were identified and phage typed. On the basis of the serotype and phage type of the excreted strains the animals could be divided into 7 groups. Characters of strains isolated from the cotted sheep were compared with those cultivated from 68 dead animals received from 44 distinct settlements. Also properties of 10 *Listeria* strains originating from 8 ill patients infected in the same region from where the carcasses of the animals were sent to the laboratory were studied. Two human *Listeria* strains were identical in serotype and phage type with some animal strains.

Serotyping, phage typing, biotyping and detection of bacteriocin production provide an aid in following the spreading of pathogenic agents. The usefulness of phage typing of *Listeria* strains in epidemiological work has only been studied by a few authors [1–8]. The purpose of the present work was to evaluate the results of phage typing and to collect further data on the occurrence of listeriae. Different samples taken from 50 healthy cotted sheep were cultured for *Listeria* and properties of the strains isolated from the samples as well as dead animals and ill patients were studied.

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Materials and methods

A total of 814 samples of 50 individually labelled healthy cotted sheep were investigated. The samples were taken between 22nd June and 31st July in an interval of 2 to 6 days. Nasal and vaginal specimens were taken with sterile swabs. Faeces were collected with sterile rubber finger stall from the rectum of each animal into a sterile dish. Milk was directly milked into sterile glass tube. Swabs, a bean-size piece of faeces or 1 ml of milk were inoculated into modified Holman's enrichment medium and the cultures were incubated at 4 °C for 3 months at the least. The first subculture was made after 2 weeks incubation onto TNSA medium and repeated on every 2nd or 3rd week. Strains were isolated, checked, serotyped and phage typed according to the usual methods [2, 3, 6]. *Listeria* strains were isolated from animal carcasses and patients in accordance with the methods prescribed for the veterinary and public health services.

Media and reagents were prepared using A. G. chemicals (Reanal, Budapest, Hungary) and Oxoid products (Basingstoke, England).

Results

Distribution of 814 samples collected from 50 animals (40 ewes and 10 tegs) and positive results are shown in Table I. All ewes excreted *Listeria* and 140 (20%) out of their 693 samples were positive. Only 8 samples (6.6%) of 5 animals out of 121 samples taken from 10 tegs contained *Listeria*.

Table I

Distribution and positive results of 814 samples from 50 healthy cotted sheep

Sample	Samples		Positive samples		Proportion of one kind of positive samples to all positive materials (148)
	No.	%	No.	%	
Nasal swabs	161	19.7	31	19.2	20.9
Vaginal swabs	175	21.4	4	2.2	2.7
Faeces	408	50.1	112	27.4	75.6
Milk	70	8.5	1	1.4	0.6
Total	814	100.0	148	18.1	—

Table II

Serotype distribution of 151 Listeria strains isolated from 50 healthy cotted sheep

Source of strains	Serotype of strains					Total
	1/2	4	5	6	not determined	
Nasal swabs	26	—	1	2	2	31
Vaginal swabs	1	—	—	2	1	4
Faeces	86	1	8	11	9	115
Milk	1	—	—	—	—	1
Total	114	1	9	15	12	151
Percent of all strains	75.4	0.6	5.9	9.9	7.9	100.0

Table III

Occurrence of Listeria strains of different serotype and phage type in 50 healthy cotted sheep

Serotype 1/2				Serotypes 1/2, 5, 6a, 6b										Other serotype and phage type entities			
Single isolation		Repeated isolations		Repeated isolations													
		Identical phage type	Different phage types	Single isolation			Identical phage type			Different phage types							
5/5*		20/7	28/8	3/1			41/9			16/3				38/12			
100000000	1**	NT 20	020000000	1	1/2	NT	1	1/2	NT	28	1/2	400000000	1	1/2	020000000	1	
400000000	1		100000000	5	5	000003736	1	5	000003736	5		NT	10		040000000	1	
NT	3		400000000	3	6b	NT	1	6a	NT	5	5	000003736	1		100000000	2	
			571000000	1				6b	000002504	1		000003737	1		400000000	2	
			NT	17					NT	2	6a	000000040	1		NT	13	
			unknown	1								NT	2		unknown	3	
														4	NT	1	
														5	NT	1	
														6a	NT	1	
														6b	NT	1	
														Unknown	000001000	1	
															040000000	1	
															NT	6	
															unknown	4	

* Number of strains/number of sheep; ** phage type and number of strains; NT = non typable with our phages

Table II demonstrates serotype distribution of listeriae. Two *Listeria* strains of different serotype were isolated from each of 3 faecal samples in the same time.

The results show that alimentary and respiratory tracts of sheep were the most important sources of listeriae.

The occurrence of *Listeria* strains of different serotype and phage type in the 50 sheep is summarized in Table III.

Five *Listeria* strains of serotype 1/2 were isolated from 5 sheep — only one from each. Twenty non-typable *L. monocytogenes* serotype 1/2 strains were cultivated from 7 animals repeatedly (at least two times), occasionally from different samples (faeces, nasal discharge, see sheep No. 18 in Table IV). From further 8 animals 28 *Listeria* strains of serotype 1/2 were isolated. It was characteristic of these animals that at least two strains of different phage type could be cultivated from each. Data of sheep No. 3 characterize this group of animals in Table IV. One sheep excreted 3 listeriae of distinct serotype — each strain was isolated only once. In 9 animals *Listeria* strains of different serotype

Table IV

Representative types of Listeria excretion observed in cases of cotted sheep

Designation of animal	*Listeria* cultured from	Date of sampling and results of examinations					
		June			July		
		22	24	26	30	3	7
	nose	·	1/2* NT**	—	·	·	·
18	vagina	·	·	·	—	·	—
	faeces	—	1/2 NT	1/2 NT	1/2 NT	—	—
	nose	·	·	·	·	·	—
3	vagina	—	·	·	·	—	·
	faeces	—	1/2 400000000	1/2 100000000	·	·	1/2 020000000
	nose	·	·	·	·	·	—
2	vagina	·	·	·	—	·	·
	faeces	6a NT 1/2 NT	1/2 NT	5 000003736	5 000003736	·	—
	nose	·	1/2 NT	1/2 NT	·	·	·
22	vagina	1/2 NT	·	·	·	—	6a NT
	faeces	6a 000000040	—	1/2 NT	—	—	1/2 NT

* Serotype
** Phage type
— *Listeria* was not present
· Not tested

occurred and certain serotypes were repeatedly observed. The phage type of the latter was the same. From the 9 sheep 41 *Listeria* strains were collected. This group is represented by animal No. 2 in Table IV. Similarly, 16 *Listeria* strains of distinct serotype were cultured from further 3 sheep; phage types of the repeatedly isolated strains were not the same. An example for this group can be seen in Table IV (sheep No. 22). Twelve sheep of the last category yielded 38 listeriae out of which 12 could not be serotyped. Phage type of 4 strains out of the 12 as well as that of 3 out of the serotyped 26 was not determined.

The members of the flock could be classified into two groups: 20 animals excreted only serotype 1/2 on one or more occasions (53 strains). Forty strains were non-typable with phages, 12 strains belonged to 5 different phage types and one strain was not tested.

From the samples of 25 further sheep at least two listeriae of distinct serotype could be isolated (98 strains). Out of them 61 belonged to serotype 1/2 and 52 were not phage-typable, 7 strains belonged to 4 different phage types and 2 were not tested. The serotype of 12 strains out of the remaining 37 ones was unknown and the phage type of 4 unserotyped strains was not investigated. The other 25 listeriae belonged to 4 serotypes (4, 5, 6a and 6b); 14 were not phage-typable, 10 belonged to 4 different phage types and one was not typed.

In summary, 45 out of the 50 sheep excreted *Listeria*, 40 out of them excreted on one or more occasions serotype 1/2 and not phage-typable strains, i.e. this type of listeriae was dominant in the stock.

In the second part of this study we compared characters of listeriae of different origin. The microorganisms were isolated from ill patients and dead animals in Borsod-Abaúj-Zemplén county. Ten *L. monocytogenes* strains were

Table V

Human listeriosis cases

Patients			Strains		Note
designation	age	residence	serotype	phage type	
29	33 years	Sajóhídvég	4b	002 010 000	mother, premature birth
30*	premature baby	Sajóhídvég	4b	002 010 000	premature baby of No. 29.
			4b	002 000 000	congenital listeriosis
31	premature baby	Gesztely	4b	004 000 004	meningitis, sepsis
			4b	000 010 000	
32*	premature baby	Bőcs	1/2	NT	congenital listeriosis
37	46 years	Szikszó	4b	NT	meningitis
44	58 years	Onga	1/2	100 000 000	meningitis
45	73 years	Kisgyőr	1/2	NT	meningitis
48	1 day	Szentistván	4b	002 100 000	sepsis

* Patient died.

cultivated from 8 human cases (Table V). Patients No. 29 and No. 30 belonging to a family outbreak yielded 3 strains one of which showed a slight difference in phage pattern from the others. The two strains isolated from patient No. 31 showed a remarkable difference.

The map shows the sources of the carcasses, the residence of the patients and finally serotype and phage type of listeriae isolated from animals (Fig. 1).

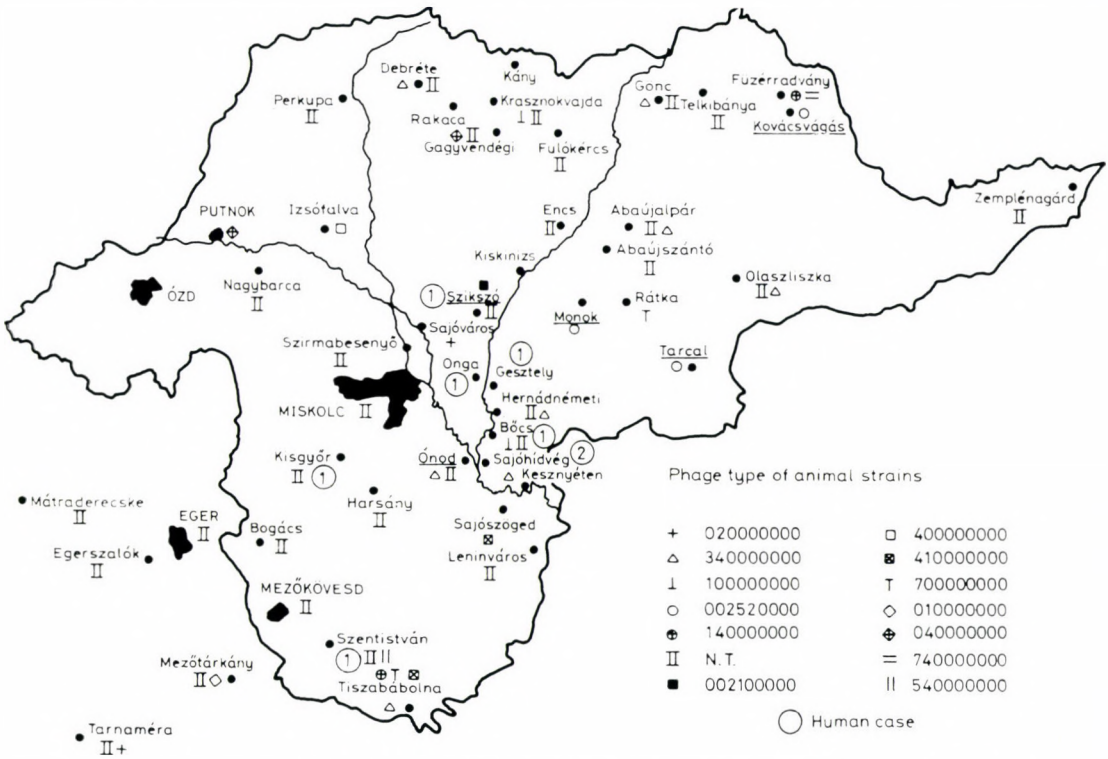

Fig. 1. Occurrence of *Listeria* strains in Borsod-Abaúj-Zemplén county. Names of localities where serotype 4 occurred are underlined

Listeria strains occurred in 47 habitations of the region of which 41 settlements belonged to Borsod-Abaúj-Zemplén county. Human cases were diagnosed in 7 localities. In 4 settlements both human and animal illnesses were observed, in further 3 (Onga, Gesztely and Sajóhídvég) only human cases occurred. In 34 places only deaths of animals were registered. Twenty-eight out of 68 carcasses originated from 28 single stocks which were kept at 25 settlements. Data for animal listeriosis are shown in Table VI.

Serotypes and phage types of *Listeria* strains isolated in course of this study are summarized in Table VII. Only 97 out of 151 listeriae isolated from healthy sheep are listed in the Table because every strain of a given serotype

Table VI

Properties of the strains isolated from 28 single animal listeriosis cases

Locality of stock	Specification of carcasses		Strains	
			serotype	phage type
Sajóvámos	lamb	brain	1/2	020 000 000
Harsány	bovine foetus	stomach content	1/2	NT
Eger IV	sheep	brain	1/2	NT
Szirmabesenyő	sheep	brain	1/2	NT
Abaújszántó	lamb	brain	1/2	NT
Bogács	lamb	brain	1/2	NT
Fulokércs	sheep	brain	1/2	NT
Mátraderecske	sheep	brain	1/2	NT
Perkupa	sheep	brain	1/2	NT
Mezőkövesd	bovine foetus	stomach content	1/2	NT
Bőcs	sheep	brain	1/2	100 000 000
Zemplénagárd	sheep	brain	1/2	NT
Szikszó	bovine foetus	stomach content	1/2	NT
Kovácsvágás	lamb	brain	4	002 520 000
Tarcal	lamb	brain	4	002 520 000
Kesznyéten	sheep	brain	1/2	340 000 000
Putnok	lamb	brain	1/2	040 000 000
Nagybarca	lamb	brain	1/2	NT
Gagyvendégi	lamb	brain	1/2	NT
Sajószöged	sheep	brain	1/2	410 000 000
Izsófalva	sheep	brain	1/2	400 000 000
Rátka	sheep	brain	1/2	700 000 000
Rakaca	sheep	brain	1/2	040 000 000
Csorba telep	sheep	brain	1/2	NT
Görömböly	sheep	brain	1/2	NT
Tiszabábolna	bovine foetus	liver	1/2	700 000 000
Tiszabábolna	lamb	brain	1/2	140 000 000
Szikszó	cattle	brain	4	002 100 000

and phage type was counted only once in each animal. Differences in phage types may be explained by the fact that the carcasses originated from 44 settlements. *Listeria* strains of serotype 1/2 belonging to 4 phage types (400 000 000, 100 000 000, 040 000 000 and 020 000 000) were found among the cotted animals and in the carcasses, but their ratio (29.6% respectively 13.2%) differed from each other. As to human listeriae, in contrast to earlier observations [5, 6], in two cases (Nos 44 and 48) the phage type of the strains (100 000 000, 002 100 000) was identical with that of the animal ones.

Discussion

In our earlier publications attention was focussed on the usefulness of phage typing of *Listeria* strains and our preliminary results were also reported [1, 3, 5]. It was described that phage-typability of the Hungarian and the French strains differed in certain extent. Besides, it was also observed that *Listeria* strains of identical serotype and phage type were not isolated from ill

Table VII

Serotype and phage type of Listeria strains of different origin

Strains isolated from healthy cotted sheep		
serotype (serovar)	phage type	No.
1/2	571 000 000	1
	400 000 000	7
	100 000 000	8
	040 000 000	2
	020 000 000	2
	NT	40
	unknown	3
4	NT	1
5	000 003 737	6
	000 003 736	1
	NT	1
6a	000 000 040	1
	NT	7
6b	000 002 504	1
	NT	4
	unknown	1
Unknown	000 001 000	1
	NT	6
	unknown	4

Strains isolated from carcasses of cattle		No.	
		epidemic	single
	700 000 000		1
1/2	340 000 000	2	
	NT		4
4	002 100 000		1

Strains isolated from carcasses of sheep		No.	
serotype (serovar)	phage type	epidemic	single
1/2	740 000 000		1
	700 000 000		1
	540 000 000		1
	410 000 000		2
	400 000 000		2
	340 000 000	1	6
	140 000 000		2
	100 000 000		2
	040 000 000		2
	020 000 000		3
	010 000 000		
	NT	8	21
4	002 520 000	4	
	NT		1

Strains isolated from patients		
serotype (serovar)	phage type	No.
1/2	100 000 000	1
	NT	2
4b	004 000 004	1
	002 100 000	1
	002 010 000	2
	002 000 000	1
	000 010 000	1
	NT	1

patients and animals, however, healthy persons excreted such strains rarely. In contrast to this, different animals (sheep, cow) excreted more frequently *Listeria* strains of the same serotype and phage type. It was also recognized that among serotypes and phage types of listeriae regular relationships existed. On the basis of these regularities it was verified that serotyping of a small part of *L. monocytogenes* strains isolated in Canada was not exact [3, 9, 10]. Last but not least a possibility was suggested to control the method of phage typing.

In the present study it was observed that intestinal and upper respiratory tracts of sheep were the main sites of harbouring and excretion of listeriae. In view of our findings on serotyping and phage typing as tools of epidemiological tracing we do not agree with one of the concept of Halifax Workshop. namely "serotyping is of limited usefulness" [11]. However, we agree with the other standpoint of the Workshop: "multiple typing systems are recommended for epidemiological studies" [11].

On the basis of the results obtained in studying a cotted stock of sheep, it may be concluded that the animals harboured *Listeria* strains of different serotype as well as phage type and even same animal failed to excrete the same strain on repeated examinations. This finding indicates that different listeriae circulated in the stock. Epidemiological tracing of *Listeria* strains is very difficult under such circumstances even when the strains have two markers. Besides, the question arises, is it worth-while to immunize a flock with a living vaccine when the members of it are infected to a great extent?

Proportion of not phage-typable listeriae was lower and the number of phage types was higher in case of carcasses than in case of cotted healthy sheep. This observation may be attributed to the fact that carcasses originated from 44 places. It is interesting that serotype 5 did not cause death of animals.

As serotype and phage type of a *L. monocytogenes* strain do not always give information about the pathogenic property of this microbe, at present there are no better means to determine virulence markers of these strains than those published earlier [6, 12].

In general, high proportion of not phage-typable *Listeria* strains urges further improvement of the method of phage typing. Also there are some open questions which should be discussed for the formation of a realistic standpoint for the correct evaluation of results of phage typing (13).

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ALTERATION OF PHAGE- AND BIOTYPES OF *LISTERIA* STRAINS

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Ethyl-methyl-sulphonate mutants of *Listeria monocytogenes* might be different from the parent strain in phage type and in splitting of lactose, maltose, melezitose, sucrose and trehalose. Differences were observed in repeated control studies in phage type and carbohydrate-decomposition of 550 *Listeria* strains isolated from a variety of sources (patients, healthy and dead animals). It has been concluded that certain carbohydrate tests are unsuitable for distinguishing biotypes of *Listeria*. An improvement of the evaluation of phage typing results is recommended.

We have reported on the properties of 550 *Listeria* strains [1–3]. Repeated test have revealed that the phage type and carbohydrate-decomposing ability of certain strains varied. The present paper gives an account of these studies.

Materials and methods

Biochemical characters, serotype, phage type, nutritional requirements and virulence markers of *Listeria* strains isolated from humans and animals in Hungary and Canada and mutants produced by EMS (ethyl-methyl-sulphonate) were studied. The methods were described in earlier publications [4–7]. EMS treatment was carried out according to Clark's method [8] and the mutants were isolated from blood agar.

Results

Listeria monocytogenes serotype 1/2a strain No. 18 which has long been used in our experiments and its mutants produced by EMS were examined. Data for the strains are summarized in Table I. Although the three strains belonged to the same serotype, in phage type and haemolytic ability they differed. One of the mutants required more amino acids for the growth on minimal

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Table I

Properties of L. monocytogenes strain No. 18 and its mutants

Properties	Strains								
No.	18			025/3			025/4/4		
Serotype	1/2a			1/2a			1/2a		
Phage type	400 000 000			NT			240 000 000		
Amino acid requirement									
DL-iso-leucine	essential			essential			essential		
L-leucine	essential			essential			essential		
DL-valine	essential			essential			essential		
L-cysteine-HCl	essential			essential			essential		
L-glutamine	non-essential			non-essential			essential		
DL-methionine	non-essential			non-essential			essential		
L-arginine-HCl	non-essential			non-essential			essential		
Haemolysis	weak beta			strong beta			very weak beta		
Anton's test	positive			positive			negative		
Acid production	1*	2	3	1	2	3	1	2	3
lactose	+	—	I	—	—	+	—	—	—
maltose	+	·	+	+	·	+	I	·	+
melezitose	+	·	+	—	·	+	—	·	+
sucrose	+	+	+	—	+	+	—	+	I
trehalose	+	·	+	—	·	+	I	·	+

* Experiment No
· = Not tested
I = Irregular.
NT = Untypable

agar; this strain became avirulent. The carbohydrate-decomposing ability of the strains was examined on three different occasions and the results were not always the same.

To obtain more data on the stability of phage type and carbohydrate-splitting, 56 *L. monocytogenes* serotype 4b strains isolated during a Canadian epidemic were examined [9]. Six strains were isolated from 3 patients, i.e. two strains from each person. Further 16 strains were isolated from family outbreaks (from mothers and their newborns), while the remaining 34 strains originated from seemingly single cases.

These *Listeria* strains on the basis of their phage pattern could be divided into two groups, but according to Audurier, this differentiation has no practical importance, i.e. all 56 *Listeria* strains can be regarded as the same in respect of their epidemiological role. The carbohydrate-decomposing ability of the strains was repeatedly studied, each test being performed three times. While the proportion of strains splitting sucrose, melezitose and glycerol was nearly the same in both phage pattern strains, that of listeriae decomposing melezitose

Table II

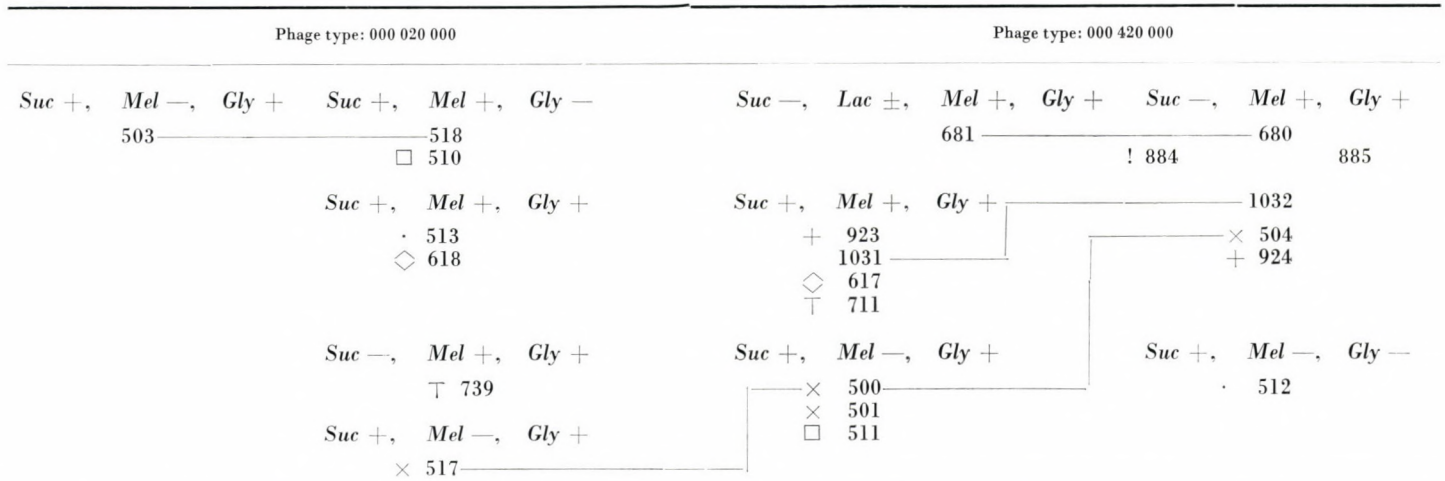
Phage type and carbohydrate-splitting ability of L. monocytogenes strains of the same serotype isolated in course of a Canadian epidemic

Phage type: 000 020 000 (16 strains)						Phage type: 000 420 000 (40 strains)					
Suc +,	*Mel* +,	*Gly* +	*Suc* —,	*Mel* +,	*Gly* +	*Suc* +,	*Mel* +,	*Gly* +	*Suc* —,	*Mel* +,	*Gly* +
	497*			619		509	617	784	504	678	907
	498			739		515	679	923	522	680	959
	513					544	682	1031	591	828	1032
	618					559	694	82—83	628	884	1087
	713					592	711		629	885	82—129
									630	924	82—130
Suc +,	*Mel* —,	*Gly* +	*Suc* +,	*Mel* +,	*Gly* —	*Suc* +,	*Mel* —,	*Gly* +	*Suc* +,	*Mel* +,	*Gly* —
	514			510			500	511		590	
	517			518			501	560			
	558										
Suc —,	*Lac* ±,	*Mel* +,	*Gly* +	*Suc* —,	*Mel* —,	*Gly* +	*Suc* +,	*Mel* —,	*Gly* —	*Suc* —,	*Mel* +,	*Gly* —
	861				503			512			700	
	886											
Suc +,	*Man* ±,	*Mel* —,	*Gly* +				*Suc* —,	*Lac* ±,	*Mel* +,	*Gly* ±		
	639							681				

Man = mannitol, *Suc* = sucrose, *Mel* = melezitose, *Gly* = glycerol, *Lac* = lactose
* Designation of strains

Table III

Phage type and carbohydrate-decomposing ability of L. monocytogenes strains isolated from the same patient or from family outbreaks



Identical signs ahead of designation of strains mark isolates from the same family outbreak. Numbers linked with lines mark strains isolated from the same patient

and glycerol but not sucrose was significantly higher in phage type 000 420 000 than in 000 020 000. In phage type 000 020 000 the proportion of bacteria cleaving sucrose and glycerol but not melezitose, and also of strains decomposing sucrose but not glycerol was higher than in phage type 000 420 000. In general, splitting of sucrose was more frequent in phage type 000 020 000 (Table II). Considering the source of the strains it was observed that pairs of strains (503–518, 680–681, 1031–1032) isolated from the same persons belonged to the same phage type but differed in the ability to decompose certain carbohydrates (Table III). In one case when three strains (500, 504, 517) were isolated from the same newborn, the phage type of one of them was 000 020 000, while that of the other two 000 420 000. In four cases (510–511, 512–513, 617–618, 711–739) out of the 7 family outbreaks the phage type of one of the two strains isolated from mothers and their newborns proved to be 000 020 000, while phage type 000 420 000 characterized the other four strains. In three out of the four cases the carbohydrate-splitting ability of the strains was also different. In further two family outbreaks (884–885, 923–924) the phage type of the strains was the same and two strains cleaved the carbohydrates in the same way, too. In a family outbreak yielding 3 strains from the newborn and one from the mother, the phage type and carbohydrate-decomposing ability of the latter were identical with those of one of the newborn strains. However, only its phage type was identical with that of the 2nd newborn strain and there were no identical properties in the case of the 3rd strain. These data support the view that phage type and carbohydrate-splitting ability of *Listeria* strains may exhibit some alterations.

Table IV

Phage type of L. monocytogenes strains of the same serotype isolated from the same patient, animal or family outbreak

Case	Strain				
	source		serotype	phage type	
1	premature baby	blood	4b	004 000 004	**
		CSF	4b	000 010 000	
2	57 years old male	spleen	4a	002 520 000	*
		brain	4a	002 500 000	
		CSF	4a	002 520 000	
3	cow	brain 1	4	002 100 000	*
		brain 2	4	001 000 000	
4	mother	vagina	4b	002 010 000	*
	premature baby	liver	4b	002 000 000	
		CSF	4b	002 010 000	

* Non-significant alteration
** Significant alteration

Table IV summarizes the Hungarian data obtained for strains isolated from the same person, animal or family outbreak. It can be concluded that more significant or less important differences can be observed in the phage type of strains isolated from the same source.

Results of repeated tests performed with the same *Listeria* strains also confirm the fact that some alterations may occur (Table V). In the case of strains 1 and 2 only non-significant differences were observed. In four examinations of strain 3 four phage types were determined which differed more or less significantly from each other. At present it is not clear whether the alterations can be attributed to the unreliability of the method or to the unstable character of the strains.

Table V

Phage types of the same L. monocytogenes strain on repeated control examinations

Designation of strain	No. of examination	Serotype	phage type	
1	1	4	006 000 000	*
	2	4	002 000 000	
2	1	1/2	010 000 000	*
	2	1/2	NT	
3	1	1/2	330 000 000	**
	2	1/2	771 000 000	
	3	1/2	331 000 000	*
	4	1/2	731 000 000	

* Non-significant alteration
** Significant alteration

In Table VI we try to illustrate the practical importance of alteration in phage type. These data were published previously [3]. If phage types in Table VI meant real differences, in the first farm the death of the four sheep would

Table VI

Phage type of L. monocytogenes strains isolated from dead animals during two epidemics

Locality of the stock (village)	Designation of animal	Strain		
		Serotype	Phage type	
Abaújalpár	33	1/2	NT	*
	46	1/2	010 000 000	
	40	1/2	340 000 000	*
	50	1/2	400 000 000	
Mezőtárkány	66	1/2	NT	*
	67	1/2	010 000 000	

* Non-significant alteration

have been caused by four *L. monocytogenes* of different origin. However, according to Audurier, the differences (NT-010 000 000 or 340 000 000—400 000 000) are non-significant. This means that not four epidemiologically distinct organisms, but at most only two different listeriae circulated in the Abaújalpár stock. On the basis of this consideration only one epidemiological unit was responsible for the Mezőtárkány outbreak.

Discussion

Osebold et al. [10] observed that *L. monocytogenes* in diffusion chambers implanted into peritoneal cavity of mice survived as long as 22 months. However, at the end of the experiment instead of the originally homogeneous *Listeria* population different mutants could be subcultured from the chambers. Thus avirulent and R mutants as well as mutants differing from the parent strain in respect of the carbohydrate-splitting ability were identified. While the parent strain cleaved trehalose, rhamnose, salicin, glycerol, slightly decomposed lactose, and irregularly sucrose, one of the mutants split only trehalose, rhamnose and salicin, another one only trehalose and gave uncertain results with the other carbohydrates. There were isolates which failed to cleave any of the above carbohydrates but caused only a slight acidity. A mutant weakly decomposed only rhamnose.

Seeliger [11] and Jones [12] were also of the opinion that the evaluation of sucrose- and lactose-splitting ability of *Listeria* strains is difficult because the same strain may give different results in repeated experiments.

Our findings are similar for sucrose, lactose, melezitose, glycerol and mannitol. These observations support the view that carbohydrate-decomposition cannot be regarded as a distinctive marker.

The first data on artificial mutants were published by Hunter et al. [13] in 1950. A wide veriety of mutants of cultural morphology, virulence, antigenic structure, carbohydrate-decomposition, haemolysis and other biochemical activities could be produced by exposure to radiophosphorus. In certain properties, including phage type, our mutants also differed from the parent strain [7].

It may be assumed that a change in carbohydrate-decomposing ability of a *Listeria* is only one sign of the alterations which occurred in the bacterium and a simultaneous change in the phage type might also be present. This assumption is supported by observations on repeated control of the same strain and of strains isolated from the same person or in connection with the same family outbreak.

In view of the above findings, it is advisable to consider a change in the present phage typing system so that non-significant alterations in the lytyc

pattern should not be reflected by the codes. On the other hand, it would be also necessary to make clear which of the alterations are non-significant and what is the reason for these differences. Experience collected up till now needs on improvement of the method of phage typing or at least the use of an explanatory dictionary to prevent false conclusions. Until these unsolved problems are not cleared up, phage typing results should be used with great caution in the epidemiological work. The results also indicate that the method suggested by us [14] for biotyping of *Listeria* can hardly be used because of the uncertain and irregular splitting of certain carbohydrates.

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EFFECT OF PHLORIDZIN ON LIPID BIOSYNTHESIS BY *CLADOSPORIUM TENUISSIMUM* COOKE

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A local strains of *Cladosporium tenuissimum* was found unsuitable as a producer of lipid. Addition of phloridzin to the fermentation media induced this organism to accumulate a moderate level of lipid, especially sterols increased highly in the presence of phloridzin. The promoting effect of phloridzin may be due to directing the sugar breakdown towards lipid biosynthesis and/or transforming the free fatty acids and oxalacetic acid to acetyl-Co A.

Much work has been carried out to study the production of lipids by fungi. The most promising organisms are the species of *Aspergillus* [1–4]; *Penicillium* [5–8] and *Fusarium* [9–11].

The purpose of this study was to determine the role of phloridzin in lipid biosynthesis using a strain of *Cladosporium tenuissimum*.

Materials and methods

The organism employed was isolated from Egyptian soil and identified by the Commonwealth Mycological Institute as *Cladosporium tenuissimum* Cooke. The organism was maintained on Czapek–Dox solid medium, spore suspensions were obtained from 7 days old cultures. The fermentation medium used was that described by Pruess et al. [12] and had the following composition (g/100 ml): glucose, 4; NH_4NO_3, 1; KH_2PO_4, 0.68; $MgSO_4 \cdot 7\ H_2O$, 0.05; $ZnSO_4 \cdot 7\ H_2O$, 0.005 and $FeCl_3 \cdot 6\ H_2O$, 0.016. The media were distributed in 250 ml Erlenmeyer flasks and sterilized at 1.5 atm for 15 min. The flasks were left to cool, the pH was initially adjusted to pH 6 and the different concentrations of phloridzin were added to the sterilized media under aseptic conditions. The culture flasks were incubated at 25 °C for 7 days after which the different estimations were carried out.

The lipids were extracted by the technique adopted by Nichols [13]. The crude lipids were dissolved in a mixture of chloroform and methanol (2 : 1, v/v) and purified as described by Folch et al. [14]. Lipids were fractionated on thin layer plates covered with silica gel G using the solvent system petroleum ether : diethylether : acetic acid (90 : 10 : 1, v/v) and detected by phosphomolybdic acid (5% in ethanol) followed by perchloric acid and heating at 110 °C for 5 min. The percentage of different fractions were estimated using EEL Scanner as described by Selim and Mousa [15]. The sugar concentrations in the metabolic solutions were estimated using the anthrone method adopted by Magnetski et al. [16]. Organic acids accumulated in the culture media were evaluated by the zone-strip technique described by Shimi et al. [17], the acid spots were detected with the reagent decided by Paskova and Munk [18]. The acid values were determined as described by Hilditch [19].

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N. B. The technique of replacement was carried out as follows: the fungus was allowed to develop its mats on the fermentation medium for 5 days at 25 °C. The metabolic solutions were poured off and replaced by equal volumes of a salt solution which had the same composition as the fermentation medium except that glucose was lacking. The salt solution was found to be suitable not only for washing the mats from their undersurfaces, but also in keeping the fungal cells in an active fresh state. Wetting the mycelial mats from the upper side was avoided by using sterile pipettes for introducing the salt solutions below the felts. The cultures were reincubated for 4 h, and the salt solution was poured off and replaced by equivalent volumes of the final replacement solution.

Results and discussion

The results recorded (Table I) showed that the addition of phloridzin to the fermentation medium of *C. tenuissimum* stimulates its metabolic activities. The maximum lipid yield was 18.4% in the presence of 10^{-2}% phloridzin

Table I

Behaviour of C-tenuissimum in the presence of different concentrations of phloridzin

Phloridzin g/100 ml	Felt weight	Sugar consumed	Lipid		
			g	%	acid value
0.0	1.543	3.0	0.051	3.3	10.6
10^{-5}	2.218	3.4	0.243	10.9	8.4
10^{-4}	2.007	3.5	0.250	12.4	8.1
10^{-3}	1.701	3.7	0.246	14.5	7.6
10^{-2}	1.513	3.8	0.278	18.4	7.4
10^{-1}	1.225	3.1	0.168	13.7	6.8

Results are given in g/100 ml of media. Initial glucose concentration: 4 g/100 ml

$$\text{Lipid (\%)} = \frac{\text{Lipid (g)}}{\text{Felt (g)}} \times 100$$

This content represent more than 5.5 times the yield accumulated in the control experiment. Of particular significance is that the sugar consumption follows the trend of lipid biosynthesis where it increased with the increase of phloridzin level achieving its maximal utilization at 10^{-2}%, after which the sugar uptake was decreased. These findings give the impression that phloridzin directed the glucose breakdown towards lipid accumulation. The previous findings differs from that of Damodaran and Rangachari [20] who found that phloridzin has no effect on sugar utilization by *Aspergillus niger*.

To throw the light on the possible effect of phloridzin on lipid biosynthesis, the performed mats of *C. tenuissimum* were grown on the final replacement solution (fermentation medium fortified with 10^{-2}% of phloridzin) and the different estimations of lipids, sugar uptake, felt formation, accumulation of organic acid in addition to the different lipid classes were carried out at inter-

Table II

Behaviour of the preformed mats of C. tenuissimum in presence of 10^{-2}% phloridzin

(a) Results at the time of replacement

Felt weighet	Sugar consumed	Lipid			Organic acids			
		g	%	acid value	gluconic	tartaric	oxalacetic	succinic
0.938	2.8	0.027	2.9	9.8	0.018	0.04	0.1	—

Results are given in g/100 ml of media

(b) Results subsequent to replacement

Incubation period (days)	Felt weight	Total sugar consumed	Lipid			Organic acids			
			g	%	acid value	gluconic	tartaric	oxalacetic	succinic
2	1.945	5.3	0.214	11.8	12.0	0.018	0.042	0.120	—
4	2.447	6.6	0.342	14.0	11.3	0.016	0.050	0.104	—
6	2.081	6.7	0.357	17.2	10.8	0.02	0.046	0.095	—
8	1.882	6.8	0.429	22.8	10.4	0.01	0.042	0.074	—
10	1.348	6.8	0.187	13.9	9.1	—	0.036	0.086	0.04

Initial glucose concentration: 8 g/200 ml of media (4 g/100 ml at the time of inoculation + 4 g/100 ml of the final replacement solution)

vals of replacement. The top part of Table II represents the state of the felt and its activity at the time of replacement (5 days), while the bottom part contain all the data determined on the changes which took place during a definite period of incubation counted from the time of replacement. Hence, the following expression has been employed in the bottom part: total sugar consumed = sugar consumed at the time of replacement + sugar consumed after a given incubation period. The calculated figures for lipid formation and sugar uptake showed a general tendency for increase with the ageing of cultures till the 8th day of replacement. These findings accords completely with that recorded in Table I and this is a confirmation to the previous explanation that phloridzin directed the sugar breakdown towards lipid accumulation.

Concerning the values recorded for the free acidity (acid value), it is obvious that the presence of phloridzin in the fermentation media of *C. tenuissimum* affect unfavourably the amount of free fatty acids and this can be attributed to the suppressive effect of phloridzin on lipase activity and/or the free fatty acids can be oxidized at position β to form acetyl-CoA which, in turn, may be transformed to the precursor of sterol, acetoacetyl-CoA [21]. As a result of the above explanation the percentage of free sterols must be increased in the produced lipids. The data (Table III) confirmed this view where the free sterols were increased gradually with the ageing of cultures reaching its maximal accumulation at the 8th day of replacement (the optimal incubation period

Table III

Quantitative estimation of different fractions of lipid produced by C. tenuissimum in the presence of 10^{-2}% phloridzin

Incubation period subsequent to replacement (days)	Lipid fraction (%)							
	Phospho-lipids	Free sterols	Free fatty acids	Glycerides		Methyl esters	Steryl esters	Squalene
				Di-	Tri-			
0	19.1	16.4	10.3	traces	35.9	3.0	10.9	4.4
2	22.5	19.7	13.1	1.3	35.9	1.0	3.6	2.9
4	21.3	23.1	12.3	1.8	36.4	1.0	2.1	2.0
6	19.0	24.2	12.0	2.5	36.0	1.3	3.0	2.0
8	19.0	26.4	11.5	2.2	35.7	1.2	2.1	1.9
10	19.2	25.1	10.3	2.2	35.7	2.7	3.0	1.8

for lipid accumulation). These results differ from that recorded by Shimi [5] who found that iodoacetate inhibits CoA during the biosynthesis of fat by *Penicillium spinulosum*.

With regard to the effect of phloridzin on the accumulation of organic acids in the metabolic solutions, the results obtained showed that tricarboxylic acid cycle as well as pentose cycle were operating in the metabolism of this organism. The detection of oxalacetic, succinic and gluconic acids in the reaction mixtures provides evidence for the operation of the two cycles. The presence of phloridzin in the final replacement solutions affects the patterns and the content of the organic acids. The amount of oxalacetic acid decreased after replacement with the ageing of the cultures till the 8th day of replacement and this can be interpreted on the basis of the fact that oxalacetic acid can be back converted to phosphoenolpyruvic acid by the action of phosphoenolpyruvate carboxykinase and give rise to acetyl-CoA leading to the increase of sterol content. Tartaric and gluconic acids reach their maximal accumulation at the 6th day of replacement and they may not be interfered with lipid biosynthesis.

The data accumulated throughout this work showed that phloridzin may influence the lipid metabolism. The addition of phloridzin to the fermentation media of *C. tenuissimum* directed the sugar breakdown towards lipid biosynthesis and/or catalyzed the transformation of the free fatty acid and oxalacetic acid to acetyl-CoA and finally to sterols.

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INTERACTION OF IMMUNOSUPPRESSIVE AGENTS AND HUMAN ADENOVIRUS INFECTION IN MICE

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It was examined whether sensitivity of mice to human adenovirus infections is affected by immunosuppressive agents. Mice treated with the lymphotropic cytostatic dianhydrodulcitol (DAD) or antilymphocytic serum (ALS) did not become susceptible to human adenovirus type 12 infection, as the virus did not replicate — not even in its component forms — in the animals. On the other hand, the effect of both DAD and ALS on the lymphoid organs of mice was intensified by human adenovirus infection. In tissue cultures the reproduction of adenovirus was facilitated by DAD.

Virus infections are known to possess immunomodulatory effects: on the one hand, they may stimulate immune responses, and on the other hand, because of disturbing the functions of leukocytes they may induce a decreased immune response to heterologous antigens [1–8]. Immunosuppression was first observed in the transitory cessation of tuberculin-positivity in measles virus infection. Later, the immunosuppressive effect of several other viruses was also detected [9–14]. Although adenoviruses are widespread in the population and have great affinity to the lymphoid system, their interactions and the pathomechanism of infection are still to be clarified. It is hardly known whether latent viruses could be activated and which are the factors promoting this [15].

As no experimental animal is known to be sensitive to human adenoviruses, we have studied in mice, an animal typically insensitive to infection, whether adenovirus was able to replicate in the form of its components or virus particles in animals treated with immunosuppressive agents like dianhydrodulcitol (DAD) or antilymphocytic serum (ALS). It was also tested whether the effect of the applied agents on the lymphoid organs was influenced by the virus infection and what effect DAD had on the replication of adenovirus in tissue culture.

Lymphoid organ weight. Relative weight of the spleen and thymus was determined as follows:

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

Student's two-sample t test was used for statistical evaluation; the accepted level of significance was $p = 0.05$.

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Materials and methods

Experimental animals. 300 CFLP, 6–8-week-old young adult mice of both sexes purchased from the Zootechnological Institute for Laboratory Animals (LATI, Gödöllő, Hungary) were used in the experiments. The interaction of adenovirus infection and DAD was rested in 100 mice; that of adenovirus and ALS in another 100 mice; 30 mice were infected with adenovirus; 25–25 animals were used in DAD and ALS treatment, and 20 mice served as uninfected and untreated controls.

Dianhydrodulcitol (DAD). DAD is a lymphotropic cytostatic agent of the alkylating group [16, 17]. The substance (Chinoin Chemical and Pharmaceutical Works Ltd., Budapest) was dissolved in distilled water and used within 30 min. Mice were treated intraperitoneally with DAD in a dose of 10–12 mg/kg body weight. For experiments with HEp-2 tissue culture, DAD was applied in a dose of 50 μg/ml.

Virus strain. Human adenovirus type 12 was propagated and titrated in HEp-2 tissue culture. Mice were infected intraperitoneally with 1500–3000 Cytopathogenic Units (CPU) of the virus.

Antilymphocytic serum (ALS). ALS was produced in rabbits against lymphocytes of mice as described earlier [18–20]. Leucoagglutinating titre of the serum was 1:256. Mice were treated intraperitoneally with 0.5 ml of the 1:1 dilution of the serum.

Tissue culture. For HEp-2 tissue culture Eagle solution containing 10% calf serum served as culture medium, and Parker-199 solution containing 3% rabbit serum as maintenance fluid. To both the culture medium and the maintenance fluid, 50 IU/ml penicillin and 51 μg/ml streptomycin were added.

Immunofluorescence. Cytological preparations of the lymphoid organs were examined according to the method of Coons and Kaplan [21]. Rabbit serum against type 12 adenovirus for direct examination was conjugated with fluorescein isothiocyanate; for indirect examinations, conjugated anti-rabbit IgG produced in goat was applied. Immunofluorescence examinations were performed with a Zeiss-Fluoval microscope at $\times 600$ magnification.

Complement fixation. Virus antigens were examined in the supernatant of lymphoid organs by complement fixation microtechnique [22].

Study of virus particles. For electron microscopic studies, parts of the lymphoid organs were prefixed, subsequently fixed in osmium tetroxide and embedded in Durcupan ACM. A JEOL JEM 100 B type electron microscope was used.

Examination of the lymphoid organs. Absolute lymphocyte count was determined from blood taken from the caudal vein under standardized conditions. After determination of the leukocyte count in Buerker's chamber, smears were stained with Giemsa and in knowledge of the WBC, the absolute lymphocyte count was determined.

Results

The following treatment with immunosuppressive agents and virus were carried out: DAD treatment + virus infection after 3 days (DAD_3V_1 group); virus infection + DAD treatment after 1 day (V_1DAD_3 group); ALS treatment + virus infection after 4 hour (ALS_4 hV group); ALS treatment + virus infection after 1 day (ALS_1V group). Mice treated only with DAD (DAD_3 group), ALS (ALS group), normal rabbit serum (NRS group), mice infected with adenovirus in two variations (V_1, V_4 group) and uninfected and untreated animals (C group) were used as controls.

In the abbreviations, the first number means the time until the second treatment, while the second number marks the time from the second treatment till sacrifice. With the controls, where only one treatment was applied (virus

infection, DAD or ALS treatment), the number marks the time from treatment to sacrifice. In some of the cases (50 animals), the animals were killed one, two or three weeks after virus infection or DAD treatment. The change of lymphocyte count was observed for two weeks in mice of groups ALS_4hV and ALS_1V. Optimal length of time and the amount of agents to be applied in treatment were determined in preliminary experiments. The applied dose of DAD caused no death.

Effect of DAD treatment and virus infection on lymphoid organs. Results are shown in Fig. 1. As compared to the untreated control group, relative thymus weight was more than 50%, relative spleen weight more than one-third, and peripheral lymphocyte count about 75% lower in DAD-treated animals (Group DAD_3). In mice infected with adenovirus, the peripheral lymphocyte count decreased after 4 h and became significant after 1 day (Group V_1). Lymphocyte count was higher on the 4th day after infection (Group V_4). Although relative thymus weight was lower on the 1st and 4th days, this change was not significant. Similarly, the higher relative spleen weight on the 1st and 4th days was not significant, either (Groups V_1 and V_4). In groups treated with DAD and infected with adenovirus (DAD_3V_1 and V_1DAD_3) relative thymus weight was significantly lower than in the untreated controls and the controls + treated with virus, and slightly lower than in the DAD-treated controls. The average relative spleen weight in groups DAD_3V_1 and V_1DAD_3 was lower than in the C and V_1, V_4 groups, but slightly higher than in the DAD-treated group. Absolute lymphocyte count was significantly lower than in the C and V groups and slightly lower than in the DAD group. Thus, adenovirus increased the effect of DAD causing thymus atrophy and lymphopenia.

In groups where the effect of DAD pretreatment and virus infection was observed through several weeks, the average relative thymus weight first decreased, then increased and reached its normal value after 2 weeks. Average relative spleen weight after an initial decrease increased significantly, reached its maximum in 10 days and was again normal after 2 weeks. Absolute lymphocyte count, after a 3–4 day gradual decrease, started to increase slowly and returned to normal after about three weeks (Fig. 2).

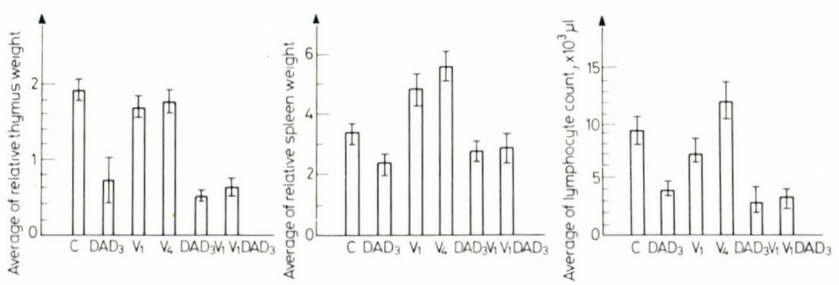

Fig. 1. Effect of DAD treatment and adenovirus infection on the lymphoid organs of mice

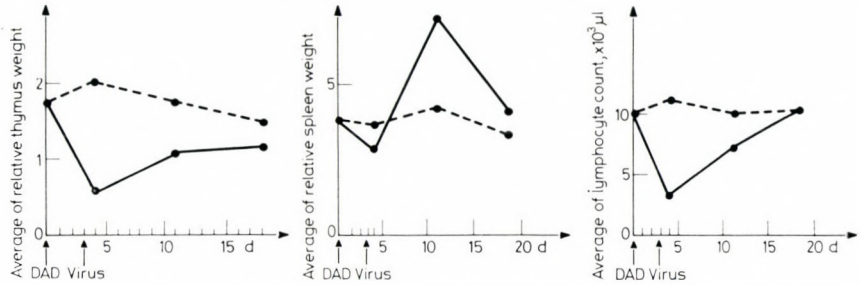

Fig. 2. Lymphoid organs of mice pretreated with DAD and infected with adenovirus. ● — — — ● untreated controls; ● ——— ● mice pretreated with DAD and infected with adenovirus

Effects of ALS treatment and adenovirus infection on the peripheral lymphocyte count. As a result of ALS treatment, a more than 50% decrease in absolute lymphocyte count was observed after 4 h. Thereafter a slow increase followed but the count did not reach its normal value even after 7 days. Also, it slightly decreased under the effect of control normal rabbit serum given in an amount and dilution similar as ALS. In the ALS_4hV and ALS_1V groups the absolute lymphocyte count decreased significantly when virus infection had been applied 4 h or one day after ALS treatment. Thus, as compared to the ALS control groups, virus infection increased the strong effect of ALS on lymphocytes. Adenovirus infection caused a decrease in lymphocyte count for 2–3 days, then it increased and reached the value of the ALS group after 7 days, but even after 15 days it failed to attain the normal value (Fig. 3).

Replication of Ad12 in lymphoid cells. From the lyphoid organs of mice infected with virus (V_1, V_4; DAD_3V_1; ALS_1V) we tried to detect the presence of virus antigens or virus particles. Immunofluorescence showed adenovirus antigens only sporadically in the cells. Neither could we reveal the presence of virus antigens with complement fixation from the supernatant of lymphoid organ homogenisates. No virus particles could be detected by electron microscopy, either.

Replication of Ad12 in HEP-2 cells treated with DAD. Applying DAD in different dilutions, it was established that the HEp-2 monolayer cell culture tolerated at most 5 μg/ml without damage. This dose of DAD was applied together with 50 CPU of adenovirus in the following combinations. DAD and the virus were incubated in test tube for 1 h, then they were put onto the tissue culture; or the tissue culture was treated with DAD, then after 1 h the virus was added; or the tissue culture was infected with virus then DAD was added after 1 h. In each experiment, DAD was applied by itself and also adenovirus infection by itself as controls, as well as untreated and uninfected tissue cultures. The amount of resulting virus antigens was determined by comple-

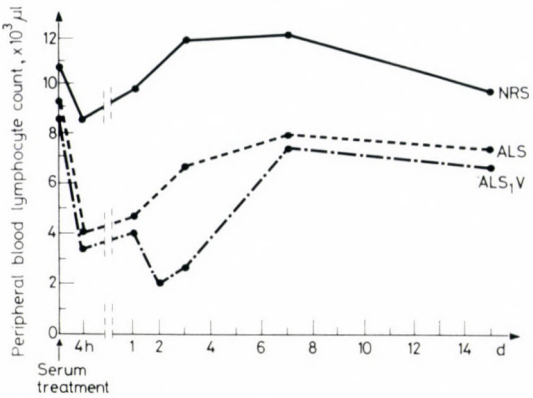

Fig. 3. Peripheral lymphocyte count in mice pretreated with ALS and infected with adenovirus

ment fixation, while infective viruses were determined by titration in tissue culture. During the experiments, the formation of both virus antigens and infective virus particles was increased with 1—2 log values by the presence of DAD in any combination.

Discussion

Viruses can be activated by immunosuppressive agents in latent virus carrying subjects [23–25]; this sometimes leads to severe consequences. According to many experiments, the thymus and T-lymphocytes play a significant role in adenovirus infections [26–31]. In our earlier experiments, when stimulating with phytohaemagglutinin the lymphoid system of mice non-susceptible to human adenovirus infections, the viruses or their antigens were able to replicate in the cells, mainly in T-lymphocytes, which fact refers to the interaction of cells and adenoviruses. In the present experiments we applied DAD and ALS as immunosuppressive agents, because both have a damaging effect on the T-system. It was assumed that these agents, due to their immune response decreasing effect, may increase the susceptibility to adenoviruses, or make the virus to replicate in otherwise non-susceptible animals. This supposition was not verified by our results, since all the treatments have failed to promote the replication of adenovirus. Virus replication was not affected by DAD treatment in vivo, but it was increased by the same agent in vitro, in tissue culture. It may therefore be assumed that due to the T-lymphocyte destroying effect of DAD, a reproduction of adenoviruses in T-cells of experimental animals is not possible. The mechanism may be the same in the case of ALS. It was, however, remarkable that adenovirus infection could increase the effect of DAD and ALS on the lymphoid organs.

Our experiments have provided data on the immunomechanism of adenovirus infection. On the basis of our earlier and present results, it is clear that adenovirus infections have a primary effect on the T-systems, and, depending on the circumstances, these cells can be mobilized or destroyed by the virus. On the other hand, if the lymphocytes are stimulated prior to virus infection, viruses will able to replicate in them [32].

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STUDIES ON ENTEROTOXIN OF *SHIGELLA DYSENTERIAE* TYPE 1 II. SYSTEMIC EFFECTS IN RABBITS OF *SHIGELLA DYSENTERIAE* 1 ENTEROTOXIN

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The enterotoxin of *Shigella dysenteriae* type 1 is an acid and heat-labile protein. It induces a gut dilatory response and increases the levels of blood glucose, serum alkaline phosphatase and serum acid phosphatase in rabbits.

Earlier we reported on the effect of aeration on the production of *S. dysenteriae* 1 enterotoxin and the suitability of animals for its biological assay [1].

Materials and methods

Organism. S. dysenteriae type 1, obtained from the Department of Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, was used to produce an enterotoxin as described earlier [1].

Animals. Apparently healthy albine white rabbits weighing 2 to 2.5 kg, were used for biological assay of enterotoxin. Rabbits were fasted for 24 h with an access to water before using them for experimental inoculation. The rabbit ileal loop method was used for assay of the toxin throughout the study [2].

Systemic effects of toxin. Three different doses were tested. Each rabbit received intravenously 1 ml of toxin with different protein concentration (200, 350 and 700 μg). The control rabbits received the same volume of phosphate buffer. Blood was drawn from each of test and control rabbits at intervals of 4, 8, 12, 36 and 48 h. The serum was collected for estimation of glucose [3]; serum alkaline phosphatase [4] and serum acid phosphatase [5].

Results

Sera collected from rabbits, after administration of toxin parenterally were used for determination of blood glucose (BG); serum alkaline phosphatase (SAP) and serum acid phosphatase (SACP).

Three doses, namely 200 and 350 and 700 μg of toxin were parenterally administered. The rabbits receiving a dose of 700 μg failed to survive more

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than 3 h, whereas other doses were tolerated up to 8 and 48 h, respectively. The BG level increased to the maximum in 12 h (185 mg/ml) with 200 μg and declined gradually thereafter reaching the normal values in 48 h (Fig. 1). Similarly, the BG level also increased with a dose of 350 μg up to 8 h and the rabbits died thereafter.

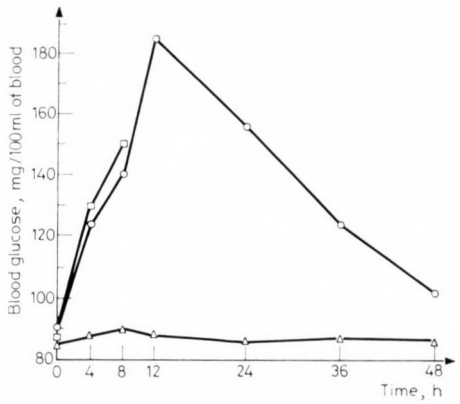

Fig. 1. Effect of parenteral administration of *S. dysenteriae* 1 enterotoxin on blood glucose level in rabbit; □——□ test dose (350 μg), ○——○ test dose (200 μg), △——△ control

The level of SAP increased with a dose of 350 and 200 μg (Fig. 2). With the former, an increase after 4 h was noticed which continued up to the 8th h, whereas with the later there was a parallel increase up to the 4th h which continued up to the 12th h and decreased thereafter gradually reaching the normal values at 48 h.

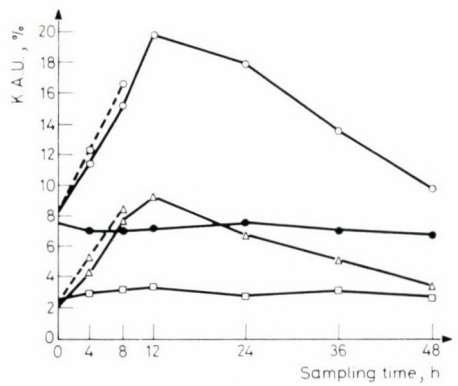

Fig. 2. Effect of parenteral administration of partially purified toxin of *S. dysenteriae* 1 on serum alkaline phosphatase and acid phosphatase in rabbit: ○- - -○ test alkaline phosphatase (350 μg/ml), ○——○ test alkaline phosphatase (200 μg/ml), ●——● control alkaline phosphatase; △- - -△ test acid phosphatase (350 μg), △——△ test acid phosphatase (200 μg), □——□ control acid phosphatase

SACP did not vary much with a dose of 350 μg and 200 μg of toxin as evidenced by an increased level with both doses (Fig. 2). With 200 μg dose the level increased after 4 h of administration, and continued so up to the 12th h and had reached normal level by the 48th h.

Discussion

There are no reports regarding the systemic effects of *S. dysenteriae* 1 toxin. When given parenterally, it was observed that a dose of 700–350 μg proved lethal. The steep rise and gradual decline in BG were suggestive of a rapid glycogenolysis in the liver and a subsequent fall after the exhaustion of the stored glycogen. However, the association of the death of animals either with a higher dosage or neurotoxicity could not be ruled out. It has been reported that *Vibrio cholerae* enterotoxin enhances glycogenolysis in disrupted liver cells in vitro resulting in a rapid loss of glycogen stored in vivo [6]. Others [7] reported similar observations on a prolonged hyperglycaemic state in dogs receiving 100 μg of cholera toxin parenterally. In an other study, a rapid rise of BG with the enterotoxin of *Escherichia coli* and *V. cholerae*, given parenterally, suggested a rapid glycogenolysis in the liver and the levels tended to show a marked decrease within 48 h due to rapid depletion of liver glycogen [8]. In an other study, the enterotoxin of *Klebsiella pneumoniae*, evoked the highest glucose level in 24 h, which declined thereafter and reached the normal level in 48 h [9]. The present observations on the BG level were not different from the above findings but these were significant at 200 μg doses.

An increased level of plasma alkaline phosphatase in dogs, as an effect of *V. cholerae* enterotoxin was also reported [7]. Similar were the observations in respect of SACP and SAP, using *K. pneumoniae* enterotoxin in rabbits administered parenterally [7]. This increase was perhaps due to a hormone like action of enterotoxin with further implication of cyclic AMP and adenyl cyclase raising SAP and SACP, as reported for cholera enterotoxin [10]. Similarly, it has been suggested that this enzyme may have its origin in the liver [11, 12] whereas others reported activation of adenyl cyclase in the intestinal mucosa with the enterotoxin of *S. dysenteriae* 1 [13]. Since SAP and SACP levels increased in the present experiments, the possibility of the toxin increasing the adenyl cyclase, thereby cyclic AMP activity, cannot be ruled out. The toxin, perhaps, caused a damage to the liver, kidney and spleen which would appear from the constantly increased level of these.

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IMMUNOFLUORESCENCE CHARACTERIZATION OF *LEGIONELLA:* NARROW SPECIFICITY OF POLYCLONAL IMMUNSERA TO VARIOUS SEROGROUPS AND SPECIES

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This study was to determine by direct fluorescent antibody staining with antibodies prepared in rabbits, the degree of cross-reactions between serogroups of *Legionella pneumophila* (1 to 6) and the other antigenic species of *Legionella (L. bozemanii, L. dumoffii,* and *L. micdadei)*, in order to increase the accuracy of diagnosis and to allow us to reduce the number of conjugates. The polyclonal antibodies were highly species and serogroup-specific without absorption for characterization of *Legionella* either in patient specimens or in isolated cultures by direct fluorescent antibody staining. No cross-reaction was observed with non-legionella bacteria isolated from sputum specimens. A battery of conjugates for different serogroups and species is necessary for increasing the accuracy of diagnosis of legionellosis.

The only standard procedure for rapid diagnosis of legionellosis is direct fluorescent antibody staining (D.F.A.) on adequate clinical samples [1, 2]. The D.F.A. is used to identify the species and serogroups of *Legionella* strains either in tissue or respiratory secretions of patients with legionellosis or in isolated cultures [3]. The number of known *Legionella* species and serogroups has continued to increase [4]. For example, two new serogroups of *Legionella pneumophila* that do not react with currently used D.F.A. conjugates have been recently isolated [5, 6]. Preliminary evidence, based on the finding that seroconversions among paired sera from patients with suspected Legionnaires disease against indirect immunofluorescence assay antigens of the new species occurred, suggests that, like *L. pneumophila*, these new species may cause a significant number of infections annually [7]. The isolation of the seventh serogroup of *L. pneumophila* from the environment and, subsequently,

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the establishment of its role in human infections further illustrates the need to include conjugates prepared against environmental isolates of *L. pneumophila* when screening clinical specimens submitted for direct fluorescent antibody testing of *Legionella* spp [5]. This finding suggests that pneumonia caused by the other *Legionella* species may be more common than previously recognized and that some suspected cases of Legionnaires disease in which seroconversion was absent may have been due to these organisms. Indeed, serodiagnosis is retrospective and as many as 10 to 20% of patients with Legionnaires disease fail to seroconvert by indirect fluorescent antibody testing (I.F.A.) [2, 8]. Moreover, the I.F.A. has been standardized only for disease due to *L. pneumophila* serogroup 1 [9]. Furthermore the value of a single high titre even in the *L. pneumophila* test is questionable in populations with high background antibody levels [10]. Like the Centers for Disease Control (CDC), Atlanta, in a previous paper, we choose to consider a test result positive only if paired sera demonstrated a fourfold increase in reciprocal titre to a value $\geq$128 [11]. To obtain a rapid diagnosis of Legionnaires disease by D.F.A. and to increase the accuracy of the test results reported in this paper, we have prepared rabbit antibodies directed against *L. pneumophila* serogroups 1 to 6 and *L. bozemanii* *L. dumoffii* and *L. micdadei* and tested it to determine whether these same antibodies are effective against antigenic determinants common to several or all of the *Legionella* species and serogroups as well as other non-legionella bacteria.

Materials and methods

Bacterial strains. The *Legionella* species, serogroups and strains designation of each antigen used for rabbit immunization and for specificity determination are listed in Table I. The bacteria were grown on buffered charcoal yeast extract (C. Y. E.) agar for 3 days as described previously [12].

Immunization. Rabbit antisera were prepared against *L. pneumophila* serogroups 1 to 6 and *L. bozemanii*, *L. dumoffii*, and *L. micdadei* as described elsewhere [12]. Briefly, heavy cell suspensions (approximately 2×10^9 colony forming units/ml) were prepared for the growth on C. Y. E. agar and killed with 2% formalin in phosphate buffered saline (PBS), pH 7.2. Immunization was performed as described in reference [12]. Indirect immunofluorescence assay titres $\geq$2000 were obtained in the 4th week of immunization with fluorescein labelled goat IgG anti-rabbit IgG.

Preparation of fluorescent antibody conjugates for D. F. A. tests. The globulin fraction of rabbit antiserum was precipitated at 47% saturation with $(NH_4)_2SO_4$. The antibodies at 20 mg/ml concentration were conjugated to fluorescein isothiocyanate (Isomer I Biomerieux) in the ratio 30 to 1 as described in reference [13]. The unfixed fluorescein was eliminated by filtration on Sephadex G 50. The conjugates displayed a F/P molar ratio of 7.9 to 10.3 as shown in Table I.

Direct immunofluorescence assay. Homologous and heterologous titrations of all conjugates were performed by D. F. A. tests with formalinized cell suspensions of relevant cultures in PBS. Air-dried bacterial smears were fixed by dipping in acetone, at room temperature for 10 min. After drying, antibodies were added and incubated in a moist chamber for half an hour at room temperature. The slides were then washed in two baths of PBS for 5 min and dried by air. After mounting with buffered glycerol (pH 9), the slides were examined with a Leitz microscope equipped with a fluorescence vertical illuminator. Titres were expressed as the lowest protein concentration giving a bright fluorescence intensity of homologous antigen.

Results

In the first phase of the study for the D.F.A. sensibility, all conjugates were tested against the homologous antigen listed in Table I. Twofold dilutions of antisera were made in PBS, from 500 μg/ml of proteins to 31.25 μg/ml. The results of testing formalinized antigens of each *Legionella* serogroup and species with homologous D.F.A. conjugates are listed in Table II. The working dilution was defined as the lowest weight of protein which gave a bright fluorescence of homologous antigen. The majority of conjugates gave a very bright staining of homologous antigen up to a concentration of 125 μg/ml of proteins.

Table I

Bacterial strains used for immunization and determination of the specificity of antibodies

Species	Serogroup	Strain	F/P molar ratio of conjugates
L. pneumophila	1	Philadelphia 1	7.9
L. pneumophila	2	Togus 1	8.2
L. pneumophila	3	Bloomington 2	9.2
L. pneumophila	4	Los Angeles 1	9.2
L. pneumophila	5	Dallas 1E	8.8
L. pneumophila	6	Chicago 2	9.1
L. bozemanii	1	Wiga	8
L. dumoffii	1	NY-23	8.7
L. micdadei	1	Tatlock	10.3

Table II

Titration of conjugates on homologous antigen

Homologous antigen of *Legionella*		Staining intensity at various antibody concentrations (μg/ml) of each conjugate				
Species	Serogroup	500	250	125	62.5	31.25
L. pneumophila	SG_1	+	+	+	—	—
	SG_2	+	+	—	—	—
	SG_3	+	+	+	—	—
	SG_4	+	+	+	—	—
	SG_5	+	+	—	—	—
	SG_6	+	—	—	—	—
L. bozemanii		+	+	+	+	—
L. dumoffii		+	+	±	—	—
L. micdadei		+	+	+	±	—

\+ Positive reaction
— Negative reaction
± Suspicious reaction

Table III

*D. F. A. specificity of Legionella antisera**

Legionella antigen		Reaction of the following *Legionella* antisera								
Species	Serogroup	*L. pneumophila* serogroup						*L. bozemanii*	*L. dumoffii*	*L. micdadei*
		1	2	3	4	5	6			
L. pneumophila	SG_1	+	—	—	—	—	—	—	—	—
	SG_2	—	+	—	—	—	—	—	—	—
	SG_3	—	—	+	—	—	—	—	—	—
	SG_4	—	—	—	+	—	—	—	—	—
	SG_5	—	—	—	—	+	—	—	—	—
	SG_6	—	—	±	—	—	+	—	—	—
L. bozemanii		—	—	—	—	—	—	+	—	—
L. dumoffii		—	—	—	—	—	—	—	+	—
L. micdadei		—	—	—	—	—	—	—	—	+

* Each conjugate was studied on heterologous antigen at its direct immunofluorescence assay concentration

In a second phase of the study for the D.F.A. specificity, the same conjugates were tested at their direct immunofluorescence assay working concentration against the heterologous antigens. The results, listed in Table III, indicate that *L. pneumophila* serogroups 1 to 6 and other species of *Legionella* can be easily distinguished with these reagents. At optimal dilution of antisera, all tests were species-specific, and for *L. pneumophila*, were also serogroup-specific without absorption, as shown in the absence of staining with heterologous antigens. The minor cross-reaction between serogroup 3 conjugate and serogroup 6 cell suspension has not, in our experience, been a problem in distinguishing these two serogroups.

Serogroup-specific reactions may arbitrarily be defined as showing at least fourfold greater titres against antigens of one homologous serogroup than against heterologous serogroups. However, when high species-specific or serogroup-specific antibodies at a titre fourfold dilution higher than the serum titre were used against heterologous antigen, no cross-reactivity appeared. With the exception of the minor cross-reactivity between serogroups 3 and 6 already noted, the D.F.A. test is group-specific.

Since screening with six or more reagents would be tedious, we experimented with the preparation of polyvalent reagents. Conjugates to *L. pneumophila* serogroups 1, 2 and 4 were combined in pool A, serogroups 3, 5 and 6 in pool B and *L. bozemanii*, *L. dumoffii*, and *L. micdadei* in pool C; each at a thirtyfold greater titre than the working concentration. For screening, each pool was extemporaneously diluted 1 : 10, i.e. the final dilution that had given optimal bright staining before pooling. In the first phase of the study, the smears were tested against the three polyvalent sera listed in Table IV: those

Table IV

Reactivity of L. pneumophila (serogroup 1 to 6) and L. bozemanii, L. dumoffii and L. micdadei with pooled conjugated reagents

Samples (*Legionella* smears)		Pools of anti-legionella conjugates		
		A	B	C
L. pneumophila	SG_1	+	—	—
	SG_2	+	—	—
	SG_3	—	+	—
	SG_4	+	—	—
	SG_5	—	+	—
	SG_6	—	+	—
L. bozemanii		—	—	+
L. dumoffii		—	—	+
L. micdadei		—	—	+

Pool A: conjugate to *L. pneumophila* serogroups 1, 2, and 4
Pool B: conjugate to *L. pneumophila* serogroups 3, 5 and 6
Pool C: conjugate to *L. bozemanii*, *L. dumoffii*, and *L. micdadei*

with positive conjugate in the initial screening were tested against each monovalent. Strong homologous staining were found to occur with each polyvalent pooled reagent.

These pools were then used to test 118 clinical samples (transtracheal aspirates, bronchoscopy specimens, pleural fluids). Culture on selective media and I.F.A. for antibody titres were also made. One case of pneumonia caused by *L. pneumophila* serogroup 5 was identified on the basis of D.F.A and culture positive specimen as well as seroconversion. Two other cases of pneumonia caused by *L. pneumophila* serogroup 1 and 6, respectively were identified only on the basis of a seroconversion. D.F.A. and culture were negative but erythromycin therapy was given before respiratory tract specimens were collected. No cross-reaction was noted with other bacterial isolates from sputum specimens.

Discussion

Some clinical laboratories "routinely" use the D.F.A. for identifying the species and serogroups of *Legionella* strains either in patient specimens or in isolated cultures [3]. However, it is the detection of antibody to *L. pneumophila* with the I.F.A. assay that has become the most common mean of establishing a diagnosis of Legionnaires disease despite the fact that results are only available retrospectively. Furthermore, the value of a single high titre in the *L. pneumophila* test may indicate past exposure to *Legionella* instead of recent infection because detectable titres can persist for months or years after clinical

illness has resolved [10]. In France, the I.F.A. and D.F.A. tests have been well studied only for the *L. pneumophila* serogroup 1 antigen. A previous work in the United States has shown that 27.6% of the samples of patients with suspected Legionnaires disease had antibodies to one or more I.F.A. assay antigens of *L. pneumophila*, *L. bozemanii*, *L. dumoffii* and *L. micdadei* when seroconversion to a reciprocal titre $\geq$128 or a single titre $\geq$256 was considered positive [7]. The fact that fewer than half of these had significant titres of antibody to *L. pneumophila* suggested widespread infection with, or exposure to, *Legionella* species other than *L. pneumophila* [9, 10]. Moreover, approximately 50% of pairs of sera from suspected or culture proven cases of Legionnaires disease show in I.F.A. either a titre rise against only one serogroup or a substantially greater titre rise against a single serogroup than against the others, comparable rises in titre to two or more serogroups can be seen in reference [9]. Preliminary studies by crossed immunoelectrophoresis have shown that strains representating the six first serogroups of *L. pneumophila* shared more than 85% of their antigens in common with *L. pneumophila* serogroup 1 [14]. In contrast, *L. bozemanii*, *L. dumoffii* and *L. micdadei* shared only 45, 53 and 43%, respectively, of their antigens in common with *L. pneumophila* serogroup 1 [14]. Other bacterial species like *Pseudomonas fluorescens*, *Pseudomonas alcaligenes* and *Bacteroides fragilis* had also an antigenic relationship to serogroup 1 of *L. pneumophila* by D.F.A. and immunodiffusion tests [15, 16]. As the number of antigenic types of *Legionellaceae* continues to grow, the need for identification of these antigens becomes increasingly important for the development of a rapid and accurate diagnostic test to screen patients for legionellosis.

Our results show that most of rabbit antisera are highly species and serogroup specific without absorption. Only a minor cross-reaction existed between *L. pneumophila* serogroup 3 and 6. Cross-reaction between these two serogroups had been noted previously [17]. The common antigens between the family *Legionellaceae* and non-legionella bacteria noted previously by immunoelectrophoretic methods [14] and immunodiffusion tests [16] are probably cytoplasmic antigens different from surface antigens detected by D.F.A. As such it may or may not present problems in identification of *Legionella* depending on the technique of characterization used. The technique of D.F.A. staining, which has the property of characterizing the surface antigens, does not show any cross-reactions between different serogroups of *Legionella* whereas the immunodiffusion technique which characterizes both surface and internal antigens, shows many such reactions. Hence, the probability of obtaining for D.F.A. staining a monoclonal antibody reacting with all serogroups and species of *Legionella* should be maximized for detecting an internal antigen but not necessarily a surface antigen.

The fact that immunization with one serogroup produces antibodies not giving cross-reactions with other serogroups by D.F.A. staining, leads us to

think that the use of monoclonal antibody for characterization of *Legionella* is not necessary. Many authors [18] have prepared monoclonal antibody in order to find an antibody directed against a common antigen to the species, one which would not cross-react with other bacteria. Their results confirm that, similarly to rabbits, mice respond to a major, or at least highly immunogenic, antigen determining the serospecificity of the bacterial strain. Moreover, the fact that the binding of their monoclonal antibody was practically totally inhibited by the polyclonal rabbit antiserum shows that both kinds of antibody react with the same antigen [18]. Other authors [19–21], too, were unable to produce monoclonal antibodies that reacted with all of the serogroups of *L. pneumophila*. On the other hand, three subtypes of *L. pneumophila* serogroup 1 have been defined by monoclonal antibody binding pattern [22].

Finally, our data suggest that a battery of polyvalent and/or monovalent conjugates for different serogroups and species is necessary for increasing the accuracy of diagnosis of legionellosis.

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SEROLOGICAL STUDIES IN *CHLAMYDIA TRACHOMATIS* ASSOCIATED PNEUMONIA OF INFANTS

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Serological results provided evidence for the clinical diagnosis of chlamydial pneumonia in 8 out of 51 cases. Elevated chlamydia-specific IgM titres (128–2048) and IgG titres (128–2048) were recorded in 5 and 8 infants, respectively. High titres of chlamydial antibodies (512–8192) in the sera of mothers of seropositive children proved genital *Chlamydia trachomatis* infection.

Chlamydia trachomatis harboured in the maternal genital tract can be transmitted from mother to infant during parturition [1]. Infection of the neonate may lead in the first life-days to conjunctivitis and/or after a few weeks to atypical pneumonia with or without conjunctivitis. Serology is a most reliable approach to verification of *C. trachomatis* as the causative agent in lower respiratory tract infection in infants between 1–6 months of age.

Materials and methods

In 1983–1984 within 10 months 1 to 3 serum samples of 51 young infants (2–16 weeks of age) with atypical pneumonia were tested for *C. trachomatis* antibodies. Detection of chlamydial antibodies was also carried out in 1 to 2 serum samples of mothers whose children had a positive serological results. Specimens arrived from several hospitals.

Ten different immunotypes of formalinized antigens produced in egg-yolk sacs, rich in elementary bodies, were used for microimmunofluorescent assay (MIF), developed by Wang and Grayston [2]. The strains of the test antigen types were as follows: A: Tunis 326, B TW-5, pooled D: IC-CAL 8, E: Bour, pooled F: IC-CAL 3, G: UW-51, H: UW-43, I:IC-CAL 6, K: UW-31. FITC conjugated antihuman Ig, IgG and IgM were Hyland products. Slides were tested under a Zeiss Laboval fluorescence microscope with a HBO 50 ultra pressure mercury lamp.

Sera positive in an 8-fold basic dilution were diluted further for the determination of IgG and IgM titres.

Nose and throat swabs and blood culture were tested as in routine. Mycoplasma growth inhibition and RS-, adeno-, herpes simplex and influenza virus complement fixing antibody tests for patients with positive chlamydial serology were carried out to exclude any other infecting agent of the lower respiratory tract.

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Results

Routine nose and throat swabs, blood culture, mycoplasma growth inhibition and virus complement fixation tests were in each case negative. Results of chlamydial MIF tests are summarized in Table I. Antibody titres of 8 infants out of 51 were as high as 128–2048. IgM was found in sera of 5 children. Two of those without IgM class antibody were sampled on the 30th and 42nd day of illness, respectively, when no detectable IgM is expected any more.

IgG titres were found considerably high in the first serum specimen of each patient, taken in the acute phase of the disease, and no appreciable increase was observed later. No IgM was demonstrated in the mothers' sera but once in a titre of 16. IgG titres were remarkably high (512–8192). Of the mothers whose paired sera were examined, two exhibited a 2-fold and one a 4-fold increase of antibody titre.

The reacting antigen-types were D, E+D and I+H. The antigen-type of mother and child was always identical.

Table I

MIF antibody titres in patients with chlamydial pneumonia syndrome

Patient	Time of test (since onset of illness, day)	Antibody titres of infants		Antibody titres of mothers		Antigen-type
		IgM	IgG	IgM	IgG	
D. G.	5	512	256			I + H
	17	256	512	16	512	I + H
J. F.	11	512	1024	<8	2048	D
	30	512	1024	<8	8192	D
	54	<8	64			D
Cs. A.*	16	128	128			D
	44	<8	128			D
				<8	2048	D
Cs. Zs.*	16	512	512			
	44	<8	512			D
O. C.	3	<8	1024	<8	512	D
	27	<8	2048	<8	1024	D
	159	<8	<8			
E. T.	7	2048	2048	not tested		I + H
	21	512	1024			I + H
	61	64	64			I + H
Gy. Zs.	42	<8	2048	<8	2048	I + H
	60	<8	1024	<8	4096	I + H
	80	<8	128			I + H
S. K.	30	<8	2048	not tested		E + D

* Twins

Discussion

The trachoma-inclusion conjunctivitis (TRIC) agents, the lymphogranuloma venereum (LGV) group of organisms and the mouse pneumonitis agent are the three biovariants of *C. trachomatis*. Serotypes A, B, Ba and C of TRIC agents cause primarily ocular disease: endemic trachoma in the tropics and inclusion conjunctivitis elsewhere; D-K serotypes are responsible for inclusion conjunctivitis, urogenital infections and atypical pneumonia of infants all over the world. Serotypes L_1, L_2 and L_3 of the LGV group are involved in the pathogenesis of lymphogranuloma venereum [3].

C. trachomatis is the causative agent of cervicitis and salpingitis in 12–37% and 30–80%, respectively, and is very often associated with infertility [4]. Cervical swabs of asymptomatic women yield *C. trachomatis* in 1–6% [5], the incidence of carriers in pregnancy is between 4–18% [6, 7]. Neonatal infection occurs in 2–6% in the United States [8], but serological evidence for acquisition of *C. trachomatis* by infants of infected mothers was provided in 70% [6]; 35% developed inclusion conjunctivitis, 20% pneumonia in this group [6]. *C. trachomatis* is responsible for lower respiratory tract infection in young infants in 27%, and for afebrile pneumonia in 74% [8].

In our studies 8 patients among 51 were positive for chlamydial antibodies. This high sequency of positivity (about 1/6 of the cases) is explained by the characteristic features of chlamydial pneumonia. Infants between 2–7 weeks of age developed symptoms of gradually worsening pertussis-like cough, tachypnoe, labial cyanosis, sometimes with conjunctivitis, but in most cases they were afebrile and in good general condition. Crepitation rales were heard and there was a radiological evidence for extended interstitial infiltration in all cases. Elevated ESR (40–80 mm/h), and WBC (11 000–22 000/μl), a mild eosinophilia (3–14%), monocytosis (7–14%) and anaemia (76.8–108.8 g/l) were the characteristic laboratory findings.

Microbiological diagnosis of chlamydial pneumonia is based on the detection of specific antibodies in blood, but serology may be completed with isolation of *C. trachomatis* from nasopharyngeal and conjunctival secretion and from rectal scraping. Our investigations were limited to serology.

Indirect MIF or ELISA are the methods of choice in chlamydial serology, being more sensitive than the complement fixation test, and suitable for identifying type-specific antibodies [9]. Chlamydial pneumonia always results in a remarkable immune response inducing usually a titre as high as 512–64 000 [10].

Serological criteria of the diagnosis are as follows: detectable IgM class antibody and/or registered 4-fold increase in IgG titre. In most cases IgM can be revealed only for about 30 days in the acute phase of the disease. In our material, however, the 61st day was the latest for detection of IgM, but in another case it could not be demonstrated already on the 44th day. IgM represents

an early response to infection, IgG in contrast may also be of maternal origin. The fact that IgM is a valuable indicator of acquired infection, underlines the importance of early sampling. In our studies no appreciable rise of IgG titres was registered, but IgM provided evidence for chlamydial pneumonia in 5 out of 8 cases. In those cases where IgM was not demonstrated, partly because of delayed sampling, characteristic symptoms and the serological proof of the mother's infection confirmed the clinical diagnosis.

Topical infections like conjunctivitis, urethritis, cervicitis, salpingitis induce a rather weak immune response, if any: a titre of 32–128 may be an indicator of an active chlamydial disease, but may also derive from a previous infection [9]. IgM can be detected rarely in local infections.

In our material all mothers but one failed to present IgM antibody, but IgG concentrations of the maternal sera were extremely high (512–8192) compared to the data published in literature. The question arises whether pregnancy, delivery or postpartum period had activated infection, that provoked a strong antibody response. These high titres, as well as the increasing titres of the paired sera in accordance with the anamnestic or present symptoms (fluor, pelvic pain) were of diagnostic value for a recently overcome or persistent genital chlamydial infection.

Since chlamydial species-specific antigens give a strong cross-reaction in the MIF, type-specific antibody can be revealed only at the end of the dilution line, but even there a reaction with more than one serotype may sometimes be observed. That is the reason why two serotypes (I+H or E+D) were responsible for the serological positivity in 4 cases.

Positive chlamydial serology in atypical pneumonia of infants is helpful in the choice of antibiotics. Cephalosporins or ampicillin introduced at admission had only transient effect, if any. A change for erythromycin led to a rapid clinical and somewhat slower radiological recovery.

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IMMUNOLOGICAL STUDY OF GLYCOLIPOPROTEIN ISOLATED FROM EXTRACELLULAR SLIME OF DIFFERENT *PSEUDOMONAS AERUGINOSA* SEROGROUPS

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Glycolipoprotein (GLP) was isolated by water–phenol extraction and ultracentrifugation from crude extracellular slime (C-ES) of 14 strains of *Pseudomonas aeruginosa*. The yields of GLP obtained between different methods were $65.6 \pm 8.4\%$ and $46.4 \pm 11.2\%$ of the dry weight of all C-ES fractions. GLP stimulated active immunity in mice against challenge with different immunotypes (or serogroups) of *P. aeruginosa*. Phenol fractions (PFr) of different *P. aeruginosa* strains behaved similarly. As compared to the corresponding C-ES in active mouse protection test, the GLP of several strains was less active, whereas that of some other strains was more protective.

In our previous paper [1] we have shown that isolated and partially purified crude extracellular slime (C-ES), induces immunity in mice against challenge with the homologous or heterologous serogroup (immunotype) of *P. aeruginosa*. Presumably, slime glycolipoprotein (GLP) or its high-molecular polysaccharide is the active component of C-ES [2–5]. In the present paper we report on the results of an immunological study of GLP and other fractions isolated from *P. aeruginosa* C-ES.

Materials and methods

Bacterial strains. Pseudomonas aeruginosa strains 170001, 170002, 170005, 170006, 170007, 170009, 170010, 170014, 170017, 170018, 170019, 170021, 170022, 170023 and Fisher's immunotypes F1–F7, were obtained from the National Institute of Hygiene, Budapest, Hungary. Strain No. 8 was isolated from a burn patient in Vishnevsky Institute for Surgery of the USSR Academy of Medical Sciences (Moscow); strain O-11 was obtained from the collection of the Tarasevich State Control Institute (Moscow, USSR); and exotoxin-producing PA-103 strain was obtained from the Department of Microbiology and Immunology, University of Louisville School of Medicine (Louisville, USA). The strains used in our investigation are described in Table I.

Crude extracellular slime (C-ES). The preparations were obtained by the method previously described [1].

Glycolipoprotein (GLP). Purified GLP was obtained from C-ES of different strains by two methods (Fig. 1).

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Table I

O-antigenic structure of P. aeruginosa strains used in this study

Strain	Compiled scheme*	Immuno-type**
170001	3a,3b,3c	—
170002	10a,10c	5
170005	2a,2d	7
170006	2a,2d,2e	—
170007	(2a),2d,2f	—
170009	6a,6c	—
170010	6a,6d	—
170014	1	4
170017	—	—
170018	—	—
170019	9a,9d	—
170021	—	—
170022	15	—
170023	12	—
O-11	11a,11b	2
8	2a,2d,2e	—
PA-103	11a,11b	2
F1	6a	1
F2	11a,11b	2
F3	(2a),2c	3
F4	1	4
F5	10a,10c	5
F6	7a,7d	6
F7	2a,2d	7

* Lányi–Bergan scheme [12] supplemented [13]
** Fisher et al. [14]

I. Aqueous C-ES solution was treated with cold 75% aqueous phenol solution; GLP was isolated [4, 6] and then centrifuged (10 000 *g*, 1 h) to remove undissolved components. At the same time, phenol fraction (PFr), in which proteins (or peptides) are supposed to concentrate, was collected [6]. GLP solution was centrifuged at 105 000 *g* for 3 h, and the resulting GLP precipitate (105–180) and supernatant (105–180) were examined.

II. At first C-ES solution was centrifuged under the same conditions, the resulting C-ES precipitate (105–180) was discarded, and GLP was obtained from the C-ES supernatant (105–180) by water–phenol extraction as described above.

Lipopolysaccharides (LPS) were obtained from the N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the USSR (Moscow). They were prepared and purified as described earlier [7].

Active mouse protection. Outbred mice weighing 18 to 20 g were immunized intraperitoneally with the preparations and, 7 days later, were challenged with *P. aeruginosa* live cultures as described earlier [1]. Two modifications of the test were used. (1) Titration of the challenge dose on immunized (test) and non-immunized (control) mice. LD_{50} and efficiency index (EI) were calculated (EI = proportion of LD_{50} for the test animals to LD_{50} for the controls; see results). (2) Titration of the immunizing dose of the antigen and determination of ED_{50} (see results). Details of this method are also given in our previous paper [1].

Chemical analysis. Protein in the preparations was determined by means of the Folin reagent [8], and carbohydrates were determined by means of the anthrone reagent [9].

Statistical analysis. The LD_{50} and ED_{50} values and fiducial limits were determined by the method of Van der Varden [10].

Results

Figure 1 gives a schematic description of the two methods of C-ES fraction. The essential difference between the methods was that using method I, C-ES was treated by phenol before ultracentrifugation, and using method II, it was treated after ultracentrifugation as described by Sensakovic et al. [4].

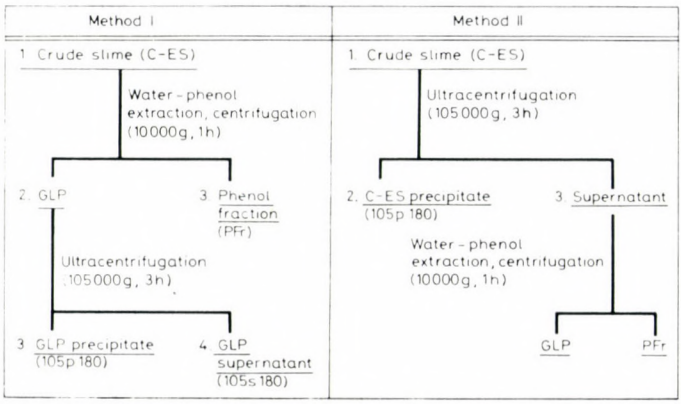

Fig. 1. Scheme of glycolipoprotein (GLP) isolation. To remove insoluble components, GLP solution was centrifuged beforehand at 10 000 *g* for 1 h

Table II

Yields of fractions in per cent of the total weight of C-ES fractions

Designation of strain	Yield in per cent				
	Method I		Method II		
	GLP-I	PFr-I	GLP-II	C-ES residue	PFr-II
170001	61.8	26.6	NS	NS	NS
170002	NS	NS	37.9	56.9	5.2
170006	88	9.3	62.5	24.5	12.1
170007	63	27	41.3	50.2	8.5
170009	63.8	28	33.9	44.8	21.3
170010	36.9	41.5	NS		
170017	NS		49.7	28.6	21.7
170018	60	32.9	NS		
170014	77.3	16.7	NS		
170019	75.9	15	52.8	24.7	22.5
170023	61.7	13.4	NS		
O-11	67.4	24.8	NS		
X ± 2 m	65.6 ± 8.4	23.5 ± 6.2	46.4 ± 11.2	38.3 ± 11.6	15.2 ± 6.4

Note. Fraction yields from strains 170005 and 170021 were not calculated. According to Method I, GLP-I solution was additionally centrifuged to remove insoluble conglomerates (10 000 *g*, 1 h) and the residue was removed. The mean specific yield was 11.1 ± 4.4% (strain variations 2.7 to 24.8%)

NS = not studied

Table III

Active cross protection of mice immunized with GLP

Strain	$LD_{50} \times 10^6$ bacterial cells and confidence limits			
	F1	F2	F3	F4
170001	347 (275–437)	692 (479–1000)	>100	398 (316–501)
170002	214 (162–281)	132 (96–182)	87 (66–115)	186 (141–246)
170005	263 (220–346)	372 (695–468)	81 (68–98)	151 (120–191)
170006	302 (219–417)	229 (166–376)	200 (145–275)	263 (191–363)
170007	115 (87–151)	93 (74–118)	151 (110–209)	132 (100–174)
170009	347 (240–501)	398 (288–550)	NS	NS
170010	562 (227–741)	1202 (891–1660)	62 (45–85)	214 (162–282)
170014	302 (240–380)	263 (191–363)	50 (38–66)	*692* (501–955)
170017	324 (224–468)	141 (102–195)	NS	214 (155–295)
170018	162 (112–234)	525 (363–759)	72 (58–91)	181 (100–174)
170019	246 (179–339)	246 (186–324)	>100	NS
170021	324 (257–407)	562 (407–776)	66 (50–87)	246 (186–324)
170022	NS	123 (93–162)	123 (93–162)	NS
170023	347 (263–471)	427 (324–562)	123 (98–155)	263 (191–363)
O-11	193 (146–254)	*501* (398–631)	62 (45–85)	246 (186–324)
Control (non-immunized animals)	23 (19–28)	25 (20–32)	6 (4–8)	62 (47–81)

Note. Each mouse was immunized with 100 μg (dry wt) of GLP using groups of 50 mice for each GLP preparation. In each group 10 immunized (test) and 10 non-immunized (control) mice were challenged with five different doses of *P. aeruginosa* live culture to establish LD_{50} values. Maximum challenge doses, depending on the strain, ranged $1600–200 \times 10^6$ cells (test) and $800–50 \times 10^6$ cells (control). Results of the two experiments were summed up. Maximum values for LD_{50} of the homologous challenge strain are printed in italics. NS = not studied

Previously we have shown [1] that the GLP yield for different *P. aeruginosa* strains varied from 8 to 34% of the C-ES dry weight. In the present paper we analysed yields on the basis of the summed weight of fractions obtained from different strains. Table II shows that GLP-I yields varied between

of different P. aeruginosa serogroups

($p = 0.05$) when challenged with strains					
F5	F6	F7	8	PA-103	170019
457 (347–603)	186 (141–246)	87 (66–126)	155 (126–145)	149 (113–189)	741 (562–477)
398 (288–550)	<100	76 (55–105)	87 (66–115)	25 (18–36)	603 (525–692)
186 (141–246)	115 (91–145)	*162* (129–204)	162 (129–204)	58 (46–72)	324 (246–427)
263 (182–380)	107 (81–141)	282 (214–372)	*257* (204–324)	58 (40–83)	646 (490–851)
<100	151 (110–209)	NS	111 (84–146)	76 (55–105)	603 (479–759)
141 (112–178)	159 (118–224)	<50	62 (49–78)	NS	NS
229 (166–316)	200 (145–275)	78 (56–102)	NS	162 (118–224)	692 (575–832)
123 (89–170)	93 (74–118)	54 (41–71)	45 (36–57)	36 (30–43)	348 (240–501)
200 (145–275)	263 (182–380)	<50	NS	NS	NS
145 (102–195)	132 (100–174)	71 (51–98)	47 (36–62)	76 (58–100)	372 (295–468)
324 (234–447)	200 (138–288)	123 (93–162)	NS	58 (48–69)	*668* (508–881)
324 (234–447)	100 (76–132)	78 (56–102)	44 (35–55)	27 (20–36)	754 (603–955)
174 (126–240)	NS	<50	91 (62–107)	NS	NS
246 (178–339)	141 (102–195)	62 (47–81)	52 (39–68)	110 (83–145)	525 (398–642)
115 (91–145)	<100	162 (123–214)	50 (40–63)	*174* (132–229)	490 (372–646)
36 (27–47)	36 (27–47)	35 (23–54)	13 (10–16)	8 (6–11)	200 (152–251)

36.9 and 88%, mean value being 65.6 ± 8.4% of the summed weight of all fractions. GLP-II yields varied between 33.9 and 62.5%, with a mean value of 46.4 ± 11.2%. Accordingly, both methods gave more or less the same yield of GLP. No significant difference existed between the mean values for PFr yields.

Chemical analysis (carbohydrate and protein content) and preliminary active protection tests showed that neither GLP-I and GLP-II, nor the corresponding PFr-I and PFr-II differed significantly.

Table III presents the results of the study of cross immunity in mice

vaccinated with GLP from different *P. aeruginosa* strains. These data are summed up for GLP obtained by means of both methods. In every experiment 10–14 test groups (50 immunized mice/group) and 1 control group (50 non-immunized mice) were used. Altogether, two experiments were performed with every challenge strain, in different seasons. According to our data [11], virulence of *P. aeruginosa* varies in different tests and especially depends on the season. Therefore, summed mean values of the two experiments and EI (animal efficiency index), are presented so as to allow a comparison with our previous data [1]. It was assumed that, if EI was less than 2 or if there was no substantial difference between LD_{50} for the test animals and LD_{50} for controls, cross protection was absent (negative result). If EI = 2.1–4.0, cross protection was low, if EI = 4.1–10.0 cross protection was of medium, and if EI > 10.1 of high degree. Besides, data for "homologous" protection (i.e., immunity to serogroup or immunotype-homologous strain) were compared to data of "heterologous" protection (i.e., immunity to serogroup or immunotype-heterologous strain).

Tables III and IV show that GLP of different *P. aeruginosa* serogroups induce active anti-*P. aeruginosa* cross immunity in mice in a high or low degree. It is interesting that GLP of different strains induced immunity to challenge

Table IV

Efficiency indexes (EI) for cross active protection of mice immunized with different serogroups P. aeruginosa GLP

Strain	EI when challenged with strains									
	F1	F2	F3	F4	F5	F6	F7	8	PA-103	170019
170001	15.1	27.7	>16.7	6.4*	12.7	5.2	2.5*	11.9*	18.6	3.7
170002	9.3	5.3*	14.5*	3*	**11.1**	—	2.2*	6.7*	3.1*	3.0
170005	11.4	14.9	13.5*	2.4*	5.2*	3.2	*4.6**	12.5*	7.3*	—
170006	13.1	9.2*	33.3	4.2*	7.3	3.0	**8.5**	19.8	7.3*	3.2
170007	5.0*	3.7*	*25.2*	2.1*	—	4.2	NS	*8.5*	9.5*	3.0
170009	*15.1*	15.9	NS	NS	3.9*	4.4	—	4.8*	NS	NS
170010	**24.4**	48.1*	10.3*	3.5*	6.3	5.6	2.2*	NS	20.3	3.5
170014	13.1	10.5*	8.3*	**11.2**	3.4*	2.5	—	3.5*	4.5*	—
170017	14.1	5.6*	NS	3.5*	5.6*	7.3	—	NS	NS	NS
170018	7.0*	21.0	12.0*	2.1*	4.0*	3.7	—	3.6*	9.5*	1.9*
170019	10.7	9.8*	>16.7	NS	9.0	5.6	3.5*	NS	7.3*	**3,3**
170021	14.1	22.5	11.0*	4.0*	9.0	2.8	2.2*	3.4*	3.4*	3.8
170022	NS	4.9*	21	NS	4.8*	NS	—	7*	NS	NS
170023	15.1	17.1	20.5	4.2*	6.8	3.9	—	4*	13.8	2.6
O-11	8.4	**20.0**	10.3*	4.0*	3.2*	—	4.6*	3.8*	**21.8**	2.5

Note. — negative, EI <2; * EI for a "heterologous" protection is substantially different from EI for a "homologous" protection. Values for the homologous strain are printed in italics those for homologous strains with maximum EI in bold type figures

with toxigenic PA-103 strain and to freshly isolated strain No. 8. The highest immunity to the challenge with PA-103 strain was induced by GLP-170001, -170010 and -170023; in this case EI for heterologous and homologous protection did not differ substantially.

When immunized mice were challenged with strain F1, the protective effect did not differ significantly in 10 out of 13 test groups. Similarly, non-significant differences were obtained for F2 in 6 out of 13, for F3 in 4 out of 12 and for F5 in 6 out of 14 test groups. When mice immunized with GLP-170010 were challenged with strain F2, the heterologous protection was substantially higher than the homologous protection. Data for immunity to challenge with strain F3, F7 and No. 8 belonging to the same O2-serogroup [12], are of certain interest, as well as data for strains 170005, 170006 and 170007 from which GLPs were isolated (see also Table I). It was evident that GLP-170006 stimulated substantially higher protection against the challenge with any of the three above-mentioned strains than GLP-170005. At the same time, GLP-170006 and -170007 elicited a more or less similar protective effect against challenge with strain F3 but not against strain No. 8. The immunogenicity of the former three GLPs was similar when the animals were challenged with heterologous strains PA-103, F6 and F4, but when they were challenged with F1 and F5, GLP-170007 was less active than GLP-170005 or GLP-170006. Comparison of GLP-170009 and GLP-170010 (both strains belong to serogroup O6) proved that they had a similar immunogenic activity against challenge with serogroup-homologous strain F1 and with heterologous strain F6 but not with strain F2.

It should be noted that GLPs of strains 170018, 170019, 170021 and 170023 which have no counterparts among the immunotypes [14], stimulate average or high protection in mice against the challenge with strains F1, F2, F3 and PA-103; low protection or no protection at all was observed against challenge with strains F4, F6 and F7.

PFr obtained in isolating GLP (see Fig. 1) was studied in comparison with GLP and C-ES. Table V shows results of protective activity of slime fractions from strains 170007 and 170009. GLPs and PFrs of both strains were equally active. However, PFr and GLP of strain 170007 and PFr of strain 170009 were less active than the corresponding C-ES.

Table VI gives data on the testing of slime preparations from different strains, the immunized mice were challenged with strain 170019. GLP and PFr did not differ in protective activity. At the same time, GLP and PFr of strain 170007 and PFr of strain 170009 were less active than the corresponding C-ES.

Tables VII and VIII show the immunogenic activity of C-ES fractions and of 170019-LPS. Titration of the immunizing dose (Table VII) showed that the ED_{50} values for C-ES, GLP, PFr and GLP supernatant were not

Table V

Active protection of mice immunized with slime fractions of P. aeruginosa strains 170007 and 170009

Fractions	Immunization with fractions of strain 170007 and challenge with strain F3		Immunization with fractions of strain 170009 and challenge with strain F1	
	LD_{50} of strain F3 $\times 10^6$ cells	EI	LD_{50} of strain F1 $\times 10^6$ cells	EI
C-ES	246 (229–363)*	47.3	1202 (724–1995)	223
GLP	115 (76–159)	22.1	741 (537–1023)	13.7
PFr	107 (71–162)	20.6	398 (263–603)	7.3
Control (non-immunized mice)	5.2 (4.6–6.0)	1	54 (39–74)	1

Note. The mice were immunized with 100 μg of each fraction. Challenge doses with strain F3 were $400\times$, $200\times$, $100\times$, $50\times$ and 25×10^6 cells for test mice and $50\times$, $25\times$, $12.5\times$, $6.2\times$ and 3.1×10^6 cells for control mice. With strain F1, the challenge doses for test mice were: $1600\times$, $800\times$, $400\times$, $200\times$ and 100×10^6 cells, and for the controls: $800\times$, $400\times$, $200\times$, $100\times$ and 50×10^6 cells. Groups of 10 mice were used for each challenge dose

*Confidence limit at $p = 0.05$

Table VI

Active protection of mice, immunized with slime fractions of different strains and challenged with P. aeruginosa strain 170019

Slime-producing stain	Fractions	LD_{50} of the challenge strain 170019	EI
170002	C-ES	851 (617–1175)*	5.6
	GLP	692 (501–955)	4.6
	PFr	372 (282–490)	2.5
170006	C-ES	646 (513–813)	4.3
	GLP	398 (288–550)	2.6
	PFr	372 (295–468)	2.5
170007	C-ES	741 (562–977)	4.9
	GLP	427 (324–562)	2.8
	PFr	427 (309–589)	2.8
170019	C-ES	1288 (977–1698)	8.5
	GLP	603 (457–794)	4.0
	PFr	692 (525–912)	4.6
Control (non-immunized) mice	—	151 (115–200)	1

Note. Titration of the challenge dose on the immunized (test) and non-immunized (control) mice. Test mice were challenged with: $1600\times$, $800\times$, $400\times$, $200\times$, $100\times$ and 50×10^6 cells: control mice were challenged with $400\times$, $200\times$, $100\times$, $50\times$ and 25×10^6 cells, 10 mice per one challenge dose; results of the two experiments are summed up

*Confidence limit at $p = 0.05$

Table VII

Active protection of mice immunized with the slime fractions of P. aeruginosa strain 170019

Fractions	ED_{50} (μg), when challenged with strain 170019; challenge dose is $3LD_{50}$	ED_{50} (μg), when challenged with strain 170019; challenge dose is $16LD_{50}$*
C-ES	6.3 (1.9–20.7)*	103.5 (42.7–247.7)
GLP	9.9 (2.9–34.5)	95.5 (52.5–173.8)
PFr	25 (10.3–60.6)	>250**
GLP residue (105p180)	2.5 (0.7–8.7)	NS
GLP supernatant (105s180)	12.6 (3.4–46.9)	NS
LPS	>250**	225.9 (116.2–367.3)

Note. Mice were immunized with 4 doses of every fraction (250, 25, 2.5 and 0.25 μg of dry weight), 10 mice per one immunizing dose. They were challenged with one dose of live culture. Titration of LD_{50} was performed on the control (non-immunized) mice in every test and quantity of LD_{50} in a challenge dose of *P. aeruginosa* live culture was estimated. Results of the two experiments are summed up

* Confidence limits at $p = 0.05$

** Individual mice survived when the maximum immunizing dose was 250 μg

NS = not studied

Table VIII

Active protection of mice immunized with the fractions of P. aeruginosa slime of strain 170019

Fractions	Challenge with strain 170019		Challenge with strain F6	
	$LD_{50} \times 10^6$ cells	EI	$LD_{50} \times 10^6$ cells	EI
C-ES	1047 (912–1202)*	7.4	800 (631–1000)	24.2
GLP	427 (309–589)	3.0	427 (339–537)	12.9
PFr	1288 (977–1698)	9.2	457 (363–575)	12.9
Residue (105p180) of GLP	214 (141–324)	—	132 (87–200)	4
Supernatant (105s180) of GLP	562 (407–776)	4.0	324 (234–447)	9.8
LPS	562 (427–741)	4.0	525 (388–692)	15.9
Control (non-immunized mice)	141 (98–155)	1	33 (29–38)	1

Note. Mice were immunized with 100 μg of every fraction and challenged with 5 different doses of the live culture ($1600\times$, $800\times$, $400\times$, $200\times$ and 100×10^6 cells, 10 mice per one challenge dose); control (non-immunized) mice were also challenged with 5 doses of the live culture ($800\times$, $400\times$, $200\times$, $100\times$ and 50×10^6 cells, 10 mice per one challenge dose). Results of the two experiments are summed up

— The fraction was not active because LD_{50} for the test mice did not substantially differ from LD_{50} for the control mice

* Confidence limit at $p = 0.05$

identical when mice were challenged with 3 LD_{50} of the homologous culture. Under the same conditions GLP residue was substantially more active than PFr. When the immunized mice were challenged with 16 LD_{50} of the homologous culture, it turned out that PFr was practically non-immunogenic and that C-ES and GLP had definite protective activity. In these tests LPS-170019 was shown to be of low immunogenicity. Titration of the challenge dose of the homologous culture (Table VIII) allowed to conclude that C-ES and PFr had the highest immunogenicity and that GLP residue was not active because the LD_{50} for mice immunized with this preparation practically did not differ from the LD_{50} for the control. Immunogenicity of GLP, GLP supernatant and LPS was nearly identical and substantially lower than immunogenicity of C-ES and PFr. When challenge was done with a heterologous strain, C-ES immunogenicity was also the highest and that of GLP residue the lowest. The rest of the preparations had similar immunogenic activity. It should be noted that under the given test conditions LPS-170019 turned out to be immunogenic enough when challenged both with homologous and heterologous F6 strains.

To find out the extent of cross-activity of PFr from different strains, in additional experiments, mice were immunized with PFr from different strains and challenged with a highly virulent strain (F3) and with a less virulent strain (F6).

As shown in Table IX, PFr had a definite cross-protective activity. Cross-protection in mice immunized with PFrs from strains 170001, 170010, 170018 and 170023 against challenge with strain F3, was the highest. When challenged with strain F6, the most active were PFr from strains 170023, 170005, 170010 and the least active was PFr from strain O-11.

Table IX

Cross active protection of mice immunized with PFr from C-ES of P. aeruginosa of different serogroups

Immunization with PFr of strain	Challenge with F3		Challenge with F6	
	$LD_{50} \times 10^6$ cells	EI	$LD_{50} \times 10^6$ cells	EI
170001	186 (148–234)	18.6	186 (141–246)	5.6
170005	*>200*	*>20*	646 (490–851)	19.6
170007	*>200*	*>20*	186 (135–257)	5.6
170009	107 (81–141)	10.7	174 (126–240)	5.3
170010	174 (126–240)	17.4	347 (263–457)	10.5
170018	162 (123–214)	16.2	263 (209–331)	8.0
170023	151 (110–209)	15.1	794 (631–1000)	24.1
O-11	62 (45–85)	6.2	93 (74–118)	2.8
Control of the challenge	10 (8–13)	1	33 (29–38)	1

Discussion

In our previous paper [1] we have shown that isolated and partly purified *P. aeruginosa* extracellular slime (crude extracellular slime: C-ES) is capable of stimulating active immunity in mice against challenge with a homologous and a heterologous strain. In course of the above work an attempt was made to find out which component of the slime was responsible for the cross-protective activity: GLP or a protein component separated from C-ES by water-phenol extraction.

In the previous paper [1] it was shown that GLP yield was 8–34% of C-ES dry weight. But it was assumed that these figures did not fully represent the actual data on GLP yield, that is, out of these figures it was not possible to conclude which portion of C-ES corresponded to GLP. This is due to the fact that in the isolation procedure the active ingredient is lost. The present work the specific yield of GLP and other C-ES fractions, was analysed as follows. The weight of all fractions obtained in the process of isolation was summed up and, on the basis of that sum, the weight of one of the C-ES fractions was calculated. It was found out that the mean value of the yield (or, rather, specific yield) of the purified GLP was $65.6 \pm 8.4\%$ (by the first method) and $46.4 \pm 11.2\%$ (by the second method) of the dry weight of all the fractions (Table II). Thus, GLP appeared to be the main component of the isolated C-ES. It should also be noted that the GLP yield of several strains was significantly lower than the mean value (strain 170010, method I), and that of some other strains higher than the mean value (strain 170006, method I). This finding may be due to a difference in the capacity of the strains to synthesize extracellular slime.

The phenol fraction, which mostly contains soluble proteins (or peptides), averages to about $^1/_5$ of C-ES dry weight. It was demonstrated that galactose was a characteristic component of C-ES and GLP, whereas it is usually absent from the corresponding LPS [15]. Uronic acids are the most typical components of GLP [1, 16], but they are also present in isolated and purified LPS of *P. aeruginosa* [17]. According to the data of Linker et al. [18], the presence of alginic acids is characteristic of *P. aeruginosa* slime and is responsible for its viscosity and low solubility. In our case C-ES and GLP of strain 170019 were the least soluble: they could be dissolved in saline only by means of a prolonged and intensive mixing.

Results for the comparison of protective activity of GLP (Tables III, IV) and C-ES [1] of different *P. aeruginosa* strains were equivocal. Table X presents data of a comparative analysis. GLPs of 170001, 170002, 170006 and O-11 were more active than the corresponding C-ES when immunized mice were challenged with 5–6 out of 9 *P. aeruginosa* strains. On the contrary, GLPs of 170005, 170007, 170009, 170021 and 170023 were less active than the cor-

Table X

Comparison of results for GLP (Table II) and C-ES (reference 1) cross protection activity according to efficiency index (EI) values

Slime producing strain	Challenge strain								
	F1	F2	F3	F4	F5	F6	F7	170019	PA-103
170001	S < G	S < G	S < G	S > G	S < G	S > G	S < G	S > G	S < G
170002	S < G	S < G	S < G	S > G	S < G	S > G	NS	S > G	S < G
170005	S < G	S < G	S > G	S > G	S = G	S > G	S > G	S > G	S > G
170006	S < G	S < G	S > G	S = G	S < G	S > G	S < G	S > G	S = G
170007	S > G	S > G	S > G	NS	NS	S > G	NS	S > G	S < G
170009	S < G	S < G	S < G	S > G	S > G	S > G	S > G	S > G	S < G
170014	S < G	S < G	S < G	S > G	S > G	S > G	S = G	S > G	S = G
170017	NS	NS	NS	NS	S > G	S > G	S = G	NS	NS
170018	S < G	S < G	S < G	S > G	S > G	S > G	S = G	S > G	S < G
170019	S < G	S < G	S = G	NS	S = G	S > G	S > G	S > G	S > G
170021	S < G	S < G	S = G	S > G	S = G	S > G	S > G	S > G	S > G
170023	S < G	S < G	S > G	S > G	S > G	S > G	S > G	S > G	S < G
O-11	S < G	S < G	S < G	S = G	S > G	S > G	S < G	S > G	S < G

Note. G = GLP, S = C-ES, S < G when EI for C-ES is less than EI for GLP, S > G when EI for C-ES is more than EI for GLP, S = G when EI is similar for both preparations. NS = not studied

responding C-ES when the immunized mice were challenged with 5–6 out of 9 *P. aeruginosa* strains. GLP and C-ES stimulate equal immunity to the challenge with some other strains. If data in Table X are analysed along the vertical lines, it is possible to make the following conclusion. When the immunized mice are challenged with strains F1 and F2, 11 out of 12 GLP preparations are more active than the corresponding C-ES, and when the mice are challenged with F4, F6 or 170019, nearly all GLP preparations are less active than the corresponding C-ES.

Data on the stimulation of GLP immunity to the challenge with toxigenic strain PA-103 are of certain interest. It was shown that 7 out of 12 GLP preparations were more active in stimulating immunity than the corresponding C-ES, and that activity of GLP-170006 and GLP-170014 did not differ from that of the corresponding C-ES.

Summing up all data in the Table X, it may be concluded that, out of 105 comparisons, in 38.6% GLP is more active than C-ES; in 50% C-ES is more active than GLP, and in 21.4% both preparations have similar protective activity.

Therefore, C-ES of GLP isolated from it, stimulate more or less actively cross-immunity in mice to *P. aeruginosa* infection not depending on serogroup or immunotype of the slime-producing strain. In some cases it is possible to increase activity of the slime preparation by means of water–phenol extraction,

but removed slime fractions (for example, PFr) are also of sufficient protective activity. We could not reveal any substantial difference between protective activity of GLP and PFr purified preparations; evidently, cross-protection in mice might be stimulated both by carbohydrate and by peptide components of *P. aeruginosa* extracellular slime.

It has been proved that a high-molecular polysaccharide component (without protein) extracted from slime has a protective activity [5] but cross-activity of such a preparation has not yet been studied. It may be assumed [19] that *P. aeruginosa* extracellular slime stimulates only type- (group-) specific immunity. Our data given in the present paper and also our previous experiments [1] allow to conclude that genus-specific protective antigen (or antigens) which can be used for the preparation of polycomponent vaccines against *P. aeruginosa* infection, are present in the slime.

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VIRULENCE FACTORS OF *ESCHERICHIA COLI*

III. CORRELATION WITH *ESCHERICHIA COLI* PATHOGENICITY OF HAEMOLYSIN PRODUCTION, HAEMAGGLUTINATING CAPACITY, ANTIGENS K1, K5, AND COLICINOGENICITY

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A total of 1156 *Escherichia coli* strains including 489 faecal, 384 urinary, 283 other extraintestinal isolates was compared for haemolysin production (Hly), mannose resistant haemagglutinating activity (MRA), presence of antigens K1 and K5 and colicinogenicity (Col). K1 capsule which was demonstrated only in a few serogroups (O1, O2, O7, O18) occurred more frequently among extraintestinal (32.1%) than among faecal (4.3%) or urinary (7.3%) isolates. In the incidence of antigen K5 there was no difference between faecal and urinary (3.3%; 3.1%) or between urinary and other extraintestinal (5.3%) isolates belonging mainly to serogroups O2, O6, O18 and O75. Col$^+$ isolates occurred frequently in all samples (23.5% of faecal, 31.7% of urinary and 43.4% of other extraintestinal strains), they being significantly more frequent in serogroups O1, O2, O7, O18 than in others. A close association existed between K1$^+$ and Col$^+$ properties, mainly (24.4%) among strains isolated from extraintestinal sources other than urine. The frequent coexistence of K1$^+$ and Col$^+$ in serogroups O1, O2, O7, O18 offers a further explanation for the extraintestinal pathogenicity of these serogroups. Neither Hly$^+$ and K1$^+$, nor Hly$^+$ and Col$^+$ were associated. MRA$^+$ and K1$^+$ correlated mainly in serogroups O1, O2 but never occurred simultaneously in serogroup O18. Connection between MRA$^+$ and Hly$^+$ was not associated with other virulence factors (K1, Col). The results showing a close connection among certain serogroups (O1, O2, O4, O6, O7, O18) and certain markers of pathogenicity (MRA, Hly, K1, Col) support the concept that *E. coli* strains have a clonal connection.

The increasing significance of *Escherichia coli* in extraintestinal infections has been emphasized by several studies [1–3]. Its importance is also corroborated by the fact that in Hungary nearly 50% of Gram-negative facultatively pathogenic isolates corresponds to *E. coli*. In view of the possible differences in the pathogenicity of individual isolates, it seemed desirable to continue our work on *E. coli* virulence factors.

In earlier studies [4, 5]. an analysis for mannose resistant haemagglutinating capacity (MRA) and haemolysin production (Hly) of *E. coli* isolates revealed that serogroup O4, O6 and O18, Hly and MRA were closely associated. As a significant proportion of *E. coli* strains causing human extraintestinal infections can be correlated with several other virulence factors i.e. their surface polysaccharide K1, K5 antigens [6–9] as well as colicinogenicity (Col) especially colicin V (ColV) production [3, 10, 11], the purpose of

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our further study was to examine the incidence of these virulence determinants among our faecal, urinary and other extraintestinal isolates, to analyse their correlation to each other and to other determinants (Hly, MRA, O antigen) studied formerly [4, 5].

Materials and methods

Escherichia coli strains were isolated from faecal samples of healthy subjects (233) or patients suffering from enteritis (256); urine of patients with pyelonephritis (155), cystitis (170), asymptomatic bacteriuria (59); and 283 from other extraintestinal sources (blood, 40; cerebrospinal fluid, 35; autopsy material, 41; wound swab, 63; umbilical cord, 8; vagina, 38; upper respiratory tract excretions, 36; placenta and lochia, 22). Fourty strains originated from H. Steinrück (Städtisches Klinikum Berlin-Buch, G. D. R.); 19 of them were isolated from CSF, 6 from blood samples, 5 from autopsy materials and 7 from faecal samples of patients with enteritis. Twenty five out of 40 strains belonged to serogroup O18. These data were included in the above-mentioned figures.

Diagnostic criteria of illnesses were described previously [4].

Serological examination of O antigens was carried out by the agglutination method of Ørskov and Ørskov [12].

Mannose resistant haemagglutination and haemolysin production were determined as described previously [4, 5].

Mannose sensitive haemagglutination (MSA) test was performed according to Duguid et al. [13].

Detection of K1 and K5 capsular antigens was carried out by phages K1 [14] and K5 [8]. Phages K1 were supplied by B. Rowe (Central Public Health Laboratory, London, U. K.) and propagated on strain U9/41. Phage K5 was obtained from D. S. Gupta. The phage was propagated on *E. coli* EH 342, received from P. H. Mäkelä (Central Public Health Laboratory, Helsinki, Finland). Phages were used undiluted and at RTD. The test strains were incubated in broth at 37 °C for 2 h, than inoculated on the surface of agar plates. The plates were allowed to dry, and the phages were dropped onto the plates. The results were read after incubation at 37 °C for 5 h, and at 4 °C overnight. Lysis was recorded as positive K1 or K5 test.

Determination of colicinogenicity was carried out according to Milch and Gyenes [15]. For colicin typing Frédéricq's indicator strains were used [16] by the method of Lewis [17] and De Alwis and Thomlinson [18].

Electron microscopic examinations were carried out as described previously [19].

Statistical analysis was carried out in 2×2 contingency tables and examined for significance by the chi^2 test [20].

Results

The incidence of K1 antigen in *E. coli* isolated from faecal samples of patients and healthy subjects is presented in Table I.

Faecal strains showed K1 positivity in 4.3% (1.6% for enteritis strains and 7.3% for healthy persons' strains). $K1^+$ strains belonged in 61.9% to serogroups O1, O2, O7, O18. The two remaining serogroups having K1 antigen were O21 and O156.

From urine 384 strains were isolated (Table II). K1 positivity occurred in 7.3% (9% for pyelonephritis, 5.9% for cystitis, 6.7% for asymptomatic bacteriuria strains). There was no significant difference in K1 positivity between faecal and urinary strains ($p > 0.05$). Twenty-two out of 28 $K1^+$ strains belonged to serogroups O1, O2 and O18. The O antigens of the remaining serogroups were O16, O23 and O83.

Table I

Serogroup distribution of K1 and K5 positive E. coli strains isolated from faecal samples

Serogroup	Patients with enteritis		Healthy subjects		Total	
	K1	K5	K1	K5	K1	K5
O1	1/9[1]	0/9	4/7	0/7	5/16	0/16
O2	1/5	1/5	2/8	2/8	3/13	3/13
O4	0/14	0/14	0/4	0/4	0/18	0/18
O6	0/13	0/13	0/6	0/6	0/19	0/19
O7	2/3	0/3	2/7	0/7	4/10	0/10
O18ac	0/20	5/20	1/6	3/6	1/26	8/26
O75	0/7	0/7	0/7	3/7	0/14	3/14
Others[2]	0/91	0/91	2/99	0/99	2/190	0/190
Sp. aggl.[3]	0/10	1/10	1/6	0/6	1/16	1/16
NT[4]	0/84	0/84	5/83	1/83	5/167	1/167
Total	4/256	7/256	17/233	9/233	21/489	16/489

[1] No. of strains K1 or K5 positive/No. of strains examined

[2] O3, O5, O8, O9, O10, O11, O12, O13, O16, O17, O17, 77, O18ab, O19, 133, O20, O21, O22, 023, O25, O26, O29, O30, O30, 116, O33, O34, O36, O40, O46, O48, O48, 87, O51, O55, O59, O61, O68, O69, O71, O73, O78, O79, O80, O81, O85, O86, O88, O89, O91, O92, O95, O98, O99, O102, O105ac, O106, O110, O111, O112ab, O112ac, O113, O121, O123, O124, O127, O128, O131, O136, O141, O142, O146, O156

[3] Spontaneous agglutination

[4] Not typable

Table II

Serogroup distribution of K1 and K5 positive E. coli strains isolated from urinary tract infections

Serogroup	Pyelonephritis		Cystitis		ABU[1]		Total	
	K1	K5	K1	K5	K1	K5	K1	K5
O1	8/10[2]	0/10	5/6	0/6	0/0	0/0	13/16	0/16
O2	2/13	0/13	4/13	0/13	2/6	0/6	8/32	0/32
O4	0/5	0/5	0/4	0/4	0/5	0/5	0/14	0/14
O6	0/12	1/12	0/18	0/18	0/2	0/2	0/32	1/32
O7	0/3	0/3	0/2	0/2	0/5	0/5	0/10	0/10
O18ac	1/5	3/5	0/3	3/3	0/3	0/3	1/11	6/11
O75	0/5	2/5	0/3	1/3	0/1	1/1	0/9	4/9
Others[3]	2/47	0/47	0/43	0/43	1/12	0/12	3/102	0/102
Sp. aggl.[4]	0/8	0/8	0/12	0/12	1/3	0/3	1/23	0/23
NT[5]	1/47	0/47	1/66	1/66	0/22	0/22	2/135	1/135
Total	14/155	6/155	10/170	5/170	4/59	1/59	28/384	12/384

[1] Asymptomatic bacteriuria

[2] No. of strains K1 or K5 positive/No. of strains examined

[3] O3, O5, O7, 16, O9, O10, O11, O15, O16, O17, O17, 77, O19, 133, O20, O21, O22, O23, O25, O29, 134, O30, O36, O42, O45, O51, O56, O57, O61, O71, O77, O78, O83, O86, O95, O96, O99, O101, O106, O107, O109, O112ab, O131, O141, O142, O149, O159

[4] Spontaneous agglutination

[5] Not typable

Table III

Serogroup distribution of K1 and K5 positive E. coli

Serogroup	Blood		CSF[1]		Autopsy material		Wound	
	K1	K5	K1	K5	K1	K5	K1	K5
O1	1/1	0/1	0/0	0/0	0/0	0/0	3/3	0/3
O2	1/1	0/1	0/0	0/0	3/4	0/4	0/4	0/4
O4	0/2	0/2	0/2	0/2	0/8	0/8	0/6	0/6
O6	0/6	0/6	0/1	0/1	0/7	0/7	0/13	0/13
O7	3/4	0/4	5/5	0/5	0/0	0/0	0/2	0/2
O18ac	6/10	0/10	19/19	0/19	2/4	1/4	0/2	1/2
O75	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Others[4]	1/5	0/5	1/3	0/3	0/10	0/10	0/14	0/14
Sp. aggl.[5]	1/3	0/3	2/4	0/4	0/0	0/0	0/4	0/4
NT[6]	0/8	0/8	0/1	0/1	0/8	0/8	2/15	0/15
Total	13/40	0/40	27/35	0/35	5/41	1/41	5/63	1/63

[1] Cerebrospinal fluid
[2] Throat, nose, sputum, ear swab
[3] Placenta, lochia
[4] O5, O8, O12, O15, O17, O18ab, O19, O20, O21, O22, O23, O33, O40, O45, O78, O82, O83, O84, O96, O105ac, O106, O107, O108, O110, O114, O118, 160, O120, O131, O134, O162
[5] Spontaneous agglutination
[6] Not typable

$K1^+$ isolates occurred most frequently (32.1%) in different extraintestinal samples (Table III), with an especially high incidence in blood, CSF, umbilical cord, vaginal swab and upper respiratory tract specimens. The differences in the incidence of K1 positivity between urinary and other extraintestinal samples, or between faecal and extraintestinal samples, were significant ($p < 0.001$).

Considering that umbilical cord and upper respiratory tract samples as well as vaginal swabs originated in an overwhelming majority from the gynaecological and perinatal wards of one hospital [4], $K1^+$ strains of these

Table IV

Distribution of K1 positive strains among isolates originating from a gynaecological and perinatal ward

	Upper respiratory tract	Umbilical cord	Vagina
No. of samples examined	4110	340	2301
No. of samples E. coli positive	254	40	262
No. of *E. coli* strains with antigen K1	10	3	13

strains isolated from extraintestinal sources

Umbilical cord		Vagina		Upper respiratory tract[2]		Miscellaneous[3]		Total	
K1	K5	K1	K5	K1	K5	K1	K5	K1	K5
1/1	0/1	3/4	0/4	4/4	0/4	1/1	0/1	13/14	0/14
1/4	0/4	5/5	0/5	5/7	0/7	0/1	0/1	15/26	0/26
0/1	0/1	0/0	0/0	0/1	0/1	0/1	0/1	0/21	0/21
0/0	0/0	0/5	0/5	0/4	3/4	0/4	1/4	0/40	4/40
0/0	0/0	2/2	0/2	0/0	0/0	0/0	0/0	10/13	0/13
1/1	0/1	3/5	2/5	1/6	5/6	2/3	0/3	34/50	9/50
0/0	0/0	0/0	0/0	0/2	1/2	0/0	0/0	0/2	1/2
1/1	0/1	1/9	0/9	2/9	0/9	0/7	0/7	6/58	0/58
0/0	0/0	1/1	0/1	0/0	0/0	0/1	0/1	4/13	0/13
0/0	0/0	4/7	0/7	2/3	0/3	1/4	0/4	9/46	0/46
4/8	0/8	19/38	2/38	14/36	9/36	4/22	1/22	91/283	14/283

materials were subjected to further analysis. It can be seen from Table IV that *E. coli* strains were isolated from 6–12% of healthy pregnants and newborns. Altogether 4–7.5% of *E. coli* strains had antigen K1. Sepsis, meningitis or other extraintestinal infections never occurred during the observation period.

K1$^+$ strains occurred mainly in serogroups O1, O2, O7, O18. The 6 remaining ones belonged to serogroups O5, O12, O33, O83.

The incidence of K5 antigen is presented in Tables I, II and III. K5 positivity occurred in 3.3% of faecal, in 3.1% of urinary tract and in 5.3% of other extraintestinal samples. The difference in their occurrence in different materials was not significant statistically.

Strains with K5 antigen occurred more frequently in serogroups O2, O6, O18ac, O75 than in others (39 out of 42 K5 positive isolates belonged to these serogroups).

The incidence of colicin producing strains (Table V) was very high in every group of materials (23.5%, 31.7% and 43.4%, respectively). Their occurrence increased significantly in the order of faecal, urinary and other extraintestinal samples. An especially high incidence was found in blood, CSF, umbilical cord and upper respiratory tract isolates. Col positivity was significantly more frequent in serogroups O1, O2, O4, O6, O7, O18 and O75 than in others ($p < 0.001$).

The incidence of colicin V producing strains (Table V) was 2% among faecal, 9.1% among urinary and 12.7% among other extraintestinal isolates. In serogroups O1, O2 and O18 the occurrence of Col V$^+$ strains was higher than in other serogroups. In serogroups O4, O6, and O75 Col V producing strains never occurred. In extraintestinal samples Col V$^+$ strains originated from

Table V

Serogroup distribution of colicinogenic E. coli strains isolated from faecal, urinary and other extraintestinal samples

Serogroup	Faeces		Urine		Extraintestinal material		Total	
	Col	Col V	Col	Col V	Col	Col V	Col	Col V
O1	9/16[1]	0/16	13/16	1/16	9/14	4/14	31/46	5/46
O2	7/13	2/13	16/32	8/32	15/26	9/26	38/71	19/71
O4	6/18	0/18	0/14	0/14	3/21	0/21	9/53	0/53
O6	7/19	0/19	5/32	0/32	12/40	0/40	24/91	0/91
O7	3/10	0/10	5/10	3/10	10/13	0/13	18/33	3/33
O18ac	8/26	0/26	6/11	2/11	34/50	6/50	48/87	8/87
O75	2/14	0/14	3/9	0/9	1/2	0/2	6/25	0/25
Others	33/190	2/190	28/102	8/102	17/58	5/58	78/350	15/350
Sp. aggl.[2]	2/16	0/16	3/23	2/23	4/13	1/13	9/52	3/52
NT[3]	38/167	6/167	43/135	11/135	18/46	11/46	99/348	28/348
Total	115/489	10/489	122/384	35/384	123/283	36/283	360/1156	81/1156

[1] No. of strains Col+, or Col V+/No. of strains examined
[2] Spontaneous agglutination
[3] Not typable

autopsy material (10), blood (4), wound swabs (5), CSF (5), vagina (6) and upper respiratory tract (6).

Correlation of the different virulence factors (e.g. MRA, Hly positivity, K1, K5 antigen, colicinogenicity) and of O antigens. There was a marked correlation between the presence of K1 antigen and colicin production in certain

Table VI

Correlation between the simultaneous presence of K1 antigen and colicinogenicity of E. coli strains

Serogroup	No. of strains isolated from										Total
	Faeces			Urine			Extraintestinal material				
	K1+ Col+	K1+ Col−	K1− Col+	K1+ Col+	K1+ Col−	K1− Col+	K1+ Col+	K1+ Col−	K1− Col+	K1− Col−	examined
O1	5	0	4	11	2	2	9	4	0	9	46
O2	1	2	6	5	3	11	12	3	3	25	71
O4	0	0	6	0	0	0	0	0	3	44	53
O6	0	0	7	0	0	5	0	0	12	67	91
O7	3	1	0	0	0	5	9	1	1	13	33
O18ac	0	1	8	1	0	5	31	3	3	35	87
O75	0	0	2	0	0	3	0	0	1	19	25
Others[1]	3	5	70	4	2	70	12	7	27	550	750
Total	12	9	103	21	7	101	73	18	50	762	1156

[1] Other serogroups (see Tables I, II, III), strains not typable and agglutinating spontaneously

serogroups of *E. coli* isolates (Table VI). K1$^+$ strains produced colicin in 57% of faecal, 75% of urinary and 80% of extraintestinal isolates. The simultaneous occurrence of these two properties in the three groups of materials was not significant. On the other hand, Col$^+$ strains that harboured K1 antigen simultaneously were more frequent (73/123) among strains belonging to extraintestinal samples than those isolated from faecal (12/115) and urinary (21/122) materials.

In serogroups O1, O2, O7, O18 more than half of the strains had one of the two properties. Strains belonging to serogroups O4 and O6 failed to have K1 and produced colicin very rarely.

As to the correlation between K1 and Col V, only 1 K1$^+$, Col V$^+$ strain occurred among 21 K1$^+$ and 10 Col V$^+$ strains in faeces, 4 K1$^+$, Col V$^+$ among 28 K1$^+$ and 35 Col V$^+$ in urinary isolates, while 16 K1$^+$, Col V$^+$ among 91 K1$^+$ and 36 Col V$^+$ in other extraintestinal isolates.

Correlation of Hly with K1 and Col. It was revealed that only 5 out of 140 K1$^+$ and 210 Hly$^+$ strains had both characteristics, mostly representing serogroup O1 (Table VII). There was no noticeable correlation between Hly and Col either; 49 out of 360 Col$^+$ strains were Hly$^+$ and 49 out of 210 Hly$^+$ strains produced colicin (Table VIII).

Simultaneous occurrence of MRA and K1 positivity. The results were equivocal (Table IX) as 5 K1$^+$, MRA$^+$ strains were found among 21 K1$^+$ and 89 MRA$^+$ isolates in faeces, 18 among 28 K1$^+$ and 91 MRA$^+$ in urine, and 21 out of 91 K1$^+$ and 89 MRA$^+$ in other extraintestinal isolates. According to these results, the K1 antigen was significantly more frequent among MRA$^+$

Table VII

Correlation between MRA, Hly and K1 positivity in E. coli strains

Serogroup	No. of strains										Total
	MRA$^+$ Hly$^+$ K1$^+$			MRA$^+$ Hly$^+$ K1$^-$	MRA$^+$ Hly$^-$ K1$^+$	MRA$^-$ Hly$^+$ K1$^+$	MRA$^-$ Hly$^-$ K1$^+$	MRA$^-$ Hly$^+$ K1$^-$	MRA$^+$ Hly$^-$ K1$^-$	MRA$^-$ Hly$^-$ K1$^-$	
	Faeces	Urine	Extra-intestinal								
O1	0	0	3	0	19	0	9	0	7	8	46
O2	0	0	0	4	14	1	11	6	11	24	71
O4	0	0	0	32	0	0	0	7	4	10	53
O6	0	0	0	45	0	0	0	21	14	11	91
O7	0	0	0	0	7	0	7	0	2	17	33
O18ac	0	0	0	28	0	0	36	3	9	11	87
O75	0	0	0	5	0	0	0	5	0	15	25
Others	0	0	0	25	0	1	32	24	40	628	750
Total	0	0	3	139	40	2	95	66	87	724	1156

Table VIII

Correlation between MRA, Hly and Col positivity in E. coli strains

Serogroup	No. of strains										Total
	MRA+ Hly+ Col+			MRA+ Hly+ Col−	MRA+ Hly− Col+	MRA− Hly+ Col+	MRA− Hly− Col+	MRA− Hly+ Col−	MRA+ Hly− Col−	MRA− Hly− Col−	
	Faeces	Urine	Extra-intes-tinal								
O1	0	0	2	1	22	0	7	0	4	10	46
O2	0	0	0	4	20	0	18	7	5	17	71
O4	5	0	3	24	0	1	0	6	4	10	53
O6	4	1	7	33	6	5	1	16	8	10	91
O7	0	0	0	0	8	0	10	0	1	14	33
O18ac	7	1	0	20	3	3	34	0	6	13	87
O75	1	2	0	2	0	1	2	4	0	13	25
Others	1	0	0	24	21	5	159	20	19	501	750
Total	18	4	12	108	80	15	231	53	47	588	1156

Table IX

Correlation between the occurrence of MRA and K1 positivity of E. coli strains in different materials

Serogroup	No. of strains isolated from										Total examined
	Faeces			Urine			Extraintestinal material				
	MRA+ K1+	MRA+ K1−	MRA− K1+	MRA+ K1+	MRA+ K1−	MRA− K1+	MRA+ K1+	MRA+ K1−	MRA− K1+	MRA− K1−	
O1	2	5	3	10	1	3	9	2	4	7	46
O2	0	3	3	6	12	2	8	0	7	30	71
O4	0	11	0	0	7	0	0	18	0	17	53
O6	0	14	0	0	21	0	0	24	0	32	91
O7	3	0	1	0	1	0	4	1	6	17	33
O18ac	0	22	1	0	5	1	0	10	34	14	87
O75	0	2	0	0	3	0	0	0	0	20	25
Others[1]	0	27	8	2	23	4	0	13	19	654	750
Total	5	84	16	18	73	10	21	68	70	791	1156

[1] Other serogroups (see Tables I, II, III), strains not typable and agglutinting spontaneously

Table X

Correlation between MRA, K1, Col and Hly in serogroup O18

No. of strains		K1		Col		Hly	
		+	−	+	−	+	−
MRA	+	37	0	37	11	26	28	9
	—	36	36	0	32	4	0	36

urinary and other extraintestinal isolates than among MRA$^+$ faecal strains. MRA positivity of K1$^+$ strains was, however, most frequent among urinary strains.

As to a correlation between serogroups and the simultaneous presence of MRA$^+$ and K1$^+$ feature, MRA positivity was found only in serogroups O1 : K1, O2 : K1, O7 : K1. It was most striking that in serogroup O18ac, K1 and MRA positivity never occurred simultaneously. In this serogroup none of the 37 MRA$^+$ strains possessed K1 antigen, while the 36 K1$^+$ strains were devoid of MRA activity (Table X). On the basis of electron microscopic examinations, 34 out of 36 O18ac, K1$^+$, MRA$^-$, and by far the greatest number of Col$^+$ strains harboured fimbrial structures. Broth cultures of 32 out of 36 strains showed a mannose sensitive haemagglutination of guinea pig erythrocytes [13].

In view of the close correlations existing between K1$^+$ and Col$^+$ strains on the one hand and between MRA$^+$ and K1$^+$ isolates on the other, it seemed interesting to analyse the relation between MRA and Col production (Table XI). The correlation between these two properties was independent from the origin of strains. MRA$^+$, Col$^+$ properties were found together in 41 out of 89 MRA$^+$ and 115 Col$^+$ faecal strains in 30 out of 91 MRA$^+$ and 122 Col$^+$ urinary isolates and in 44 out of 89 MRA$^+$ and 123 Col$^+$ extraintestinal samples ($p > 0.02$ and $p > 0.05$, respectively).

To find out whether correlations between MRA–K1, K1–Col, MRA–Col mean also an MRA–K1–Col correlation, the occurrence of these three properties was also analysed (Table XII). They were found together in 5 out of

Table XI

Correlation between the occurrence of MRA and Col of E. coli strains in different materials

Serogroup	No. of strains isolated from									Total	
	Faeces			Urine			Extraintestinal material				
	MRA$^+$ Col$^+$	MRA$^+$ Col$^-$	MRA$^-$ Col$^+$	MRA$^+$ Col$^+$	MRA$^+$ Col$^-$	MRA$^-$ Col$^+$	MRA$^+$ Col$^+$	MRA$^+$ Col$^-$	MRA$^-$ Col$^+$	MRA$^-$ Col$^-$	examined
O1	6	1	3	10	1	3	8	3	1	10	46
O2	3	0	4	9	9	7	8	0	7	24	71
O4	6	5	0	0	7	0	3	15	0	17	53
O6	6	8	1	2	19	3	10	14	2	26	91
O7	3	0	0	0	1	5	5	0	5	14	33
O18ac	8	14	0	1	4	5	2	8	32	13	87
O75	1	1	1	2	1	1	0	0	1	17	25
Others[1]	8	19	65	6	19	68	8	5	31	521	750
Total	41	48	74	30	61	92	44	45	79	642	1156

[1] Other serogroups (see Tables I, II, III), strains not typable and agglutinating spontaneously

Table XII

Correlation between MRA, K1 and Col positivity in E. coli strains

Serogroup	No. of strains										Total
	MRA+ K1+ Col+			MRA+ K1+ Col−	MRA+ K1− Col+	MRA− K1+ Col+	MRA− K1− Col+	MRA− K1+ Col−	MRA+ K1− Col−	MRA− K1− Col−	
	Faeces	Urine	Extra-intes-tinal								
O1	2	9	7	3	6	7	0	3	2	7	46
O2	0	5	8	1	7	5	13	7	8	17	71
O4	0	0	0	0	9	0	0	0	27	17	53
O6	0	0	0	0	18	0	6	0	41	26	91
O7	3	0	4	0	1	5	5	2	1	12	33
O18ac	0	0	0	0	11	32	5	4	26	9	87
O75	0	0	0	0	3	0	3	0	2	17	25
Others	0	1	0	1	21	18	146	13	42	508	750
Total	5	15	19	5	76	67	178	29	149	613	1156

489 faecal, in 15 out of 384 urinary and in 19 out of 283 other strains isolated from extraintestinal sources. It can also be seen from Tables X and XII that in serogroup O18ac, 32 out of 36 $K1^+$ strains were Col^+ all the 36 were MRA^-, and all the 37 MRA^+ strains were $K1^-$, and 26 out of them were Col^-. This sharp separation of two properties (i.e. K1–MRA, Col–MRA) failed to occur among strains of other serogroups (27.8% of $K1^+$ strains were MRA^+ and 42.7% of MRA^+ isolates produced colicin).

The simultaneous occurrence of MRA–Hly–K1 and MRA–Hly–Col properties are shown in Tables VII and VIII. Association of MRA–Hly–K1 was infrequent. On the contrary, MRA and Hly positivity were found frequently together, especially in serogroups O4, O6 and O18. In case of serogroup O18 : K1, MRA or Hly positivity had never occurred (Tables VII, X). Strains which were carriers for MRA–Hly–Col were also very rare (36 out of 1156), since Hly–Col properties hardly occurred simultaneously.

Only two strains (belonging to serogroup O1) out of 1156 possessed all the four properties (Hly–MRA–K1–Col).

Discussion

The capsular structures of *E. coli* bacteria have been considered important virulence factors of extraintestinal infections [21, 22]. The outcome of bacteriaemia, meningitis, or pyelonephritis of an infection caused by *E. coli* will be worse if the agent contains K1 antigen [23]. Our results concerning on the incidence of *E. coli* $K1^+$ strains corresponded to literary data [7, 24] as

K1 isolates occurred more frequently in extraintestinal [32.1%] than in faecal [4.3%] or urinary [7.3%] isolates. The finding that we frequently found K1 antigen in strains isolated from blood (13 out of 40) is slightly inconsistent with the observation of Sarff et al. [7]. Moreover, in contradiction to Achtman's data cited by Ørskov and Ørskov [26] we isolated strains belonging to serogroups O1 : K1 and O2 : K1 from the blood of septic patients (Table III).

It has also been known [7, 23, 25] that $K1^+$ strains occur mainly in serogroups O1, O2, O7, O18 and less frequently in serogroups O16, O83, O156 [7]. However, we identified strains harbouring antigen K1 in serogroups O5, O12, O21, O23 and O33 as well.

In contrast to Robbins et al. [6], we failed to show antigen K1 in serogroup O6. In agreement with the observations of Sarff et al. [7] and Pluschke et al. [25], the great majority of our isolates originating from CSF belonged to serogroup O7 : K1 and O18 : K1, while in contrast with their findings we failed to show antigen K1 in O7 strains isolated from pyelonephritis.

The high incidence of $K1^+$ isolates among strains originating from the gynaecological and perinatal ward of a hospital (Table IV) draw attention to that many newborns were colonized by potentially invasive K1 strains and that there is a possibility of spreading the infections from mother to infant, and of cross infecting infants via the hands of the nursery staff. Similar observation were described by Sarff et al. [7], and Glode et al. [27]. It is curious that none of those 13 newborns who harboured strains with antigen K1 in their umbilical cord and upper respiratory tract developed *E. coli* meningitis or sepsis and none had become ill of the babies of those 13 mothers who carried $K1^+$ strains in their vagina. However, in another hospital a mother having a rectal fistula and harbouring *E. coli* O18ac : K1 in her vagina infected her infant, who developed a fatal meningitis.

Data in the literature and the present results indicated that K1 capsule which is possessed only by a few serogroups (O1, O2, O7, O18) plays an important role in the pathogenicity of *E. coli* especially in extraintestinal infections.

Gupta et al. [8] stated that antigen K5 occurred among the most frequent K antigens in strains from extraintestinal diseases. In the opinion of Jann and Jann [9] the receptors of K5 antigens play a very important role in the pathogenicity of *E. coli*. The present studies have failed to confirm these results. We were unable to show any difference in the incidence of $K5^+$ strains between faecal and urinary (3.3%; 3.1%) or between urinary and other extraintestinal (3.1%; 5.3%) isolates.

In agreement with Gupta et al. [8] our $K5^+$ strains belonged to serogroups O2, O6, O18 and O75, but in contrast to their findings antigen K5 was absent from our strains belonging to serogroup O10. According to Pluschke et al. [25] and Kusecek et al. [28] O18 : K5 bacteria were rarely isolated from

neonatal meningitis, but they were common in faeces and UTI. We had a similar observation. We confirmed the pathogenic role of O18ac : K5 isolates only in 2 out of 42 patients harbouring $K5^+$ strains (one of these $K5^+$ strains was isolated from necropsy material, the other from a subphrenical abscess).

According to Davies et al. [3] the frequency of colicin producing strains ranged between 31–51% among faecal, urinary and blood isolates. The observations of Murray et al. [29] were similar to those of Davies et al. [3] in that 26 out of 124 *E. coli* originating from diarrhoeal stools produced colicin. Similar results were described by Csiszár et al. [30] in Hungary, as 48.9% of their strains isolated from urine of pregnant women with asymptomatic bacteriuria proved to be colicin producers.

In the present study, Col^+ isolates occurred also frequently in all groups of samples, especially in extraintestinal specimens other than urine.

Several studies have shown the Col V plasmid to be one of the virulence factors of *E. coli* [31–33] and that the virulence was independent of colicin V production [10, 34, 35]. In our material Col V producer strains occurred significantly more frequently among urinary and other extraintestinal isolates than those isolated from faecal flora. These data support earlier literary data; for example Minshew et al. [36] also found that colicin V producers occurred with high frequency in isolates from other extraintestinal sources (16%). Agüero et al. [37] reported 53% Col V^+ strains among blood isolates, and 25% Col V^+ strains among CSF isolates. On the other hand, Davies et al. [3] found that the proportion of Col V^+ strains from urine (60%) was not significantly greater than that of Col V^+ strains among Col^+ faecal isolates (43%).

In contradiction to these data, our survey revealed a significant difference in Col V positivity of strains isolated from urine (28% of Col^+ strains) as compared to Col V positivity in faecal strains (8% of Col^+ strains).

Col V^+ strains occurred mainly in serogroups O1, O2, O7 and O18ac in our material. We have never found Col V producers in serogroups O4, O6 and O75. This was in agreement with the results of Smith and Huggins [32] and Milch et al. [35] who found that Col V producer strains were common in serogroup O18ac as well as in serogroups O1, O2, O7. Nowicki [38] has also observed that the frequency of Col V^+ strains in serogroups O6 and O75 was very low.

A close association between $K1^+$ Col^+ properties was observed in our study. The two properties occurred simultaneously in 2.4% of faecal, in 5.2% of urinary and mainly among strains isolated from extraintestinal sources other than urine [24%]. The high incidence of $K1^+$ isolates in CSF, umbilical cord of newborns and in the vagina of pregnant women explains the possible source of meningeal infection of the newborns by *E. coli*. Moreover the frequent coexistance of K1 and Col in serogroups O1, O2, O7, O18 offers a further explanation for the extraintestinal pathogenicity of these serogroups.

The phenomena that Col positivity of K1$^+$ strains was independent of the site of infection and K1 positivity of Col$^+$ strains was most frequent among isolates of other extraintestinal materials followed from the high incidence of Col$^+$ strains in every material and the high incidence of K1$^+$ strains in other extraintestinal samples. Also the high incidence of MRA positivity among K1$^+$ strains isolated from UTI was probably due to the fact that UTI strains very frequently harbour mannose resistant adhesins.

The findings were remarkable that there was no association between Hly positivity and K1 positivity, and that MRA positivity and K1 positivity of our strains correlated mainly in serogroups O1 and O2, while in serogroup O18ac MRA$^+$ and K1$^+$ properties had never occurred simultaneously.

The fact that K1$^+$ and MRA$^+$ strains harbour Col V plasmid with a high frequency has been shown by Agüero et al. [37]. However, the present work has revealed that this rule is valid only for serogroups O1 and O2 and sometimes for serogroup O7 (Tables X and XII). The overwhelming majority of O18ac : K1 strains produce colicin, but MRA$^+$ O18ac strains have no K1 antigen and rarely produce colicin. Thus, in serogroup O18 the two properties (MRA–K1) are strictly separated from each other.

In a previous study [5] a marked correlation was revealed between MRA and Hly positivity of certain serogroups. In the present examinations there was no definite correlation between either MRA–Hly–K1 or MRA–Hly–Col. Accordingly, the close connection between MRA–Hly shown in the previous study [5] is not associated with other virulence factors (i.e. K1, Col). This could not be attributed to the incompatibility of MRA–K1, as in serogroup O1 and O2 they very frequently occurred simultaneously; nor to the incompatibility of MRA–Col since half of our MRA$^+$ strains were Col$^+$ as well.

Another interesting observation was that, in agreement with Achtman et al. [39], strains belonging to serogroups O1, O7 and O75 produced haemolysin rarely (13 out of 104 — see Table VII). Our findings agreed with those of Achtman et al. [39] in many other respects, too: their O18ac : K1 strains belonging to membrane pattern 9 and originating from England and Finland were also Col$^+$ and Hly$^-$ (see our results in Tables X and VII). We supposed that our strains rather belonged to their membrane pattern 9 than to their membrane pattern 6. We have to remark that 25 out of our 32 O18ac : K1, Col$^+$ strains originated from the GDR. Our supposition was verified by the examination of G. Seltmann (personal communication) according to which both the isolates originating from Hungary and GDR belonged to the same OMP pattern 9 as O18ac : K1 strains of Achtman et al. Hly positivity of the strains of Achtman et al. [39] was characteristic of O18ac : K1, belonging to membrane pattern 6, that had originated from the USA. None of our O18ac : K1 strains were Hly$^+$.

Their O18ac : K5 strains were Hly$^+$ and Col$^-$, similar to ours (see

Tables VII, X, XII). On the basis of their and of our data, we should like to emphasize that there is no correlation between Hly–Col, while there is a close association between Kl–Col properties.

In agreement with the data of Achtman et al. [39] 5 out of 46 *E. coli* O1 strains failed to produce detectable amounts of indole in Kovács's indole test [40] after 24 h incubation, but after 72 h, the Kovács test was positive. If examined by the xylene extraction technique [40], indole positivity could be demonstrated after 24 h. Similarly to us Achtman et al. [39] have found that strains belonging to serogroups O4 and O6 were MRA^+, Hly^+ and Col^-.

Briefly, results showing a close connection among certain serogroups (O1, O2, O4, O6, O7, O18) and certain markers of pathogenicity (MRA, Hly, Kl, Col) support the concept of many workers [2, 26, 39] that *E. coli* strains have a clonal connection.

Examinations concerning the comparison of different virulence markers with LD_{50} of strains in mice and with other in vivo tests are in progress.

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FERRIC AMMONIUM CITRATE DECOMPOSITION — A TAXONOMIC TOOL FOR GRAM-NEGATIVE BACTERIA

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The iron uptake test of Szabó and Vandra has been modified and used for the differentiation of Gram-negative bacteria. Nutrient agar containing 20 g per litre of ferric ammonium citrate was distributed into narrow tubes and solidified so as to form butts and slants. Considering the localization of the rusty-brown coloration produced after seeding and incubation, 2367 strains were classified into four groups. (1) Unchanged medium: *Escherichia coli*, *Shigella* spp., *Yersinia* spp., *Hafnia alvei* and *Morganella morganii* 100% each, *Klebsiella* spp., 50%, *Enterobacter cloacae* 37%, *Proteus vulgaris* 59%, *Acinetobacter* spp. 42%, *Pseudomonas fluorescens* 19%, some other bacteria 2–12%. (2) Rusty-brown slant, unchanged butt: *Salmonella* subgenera II, III and IV 98%, *Citrobacter freundii* 65%, *E. cloacae* 55%, *P.vulgaris* 41%, *Proteus mirabilis* 98%, *Providencia rettgeri* 100%, urease-negative *Providencia* 96%, *Acinetobacter* spp. 58%, *Pseudomonas aeruginosa* 100%, *P. fluorescens* 81%, UFP (unclassified fluorescent pseudomonads) 100%, other *Pseudomonas* spp. 55%. (3) Unchanged slant, brown butt: *S. typhi* 88%, *Salmonella* subgenus I 3%, *Klebsiella* spp. 31%, some other bacteria 2–3%. (4) Rusty-brown slant, brown butt: *Salmonella* subgenus I 75%, *C. freundii* 20%, *Klebsiella* spp. 12%, some other bacteria 1–5%. Colour reactions in ferric ammonium citrate agar are associated with the accumulation of ferric hydroxide: bacteria giving positive reactions on the slant took up as an average, 63 times more iron than those with negative test. The localization of colour reaction correlated partly with aerobic and anaerobic citrate utilization or decomposition in Simmons' minimal and in Kauffmann's peptone water medium.

In 1963 Szabó and Vandra [1] elaborated a test for the intrageneric differentiation of acid-fast bacteria. If cultured on Löwenstein–Jensen egg medium containing 2% iron ammonium citrate, certain species of rapidly growing mycobacteria accumulated iron and produced rusty-brown colonies. The value of the "iron uptake" test was confirmed by Tison et al. [2] and Wayne and Doubek [3]. Pattyn and van Ermengen [4] have found that on peptone water agar the reaction proceeds even more rapidly than on Löwenstein–Jensen medium. The "iron uptake" test of Szabó and Vandra has attracted world-wide attention in the identification of mycobacteria [5].

A constant need for new taxonomic labels for an improved characterization of microorganisms has led us to test the value of this method in the classification of Gram-negative bacteria.

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Materials and methods

Bacterial strains. Reference strains maintained in the Hungarian National Collection of Medical Bacteria, Budapest [6] and fresh isolates obtained from Hungarian Public Health laboratories were used (Table I). The cultures represented different biochemical, serological and phage-pattern entities and were isolated from a wide variety of sources.

Ferric ammonium citrate agar and test procedure. Lab-Lemco beef extract (Oxoid), 4 g; yeast extract (Oxoid No. L21), 4 g; Tryptone (Oxoid No. L42), 8 g; agar (Oxoid No. 1), 10 g; tap water, 1000 ml; after sterilization at 121 °C for 30 min, 20 g ferric ammonium citrate (brown, hydrated) was dissolved by boiling in 20 ml distilled water and added to the medium; the pH was adjusted to 7.4–7.6 with N NaOH. The medium was distributed aseptically in 100 × 10 mm tubes and cooled to form deep (25 mm) buts and slants (35 mm). Inoculation was performed by stabbing into the butt and streaking the slant. Incubation lasted for 3 days at 37 °C (at 30 °C for some non-fermenters).

Other citrate media. Simmons' liquid, Christensen's solid and Kauffmann's liquid citrate media were prepared as described [7, 8]. Anaerobic incubation in Simmons' and Kauffmann's citrate media was performed by covering the media with a layer of liquid paraffin immediately after removal from the sterilizer.

Determination of iron content of the cultures. Strains chosen for iron content assay were grown in Roux flasks with ferric ammonium citrate agar. The surface growth was harvested and washed four times in distilled water by centrifugation at 4000 rpm for 2 h for each washing. The packed cells were dried with three changes of acetone at 105 °C. One hundred mg of dried bacteria were mixed with 10 drops of cc. H_2SO_4 and 10 ml of cc. HNO_3, then under gentle heating cc. HNO_3 was added at small portions until a clear solution was obtained. Nitrous gases were left to evaporate and the sample was cooled to room temperature [9].

Iron content was determined using the thiocyanate method [9]. The sample was evaporated to dryness. After cooling, the residue was wetted with 0.5 ml cc. HNO_3 then taken up in 100 ml distilled water. To 50 ml of appropriately diluted (1 : 10–1 : 1000) sample 0.2 ml of 3.2% $KMnO_4$ (w/v) was added. After mixing and leaving to stand for 5 min, 2 ml 20% (w/v) KSCN solution was added. A blank solution prepared in a similar way without sample was titrated with standard iron solution (ferrous ammonium sulphate equivalent with 0.05 mg iron per ml) until the sample and blank were identical in colour. The amount of Fe in the sample was calculated by considering the volume of standard iron solution used.

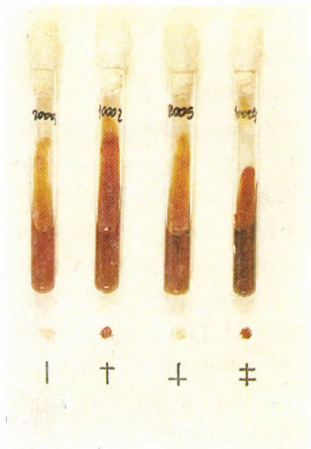

Fig. 1. Growth of bacteria in ferric ammonium citrate agar. Tubes from left to right: *S. flexneri*, *S. paratyphi-A*, *K. pneumoniae*, *S. bispebjerg*. Spots seen under the tubes were prepared by scraping off the growth from the slant of the corresponding tube and streaking it on a strip of filter paper. Results are designated by signs in the lower row: | unchanged slant and unchanged butt; † rusty-brown growth on slant, unchanged butt; ⊥ unchanged slant, rusty-brown colour reaction along the stab; ‡ rusty-brown growth on slant, brown to greyish black colour reaction along the stab

Results

Reactions of Gram-negative bacteria in ferric ammonium citrate agar. Figure 1 shows a negative and three kinds of positive test in ferric ammonium citrate agar. The same colour as that of the uninoculated tube (yellow) or a slight brownish coloration in the butt was interpreted as a negative test and was recorded as |. A rusty brown growth on the slant with unchanged butt was designated †. Two other kinds of reaction were brown coloration along the stab or throughout the butt (╪) and a variable shade of brown colour reaction both on the slant and in the butt (‡). Changes in colour on the slant could be demonstrated by scraping off the growth and streaking it on a piece of filter paper; a positive test was characterized by a rusty brown colour of the spot.

Table I shows that *E. coli*, *Shigella* spp., *Yersinia* spp., *H. alvei* and *M. morganii* and part of other organisms, although grew well in the medium, failed to develop a colour reaction. The 6 negative *Salmonella* subgenus I strains included *S. abortus-ovis* and *S. typhi-suis*. Most *E. coli* and *H. alvei* and about 10% of *C. freundii* strains exhibited a slight diffuse brownish coloration in the butt after 2 days, which was easily distinguishable from a positive reaction characterized by a marked brown, dark-brown or even greyish-black colour along the stab. A rusty-brown growth on the slant was exhibited mainly by *Salmonella* subgenera II, III and IV, *C. freundii*, *Providencia* and *Pseudomonas*. *P. mirabilis* and part of *P. vulgaris* and *Acinetobacter* isolates gave a similar reaction, but usually only after incubation for 2 days. Most *S. typhi* and some other *Salmonella* subgenus I strains including *S. abortus-equi* and some *S. cholerae-suis* isolates, caused a rusty brown colour reaction along the stab after 1 or 2 days. A brown colour of the slant and of the butt (‡) was characteristic mainly of subgenus I *Salmonella* strains. Variable reactions were recorded for different isolates of *C. freundii*, *K. pneumoniae*, *E. cloacae*, *P. vulgaris*, *Acinetobacter* and *Pseudomonas* spp. other than *P. aeruginosa* and UFP.

Table II shows the number of strains giving positive tests after different periods of incubation. For all strains examined, 60.2% of the reactions occurred within 1 day. If taxa exhibiting delayed reactions frequently or regularly *(S. typhi, P. vulgaris, P. mirabilis* and *Acinetobacter)* are disregarded, 89.1% of the positive tests appeared already after 1 day incubation. Two-day readings gave evidence of positive test in 96.5% of all strains.

Correlation between reactions in ferric ammonium citrate agar and citrate decomposition in other media. In the course of testing various bacteria in ferric ammonium citrate medium it became obvious that positive tests were given mostly by those organisms which were able to decompose citrate in other media, as Simmons' citrate (free from organic nitrogen source), Christensen's

Table I

Behaviour of Gram-negative bacteria in ferric ammonium citrate agar

Genus	Species, subgenus or biogroup	No. of strains	Results in ferric ammonium citrate agar*							
					†		↓		‡	
			No.	%	No.	%	No.	%	No.	%
Escherichia	*coli*	272	272	100.0	—	—	—	—	—	—
Shigella	*boydii*	33	33	100.0	—	—	—	—	—	—
	dysenteriae	30	30	100.0	—	—	—	—	—	—
	flexneri	145	145	100.0	—	—	—	—	—	—
	sonnei	95	95	100.0	—	—	—	—	—	—
Yersinia	*enterocolitica*	11	11	100.0	—	—	—	—	—	—
	pseudotuberculosis	5	5		—		—		—	
Salmonella	*typhi*	59	2	3.4	2	3.4	52	88.2	3	5.1
	subgenus I	269	6	2.2	53	19.7	8	3.0	204	75.1
	subgenus II	13	—		13		—		—	
	subgenus III	67	2	3.0	65	97.0	—	-	—	—
	subgenus IV	1	—		1		—		—	
Citrobacter	*freundii*	65	8	13.3	42	64.6	2	3.1	13	20.0
Edwardsiella	*tarda*	1	—		—		1		—	
Klebsiella	*pneumoniae*	137	70	51.1	11	8.0	40	29.2	16	11.7
	oxytoca	8	2		—		5		1	
Enterobacter	*cloacae*	54	20	37.0	30	55.5	4	7.4	—	—
	aerogenes	1	—		—		1		—	
Serratia	*marcescens*	3	—		3		—		—	
Hafnia	*alvei*	51	51	100.0	—	—	—	—	—	—
Proteus	*vulgaris*	112	66	58.9	46	41.1	—	—	—	—
	mirabilis	359	7	1.9	352	98.1	—	—	—	—
Morganella	*morganii*	110	110	100.0	—	—	—	—	—	—
Providencia	*rettgeri*	44	—	—	44	100.0	—	—	—	—
	urease-negative	82	—	—	79	96.4	2	2.4	1	1.2
Pseudomonas	*aeruginosa*	195	—	—	195	100.0	—	—	—	—
	fluorescens	37	7	18.9	30	81.1	—	—	—	—
	UFP**	50	—	—	50	100.0	—	—	—	—
	other	20	9	45.0	11	55.0	—	—	—	—
Acinetobacter	*calcoaceticus*	19	3	15.8	16	84.2	—	—	—	—
	lwoffii	19	13	68.4	6	31.6	—	—	—	—
Total		2367	967	40.8	1049	44.3	115	4.8	238	10.1

* Time of incubation, 3 days; for explanation of signs, see Fig. 1

** Unclassified fluorescent pseudomonads [10, 11]

Table II

Positive results in ferric ammonium citrate agar after different periods of incubation

Organism	Sign*	No. of strains	Positive within		
			1 day	2 days	3 days
S. typhi	†	2	2	—	—
	⸸	52	19	26	7
	‡	3	—	—	3
Salmonella subg. I	†	53	47	6	—
	⸸	8	—	6	2
	‡	204	179	20	5
Salmonella subg. other	†	79	72	7	—
C. freundii	†	42	42	—	—
	⸸	2	—	2	—
	‡	13	1	12	—
K. pneumoniae	†	11	11	—	—
	⸸	40	40	—	—
	‡	16	16	—	—
E. cloacae	†	30	29	—	1
	⸸	4	1	3	—
P. vulgaris	†	46	—	41	5
P. mirabilis	†	352	1	349	2
P. rettgeri	†	44	44	—	—
Providencia urease-neg.	†	79	77	2	0
	⸸	2	—	1	1
	‡	1	—	1	—
P. aeruginosa	†	195	195	—	—
Pseudomonas UFP	†	50	50	—	—
P. fluorescens	†	30	4	7	19
Pseudomonas other	†	11	7	—	4
A. calcoaceticus	†	16	—	16	—
A. lwoffii	†	6	—	6	—
Total No.		1391	837	505	49
per cent			60.2	36.3	3.5

* For explanation of signs, see Fig. 1

citrate (containing a low amount of yeast extract) and in Kauffmann's citrate (rich in peptone). To study the correlation, Simmons' and Kauffmann's citrate were inoculated with strains representing different species and incubated not only aerobically but also anaerobically.

Table III shows that bacteria unable to utilize citrate were negative in ferric ammonium citrate agar. The 16 *E. coli* strains which decomposed citrate in 14 days in Kauffmann's medium, were also negative. Most citrate-utilizers (*Salmonella*, *Citrobacter*, *P. mirabilis*, *P. rettgeri*, urease-negative

Providencia, *P. aeruginosa* and UFP) gave some kind of positive reaction in ferric ammonium citrate agar. Part of pseudomonads *(P. aminovorans*, *P. diminuta*, *P. paucimobilis*, *P. vesicularis)* were negative in all citrate media, others *(P. cepacia*, *P. putrefaciens*, *P. pseudoalcaligenes*, *P. pickettii)* gave divergent results in Simmons' and in ferric ammonium citrate agar. The behaviour of *A. calcoaceticus* and *A. lwoffii* in ferric ammonium citrate agar (84.2 and 31.6% positive) corresponded to that of literary data on citrate utilization (94 and 40% positive, respectively).

Isolates belonging to other taxa frequently exhibited diverse reactions. *H. alvei* which produces a delayed positive test in Simmons' citrate in about 60% [8], was uniformly negative in ferric ammonium citrate agar (Tables I and III). *K. pneumoniae* is known to grow in Simmons' citrate in 98% and *E. cloacae* in 86–100% (8, 12); yet, they were negative in ferric ammonium citrate agar in 51.1 and 37.0%, respectively. In contrast, for *P. vulgaris*, which utilizes citrate in Simmons' medium only in 10–24% [8, 12], the ferric ammonium citrate agar proved to be more sensitive (positive in 41.1%, Table I).

It seemed that a colour reaction on the slant of ferric ammonium citrate agar corresponds to aerobic, and that in the butt to an anaerobic breakdown of citrate. This correlation was evident in case of oxidative bacteria *(Pseudomonas*, *Acinetobacter)*, which caused a † reaction in ferric ammonium citrate agar and decomposed citrate only in aerobically incubated tubes of Simmons' and Kauffmann's medium (Table III).

With *Enterobacteriaceae*, there were also some correlations. *S. typhi* only attacked citrate in peptone water (Kauffmann's medium) and usually gave an anaerobic type (⸸) reaction in ferric ammonium citrate agar. Most *Salmonella* subgenus I strains decomposed citrate in Simmons' and in Kauffmann's medium both aerobically and anaerobically and exhibited, accordingly, an aerobic + anaerobic type reaction (‡) in ferric ammonium citrate agar.

For *C. freundii*, *K. pneumoniae* and *E. cloacae* there seemed to be no correlation between aerobic or anaerobic citrate breakdown and the type of reaction in ferric ammonium citrate medium. It was interesting that *P. vulgaris* and *P. mirabilis* decomposed citrate in Simmons' only aerobically and in Kauffmann's only anaerobically, whereas *P. rettgeri* and urease-negative *Providencia* strains did so aerobically and anaerobically in both media.

Absence of correlation between reactions in ferric ammonium citrate agar and H_2S production. Although ferric ammonium citrate agar contained no sulphur source except small amounts of cystine, cysteine and methionine in the peptone, it seemed interesting to check whether or not the colour reaction in the butt was associated with the ferrous sulphide produced as a result of H_2S formation.

If ferrous sulphate, ferric sulphate or ferrous ammonium sulphate were substituted for ferric ammonium citrate in the basal medium, salmonellae

Table III

Comparison of reactions in ferric ammonium citrate agar, Simmons' citrate and Kauffmann's citrate

Organism	No. of strains	Ferric ammonium citrate agar*		Simmons' citrate positive**		Kauffmann's citrate positive***	
		sign	No.	aerobic No.	anaerobic No.	aerobic No.	anaerobic No.
E. coli	20	|	20	—	—	—	—
S. boydii	5	|	5	—	—	—	—
S. dysenteriae	5	|	5	—	—	—	—
S. flexneri	5	|	5	—	—	—	—
S. sonnei	3	|	3	—	—	—	—
Y. enterocolitica	11	|	11	—	—	—	—
Y. pseudotuberculosis	5	|	5	—	—	—	—
S. typhi	41	|	1	—	—	1	1
		†	5	—	—	4	5
		ǂ	35	—	—	34	35
Salmonella subg. I	20	|	1	—	—	—	—
		†	5	2	—	2	3
		ǂ	2	—	1	2	2
		‡	12	12	12	12	12
C. freundii	11	|	7	3	3	2	3
		†	4	4	3	3	3
E. tarda	1	ǂ	1	—	—	1	1
K. pneumoniae	145	|	72	67	53	26	31
		†	11	11	9	9	10
		ǂ	45	45	45	45	45
		‡	17	17	17	17	17
E. cloacae	54	|	20	19	18	17	18
		†	30	29	28	23	25
		ǂ	4	4	4	4	4
H. alvei	20	|	20	6	2	18	20
P. vulgaris	10	|	2	—	—	1	2
		†	8	7	—	—	8
P. mirabilis	10	†	10	10	—	—	8
M. morganii	20	|	20	—	—	—	—
P. rettgeri	20	†	20	20	20	20	19
Providencia urease-neg.	20	†	20	13	14	18	17
P. aeruginosa	10	†	10	10	—	10	—
Pseudomonas UFP	10	†	10	10	—	10	—

* Read after 3 days incubation; for explanation of signs, see Fig. 1

** Simmons' liquid ammonium citrate medium with 0.1% ammonium dihydrogen phosphate and 0.5% sodium citrate read after 4 days incubation. Positive test: growth and alkaline reaction in aerobic tubes; growth and/or acid reaction or decolorization of the indicator in anaerobic tubes

*** Kauffmann's liquid medium with 1% peptone and 1% sodium citrate read after 14 days incubation. Decomposition of citrate: absence of voluminous precipitate with lead acetate reagent

showed but a faint thin line of blackening along the stab, in contrast to a marked dark-brown or greyish-black coloration in the presence of ferric ammonium citrate. *C. freundii*, *P. mirabilis* and *P. vulgaris* which produce H_2S in the presence of a sufficient sulphur source (thiosulphate), developed no colour reaction when the basal medium contained iron salts other than citrate. A further proof that the colour reaction in ferric citrate agar is independent of H_2S production was that many *Klebsiella* and *Enterobacter* strains produced a rusty-brown colour in the butt of ferric ammonium citrate agar, but were uniformly

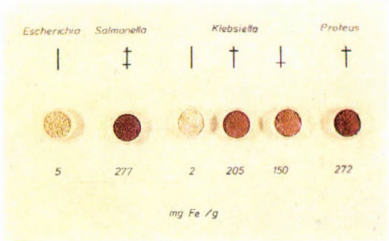

Fig. 2. Iron accumulation by different bacterial cultures grown on plates of ferric ammonium citrate agar. Upper row: sign of result (see Fig. 1). Middle row: washed and acetone-dried bacteria in plastic containers. Lower row: amount of iron (mg Fe) accumulated by 1 g dry bacteria

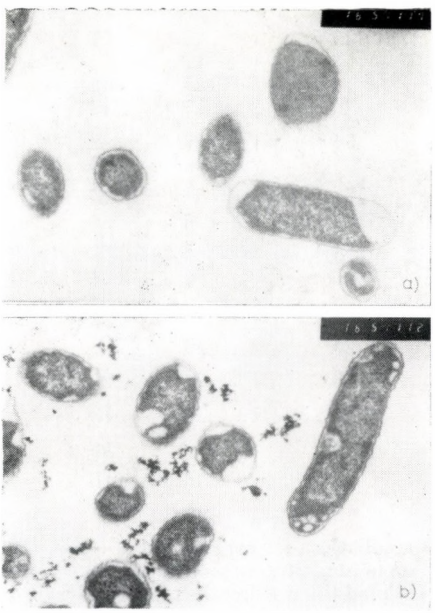

Fig. 3. Electron micrograph of washed cells of *P. rettgeri* strain 65001 grown in liquid basal medium (a) without, and (b) with, ferric ammonium citrate. Cytoplasmic membrane and structure of cytoplasm are intact; no difference in electron density and vacuolization of cytoplasm. Micrograph (b) shows that iron precipitates are confined to intercellular spaces. ×12 500

negative in thiosulphate-iron agar and exhibited no colour reaction in the basal medium supplemented with iron salts other than citrate.

Binding of iron by bacteria grown on iron ammonium citrate agar plates. Figure 2 shows that after thorough washing, bacteria that failed to develop a colour reaction in ferric ammonium citrate agar, contained iron in small amounts. Organisms positive on the slant or on the slant and in the butt, accumulated considerable amounts of iron (on the average 63 times more than those with negative test). The *K. pneumoniae* strains which produced a colour reaction only in the butt of ferric ammonium citrate agar, in Roux flask cultures took up a considerable amount of iron.

Figures 3a and 3b show electron micrographs of *P. rettgeri* grown in iron-free and iron-containing medium. The iron precipitate is confined to intercellular spaces.

Discussion

Although certain bacteria differ in the result of ferric ammonium citrate and other citrate tests, it may be assumed that all positive reactions are associated with metabolism of the same substrate. It is known that the ability of some bacteria to attack citrate varies with the medium used, e.g. *S. typhi* is usually positive in Kauffmann's but invariably negative in Simmons' citrate; *E. coli* is negative in Simmons' but in about 16% positive in Christensen's medium.

The products of citrate breakdown depend on the mode of incubation and on the kind of nitrogen source [7, 8, 13]. Under anaerobic conditions, citrate is degraded to lower acids which maintain an acid environment in the medium. In open tubes of Kauffmann's citrate at first an acid reaction occurs, since bacteria — utilizing the energy provided by an abundance of peptone — produce a low oxidation-reduction potential favourable for the accumulation of lower acids; on prolonged incubation, however, as a result of further degradation of the acids and ammonia production from the peptone, the pH shifts to alkaline. In the absence of organic nitrogen (Simmons' medium) or at low organic nitrogen content (Christensen's medium) aerobic degradation of citrate results in the production of carbonates shifting the pH toward alkalinity. In the classical citrate media splitting of the substrate is detected with pH indicators; in liquid media the result can be confirmed by adding lead acetate which forms a precipitate with unattacked citrate.

In ferric ammonium citrate agar, breakdown of the citrate complex results in a — probably extracellular — accumulation of insoluble iron compounds (mainly ferric hydroxide and perhaps also ferric oxyhydroxide). Accordingly, a new indicator system is involved which may be responsible for

differences in the results obtained with classical citrate media. *S. typhi* behaves in ferric ammonium citrate medium as expected: growing with an abundance of peptone, it decomposes the substrate. In contrast, *H. alvei* which is partly positive in Simmons' and mostly delayed positive in Kauffmann's, fails to develop a distinct colour reaction in ferric ammonium citrate agar. Negative results in ferric ammonium citrate agar for a number of *C. freundii*, *K. pneumoniae* and *E. cloacae* strains which decompose citrate in the classical media might be associated with a difference in the utilization of the iron complex and of the sodium salt of citric acid. The same difference may account also for the fact that "aerobic" (†), "anaerobic" (⸸) and "aerobic + anaerobic" (‡) reactions of *C. freundii*, *K. pneumoniae* and *E. cloacae* showed practically no correlation with the results in aerobically and anaerobically incubated Simmons' and Kauffmann's media.

Ferric ammonium citrate agar may be useful in routine identification and in taxonomic studies. The medium is easy to prepare and, if sealed airtight, can be stored for months. From data in Table I it is evident that it cannot be used as a substitute for Simmons' citrate, mainly because of the numerous negative tests with *Citrobacter*, *Klebsiella* and *Enterobacter*. On the other hand, ferric ammonium citrate agar is suitable for differentiation of *S. typhi*, *Salmonella* subgenus I and other *Salmonella* subgenera. The uniform behaviour of *P. rettgeri* and urease-negative *Providencia* strains is not only a diagnostic feature, but also a further proof that it is justified to classify these bacteria in one genus. As individual strains of different bacteria give fairly constant results in ferric ammonium citrate agar, the medium can be used for differentiation of "iron biovariants" within *C. freundii*, *K. pneumoniae*, *E. cloacae*, *P. vulgaris* and *Acinetobacter*.

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EFFECT OF METAL IONS ON THE ANTIMICROBIAL ACTIVITY OF 5-SULPHOSALICYLIC ACID

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(Received August 6, 1984)

Bis-(5-sulphosalicylato)-diaquo chelates of VO(II), Cu(II), Ni(II), Co(II), Fe(II) and Mn(II) exerted antifungal activities. The chelates have been characterised for elemental, thermal and infra-red spectral studies and were found to be more fungicidal than the chelating agent. The antifungal activity was found to be in the order: Cu(II) $\simeq$ Ni(II) > Co(II) > > Fe(II) > Mn(II) > VO(II).

The salicylates are drugs of great antiquity. The bioability of salicylates at their sites of action can be enhanced or reduced by interaction with other drugs. Several studies were carried out of the biochemical and pharmacological effects of these antimicrobial agents given with other drugs.

The metal ions have a considerable effect on the antimicrobial activity of salicylates, especially of salicylic acid. Salicylic and nuclear substituted salicylic acids form stable chelates with a number of metal ions. Recently we have investigated the antimicrobial reactivity of metal chelates of salicylic-, 4-amino-salicylic- and 3,5-dinitro-salicylic acids [1–6].

A survey of the literature shows that little work has been done on the preparation and characterization of metal chelates formed by 5-sulphosalicylic acid (5-SSA). Neither has the role of biologically occurring metals on the antimicrobial activity of 5-SSA been studied. With this objective in view we have studied the effect of various metal ions on the antimicrobial activity of 5-SSA. The present report deals with the antifungal activity of 5-SSA chelates of VO(II), Cu(II), Ni(II), Co(II), Fe(II) and Mn(II). These chelates are prepared and characterised by elemental, thermal and infra-red spectral studies. The antifungal activity is then correlated to the covalency in M-L bond.

Materials and methods

The chemicals used were of BDH AnalaR or equivalent quality. The complexes were prepared following methods described previously [7].

The metal content of all the complexes was determined by standard complexometric titration. In addition, carbon and hydrogen analyses were carried out using Coleman CHN-analyser 29.

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Physical measurements were made as described previously [8, 9]. The water content of the complexes was determined by thermogravimetric analysis (Table II). Thermogravimetry (TG) was carried out on a Stanton recording thermobalance (HT-model) of one mg sensitivity in static air with a heating rate of 4 °C/min. All the samples were packed as uniformly as possible in platinum crucible of appropriate size. The same platinum crucible was used throughout the experiments.

Differential thermal analysis (DTA) assembly with temperature programmer of F and M Scientific 240 Hewlett Packard and thermocouple platiner II of Engelhard Ltd., USA were used. DTA curves were recorded by Rikadenki Kogyo Co. Ltd. recorder in static air at a heating rate of 4 °C/min. Aluminium was used as the reference standard.

The presence of ligand and the water molecules in the complexes was identified by infra-red spectroscopy using a Perkin Elmer Grating IR-Spectrophotometer Model 377, by the KBr disk technique. The 4000–400 cm^{-1} region was scanned at fifteen min. Far IR (500–50 cm^{-1}) were scanned on Polytec FIR-30 Fouries Far Infra-red Spectrometer.

The instruments were capable of recording frequencies with an accuracy of ± 2 cm^{-1}.

The characteristic IR band positions are presented in Table III.

Fungicidal activity was evaluated against five fungi (Table IV) by the agar-plate technique [10]. The number of replications was three.

Results and discussion

Stoichiometry and behaviour. The prepared chelates, their analysis, colour, yield in per cent and abbreviated names are reported in Table I. The chelates are stable in air and insoluble in common organic solvents except DMF in which they are easily soluble. The analytical data recorded in Table I indicate the formation of ML_2—2 H_2O chelate in each case, where M stands for VO(II), Cu(II), Ni(II), Co(II), Mn(II) and Fe(II) and L for the anion of 5-SSA.

Table I

Physical and analytical data for bis-(5-sulphosalicylato)-diaquo metal chelates of bivalent metal ions

Complex[a]	Colour	Analytical data[b], per cent found		
		M	C	H
(I) $VO(5SSA)_2 \cdot 2\ H_2O$	Dark green	12.41	32.08	2.20
(II) $Cu(5SSA)_2 \cdot 2\ H_2O$	Olive green	11.91	30.98	2.31
(III) $Ni(5SSA)_2 \cdot 2\ H_2O$	Green	11.82	31.27	2.20
(IV) $Co(5SSA)_2 \cdot 2\ H_2O$	Pink	10.98	31.16	2.34
(V) $Mn(5SSA)_2 \cdot 2\ H_2O$	Buff	10.41	31.89	2.21
(VI) $Fe(5SSA)_2 \cdot 2\ H_2O$	Coffee	11.04	31.92	2.23

[a] %Yield: I(60), II(76), III(70), IV(55), V(65), VI(70)
[b] %M(cal): I(12.51), II(12.25), III(11.14), IV(11.18), V(10.52), VI(10.66)
%C(cal): I(31.40), II(31.60), III(31.57), IV(32.12), V(32.12), VI(32.07)
%H(cal): I(2.24), II(2.25), III(2.28), IV(2.27), V(2.29), VI(2.29)

Earlier data of optical, magnetic and X-ray K-absorption studies indicated distorted octahedral stereochemistry [6].

Because of the insolubility of chelates in common organic solvents single crystal growth is not possible. In absence of X-ray structure analysis it is hoped that the infra-red spectral study will throw some light on the structure of these chelates of 5-SSA.

Thermogravimetric analysis. Thermogravimetric analyses were carried out with a view to investigate the number and nature of water molecules present in the chelates. Data recorded in Table II indicate that the first weight loss observed in the 80–260 °C temperature range was due to the loss of two water molecules. The observed dehydration temperature range indicated that they are present as co-ordinated water molecules [11]. The presence of co-ordinated water molecules found further conformation by the infra-red spectra studies. The loss of two water molecules in a single step indicates that they are equally bound to the metal ion.

Table II

Thermogravimetric analysis of bis-(5-sulphosalicylato)-diaquo chelates of bivalent metal ions

Chelate	Temperature range, °C	Residual product	Per cent weight loss for two H_2O		DTA Peak* °C
			calc.	obs.	
$VO(5SSA)_2 \cdot 2\,H_2O$	110–260	$VO(5SSA)_2$	6.72	6.25	220 Endo.
$Cu(5SSA)_2 \cdot 2\,H_2O$	100–240	$Cu(5SSA)_2$	6.77	6.50	210 Endo.
$Ni(5SSA)_2 \cdot 2\,H_2O$	80–220	$Ni(5SSA)_2$	6.83	7.00	200 Endo.
$Co(5SSA)_2 \cdot 2\,H_2O$	100–230	$Co(5SSA)_2$	6.83	6.75	210 Endo.
$Fe(5SSA)_2 \cdot 2\,H_2O$	90–210	$Fe(5SSA)_2$	6.87	6.50	200 Endo.
$Mn(5SSA)_2 \cdot 2\,H_2O$	80–210	$Mn(5SSA)_2$	6.88	7.00	190 Endo.

* Endo. = endotherm

Infra-red spectras. The infra-red spectrum of solid 5-SSA is almost identical to that of its chelates in the region 2000–625 cm^{-1}. The frequencies of most interest with regard to structure are the C—O and O—H vibrations. The γ C=O band at 1665 cm^{-1} is shifted to a lower frequency ($\simeq$1630 cm^{-1}) in all the chelates, showing that chelation has taken place through the carboxyl group [12, 13]. Appearance of the new band in the neighbourhood of 840 cm^{-1} suggested the presence of co-ordinated water molecules [14–16]. A band at 335 cm^{-1} confirmed the presence of co-ordinated water [17]. The δ O—H peak at 1350 cm^{-1} remained almost at the same position for both 5-SSA and its chelates; this show that there was no loss of protons by the phenolic OH group on chelation.

The increase in the difference between γ COO (asym) and γ COO (sym), Δ C=O, has been taken as a measure of increasing covalence of M—L bond [18]. The 5-SSA chelates presented a band at $\simeq$1640 cm^{-1} for γ COO (asym)

and at $\simeq$1420 cm^{-1} for γ COO (sym). Thus the covalent character of the M—L bond (Table III) followed the order VO(II)-5SSA > Cu(II)-5SSA > > Ni(II)-5SSA > Co(II)-5SSA $\simeq$ Fe(II)-5SSA $\approx$ Mn(II)-5SSA.

Trends in positions and separation between antisymmetric and symmetric carboxylate stretching band were useful for assigning the co-ordination type of the carboxylate group. γ COO (sym) greater than 1414 cm^{-1} may be assumed as strongly indicative of bridging bidentate. Carboxylate values were in the 1400–1414 cm^{-1} spectral range of monodentate and those lower than 1400 cm^{-1} of bidentate carboxylate [19–24]. On the basis of these considerations in all our metal chelates of 5-SSA we may assume the presence of carboxylate which acts as bridging bidentate, an observation consistent with the polymeric structure suggested earlier [25].

VO(II)-5SSA exhibited a band at 850 cm^{-1} coupled with $\delta\gamma$ (H_2O) which may be attributed to the V—O stretching. The value is abnormally low, and has earlier been explained [26] in terms of the V—O—V—O bridging in polynuclear six co-ordinated oxovanadium complexes.

Antifungal activity. The results showed marked variations in the activity of 5SSA as influenced by metal ions. In each case an increase in activity was observed in comparison to parent 5SSA. Against *Cunning hamella*, 5SSA itself was found to be inactive, while its Cu(II), Co(II) and VO(II) chelates showed measurable activity. These chelates exhibited an equal activity against *C. hamella*. Cu(II) and Co(II) chelates showed activity against all the fungi used, while Mn(II)-chelate was found to be active only against *Rhizopus nigricans* and *Penicillium chrysogenum*. In general, Cu(II)-chelates showed maximum activity against all the fungi used. It appears, therefore, that metals have some role in the antimicrobial activity of 5SSA.

Increased activity due to the formation of metal chelates support the idea of Perrin [27], Foye et al. [28–30] and our earlier results [1–6], in which it was suggested that the mode of action of salicylates is dependent upon the presence of certain metal ions. The chemistry of salicylates and the potential binding sites for cations may be responsible for the activity.

Table IV shows that the fungicidal activity increases roughly in the sequence Cu(II) $\simeq$ Ni(II) > Co(II) > Fe(II) > Mn(II) > VO(II). Except for VO(II), this is the order exhibited in spectrochemical series and covalency in M—L bond.

From the above it is clear that VO(II), Cu(II), Ni(II), Co(II), Fe(II) and Mn(II) chelates of 5SSA were more active than 5SSA itself against all the fungi under investigation. This was probably due to the chelation. As a consequence, the structural variation leads to new physical and chemical properties, alters the reactivity of a molecule, which in turn may cause changes in distribution, in cells and tissues and in excretion pattern. A correlation of antifungal activity and the structure of chelates has also been found. This

Table III

Characteristic IR group frequencies for bis-(5-sulfosalicylato)-diaquo complexes

		Bis-(5-sulphosalicylato)-diaquo complexes of					
	5 SSA	Cu(II)	Ni(II)	Co(II)	Mn(II)	Fe(II)	VO++
γ COO^- (asym.)	1665 (s)	1648 (s)	1650 (s)	1640 (s)	1630 (s)	1635 (s)	1650 (s)
γ COO^- (sym.)	1445 (s)	1420 (s)	1430 (s)	1430 (s)	1420 (s)	1425 (s)	1420 (s)
γ (O—H)	2400–3600 (3200) (sb)	2800–3750 (3475) (sb)	2900–3690 (3450) (sb)	3000–3715 (3510) (sb)	2900–3670 (3400) (sb)	2700–3690 (3480) (sb)	2800–3680 (3520) (sb)
δ (O—H)	1350 (s)	1345 (s)	1350 (s)	1350 (s)	1345 (s)	1350 (s)	1360 (s)
$\delta\gamma$ (H_2O)	—	850 (s)	840 (s)	830 (s)	830 (s)	825 (s)	870 (s) * 860 (sh)
γ (M—O) + (C—C)	—	530 (w)	520 (mb)	495 (w)	490 (m)	505 (m)	510 (w)
γ (M—O) + ring before	—	440 (sb)	420 (w)	430 (m)	405 (sh)	425 (w)	430 (sh)
γ (M—OH_2)	—	335 (sb)	330 (bs)	330 (s)	335 (b)	330 (s)	332 (s)
Δ C=O	—	228	220	210	210	210	230

Δ C=O = γ COO asym. — γ COO sym.; s = sharp, sb = sharp and broad, sh = shoulder, m = medium, mb = medium and broad, w = weak, * coupled with γ V—O

Table IV

Antifungal screening of bis-(5-sulphosalicylato)-diaquo chelates of bivalent metal ions

Fungi	Bis-(5-sulphosalicylato)-diaquo chelates*						
	5SSA	VO(II)	Cu(II)	Ni(II)	Co(II)	Fe(II)	Mn(II)
Cunning hamella	—	9	8	—	8	—	—
Rhizopus nigricans	16	18	19	22	19	20	22
Penicillium chrysogenum	14	16	24	24	20	18	20
Aspergillus niger	16	—	18	18	16	16	—
Mucer sp.	14	16	16	16	20	—	—

* Antifungal activity in terms of mean diameter (mm) of inhibited zone appearing around the cup; the solvent (DMF) had no antifungal activity

information might help biochemists, physiologists and pharmacists in finding new and useful agents, but the study still needs confirmation by clinical trials.

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CYTOTOXICITY OF LYMPHOCYTES AND ANTIBODIES AGAINST AUTOLOGOUS TUMOR CELLS IN PATIENTS WITH MYELOID LEUKAEMIAS AND PRELEUKAEMIC DISORDERS

III. STAGE-DEPENDENCE OF ONCOVIRUS-SPECIFIC IMMUNE RESPONSE

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Lymphocyte and plasma samples from the quiescent and blastic phase of chronic granulocytic leukaemia (CGL) and from the blastosis and remission of acute myeloid leukaemia (AML), were compared for cytotoxic activity. Target cells were collected from the blastic phases of diseases. ^{51}Cr-release tests showed that the lymphocytes and plasma samples from blastic crisis of CGL had no cytotoxic activity for autologous blast cells. In contrast, cryopreserved lymphocytes and plasmas from the quiescent phase of CGL proved to be cytotoxic for the autologous tumor cells, and their effect could be blocked by native gp70 antigens of gibbon ape leukaemia virus (GaLV) and baboon endogenous virus (BaEV). A blocking effect was less frequently exerted by carbohydrate-free gp70 and p15(E) antigens. A similar relationship was found between the blastosis and remission stage of AML, however, out of the antigens of BaEV only the native gp70 showed a marked blocking effect.

Retroviruses isolated from human leukaemias are closely related to previously characterized primate type-C viruses. The first isolation from a patient with acute myeloid leukaemia resulted in a virus termed HL-23 V [1], which proved to be a complex of two viruses [2], one related to GaLV [3] and the other to BaEV [4]. Similar isolations were reported from different types of acute leukaemias [5, 6], even with an evidence for transforming ability [6]. The other branch of attempts to prove the aetiopathogenetic role of these viruses is the search for immune response to their antigens in patients with different types of leukaemias [7]. In a previous report we have demonstrated that lymphocytes and blood plasma samples from patients with myeloproliferative diseases exhibit cytotoxic effect against autologous tumor cells [8] and the cytotoxicity of lymphocytes and antibodies can be inhibited by

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the envelope glycoprotein (gp70) of BaEV and GaLV. Cytotoxic activity was found frequently in the quiescent phase of CGL, but very rarely in the progressive phase of AML. However, lymphocyte and plasma samples from patients being in the remission of AML or in the blastic crisis of CGL have not been studied. Preliminary data showed a difference between cytotoxic activity of lymphocytes from the quiescent phase and that of lymphocytes from the active phase of CGL and AML, too [9]. In addition, results of Tóth et al. [10] showed a significant decrease of the titres of antibodies reacting with primate oncoviral antigens in patients with potentially preleukaemic disorders prior to the manifestation of overt leukaemic disease. For clarification of the role of oncovirus expression in the aetiopathology of myeloproliferative diseases, the cytotoxic activity of lymphocytes and antibodies against autologous tumor cells was comparatively studied in the quiescent and the progressive phases of AML and CGL. The oncovirus specific nature of the reaction was proved by the blocking effect of purified oncovirus antigens.

Materials and methods

Cell separation. Separation of cells from heparinized blood samples was carried out on Ficoll-Uromiro gradient (Flow Laboratories Ltd. Irvine, UK) as described previously [8]. Blood plasma, lymphocytes and myeloid elements were separately aspirated. As revealed by haematological investigation, the purity of cell fractions proved to be 95–100%.

Cryopreservation procedure. A suspension of separated lymphocytes was prepared by adding the cells to RPMI 1640 medium (Gibco Bio-Cult Ltd., Paisley, UK) supplemented with 20% heat inactivated fetal calf serum (Gibco Bio-Cult Ltd., Paislay, UK). The portion to be cryopreserved was placed at a concentration of $5–10 \times 10^6$ cells/ml in 10% DMSO on ice in individual 2 ml glass tubes. Then the following cooling protocol was utilized: I. starting temperature 2 °C to 4 °C; II. cooling rate 1 °C/min to —30 °C; III. transfer to the liquid nitrogen storage container. When leukaemic disease of a patient had developed into progressive phase, cryopreserved lymphocytes were thawed for cytotoxic assay. After a rapid thawing (37 °C water bath without agitation until all ice crystals melted) the samples were placed at room temperature for further manipulations. The cells were washed twice with 50 ml RPMI 1640 medium containing 20% FCS. The viable cell count was then determined and the suspension adjusted to the appropriate concentration for cytotoxic test. The trypan blue viability of the cooled and thawed cell samples was 80 $\pm$ 10%.

Cytotoxic test. For study of cell-mediated and complement-dependent antibody cytotoxicity the ^{51}Cr-release technique was used, as previously described [8]. Viability of target cells and lymphocytes was controlled by trypan blue dye exclusion, and they were used in further experiments only in the case above 90%. For investigation of cell-mediated cytotoxicity, 5×10^6 lymphocytes and 5×10^4 ^{51}Cr-labelled autologous target cells were incubated for 4 h. The amount of ^{51}Cr released into the supernatant was measured by use of a scintillation counter. The per cent cytotoxicity for each sample was calculated as described by Oren et al. [11]. The background control was the percentage of cytotoxicity in tubes containing only target cells. For investigation of complement-dependent antibody cytotoxicity, 5×10^4 ^{51}Cr-labelled target cells were added to 0.5 ml of heat inactivated (56 °C, 30 min) plasma sample diluted to 1 : 10. After incubation at 37 °C for 30 min, 0.1 ml of non-toxic guinea pig serum (Human Institute, Budapest, Hungary) was added as a source of complement, and incubation was continued for an additional 30 min. After centrifugation the supernatant fluid was counted for released radioactivity. Target cells incubated in the presence of complement served as background controls.

Purification of gp70 and p15(E) antigens. The baboon endogenous virus propagated on A204 cells (Lot No. 990A) was from the Frederick Cancer Research Center (Frederick, MD, USA) and the gibbon ape leukaemia virus propagated on NC37 cells (Lot No. 18-76)

was from Pfizer Laboratory (Maywood, NJ, USA). Both viruses were provided in purified form through the Office of Resources and Logistics, National Cancer Institute (Bethesda, MD, USA). A method for isolating pure viral envelopes from C-type RNA viruses [12] was combined with methods for preparation of envelope antigens from Rauscher virus [13, 14] and from endogenous primate retroviruses [15]. The purification procedure used by us was described in details elsewhere [16].

Glycosidase treatment of viral gp70-s. The procedure as described by Ohno et al. [17] was followed: 40 μg of a glycosidase mixture (Miles, Frankfurt, FRG) were added to 400 μg of BaEV or GaLV gp70 in 50 mM sodium citrate buffer (pH 4.0). The efficacy of the glycosidase treatment was controlled by SDS-PAGE. The glycosidase treated antigens proved to be free of carbohydrate.

Inhibition of cytotoxic reaction by oncoviral envelope antigens. Before adding to target cells, lymphocyte or plasma samples were incubated with 20 ng of appropriate native and glycosidase treated gp70 as well as p15(E) antigens at 37 °C for 30 min. Degree of inhibition was expressed as per cent of total cytotoxic activity (cpm) measured in the absence of antigens.

Results

Tables I and II show results of cellular cytotoxic studies in 6 patients with CGL. ^{51}Cr-labelled myeloblasts collected from the blastic phase of the disease served as target cells. A remarkable difference was found between the cytotoxic effect of autologous lympocytes from the blastic crisis and that of lymphocytes from the quiescent phase. Cytotoxic activity of lymphocytes from the blastic crisis could be detected only in two cases. Previous incubation of lymphocytes with native and glycosidase-treated gp70 as well as p15(E) antigens of GaLV and BaEV exerted no blocking effect on their cytotoxic activity. On the contrary, considerable cytotoxic effect of cryopreserved lymphocytes against the same target cells could be demonstrated in each case. Blocking activity of native GaLV and BaEV gp70 antigens varied from 20 to 80%. Glycosidase treatment of these antigens significantly reduced or totally abrogated their blocking effect. Blocking of cytotoxicity by the p15(E) antigen was observed in one case.

Table I

Cell-mediated cytotoxicity in patients with CGL. Cytotoxic activity of lymphocytes from the blastic crisis

Patient	Leukocytes				% cpm	Cytotoxicity, % inhibition					
	G/1		% blast			GaLV			BaEV		
	B	Q	B	Q		gp70 native	gp70 glyc	p15(E)	gp70 native	gp70 glyc	p15(E)
1	28	24	78	2	15	0	0	0	0	0	0
2	34	13	28	2	0	.	.	.	.	.	.
3	155	19	93	3	0	.	.	.	.	.	.
4	30	12	45	2	0	.	.	.	.	.	.
5	14	3	30	0	0	.	.	.	.	.	.
6	24	5	30	0	10	0	0	0	0	0	0

Abbreviations: B = blastic crisis, Q = quiescent phase, glyc = glycosidase-treated antigen

Table II

Cell-mediated cytotoxicity in patients with CGL. Cytotoxic activity of lymphocytes from quiescent phase

Patient*	% cpm	Cytotoxicity, % inhibition					
		GaLV			BaEV		
		gp70		p15(E)	gp70		p15(E)
		native	glyc		native	glyc	
1	50	80	45	30	70	25	0
2	48	20	12	0	52	15	0
3	68	56	30	0	32	14	0
4	60	38	25	0	50	0	0
5	51	30	15	0	22	0	0
6	72	75	30	0	45	15	0

* For haematological data of patients see Table I

Studies on complement-dependent humoral cytotoxicity were carried out with plasma samples from the same patients, too (Table III). Plasma samples from the blastic crisis showed no cytotoxic activity. On the other hand, cytotoxic activity could be detected in each plasma sample from patients being in the quiescent phase of CGL, but the degree of humoral cytotoxicity proved to be lower than that of cellular cytotoxicity. Complement-dependent antibody cytotoxicity was intensively inhibited by the native gp70 antigens of GaLV and BaEV, whereas the glycosidase-treated gp70 antigens showed inhibitory activity of lower degree. Blocking activity of p15(E) antigen of GaLV was observed only in two cases.

Similar experiments were carried out with materials from patients with acute myeloid leukaemia. Myeloblasts from the progressive phase were used

Table III

Complement-dependent antibody cytotoxicity of plasma samples from the quiescent phase of CGL

Patient*	% cpm	Cytotoxicity, % inhibition					
		GaLV			BaEV		
		gp70		p15(E)	gp70		p15(E)
		native	glyc		native	glyc	
1	48	80	58	32	70	25	0
2	40	65	34	30	60	15	0
3	37	60	32	0	24	0	0
4	30	28	20	0	28	0	0
5	27	25	0	0	18	0	0
6	40	70	35	0	66	30	0

* For haematological data of patients see Table I

as target cells. Tables IV and V show the difference, observed in the cytotoxic activity of lymphocytes from the progressive phase and from remission. Cytotoxic activity of the progressive phase lymphocytes was found in 2 cases. Cytotoxicity could be blocked only by the native gp70 antigens in patients No. 1. In contrast, a high-level cytotoxic effect of remission lymphocytes was shown in each case. Blocking activity of native gp70 antigens of GaLV and BaEV could be demonstrated in each case, but it disappeared after glycosidase treatment of the BaEV gp70. Similarly, p15(E) of GaLV inhibited the cellular cytotoxicity in most cases, whereas the BaEV p15(E) exerted no inhibitory activity.

Complement-dependent humoral cytotoxic activity of plasma samples from the same AML patients is summarized in Table VI. Plasmas taken from patients being in the progressive phase of AML showed no cytotoxicity against

Table IV

Cell-mediated cytotoxicity in patients with AML. Cytotoxic activity of lymphocytes from the progressive phase

Patient	Leukocytes					Cytotoxicity, % inhibition					
	G/l		% blast		% cpm	GaLV			BaEV		
						gp70		p15(E)	gp70		p15(E)
	B	Q	B	Q		native	glyc		native	glyc	
1	0.7	7	76	8	25	50	0	0	40	0	0
2	9.2	4	81	5	80	0	0	0	0	0	0
3	7.6	2.8	49	4	0	.	.	.	.	.	.
4	5	2	40	2	0	.	.	.	.	.	.
5	7	3.2	70	4	0	.	.	.	.	.	.

For abbreviations see Table I

Table V

Cell-mediated cytotoxicity in patients with AML. Cytotoxic activity of lymphocytes from the remission

Patient*	% cpm	Cytotoxicity, % inhibition					
		GaLV			BaEV		
		gp70		p15(E)	gp70		p15(E)
		native	glyc		native	glyc	
1	70	90	50	48	70	0	0
2	68	85	70	72	66	0	0
3	48	25	20	25	65	0	0
4	45	40	18	10	60	0	0
5	42	32	15	0	25	0	0

* For haematological data of patients see Table I

Table VI

Complement-dependent antibody cytotoxicity of plasma samples from the remission of AML

Patient*	% cpm	Cytotoxicity, % inhibition					
		GaLV			BaEV		
		gp70		p15(E)	gp70		p15(E)
		native	glyc		native	glyc	
1	90	70	40	15	80	0	0
2	45	80	0	0	35	0	0
3	40	75	0	0	32	0	0
4	38	45	0	0	20	0	0
5	37	35	14	0	20	0	0

* For haematological data of patients see Table IV

autologous myeloblasts. However, cytotoxic activity of plasma samples collected in the remission phase of the disease varied between 37 and 90%. Inhibitory effect of native GaLV and BaEV gp70 antigens was demonstrated in each case, but in the majority of cases it could be abrogated by glycosidase treatment. Blocking effect of GaLV p15(E) antigen was found only in patient No. 1.

Studies on 15 healthy humans gave consequently negative results. In control experiments cryopreservation of lymphocytes and plasma samples from the blastic crisis of CGL did not influence the degree of their cytotoxic activity.

Discussion

Lymphocytes and plasma samples from the blastosis of CGL and AML had no cytotoxic activity against autologous blast cells. However, cryopreserved lymphocytes and plasmas, collected from the quiescent or remission phase of CGL and AML, respectively, proved to be cytotoxic for autologous blast cells and their effect could be blocked by envelope antigens of primate type-C oncoviruses. Both native and carbohydrate-free gp70, as well as p15(E) of GaLV exerted inhibitory effect on the cytotoxic activity of lymphocytes and antibodies from AML patients. However, out of the BaEV envelope antigens only the native gp70 possessed blocking activity. The virus-specific nature of the immune response to the carbohydrate part of gp70 envelope antigen is questionable. From this point of view it should be noted that DNS sequences related to the genome of BaEV exist in normal human cells [18] and may be expressed without malignant transformation [19]. On the opposite, expression of GaLV-related sequences was detected mainly in malig-

nancies of the human haematopoietic system [20]. Hence, GaLV-like agent(s) may have a role in the aetiology of some forms of human AML. This assumption seems to be supported by the fact that the development of AML in patients suffering from preleukaemic disorders was connected with an expression of antigen related to the GaLV envelope polipeptides [10]. The difference in frequency and degree of cytotoxic activity to GaLV-related antigens in the various phases of AML could be explained as follows.

(1) It seems reasonable to assume that large amounts of GaLV-related antigens shedding from the surface of leukaemic myeloblasts may block the cellular and humoral effector mechanisms.

(2) Another explanation may be the immunosuppressive effect exerted by leukaemia viruses [21] or by the proliferation of leukaemia cells in the immunopoietic system [22].

Cytotoxicity of lymphocytes and plasma samples collected from the quiescent phase of CGL and cryopreserved until the blastic phase exhibited oncovirus-specificity for BaEV, too. Thus, AML and CGL seem to have different oncovirus aetiology. Nevertheless, oncovirus-like antigens may have a role in the pathogenesis of both AML and CGL, presumably by induction of immune response resulting in the elimination of tumor cells.

Further studies are needed to identify the lymphocyte subpopulations reacting with the oncovirus antigens above, or in combination with antibodies and/or macrophages.

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REGULATION OF GLUTAMATE DEHYDROGENASE ACTIVITY AND AMMONIA PRODUCTION IN A NITROGEN FIXING CYANOBACTERIUM

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A glutamate auxotroph was obtained in *Nostoc muscorum* by induced mutagenesis with nitrosoguanidine. The metabolic pathway leading to glutamate synthesis was traced by selecting several enzymes. The strain was found to be lacking glutamate dehydrogenase. Other enzymes, however, were normal in their activity including isocitric dehydrogenase, glutamine synthetase and glutamate synthase. Nitrogen metabolism of the auxotroph and wild type was compared. The strain released exceedingly high amounts of ammonium in the medium.

Till recently not much attention has been devoted to the enzymes involved in primary amination in nitrogen fixing cyanobacteria. Stewart's team studied glutamine synthetase from *Anabaena cylindrica* and *Nostoc* sp. [1]. Earlier, glutamate dehydrogenase activity had been reported in *Anabaena variabilis* [2] and was later studied by Batt and Brown [3]. There have been some reports on the role of glutamate synthase in the regulation of nitrogenase activity by modulating glutamine levels in photosynthetic bacteria [4–6]; as found recently in *Rhodospirillum rubrum* [7]. The role of various enzymes has briefly been mentioned in several photosynthetic prokaryotes [8–10].

The present investigation was made on *Nostoc muscorum* which can conveniently be handled in liquid cultures and in which a system of genetic transfer has recently been reported [11–14]. The biochemical control mechanism has been studied in apparently a structural gene mutant strain lacking glutamate, correlating it with other enzymes of primary amination including glutamic dehydrogenase and glutamine synthetase. This system of *N. muscorum* could efficiently be used for understanding genetic regulatory mechanisms underlying nitrogen fixation and various pathways of nitrogen metabolism.

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Materials and methods

Strain. Glu-NM, a N-methyl-N-nitro-N-nitrosoguanidine induced mutant progeny of the parent cyanobacterium *N. muscorum* (NM-N), was isolated in this laboratory with the techniques described in references [11, 12]. *Glu*-NM was grown continuously in liquid cultures in medium BG-II [15] omitting nitrate and fortified with 4 μg ml^{-1} of glutamate. The composition of medium BG-II was in grams per litre: K_2HPO_4, 0.04; $MgSO_4 \cdot 7\ H_2O$, 0.075; $CaCl_2 \cdot 2\ H_2O$, 0.036; citric acid, 0.006; EDTA (disodium magnesium salt), 0.001; Na_2CO_3, 0.02; trace metal mix A_5, 1 ml. After autoclaving and cooling, the pH of the medium was 7.4. The strain was grown in a B.O.D. incubator at 27 $\pm$ 2 °C under illumination (2800 lux) with fluorescent light. Growth was monitored in liquid cultures as described earlier [11] and photometric measurements were done with Beckman DU spectrophotometer and EC Spectrophotometer (1 cm path length). The specific growth rate constant k has been described in Log_{10} units day^{-1}.

Osmotic shock. The procedure used was with slight modifications essentially the same as used by Smith and Wyatt [16] and Broad and Smith [17] in *Escherichia coli*. The cyanobacterial cells, primarily from the stationary phase of growth, were treated with lysozyme (E. Merck, highest purity, 2 μg ml^{-1}) for 24 h and then centrifuged at 5000 rpm for 15 min and the pellet was resuspended in 500 ml of 0.03 M Tris–HCl buffer pH 7.5 containing 35% (w/v) sucrose at room temperature. EDTA (10 mg ml^{-1}) was added to yield 1 mM. The cells were stirred constantly for 10 min at room temperature followed by centrifugation at 5000 rpm for 15 min. The pellet was resuspended in 500 ml cold water and stirred for 5 min and centrifuged again. The resultant supernatant ("osmotic shock supernatant") was used as a source of glutamate dehydrogenase and other enzymes. The supernatant was frozen overnight and concentrated by drying at low temperature (in an ice bucket) under suction employing anhydrous $CaCl_2$.

Protein and pigment estimation. Protein was determined by the method of Lowry et al. [18] after acidifying the samples with trichloracetic acid and digesting the precipitated protein with alkali. Bovine serum albumin was used as a standard reference protein. Phycocyanin was measured by its absorbance at 625 nm after liberation from the cyanobacterium by freezing and thawing. The phycocyanin content was determined on the residue remaining after acetone fractioning as reported by Allen [19], using the specific extinction coefficient of 7.5 at 625 nm [20]. Chlorophyll-a and carotenoids were extracted with 80% acetone (v/v) in water. The absorbance of the acetone extract was measured at 460 nm and 665 nm. The amount of these two pigments was calculated using the specific absorption coefficient 82.04 for chlorophyll-a at 665 nm [21], and 200 for total carotenoids at 460 nm.

Nucleic acid determination. DNA estimation was done by the diphenylamine reaction [22], and RNA was determined by the orcinol reaction [23].

Enzyme assay. Isocitric dehydrogenase was assayed as described by Kornberg [24]. Specific activity was expressed as units per mg of protein. Glutamate synthase and glutamate dehydrogenase activity were determined as described by Prusiner et al. and adopted by Shanmugam et al. [25]. Activities were determined in freshly prepared concentrated supernatant fractions by following the rate of NADPH oxidation at room temperature. Glutamine synthetase activity was determined according to Shanmugam et al. [25] as described in Materials and methods and by measuring the amount of r-glutamylhydroxymate. Nitrate nitrite and ammonia were determined as described by Ohmori and Hattori [26] and Kodama [27]

Results

Ammonium production. An excess of ammonium was detected in the cultures of *Glu*-NM in comparison to the wild type NM-N (Table I). This ammonia was accumulating and the growth of the cyanobacterium was highly affected (0.09 D day^{-1}) in comparison to the normal strain NM-N. This inhibition of growth was, however, not due to excess NH_4^+ as tested by supplying ammonium to the culture. In the presence of glutamate, although the ammonium level in the cultures remained the same, the growth of the cyanobacterium

was brought to normal (0.50 D day^{-1}). The amount of nitrite and nitrate was almost same in NM-N and *Glu*-NM. The strain *Glu*-NM was continuously releasing ammonia for about 7 days in a non-dividing state and ammonium concentration was increasing at a rate of 12%/day^{-1}.

Enzyme levels. Glutamate dehydrogenase activity (GDH) was absent in *Glu*-NM irrespective of the nitrogen supply to the culture (Table II). Ammonium ions increased the relative activity of GDH by 36% in NM-N whereas glutamine and glutamate decreased the relative activity of GDH in NM-N by about 38%.

Isocitric dehydrogenase (IDH) activity was equally represented by NM-N and *Glu*-NM under variable conditions of nitrogen supply. Neither NM-N nor *Glu*-NM had lost the ability to synthesize glutamine synthetase (GS) and

Table I

Comparative amounts of NO_2^-, NO_3^- and NH_4^+ present in the growing culture of NM-N and Glu-*NM*

Strain	NO_2^-	NO_3^-	NH_4^+	Specific growth rate D-day^{-1}
	Concentration in μmol mg protein-1			
Glu-NM	18	15	425	0.09
NM-N	16	14	18	0.50
Glu-NM + glutamate*	18	20	428	0.51

* Amount of glutamate added, 4 μg ml^{-1}

Table II

Specific activities of GDH, isocitric dehydrogenase glutamine synthetase and glutamate synthase in NM-N and Glu-*NM grown on various nitrogenous substances*

Strain	State of culture	Glutamate dehydrogenase GDH	Isocitric dehydrogenase	Glutamine synthetase	Glutamate synthase
		Specific activity in nmol min^{-1} mg $protein^{-1}$			
NM-N	$-NH_4$	8	280	640	628
	$+NH_4$	11	282	20	640
	+Glutamate	5	279	672	15
	+Glutamine	5	280	74	648
Glu-NM	$-NH_4$	ND	280	682	629
	$+NH_4$	ND	292	18	632
	+Glutamate	ND	284	681	13
	+Glutamine	ND	274	64	647

ND = not detectable

Concentrations of NH_4^+, glutamate and glutamine added were kept constant throughout the experiment (4 μg/ml). Higher or lower concentrations did not influence the observations

glutamate synthase (GOGAT) under normal conditions i.e. during growth without ammonium, while NH_4^+ inhibited the synthesis of glutamine synthetase in both strains. NM-N gave only 3.1% and *Glu* NM only 2.8% of the normal activity of glutamine synthetase in the presence of NH_4^+. Glutamine also lowered the activity of glutamine synthetase to 11.5 and 9.9% in NM-N and *Glu*-NM, respectively.

Glutamate synthase was found to have normal activity in both *Glu*-NM and NM-N. Glutamate, however, inhibited the synthesis of glutamate synthase in *Glu*-NM as well as NM-N. Both strains exhibited a relative decrease of glutamate synthase by 97–98%. Ammonium and glutamine did not inhibit glutamate synthase activity in either strain.

Glu-NM had not lost the ability to fix nitrogen as it was able to grow without combined nitrogen source under aerobic conditions and its slow growth was restored to normal after glutamate had been supplied. No variation was observed in heterocyst frequency (data not shown) in *Glu*-NM and NM-N.

Macromolecules. Protein synthesis and DNA and RNA contents were considerably low in *Glu*-NM (Table III). Carotenoids were almost unaffected in *Glu*-NM in comparison to NM-N. The bile protein phycocyanin was reduced to half in *Glu*-NM, and restored to normal on supplying glutamate or glutamine.

Table III

Relative composition of Chlorophyll and macromolecules in NM-N and Glu-NM

Macromolecule	NM-N	NM-2
	mg/mg dry wt	
Total protein	0.54	0.12
RNA	0.34	0.11
DNA	0.02	0.003
Chlorophyll-a	0.052	0.041
Carotenoids	0.021	0.023
Phycocyanin	0.31	0.16

Discussion

The present investigation revealed interesting facts concerning multiple pathways of nitrogen metabolism in relation to gene expression. The distinctive role of glutamate dehydrogenase, glutamine synthetase and glutamate synthase has been clearly emphasized in heterotrophic bacteria, photosynthetic bacteria and cyanobacteria [5, 9, 25, 28–34]. Isocitric dehydrogenase is the key enzyme in the krebs cycle for the conversion of isocitric acid into

alpha-ketoglutaric acid and this marks the turning point for the synthesis of glutamic acid through reductive amination reactions. It was interesting that a mutation resulting in the absence of glutamic acid dehydrogenase activity still produced a glutamate requiring phenotype in the background of glutamine synthetase and glutamate synthase activity. *Glu*-NM is apparently a structural gene mutant for glutamate dehydrogenase and is non-supplementable. NH_4^+ formed during active nitrogen fixation is not able to aminate alpha-ketoglutaric acid for paucity of enzymatic activity of glutamate dehydrogenase. Detection of isocitric dehydrogenase accounts for and confirms the regular synthesis of alpha-ketoglutaric acid, the substrate for reductive amination and formation of glutamic acid. As NO_2^- and NO_3^- were excreted in a normal fashion by NM-N and *Glu*-NM, there could be no block in nitrite and nitrate reductase activity. Moreover, NH_4^+ supplemented culture did not restore the growth of *Glu*-NM to normal levels. Because of the genetic lesion in the production of glutamate in *Glu*-NM, further reactions including transamination cannot take place and may be responsible for the decline in total protein and DNA. The protein contents (in terms of per unit amount) are normalized on supplying glutamate.

Although glutamate synthase was present, glutamate could not be synthesized further because of a failure of the formation of glutamine through the primary reaction. There was, however, no genetic lesion observed in the activity of glutamate synthase and glutamine synthetase which was considered to be an effective modulator of nitrogenase activity [7, 8, 10, 25, 32]. An interesting point to be noted was the differential inhibition of glutamine synthetase and glutamate synthase by NH_4^+ and glutamate in *Glu*-NM and NM-N. NH_4^+ inhibited glutamine synthetase without inhibiting glutamate synthase. Glutamate inhibited glutamate synthase without inhibiting glutamine synthetase. Glutamate dehydrogenase was non-inducible as observed continuously by supplying alpha-ketoglutaric acid and ammonium externally and seemed to be unrelated to nitrogen fixation regulation as such. Implication of the deterrant activity of glutamate dehydrogenase on the concentration of phycocyanin remained unaccounted for, although some intermediary metabolism involving the incorporation of nitrogen into this pigment protein could be imagined.

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EFFECT OF SOME NEW CROWN ETHERS ON PLANT-RELATED BACTERIA AND THEIR POSSIBLE MODE OF ACTION

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Crown ethers are of growing acceptance and application in various fields of chemistry [1]. As some modified crown ethers [2, 3] influence considerably the structure of model membranes [4], it is reasonable to assume that they may show marked membrane damaging effect also in living organisms.

The purpose of our work was to determine the influence of these substances on various plant related bacteria and to correlate this biological activity to their membrane damaging effect.

Materials and methods

Plant related bacteria and crown ether derivatives are shown in Tables I and II and in Fig. 1. Dipalmitoyl-phosphatidylcholine (DPPC) and dipalmitoyl-phosphatidyl-ethanolamine (DPPE) were purchased from Sigma and was applied without additional purification.

Media and cultures. The bacteria were maintained and cultured in the following broth: Bacto Peptone (Difco), 10 g; Yeast Extract (Difco), 1 g; glucose, 3 g; glycerol, 1 g; KH_2PO_4, 0.55 g; KCl, 0.425 g; $MgSO_4$, 0.125 g; $CaCl_2$, 0.125 g; NaCl, 0.1 g; Fe(III)citrate, 0.005 g; Hoagland-Arnon's microelement solution 1 ml; water to 1 litre. Solid media were prepared by adding 15 g agar (Oxoid) per litre of broth.

Standardized bacterial suspension for the determination of minimal inhibitory concentration (MIC) was prepared by washing a 20 h solid medium culture with 5 ml sterile liquid broth. The optical density of cell suspension was determined in 1 cm cuvette at 550 nm wavelength using sterile broth as blank. The suspension was diluted with sterile broth to give a final optical density of 0.4–0.5.

Determination of biological activity. The appropriate quantities of crown ether derivatives were dissolved in 0.2 ml acetone and the solutions were mixed at 45–50 °C with portions of 30 ml agar medium. The mixture was poured into two Petri dishes of 9 cm diameter. Each Petri dish was inoculated with the standardized suspensions of 23 bacteria presented in Table I with a multipoint inoculator (inoculum volume 2–5 μl). The Petri dishes were incubated at 27 °C and the bacterial growth was read after 48 and 96 h. The antibiotic polymyxin [5, 6] and the pesticide tridemorph [7] both exerting membrane damaging effect served for comparison.

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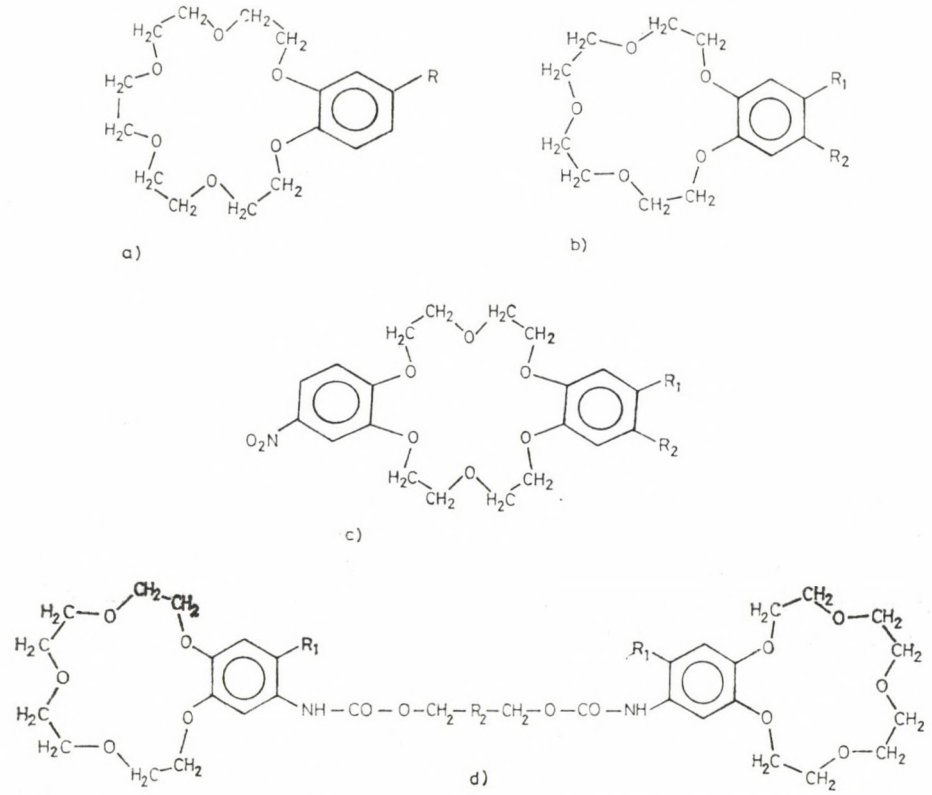


Fig. 1. a—d. General chemical structures of modified crown ethers

Determination of permeability of DPPC liposomes. Liposomes were formed by sonification from DPPC in 0.16 M KCl solution containing tracer amount of ^{42}K. After overnight equilibration at room temperature the liquid dispersion was passed down a columne of Sephadex G-50 (1.5 × 30 cm) to remove the excess tracer not trapped within the liposomes. The liposomes were eluted from the column with 0.16 M KCl solution (flow rate, 0.5 ml min^{-1}), 3 ml portions of eluted liposomes were dialyzed against 10 ml 0.16 M KCl. The efflux rate was measured for consecutive 15 min periods for 2 h. At the end the ^{42}K content of liposomes was measured using a gamma-scintillation counter. The crown ethers exhibiting marked biological activity were added to the DPPC liposomes at a molar ratio 1 : 100. The permeability time constants were calculated as described [8].

Determination of melting properties of DPPC and DPPE by Differential Scanning Calorimetry (DSC). The crown ether derivatives and the phospholipids were dissolved in chloroform at a molar ratio 1 : 100. The chloroform was evaporated in nitrogen atmosphere, then water was added to the crown ether–phospholipid mixtures in a weight ratio 4 : 1. The samples were mixed for 30 min vigorously in a Vortex mixer. The measurements were carried out in a Du Pont Thermoanalyser 990 at a heating rate of 5 °C per min. The temperature of pretransition (T_p) and that of main transition (T_m), the half width of main transitions ($\Delta T_{1/2}$) characterizing the correspondence and the enthalpy of phase transition (ΔH mJ mol^{-1}) were determined.

Results and discussion

Under our experimental conditions only two of the crown ether derivatives showed in vitro biological activity (Table III). The evaluations after 48 and 96 h resulted in identical behaviour patterns. The crown ethers inhibited only the growth of *Corynebacterium* species (species 5–10 in Table III), for these species they were more effective than the antibiotics polymyxine and the

Table I

Plant related bacteria

	Species	Code No.	Source
1	*Agrobacterium radiobacter*	K-84	soil
2	*A. tumefaciens*	0	grape vine
3	*A. tumefaciens*	C-58	cherry
4	*A. tumefaciens*	B-6	tomato
5	*Corynebacterium betae*	CN 101	sugar beet
6	*C. fascians*	B-27	pelargonium
7	*C. flaccumfaciens*	8	bean
8	*C. michiganense*	36/3	tomato
9	*C. nebraskense*	CN 101	maize
10	*C. oortii*	B-11	tulip
11	*Erwinia uredovora*	VfR3	*Vicia faba rust*
12	*E. carotovora* pv. *atroseptica*	G-128	potato
13	*E. carotovora* pv. *carotovora*	CCM 1008	potato
14	*Pseudomonas fluorescens*	K-25	soil
15	*P. lachrymans*	31	cucumber
16	*P. phaseolicola*	67	bean
17	*P. mors-prunorum*	SR-2	pear
18	*Rhizobium japonicum*	B-37	soya
19	*Xanthomonas alfalfae*	KX-1	lucerne
20	*X. campestris*	2D 510	cabbage
21	*X. carotae*	B-39	carrot
22	*X. phaseoli* var. *fuscans*	ERA 4	bean
23	*X. vesicatoria*	53	tomato

pesticide tridemorph. As corynebacteria are Gram-positive and all other species Gram-negative, it is probable that the biological action of crown ethers depends of the type of cell wall or membrane.

The same crown ethers increased considerably the K^+ efflux from DPPC liposomes (Fig. 2), the permeability time constants being about 30 times higher in the presence of crown ether derivatives than in the control (Table IV). These findings indicate that the bioactive crown ethers interact strongly with DPPC liposomes, they decrease the organization of lipid molecules, which results in an enhanced permeability.

The DSC data support our previous conclusions (Fig. 3). The bioactive crown ether derivatives modify each melting parameter of DPPC and DPPE

Table II

Chemical structure of modified crown ethers

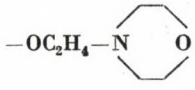
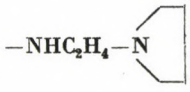
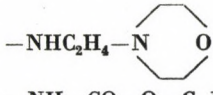
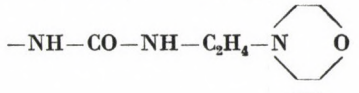
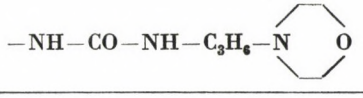
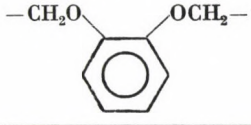
General structure*	Compound	R	
a	1	H	
	2	NO_2	
		R_1	R_2
b	3	H	H
	4	NO_2	H
	5	COOH	H
	6	$-CO-CH_3$	H
	7	NO_2	NH_2
	8	NO_2	$-OCH_3$
	9	NO_2	$-OCH(CH_3)_2$
	10	H	$-OC_2H_4OC_2H_4N(C_2H_5)_2$
	11	H	
	12	H	
	13	H	
	14	NO_2	$-NH-CO-O-C_{16}H_{33}$
	15	NO_2	$-NH-CO-NH-C_{16}H_{33}$
	16	NO_2	
	17	H	
c	18	NO_2	H trans
	19	N	NO_2 cis
d	20	H	$-CH_2OCH_2-$
	21	H	$-CH_2SCH_2-$
	22	NO_2	$-CH_2SCH_2-$
	23	H	$-C_3H_6-$
	24	NO_2	$-C_3H_6-$
	25	H	

Table II (continued)

General structure*	Compound	R
	26	NO_2
	27	NO_2
	28	NO_2

* See Fig. 1

Table III

Minimal inhibitory concentration (mg/l) of some membrane damaging agents

Species*	Crown ethers**		Polymyxin	Tridemorph
	Comp. 26	Comp. 27		
1	>100	>100	10—100	>100
2	>100	>100	10—100	>100
3	>100	>100	10—100	>100
4	>100	>100	10—100	>100
5	1— 10	1— 10	10—100	>100
6	10—100	1— 10	>100	10—100
7	1— 10	10—100	10—100	10—100
8	1— 10	1— 10	>100	10—100
9	10—100	1— 10	>100	10—100
10	1— 10	1— 10	10—100	>100
11	>100	>100	>100	>100
12	>100	>100	10—100	>100
13	>100	>100	10—100	>100
14	>100	>100	>100	>100
15	>100	>100	1— 10	>100
16	>100	>100	10—100	>100
17	>100	>100	10—100	>100
18	>100	>100	10—100	>100
19	>100	>100	10—100	>100
20	>100	>100	10—100	>100
21	>100	>100	10—100	>100
22	>100	>100	10—100	>100
23	>100	>100	1— 10	>100

* See Table I
** See Table II

Table IV

Permeability time constants (10^{-4} s^{-1}) of DPPC liposomes

Compound*	Molar ratio	
	1 : 100	1 : 200
Control	0.3	0.3
Comp. 26	11.9	8.3
Comp. 27	8.6	not determined

* See Table II

Table V

Effect of bioactive crown ethers on the melting properties of DPPC and DPPE

Compounds*	T_p °C	T_m °C	$T_{1/2}$ °C	ΔH (mJ/mol)
DPPC	35.5	41.5	1.3	56.0
DPPC + Comp. 26	32.2	40.2	1.5	51.0
DPPC + Comp. 27	disappeared	40.1	1.7	40.3
DPPE		63.2	1.9	52.0
DPPE + Comp. 26		62.9	2.3	47.4
DPPE + Comp. 27		63.0	2.6	45.3

* See Table II

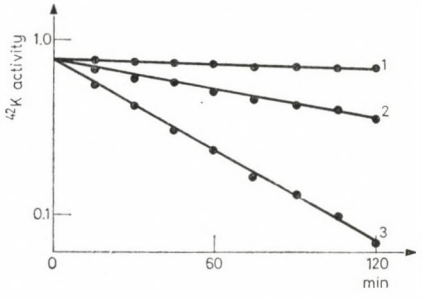


Fig. 2. Effect of crown ether derivatives on the ^{42}K efflux of DPPC lyposomes. (1) DPPC control; (2) DPPC + crown ether Comp. 27 (100 : 1); (3) DPPC + crown ether Comp. 26 (100 : 1)

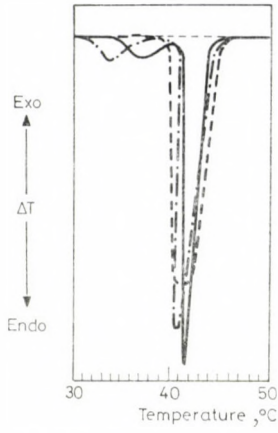


Fig. 3. Influence of bioactive crown ethers on the melting properties of DPPC. ——— DPPC control; –·–·–·– DPPC + crown ether Comp. 26; – – – – DPPC + crown ether Comp. 27

(Table V). This observation supports that the crown ethers influence not only the organization of apolar fatty acid chains but also they interact with the polar head groups of phospholipids.

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EFFECT OF DIPHTHERIA AND TETANUS TOXOIDS ON THE GROWTH OF, AND IMPRINTING DEVELOPED IN, *TETRAHYMENA*

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The growth of *Tetrahymena* was influenced by both diphtheria and tetanus toxoids; depression occurred at a high and stimulation at a low concentration. Pretreatment with the stimulating concentration of diphtheria toxoid caused a lasting modification of growth rate and development of imprinting that resulted in an enhanced response of the cell at reexposure to the toxoid. Tetanus toxoid failed to induce either switch over of cell function or imprinting.

Tetrahymena is a highly useful tool for receptor research [1]. It is capable of binding hormones of higher organisms and develop hormonal imprinting at the first encounter with the hormone. As a result, receptors become amplified and both binding and cellular response will be more marked following further exposures [2–4]. The memory is transmitted through more than 500 subsequent generations [5]. In addition, *Tetrahymena* harbours a number of hormones characteristic of higher organisms [6–9].

Our earlier experiments have demonstrated that not only hormones induce imprinting but all those molecules of defined spatial configuration that provoke receptor formation in *Tetrahymena*, namely albumin [4], digoxin [11], benzpyrene [10] and the toxin of *Anemonia sulcata* [12].

Besides imprinting another characteristic reaction of *Tetrahymena* is a lasting growth stimulating activity at the first encounter with the "foreign" material [4]. This may have implications to evolution, since the information encoded by *Tetrahymena* may be inherited by a great number of descendants [13].

In the present study the effect of diphtheria and tetanus toxoids on *Tetrahymena* as well as development of imprinting were assayed. The choice of these substances was motivated by the facts that toxins may be present in the natural environment of *Tetrahymena*, and that being protein-like materials, they fully meet the configuration requirements. In addition, it seemed interesting to obtain data on materials probably harmful for *Tetrahymena*.

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The toxoid is antigenic, i.e. it has the chemical structure of the toxin, and although it does not kill the organism, the mechanism of its recognition is similar to that of the toxin.

Materials and methods

Cultures of Tetrahymena piriformis GL were incubated for 2 days at 28 °C in a 1% Bacto Tryptone medium completed with 0.1% yeast extract.

Assay of cell growth rate. The tolerable concentrations (i.e. sublethal concentrations) of purified diphtheria toxoid (Human, Budapest; protein-nitrogen content, 0.79 $\mu g/L_f$) and purified tetanus toxoid (Human, Budapest; protein-nitrogen content 0.66 $\mu g/L_f$) were 0.43 L_f/ml and 0.23 L_f/ml, respectively. Two-fold dilutions of the toxoid were prepared up to the endpoints 0.027 L_f/ml and 0.0035 L_f/ml (D_1–D_5 and T_1–T_7, respectively). The multiplication of *Tetrahymena* was assayed with the capillary method in four subsequent experiments using the same concentrations and starting from single-cell cultures. In the capillary culture one cell was grown in 30 μl medium for 17 h at 25 °C and the growth rate was checked.

The fifth dilution of each toxoid was chosen (D_5 = 0.027 L_f/ml and T_5 = 0.014 L_f/ml) for the development of imprinting. Two-day cultures were treated with these concentrations for 24 h, the controls were incubated without toxoids. After this pretreatment the cultures were washed, kept in normal medium for 48 h and reexposures to the toxoids followed in capillary single-cell cultures. This time one third of the controls was left unexposed (C/C), while another third was submitted to treatment with concentrations D_5 and T_5 of diphtheria and tetanus toxoids (C/D, C/T). Part of the capillary single-cell cultures prepared from the toxoid-treated original culture was not submitted to another treatment (D/C, T/C) while another part was reexposed to the same dose of toxoid (D/D, T/T).

The experiments were repeated four times. The mean values of the treated groups were related to those of the controls as 1. Statistical significance was calculated according to Student's *t*-test.

Assay of diphtheria toxoid-binding capacity. Diphtheria toxoid was conjugated with fluorescein-isothiocyanate (FITC, BDH, England). The two-day-old cultures were divided into three groups: (1) untreated; (2) treated with 0.054 L_f/ml of diphtheria toxoid (dilution D_4); (3) treated with 0.027 L_f/ml of unconjugated diphtheria toxoid (dilution D_5). Groups (2) and (3) were exposed for 24 h. Subsequently the cultures were inoculated into a normal medium and incubated for 48 h. One drop of culture was placed on a slide and FITC conjugated diphtheria toxoid was added. The toxoid-binding capacity of *Tetrahymena* was assayed with a Zeiss Fluoval cytofluorimeter combined with HP-41 computer. The rates of FITC-conjugated diphtheria toxoid-binding capacity of the test groups were related to the mean values of the control groups regarded as 100%. A computer programme was developed for calculation of statistical significance according to Student's *t*-test and for variance analysis.

Results and discussion

Both diphtheria and tetanus toxoids are high molecular weight (70 000–150 000 dalton) protein [14]. Diphtheria toxin is a general cell poison, an inhibitor of protein elongation in eukaryote cells. The tetanus toxin exhibits its action on neurons. Both toxins can be transformed to toxoids by formalin treatment without loss of their antigenic structure. In these experiments formalin treated toxins (toxoids) were used.

Our results demonstrate that a large dosis of both toxoids depresses the growth of *Tetrahymena*, while a low concentration stimulates it. The effect appears to be dose-related (Figs 1 and 2). A comparatively small concentra-

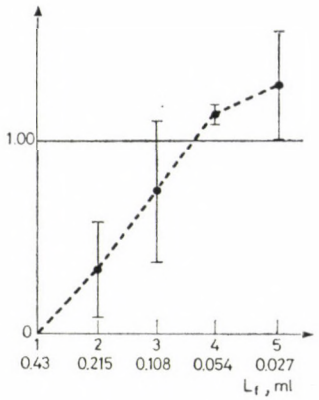

Fig. 1. **Dose-effect curve of diphtheria toxoid**

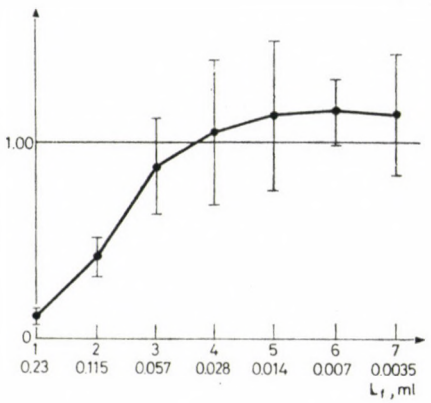

Fig. 2. **Dose-effect curve of tetanus toxoid**

tion of the toxoids affects the growth of *Tetrahymena*, but this effect cannot be attributed to a metabolism of the toxoids by the organism, since stimulation is achieved by a small concentration while inhibition by a larger dose.

The doses of toxoids used for development of imprinting were those that enhanced the growth of *Tetrahymena* already at the first exposure. The diphtheria toxoid provoked a long term effect (Fig. 3). After the first treatment a 48-hour incubation in normal medium and a 24-hour incubation in single cell culture followed; one of the culture-groups remained untreated. This group also demonstrated a significantly higher growth rate than the control group, and exceeded, though not significantly, its earlier growth rate measured immediately at the first encounter with the toxoid. Tetrahymenae multiply 4 to 5 times a day. The results of the second treatment were measured after the 15th cell-division, that means that an increase of the growth rate remained

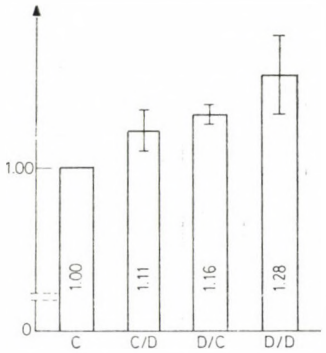

Fig. 3. Effect of diphtheria toxoid on the growth of *Tetrahymena*. C = control, D = toxoid-treated; C/D = $p < 0.01$, D/C = $p < 0.05$, C/D–D/D: x = $p < 0.1$

appreciable after this long sequence of generations. The second exposure to diphtheria toxoid resulted in a further increase of growth, that was more than twice as high as the growth rate after the first treatment and remarkably exceeded the growth rate measured 72 h after the first treatment in cultures without a second exposure. Though the difference between these two growth rates did not show a mathematical significance, it appears to be remarkable from the biological point of view. The diphtheria toxoid seems to have induced the switch over of cell division and development of imprinting as well.

Tetanus toxoid did not evoke either imprinting or an appreciable change in the cell function (Fig. 4). There were great deviations and not even the mean values differed remarkably from those of the control. These experiments demonstrate that *Tetrahymena* reacts differently to treatment with proteins of similar molecular weight as far as switch over of cell-function or imprinting is concerned. The finding may be attributed to different molecular structures of the toxoids. On the other hand, the "attention" of tetrahymenae may be

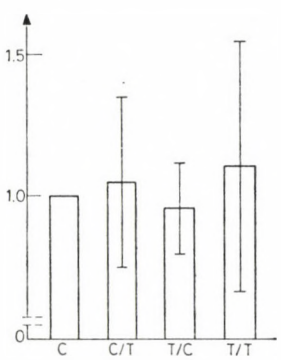

Fig. 4. Effect of tetanus toxoid on the growth of *Tetrahymena*

attracted more by diphtheria toxoid, which is a general cell poison, than by tetanus toxoid that accounts for a highly specific effect. Our study has left this question open.

A reduction of the binding of FITC-conjugated diphtheria toxoid ensued as a consequence of pretreatment (imprinting) with both concentrations applied (Fig. 5). That is, imprinting involved not only an altered cell-function but binding of toxoid as well, however, the latter in a reverse sense. This means that, as several earlier studies have proved [15, 16] binding and cell

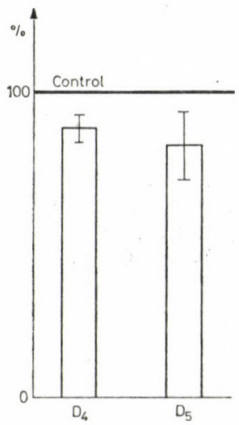

Fig. 5. FITC-conjugated diphtheria toxoid binding by pretreated tetrahymenae and control. Statistically significant difference related to the contol: $D_4 = p < 0.01$, $D_5 = p < 0.1$

growth effect do not always run parallel. It may be assumed that fewer binding sites might be sufficient to evoke greater reaction, as an additional aspect of imprinting.

This study supports the previous observation that (i) *Tetrahymena* develops not only hormone receptors, but generally also receptors for alien materials active on membrane level as evidenced by imprinting in the organism, and (ii) *Tetrahymena* selects materials of similar molecular weight and responds differently to them [1].

The receptor memory of *Tetrahymena* is a phenomenon similar to the immunological memory [7]. Diphtheria toxoid which is an antigen, supports this consideration. At present there is no explanation why tetanus toxoid failed to develop receptor memory.

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COMPARATIVE LABORATORY STUDIES ON α–METHOXYIMINO FURYL- AND PHENYLACETAMIDO CEPHALOSPORINS: STRUCTURE-ACTIVITY RELATIONSHIPS

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Eleven new cephalosporins (three phenylacetamido and eight furylacetamido) containing a methoxyimino group on the 7 β-acyl side chain and having various substituents at their 3-positions, exhibited similar qualitative, but differing quantitative in vitro antibacterial spectra compared to that of cefuroxime, the first therapeutically used α-methoxyimino cephalosporin. The *syn*-isomers and the α-acyl substituted compounds are more active than either the *anti*-isomer or the β-acyl substituted compounds. Compounds containing substituted tetrazole rings at the 3-position are likewise more active than those containing other types of substituents in this position. In vivo (mouse) the heterocyclic furylacetamido compounds are more efficacious (protective) than the aromatic phenylacetamido compounds. The furylacetamido α-methoxyimino cephalosporins containing at the 3-position the tetrazole group carrying an acidic function possess favorable pharmacokinetic properties, i.e., higher serum levels and prolonged biological half-lives in mouse and squirrel monkey and extensive binding to serum proteins.

The first clinically useful parenteral semisynthetic cephalosporin containing the α-methoxyimino group attached to the furylacetamido side chain at the 7-position of the cephem nucleus was cefuroxime, a compound having a wider spectrum of antibacterial activity and greater stability to most beta-lactamases [1–4] than older cephalosporins. On the basis of these improved properties over the first-generation cephalosporins (cephalothin, cephaloridine, cephalexin, cephradine, cephapirin, cephacetrile and cefazolin), cefuroxime is justifiably classified as a second-generation cephalosporin, together with cefamandole, cefoxitin, cefonicid, cefmetazole, cefaclor and ceforanide [4–6].

The success of cefuroxime which contains the unique α-methoxyimino side chain has led us to design and synthesize a series of new cephalosporins with this side chain, and study their basic biological properties in the laboratory in comparison with those of cefuroxime and cefazolin. Figure 1 shows the chemical structures of these new cephalosporins. Three of them (Nos I–III) contain the phenylacetamido side chain and eight (Nos IV–XI) contain the

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Compounds		RCONH-[S, N, O, CH$_2$A, COOH]	
No.	SK&F No.	R	A
I	79384	C(=N-OCH$_3$)-	-OCOCH$_3$
II	79395	C(=N-OCH$_3$)-	-S-[N-N, N, N, CH$_3$]
III	80196	C(=N-OCH$_3$)-	-S-[N-N, N, N, CH$_2$SO$_3$H]
IV	81367	C(=N-OCH$_3$)-	-OCOCH$_3$
V	81147	C(=N-OCH$_3$)-	-S-[N-N, N, N, CH$_3$]
VI	81366	C(=N-OCH$_3$)-	-S-[N-N, N, N, CH$_3$]
VII	81409	C(=N-OCH$_3$)-	-S-[N-N, N, N, CH$_2$SO$_3$H]
VIII	81292	C(=N-OCH$_3$)-	-S-[N-N, N, N, C$_2$H$_4$COOH]
IX	82464	C(=N-OCH$_3$)-	-S-[N-N, N, N, C$_2$H$_4$NHSO$_3$H]
X	81903	C(=N-OCH$_3$)-	-S-[N-N, N, N, C$_2$H$_4$NHSO$_2$NH$_2$]
XI	82259	CH$_2$-C(=N-OCH$_3$)-	-S-[N-N, N, N, CH$_3$]
XII	Cefuroxime	C(=N-OCH$_3$)-	-OCONH$_2$

Fig. 1. Chemical structures of twelve methoxyimino furyl- or phenylacetamido cephalosporins

furylacetamido group at the 7-position of the aminocephalosporanic acid nucleus. At the 3-position of the dihydrothiazine ring, the carbamoyloxymethyl chain of cefuroxime is replaced by the acetoxymethyl chain in two compounds (Nos I, IV) and by a substituted tetrazole ring in nine compounds (Nos II, III, V–XI). The methoxyimino group is in the *syn*-configuration, except in compound No. VI. where it is in the *anti*-configuration. The methoxyimino group is attached to the α-carbon of the acyl chain in all cases except compound No. XI where it is on the β-carbon. These chemical modifications caused substantial changes in the in vitro, in vivo and pharmacokinetic properties of the compounds as compared to cefuroxime to which some appear to be superior. The results of this study are summarized here.

Materials and methods

Cephalosporins. Cefuroxime was kindly supplied as a research sample by Glaxo Laboratories Ltd. Cefalozin (Smith Kline and French Laboratories) was a commercial preparation. All the other compounds were synthesized by members of the Medicinal Chemistry Department, Smith Kline and French Laboratories. Their chemical structures are presented in Fig. 1.

Organisms. The bacterial strains used for the MIC and ED_{50} determinations are those regularly employed in our laboratory for the primary testing of cephalosporins and penicillins [7, 8] and are, for the most part, clinical isolates obtained from various geographical locations in the United States.

Animals. Webster-derived CDl male albino Swiss mice (Charles River Breeding Laboratories) with average weights of 18 to 21 g were used for the efficacy and serum level studies. Squirrel monkeys, each weighing 0.8 to 1.1 kg, were used for serum level studies [9, 10].

Microbiological assay. All serum and urine samples were assayed for antibiotic concentrations by the disc agar-diffusion method, using *Bacillus subtilis* ATCC 6633 as the indicator organism. For serum level assays, standards and test samples were diluted using appropriate pooled animal sera devoid of background activity. For urine assays, the diluent was 1% phosphate buffer, pH 6.0. Assay plates were incubated overnight at 30 °C. Zone diameters were measured with a Fisher-Lilly zone reader.

In vitro binding to serum proteins. The percentage of antibiotic activity bound by serum proteins (mouse, squirrel monkey, or human) was estimated by comparing standard dose-response assay curves obtained for the compounds diluted in buffer (pH 6.0) or in serum. A disc agar-diffusion assay was employed for these determinations.

Activity and efficacy tests [7, 9, 10]. (a) *In vitro assays.* The minimum inhibitory concentrations (MICs) were determined by an agar dilution technique, on Trypticase Soy agar buffered to pH 6 by the addition of 10% McIlvaine's citric acid-phosphate buffer. Serial twofold dilutions of freshly prepared standard cephalosporin solutions were incorporated into the melted Trypticase Soy agar and poured into Petri dishes. Plates were inoculated with the aid of a Steers' inocula replicating device [11] with overnight-grown bacterial cultures (Table I) diluted to contain approximately 10^4–10^6 colony forming units. The MIC values in μg/ml were determined, after 18 h of incubation at 37 °C, as the lowest concentration of compound that inhibited growth (colony formation). Cefuroxime and cefazolin were routinely included as control compounds.

(b) *In vivo efficacy studies.* Strains of *Escherichia coli* and *Klebsiella pneumoniae* diluted in 5% hog gastric mucin were used to intraperitoneally infect groups of ten male albino Swiss-Webster mice weighing 18–21 g, with the number of bacterial cells which produced uniformly lethal infections in non-treated animals. Infected mice were treated subcutaneously at one and five h post-infection with fourfold concentration increments of the cephalosporins in isotonic sodium chloride solution. Death and survivors were recorded for a period of three days. The total dose of each compound that protected 50% of infected mice (ED_{50}) was calculated by the method of Litchfield and Wilcoxon [12].

Pharmacokinetic studies. After subcutaneous injection of 20 mg/kg of the cephalosporins, serum and urinary antibiotic levels during the first 4 h after dosing were determined in duplicate pooled groups of ten mice. Serum levels were also determined in squirrel monkeys injected intramuscularly with 20 mg/kg of drug. The serum samples were kept frozen (—20 °C) until assayed. The apparent serum half-life was determined from a semi-log plot of antibiotic concentrations versus time.

Urinary antibiotic recovery during the first four-hour period after dosing was determined in duplicate groups of ten mice. Mice were placed in metabolism cages and urine samples were collected in containers packed in dry ice. Urine volumes were measured and the samples were stored at —20 °C prior to assay. The disc agar-diffusion assay, described above, was employed to determine drug concentration in blood and urine samples.

Results

In vitro studies. In Table I, the in vitro antibacterial activities (MIC values in μg/ml) of the eleven methoxyimino cephalosporin analogues are compared with those of cefuroxime and cefazolin against 15 representative

Table I

In vitro activity of twelve methoxyimino furyl- or phenylacetamido cephalosporins and cefazolin against fifteen bacterial strains

Bacterial strains / Compounds	Minimal inhibitory concentration (μg/ml)												
	I 79384	II 79395	III 80196	IV 81367	V 81147	VI 81366	VII 81409	VIII 81292	IX 82464	X 81903	XI 82259	Cefuroxime	Cefazolin
Staphylococcus aureus SK&F 23390	0.4	0.4	1.6	0.8	0.8	6.3	1.6	1.6	3.1	0.8	1.6	0.8	0.2
*Staphylococcus aureus** HH 127	0.4	0.4	1.6	1.6	1.6	6.3	1.6	3.1	3.1	0.8	1.6	0.8	0.4
*Staphylococcus aureus*** Villaluze SKF 70390	>200	200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	200
Streptococcus faecalis HH 34358	100	50	25	50	12.5	50	50	50	25	12.5	50	12.5	6.3
Escherichia coli[w] SKF 12140	12.5	6.3	6.3	3.1	1.6	25	0.8	1.6	1.6	1.6	100	3.1	0.8
*Escherichia coli** HH 33779	12.5	6.3	6.3	3.1	3.1	25	1.6	3.1	1.6	3.1	100	6.3	1.6
Klebsiella pneumoniae SKF 4200	3.1	1.6	3.1	1.6	0.8	12.5	0.4	0.8	0.4	1.6	50	1.6	0.8
*Klebsiella pneumoniae** SKF 1200	12.5	6.3	3.1	1.6	1.6	12.5	0.4	0.8	0.4	1.6	50	1.6	1.6
Salmonella paratyphi ATCC 12176	6.3	1.6	1.6	3.1	0.8	12.5	0.4	0.8	0.4	1.6	50	3.1	0.8
Proteus mirabilis PM 444	N.D.	N.D.	3.1	3.1	1.6	25	0.2	0.2	0.2	1.6	200	1.6	3.1
*Morganella morganii** # 179	6.3	3.1	6.3	6.3	1.6	12.5	1.6	1.6	1.6	1.6	>200	50	200
*Serratia marcescens** ATCC 13880	25	12.5	12.5	12.5	3.1	50	3.1	3.1	3.1	6.3	>200	12.5	>200
*Enterobacter aerogenes** ATCC 13048	12.5	12.5	12.5	6.3	3.1	25	3.1	3.1	3.1	6.3	100	6.3	1.6
*Enterobacter cloacae** HH 31254	6.3	3.1	3.1	3.1	1.6	12.5	0.8	1.6	0.8	1.6	100	3.1	0.8
*Pseudomonas aeruginosa** HH 63	>200	200	200	>200	200	>200	100	200	>200	200	>200	>200	>200

* β-Lactamase producer; [w] weak producer
** Methicillin resistant & β-lactamase producer
N.D.: Not done

bacterial strains regularly used in our laboratory for primary evaluation of cephalosporins and penicillins. All compounds containing the methoxyimino group in *syn*-configuration and in the α-position have the same qualitative antibacterial spectra characteristic of the second-generation cephalosporins and represented here by cefuroxime. These compounds have acceptable activity against *S. marcescens* and the indole-positive *M. morganii*, strains which are insensitive to the first-generation cephalosporins, i.e. cefazolin. The overall activity of compound No. VI, which contains the methoxyimino group in the *anti*-configuration is reduced. The activity of compound No. XI containing the methoxyimino group attached to the β- and not to the α-carbon atom of the acetyl group is especially weaker against Gram-negative bacilli. These are group specific general features of the structure activity relationships.

All these compounds have equally good activity (low MIC values) against the penicillinase-producing and non-penicillinase producing *S. aureus* strains, Compound No. X has the same level of activity as cefuroxime (MIC, 0.8 μg/ml), and compounds Nos I and II are somewhat better (MICs 0.4 μg/ml). Cefazolin as expected [13] is even more active, especially against the non-penicillinase producing *S. aureus* strains. Like all other cephalosporins, none of these compounds has activity against methicillin-resistant *S. aureus* strains. Again, as is characteristic of the presently known and used cephalosporins, none of the compounds in Table I has acceptable activity against *S. faecalis* strains.

Against Gram-negative bacteria, in general, the *syn* α-methoxyimino-phenylacetamido compounds (Nos I–III) are more active then the non-methoxyimino counterparts [14] but are somewhat less active than the *syn* α-methoxyimino-furylacetamido cephalosporins (Nos IV–XII). Compounds containing the acetoxymethyl (Nos I, IV) or carbamoyloxymethyl (No. XII) groups at the 3-position are somewhat less active than compounds which contain a substituted tetrazole-thiomethyl ring. These latter compounds, especially those carrying a strongly acidic function and a large electronegative charge at the N_1-tetrazole ring (Nos VII, VIII, IX) have even lower MIC values against the Gram-negative bacteria than cefuroxime and cefazolin. Although activity against *P. aeruginosa* is not expected from the first- and second-generation cephalosporins, some of these new compounds (Nos II, III, V, VII, VIII, X) exhibited traces of in vitro activity against this difficult-to-treat pathogen.

All of these new cephalosporins, like cefuroxime, have basically the same level of in vitro activity against the beta-lactamase producing or non-beta-lactamase producing variants of the same genus of the Gram-negative bacilli in the test.

In vivo experimental chemotherapeutic efficacy studies. The therapeutic efficacy of these new cephalosporins, with the exception of compounds Nos VI and XI which were found weakly active in the in vitro test, was compared along with that of cefuroxime and cefazolin in acute systemic experimental

Table II

Subcutaneous efficacy of twelve methoxyimino furyl- and phenylacetamido cephalosporins and cefazolin in experimental acute infections of mice

Bacterial strains / Compounds	ED_{50} (mg/kg)													
	I 79384	II 79395	III 80196	IV 81367	V 81147	VI 81366	VII 81409	VIII 81292	IX 82464	X 81903	XI 82259	Cefuroxime	Cefazolin	Challenge LD_{50}**
*Escherichia coli** SK&F 12140	>50													222
		43												73
			>50											250
				29								6.25	6.25	386
					6.25									88
						N.D.					N.D.			—
							6.25							244
								4.4						386
									1.56					166
										1.02			4.0	130
Klebsiella pneumoniae SK&F 4200	>50												12.5	142
		>50												681
			45									4.0	4.5	214
				>50										150
					5.5									88
						N.D.					N.D.			—
							3.9							57
								1.82				4.6		150
									0.78					77
										7.2				62

* Weak β-lactamase producer
** Excess dose of inoculum required to kill 50% of the mice
N.D.: Not done

infections caused by *E. coli* or *K. pneumoniae* in mice. The ED_{50} values of the α-methoxyimino-phenylacetamido cephalosporins (Table II) are, in general, inferior to those of the α-methoxyimino furylacetamido cephalosporins in spite of their relatively similar MIC values. In *E. coli* infections the furylacetamido compounds exhibited the same (Nos VII, VIII) or better (Nos IX, X) protective power, as expressed by the ED_{50} values, than cefuroxime or cefazolin. In *K. pneumoniae* infections compound No. X was found to be weaker, compound No. VII equivalent and compounds Nos VIII and IX better than cefuroxime. Cefazolin, for unknown reasons, is not very effective in mouse *K. pneumoniae* experimental infections.

Basic pharmacokinetics in mice and squirrel monkeys. The antibiotic concentrations in serum and elimination half-lives, as well as the urinary recovery of five selected α-methoxyimino furylacetamido cephalosporins were determined in mice dosed with 20 mg/kg of compound (Table III). Compounds containing at the 3-position the tetrazole moiety with strong acidic function (Nos VII, IX), produced higher and more prolonged serum levels than the other compounds. This finding is in agreement with our earlier findings with SK&F 75073 (cefonicid), SK&F 80303 and SK&F 88070, cephalosporins which contain the same acidic functional group at the 3-position [9, 10, 15]. Cefuroxime has the shortest elimination half-live but the highest urinary recovery in 4 h.

In squirrel monkey only compounds found to be long-acting in mice (Nos VII, IX), were studied. After 20 mg/kg intramuscular injection, these compounds produced higher serum peak concentrations, somewhat shorter half-lives but higher urinary recoveries in 4 h in squirrel monkey (Table IV) than was observed in mice (Table III).

Table III

Serum peak concentrations, half-lives and urinary recoveries following subcutaneous injections to mice of selected methoxyimino-furylacetamido cephalosporins at 20 mg/kg and their in vitro binding to serum proteins

	Compounds	Peak conc. (μg/ml)	Half lives (min)	Urine recovery (%), 4 h	Serum Binding (%)	
					mouse	human*
V	SK&F 81147	38	18	28	31	56
VII	SK&F 81409	52	135	10	76	90
IX	SK&F 82464	80	80	36	69	72
X	SK&F 81903	19	18	11	26	50
XII	Cefuroxime	38	14	60	35	30

* Binding to human serum proteins of compounds III (SK&F 80196) and IV (SK&F 81367) was 91% and 38%, respectively

Table IV

Serum peak concentrations and half-lives in squirrel monkeys following intramuscular injections of compounds No. VII and No. IX at 20 mg/kg and their in vitro binding to serum proteins

	Compounds	Peak conc. (μg/ml)	Half lives (min)	Urine recovery (%), 4 h	Serum binding (%)	
					mouse	human
VII	SK&F 81409	170	94	76	87	90
IX	SK&F 82464	125	50	69	62	72

In vitro binding to serum proteins of the compounds listed in Tables III and IV varies. Cefuroxime has a relatively low serum protein-binding quality. Compounds with acidic functions at the 3-position have higher serum protein binding properties which appears to play a role in their favorable serum-level profiles. In general, the serum protein binding of the studied compounds displays a species specificity. Their binding to mouse serum proteins is less than to squirrel monkey serum proteins which is less than to human serum proteins.

Discussion

The introduction of the α-methoxyimino group into the aromatic or heterocyclic acyl moiety at the 7-position of the cephalosporin molecule significantly increases the antibacterial activity and beta-lactamase stability of these compounds. Originally it was found in a fermentation product. Nocardicin A was the first natural product antibacterial beta-lactam containing an oxyimino group at the α-position of the phenylacylamino moiety [16, 17]. There is at the α-position a hydroxyl group in cefamandole and cefonicid; an amino group in ampicillin and all oral cephalosporins; a carboxyl group in carbenicillin, ticarcillin, temocillin, and moxalactam; a sulphonic acid group in sulbenicillin and cefsulodin and a ureido group in azlocillin, mezlocillin, piperacillin and cefoperazone.

Nocardicin A having the unique α-hydroxyimino group in the α-carbon served as the prototype for the synthesis of the hydroxyimino- and alkoxyimino-substituted cephalosporins [10, 18, 19, 23]. Attachment of the α-methoxyimino group to the 7-furylacetamido substituent produced cefuroxime with its broad antibacterial spectrum and high level of resistance to most beta-lactamases. Cefuroxime additionally contains a carbamoyloxymethyl group at the 3-position, that was replaced in our new compounds by substituted tetrazole moieties. Some of these new compounds possess not only improved pharmacokinetic properties (higher and prolonged serum levels), usually conveyed by substituents at 3-position but they also have increased antibacterial

potencies, which are usually attributable to the 7-substituents. Some minimal in vitro activity was observed against *P. aeruginosa.*

Spectacular spectrum broadening, high level of beta-lactamase stability and extraordinary antibacterial potency were obtained when the α-methoxyimino or, in general, an oxyimino group was attached in *syn*-configuration to the α-carbon of the acetamido side chain located at the 7-position. A host of such new cephalosporins containing a variety of substituents at the 3-position of the beta-lactamdihydrothiazine nucleus are described as third or fourth generation cephalosporins [20, 21]. The marketed and more extensively studied ones have been the subject of many recent papers [22–24]. These third and fourth generation cephalosporins are used for the treatment of infections caused by bacteria insensitive to the first- and second-generation cephalosporins. They should be reserved for the therapy of such infections, where they are uniquely potent and life-saving agents. They are costly and the spectacular in vitro activity is often not associated with reduced doses. For the treatment of common Gram-negative and most staphylococcal infections the first and second-generation compounds serve a useful purpose. Cefuroxime has many advantageous properties [25], but, as is demonstrated in this study, even better compounds can be produced within the same class of cephalosporins.

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Acta Microbiologica Hungarica 33 (2), pp. 141—146 (1986)

ANTIBACTERIAL ACTIVITY OF PRIMYCIN AGAINST MULTIPLE STRAINS OF GRAM-POSITIVE BACTERIA

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Crystalline primycin was found to be very active in broth dilution assay against *Staphylococcus aureus* (50 strains), *Staphylococcus epidermidis* (77 strains), *Streptococcus faecalis* (76 strains) and one strain of *Listeria monocytogenes* with minimal inhibitory concentrations (MIC) of 0.12–0.5 μg/ml. The activity was influenced by the pH of the liquid medium with greater activity (lower MICs) at pH 8 against the majority of strains, than at pH 6 or 7.3. In disc agar diffusion assay *Bacillus subtilis* ATCC 6633 proved more sensitive than staphylococcus strains.

Primycin is a mixture of homologous antibiotics produced by a strain initially described as *Streptomyces primycini* [1] and isolated from the fermentation material as an amorphous yellowish powder. Later the strain was re-isolated and re-classified as *Micromonospora galeriensis* [2]. I have coined the name primycin. The first part of the name originates from the Latin word "primus" to denote that this was the first original antibiotic discovered, isolated and produced in Hungary. The second part of the name "mycin" relates to the producing microorganism, which is a new *Streptomyces* sp. The chemical structure of the major component of primycin (Fig. 1) has been elucidated [3–7]. To date, it is the largest (36-membered), polyhydroxylated non-polyene macrolide antibiotic, uniquely containing a guanidine and a D-(—)-arabinose moiety.

I obtained primycin in crystalline form (Fig. 2) from a mixture of dimethylsulfoxide, methanol and water at 37 °C over a period of about 20 days [8]. It is a snow-white, needle-form crystalline material with a melting point of 202–206 °C (decomp.), in contrast to the industry-produced material, the melting point of which is 178–180 °C (decomp.) [7]. The in vitro antibacterial studies described in this paper were performed with this crystalline material.

Originally, primycin was described as being active against *S. aureus* [1] and highly active against human pathogenic, including polyresistant, as well

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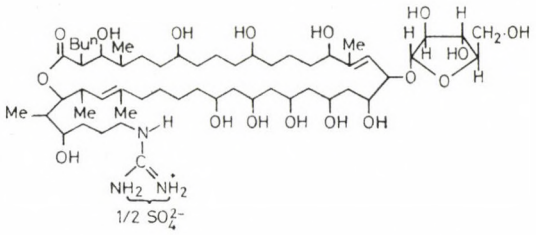

Fig. 1. Chemical formula of primycin

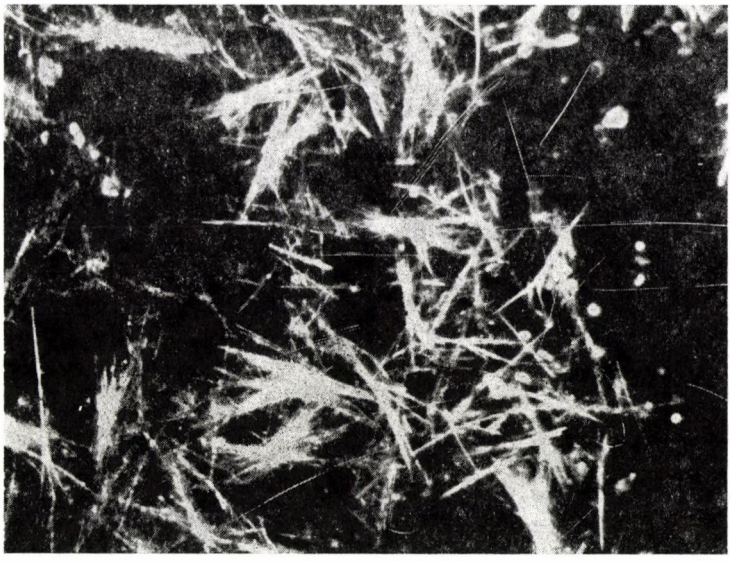

Fig. 2. Crystals of primycin from dimethylsulfoxide/methanol/water

as non-pathogenic mycobacterial strains [9]. Later it was also found to be inhibitory to *Euglena gracilis* and *Astasia longa* [10], as well as *Candida albicans* and *Trichophyton mentagrophytes* [8].

The purpose of the study described in this paper was to examine the in vitro activity at three pH levels of the highly purified crystalline primycin against a large number of Gram-positive bacteria some of which have not been tested earlier. Because primycin is a week base, it was surmised that MIC values would vary with the pH of the medium [11]. In addition, the activity and diffusibility of primycin into solid medium was studied.

Materials and methods

Primycin. For the assays described here the crystalline primycin preparation was used. The stock solutions were prepared in dimethylsulfoxide (DMSO) and further diluted in sterile deionized water.

Bacterial strains. Of the 50 *Staphylococcus aureus* strains, seven are often used laboratory strains: ATCC 29923, 209P, 910R, villaluze; SK&F Nos 127 (670), 671, and 674 [12, 13]. The rest of the *S. aureus*, all of the *Staphylococcus epidermidis* strains and the majority of the *Streptococcus faecalis* strains were recent clinical isolates from various geographical areas in the United States. The *Listeria monocytogenes* tested is a laboratory strain. It is widely used as a model for studying cell-mediated immune mechanisms in animal experiments. *Bacillus subtilis* ATCC 6633 is the strain extensively used in antibiotic assays.

In vitro assays. Minimum inhibitory concentrations (MICs) were determined by the conventional broth-dilution technique adding the serial doubling concentrations of primycin to the semisynthetic peptone-glucose broth [14]. This medium was buffered to pH 6.0, 7.3. or 8.0 by the addition of 10% McIlvaine's citric acid-phosphate buffer [15]. The media were inoculated with the appropriate dilutions of strains grown in the same medium for 18 h at 37 °C. The MICs were established by visual observation of the cultures grown overnight a, 37 °C. The MIC values represent the dilutions where no visible growth could be observedt

For the disc agar diffusion assay, the antibiotic susceptibility discs (Schleicher–Schuell Inc., 6.35 mm diameter) were saturated prior to the test with the appropriate concentrations of primycin and placed on the surface of the Penassay seed agar plates seeded with spores of *Bacillus subtilis* ATCC 6633 or with the titrated dilutions of the staphylococcus cultures. Plates were incubated overnight at 30 °C and the inhibition zones were measured in mm in duplicate experiments [16].

Bactericidal activity. MBC values were determined by plating 0.05 ml of each turbidity-free tube from the MIC dilution series on Penassay seed agar plates. The MBC was considered to be the lowest concentration that resulted in no more than 1–2 colonies after incubation at 37 °C for 24 h.

Results and discussion

Table I demonstrates the number of strains in each species, and the number of strains the growth of which was inhibited (MIC) at various concentrations (0.5, 0.25 and 0.12 μg/ml) of primycin at the stated pH of the medium (pH 6, 7.3 or 8). In general, all the cocci (staphylococci and streptococci) are, with little variation, very sensitive to primycin, having MICs between 0.12 and 0.5 μg/ml. Since there was only one *L. monocytogenes* strain included, no generalization can be made as for the distribution of susceptibility of strains. This laboratory strain has a MIC value for primycin of 0.25 μg/ml, which appears to be not pH-dependent.

Table I

Minimum inhibitory concentrations (MICs) in μg/ml of primycin against multiple strains of Gram-positive bacteria at three pH values

Bacteria	Total No. of strains	μg/ml	No. of strains inhibited at 0.5			0.25			0.12		
		pH 6	7.3	8	6	7.3	8	6	7.3	8
S. aureus	50	50	50	50	50	50	50	2	None	26
S. epidermidis	77	77	77	77	72	77	77	None	51	65
S. faecalis	76	26	74	70	None	13	29	None	6	10
L. monocytogenes	1	1	1	1	1	1	1	None	None	None

All fifty *S. aureus* strains were inhibited by 0.25 μg/ml of primycin at all three pH values. At 0.12 μg/ml two strains were inhibited at pH 6, no strains were inhibited by pH 7.3 whereas 36 strains were inhibited at pH 8. The two strains which were inhibited by 0.12 μg/ml at pH 6 suggest that not only the state of ionization of primycin but also that of primycin-receptor(s) in these two strains are different than the other *S. aureus* strains tested.

All 77 *S. epidermidis* strains were inhibited by 0.5 μg/ml of primycin at all three pHs. At 0.25 μg/ml all strains were inhibited at pH 7.3 and 8. However, only 72 strains were inhibited at pH 6. This pH-dependent susceptibility was more prominent at 0.12 μg/ml, where no inhibition was observed at pH 6, 51 strains were inhibited at pH 7.3, and 65 strains were inhibited at pH 8.

Primycin also showed definite pH-dependent activity against 76 strains of *S. faecalis*. At 0.5 μg/ml concentration, 70 strains were inhibited at pH 8, 74 strains were inhibited at pH 7.3 and 26 strains were inhibited at pH 6.0. At 0.25 μg/ml concentration, 29 strains were inhibited at pH 8, 13 were inhibited at pH 7.3 and none was inhibited at pH 6. The same tendency of susceptibility was obtained at 0.12 μg/ml; at pH 8 ten strains, at pH 7.3 six strains and at pH 6 no strains were inhibited.

L. monocytogenes was inhibited at all three pHs by 0.5 and 0.25 μg/ml, but not at 0.12 μg/ml primycin.

This pH-dependent activity of primycin can be explained by the weak basic character of the molecule which has a different level of ionization at various pH levels resulting in variable penetration into the bacterial cell and attachment to the receptor(s); this is a general finding for all antibiotics (and drugs) which are weak acids or bases [17].

Primycin was found to be bactericidal to these Gram-positive cocci at the bacteriostatic concentrations which is independent of the bacterium's resistance to other antibiotics. This is probably due to primycin's mode of action. Primycin affects bacterial cells in two ways. First, it is an ionophore binding strongly to the cell membrane of the Gram-positive bacteria [18]. Primycin is also a potent ionophore in other cell membranes where it has been shown to affect the neuromuscular junction [19], acetylcholine-releasing sites [20], erythrocyte membrane and lipid bilayer [21], the inner membrane of mitochondria [22, 23], and striated muscle [24]. Secondly, primycin interacts with DNA, thereby affecting growth and reproductive processes of the bacterial cells [10, 25–27]. This activity is responsible for primycin's ability to influence the induction of tryptophan pyrrolase [28].

Primycin is toxic to laboratory animals [1, 9, 29], therefore its use is limited to topical application in such areas as ophthalmology [30], gynecology [31], urology, i.e., non-gonorrhoeal urethritis [32] and urogenital tuberculosis [33]. In addition to its antibacterial activity, primycin has exhibited antiinflammatory and antiallergenic properties, especially in the con-

junctiva. Sensitization to primycin was not observed or reported even after more than 50 days of topical application [34].

In the disc agar diffusion assay, the activity of primycin was tested against *B. subtilis* ATCC 6633, and *S. aureus* strains 910R and 209P. Table II shows that these staphylococcus strains can be used in this assay, although *B. subtilis* ATCC 6633 is still somewhat more sensitive for assaying primycin in samplable biological materials and for quality control of laboratory samples. In spite of the large molecular weight, primycin diffuses fairly well in the agar.

Table II

Diameters of inhibition zones in mm of primycin on these bacterial strains

Primycin (μg) content of discs	Diameter of inhibition zones in mm		
	B. subtilis ATCC 6633	*S. aureus* 910R	*S. aureus* 209P
10	26	18	19
5	23	16	16
2.5	20	13	13
1.25	18	12	12
0.6	17	11	10
0.3	14	10	8
0.1	10	0	0

Because of the increasing interest in the drug delivery systems [35], primycin (with its higher activity against mycobacteria and Gram-positive cocci at pH 8) may be a candidate for use in the preparation of improved topical formulations. Primycin's anecdotal „virucidal" activity requires careful evalution [36]. Recently other antibiotics with a large macrolide structure related to primycin were reported [37].

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Note added in proof

During the process of this paper primycin was marketed for topical use by Chinoin Pharmaceutical and Chemical Works with the trademark of Ebrimycin®.

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SEROLOGIC AND PROTECTIVE CROSS-REACTIVITY OF ANTISERA TO *PSEUDOMONAS AERUGINOSA* EXTRACELLULAR SLIME GLYCOLIPOPROTEIN

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Antisera to extracellular slime glycolipoprotein of *Pseudomonas aeruginosa* of different O serogroups (immunotypes) show cross reactivity in passive haemagglutination (PHA) and agar gel immunoprecipitation test; cross reactivity is more distinct in PHA. Antisera to glycolipoprotein also have marked cross activity in mouse passive protection test against intraperitoneal challenge with 24 *P. aeruginosa* strains of different O groups including the toxigenic strain PA-103. On the basis of the results, glycolipoprotein producer *P. aeruginosa* strains may be selected in order to make a preparation for active and passive immunization against pseudomonas infection.

In our previous paper [1] it was proved that purified glycolipoprotein (GLP) of *Pseudomonas aeruginosa* extracellular slime stimulates active immunity in mice to the intraperitoneal challenge with O serogroup (immunotype) homologous or heterologous *P. aeruginosa* strains. It was also shown that sheep antiserum to partially purified extracellular slime [2] stimulates passive immunity against the challenge with homologous *P. aeruginosa* strain in mice, and anti-GLP serum stimulates reaction between *P. aeruginosa* and macrophages [3].

In this paper we present data on the cross serologic and protective activity of antisera to GLP isolated from various *P. aeruginosa* strains.

Materials and methods

Strains. We used *P. aeruginosa* strains 170001, 170005, 170006, 170007, 170009, 170010, 170014, 170017, 170018, 170019, 170021, 170022, 170023 and also Fisher's F1–F7 immunotypes received from the National Institute of Hygiene, Budapest, Hungary; strain No. 8 from the Vishnevsky Institute for Surgery of the USSR Academy of Medical Sciences, Moscow, USSR; strain OII from the Tarasevich State Research Institute for Standardization and Control of Medical and Biological Preparations, Moscow, USSR, and the toxin-producing strain PA-103 from the Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, WA, USA. The O-antigen structures of the strains is given according to the Lányi–Bergan system [4] as in our previous paper [1].

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GLP was isolated from crude *P. aeruginosa* extracellular slime, as described earlier [1]. Chinchilla rabbits weighing 2 to 3 kg were immunized with GLP preparations according to the following schedule: first immunization, 1 mg in complete Freund adjuvant subcutaneously; second and third immunization, 2 mg each intravenously, the interval between immunizations was 3 to 4 days; a repeated immunization cycle consisting of 2 intravenous injections (4 and 6 mg of the preparation) with the same interval was performed four weeks later. On the 7th day after the last immunization, the rabbits were bled, and the serum was stored at—30 °C.

Antisera were studied by passive haemagglutination test (PHA), by immunoprecipitation in agar gel by the Ouchterlony method [5] and in mouse passive protection tests [2].

PHA was performed by means of Takátsy's micromethod [6]. Formalinized sheep erythrocytes were sensitized with GLP: optimum sensitive dose was 125 μ. Antisera were diluted with buffered saline (pH 7.2–7.4), from 1 : 100 to 1 : 51 200; 0.05 ml of a 25% suspension of erythrocytes sensitized with GLP was added to every dilution. The mixture was kept in an incubator at 37 °C for 2 h, then the reaction was read. The last serum dilution showing a clear haemagglutination, estimated as 4+, was accepted as the titre.

Results and discussion

Table I sums up the PHA results. GLP of some strains exhibited a cross antigenic activity, independent of the O serogroup of the given strains. Comparing these data with those for the lipopolysaccharide (LPS) analysis performed by means of PHA [7], it may be concluded that GLP, unlike the corresponding LPS, has no strict O antigen specificity. There are antigenic relationship between GLP of the strains of different O serogroups. However, certain GLPs do not cross-react. Accordingly, GLP differs in antigenic specificity from LPS and it may be possible to work out an individual antigenic scheme for GLP.

Antigenic relationships between crude slime or its purified fractions, on the one hand, and the corresponding LPS, on the other, were shown by immunoprecipitation (Table II). It turned out that GLPs were more specific by immunoprecipitation than PHA. GLP immunoprecipitation data conform to the O antigen specificity though several cross-reactions were observed which did not occur between the corresponding LPS [7]. The absence of a strict correspondence between the results may be attributed, firstly to a different sensitivity of these tests for common antigenic determinants, and secondly, it is possible that in PHA and in immunoprecipitation different antigenic determinants react. Evidently GLP of different strains contain different sets of these antigenic determinants. Results of the immunoprecipitation test also support our view that a separate system is practical for the classification of slime antigens. Cross-reactions between GLP of different O serogroups seem to have been proved chemically: slime preparations of different *P. aeruginosa* strains contain common components — algine acids [8] — which evidently can cause cross-reactions.

Tables III and IV give results of passive protection tests on mice immunized with anti-GLP sera. The result was considered positive (presence of cross-protection) if the value of the corresponding antiserum EI was sub-

Table I

Results of the cross PHA with antisera to P. aeruginosa GLP

Anti-GLP sera to strains	Titres* of antibodies to GLP of strains											
	170001	170005	170006	170007	170009	170010	170014	170017	170018	170019	170023	OII
170001 (O3)**	204 800	0	400	0	0	800	0	0	100	800	400	0
170005 (O2)	100	12 800	12 800	1 600	0	0	0	0	0	0	100	0
170006 (O2)	0	100	25 600	400	0	0	0	0	0	0	0	0
170007 (O2)	1 600	800	3 200	25 600	0	200	800	200	0	800	800	200
170009 (O6)	400	0	800	400	12 800	3200	400	0	100	100	0	0
170010 (O6)	400	0	200	400	400	3200	400	0	0	1000	800	0
170014 (O1)	400	0	0	0	100	0	12 800	0	0	0	1600	0
170017 (O8)	200	0	0	0	0	0	800	1600	0	0	400	0
170018 (O9)	0	0	0	0	0	0	0	0	6400	0	0	0
170019 (O9)	6 400	0	3 200	1 600	100	0	1 600	0	0	6400	800	100
170023 (O12)	800	0	400	200	0	400	400	0	100	200	6400	0
OII (O11)	0	0	0	0	0	100	0	100	0	0	0	3200

* Reciprocal values
** Bracketed figures: O serogroups according to Lányi and Bergan's system [4]

Table II

Results of cross immunoprecipitation in agar gel with antisera to P. aeruginosa GLP

Anti-GLP serum	GLP of strains											
	170001	170005	170006	170007	170009	170010	170014	170017	170018	170019	170023	OII
170001	++	−	−	−	−	+	−	−	−	+	+	−
170005	−	+++	++	+	−	−	−	−	−	+	+	−
170006	−	+	++	+	−	−	−	−	−	+	+	−
170007	−	+	−	+	−	+	−	−	−	+	+	−
170009	−	−	−	−	++	+	−	−	−	+	+	−
170010	+	−	−	−	−	++	−	−	−	+	+	−
170014	−	−	−	−	−	−	+	−	−	−	−	−
170017	−	−	−	−	−	−	+	+	−	−	−	−
170018	−	−	−	−	−	−	−	−	+	+	+	−
170019	−	−	−	−	−	−	−	−	−	++	+	−
170023	++	−	−	−	−	−	−	−	−	+	+	−
OII	−	−	−	−	−	−	−	−	−	+	+	+

O serogroups of the strains: see Table I
Crosses denote the number of precipitation lines

Table III

Passive protection with anti-GLP sera of mice challenged with different P. aeruginosa strains

Anti-GLP serum	LD_{50} and its confidence limits ($\times 10^6$ cells) when challenge is performed with the strains:				
	F1 (O6)	Fe (O11)	F3 (O2)	F4 (O1)	F5 (O10)
170001 (O3)	919 (695–1211)	282 (214–373)	54 (41–71)	360 (246–528)	528 (400–695)
170002 (O10)	650 (578–919)	373 (264–528)	81 (61–123)	162 (123–246)	400 (283–566)
170005 (O2)	919 (695–1211)	348 (264–525)	649 (460–919)	566 (386–828)	649 (492–857)
170006 (O2)	649 (491–857)	282 (214–373)	373 (264–528)	193 (127–255)	649 (443–951)
170007 (O2)	132 (100–199)	800 (695–1114)	132 (87–174)	303 (214–429)	264 (187–374)
170009 (O6)	>1600	400 (303–528)	66 (50–87)	516 (391–782)	400 (296–649)
170010 (O6)	697 (493–984)	373 (255–546)	81 (62–123)	400 (296–650)	400 (296–649)
170014 (O1)	230 (162–324)	1054 (800–1600)	107 (181–162)	1055 (800–1600)	246 (174–348)
170017	400 (303–528)	491 (336–719)	43 (33–57)	528 (400–695)	214 (163–283)
170018	325 (246–429)	373 (255–546)	38 (29–50)	459 (348–605)	246 (187–325)
170019 (O9)	209 (148–295)	527 (361–970)	100 (71–141)	377 (283–566)	348 (246–492)
170023 (O12)	459 (325–649)	527 (361–970)	47 (33–66)	152 (115–230)	187 (123–246)
OII (O11)	283 (214–373)	1300 (706–1901)	41 (31–53)	528 (400–695)	241 (182–318)
Control-1 (normal serum)	200 (152–264)	123 (87–174)	20 (14–30)	71 (44–113)	141 (107–186)
Control-2 (non-immunized mice)	61 (39–105)	47 (30–73)	9 (7–12)	50 (38–66)	35 (17–76)

Table III (continued)

Anti-GLP serum	LD_{50} and its confidence limits ($\times 10^6$ cells) when challenge is performed with the strains:				
	F6 (O7)	F7 (O2)	F8 (O3)	1700021 (O4)	170022 (O15)
170001 (O3)	107 (81–162)	123 (87–174)	107 (76–152)	1047 (842–1303)	3428 (2427–4842)
170002 (O10)	174 (129–246)	200 (141–283)	132 (87–174)	1393 (986–1968)	2985 (2113–4217)
170005 (O2)	207 (146–293)	209 (148–295)	241 (138–318)	1380 (1076–1770)	4353 (3436–6855)
170006 (O2)	123 (71–152)	303 (214–428)	263 (230–300)	562 (436–725)	4529 (3206–6397)
170007 (O2)	214 (152–303)	459 (303–605)	283 (214–373)	1820 (1479–2264)	1585 (1149–2188)
170009 (O6)	695 (492–985)	61 (35–76)	141 (107–187)	1820 (1445–2291)	3631 (2951–4467)
170010 (O6)	400 (283–566)	107 (76–152)	100 (71–142)	1698 (1380–2089)	3483 (2471–6823)
170014 (O1)	649 (578–918)	246 (187–325)	141 (125–165)	1600 (1094–2339)	4853 (3436–6855)
170017	264 (187–373)	141 (100–200)	174 (123–246)	1941 (1327–2839)	3483 (2630–4571)
170018	132 (100–200)	<50	200 (141–283)	1380 (1076–1770)	3673 (2600–5188)
170019 (O9)	429 (303–605)	141 (100–200)	132 (100–200)	1047 (842–1303)	3388 (2673–4295)
170023 (O12)	800 (566–1130)	<50	123 (87–174)	1698 (1380–2089)	2951 (2286–3807)
OII (O11)	132 (100–199)	230 (162–324)	209 (159–276)	2265 (953–3311)	4529 (3206–6397)
Control-1 (normal serum)	186 (123–246)	62 (36–77)	41 (28–59)	528 (400–695)	1132 (774–1656)
Control-2 (non-immunized mice)	27 (17–40)	35 (27–47)	15 (12–17)	245 (129–316)	427 (252–632)

stantially higher than that of normal rabbit serum EI. It may be concluded that the results showed cross-protection of mice immunized with different antisera. It should be noted that results of the challenge with different strains of one and the same O serogroup (or immuno-type) are not identical. Thus, strains F3, F7, 170007 and No. 8 of serogroup O2 are highly virulent for mice, especially strains F3 (Table III), but protection of mice against the challenge with these strains is not the same. While mice of all the 13 test groups are resistant to challenge with strain No. 8, mice in 8 out of 13 test groups are resistant to strain 170007, including three test groups in which mice were injected with serogroup-homologous antisera. These data also show an absence of correlation between serogroup (immunotype) and protective activity. Evidently, various strains of one and the same serogroup contain different amounts of the general (species specific) protective antigen (antigens) localized in the slime. Results of the passive mouse protection tests show that different anti-GLP sera protect mice against the challenge with 11–13 out of 14 heterologous *P. aeruginosa* strains, but anti-GLP 170018 serum protects mice against the

Table III (continued)

Anti-GLP serum	LD_{50} and its confidence limits ($\times 10^6$ cells) when challenge is performed with the strains:				
	170001 (O3)	170015 (O11)	170007 (O2)	PA-103 (O11)	170023 (O12)
170001 (O3)	1047 (842–1303)	359 (240–523)	286 (209–390)	457 (375–569)	912 (641–1297)
170002 (O10)	800 (566–1130)	428 (293–627)	360 (246–528)	NS	NS
170005 (O2)	800 (566–1130)	324 (225–466)	800 (566–1130)	371 (302–460)	977 (701–1363)
170006 (O2)	607 (429–857)	892 (538–889)	695 (492–985)	347 (254–473)	1288 (946–1754)
170007 (O2)	214 (111–330)	333 (219–456)	528 (400–695)	346 (256–470)	1478 (1094–2000)
170009 (O6)	837 (598–1183)	303 (214–429)	324 (225–466)	347 (259–465)	1698 (1318–2191)
170010 (O6)	746 (528–1054)	650 (445–951)	377 (283–566)	427 (324–548)	1698 (1380–2103)
170014 (O1)	800 (566–1130)	214 (152–303)	246 (187–325)	347 (254–473)	1288 (953–1744)
170017	650 (460–918)	303 (214–429)	400 (296–650)	347 (270–446)	1479 (1122–1950)
170018	857 (607–1211)	246 (187–325)	325 (246–429)	324 (245–427)	977 (701–1363)
170019 (O9)	607 (429–857)	209 (148–295)	359 (240–523)	427 (336–542)	1585 (1216–2067)
170023 (O12)	857 (607–1281)	324 (225–466)	303 (214–429)	282 (202–393)	2951 (2285–3807)
OII (O11)	857 (607–1211)	697 (476–1023)	325 (246–429)	263 (184–376)	1288 (953–1744)
Control-1 (normal serum	152 (115–230)	141 (100–200)	200 (141–283)	162 (113–234)	507 (383–696)
Control-2 (non-immunized mice)	71 (44–113)	35 (25–50)	50 (31–82)	54 (30–96)	123 (89–170)

O serogroups by Lányi and Bergan's system [4] are given in brackets; strains 170017 and 170018, described in Lányi's original scheme as belonging to groups O8 and O9, respectively, have been shown to contain deficient O antigens. Each experiment was performed on 11–13 test groups (11–13 different antisera) and on 2 control groups (control 1 = mice immunized with pooled normal rabbit serum, control 2 = non-immunized mice). Mice were challenged with different doses of *P. aeruginosa* live culture: 50 animals in every group, 10 mice per 1 dose of live culture

NS = not studied

challenge with only 8 out of 14 heterologous strains. It is interesting to note that crude slime [9] and GLP [1] and also anti-GLP sera protect mice against a challenge with the toxigenic strains PA-103 (Tables III and IV). But antiserum to strains OII GLP (O11 serogroup) do not protect mice (when compared with the normal serum) against challenge with the toxigenic strain PA-103 which is also of the O11 serogroup. Nevertheless, isolated slime or purified GLP of strain OII, when used for active immunization of mice, stimulate resistance to the challenge with the toxigenic strain PA-103 [1, 9]. We could presume that strain OII slime and the corresponding GLP are immunogenic for mice and weakly or not at all immunogenic for rabbits and,

Table IV

Efficiency indexes (EI) of antisera to GLP of different P. aeruginosa strains

Anti-GLP sera	EI after challenge with strains														
	F1	F2	F3	F4	F5	F6	F7	170001	170007	170015	170021	170022	170023	No. 8	PA-103
170001	15	6	6	7.2	15.1	—	—	*14*	—	10.2	4.2	8	—	7.1	8.5
170002	10.6	7.9	9	3.2	*11.4*	—	5.7	11	—	12.2	5.4	6.9	NS	8.8	NS
170005	15	7.5	*72.2*	11.3	18.5	—	*5.9*	11	*16*	9.2	5.6	11.3	7.9	*16*	6.8
170006	10.6	6.0	*41.4*	3.8	18.5	—	*8.6*	8.5	*13.9*	25.5	—	10.6	10.3	*17.5*	6.4
170007	—	17	*14.6*	7.1	7.5	—	*13*	—	*10.5*	9.5	7.4	—	12	*19*	6.4
170009	*>26.2*	8.5	7.3	10.3	11.4	25.7	—	11.7	—	8.6	7.4	8.5	13.8	9.4	6.4
170010	11.4	7.9	9	8.0	11.4	14.7	—	10.5	7.5	18.5	6.4	8.1	13.8	6.6	7.9
170014	—	22.4	11.8	*21.1*	—	24	7	11	—	—	6.5	11.3	10.3	9.4	6.4
170017	6.5	10.4	4.7	10.5	—	—	4	9.1	8	8.6	7.9	8.1	12	11.6	6.4
110018	—	7.9	—	9.2	—	—	—	12	—	—	5.6	8.6	7.9	13.3	6
170019	—	11.2	11.1	7.5	9.9	15.8	4	8.5	—	—	4.2	7.8	12.8	8.8	7.9
170023	7.5	11.2	5.2	3	—	29.6	—	12	—	9.2	6.9	6.9	*23.9*	8.2	—
OII	—	*27.6*	4.5	10.5	—	—	6.5	12	—	*19.9*	9.2	10.6	10.3	14.2	—

Note. Figures printed in italics: antiserum EI (i.e., LD_{50} in the test) does not substantially differ from normal serum EI (i.e., LD_{50} in the control 1, see also Table III)

$$\text{Efficiency index (EI)} = \frac{\text{test } LD_{50}}{\text{control—1 } LD_{50}}$$

NS = not studied

accordingly, anti-GLP OII rabbit serum does not provide passive protection for mice. We cannot, however, exclude another version, namely that there is no correlation between value of protection against *P. aeruginosa* toxigenic strains belonging to certain serogroups.

Eliciting passive immunity in mice by injecting anti-GLP sera is evidently due to the presence in such sera of specific opsonins [3] which give protection also against *P. aeruginosa* toxigenic strains. From these data it may be concluded that antiserum (or immune plasma) to the slime antigens can completely substitute antitoxic serum obtained after immunization with *P. aeruginosa* exotoxin A. Thus, anti-GLP serum (or immune plasma) can have both anti-infectious and antitoxic effects.

Results of passive protection tests in mice immunized with anti-GLP sera, compared with the results of active protection tests in mice immunized with GLP [1] have shown (Table V) that in 34.7% out of 98 test variations GLP is more active than anti-GLP serum; on the contrary, anti-GLP serum is more active than GLP in 42.9%, and the activity of the preparations is similar in 22.4%. Reading data of Table V along the vertical lines, we can conclude that, being challenged with F1, F2 and F3, GLP is more active than anti-GLP serum in 7–9 out of 13 cases. When challenged with strain F4, anti-GLP serum turned out to be more active than GLP in all the variations. When challenge was made with strains F5, F7 and No. 8, GLP was more active than anti-GLP serum only in 1–4 out of 10–13 cases. When challenged with strain F7, activity of both preparations was similar in 6 out of 13 cases, and when challenged with other strain, in 1–4 out of 11–13 cases.

Reading Table V along the horizontal lines we can conclude the following: GLPs of strains 170001, 170018, 170023 were more active than the corresponding antisera. But antisera to GLPs 170005, 170007, 170009 and 170014, were more active than the corresponding GLP. It was shown that GLPs 170002, 170006, 170019 and the corresponding antisera were equally active when challenge was done with 3–5 out of 8 *P. aeruginosa* strains. Analysis of the data given in Table V have shown that GLPs of different strains had different immunogenicity for mice (were able to stimulate active immunity in them) and for rabbits (were able to stimulate production of protective antibodies). On the basis of these experimental data we may select *P. aeruginosa* strains producing GLP which could be used for preparation of vaccine (for active immunization), antiserum or immune plasma (for passive immunization).

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Table V

Comparison of GLP EI and anti-GLP sera EI

GLP-producing strain	Challenge strain								Total for GLP of every strain	
	F1	F2	F3	F4	F5	F6	F7	No. 8		
170001	G = A	G > A	G > A	G = A	G = A	G > A	G > A	G > A	G > A 5 G < A 0	G = A 3
170002	G = A	G = A	G > A	G = A	G = A	G = A	G < A	G < A	G > A 1 G < A 2	G = A 5
170005	G < A	G > A	G < A	G < A	G < A	G > A	G = A	G < A	G > A 2 G < A 5	G = A 1
170006	G > A	G > A	G < A	G = A	G < A	G > A	G = A	G = A	G > A 3 G < A 2	G = A 3
170007	G > A	G < A	G = A	G < A	G < A	G > A	G < A	G < A	G > A 2 G < A 5	G = A 1
170009	G < A	G > A			G < A	G < A	G = A	G < A	G > A 1 G < A 4	G = A 1
170010	G > A	G > A	G = A	G < A	G < A	G < A	G > A		G > A 3 G < A 3	G = A 1
170014	G > A	G < A	G < A	G < A	G > A	G < A	G < A	G < A	G > A 2 G < A 6	G = A 0
170017	G > A	G < A	G > A	G < A	G > A	G > A	G < A		G > A 4 G < A 3	G = A 0
170018	G > A	G > A	G > A	G < A	G < A	G > A	G = A	G < A	G > A 4 G < A 3	G = A 0
170019	G > A	G = A	G > A		G = A	G < A	G = A		G > A 2 G < A 1	G = A 3
170023	G > A	G > A	G > A	G = A	G > A	G < A	G = A	G < A	G > A 4 G < A 2	G = A 2
OII	G > A	G = A	G > A	G < A	G > A	G = A	G < A	G < A	G > A 3 G < A 3	G = A 8
Total for every challenge strain	G > A = 9 G < A = 2 G = A = 2	G > A = 7 G < A = 3 G = A = 3	G > A = 7 G < A = 3 G = A = 2	G > A = 0 G < A = 7 G = A = 4	G > A = 4 G < A = 6 G = A = 3	G > A = 6 G < A = 5 G = A = 2	G > A = 2 G < A = 5 G = A = 6	G > A = 1 G < A = 8 G = A = 1	Total: G > A, 34 (34.7%) G < A, 42 (42,9%) G = A, 22 (22.4%)	
Number of test groups	13	13	12	11	13	13	13	10	Number of variations, 98	

G = GLP, A = anti-GLP serum
G > A when EI of GLP > EI of anti-GLP
G = A when EI of GLP = EI of anti-GLP
G < A when EI of GLP < EI of anti-GLP

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CURRENT STATE AND TENDENCIES OF ANTIBIOTIC RESISTANCE IN HUNGARY

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This survey is based on data for 245 903 isolates reported by Public Health Network laboratories in 1983. Facultatively pathogenic Gram-negative bacteria comprised two-third of the isolates, and — except *Escherichia coli* — were resistant in a high percent to the most frequently used antibiotics. Oxacillin and vancomycin were the most effective against *Staphylococcus aureus* being in 94.7% resistant to penicillin. In contrast to other streptococci, all *Streptococcus pyogenes* strains were sensitive to penicillin. The majority of the Gram-positive strains were resistant to tetracycline. A comparison to results reported earlier (1974 to 1983) showed an increasing resistance rate mainly to ampicillin, carbenicillin, co-trimoxazole and gentamicin, which were introduced in therapy during this period. Resistance rate of almost all species has increased to gentamicin, e.g. that of *Proteus mirabilis* has risen tenfold. Emergence of *Haemophilus influenzae* resistant to ampicillin, and increasing resistance rates of *P. mirabilis* and *Streptococcus pneumoniae* to almost all drugs are remarkable findings. The increasing or variable usage of drugs that have been used for a long time did not influence resistance markedly. In some instances the resistance rates even diminished, e.g. the tetracycline resistance of agents associated with enteric diseases. A restricted use of chloramphenicol reflected in a decreased resistance of some species. Multiresistant Gram-negative strains — which are resistant to all drugs frequently used in Hungary — were isolated in 12.7% from a representative clinical material. The frequent occurrence of multiresistant *P. mirabilis* and *Acinetobacter* isolates is a new phenomenon. Surprisingly, the percentage of multiresistant *E. coli* strains was very low. Amikacin and netilmicin were found to be the most effective against multiresistant isolates.

Infections due to drug-resistant organisms are an increasing problem all over the world [1–3]. In recent years, there has been a great emphasis on the importance of antibiotic resistance surveillance [3–6]. In Hungary, reliably collected and computerized data on the sensitivity of important human pathogens to the most frequently used antibiotics, have been available since 1974. In accordance with a recommendation of WHO, in the following we report on these data. (1) Present status of antibiotic resistance in Hungary according to nation-wide data reported in 1983. (2) Tendencies of antibiotic resistance in relation to antibiotic consumption during a ten-year period (1974–1983). (3) Occurrence of multiresistant pathogens in a representative clinical material, and their sensitivity to drugs that are available only in a limited amount in this country.

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Materials and methods

Bacterial strains. In 1974 about 130 000 isolates were examined, and this number increased up to 245 903 by 1983. The strains were isolated from clinical specimens and were identified by standard methods [7]. The occurrence of multiresistant pathogens isolated from miscellanous clinical material of the Department of Bacteriology, National Institute of Hygiene, was investigated during a 5-month period (September 1, 1983–January 31, 1984).

Antibiotic sensitivity testing. Sensitivity spectrum, as a rule, was tested and reported whenever the isolate was considered to be an agent associated with the infection. A standardized disc diffusion method was used in all laboratories. The discs were manufactured by Human Institute for Serobacteriological Products and Research, Budapest. Antibiotic sensitivity was examined on modified Mueller–Hinton medium; for interpretation of zone sizes a set of control strains was used [7].

Statistical data. The survey was based on data reported yearly by 24 laboratories of the Public Health Network in Hungary. Repeated isolations from the same patient were scored as separate ones. Data on antibiotic consumption were estimated and kindly supplied by the Ministry of Health.

Results

1. *Resistance rates of most important pathogens in Hungary in 1983*

The latest and most detailed data regarding antibiotic resistance rates are summarized in Tables I, II and III. From the first columns of the Tables it can be concluded that in Hungary two-third of the isolates belongs to the Gram-negative species. In general it may be assumed that the frequency of strains which are resistant to individual antibiotics is very high, mainly in case of facultatively pathogenic Gram-negatives. As seen in Table I, most *S. aureus* were resistant to penicillinase-sensitive penicillins and part of them to oxacillin, whereas most other drugs were relatively effective except tetracycline. *S. pyogenes* strains have retained their sensitivity to penicillin in contrast to other streptococci. The appearance of penicillin-resistant *S. pneumoniae* has a great clinical importance. Streptococci were resistant to tetracycline and co-trimoxazole in a high percentage. *S. faecalis* was found to be the most resistant Gram-positive species. *H. influenzae* was infrequently resistant to drugs considered effective against it, however, emergence of ampicillin-resistant strains is an alarming phenomenon.

Among non-fastidious Gram-negative bacteria (Table II), *E. coli* which comprised half of these organisms, exhibited the lowest, and *P. aeruginosa* the highest resistance rate. Surprisingly, the resistance rates of *P. mirabilis* were similar to that of other *Proteeae.*

The number of resistant strains among bacteria causing enteric diseases (Table III) was not too high, only co-trimoxazole resistance in *S. sonnei* is notable.

Table I

*Resistance rates of Gram-positive pathogens, H. influenzae and N. meningitidis in 1983**

Species	No. of strains	Penicillin	Oxacillin	Ampicillin	Carbenicillin	Cephaloridine	Erythromycin	Oleandomycin	Lincomicin	Vancomycin	Gentamicin	Kanamycin	Chloramphenicol	Tetracycline	Cotrimoxazole	Nitrofurantoin
Staphylococcus aureus	25 834	94.7	16.5	92.8	92.7	24.0	17.9	15.2	8.5	6.3	15.0	9.1	12.2	50.3	7.5	4.1
Staphylococcus coagulase-negativ	2 684	71.4	16.6	67.5	72.8	12.2	34.9	29.0	23.4	4.9	18.9	29.3	31.9	69.2	36.8	3.9
Streptococcus pyogenes	6 698	0	2.7	1.6	2.2	4.4	2.0	4.5	2.9	4.5	51.6	85.5	3.6	65.0	68.1	7.2
Streptococcus β-haemolytic other	6 922	15.3	15.1	2.9	4.0	9.2	5.0	6.8	10.4	10.0	67.5	89.7	10.5	73.6	74.3	17.2
Streptococcus pneumoniae	4 915	20.2	33.4	9.5	6.5	6.9	27.9	34.6	17.8	5.9	60.8	85.0	15.3	69.9	51.5	18.7
Streptococcus α-haemolytic other	5 251	35.5	39.7	7.2	7.7	19.3	19.9	21.9	21.2	7.3	61.5	93.1	15.7	65.8	58.5	20.3
Streptococcus faecalis	20 656	96.7	95.0	10.9	14.6	96.0	51.2	61.2	94.0	8.8	67.7	94.9	53.0	85.7	84.8	9.7
Streptococcus other	1 522	45.6	39.5	19.6	10.7	44.5	19.0	21.8	49.2	4.8	41.4	88.9	25.7	66.1	59.0	17.2
Haemophilus influenzae	2 011	96.6[1]	94.3	8.4	9.2	71.8	17.1	91.3	89.2	84.0	7.3	9.6	3.7	22.6	24.2	2.4
Neisseria meningitidis	44	0	36.7	0	15.3	30.8	9.1	.	90.9	.	10.0	.	2.4	7.1	27.1	.

* Percentage of resistant and moderately sensitive strains
[1] Moderately sensitive 37.7%

Table II

*Resistance rates of facultatively pathogenic Gram-negative bacteria in 1983**

Species	No. of strains	Ampicillin	Carbenicillin	Cephaloridine	Gentamicin	Kanamycin	Chloramphenicol	Tetracycline	Cotrimoxazole	Nitrofurantoin	Nalidixic acid
Escherichia coli	72 293	61.1	44.5	77.8	6.3	20.1	29.7	54.7	24.8	6.6	9.3
Klebsiella–Enterobacter group	28 187	91.5	80.4	90.3	24.1	30.3	44.7	57.3	36.0	22.5	23.0
Citrobacter	3 094	79.0	49.0	94.4	30.6	39.1	39.7	59.1	47.6	15.5	27.3
Proteus mirabilis	17 582	72.7	64.5	98.6	42.5	53.9	67.5	99.3	60.3	67.1	57.7
Proteus vulgaris	2 108	89.3	61.7	98.0	31.7	48.2	58.4	88.9	55.8	70.7	40.2
Proteus other	4 012	92.3	73.1	97.1	53.6	60.7	68.3	87.0	68.8	64.4	54.4
Enterobacteriaceae other	3 636	71.5	50.2	87.8	12.1	20.0	37.9	52.7	27.7	21.4	22.5
Pseudomonas aeruginosa	9 737	98.9	80.4	99.6	29.8	94.2	96.6	87.2	97.4	97.3	97.7
Pseudomonas other	1 752	94.3	76.7	98.2	26.2	46.3	79.8	66.4	69.2	91.1	78.5
Acinetobacter calcoaceticus	3 138	79.5	58.0	94.6	46.2	52.0	75.5	65.4	45.2	82.2	38.0

* Percentage of resistant and moderately sensitive strains

Table III

*Resistance rates of enterobacteria associated with enteric diseases**

Species	No. of strains	Ampicillin	Carbenicillin	Gentamicin	Kanamycin	Chloramphenicol	Tetracycline	Cotrimoxazole	Nitrofurantoin	Nalidixic acid
Salmonella typhi	117	1.7	0	0	0	0	11.1	2.0	2.4	10.8
Salmonella other	13 184	7.4	5.2	0.9	3.8	3.5	20.1	8.0	20.3	5.6
Shigella flexneri	948	19.7	20.5	2.2	6.7	11.1	20.8	11.8	1.3	1.6
Shigella sonnei	5 490	31.6	7.9	1.0	2.5	12.0	19.5	62.9	1.5	1.6
Shigella other	19	19.4	0	0	16.7	7.1	16.7	0	0	0
Escherichia coli O111	280	27.6	9.8	0.4	9.1	6.0	20.3	4.2	6.5	4.6
E. coli associated with infantile enteritis other	1 075	51.5	26.4	2.9	16.0	16.6	45.1	15.7	5.2	3.3
E. coli O124	1 857	18.7	13.9	1.5	5.9	10.4	14.0	9.7	4.7	2.8
Yersinia enterocolitica	963	86.2	94.1	1.0	0.3	0.8	5.7	19.3	12.5	4.1

* Percentage of resistant and moderately sensitive strains

2. *Tendencies of antibiotic resistance in relation to antibiotic consumption during a ten-year period (1974–1983)*

The data concerning the consumption of antibiotics frequently used in Hungary between 1974–1983 are shown in Fig. 1. Cephalosporins are not included in this study, because until the introduction of more effective derivatives, only cephaloridine disc was used as a representative of first generation cephalosporins. Polymyxins have also been omitted, since sensitivity of Gram-negative bacteria to them remained practically unchanged.

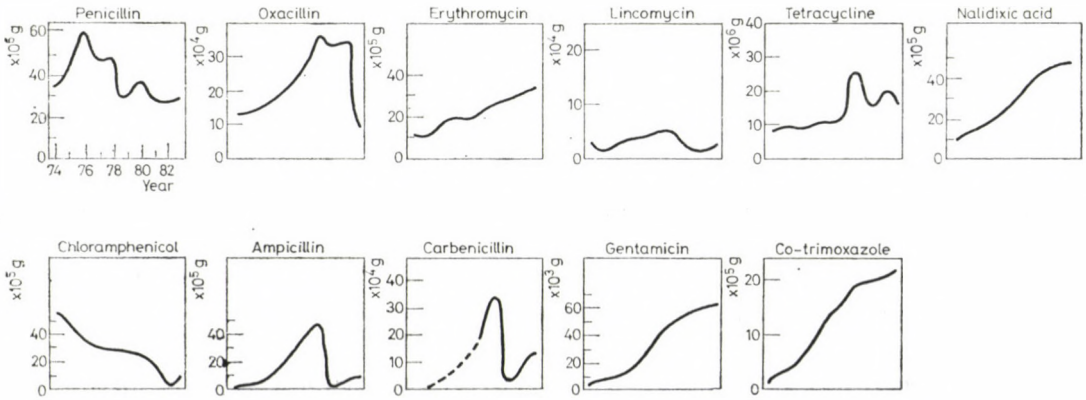

Fig. 1. **Consumption of frequently used antibiotics in Hungary, 1974–1983**

Taking into account the differences in dosage, instead of comparing the absolute amount of antibiotics consumed, it seems more reliable to point out the tendencies of consumption.

According to their consumption the antibiotics may be divided into three groups. (i) There are drugs (penicillin, oxacillin, erythromycin, lincomycin, nalidixic acid) which have been widely used for a long time, and their consumption has not changed as markedly as that of the others (i.e. increase or decrease did not exceed fourfold). (ii) Chloramphenical, the single member of the second group, is the only drug the consumption of which has diminished evenly due to recommendations discouraging its use. (iii) Ampicillin, carbenicillin, co-trimoxazole and gentamicin belong to the third group. These drugs have been introduced in the course of the last few years and their consumption has increased markedly. Unfortunately, we have no data on the usage of carbenicillin in the first years of the survey, but it may be assumed that the course of its graph rises in the same manner as that of the others. The fall in the curves for ampicillin and carbenicillin is due to difficulties in their import in the early eighties.

Relationship between consumption of, and resistance rates to, antibiotics will be evaluated according to this grouping. Since presenting graphs for every antibiotic and every species is not possible in this paper, a brief information will be given about the drugs studied, and only some remarkable findings will be illustrated.

Penicillin. Fluctuation in consumption of penicillin had no influence on the resistance rates of Gram-positive cocci. The incidence of penicillinase-producing *S. aureus* has increased only slightly (88% in 1974 to 95% in 1983). Penicillin resistance rate of streptococci other than the uniformly sensitive *S. pyogenes* has not changed, with exception of *S. pneumoniae* which showed a moderate increase followed by a decrease from the beginning of 1982 (Fig. 4).

Oxacillin. Sensitivity of *S. aureus* to this drug has not been influenced by the variable consumption of oxacillin, the percentage of resistant strains was about 16% as at present (Table I).

Erythromycin. In spite of the moderately increasing erythromycin usage the resistance rate of *S. aureus* has diminished (27% in 1974 to 18% in 1983). There was no change in resistance rate of streptococci, but a rising trend was observed for *S. pneumoniae* (6% in 1974 to 28% in 1983; Table I and Fig. 4).

Lincomycin. No remarkable change was found in resistance rates of Gram-positive cocci, save *S. pneumoniae* which exhibited a rising trend to lincomycin, too (4% in 1974 to 18% in 1983; Table I and Fig. 4).

Tetracycline. Tetracycline-resistant strains occurred in almost all species at a permanently high percentage during this period (Tables I, II and III). The moderately increasing consumption of this drug resulted in a higher frequency of resistant strains of haemolytic streptococci and — to a lower extent — of *H. influenzae.* There is a declining tendency in resistance of *E. coli, Klebsiella–Enterobacter* group and *Citrobacter.* Although tetracycline is not the drug of choice in enteric diseases, it is of interest that the resistance rates of agents responsible for enteric infections diminished markedly. As sensitivity of these bacteria has been surveyed since 1971, early data (1971) can be compared to the latest ones (1983): *Salmonella* 56% vs 20%; *S. flexneri* 56% vs 21%; *S. sonnei* 63% vs 20%; *Shigella* other 66% vs 17%; *E. coli* O111 87% vs 20%; *E. coli* other serogroups associated with infantile enteritis 69% vs 45%; *E. coli* O124 44% vs 14%.

Nalidixic acid. In spite of a moderately increasing nalidixic acid consumption the sensitivity of bacteria associated with enteric infections and most of those causing urinary infections *(E. coli, Klebsielleae* and *Citrobacter)* remained practically unchanged. Only *P. mirabilis* exhibited an increased resistance rate.

Chloramphenicol. Resistance rates of *S. aureus* and the following enteric pathogens diminished parallel with the restricted use of chloramphenicol; respective data for 1971 and 1983 are as follows: *S. aureus* 47% vs 12%;

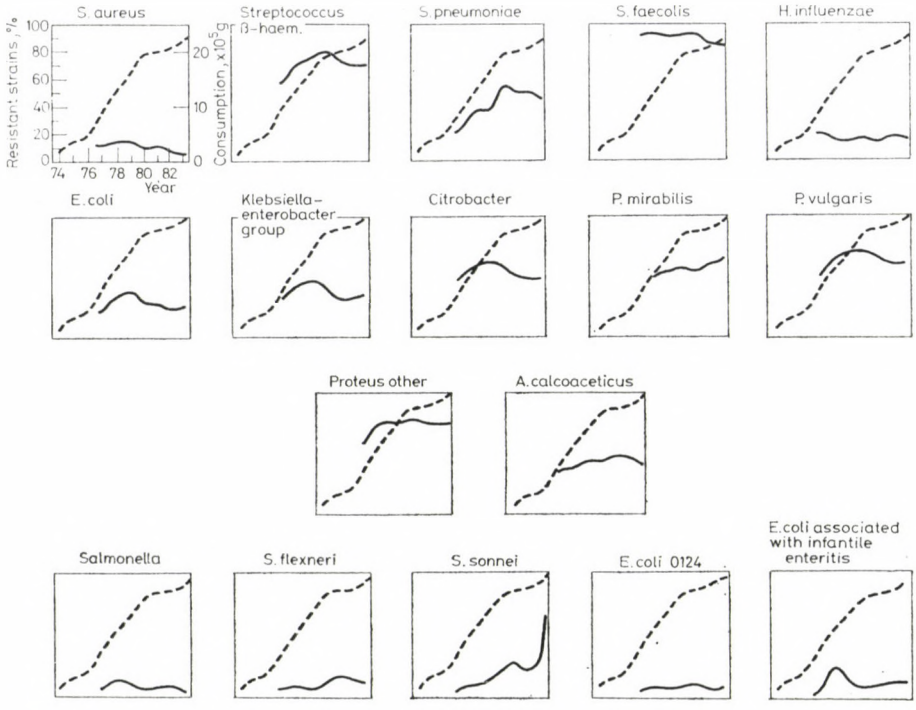

Fig. 2. **Tendencies of co-trimoxazole resistance rates (continuous lines) and consumption (dotted lines), 1974–1983**

S. typhi 7% vs 0%; *S. flexneri* 23% vs 11%; *S. sonnei* 30% vs 12%; *E. coli* O111 61% vs 6%; *E. coli* other serogroups associated with infantile enteritis 46% vs 17%. An inverse relationship is apparent between the amount of chloramphenicol used and resistance rate of *P. mirabilis* (Fig. 4).

Ampicillin is one of the drugs which were introduced in therapy in the course of the last decade. There was no association between the variable consumption and the resistance of streptococci which — including *S. faecalis* — were sensitive in a high percentage to this drug. The increasing resistance of *H. influenzae* is remarkable. From 1974 onwards there was no change in the resistance of *E. coli*, *Klebsiella–Enterobacter* and indole-positive *Proteus* strains (Table II). However, the resistance rate of *P. mirabilis* has increased (Fig. 4). The resistance rates for agents of enteric diseases showed a declining tendency during the last three years.

Carbenicillin. A slight increase appears in the resistance rate of *S. pneumoniae* (Fig. 4), whereas the other streptococci retained their sensitivity to carbenicillin (Table I). An increasing trend in resistance of *P. mirabilis* and *P. vulgaris* is remarkable (*P. mirabilis* 38% in 1976 to 65% in 1983; *P. vulgaris* 39% in 1976 to 62% in 1983; Fig. 4). There was no change in a fairly

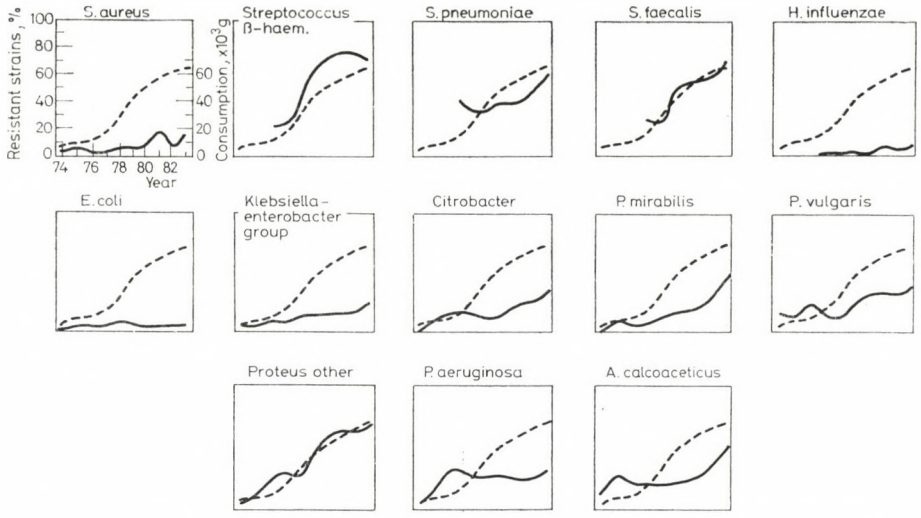

Fig. 3. **Tendencies of gentamicin resistance rates (continuous lines) and consumption (dotted lines), 1974–1983**

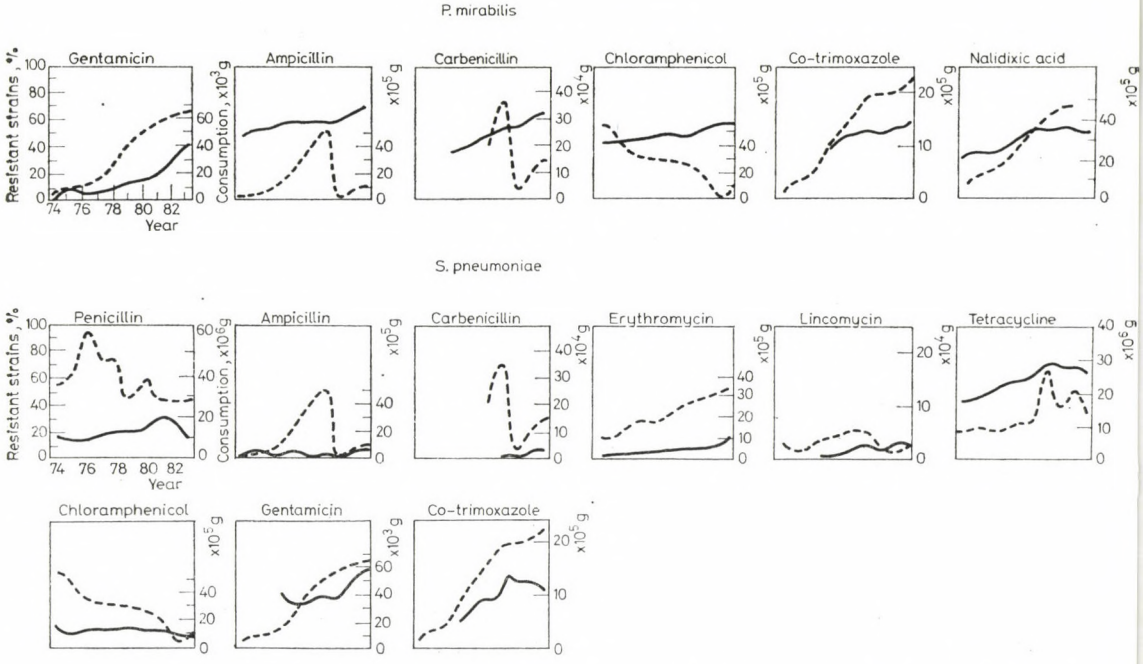

Fig. 4. **Tendencies of antibiotic resistance rates of *P. mirabilis* and *S. pneumoniae* 1974–1983 (resistance: continuous lines, consumption: dotted lines)**

great number of resistant strains of the other enterobacteria (Table II). *Acinetobacter* and especially *P. aeruginosa* strains were resistant in a high percentage from the beginning, and this rate fluctuated with slightly increasing trend.

Co-trimoxazole. Following introduction of co-trimoxazole in the middle of the seventies its consumption has risen rapidly (Fig. 2). Only *P. mirabilis* exhibited an evenly increasing resistance rate. The graphs for several species, except *H. influenzae*, are similar to each other: after a peak they show a slight decline. The same finding is true for pathogens causing enteric diseases. An alarming increase was revealed in the frequency of co-trimoxazole-resistant *S. sonnei* (20% in 1982 to 62% in 1983; Fig. 2).

Gentamicin. Being more effective than any other drugs available in Hungary, at the beginning gentamicin was considered as an outstanding antibiotic. Due to the widespread usage of gentamicin in hospitals the resistance rate of almost all species has risen markedly (Fig. 3). It is interesting that the low resistance rate of the two most frequently isolated species, *S. aureus* and *E. coli* changed the least of all. (Considering that these two species comprise more than 40% of all isolates, these relatively low resistance rates mean a considerable number of insusceptible strains.)

As it is shown in Fig. 4, an increasing resistance to almost all antibiotics could be observed only in two species: *P. mirabilis* and *S. pneumoniae.*

3. *Occurrence of multiresistant isolates in a representative clinical material*

The term multiresistant is used hereby to refer to Gram-positive or Gram-negative isolates which are resistant to all antibiotics listed in Tables I and II.

Among the Gram-positive species only a small number of *S. faecalis* strains fell into this category. The majority of the *S. faecalis* isolates were sensitive only to ampicillin, carbenicillin and nitrofurantoin.

The great majority of multiresistant isolates belonged to the Gram-negative species. Their occurrence and sensitivity to drugs available in a limited amount in Hungary will be discussed in the following.

As shown in Fig. 5, 166 out of 1308 strains (12.7%) proved multiresistant. Percentage of multiresistant isolates was the highest in *P. aeruginosa*, followed in the order of frequency by *P. mirabilis*, *Acinetobacter*, other *Proteeae*, other *Enterobacteriaceae* (identified only on a family level), *Citrobacter* and the *Klebsiella–Enterobacter* group. There were few multiresistant isolates among *E. coli*.

Sensitivity of 93 multiresistant isolates to antibiotics recently available are summarized in Table IV. Amikacin and netilmicin had an outstanding efficacy, and about half of the strains were sensitive to cefuroxime and cefoxitin.

Table IV

Sensitivity of multiresistant Gram-negative bacteria

Species	No. of strains	Tobramycin			Amikacin			Netilmicin		
		S	M	R	S	M	R	S	M	R
E. coli	4	1	—	3	3	—	1	3	—	1
Klebsiella–Enterobacter other	12	1	3	8	11	1	—	9	1	2
Citrobacter	1	—	—	1	1	—	—	1	—	—
P. mirabilis	36	1	2	33	34	1	1	34	1	1
Proteus other	16	1	—	15	15	1	—	13	2	1
Enterobacteriaceae other	3	1	—	2	2	—	1	2	—	1
Acinetobacter	3	1	—	2	2	1	—	2	1	—
P. aeruginosa	18	—	—	18	18	—	—	18	—	—
Total	93	6	5	82	86	4	3	82	5	6

S = sensitive; M = moderately sensitive; R = resistant

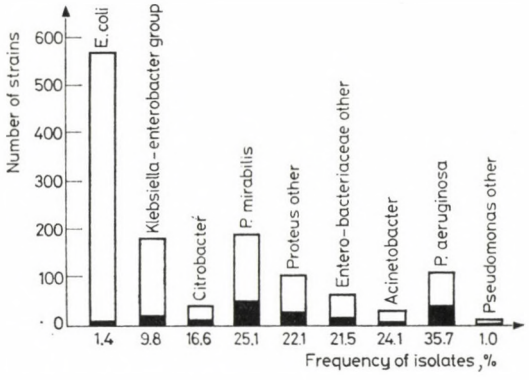

Fig. 5. **Occurrence of multiresistant Gram-negative bacteria in a representative clinical material (columns: number of strains, closed columns: number of multiresistant strains)**

Azlocillin was found to be most active against *P. aeruginosa*. Only few multiresistant strains were sensitive to tobramycin, cefamandole and mezlocillin.

Source of the multiresistant strains is presented in Fig. 6. More than half of the strains were isolated from urine.

to antibiotics recently introduced in Hungary

Azlocillin			Mezlocillin			Cefamandole			Cefuroxime			Cefoxitin		
S	M	R	S	M	R	S	M	R	S	M	R	S	M	R
—	—	4	—	—	4	1	1	2	2	1	1	2	1	1
—	—	12	—	—	12	—	2	10	9	1	2	9	1	2
—	—	1	—	—	1	—	—	1	—	—	1	—	1	—
1	—	35	2	—	34	—	8	28	20	16	—	29	7	—
1	—	15	1	—	15	5	3	8	10	4	2	10	3	3
—	1	2	1	—	2	1	1	1	2	—	1	2	—	1
—	—	3	—	—	3	—	—	3	1	—	2	1	—	2
10	5	3	—	3	15	—	—	18	—	—	18	—	—	18
12	6	75	4	3	86	7	15	71	44	22	27	53	13	27

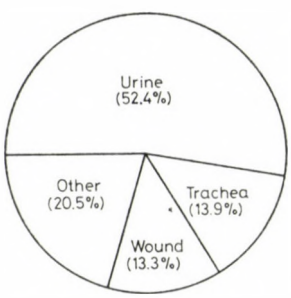

Fig. 6. Source of multiresistant Gram-negative bacteria

Discussion

Similarly to literary data [2, 8–10] a predominance of Gram-negative opportunistic pathogens was observed in Hungary. Comparison of our data to those obtained in other countries contributes to evaluation of the present resistance-situation. Among WHO [3–6] and other publications [11–15] only some are based on such a great number of nation-wide collected data. Reports from Switzerland and FRG [16–17] may be compared to our report. The most frequently isolated species — *E. coli* and *S. aureus* — were chosen for comparison (Tables V and VI).

Although these data compared in the Tables were reported earlier, they suggest that the resistance rates of these two relatively sensitive species are much higher in Hungary than in the above countries.

Table V

Frequency in percentage of antibiotic-resistant S. aureus in Hungary, Switzerland and FRG

Antibiotic	Hungary, 1979 n = 26 614	Switzerland, 1979 n = 4022	FRG, 1981 n = 800
Penicillin	88.7	68.3	69
Oxacillin (methicillin)	12.6	3.8	1
Ampicillin	67.9	55.0	68
Erythromycin	17.8	10.7	15
Gentamicin	4.8	2.3	9
Kanamycin	16.1	10.9	18
Chloramphenicol	20.0	7.8	7
Tetracycline	40.6	17.0	21
Co-trimoxazole	15.7	12.0	10

Table VI

Frequency in percentage of antibiotic-resistant E. coli in Hungary, Switzerland and FRG

Antibiotic	Hungary, 1979 n = 56 651	Switzerland, 1979 n = 7814	FRG, 1981 n = 1900
Ampicillin	58.6	30.3	22
Carbenicillin	44.7	23.6	17
Gentamicin	2.9	1.3	1
Kanamycin	19.0	13.5	12
Chloramphenicol	28.2	19.7	12
Tetracycline	58.9	33.3	33
Co-trimoxazole	34.1	12.0	10
Nitrofurantoin	9.3	15.5	10
Nalidixic acid	10.6	3.4	13

The high rate of co-trimoxazole resistant streptococci in this country needs some explanation. It is likely to be due to use of a Mueller–Hinton medium for sensitivity testing which is not entirely free from sulphonamide and trimethoprim inhibitors [18]. It may be assumed that the co-trimoxazole sensitivity testing of streptococci is much more influenced by the medium than that of any other species.

A relationship between the antibiotic consumption and resistance rates was observed mainly in case of drugs that were introduced or withdrawn during the period examined. For instance, due to introduction and frequent use of gentamicin, the resistance rate for almost all species has risen markedly,

especially that of *P. mirabilis* which has increased tenfold. The restriction in the usage of chloramphenicol was associated with a decreased resistance of certain bacteria. The increasing or variable usage of drugs available throughout the survey period influenced but slightly the previous resistance-situation. Hence it may be concluded that only a marked change in consumption would cause an alteration involving the resistance of a nation-wide bacterial population. In contrast, use of particular antibiotics in hospitals is frequently associated with local changes in resistance rates [19–21].

The tendency of increase or decrease in resistance varied with different species. Some remarkable findings were as follows. Emergence of antibiotic-resistant pneumococci has been observed throughout the world [22–25], but the increasing number of resistant *P. mirabilis* seems to be a Hungarian speciality. The appearance of ampicillin-resistant *H. influenzae* is of great clinical importance. The dramatic increase in co-trimoxazole-resistant *S. sonnei* within a year can be explained by an epidemic caused by shigellae belonging to the same phage and colicin type (Milch, H., personal communication). A marked diminution in the number of tetracycline-resistant salmonellae is likely to be due to a restriction of use of this drug in animals. In general, a trend of decrease was observed in resistance of bacteria causing enteric diseases. This may be associated with the fact that patients with enteritis are treated with antibiotics less frequently than ten years ago.

The great number of multiresistant isolates is an alarming phenomenon. Investigations were focussed on the most resistant bacterial population, i.e. on strains which were resistant to every drug widely used in Hungary. Multiresistant Gram-negatives were isolated in 12.7% from a representative clinical material. As it could be predicted from the results discussed earlier, multiresistant *P. mirabilis* strains were isolated most frequently. As 80% of *P. mirabilis* strains origined from urine, this means a problem mainly in the treatment of urinary tract infections. It may be concluded that there is a need of new antibiotics active against multiresistant strains. Some of these new agents are available in Hungary for life-threatening infections, and among them amikacin and netilmicin proved the most effective against "problem bacteria".

The following factors may be regarded responsible for the present situation:

(1) Antibiotics are widely prescribed without any laboratory examinations.

(2) Inappropriate use of antibacterial drugs, e.g. in viral infections or in mild bacterial infections.

(3) A narrow spectrum of available and widely used antibiotics.

(4) Unsatisfactory hygienic level in hospitals enhancing the spread of antibiotic-resistant bacteria.

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BOOK RECEIVED

Jeljaszewicz, J. (ed.): The Staphylococci. Proceedings of the Vth International Symposium on Staphylococci and Staphylococcal Infections. Gustav Fischer Verlag, Stuttgart—New York. 1985. pp. 706, 155 figures and 186 tables.

The international-recognized symposia on staphylococci and staphylococcal infestions have been organized since 20 years in Warsaw. At the 1985 symposium, 250 scientists participated from all over the world. Data presented at the symposium were largely unpublished. The book is a unique collection of the papers delivered at thy symposium, from the most varied topics.

The volume is divided into 10 parts. The first eight papers of the first part are dedicated — under the title of memorial lectures — to the memory of the forerunners of staphylococcus-investigation. These papers are followed by the paper of T. Kereselidze (WHO) about problems associated with hospital infections and by five other papers on toxic shock syndroma. Following this, the volume consists of 9 chapters, each treating different topics of staphylococcus research.

Papers of Chapter 1 deal with the taxonomy of *Micrococcaceae*, with some of the articles centering on the differentiation of the genus *Micrococcus* from *Staphylococcus*. There are a number of studies here on different kinds of staphylococci isolated from animals like birds or flies; others introduce the most recent methods for determining, typing and testing the staphylococci. Chapter 2 includes essays on different aspects of cell envelope, binding properties of different staphylococcus strains (fibronectin, clumping factor etc.), and in general, it deals with the characteristics of the surface components of the bacterium and with the role of staphylococcal capsular material in the pathogenicity. The essays of Chapter 3 deal with biologically active substances staphylococci may produce (extracellular products) ranging from different staphylococcal toxins (alpha-, entero-, exfoliative-, toxic shock toxin, etc.) to the several extracellular enzymes of varying specificities. The essays of Chapter 4 present up-to-date data on the in vitro activity and on the clinical efficacy of some antibiotics in the therapy of staphylococcal infections. Chapter 5 deals with the different reactions of the host organism to staphylococcal infection. The papers include studies on the antibiotic resistance of staphylococci, cellular and humoral immune response of the host to the staphylococcal infections and on the treatment of specific infections due to *Staphylococcus aureus*. Chapter 6 gives an insight into the increasing problem of coagulase-negative staphylococci, treating the questions of antibiotic resistance, adherence to smooth surfaces and the variability in species-virulence, etc. Papers in Chapter 7 deal mainly with experimental infections, and the pathogenesis and pathomechanism of staphylococcal infections. The reader gets an insight from Chapter 8 into the problems of the genetics and mechanism of antibiotic resistance to staphylococcal strains. Papers here deal with such refined methods as gene fusion, expression of cloned genes and the localization of antibiotic resistance genes. The last chapter deals with epidemiology and ecology. The papers are handling such problems as the epidemiology of staphylococcal infections and food poisoning outbreaks in different countries, the mutual transfer of drug resistance between staphylococcus strains from humans and animals, the role and usefulness of phage typing etc.

The volume elaborates a wide range of problems connected to staphylococci, and therefore it can be useful to almost all scientists working in the fields of microbiology, epidemiology, antibiotic research and drug production and to medical practitioners as well, and the book would be a fine addition to all medical libraries.

I. Nász

RESEARCH ON DIETARY FIBRES

A Joint Study of Medicine, Nutrition and Industry

Symposium on the Possibilities of the Chemical Determinations
of the Dietary Fibres and their Nutritional and Dietetic Roles
September 23–24, 1983, Pécs, Hungary

Edited by
Cs. Ruzsa, T. Jávor and *Gy. Mózsik*

In English. 1986. XIV + 222 pages. 1 photos, 52 figures. 44 tables. 17×25 cm.
Hardcover $ 24.00/DM 58,–/£ 17.00
ISBN 963 05 4254 4

The basic changes in the living conditions of the Hungarian population during the past three decades resulted in an unfavourable tendency in nutrition. Researchers have detected that the dietary fibre consumption is strikingly low in Hungary which favours the development the so-called civilization diseases like disorders of the colon, diabetes, hypertension, etc. The present state of dietary fibre consumption in Hungary was the topic of the symposium held in Pécs where experts in medicine, nutrition and food industry discussed the role of dietary fibres in the development of different illnesses as well as their therapeutical effect. The round-table conference of the symposium has determined the main lines of research.
The book may be useful to specialists dealing with the problems of dietary fibre in the most different fields: medicine, industry and nutritional science.

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TESTING METHODS IN FOOD MICROBIOLOGY

Edited by

I. KISS

In English. 1984. 447 pages, 78 figures, 58 tables. 17×25 cm
Hardback ISBN 963 05 3224 7

This manual follows a consistent and systematic approach nad provides a detailed description of the exact methods of the quantitative and qualitative determinations, in order to prepare the ground for the unification of the various methods used in this field and to render the comparison of data originating from various sources possible.

The main chapters of the book discuss the equipment of the microbiological laboratory, the various laboratory procedures, the most widespread and dangerous putrefying organisms encountered in food industry and their morphological and biochemical identification, the mathematical principles of qualification and decision making, the evaluation and interpretation of measurement results, the investigation of foods and additives, the environmental factors and the culture media, all essential for getting reliable and generally valid results. The book concentrates upon simple and effective methods, easy to realize in an average routine laboratory but furnishing the food industry with a great quantity of useful data and used with excellent results by the uthors themselves.

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CYTOTOXICITY OF HUMAN PERIPHERAL LEUKOCYTES ON ADENOVIRUS-TRANSFORMED CELL CULTURE

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(Received July 2, 1984)

Cytotoxicity of natural and adenovirus-infected mononuclear leukocytes of healthy individuals has been examined on premanent rat kidney cell line transformed by human adenovirus DNA as target. Mononuclear leukocytes of healthy individuals are cytotoxic cells which induce an average 24.5% growth inhibition on target cells. The cytotoxic activity of cells is significantly increased by the human adenovirus infection of effector mononuclear leukocytes, while the cytotoxic effect is significantly decreased by the simultaneous application of virus infection and anti-adenovirus serum. The anti-serum applied simultaneously with non-infected effector cells produces an intensive cytotoxic effect on the target cell culture. Though cytotoxicity of the effector cells was increased also by phytohaemagglutinin and *Haemophilus influenzae* endotoxin treatment of mononuclear leukocytes, this effect was not significant. The effector cells were not cytotoxic for control primary rat kidney cell culture.

According to last year's experimental results supported by animal experiments and observations in humans [1–9], natural cytotoxicity has an important role in protection against tumours and virus infections.

Natural killer cells (NK) belong to non-adherent, non-phagocyting lymphocytes, though, according to some data, macrophages also play an important role in the cytotoxicity [10–11]. Studies on cytotoxicity against cells infected or transformed by adenoviruses refer so far to animal experiments; observations in humans are of limited number [7]. In our experiments, the cytotoxicity of peripheral mononuclear leukocytes of healthy individuals was examined on rat kidney permanent cell line transformed by adenovirus type 6 DNA belonging to the subgenus C of adenoviruses.

Materials and methods

Effector cells. Blood anticoagulated with heparin was taken from healthy individuals by vein-puncture. Mononuclear leukocytes of the blood were separated on Ficoll-Uromiro gradient [12]. The cells were washed three times in Hanks solution, the cell number was set to 5×10^6/ml in Eagle MEM solution containing 10% inactivated fetal calf serum. One part of the cells was cultivated untreated for 72 h as control, the other part was infected with a 10^4 CPD_{50} amount of human adenovirus type 5, which serotype belongs to subgenus C. A smaller part of the lymphocyte cultures was treated by a 3000-fold final dilution of phytohaemagglutinin (PHA) Difco

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PHA—P, while the other part was treated and incubated with a 0.1 mg/ml amount of *Haemophilus influenzae* endotoxin.

Target cells. Rat kidney cell line N-68 transformed with human adenovirus type 6 DNA was cultured in test-tubes of uniform size and shape to 75% confluence. The cell number was $4–5 \times 10^3$/tube, and 10% inactivated fetal calf serum in Eagle MEM solution was used as a medium.

Cytotoxicity reaction. Half ml of the lymphocyte cultures incubated for 72 h was measured to the target cell cultures, then cultured for 72 h at 37 °C. The optimal length of the cytotoxicity reaction and the ideal 100 : 1 effector : target cell proportion were determined in pre-experiments. In case of each test, at least three parallel experiments were made. For measuring the cytotoxicity reaction, a photometric method was applied [13]. After the 72 h incubation period, the cells were washed in 0.01 M pH 8.6 borate buffer, then they were fixed in 5% formalin overnight. After draining off the formalin, cells were rinsed in the buffer mentioned above, then they were stained in 0.1 ml 1% methylene blue (0.1 N borate buffer, pH 8.6). Superfluous stain was removed with borate buffer and the tubes were dried. The stain from the cells was eluted with 0.2 ml 0.1 N hydrochloric acid at 37 °C for 40 min and then the suspension was diluted with distilled water at 1 : 6 proportion. Absorption of the stain dilution was measured in the Unicam spectrophotometer at 660 nm. Results were calculated as follows:

$$\text{Inhibition index: } \frac{(A_{tar} + Ly) - A_{Ly}}{A_{tar}}$$

where (A_{tar} + Ly) is the absorbance of target cells treated with effector cells; A_{Ly} is the absorbance of mononuclear cells; and A_{tar} is the absorbance of untreated target cell cultures.

Results were mathematically evaluated with the help of Student's *t* test.

Results

Among mononuclear leukocytes of healthy individuals there are cytotoxic cells which cause an average 24.5% growth inhibition in target cells transformed by adenoviruses. The degree of cytotoxic effect, otherwise, ranges within wide limits (—5 and 95%), i.e. there was a patient whose mononuclear leukocytes increased the growth of the target cells. The cytotoxic activity of the cells was significantly increased by the infection of the effector cells with adenovirus type 5 during the 3 day preincubation period, as can be seen in Table I ($p < 0.01$). When serum against adenovirus was applied si-

Table I

Cytotoxic effect of differently treated and control effector cells on target cells

Treatment of effector cells	Treatment of target cells	Cytotoxicity (inhibition index) average	SD	P
Untreated control	nil	24.4	9	.
Phytohaemagglutinin	nil	27.8	7.2	NS
Endotoxin	nil	31.6	9.7	NS
Adenovirus type 5	nil	35.1	12	< 0.01
Adenovirus type 5	serum against adenovirus	— 14.1	7.5*	< 0.01
Untreated	serum against adenovirus	48.3	18	< 0.002
Untreated	primary rat kidney cell culture control	2.2	0.9	

* Increasing effect of target growth

multaneously with the effector cells infected by virus, the cytotoxic effect of the effector cells was significantly decreased. When, however, simultaneously with adding uninfected effector cells to the target cells, 1 : 5 diluted rabbit serum against adenovirus was added, the cytotoxic activity of the cells was significantly increased by the serum treatment ($p < 0.002$). The PHA treatment of effector cells during pre-incubation increased the cytotoxic effect of the cells, this increase was, however, insignificant. Similarly, the cytotoxicity of the effector cells was not significantly increased by the 0.1 mg/ml concentration of the *H. influenzae* endotoxin treatment. None of the effector cells displayed cytotoxic activity for the primary rat kidney cell culture applied as control. Results obtained in the different systems are shown in Table I.

Discussion

Our experiments indicate that the peripheral mononuclear leukocytes of healthy individuals display cytotoxic activity and, to detect this, rat kidney cells transformed by human adenovirus are suitable targets. The assumption that the effector belongs to natural killer cell population seems to be verified by the fact that the efficiency of the lysis is not influenced significantly by PHA. According to literary data, the activity of NK cells is increased by interferon produced during virus infection, though it did not play a role in our experiments, as, according to our present kowledge, interferon is produced by adenoviruses only in chicken embryo cells. Cytotoxicity increasing the effect of antibodies, which was also mentioned by others [14], seems to prove the fact that macrophages and cytotoxic secondary T-cells also play a role in the reaction. According to several data, NK and K cells, though they are not identical, belong to the same population as lymphocytes. The effector cells infected by adenovirus type 5 carry on their surface adenovirus-specific antigens, therefore the negative effect of adenovirus-antibodies is presumably the result of the toxic or lytic effect on effector cells [15].

The effect of *Escherichia coli* endotoxin increasing cytotoxicity has already been known, however, that endotoxin recently isolated from *H. influenzae* [16] exerts the same effect, has not yet been described.

The increased cytotoxic effect of effector cells infected by adenovirus is very interesting. Adenoviruses are not multiplied by human, non-stimulated lymphocytes, which are non-permissive; they can, however, become virus-carriers and this state can be prolonged [17–19]. It may be assumed that the nature of virus carrier lymphocytes changes and they produce cytotoxic mediators; or that cytotoxic T-lymphocytes are evolved during pre-incubation after virus infection or during the further 3 days of the cytotoxic reaction [20, 21]; the two phenomena may occur simultaneously.

In our present experiment a method was applied which is relatively exact, needs no isotope and is suitable for measuring the cytotoxic reaction in case of the adhesive target cells [13].

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STABILITY OF CEFATRIZINE IN AQUEOUS SOLUTION AND IN SERA

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Cefatrizine (SK & F 60771; BL—S640), like most other phenylglycine-type cephalosporins, has a tendency to lose potency in aqueous solutions and in normal sera even at low temperatures. Cefatrizine can be stabilized during storage by sodium metabisulphite ($Na_2S_2O_5$), a reducing agent partially in tap water, better in deionized water, and to a lesser degree in citric acid-phosphate buffer (pH 6). Although this partial stabilizing effect of sodium metabisulphite is temperature-dependent, storage at 4 °C gives better results than storage in the frozen state (—20 °C). In these aqueous solutions and in sera, the potency of cefatrizine can be preserved even at room temperature for up to four weeks by the addition of 0.1 ml of 2 N hydrochloric acid to each 2 ml of aqueous solutions or sera.

Cefatrizine (SK&F 60771; BL-S640) is an orally absorbed cephalosporin antibiotic. Chemically, cefatrizine is 7-[D-α-amino-α-(4 hydroxyphenyl) acetamido]-3[(1H-1,2,3,-triazole-5-yl)-thio]methyl-3-cephem-4-carboxylic acid. It differs from the other members of the orally active aminocephalosporins in that it contains at the 3-position a thio-triazole moiety (Fig. 1). In addition, cefatrizine has a broader in vitro antibacterial spectrum of activity than other orally active aminocephalosporins [1–9]. Cefatrizine is water soluble, can be administered orally and parenterally and has favourable pharmacokinetic properties [10–13].

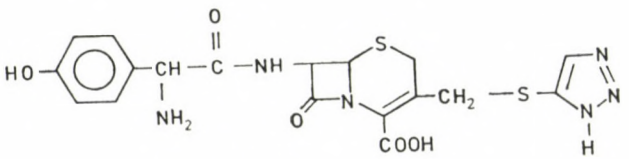

Fig. 1. Structure of cefatrizine

Cefatrizine gradually loses antibacterial activity in solutions. The rate of loss of potency in solutions and sera is influenced by time, temperature, quality of water (presence of ions), composition of buffers and foremost, the ambient pH about or below 4.0.

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The present paper describes procedures by which the activity of cefatrizine in solutions and sera can be stabilized and maintained for a longer period of time, permitting realistic biologic determination of potency in samplable biological fluids and in materials for quality controls.

Materials and methods

Cefatrizine (SK & F 60771; BL—S640) used in these experiments was a 98% pure preparation synthesized by members of the Medicinal Chemistry Department, Smith Kline & French Laboratories [14]

In all experiments, stock solutions of cefatrizine were freshly prepared by dissolving 4 mg of the compound in 50 ml of deionized water and sterile filtered.

In the experiments with aqueous solutions, the final dilutions [4 μg/ml] of cefatrizine were prepared in tap water, in deionized water or in McIlvaine's citric acid-phosphate buffer (pH 6). Aliquots of these solutions were treated with 2 N hydrochloric acid, and other aliquots with 2% freshly prepared sodium metabisulphite ($Na_2S_2O_5$) solutions). Non-treated aliquots of each aqueous solution served as controls.

Neither the sodium metabisulphite nor the hydrochloric acid containing solutions nor the "natural" sera (without cefatrizine) produced inhibition zones per se. Hydrochloric acid was used for the temporary stabilization of streptozotocin in blood [15].

For serum-stability studies, the stock-solution was further diluted with pooled sera (human, mouse, dog) to a final concentration of 4 μg/ml. At this concentration (equals to about 0.1 μg/disc), selected on the basis of convenience, the majority of the phenylglycine-type cephalosporins produce inhibition zones of about 20 mm, using the very sensitive *Bacillus subtilis* ATCC 6633 strain (spore suspension) as indicator.

All the final dilutions were dispensed in smaller aliquots in small plastic tubes to be kept at various temperatures, i.e., 37 °C, 30 °C, room temperature (RT), 4 °C and frozen (—20 °C). The frozen aliquots were dispensed so that for each assay there was a separate tube to be thawed and used only for one occasion. During dilutions and placing the discs all solutions of cefatrizine were kept in ice. After taking the zero time samples for saturation of discs, the tubes containing the aliquot amounts were kept at the temperatures specified above or frozen immediately, and further samples were taken at 3, 5 and 24 h as well as at 1, 2 and 4 weeks after zero time.

The samples were assayed for antibiotic activity (potency) using the disc agar diffusion method with *B. subtilis* ATTC 6633 as the indicator organism, mixed in Penassay seed agar. The susceptibility filter paper discs with a diameter of 6.35 mm (Schleicher & Schuell, Inc.) were treated with 30 μl of solutions to be assayed. The disced plates were incubated at 30 °C overnight (about 18 h) and the diameters of the inhibition zones read and recorded [16, 17].

In all assays three discs on duplicate plates were used and the data given in the tables represent the average values of the inhibition zone diameters of the six discs.

Results

Stability studies on cefatrizine in aqueous environments. Cefatrizine gradually loses potency in aqueous milieu. In tap water the degradation is very rapid at 37 and 30 °C and even at room temperature (RT). Lower temperatures (4 °C and —20 °C) aid in maintaining potency for about 24 h, but thereafter the potency tends to diminish, more rapidly at 4 °C than at the frozen state. In deionized water, the loss of potency is slower even at higher temperatures with lower temperatures (4 °C and —20 °C) having a stabilizing effect on the potency for about 2 weeks. In critic acid-phosphate buffer, the stability at

Table I

Loss of activity of cefatrizine in different aqueous solutions at various temperatures

Solvent	Tap water					Deionized water					Buffer (pH 6)				
Temperature	37°	30°	RT	4°	−20°	37°	30°	RT	4°	20°	37°	30°	RT	4°	−20°
Sampling															
0 h	19*	19	19	19	19	19	19	19	19	19	20	20	20	20	20
3 h	0	9	17	19	—	19	19	19	20	—	19	20	20	20	—
5 h	0	0	12	17	—	19	19	19	19	—	18	19	19	20	—
24 h	0	0	0	17	17	16	17	18	19	19	±	16	17	19	16
1 week	0	0	0	13	16	0	0	13	18	17	0	0	0	18	15
2 weeks	0	0	0	0	15	0	0	0	18	17	0	0	0	17	15
4 weeks	0	0	0	0	12	0	0	0	14	17	0	0	0	16	15

* Numbers denote diameters of inhibition zones in mm
RT = Room temperature
± Trace of activity
— Not done

higher temperatures (37 °C, 30 ° C and RT) is not as good as in deionized water, and, interestingly enough, at 4 °C the stability is somewhat better than in the frozen state when kept at least for 4 weeks (Table I).

Sodium metabisulphite at the employed concentration provides certain stabilizing effects on cefatrizine in aqueous solutions but it is not dramatic; apparently stabilization is better in deionized water than in tap water or in citric acid-phosphate buffer (Table II).

Diluted hydrochloric acid added to aqueous solutions of cefatrizine proved to be the most effective means of maintaining potency. Hydrochloric acid was clearly effective at each temperature for the 4 week experimental period

Table II

Partial stabilization of cefatrizine in different aqueous solutions at various temperatures by addition of 0.1 ml of 2% $Na_2S_2O_5$ to 2 ml of solutions

Solvent	Tap water					Deionized water					Buffer (pH 6)				
Temperature	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°
Sampling															
0 h	19*	19	19	19	19	19	19	19	19	19	20	20	20	20	20
3 h	18	19	18	19	—	19	19	79	19	—	20	20	19	20	—
5 h	18	18	18	19	—	19	18	19	19	—	18	18	18	20	—
24 h	17	17	17	18	15	18	18	18	19	13	±	15	17	19	16
1 week	±	12	13	16	±	10	15	15	17	12	0	0	±	17	16
2 weeks	0	0	10	16	0	0	12	12	17	±	0	0	0	16	15
4 weeks	0	0	8	15	0	0	±	±	14	0	0	0	0	14	14

* Numbers denote diameters of inhibition zones in mm
RT = Room temperature
± Trace of activity
— Not done

(Table III). Again, storage at 4 °C produced better stability results than those obtained at the frozen condition. Because it is cheaper to store solutions at 4 °C than at the frozen state, this phenomenon deserves further exploration. Here again, the hydrochloric acid added to the buffer containing phosphate ions, at least at higher temperatures (37 °, 30 °C and RT), has less stabilizing effect than in deionized water. The same phenomenon can be seen in the experiments with the addition of sodium metabisulphite (Table II).

Table III

Stabilization of activity of cefatrizine in different aqueous solutions at various temperatures by addition of 0.1 ml of 2 N HCl to 2 ml of solutions

Solvent	Tap water					Deionized water					Buffer (pH 6)				
Temperature	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°
Sampling															
0 h	20*	20	20	20	20	20	20	20	20	20	21	21	21	21	21
3 h	20	20	20	20	—	20	20	20	20	—	21	21	21	20	—
5 h	19	19	20	20	—	20	19	20	20	—	20	20	20	20	20
24 h	19	19	19	20	18	20	19	19	20	17	20	20	20	20	20
1 week	19	20	19	20	17	20	20	20	20	17	16	19	22	21	19
2 weeks	17	18	18	19	15	15	19	19	20	13	8	16	19	20	17
4 weeks	20	17	18	18	16	18	18	19	19	±	0	12	16	20	16

* Numbers denote diameters of inhibition zones in mm
RT = Room temperature
± Trace of activity
— Not done

Stability studies of cefatrizine in sera. There is a time-related loss of potency of cefatrizine in "normal" sera (pH ~ 8); this loss depends on the temperature at which the sera are kept (Table IV). The loss of potency seems to start immediately as the antibiotic is dissolved in or diluted with the pooled sera. At 37 °C, 30 °C and room temperature (~ 18–22 °C) all activity disappears after 24 h. At 4 °C (cold-room temperature) the loss of potency is somewhat slower but after one week of storage the activity is diminished or barely demonstrable. The freezing (—20 °C) of cefatrizine in human sera results in a loss of potency detectable at 24 h and further loss is observed at 4 weeks. Similar results are observed in mouse and dog sera, albeit mouse serum appears to have less degrading effect on cefatrizine than human or dog sera.

In contrast to the results obtained with "normal" sera, when cefatrizine was dissolved in acidified sera with a final pH of about 4.0, the potency was stabilized, especially at room temperature and below (Table V).

Comparing the data in Table IV and Table V it is evident that acidification of sera with the addition of 0.1 ml of the 2 N hydrochloric acid to each 2 ml of sera almost completely stabilizes the potency of cefatrizine during storage and while performing the bioassay at room temperature and below.

Table IV

Loss of activity of cefatrizine in sera from different species at various temperatures

Serum*	Pooled human					Pooled mouse					Pooled dog				
Temperature	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°
Sampling															
0 h	17**	17	17	17	17	18	18	18	18	18	19	19	19	19	19
3 h	15	17	17	18	—	17	18	18	18	—	16	18	18	19	—
5 h	13	15	16	17	—	15	17	18	18	—	14	16	17	18	—
24 h	0	±	±	13	14	0	±	±	16	16	0	0	0	16	15
1 week	0	0	0	±	13	0	0	0	14	16	0	0	0	7	14
2 weeks	0	0	0	0	12	0	0	0	12	12	0	0	0	0	12
4 weeks	0	0	0	0	12	0	0	0	0	12	0	0	0	0	12

* pH of sera = ~ 8
** Numbers mean inhibition zones in mm
± Trace of activity
— Not done

Table V

Stabilization of activity of cefatrizine in sera from different species at various temperatures by addition of 0.1 ml of 2 N HCl per 2 ml of serum

Serum*	Pooled human					Pooled mouse					Pooled dog				
Temperature	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°	37°	30°	RT	4°	−20°
Sampling															
0 h	19**	19	19	19	19	19	19	19	19	19	20	20	20	20	20
3 h	19	19	19	19	—	19	19	20	19	—	19	20	20	20	—
5 h	19	19	19	19	—	19	19	19	19	—	19	19	19	19	—
24 h	18	19	18	17	19	18	18	18	19	17	18	19	18	19	17
1 week	16	19	19	19	19	18	19	20	19	17	18	19	18	19	17
2 weeks	12	16	19	19	19	14	19	20	19	17	14	19	19	18	17
4 weeks	0	14	18	18	18	±	17	18	19	17	±	18	19	20	17

* pH of sera = ~8
** Numbers mean inhibition zones in mm
± Trace of activity
— Not done

Discussion

Cefatrizine belongs to the group of orally active phenylglycine-type cephalosporins. Many members of this group, especially cephaloglycin [18], but to a lesser degree cephalexin and cephradine, may lose activity during bioassay in aqueous solutions as well as in frozen sera [19]. Cefatrizine was also found to show a certain degree of instability under the same circumstances. This loss of activity or degradation is influenced by the substituent at the 3-position of the cephem nucleus and is, among other factors, pH-dependent. The physiochemical basis of this instability of cefatrizine was studied by Tsuji et al. [20].

O'Callaghan [21] has described that serum protein can cause irreversible degrading effects on certain beta-lactam antibiotics and that this decomposition is pH-dependent and occurs more slowly at acidic pH. It is known that the pH of the serum (plasma) increases. Within two hours after being separeted from the blood clot, the serum pH reaches 8–8.5 due to the loss of carbon

dioxide [22]. At this alkaline pH, any antibiotics, including beta-lactams like cefatrizine, start to degrade even at low temperatures. As described in this paper, acidifying the serum (plasma) prevents the loss of activity of cefatrizine even at room temperature. Broughall et al. [23] found that the stability of cefatrizine at 37 °C was considerably increased by adding sodium dodecyl sulphate to serum to a final concentration of 1% and adjusting the pH simultaneously to < 6.0. Using this method they found significantly higher serum concentrations and urinary recoveries than reported earlier [11]. This complex procedure is, however, recommended only for pharmacokinetic studies and not for everyday assays for unstable cephalosporins. The application of the simple method described in this paper, i.e. acidifying serums, urine samples or aqueous solutions with 2 N hydrochloric acid (pH about 4.0) makes microbiological assay of cefatrizine [24, 25], and probably other unstable beta-lactam antibiotics, more reliable and realistic.

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CHARACTERIZATION OF HOSPITAL AND COMMUNITY STRAINS OF *STAPHYLOCOCCUS AUREUS* FOR RESISTANCE TO ANTIMICROBIAL DRUGS, METALLIC IONS, DISINFECTANTS, THERMAL INJURY AND SOLAR RADIATION

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One hundred and sixty strains of *Staphylococcus aureus* isolated from various clinical specimens were classified into groups: hospital staphylococci (HS) or community staphylococci (CS), based on the clinico-ecological circumstances of isolation. Fifty strains from both groups were tested for the minimum inhibitory concentration (MIC) of penicillin G, streptomycin, tetracycline, chloramphenicol, mercuric chloride, disodium hydrogen arsenate, silver nitrate and sodium bisulphite. Four representative strains from each group were further studied for resistance to "in-use" dilutions of 4 disinfectants (Dettol, Izal, Savlon and Chlohexidine), heat stress and the effect of solar radiation in sand cultures. All HS and 31.8% of CS were resistant to penicillin and ampicillin and produced penicillinase. HS had higher MICs of antibiotics and metallic ions and longer bactericidal times with disinfectants than CS. Resistance to thermal stress varied within each group but survival in sand cultures under solar radiation appeared to be influenced by multiple factors to which community staphylococci were probably better adapted.

Staphylococcus aureus is responsible for many of the common bacterial infections of man. In the hospital environment, drug-resistant strains pose serious problems of post-operative infections. Multiple drug resistance and increased virulence are the usual phenotypic markers of hospital staphylococci [1].

Community strains of organisms are usually more susceptible to many antibiotics than hospital strains [2]. Widespread use of antibiotics in hospital practice has a selective effect on the emergence of drug resistance in bacteria [3]. Other selective pressures such as environmental contamination by metals may also promote resistance to metals and metal-based disinfectants in bacteria [4]. In the community, antibiotics and metal -based disinfectants are not used as extensively as in hospital practice.

In this, study, hospital and community strains of *Staphylococcus aureus* from clinical specimens were characterized.

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Materials and methods

Staphylococcal strains. *S. aureus* strains were clinical isolates from patients seen during January—May, 1984 at the University of Calabar Medical Centre and the University of Calabar Teaching Hospital, Calabar, Nigeria. Organisms were identified according to Kloos and Schleifer [5]. The basis of ecological designation of each isolate was primarily on the circumstances of its isolation, and partly on the antimicrobial susceptibility pattern. HS designation was used for strains isolated from in-patients after 3 weeks of hospitalization, if they showed multiple drug resistance. CS was for strains isolated from in-patients within 2 weeks of hospitalization or from out-patients who had no history of hospitalization and had not received chemotherapeutic agents 2 months preceeding their visit to the hospital.

Antimicrobial susceptibility tests. The sensitivity of staphylococci to antimicrobial drugs was determined by the disk diffusion method using Multodisks 1789E (Oxid) on sensitivity test agar (Gibco Diagnostics, Madison, Wis.), with Oxford *S. aureus* as the control organism. Strains with zones of inhibition $\leq$ 5 mm less than that of the control organism were regarded as sensitive. Resistance was indicated by a zone of inhibition $>$ 5 mm smaller than that of the control organism.

Twenty seven HS and 23 CS were randomly selected for tests to determine the MIC of penicillin G, streptomycin, tetracycline and chloramphenicol by the agar dilution method [6]. Test plates inoculated with Trypticase Soy Broth (TSB) sispensions of staphylococci (ca. 10^5 colony-forming units c. f. u./ml) were incubated at 37 °C for 48 h. The lowest concentration of antibiotic producing $\leq$ colonies was taken as the MIC.

Penicillinase test. Overnight TSB cultures were tested for penicillinase production by the rapid iodometric method [7].

Resistance to metalic ions and bisulphite. The MICs of $HgCl_2$, Na_2HAsO_4, $AgNO_3$ and $NaHSO_3$ were determined by the broth dilution method in TSB. Aqueous solutions of salts were filter-sterilized (0.45 μm filter, Milipore Corp., Bedford, Mass.) and diluted to contain the following concentrations of Hg^{++}, $HAsO_4^-$, Ag^+ and HSO_3^-: 0, 12.5, 25, 50, 100, 200, 300, 400, 500 μg/ml, after addition of TSB suspension of staphylococci (ca. 10^5 c. f. u./ml final concentration). Cultures were incubated at 3 °C for 5 days before they were examined for growth (turbidity).

Resistance to germicidal action of disinfectants. Fifty tandomly selected HS and CS were screened to determine the sterilizing times for „in-use" dilutions of 4 disinfectants: Dettol (chloroxylenol, Reckitt and Coleman Ltd., UK), 2.5%; Izal (carbolic acid, Sterling—Izal Ltd., UK), 1/600; Savlon (chlorhexidine + cetrimede, ICI Ltd., UK), 1/200; and chlorhexidine diacetate, (Sigma Chemical Co., St. Louis, Mo.), 0.02%. Chlorhexidine diacetate was dissolved in 0.1 M NaOAc buffer, pH 4.6. Suspensions of staphylococci in saline and in TS—ES were tested by adding 0.2 ml (ca. 10^9 c. f. u./ml) to 1 ml of disinfectant in sterile 1/2 — oz screw-cap bottles and shaking. At intervals of 1, 2.5, 5, 10, 15, 30, 45, 60, 70, 90 min, a loopful (0.01 ml standard loop) of the inoculated disinfectant was sub-cultured into 10 ml of sterile TSB and shaken. Sub-cultures incubated at 37 °C for 5 days were examined for turbidity. The first exposure time showing no growth was the sterilizing time.

Estimation of thermal injury. Two ml of TSB suspension of 8 representative strains (ca. 10^8 c. f. u./ml) dispensed into 5 sterile cotton-plugged tubes (100 $\times$ 13 mm) were exposed in a water bath at 60 °C. Tubes were removed at intervals and rapidly cooled in an ice bath. Serial tenfold dilutation in 0.1 M potassium phosphate buffer (PB; pH 7.2) were plated on trypticase soy agar (TSA) spread plates using 0.1 ml inoculum. Colonies were counted after 3 days incubation at 37 °C.

Effects of solar radiation on sand cultures. Cotton-plugged tubes (150 $\times$ 25 mm) containing 5 g of hot-sir-sterilized sand were inoculated with 1 ml of TSB suspension of staphylococci (ca. 10^8 c. f. u./ml). Control samples received sterile TSB. Plugs were screened with aluminium foil and sealed with masking tape. Tubes were weighed and incubated in the open lawn for 3 weeks. Daily atmospheric temperatures were taken. Percentage residual moisture in control tubes were determined weekly. The populations of viable survivors were determined weekly on TSA spread plates. Tenfold serial dilutions were prepared from sand cultures suspended with 45 ml of sterile PB. Colonies were counted after incubation at 37 °C for 3 days.

Results

The clinical sources and antimicrobial resistance pattern of 160 *S. aureus* strains are shown in Table I. Of this number, 94 (58.7%) were designated HS while 66 (41.3%) were CS. For both strains, the observed incidence of antimicrobial resistance was highest with streptomycin and penicillin for which a combined total of 124 (78%) and 115 (72%) strains, respectively, were resistant. All 94 (100%) HS were penicillinase producers and resistant to penicillin and sterptomycin. Resistance to ampicillin was the same as to penicillin. Of the CS, 21 (31.8%) were resistant to penicillin and produced penicillinase while streptomycin resistance was shown in 30 (45.5%). Among the 160 isolates erythromycin and gentamicin resistance was observed in a total of 14 (8.8%) and 8 (5%) strains, respectively. All staphylococci were uniformly sensitive to cloxacillin (data not shown) and clindamycin.

Table I

Distribution of antimicrobial resistance pattern in hospital and community strains of S. aureus according to source Total number of strains: HS, 94; Cs, 66

Sources	Strain	No. of strains	No. resistant to								
			Pc	Sm	Tc	Cm	Em	Em	Cot	Ft	Gm
Urinary tract infection	HS	47	47	47	47	43	34	5	18	13(28)	3
	CS	9	4	4	5	3	1	0	5	1(11)	0
Wound infections	HS	22	22	22	22	17	16	2	14	ND	1
	CS	11	3	3	3	2	2	0	3	ND	0
Eye infections	HS	7	7	7	7	5	5	0	2	ND	1
	CS	12	5	5	6	4	0	0	3	ND	0
Blood cultures	HS	5	5	5	5	4	3	0	0	ND	0
	CS	4	1	1	1	0	0	0	1	ND	0
Miscellaneous infections	HS	13	13	13	13	12	11	5	9	ND	1
	CS	30	8	8	15	5	4	2	2	ND	2

** Pen = penicillin G; Sm = streptomycin, Tc = tetracycline, Cm = chloramphenicol, Em = erythromycin, Cot = co-trimoxazole, Ft = nitrofurantoin, Gm = gentamicin, All strains were uniformly sensitive to cloxacillin and clindamycin.
ND = Not done

The MICs of antibiotics, metallic ions and bisulphite for HS and CS are shown in Table II. For HS, the MIC values were in the higher ranges. For instance MIC of penicillin, streptomycin, tetracycline and chloramphenicol for most strains was 64 μg/ml while 22 others had a MIC of 128 μg/ml for streptomycin. No strain had MICs $\leq$ 8 μg/ml of any antibiotic. Conversely, for most CS the MIC of streptomycin, tetracycline and chloramphenicol was 4 μg/ml while that of penicillin for 17 others was $\leq$ 0.25 μg/ml. Fewer strains had MICs within 8–32 μg/ml range.

Hg^{++} and Ag^{+} were more inhibitory than $HAsO_4^{-}$ or bisulphite. Most HS were inhibited by Hg^{++} and Ag^{+} at concentrations of 100 to 200 μg/ml

Table II

The minimal inhibitory concentrations (μg/ml)

Total number of

Strain	Penicillin G							Streptomycin					
	0.1	0.25	4	8	16	32	64	4	8	16	32	64	128
HS	0	0	0	0	2	7	18	0	0	0	2	3	22
CS	5	12	0	4	2	0	0	8	7	5	3	0	0

Strain	Hg^{++}							$HAsO_4^-$					
	12.5	25	50	100	200			12.5	25	50	100	200	400
HS	0	2	4	5	16	—	—	0	0	0	5	10	12
CS	8	10	5	0	0	—	—	0	2	15	6	0	0

and at 200 μg/ml or more of $HAsO_4^-$ and bisulphite. Most CS were inhibited by metallic ions at concentration of 50 μg/ml or less, and bisulphite at 100 μg/ml or less.

Table III shows the bactericidal of disinfectants for saline and TS-BS suspensions of staphylococci. Izal was the least potent and killed the bacteria in saline suspensions of 3 strains (HS 44, HS 3101 and CS 166) in 15 min or less, and TS-BS suspensions in over 60 min. Both suspensions of CS 3540 were killed in 15 min. Dettol was effective in most saline suspensions in 5 min or less, and in TA-BS in 15 min or less except HS 3101 and CS 166 which were killed in

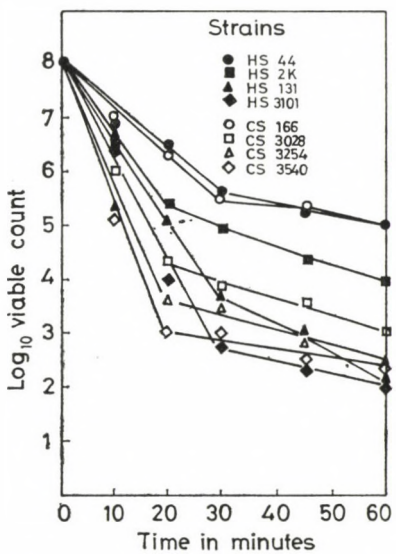

Fig. 1. The effect of thermal stress on the viability of HS and CS cultures in TSB

of antibiotics, metallic ions and bisulphite on S. aureus

strains: HS, 27; CS, 23

Tetracycline						Chloramphenicol					
2	4	8	16	32	64	4	8	16	32	64	128
0	0	0	2	5	20	0	0	4	3	20	0
0	12	7	4	0	0	11	7	4	1	0	0

Ag^+					$NaHSO_3$						
12.5	25	50	100	200	12.5	50	100	200	300	400	500
0	0	6	18	3	0	0	2	3	15	4	3
0	11	12	0	0	0	13	10	0	0	0	0

Table III

Bactericidal time in minutes by "in use" concentrations of disinfectants on S. aureus strains

Designation ot strain	Suspending medium	Dettol (2.5%)	Izal (1/600)	Savlon (1/200)	Chlorhexidine (0.02%)
HS 44	Saline	5	5	ND	ND
	TS-BS	15	90	5	15
HS 131	Saline	2.5	10	ND	ND
	TS-BS	5	30	< 1	< 1
HS 3101	Saline	5	15	ND	ND
	TS-BS	30	90	5	10
HS 2K	Saline	2.5	15	ND	ND
	TS-BS	5	45	< 1	< 1
CS 166	Saline	5	5	ND	ND
	TS-BS	30	70	5	5
CS 3028	Saline	< 1	5	ND	ND
	TS-BS	5	10	< 1	1
CS 3254	Saline	< 1	15	ND	ND
	TS-BS	5	30	< 1	1
CS 3540	Saline	< 1	15	ND	ND
	TS-BS	2.5	15	< 1	1

ND = Not done

30 min. For Savlon and chlorhexidine, bactericidal times were identical for most staphylococci except that the former killed HS 44 and HS 3101 faster than the latter.

The data presented in Fig. 1 show the response of *S. aureus* strains to heat stress. All strains were killed at constant exponential rates. Decimal reduction times (D-values) ranged from 4–12.5 min. Most CS had D-values of 5.4 or less, while HS had D-values of 7–12.5 min. Survival curves were generally biphasic in nature.

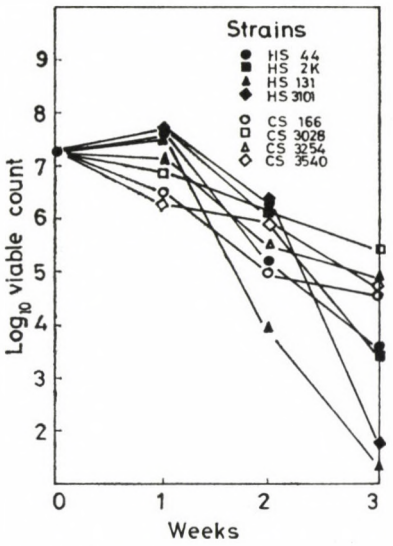

Fig. 2. The pattern of survival of HS and CS in sand cultures exposed to solar radiation

The effect of exposure to solar radiation on viability of strains in sand cultures are shown in Fig. 2. Approximately 0.5 log increases were detected in CS in week. 1. Subsequently, the order of death through week 3 was either gradual and linear (HS 44 and HS 2K) or almost exponential (HS 131 and HS 3101). For CS, decreases of 0.1—1 log occurred in week 1. Most strains declined equally through week 3 and hence survival rates were generally higher among CS (Fig. 2).

Table IV

Changes in the mean atmospheric temperature and percentage residual moisture content of sand cultures of staphylococci

Weeks	Mean temperature, °C	Residual moisture %
0	39.0	100.
1	39.4	73.2
2	40.0	32.5
3	43.5	7.4

Variations in the atmospheric temperature and changes in the percentage residual moisture of sand cultures are shown in Table IV. The overall temperature increase of 5.5 °C observed during incubation was matched by a total moisture loss of about 93%.

Discussion

S. aureus is often involved in hospital-acquired infections and may sometimes account for 15 to 20% of nosocomial infections [8]. Multiple drug resistance is an important characteristic of hospital staphylococci [1]. Sabramanyan and Agarwal [3] have observed 94% incidence of penicillin and ampicillin resistance in hospital staphylococci. This study showed an incidence of resistance to penicillin and ampicillin of 100% for hospital strains. Although it is valid to relate the high incidence of resistance to the selectional pressures peculiar to the ecology of nosocomial strains, the observed penicillin and ampicillin resistance of 35% for strains of non-nosocomial antecedent appears remarkable. These data suggest that about one-third of infections due to CS would be resistant to these drugs and hence treatment failures in clinical infections involving these strains would be predictably high. Equally interesting is the observetion that erythromycin, gentamicin, cloxacillin and clindamycin appeared to be the drugs of choice for most *S. aureus* infections irrespective of the ecological source of the causative strain. Under the present situation of restricted importation of pharmaceuticals in Nigeria, it is reassuring that this pattern of sensitivity makes a reasonable range of drugs available for infections by either of these strains.

Further, it was observed that HS was resistant to higher concentrations of more antibiotics and metallic ions that CS but the significance of the relationship between resistance to these agents is not clerar. Some studies have noted the correlation of drug resistance with that of metallic ions in bacteria. Nakahara et al. [9] have observed that the frequency of metal resistance in clinical isolates of *Pseudomonas aeruginosa* was the same as, or higher than, antibiotic resistance. Resistance to metallic ions or antibiotics may be mediated by plasmids [10]. Weiss et al. [11] have observed plasmid-mediated resistance to Hg-based disinfectants in staphylococci. Also, co-elimination of resistance to Hg^{++} and penicillin has been noted in staphylococci [12]. The high resistance of HS to metallic ions, especially Hg^{++}, seemed to agree with the observation [4] that Hg^{++} resistance probably results from the use of Hg in disinfectants in hospital practice. This relationship might not exist for Ag or $HAsO_4$. Bisulphite at 500–1500 μg/ml is used for preserving food and hospital medications [13]. The observation that 3 HS had MIC of bisulphite of 500 μg/ml, probably suggested that only marginal increases in the resistance of such strains would render bisulphite preservation ineffective at the lower practical concentration.

No ready explanation appeared available for comparable sterilizing times of Izal for TS-BS and saline suspensions of CS 3540; except that the strain usually formed granulations in saline which might thus prevent rapid penetration of disinfectant into cell aggregates. The protective effect of TS-BS against

disinfectant action appeared similar to that provided by organic matter such as slime for bacterial organisms in clinical specimens [14] and might be an important factor in surface decontamination where HS may be protected by serous exudates.

Preliminary studies with "in-use" dilution of Savlon (1/60) showed bactericidal times of < 1 min for saline and TS-BS suspensions. Hence, subsequently, Savlon was tested at a high abuse dilution (1/200) that might simulate practical situations.

The biphasic survival curves observed in this study are typical of heat resistance studies on *Salmonella arizonae* [15], and represent cell populations heterogeneous for heat tolerance [16]. However, the variability of heat resistance among the same ecological strains seemed to preclude generalization with respect to heat tolerance.

It is uncertain if viability of HS and CS in sand cultures was related to the initial growth patterns observed early in the incubation period. Actively multiplying cells are more susceptible to heat injury than dormant cells. However, the rapid die-off rate of HS in sand cultures did not appear to have been influenced remarkably by heat since only marginal tamperature increases occurred. Rather it seemed likely that a moisture loss of about 25% was more critical and that desiccation probably had greater impact on survival than minimal temperature changes. Perhaps equally important was the effect of sunlight whose ultra-violet (UV) radiation might have contributed to population declines. It may be concluded that survival of sand cultures under radiant sunlight was probably influenced by multiple factors to which CS appeared better adapted than HS.

These observations probably reflected differences in the subtle adaptive changes in two strains of *S. aureus* as related to the peculiarities of their separate ecological pressures.

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EFFECT OF THE VIRULENCE ASSOCIATED 47 MEGADALTON PLASMID OF *YERSINIA PESTIS* ON PERMEABILITY TO GENTIAN VIOLET AND SENSITIVITY TO NOVOBIOCIN

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The loss of the 47 megadalton (Mdal) plasmid of *Yersinia pestis* resulted in increased gentian violet uptake and increased sensitivity to novobiocin. The addition of calcium caused a decrease in gentian violet uptake and a decrease in novobiocin sensitivity independent of the plasmid. Differentiation of cells bearing and lacking the plasmid was accomplished using novobiocin in the presence of calcium at 26 °C. The novobiocin resistance levels of cells lacking the 47 Mdal plasmid and cells with the plasmid, but with an insertion in or a deletion in, or encomposing, the calcium dependence region of this plasmid, indicated that the resistance to novobiocin was not associated with the calcium dependence region, but was associated with a separate 47 Mdal plasmid region. Examination of *Yersinia pseudituberculosis* and *Yersinia enterocolitica* isolates demonstrated that functionally similar sites are present on the plasmids that encode for calcium dependence in these species.

Yersinia pestis, the causative agent of bubonic and pneumonic plague, is a facultative intracellular parasite (for review see reference 1). Virulent strains harbor a 47 megadalton (Mdal) plasmid [2–4], require Ca^{++} for growth at 37 °C [5] and produce the V and W antigens [6, 7]. Avirulent mutants which are obtained by prolonged growth at 37 °C in the absence of Ca^{++}, either lack this plasmid [3] or have a mutation in or encompassing what has been termed the calcium dependence region of this plasmid [4]. Incubation temperature and this plasmid both influence the rate of phagocytosis of *Y. pestis* by macrophages and the plasmid is required for growth within macrophages in vivo and, under some conditions, in vitro [8–10]. The incubation temperature influences the composition of the lipopolysaccharide (LPS) and the outer membrane protein composition of *Y. pestis* [11]. The 47 Mdal plasmid also influences the lipopolysaccharide composition at 37 °C [12]. Since these changes in the *Y. pestis* cell envelope occur as a result of alteration in parameters reflected in the

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host-phagocyte interactions, it is important to determine how these changes affect bacterial cell function.

In other bacteria, good correlations have been found between the structure of the cell envelope, the uptake of gential violet [13, 14] and sensitivity to novobiocin [15, 16]. Changes in phospholipid content and changes in the lipopolysaccharide structure have been related to changes in resistance to novobiocin [16–18]. Gentian violet and novobiocin are grouped with other hydrophobic substances which some believe pass through the outer membrane via non-porin mediated mechanisms, perhaps by dissolving into the hydrophobic regions of the cell envelope [19, 20]. One might suspect that in conjunction with changes in the cell envelope which result from changes in temperature, Ca^{++} availability and plasmid content in *Y. pestis*, changes would also occur in cell permeability. We examined this possibility.

Here we will show that incubation temperature and calcium influence gentian violet uptake and novobiocin sensitivity, and that cells bearing the 47 Mdal plasmid take up gentian violet more slowly and are resistant to higher levels of novobiocin than cells lacking this plasmid. Furthermore, we show the site for high level novobiocin resistance is separate from the region of this plasmid involved in calcium dependence, and that similar relationship between novobiocin resistance and the plasmid associated with calcium dependence are found in two other human pathogens in this genus, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*.

Materials and methods

Bacterial strains and culture conditions. *Y. pestis* strains EV76 and KIM were obtained from Dr. R. R. Brubaker (Michigan State University, East Lansing, Michigan). The sources of other strains used in this study are provided in the footnotes to the appropriate tables. Mutans lacking the 6 and the 61 Mdal plasmids, but bearing the 47 Mdal plasmid (EV76S11 and EV76S13) were obtained from. *Y. pestis* strain EV76 following a 2 day incubation at 37 °C on blood agar base plates containing 2.5 mM Ca^{++} and 14 μg/ml gentian violet (crystal violet, Allied Chemical, Morristown, NJ). Strain EV76C1, which lacks the 47 Mdal plasmid but retains the 6 and 61 Mdal plasmids, was selected from EV76 on magnesium oxalate agar plates [21] other strains lacking this plasmid or the corresponding plasmids in *Y. enterocolitica* or *Y. pseudotuberculosis* were selected using the same procedure. Plasmid content in each case was determined by isolating the plasmids by the method of Holmes et al. [22] and analysing them by agarose gel electrophoresis as described by Meyers et al. [23]. These gels are not presented in this report. Plating medium was prepared in all cases using 40 g blood agar base per liter deionized water. Gentian violet and novobiocin (monosodium salt) were dissolved in water, sterilized by filtration and, if indicated, added to sterilized blood agar base medium immediately prior to dispensing. Novobiocin was prepared on the day used. Plates were scored after 5 days incubation at 26 °C unless otherwise specified. Stock cultures were kept as suspensions of cells at —20 °C in 40% glycerol 60% 0.1 M phosphate buffer pH 7.0. Cells were subcultured on blood agar base (BBL Microbiology Systems) at 26 °C before inoculation of liquid medium. Cultures were grown with aeration in Erlenmeyer flasks at 1/10 the nominal flask volume in the medium of Higuchi et al. [24] as modified by Brubaker [25]. The optical density was monitored at 620 nm.

Gentian violet uptake. Total uptake of gentian violet was determined by methods similar to those described by Guymon and Sparling [14]. Samples (3.8 ml) were removed from cultures, filtered gentian violet (0.2 ml) was added to a final concentration of 10 μg/ml and incubation

was continued for an additional 10 min at 26 °C or 37 °C for the determination of the total uptake or at 0 °C for the determination of the type I uptake (binding). The suspensions were cooled quickly in an ice bath to prevent further uptake and the cells were pelleted by centrifugation at 12 000 *g* for 10 min at 4 °C. The amount of dye remaining in the supernatant fluid was determined by measuring absorbance at 590 nm. To determine the kinetics of gention violet uptake, the dye was added to actively growing cultures, samples were transferred to ice-cold tubes at the times indicated and handled as described above. When cultures were shifted from 26 °C to 37 °C, gentian violet was added 10 min after shift.

Results

Gentian violet uptake by EV76 and EV76Cl cells. Cells of both the EV76 and EV76Cl stains, grown at 26 °C or 37 °C in either the presence or absence of calcium, bound approximately equal quantities of gentian violet at 0 °C (15–16% of the total dye in solution). Both EV76 and EV76Cl cells took up gentian violet at higher temperatures (Fig. 1a, 1b). The rate of gentian violet uptake was greater at 37 °C than at 26 °C both in the presence and absence of calcium. EV76Cl cells took up gentian violet more rapidly than EV76 cells at both 26 °C and 37 °C. At both temperatures, Ca^{++} reduced the rate of uptake by EV76 and EV76Cl cells. If cells were washed to remove Ca^{++}, however, this decrease in the rate of gentian violet uptake was not detected.

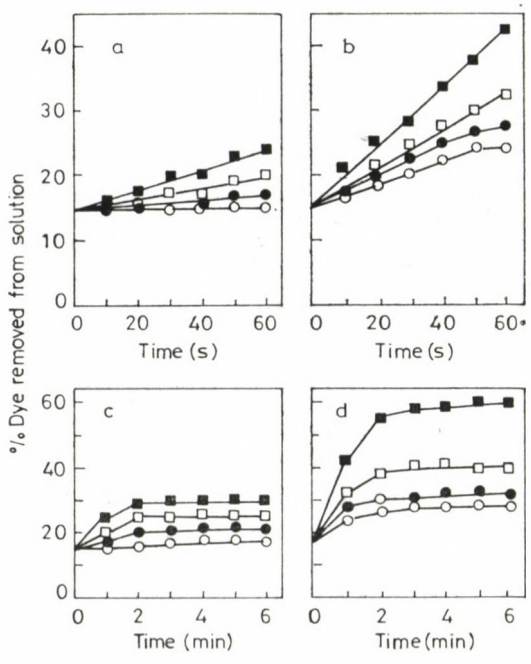

Fig. 1. Uptake of gentian violet by *Y. pestis* EV76 cells grown with (○) and without (●) Ca^{++}, and EV76 Cl grown with (□) and without (■) Ca^{++} at 26 °C (a, c) and at 37 °C (b, d). Uptake is expressed as % dye removed from solution per optical density unit of bacterial cells

The total gentian violet uptake by EV76Cl cells was greater than that of EV76 cells under similar conditions. Furthermore, the total uptake of gentian violet by EV76 cells at 37 °C in the absence of Ca^{++} depended on prior incubation period at 37 °C (Fig. 1c, 1d). A reduction in the extent of gentian violet uptake began 3 h after shift to 37 °C and resulted in a new level of total gentian violet uptake (25%) by 7 h. This variation in gentian violet uptake was not observed with EV76Cl cells. Type I uptake (binding) did not change with time.

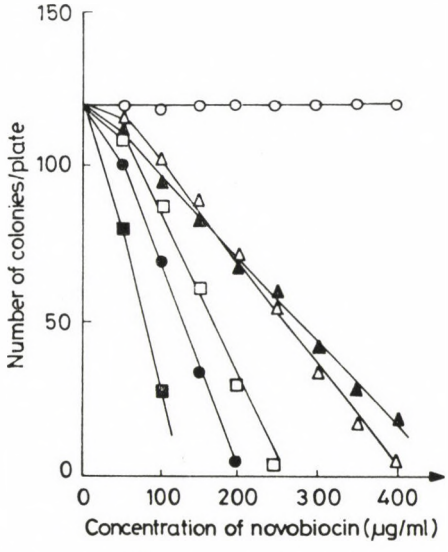

Fig. 2. Colony formation by cells of *Y. pestis* on plates with selected concentration of novobiocin in the presence and absence of added 2.5 mM Ca^{++}. Open symbols strain EV76, closed symbols EV76Cl, triangles 37 °C with Ca^{++}, circles 26 °C with Ca^{++}, squares 26 °C no added Ca^{++}. A culture grown at 26 °C was diluted and a volume giving 120 colony forming units on blood agar base without novobiocin (26 °C) was plated on each medium and incubated at the indicated temperature for 5 days

Novobiocin sensitivities of EV76 and EV76Cl cells. The novobiocin sensitivities of these strains were determined using the same variations in temperature and calcium availability used in gentian violet uptake studies. EV76 cells were more resistant to novobiocin than EV76Cl cells at 26 °C (Fig. 2). In the presence of Ca^{++}, 100% recovery of EV76 cells was common at 400 μg/ml of novobiocin, whereas all EV76Cl cells were killed at 300 μg/ml novobiocin. In the absence of Ca^{++}, EV76 and EV76Cl cells were inhibited by 250 μg/ml and 125 μg/ml of novobiocin, respectively. In the presence of Ca^{++}, EV76Cl cells were more resistant to novobiocin at 37 °C than at 26 °C, whereas the EV76 cells were more sensitive to novobiocin at 37 °C. Novobiocin resistance in the absence of Ca^{++} at 37 °C was not determined since EV76 cells do not grow

under these conditions. The obvious differences in resistance to novobiocin of EV76 cells in the presence and absence of calcium at 26 °C suggested an optimal concentration of Ca^{++} should exist for the novobiocin resistance of these cells. This optimum was found to be in the range of 1.5–5 mM (Fig. 3). The only known genetic difference between strains EV76 and EV76Cl is the absence of the 47 Mdal plasmid in strain EV76Cl [3]. Cells of *Y. pestis* (KIM) with and without the 47 Mdal plasmid, showed the same effect of this plasmid on novobiocin resistance, but at a different novobiocin concentrations (450 μg/ml). Loss of the 6 and 61 Mdal plasmids did not alter novobiocin resistance levels (Table I)

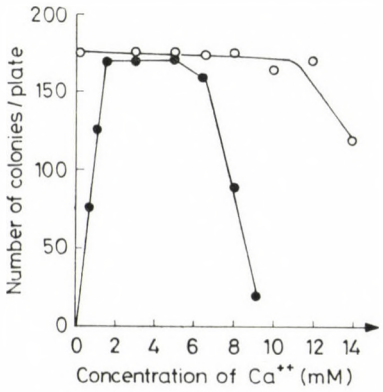

Fig. 3. Effect of Ca^{++} concentration on the colony forming ability of *Y. pestis* EV76 at 26 °C on medium containing 400 μg/ml of novobiocin (●) and on medium without novobiocin (○)

Table I

Plating efficiency of derivatives of Y. pestis EV76 on blood agar base plates containing 350 μg/ml novobiocin, and 2.5 mM Ca^{++}

Strain	Ca++ dependence	Plasmid (Mdal)	Plating efficiency (%)
EV76	+	61; 47; 6	100
EV76Cl[1]	—	61,6	0
EV76S11[1]	+	47	100
EV76S12[1]	—	—	0
EV76S13[1]	+	47	100
EV76S14[1]	—	—	0
EV766[2]	—	61; 6	0
EV7618[2]	—	61; 16[3]; 6	100
EV7621[2]	—	61; 2.5[3]; 6	100
EV7651[2]	—	61; 48.5[3]; 6	100

[1] Strains derived in this laboratory from EV76 (EV76Cl, EV76S11, and EV76S13) or from EV76S11 (EV76S12) or EV76S13 (EV76S14)

[2] Strains obtained from D.A. Portnoy, Department of Medical Microbiology, Stanford Unversity, Stanford, CA 94304

[3] Plasmid bearing insertions in the calcium dependence region of the 47 Mdal plasmid (EV7651) or deletions in or encompassing the calcium dependence region (EV7618, EV7621)

Novobiocin sensitivity of cells with mutations in the calcium dependence region of the 47 Mdal plasmid. To determine if the difference in novobiocin resistance related directly to the calcium dependence region of the 47 Mdal plasmid, we examined a number of additional derivatives of strain EV76. Some lacked the 47 Mdal plasmid and others had insertions in or deletions in or encompassing the calcium dependence region of this plasmid. Strains which lacked the 47 Mdal plasmid had lost high level resistance to novobiocin, whereas strains with mutations in or delections in or encompassing the calcium dependence region maintained high level novobiocin resistance. This indicated that a separate region of the plasmid was involved in novobiocin resistance (Table I).

Novobiocin resistance in Y. pseudotuberculosis and Y. enterocolitica. Representative *Y. pseudotuberculosis* and *Y. enterocolitica* isolates and direct calcium independent derivatives, which in each case lacked the plasmid associated with calcium dependence [2, 4, 26, 27], were examined to determine if these plasmids exerted a similar influence on novobiocin resistance in these microorganisms. This was found to be the case, although the levels of novobiocin discriminating between plasmid bearing and plasmid lacking cells varied (Table II).

Table II

Plating efficiency of Y. pseudotuberculosis and Y. enterocolitica isolates and derivatives of these isolates, in the presence of novobiocin and 2.5mM Ca^{++}

Strain	Novobiocin concentration (μg/ml)	Calcium dependence associated plasmid	Plating efficiency (%)
Y. pseudotuberculosis			
PB[1]	750	+	100
PB1Cl[3]	750	—	20
Y. enterocolitica			
8272[2]	950	+	100
8272Cl[3]	950	—	0
8072[2]	950	+	100
8072Cl[3]	950	—	0

[1] Strain obtained from R.R. Brubaker, Department of Microbiology and Public Health, Michigan Ttata University, East Lansing, Michigan

[2] Strain obtained from Dr. T.F. Wetzler, Department of Environmental Health, University of Washington, Seattle, Washington

[3] Strains derived in this laboratory from *Y. pseudotuberculosis* PB1 (PB1Cl) or *Y. enterocolitica* 8272 (8272Cl or 8072 (8072Cl)

Discussion

The apparent uptake of gentian violet by bacteria is the sum of at least two processes. Type I uptake, which occurs at 0 °C, is rapid, energy independent and presumably involves non-specific ion binding to the negatively

charged cell surface [13, 14]. Type I binding was similar with EV76 and EV76Cl cells grown at 26 °C or 37 °C and was independent of calcium. This is consistent with earlier results reported for *E. coli* [13] and suggests that EV76 and EV76Cl cell envelopes have similar surface charges. In addition to type I uptake, type II uptake, which represents transport of the dye to the cytoplasm and terminates when cytoplasmic sites are filled [13, 14, 17, 28], occured in *Y. pestis* at higher temperatures (26 °C and 37 °C). The effects of temperature are consistent with temperature induced phase transition in lipid membranes previously reported to occur in *E. coli* [13] and are also consistent with temperature dependent changes which occur in *Y. pestis* outer membrane proteins and LPS [12]. Calcium, which reduces gentian violet uptake in *E. coli* [28], also reduced the uptake of the dye by *Y. pestis*. Type II uptake was greater in cells lacking the 47 Mdal plasmid than in cells bearing this plasmid. This clearly indicated that the permeability to this dye is influenced by the 47 Mdal plasmid.

EV76 cells grown at 26 °C, then transferred to 37 °C in the absence of calcium, had an immediate but slight increase in total dye uptake. However, total dye uptake decreased from three to seven hours post shift. The decrease began at approximately the time these cells cease growth under these conditions [29], suggesting that his reduction is a secondary consequence of earlier events. Since these cells fail to divide but elongate [30], one explanation would be that type I uptake changes because the total surface available for dye binding per optical density unit changes. However, no difference in type I uptake (binding) occurs, the change, therefore, is in type II uptake.

The novobiocin sensitivities of strains EV76 and EV76Cl correlated well with the rates of gentian violet uptake under each condition examined. Novobiocin sensitivities of EV76 and EV76Cl cells were similar at 37 °C in the presence of calcium. Sensitivity in the absence of calcium at 37 °C was not examined since EV76 cells do not continue to proliferate under these conditions. EV76Cl cells grown at 26 °C were more sensitive to novobiocin than EV76 cells and, at 26 °C, calcium increased novobiocin resistance more significantly with EV76 cells than with EV76Cl cells. Optimal novobiocin resistance of EV76 cells occurred at 1.5–5 mM calcium and higher calcium levels were inhibitory to growth in the presence or absence of novobiocin. Release of periplasmic enzymes and lipopolysaccharide occurs at high calcium concentrations in other bacteria [31]. This possibility was not directly examined with *Y. pestis*, but could account for the observed inhibitory effects of high calcium levels. *Y. pestis* strain KIM and a 47 Mdal plasmid lacking, but otherwise isogenic, derivative of strain KIM showed similar correlations of novobiocin resistance with the presence of the plasmid (data not shown). There was no correlation between mutations in the calcium dependence region of the 47 Mdal plasmid and altered sensitivity to novobiocin. This suggests that the site on the 47 Mdal plasmid which influences novobiocin resistance, perhaps by altering per-

meability, is distinct from the calcium dependence region. The 47 Mdal plasmid has been shown to influence the LPS structure of *Y. pestis* [12] but whether this relates to the calcium dependence region or to the site(s) affecting novobiocin resistance is unknown. We are now examining these possibilities.

Since calcium also affects gentian violet uptake by *E. coli* [28] it may be coincidental that the uptake of gentian violet, and presumably novobiocin, are affected by calcium and are influenced by a site on the same plasmid which bears the calcium dependence region. This raises the possibility, however, that more than one region of this plasmid may harbour sites which influence the response to calcium, even though only one such region has been identified [4]. A mutant with a lesion in the site influencing permeability, but with an intact calcium dependence region will be required to test this possibility and to determine if the site influencing permeability also influences virulence. We have been unable to isolate this type of mutant. We continue to seek such a mutant, but it is possible that such a mutant is lethal, or that the site influencing permeability is in a region required for the maintenance or replication of this plasmid.

The 47 Mdal plasmid of *Y. pestis* and corresponding plasmids in *Y. pseudotuberculosis* and *Y. enterocolitica* apparently bear similar functional sites. The concentration of novobiocin optimal for differentiation of cells with and those without these plasmids varies considerably among the strains tested. This suggests, as one might expect, that the genetic background in which the function is expressed also influences the final phenotype.

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A PERMEABILITY MUTANT OF *YERSINIA PESTIS* WITH INCREASED SUSCEPTIBILITY TO PHAGOCYTOSIS WHICH RETAINS POTENTIAL FOR INTRAPHAGOCYTIC GROWTH AND VIRULENCE

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Gentian violet resistance was used to select mutants of *Yersinia pestis* with altered cell envelope permeability. Mutants in one class lacked the 6 and 61 megadalton (Mdal) plasmids, but retained the 47 Mdal plasmid associated with calcium dependence. Mutants in a second class retained all three plasmids. One mutant in the latter class, EV76S7, lacked major outer membrane protein J and yielded reduced levels of minor outer membrane proteins A and C. Protein J is known to interact differently with lipopolysaccharide (LPS) from cells bearing and cells lacking the 47 Mdal plasmid following growth at 37 °C. The 47 Mdal plasmid is known to influence gentian violet uptake, novobiocin sensitivity, susceptibility to phagocytosis, the growth in macrophages and the virulence of *Y. pestis*. Gentian violet uptake was lower and novobiocin sensitivity higher in the mutant than in the parental strain, but the underlying effects of the 47 Mdal plasmid on these parameters had not changed. EV76S7 cells were phagocytized to a greater extent than the parental cells following growth at 37 °C, but survival and growth within peritoneal phagocytes and the LD_{50}'s of cells with and without the 47 Mdal plasmid were not altered by the loss of protein J, we conclude that protein J—LPS interactions are not required for virulence, and that the reduced virulence of cells lacking the 47 Mdal plasmid does not result from the altered protein J—LPS interactions whch are known to result from loss of the plasmid.

The outer membrane of Gram-negative bacteria acts as a permeability barrier. In addition to influencing the uptake of required substrates [1, 2] the outer membrane contributes to resistance to some antibiotics, dyes, and detergents by regulating passage of these molecules [3, 4]. The outer membrane is in direct contact with the host and can influence the rate of phagocytosis and the ability to survive in phagocytic cells [5, 6]. In the latter case it can provide the bacterium with some protection from the lysosomal enzymes [7]. The lipopolysaccharide (LPS) of the outer membrane is toxic to the host [8], but relatively little is known about the role of the outer membrane proteins in pathogenesis.

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Bacteria are known to alter their membrane protein [9, 10] and lipopolysaccharide [11, 12] composition in response to environmental changes, but the manners in which pathogenic bacteria alter the outer membrane in response to the environments provided by the host are, in general, unknown. The incubation temperature of *Yersinia pestis* has been shown to be an important factor in the expression of several determinants of pathogenicity [13], cell invasiveness [6, 14] and nutritional requirements [15, 16]. These properties may be associated with changes in the outer membrane [11, 14, 17–19] and at least one temperature dependent characteristic is influenced by a 47 Mdal plasmid. *Y. pestis* cells which bear this plasmid differ from those which lack this plasmid in terms of LPS composition [11], gentian violet uptake, novobiocin sensitivity [20], susceptibility to phagocytosis following growth at 37 °C, ability to grow in macrophages and LD_{50} in mice [21]. The specific characteristic(s) associated with this plasmid which related directly to growth in macrophages and to virulence has not been established. To test the possibility that the survival in the macrophages relates to specific LPS-protein interactions which might directly after permeability to the lysosomal components and accordingly to the killing by the macrophage [7], we selected mutants from a strain bearing the 47 Mdal plasmid which had altered (decreased) permeability to gentian violet, by using this dye as a selective agent. None of these mutants had permeability changes identical to those associated with loss of the 47 Mdal plasmid. Some mutants had lost the 6 and the 61 Mdal plasmids, whereas others had retained the 6, 47 and 61 Mdal plasmids. One of the latter mutants (EV76S7) lacked the peptidoglycan associated protein J and had reduced levels of outer membrane proteins A and C. Protein J is a major outer membrane in *Y. pestis* which is known to interact differently with the LPS from cells bearing and those lacking the 47 Mdal plasmid following growht at 37 °C in the absence of calcium [11]. To determine if the protein J-LPS interactions enhanced virulence in 47 Mdal plasmid bearing cells or if such interactions reduced the virulence of cells lacking this plasmid, we compared this mutant and a direct derivative of this mutant which lacked the 47 Mdal plasmid to the parental strain (EV76) and to an isolate isogenic to the parent except for a lack of the 47 Mdal plasmid (EV76Cl).

Materials and methods

Bacterial strains and culture conditions. Y. pestis EV76 (Pgm^- Pur^+ Fra^+ Vwa^+ Cal^+), was obtained from Dr. R. R. Brubaker (Michigan State University). EV76S7 was selected from strain EV76 at 37 °C using blood agar base (BBL Microbiology Systems) containing 2.5 mM Ca^{++} and 8 μg/ml gentian violet. EV76Cl and EC76S71 (both of which lack the 47 Mdal plasmid) were selected from strains EV76 and EV76S7, respectively, using magnesium oxalate agar at 37 °C [16]. Cells were grown in an enriched casein hydrolysate medium as described by Darveau et al [18] if they were to be used for cell envelope isolation, or in a complex defined medium [22] if they were to be used for dye uptake, antibiotic sensitivity, or animal studies.

Isolation of the cell walls and peptidoglycan fractions. Frozen cells, wet weight between 3 and 4 g, were resuspended in 20 ml of 10 mM Tris-hydrochloride buffer (pH 8.0) containing 1 mK DL-dithiothreitol and 1 mM EDTA. Pancreatic RNase and DNase were added to the suspension which was then passed three times through a French pressure cell at 15 000 to 20 000 lb/in^2. This and all subsequent steps were carried out at 4 °C. After centrifugation of the lysate at 1300 *g* for 10 min, the pellet of unbroken cells was discarded. The supernatant was centrifuged at 45 000 *g* for 30 min, and the resulting supernatant was discarded and the pellet was washed with 5 ml of the breakage buffer, then with 5 ml of deionized water. If the cell wall preparation was to be used for the two-dimensional analysis, samples were not frozen until they had been placed in IEF sample buffer. If the cell wall preparation was to be used for SDS—PAGE, it was suspended in deionized water and stored at —20 °C. Cell walls prepared by this procedure from EV76 cells yielded electrophoretic protein patterns similar to those obtained from sucrose gradient purified outer membranes [18]. Peptidoglycan was extracted from unwashed cell wall preparations which were resuspended in breakage buffer to a protein concentration of 20 mg/ml as determined by the method of Lowry et al. [23] with the modifications of Herbert et al. [24]. A two-fold concentration of the extraction buffer of Mizuno and Kageyama [25] was added to give a final protein concentration of 10 mg/ml in the normal strength buffer. The suspension was incubated at 35 °C for 30 min then centrifuged at 45 000 *g* for 30 min at 10 °C. The pellet of insoluble peptidoglycan was washed and stored at —20 °C.

Electrophoretic analysis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS—PAGE) was carried out as described by Hui and Hurlbert [26]. Two-dimensional analysis with isoelectric focusing (IEF) in the first dimension and SDS—PAGE in the second dimension was performed using the modifications of earlier procedures [27, 28] described by Darveau et al. [11] except that ampholines (2% total) of the pH ranges of 3.5–5, 5–8, and 9–11, were used at a ratio of 1 : 1 : 2, respectively. Gels (2 mm × 11 cm) were focused at 0.15 watt/tube for 8 h, 60 μg of protein was applied to each gel. The effective pH gradient obtained ranged from 3.8–7.4.

Gentian violet uptake and sensitivity to novobiocin and deoxycholate. The uptake of gentian violet consists of two processes known as type I and type II [29]. Type I represents the binding of the dye to the cell envelope at 0 °C, whereas type II represents the transport of the dye into the cytoplasm at higher temperatures. Both types of uptake were determined as described by Guymon et al. [30]. The total concentration of gentian violet was 10 μg/ml. To determine novobiocin or deoxycholate sensitivity, cells were plated on blood agar base containing different concentrations of novobiocin or deoxycholate (sodium salt, Sigma Chemicals). Colony formation was observed after 5 days incubation at 26 °C. In each case the lowest concentration which completely inhibited growth was considered the minimum inhibitory concentration (MIC).

Animal studies. Outbred Swiss Webster mice, 6–8 weeks old, were obtained from Washington State University mouse colony. All mice were injected intraperitoneally with 0.4% solution of $FeSO_4 \cdot 7H_2O$ in 0.87% NaCl (40 μg/mouse) 30 min prior to intraperitoneal injection of bacteria. Saline suspensions of 10 to 10^8 bacteria were injected intraperitoneally and the numbers of mice dying within 14 days were noted. The LD_{50} was calculated according to Reed and Muench [31]. For quantitation of intraperitoneal bacterial survival and localization saline suspensions of bacteria (10^6 bacteria/0.1 ml saline) were injected into iron compromised mice. The peritoneal cavities were lavaged at the indicated times with 6 ml of cold Dulbecco's phosphate buffered saline (PBS) pH 7.3–7.4 and the peritoneal lavage fluids examined to determine bacterial survival and localization as described by Charnetzky and Shuford [21].

Results

We selected a number of gentian violet resistant mutants of *Y. pestis* strain EV76, all of which had reduced gentian violet uptake. Some of these mutants lacked the 6 and the 61 Mdal plasmids, but retained the 47 Mdal plasmid. Other mutants retained all 3 plasmids and were presumed to have mutations resulting in cell envelope changes. The subject of these studies was one mutant which retained all 3 plasmids (EV76S7).

EV76S7 lacks outer membrane protein J. Two dimensional analysis (IEF X SDS-PAGE) of cell walls from EV76 and EV76S7 cells grown at 37 °C

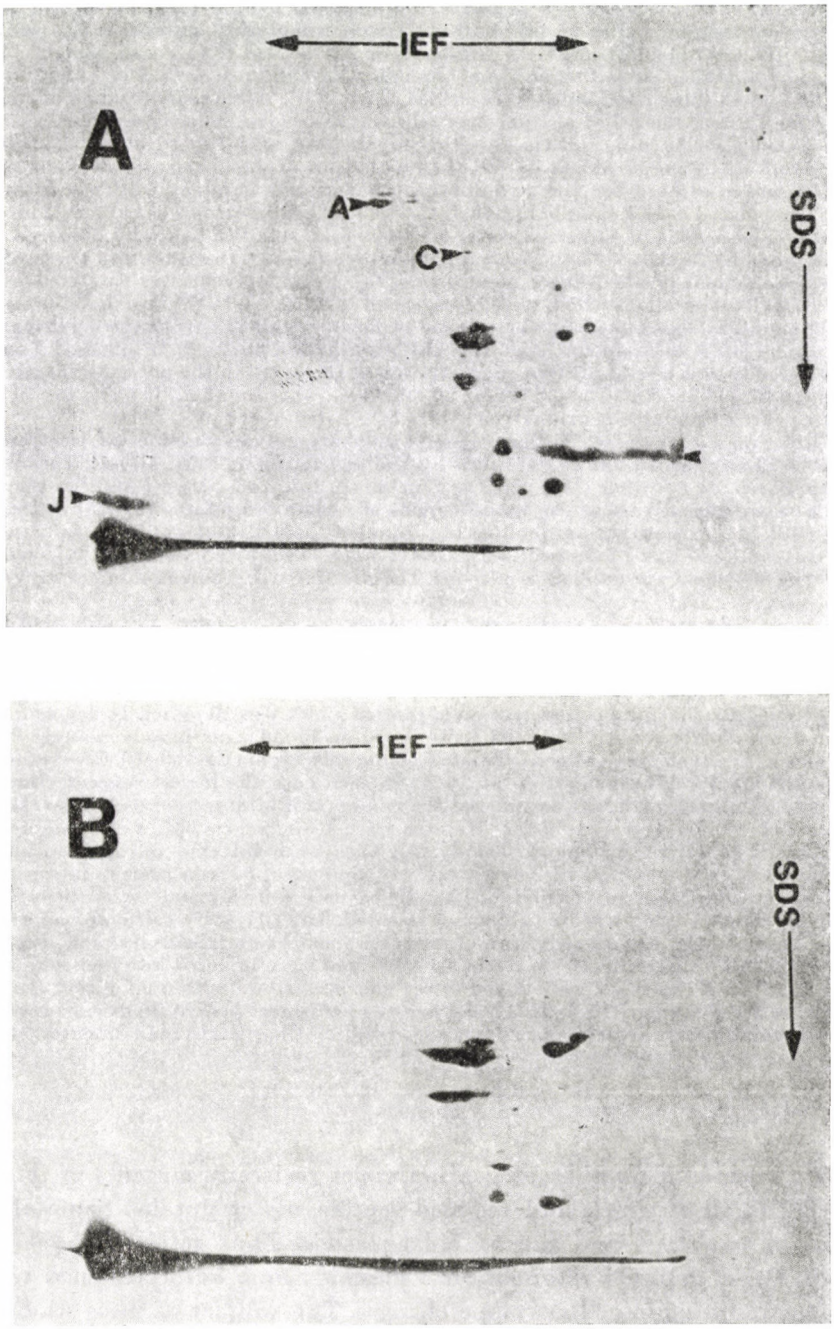

Fig. 1. Two-dimensional analysis of the cell wall proteins of *Y. pestis* strain EV76 (panel/A) and EV76S7 (panel B). The first dimension (horizontal) was isoelectric focusing, the second dimension (vertical) was SDS—PAGE. The arrow to the right of panel A indicates the position of the unheated form of protein J

(Fig. 1) revealed that protein J was absent, and proteins A and C barely detectable in EV76S7. Proteins A and C are synthesized only at 37 °C [18], protein J, which is present at 26 °C and 37 °C was also absent in EV76S7 at 26 °C (data not presented). Protein J. a peptidoglycan associated protein in *Y. pestis*, was not evident in the peptidoglycan fraction of EV76S7 cells grown at 26 °C (data not presented) or 37 °C (Fig. 2). Since protein J is one of the proteins which interacts differently with LPS from cells with and cells without the 47 Mdal plasmid, we isolated a derivative of EV76S7 which lacked the 47 Mdal plasmid as well as protein J (EV76S71). We compared EV76S7 and EV76S71 to strain EV76 and a direct derivative of EV76 which lacks the 47 Mdal plasmid, EV76Cl, to determine the effects of the loss of protein J on the uptake of gentian violet, novobiocin and deoxycholate sensitivities and virulence.

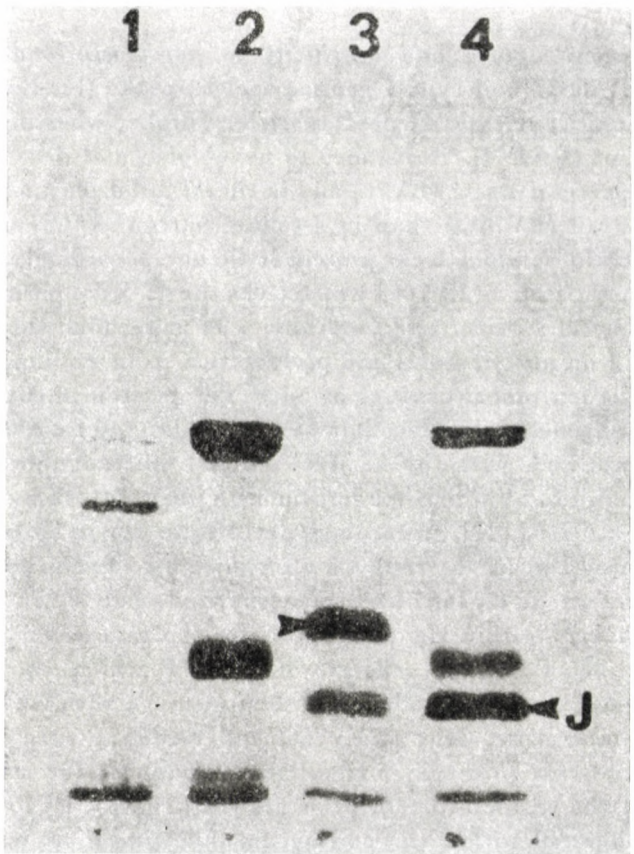

Fig. 2. SDS—PAGE analysis of proteins obtained with the insoluble peptidoglycan fraction from EV76S7 cells (lanes 1 and 2) and EV76 cells (lanes 3 and 4) grown at 37 °C with calcium. Samples were solubilized for 5 min at either 100 °C (lanes 2 and 4) or 24 °C (lanes 1 and 3) in sample buffer prior to analysis. Each lane received 30 μg of protein. The arrow to the left of lane 3 indicates the position of protein J in unheated samples

Table I

Gentian violet uptake, and sensitivity to novobiocin and deoxycholate by strains of Y. pestis

Characteristic	Strain[a]			
	EV76	EV76Cl	EV76S7	EV76S71
Type I gentian violet uptake[b], 26° or 37 °C	16	16	11.7	12
Total gentian violet uptake[b], 26 °C	24.6	30	12.2	12
Total gentian violet uptake[b] 37 °C	25—32	62.5	12.0—14.5	15
Novobiocin (MIC at 26 °C in μg/ml)	250	120	150	90
Deoxycholate (MOC at 26 °C in μg/ml)	4.000	3.600	2.800	2.300

[a]Cells were grown at 26 °C, and assayed for gentian violet uptake and novobiocin and deoxycholate sensitivities as described in Materials and methods

[b] Gentian violet uptake expressed as percent dye removed from solution per optical density unit of bacterial cells

Gentian violet uptake and sensitivity to novobiocin and deoxycholate. EV76S7 and EV76S71 had type I gentian violet uptake (binding). EV76 and EV76Cl had significant type II gentian violet uptake, whereas EV76S7 and EV76S71 did not (Table I). Resistance to novobiocin and deoxycholate were greater in the parental strain (EV76) and in the 47 Mdal plasmid lacking derivative of this strain (EV76Cl) than in J-counterparts (EV76S7 and EV76S71, respectively). EV76S7, which lacks protein J, did not, accordingly, have exactly the same characteristics as EV76Cl which lacks the 47 Mdal plasmid.

Intraperitoneal survival and localization. To determine the effect of the loss of protein J on phagocytosis and proliferation in macrophages, cells were injected into the peritoneal cavities of mice. The peritoneal cavities were lavaged at selected times and intracellular, extracellular and total viable bacteria were enumerated. Cells with the 47 Mdal plasmid, but not those lacking this plasmid, are known to acquire some resistance to phagocytosis by macrophages during growth at 37 °C [21]. Accordingly, cells were grown both at 26 °C and 37 °C prior to use. During the first 3 h after injection of cells grown at 37 °C, a higher percentage of EV76S7 were phagocytized than EV76 and a higher percentage remained within the phagocytes for the remainder of the experiment. The portion of EV76S7 cells grown at 26 °C which were intracellular was slightly higher at each sampling time than was seen with EV76 cells grown at the same temperature. Cells of EV76Cl and EV76S71 were essentially all phagocytized and were primarily intracellular throughout the sampling period independent of the temperature of prior growth (Fig. 3). EV76 and EV76S7 cells both proliferated within the peritoneum following a ca. 6 h lag if they had been grown at 37 °C and both proliferated after a ca. 6 h lag if they had been grown at 26 °C (Fig. 4). Neither EV79S71 cells (Fig. 4) nor EV76Cl cells (not shown) were able to proliferate in the peritoneum following growth at either temperature. Comparison of Figs 3 and 4 indicates that both EV76 and

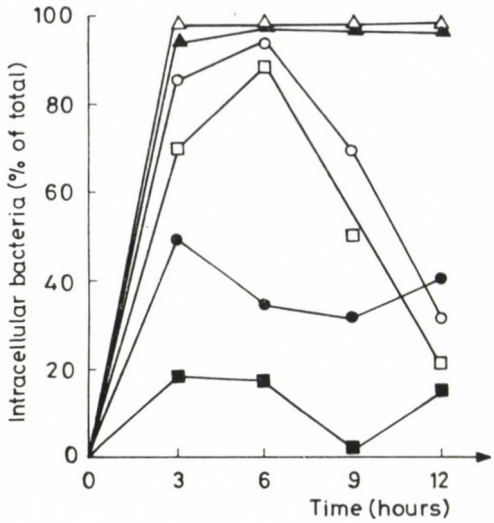

Fig. 3. Location of viable cells of *Y. pestis* strains EV76 (squares), EV76S7 (circles) and EV76S71 (triangles) following injection into the peritoneal cavity of iron compromised mice. Cells were grown at either 26 °C (open symbols) or 36 °C (closed symbols) then washed and resuspended in saline prior to injection. Peritoneal cavities were lavaged and viable intracellular and extracellular bacteria determined. Values for EV76C1 cells (not shown) are identical to those shown for EV76S71 cells

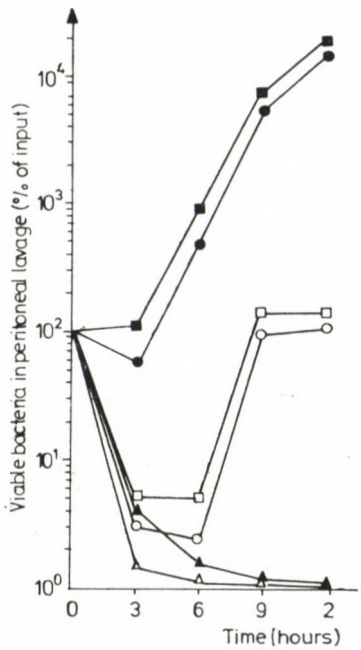

Fig. 4. Growth of *Y. pestis* in the peritoneal cavity of iron compromised swins Webster mice. Cells of strain EV76 (squares) EV76S7 (circles) and EV76S71 (triangles) were grown at either 26 °C (open symbols) or 36 °C (closed symbols), then washed and resuspended in saline prior to injection. Values for EV76C1 (not shown) were indistinguishable from those presented for EV76S71

EV76S7 cells grew within peritoneal phagocytes, whereas the derivative of each strain which lacks the 47 Mdal plasmid, EV76Cl and EV76S71, respectively, did not. We determined the LD_{50}'s of these strains to determine if loss of protein J affected virulence at another point in the infection. Mouse (iron compromised) LD_{50}'s of less than 10 bacteria were found for both EV76 and EV76S7, whereas the LD_{50}'s of their 47 Mdal plasmid lacking derivatives (EV76Cl and EV76S71, respectively) were greater than 10^8.

Discussion

Gentian violet has been used as an indicator of permeability by a number of authors [3, 29, 30, 32, 33]. We used this dye as a selective agent for the isolation of permeability mutants of *Y. pestis*. Isolates in one class of mutants lacked the 6 and the 61 Mdal plasmids, but retained the 47 Mdal plasmid. The 47 Mdal plasmid has been shown to affect the permeability barrier [20], this observation suggests that the 6 and/or 61 Mdal plasmids also influence the barrier function of the cell envelope. The 6 Mdal plasmid has been associated with virulence and with the production of pesticin, coagulase and fibrinolysin [34]; cells lacking this plasmid should be of reduced virulence. The virulence of these isolates could not be tested directly since the parental strain, which is Pgm^-, is virulent only in iron compromised mice and pesticin negative strains are also virulent in this animal model. Isolates in the second class of mutants retained all three of these plasmids. One of the latter mutants (EV76S7) lacked a major peptidoglycan associated outer membrane protein, J, and had reduced levels of minor outer membrane proteins A and C. The LPS of cells bearing and lacking the 47 Mdal plasmid differs at 37 °C and protein J interacts differently with these LPS's following incubation at 37 °C in the absence of Ca^{++} [11]. Gentian violet uptake and novobiocin resistance are influenced by this plasmid [20], but although both characteristics in EV76S7 differed from those of EV76, they were not identical to those of EV76Cl. Accordingly, protein J-LPS interactions cannot be entirely responsible for the changes in these parameters associated with the loss of the 47 Mdal plasmid.

EV76S7 is functionally similar to several classes of Env^- mutants of *E. coli* which lack significant type II gentian violet uptake [29]. Janzer et al. [32] isolated Env^- mutants by selection for growth on hemin, all of which had increased permeability to gentian violet and novobiocin. They concluded that a single mutation altered the barrier against gentian violet and novobiocin. This may be true for one type of mutant which decreases the effectiveness of the outer membrane barrier function, but one can apparently reduce gentian violet uptake, but increase novobiocin (and deoxycholate) sensitivity as seen with EV76S7. Our results do not provide an explanation for this and

it is not easily explainable in terms of current hypotheses for the passage of such hydrophobic compounds through the cell envelope [3]. Sanderson et al. [33] isolated rough mutants of *Salmonella typhi-murium*, and found they were more sensitive to novobiocin and deoxycholate than the smooth parents, indicating the "O" side chains are important in restricting internalization of these compounds. The sensitivity of EV76S7 to novobiocin and deoxycholate might be interpreted as suggesting that EV76S7 is rough. The reduced type I gentian violet uptake (binding) suggests a change in the cell surface and EV76S7 colonies appear somewhat rough when compared to EV76 colonies. *Y. pestis* EV76, however, has been reported to have a rough type LPS [11]. If this is the case, EV76S7 may have a shortened LPS core region. We have not examined this possibility.

We compared the effects of the loss of protein J on other parameters known to be affected by the loss of the plasmid. Phagocytosis of EV76S7 cells was more efficient than of EV76 cells; this probably relates directly to the difference in cell surfaces indicated by the gentian violet (type I) uptake studies. EV76S7 cells remained dependent upon calcium for growth at 37 °C and the calcium independent isolate lacking the 47 Mdal plasmid (EV76S71) was as readily obtained as the corresponding derivative of EV76. EV76S7 cells, like EV76 cells, multiplied within the phagocytes and had low, but comparable, LD_{50}'s. The derivatives which lacked the 47 Mdal plasmid (EV76S71 and EV76Cl) did not proliferate in the phagocytes and had high Ld_{50}'s. We conclude that protein J neither enhances nor reduces the ability to proliferate within phagocytes or the virulence of *Y. pestis* in iron compromised mice. The 47 Mdal plasmid is known to affect the cell envelope characteristics and the virulence of *Y. pestis*. These may be related, but it is clear that the specific permeability changes seen in EV76S7 and the specific protein J-LPS interactions are not the important factors in virulence.

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THE CALCIUM DEPENDENCE REGION OF THE 47 MEGADALTON PLASMID OF *YERSINIA PESTIS* IS REQUIRED FOR GROWTH WITHIN MACROPHAGES

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Yersinia pestis cells bearing the 6,47 and 61 megadalton (Mdal) plasmids have been shown previously to be more resistant to novobiocin and to take up less gentian violet than cells lacking the 47 Mdal plasmid. Mutants with lesions in the calcium dependence region of the 47 Mdal plasmid, which are known to have resistance to high levels of novobiocin, were found to have rates of gentian violet and deoxycholate sensitivities identical to cells with intact plasmids. Cells bearing the 6, 47 and 61 Mdal plasmids, but not cells without the 47 Mdal plasmid, were known to grow within peritoneal macrophages. Cells with mutations in the calcium dependence region of the 47 Mdal plasmid had greatly reduced virulence in iron compromised mice and were unable to proliferate in the peritoneal macrophages of these mice. Cells lacking both the 6 and 61 Mdal plasmids, selected in the presence of gentian violet, were found to have reduced gentian violet uptake but unexpectedly had increased sensitivity to novobiocin and deoxycholate. These cells had an LD_{50} in iron compromised mice similar to that of cells with all three plasmids and proliferated in the peritoneal macrophages of iron compromised mice.

Virulent isolates of *Yersinia pestis* require Ca^{++} for prolonged growth at 37 °C [1, 2] and upon culturing on a medium lacking Ca^{++} yield avirulent, calcium independent mutants [3]. Burrows et al. [4] noted that aeration at 37 °C in highly enriched medium favours the production of V and W antigens by the calcium dependent, but not calcium independent strains. Calcium independent isolates which still produce the V and W antigens, however, have been isolated [5]. Other virulence determinants, including the fraction 1 antigen, pesticin I, pigmentation, and the ability to synthesize purines de novo are independent of calcium dependence [6]. Calcium dependence correlates with the presence of a 47 megadalton (Mdal) plasmid [7, 8] and more specifically with a limited, but not precisely defined, region of this plasmid [9]. The 47 Mdal plasmid has been associated with the ability to grow within mouse peritoneal macrophages [10. 11], with a change in the LPS of cells grown at 37 °C [12], and with increased resistance to novobiocin and reduced rates of gentian violet uptake [13].

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Novobiocin resistance has been correlated to a region of the 47 Mdal plasmid separate from that related to calcium dependence [13]. It was not clear, however, which region of the plasmid was involved in growth within macrophages or which influenced the rate of gentian violet uptake. In this study we examined gentian violet uptake by, and novobiocin and deoxycholate sensitivity of, strains having mutations in or encompassing the calcium dependence region of the 47 Mdal plasmid, and a strain bearing only the 47 Mdal plasmid. We also examined the LD_{50}'s of these strains and their growth within peritoneal phagocytes in vivo. These studies were designed to determine if permeability to gentian violet and sensitivity to deoxycholate related to the site influencing novobiocin resistance or to the site influencing calcium dependence and to determine what influence, if any, a mutation in the calcium dependence region has on the LD_{50}, the temperature dependent resistance to phagocytosis and the ability to grow within the peritoneal phagocytes.

Materials and methods

Bacterial strains and culture conditions. The strains used are listed in Table I. Strain EV76S10 was obtained from strain EV76 using blood agar base (BBL Microbiology Systems, Cockeysville, MD) plates containing 14 μg gentian violet per ml as previously described [11]. Stock cultures were maintained in 40% glycerol — 60% phosphate buffer (0.1 M, pH 7.0) at —20 °C. Cells were subcultured on blood agar base plates at 26 °C then transferred to a defined medium [14, 15] and grown as described previously [13].

Gentian violet uptake, and senstivity to novobiocin and deoxycholate. The initial (Type I) and total (Type II) uptake of gentian violet were determined at 26 ° or 37 °C as described by Guymon et al. [6] using 10 μg/ml of gentian violet. Novobiocin and deoxycholate sensitivities were determined using blood agar base plates containing different concentrations of novobiocin or deoxycholate (sodium salt, Sigma Chemical Company) as previously described [13]. The colonies were scored after 5 days incubation at 26 °C.

Animal studies. Outbred Swiss Webster mice (6–8 weeks old) obtained from Washington State University mouse colony were injected intrapritoneally with 40 μg iron 30 min prior to intraperitoneal injection of bacteria. The experiment was done 3 times and a total of 7 mice were injected per each bacterial dilution. The numbers of dead mice were scored after 14 days and the LD_{50}'s calculated according to Reed and Muench [16]. Saline suspensions of bacteria (10^6 bacteria/0.1 ml) were injected into iron compromised mice and peritoneal lavage fluids taken at selected times after infection were used to determine the numbers of intracellular and extracellular bacteria as described previously [10].

Results

Gentian violet uptake, and sensitivity to novobiocin and deoxycholate. Strains of *Y. pestis* with different plasmid contents were compared for sensitivity to novobiocin and deoxycholate and for the uptake of gentian violet (Table II). Cells with mutations in or encompassing the calcium dependence region were idential to one another and to cells bearing an intact 47 Mdal plasmid, in their interactions with these agents. Cells bearing the 47 Mdal, but not the 6 or 61 Mdal plasmids (EV76S10), bound and took up less gentian

violet, but were more sensitive to novobiocin and deoxycholate than cells bearing the 6, 47 and 61 Mdal plasmids. This suggests that a site or sites on the 6 or 61 Mdal plasmids, as well as a site on the 47 Mdal plasmid, influence permeability.

Table I

Y. pestis strains used

Strain	Ca^{++} Dependence	Plasmid size (Mdal)
EV76S10[a]	+	47
EV7612[b]	—	61,48.5[c],6
EV7618[b]	—	61,16[d],6
EV7651[b]	—	61,48.5[c],6

[a] Strain derived in this laboratory from EV76

[b] Strains obtained from D. A. Portnoy, Department of Medical Microbiology, Stanford University, Stanford, CA 94304

[c] The 47 Mdal plasmid modified by a 2.2-Kb insertion into one of two adjacent fragments, Bam-5 (EV7612) and Bam-8 (EV7651), of the calcium dependence region

[d] The 47 Mdal plasmid modified by an extensive deletion encompassing the calcium dependence region

Table II

Gentian violet uptake, sensitivity to novobiocin and deoxycholate, and LD_{50} of strains of Y. pestis with different plasmid contents

Characteristic	Strain[a]			
	EV76S10	EV7612	EV7618	EV7651
Type I gentian violet uptake[b], 26 ° or 37 °C	11.5	15.9	16	15.6
Total gentian violet uptake[b]: 26 °C	11.8	24.8	24.8	24.5
Total gentian violet uptake[b]; 37 °C	11.6–13.8	31.9	31.9	31.5
Novobiocin (MIC at 26 °C in μg/ml)	140	250	250	250
Deoxycholate (MIC at 26 °C in μg/ml)	2.700	4.000	4.000	4.000
LD_{50}	10^2	$> 10^8$	$> 10^8$	$> 10^8$

[a] Cells were grown at 26 °C or 37 °C and assayed for gentian violet uptake and novobiocin and deoxycholate sensitivities, and LD_{50} as described in Materials and methods

[b] Gentian violet uptake expressed as percent dye taken per optical density unit of bacterial cells

LD_{50}. To determine whether the calcium dependence region of the 47 Mdal plasmid had a role in virulence, we determined the LD_{50} of the strains listed in Table I. Strains having an insertion (EV7612, and EV7651) or deletion in or encompassing (EV7618) the calcium dependence region of the 47 Mdal plasmid had LD_{50}'s of more than 10^8 bacteria and in this regard were similar to EV76Cl [11] and KIM1 [10], both of which lack the 47 Mdal plasmid. Strain EV76S10 had an LD_{50} of 10^2, which is close to that reported for EV76 [11] and KIM [10] which bear the 6, 47 and 61 Mdal plasmids. This data clearly indicated that the calcium dependence region of the 47 Mdal plasmid influences virulence.

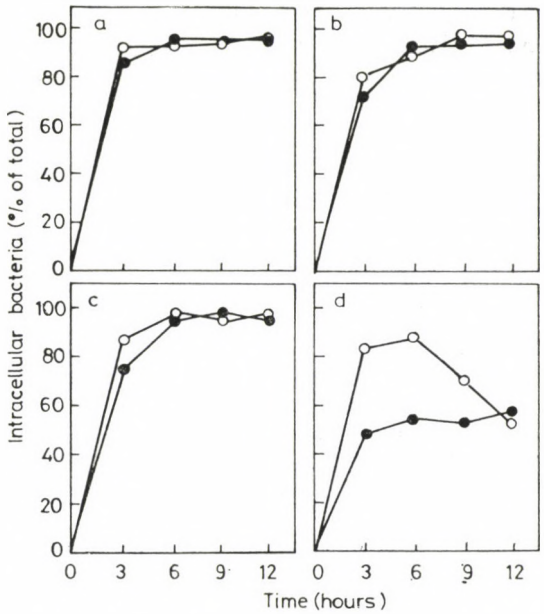

Fig. 1. Phagocytosis of *Y. pestis* strains having different plasmid contents by the peritoneal phagocytic cells of iron compromised swiss Webster mice. Closed symbols represent cells grown at 37 °C. Open symbols represent cells grown at 26 °C. The strains are (a) EV7612, (b) EV7618, (c) EV7651, (d) EV76S10

Peritoneal survival and localization. EV76 cells, but not EV76Cl cells, develop a degree of resistance to phagocytosis during growth at 37 °C [11]. EV7612, EV7618, and EV7651 cells were identical to EV76Cl, in that most were taken up by the phagocytic cells by 3 h post infection and remained intracellular throughout the experiment. As with EV76Cl, these events were independent of the temperature at which they had been grown prior to infection (Fig. 1a, b, and c, respectively). The susceptibility of EV76S10 to phagocytosis was reduced in cells grown at 37 °C (Fig. ad). In this regard EV76S10 was similar to EV76 [11].

Comparing the intraperitoneal survival and growth of these strains, we found that EV7612, EV7618, and EV7651 were identical to each other and similar to EV76Cl [11] and KIM1 [10]. The majority of these cells were killed following phagocytosis and the surviving cells did not increase in number during the course of experiment (Fig. 2a, b and c). As previously reported, EV76S10 cells, like EV76 cells [11], remained primarily extracellular if they had been grown at 37 °C and increased in number beginning 3 h post injection, whereas the majority of these cells grown at 26 °C were phagocytized and killed initially and the surviving cells increased in number by 6 h post injection (Fig. 2d).

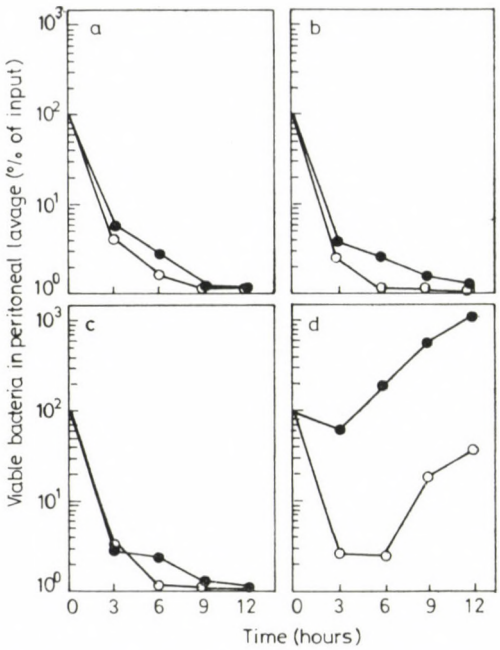

Fig. 2. Growth of *Y. pestis* strains having different plasmid contents in the peritoneal cavity of iron compromised Swiss Webster mice. Closed symbols represent cells grown at 37 °C. Open symbols represent cells grown at 26 °C. The strains are: (a) EV7612, (b) EV7618, (c) EV7651, (d) EV76S10

Discussion

Gential violet, novobiocin and deoxycholate are hydrophobic compounds [17–19] having their targets inside the outer membrane [18]. Thus it is reasonable to use them as an indicators of the permeability changes of the cell envelope. They have similar patterns of effects on many [18] but not all [11], bacteria. EV76S10, like EV76S7 which was also selected in the presence of gentian violet [11], is unusual in that mutation, or mutations, which reduce its permeability to gentian violet, allow it to remain permeable to novobiocin and deoxycholate. In this respect it differs from both strains bearing and strains lacking the 47 Mdal plasmid. This suggests that a 6 or 61 Mdal plasmid encoded factor also influences permeability. Consistent with this possibility, cells lacking the 6 Mdal plasmid have reduced potential for palmitic acid uptake [20]. Those cells with mutations in or encompassing the calcium dependence region of the 47 Mdal plasmid were identical to cells bearing an intact 47 Mdal plasmid [11] in their interaction with the three agents listed. This provides additional evidence that a site on the 47 Mdal plasmid other than that for calcium dependence influences the permeability of *Y. pestis*.

Strains lacking the 6 Mdal plasmid, like Pgm$^-$ mutants, should be virulent in iron compromised mice [8], but it is not known if any 61 Mdal plasmid encoded factor is involved in virulence. EV76S10, which lacks both the 6 and 61 Mdal plasmids, has an LD_{50} in iron compromised mice close to those reported for strains which possess these plasmids [10, 11] and like these strains proliferates within the peritoneal macrophages. If a 61 Mdal plasmid encoded factor is involved in virulence, then this factor, like any 6 Mdal plasmid encoded factor, is not required for pathogenicity or growth within macrophages in this system.

More than one virulence determinant might be carried on a given plasmid and a mutation could alter the phenotype without influencing virulence. The observation that the 47 Mdal plasmid contains a region separate from the calcium dependence region which alters cell permeability [13] raised the possibility that this region, rather than the region involved in calcium dependence, could be enhancing survival in macrophages, and hence virulence, by reducing permeability to macrophage products. If this were the case, the calcium dependence region might not encode for a factor involved in the pathogenicity of this organism. Cells with unaltered permeability, but with mutations in the calcium dependence region of the 47 Mdal plasmid had LD_{50}'s identical to those previously reported for cells of which lack the 47 Mdal plasmid [10, 11] and were unable to proliferate in the macrophages, demonstrating that the calcium dependence region of this plasmid is required for virulence and for growth in the peritoneal macrophage. We could not test the possibility that the region influencing permeability is involved in susceptibility to phagocytosis, the ability to grow in macrophages, or in some other manner influences the virulence of *Y. pestis*. Mutants with an intact calcium dependence region, but with an alteration in this site, are required and no such mutant is available at this time. Some of our mutants have altered permeability, but none are identical in this regard to mutants lacking the 47 Mdal plasmid.

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THE EFFECTS OF CANNABISPIRO COMPOUNDS AND TETRAHYDROCANNABIDIOLIC ACID ON THE PLASMID TRANSFER AND MAINTENANCE IN *ESCHERICHIA COLI*

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Some cannabispiro compounds and tetrahydrocannabidiolic acid were tested for antibacterial plasmid curing activity and inhibition of plasmid transfer. MIC values of the compound were above 1500 μg/ml. Cannabispirol and tetrahydrocannabidiolic acid eliminated the F'lac plasmid from *Escherichia coli*, but acetylcannabispirol, cannabispirone and cannabispirenone were ineffective as curing agents. Each compound, except acetyl-cannabispirol, selectively killed plasmid carrying bacteria. The compounds inhibited R144 plasmid transfer from *E. coli* into *E. coli* cells via inhibition of mating pair formation, zygotic killing and inhibition of transconjugal DNA synthesis in a lesser extent. All of the cannabispiro compounds and tetrahydrocannabidiolic acid inhibited the transformation with pBR322 plasmid DNA when the bacteria were pretreated with the compounds, via inhibition of the DNA penetration or decreasing the synthesis of plasmid DNA during bacterial growth. Although each of the compounds, except acetyl-cannabispirol, had a weak antibacterial effect which was more definite on plasmid carrying bacteria than plasmidless ones, and inhibited intercellular plasmid transfer and transforming activity of plasmid DNA, only two of them were able to cure F'lac plasmid showing that plasmid elimination is a complex process which strictly depends on the stereochemical configuration of curing agents.

The antimicrobial activity of compounds isolated from *Cannabis sativa* has been known for many years [1, 2]. Some tricyclic psychopharmacons, phenothiazones, dibenzoazepines and dibenzocyclopentenes also exert antibacterial effects [3–5]. Besides antibacterial effects, such derivatives inhibit bacterial plasmid replication [4–6].

Amongst the cannabinoids, Δ^8-THC, Δ^9-THC, cannabinol, cannabidiol, cannabidiolic acid and tetrahydrocannabidiolic acid have been tested for inhibition of plasmid replication but only cannabidiolic acid displays moderate effect, while tetrahydrocannabidiolic acid eliminates the F'lac plasmid of

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Escherichia coli at a fairly high frequency [6]. The results were interesting because the most effective cannabinoid was a pure sample of tetrahydrocannabidiolic acid. This compound contains only one aromatic ring and it is striking that it has no cationic side-chain, which has been assumed to be important for the plasmid curing effects of other tricyclic psychopharmacons [7].

Since we were interested in the relationship between the chemical structure of the cannabinoids and their inhibitory effects on plasmid replication, it seemed to be worthwile to analyse tetrahydrocannabidiolic acid for effective impurities and to compare the effects with those of some new derivatives.

In this report we describe results of studies on the chemical structure-dependent antibacterial and plasmid curing effects of some newly discovered cannabispiro compounds, cannabispirenone, cannabispirol, and acetylcannabispirol [8], together with tetrahydrocannabidiolic acid in model experiments.

Materials and methods

Compounds. Cannabispirol, acetylcannabispirol, cannabispirone and cannabispirenone were prepared by the method of Shoyama and Nishioka [8]. Cannabidiolic acid was provided by Professor K. Watanabe, Narcotics Laboratory Section, United Nations Laboratories, Vienna, Austria. Tetrahydrocannabidiolic acid was produced by Alkaloida, Tiszavasvári, Hungary. The chemical structure of the compounds are shown in Fig. 1.

Bacterial strains. Escherichia coli K12 LE140 F'lac, *E. coli* K12 HB101 or *E. coli* C600 R144 drd-3-as donor, and *E. coli* K12 W1 azir as recipient in plasmid transfer experiments.

Culture media. MTY broth and MTY agar were prepared according to Alföldi et al. [9]. Eosin methylene blue agar was used for the detection of lac^- colonies resulting from curing of the F'lac plasmid.

Elimination of F'lac plasmid. The procedure was carried out as described by Mándi et al. [10]. In these experiments the effects of various cannabispiro compounds and tetrahydrocannabidiolic acid were tested. The applied concentrations were 0–1500 μg/ml.

Gas chromatographic and spectrometric analysis of tetrahydrocannabidiolic acid. The field-desorption mass spectrum (FD—MS) was taken with a JEOL JMS DX-300 instrument using a direct inlet system. The proton magnetic resonance (^{1}H-NMR) spectrum was recorded on a JEOL FXX-100 spectrometer with tetramethyl- silane as internal standard. The gas chromatographic and mass spectrometric (GC—MS) analysis was conducted under the following conditions. GC conditions: Shimadzu GC—MS 7000 instrument, column, 1.5% OV-17 on Shimalite W (80–100 mesh), 1 m × 5 3 mm glass column; carrier, He 30 ml/min; column temperature, 230 °C; injection temperature, 250 °C. MS conditions: accelerating voltage, 3 kV; ionizing current, 60 μA, ionizing energy, initial 20 eV, jump 70 eV; separator temperature 250 °C Tetrahydrocannabidiolic acid methyl ester was prepared as described by Shoyama et al. [11].

Inhibition of R-plasmid transfer. For mating experiments, *E. coli* K12 C600 was used as donor and *E. coli* K12 W1 azir served as recipient. Cultures of these two strains were prepared in MTY broth incubated at 37 °C for 18 h. From these cultures 100-fold dilutions were prepared in MTY broth and incubated at 37 °C for 6 h until an absorbance of 0.4 was reached at 620 nm. Donor and recipient cultures were diluted 10-fold in MTY broth, and equal volumes were mixed and incubated without shaking in the presence of different concentrations of the cannabis preparations. These experiments were designed to study the activities of the cannabinoids on conjugation.

In the experiments we added the cannabinoids to the donors 10 min prior to mating, in order to determine the effects of the drugs on the formation of mating pairs. In other experiments the cannabinoids were added 10 min after the addition of donors to the recipients. This was done to determine the effects of these agents after mating pairs had been formed. All tubes were incubated at 37 °C for 120 min.

Following the incubation, samples were diluted in 0.98% saline and 0.1 ml dilutions were plated onto selective media. MTY agar containing 50 μg kanamycin and 600 μg sodium azide per ml was used for the determination of transconjugants. Neither donor cells nor recipient cells had the ability to grow on this selective medium, whereas, the sodium azide-resistant recipient which acquired the kanamycin-resistance plasmid from the donor would grow on this selective medium. Thus, the effects of the cannabis agent upon this R-plasmid transfer could be compared with those of the controls and quantified. All plates were incubated at 37 °C for 48 h and the colonies were counted. From these data, the relative inhibition of plasmid transfer by the cannabis drugs was calculated.

Transformation with pHC624 plasmid DNA in the presence of various tricyclic compounds. *E. coli* HB101 strain was grown in MTE medium at 37 °C until the spectrophotometric absorbance at 620 nm reached 0.5. Fifty ml of the culture were centrifuged at 4000 rpm for 15 min. The sediment was washed in 0.1 M $MgCl_2$, then resuspended in 3.0 ml 0.1 M $CaCl_2$ and the suspension was distributed into 200 μl aliquots. The cells were pretreated with 100 μg cannabispirol, acetylcannabispirol, cannabispirone, cannabispirenone, cannabidiolic acid or tetrahydrocannabidiolic acid for 5 min at 37 °C, and 1 μg pHC624 (which is a pBR 322 plasmid derivative carrying ampicillin resistance) plasmid DNA was then added to each sample. The samples were incubated at 4 °C for 60 min, heat shock was then applied at 42 °C for 2 min, and a further 60 min incubation was carried out at 37 °C. The samples were centrifuged at 4000 rpm for 15 min and the pellet was then resuspended in 200 μl MTE medium. From the different dilutions of the sample 50–100 μl aliquots were plated onto 100 μg/ml ampicillin-containing MTE agar. The plates were incubated at 37 °C for 24 h and the numbers of ampicillin-resistant transformants were then counted and recorded.

Results

The minimal inhibitory concentrations (MIC) of cannabispirol, acetylcannabispirol, cannabispirenone and cannabispirone were first studied. It can be seen from the number of viable cells grown in the presence of these compounds that all of them have only a weak antibacterial effect and the MIC values were found above 1500 μg/ml, however, they could not be determined exactly because of their precipitation above these concentrations (Table I). The cannabispiro compounds and tetrahydrocannabidiolic acid did not cause plasmid elimination, if stock solutions were prepared in Tween 80.

To check the effect of the solvent on the biological effects of the compounds, the previously used ethanol-DMSO-water solvent was applied. The results obtained with this solvent were better, since only one of the four compounds tested proved effective in plasmid elimination. Tetrahydrocannabidiolic acid, which served as control, was also active under the same conditions. Surprisingly, cannabispirol exerted plasmid curing activity but the other compounds apart from the tetrahydrocannabidiolic acid were ineffective. The cannabispirol and tetrahydrocannabidiolic acid preferentially killed plasmid carrying bacteria but plasmidless cells were less sensitive. Acetylcannabispirol, cannabispirone or cannabispirenone were unsuitable to distinguish between the plasmid containing and plasmidless cells (Table II).

Cannabispirol and tetrahydrocannabidiolic acid only had curing effect which decreased the MIC values of gentamicin. The minimal inhibitory concentration of gentamicin was 1.2 μg/ml in MTY which decreased to 0.9 μg/ml in the presence of 200 μg/ml cannabispirol or tetrahydrocannabidiolic acid.

Table I

Elimination pf F'lac plasmid of E. coli in the presence of cannabispirol, acetylcannabispirol, cannabispirone, cannabispirenone or tetrahydrocannabidiolic acid

Compounds μg/ml	Plasmid elimination %	No. of viable cells × 10	MIC μg/ml
Cannabispirol			
0	0	29.2	
100	0	27.0	
200	0.2	22.5	
400	0.01	6.3	
800	63.0	0.34	
1000			
1500			1500 <
Acetylcannabispirol			
0	0	54.2	
100	0	40.0	
200	0	45.0	
400	0	45.1	
800	0.1	42.4	
1000			
1500			1500 <
Cannabispirone			
0	0	43.0	
100	0	40.5	
200	0.1	41.4	
400	0.0	19.6	
800	0.0	15.9	
1000			
1500			1500 <
Cannabispirenone			
0	0	48.4	
100	0	41.0	
200	0.15	32.2	
400	0.0	31.2	
800	0.0	32.0	
1000	0.0	30.0	
1500	0.0	21.5	1500 <
Tetrahydrocannabidiolic acid			
0	0	38.6	
100	0	32.0	
200	4.2	27.5	
400	23.0	25.6	
800	0.5	20.5	
1000	0	14.2	
1500			1500 <

Ten mg of the compound were dissolved in 0.2 ml ethanol + 0.3 ml DMSO + 0.5 ml distilled water

All the compounds except tetrahydrocannabidiolic acid were chromatographically pure. The impurities and the chemical composition of this biologically effective compound were determined as follows. The FD-MS of this compound showed molecular ions at m/z 362 (100%) and m/z 360 (13%),

Table II

Sensitivity of plasmid carrying and plasmidless derivatives of E.coli K12 LE 140 F'lac to some cannabispiro compounds and tetrahydrocannabidiolic acid

Compounds	μg/ml	No of viable cells × 10-	
		F'lac	lac⁻
Cannabispirol	0	26	20
	400	12	22
	1000	0.2	5.0
Acetylcannabispirol	0	24	18
	400	20	20
	1000	23	19
Cannabispirone	0	21	24
	400	22	20
	1000	18	15
Cannabispirenone	0	24	22
	400	22	24
	1000	24	20
Tetrahydorcannabidiolic acid	0	22	20
	400	11	16
	1000	3	9

The plasmid carrying (F'lac) and plasmidless (lac⁻) strains were grown in MTY broth overnight, the cultures were centrifuged and washed in 0.98% sodium chloride and resuspended when the number of colony formers were 2.0×10^8/ml. The bacterial suspensions were distributed into 1.0 ml aliquots and the tested compounds were given to them at 0, 400 and 1000 μg/ml final concentrations. The samples were incubated at 37 °C for 120 min then the numbers of colony formers were determined of each sample on EMB agar plates

Table III

Effects of cannabispiro compounds on R144 plasmid transfer after pretreatment of the donor E. coli strain prior to mating with E. coli K12 W1 azir as recipient and treatment of preformed mating pairs

Compounds	Treatment	No. of transconjugants × 10^4	No. of donors × 10^8
Cannabispirol	donor pretreatment	0.18	104
	pair treatment	0.22	135
Acetylcannabispirol	donor pretreatment	0.34	102
	pair treatment	0.38	128
Cannabispirone	donor pretreatment	0.23	84
	pair treatment	0.29	112
Cannabispirenone	donor pretreatment	0.19	127
	pair treatment	0.29	82
Tetrahydrocannabidolic acid	donor pretreatment	0.06	96
	pair treatment	0.069	120
Control		0.93	116

Each of the compounds was used at 100 μg/ml final concentration (10 mg of the compound was dissolved in 0.2 ml ethanol + 0.3 ml DMSO + 0.5 ml distilled water).

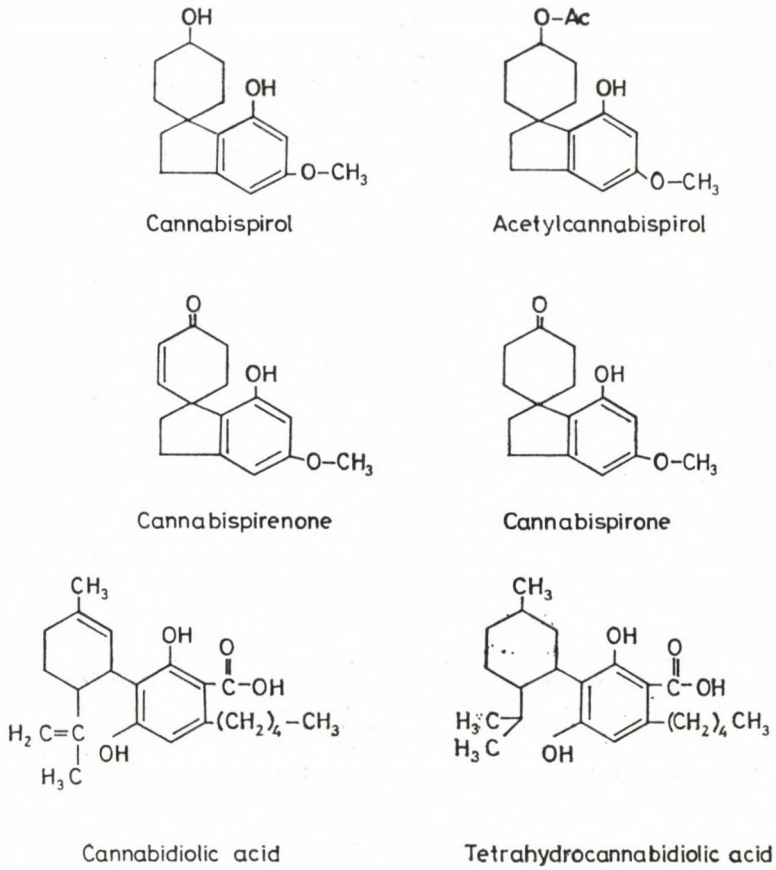

Fig. 1. Chemical structures of some cannabispiro compounds and cannabinoids

suggesting that it might be a mixture of tetrahydrocannabidiolic acid and dihydrocannabidiolic acid. Treatment with diazomethane in ether afforded the corresponding methyl ester. The MS spectrum (during GC-MS analysis) depicted in Fig. 2 was in good agreement with that of tetrahydrocannabidiolic acid methyl ester. However, the ^{1}H-NMR spectrum showed that this methyl ester was a mixture of three compounds, with three aromatic proton signals and three chelated hydroxyl proton signals.

Mechoulam and Gaoni [12] demonstrated that the Δ^8 double bond in cannabidiol was hydrogenated in preference to the Δ^1 double bond, to give dihydrocannabidiol, and further hydrogenation occurred predominantly to the tetrahydrocannabidiol in which the methyl group at C_1 is equatorial. On the basis of the above evidence, it is speculated that the major compound in the mixture may be the tetrahydrocannabidiolic acid having an equatorial methyl group at C_1 while the minor component is the isomer having an axial

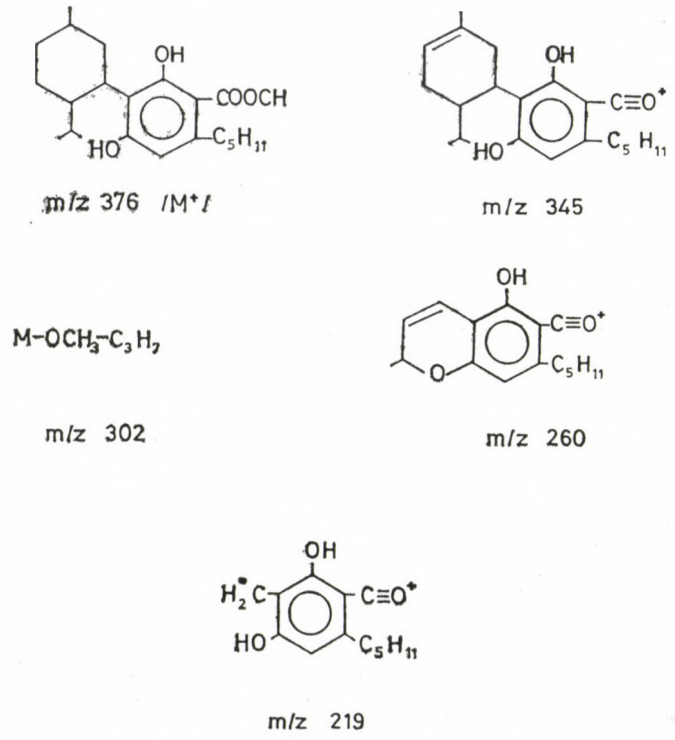

Fig. 2. MS fragmentation pattern of tetrahydrocannabidiolic acid methyl ester or GC—MS

methyl group and the trace component may be dihydrocannabidiolic acid, as shown in Fig. 3.

The effects of the three different derivatives were measured simultaneously because we could not separate any of them from the mixture. However, this question is of interest as concerns the relation of structure and activity. The individual components should be separated and investigated in the near future.

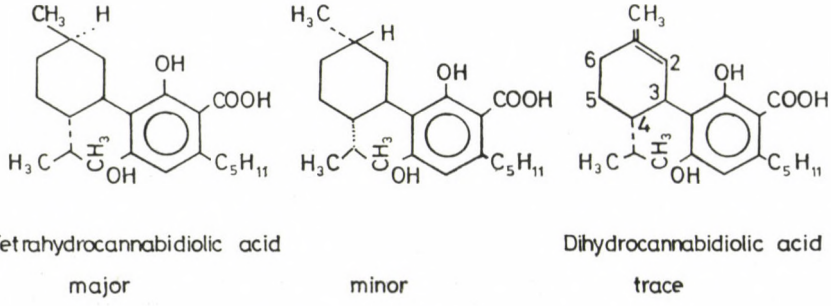

Fig. 3. Probable components of tetrahydrocannabidiolic acid mixture

Table III shows that pretreatment of the donor cells with the compounds inhibited plasmid transfer. Plasmid transfer was also inhibited in the preformed mating pairs partly because of the sensitivity of donor cells and zygotic killing and partly via inhibition itself transconjugal DNA synthesis, while the number of recipient cells did not change during the experiment (Table IV). The results show that the inhibition of pair formation also had some importance in the

Table IV

The viability of mating pairs, donors and recipient cells in the presence of some cannabispirol compounds and tetrahydrocannabidiolic acid on E. coli conjugation system

Compounds μg/ml		No. of transconjugants $\times 10^7$	Donors $\times 10^7$	Recipients $\times 10^7$
Cannabispirol	100	3.9	12.9	28.4
Acetylcannabispirol	100	7.8	26.0	29.1
Cannabispirone	100	4.2	14.5	30.7
Cannabispirenone	100	4.8	18.2	33.4
Tetrahydrocannabidiolic acid	100	2.6	16.0	30
Control	—	8.0	25.0	32

The transconjugant, donor and recipient cells were grown in MTY broth for 8 h, then the cultures were distributed into 2.0 ml aliquots and the tested compounds were given to them at the same concentration which was used for inhibition of plasmid transfer (100 μg/ml); the samples were incubated at 37 °C for 120 min, the number of colony formers was determined on MTY agar plates.

Table V

Transforming capacity of pHC624 plasmid DNA in the presence of cannabispirol, acetylcannabispirol cannabispirenone, cannabispirone, cannabidiolic acid or tetrahydrocannabidiolic acid

Cpouomnds	No. of transformed bacterial cells $\times 10^2$	
	in the presence of compounds	after DNA pretreatment $\times 10^2$
Control	43.5	44.8
Cannabispirol	3.3	40.5
Acetylcannabispirol	6.2	45.0
Cannabispirenone	22.4	42.2
Cannabispirone	28.4	45.6
Cannabidiolic acid	4.5	39.4
Tetrahydrocannabidiolic acid	2.8	40.6

Ten mg of the compound were dissolved in 0.2 ml ethanol + 0.3 ml DMSO + 0.5 ml distilled water. Each compound was used at 100 μg per 200 μl sample, which contained 2×10^9 bacterial cells. One μg of pHC624 DNA was treated with 100 μg of cannabispiro compounds at 4 °C for 5 min, then DNA was ethanol precipitated and centrifuged. The pellet was dissolved in 50 μl 0.01 M $MgCl_2$ than the transformation was carried out as described in the methods

lowering of intercellular plasmid transfer. There were no large differences between the effects of the tested cannabispirol compounds and tetrahydrocannabidiolic acid.

In other experiments the transforming capacity of pHC624 plasmid DNA was tested on *E. coli* cells which had been pretreated with cannabispiro compounds or tetrahydrocannabidiolic acid. The inhibition of the transforming activity of plasmid DNA was more specific in revealing differences between the effects of the various cannabispiro derivatives and tetrahydrocannabidiolic acid (Table V). Tetrahydrocannabidiolic acid and cannabispirol inhibited this process more effectively than did cannabispirone and cannabispirenone. Acetylcannabispirol was an exception in this respect since it moderately inhibited the transformation, but was ineffective in curing experiments. This compound may possibly be more easily metabolized by bacteria than the others, which could be the reason for its being ineffective in plasmid elimination. Pretreatment of transforming plasmid DNA with the cannabispiro compounds or tetrahydrocannabidiolic acid did not decrease the transforming activity of plasmid DNA itself, indicating that the compounds are not able to make complexes with plasmid DNA, but rather they inhibit the synthesis of new DNA during the multiplication process or inhibit the penetration of plasmid DNA into bacteria.

Discussion

Among the cannabinoids, cannabidiolic acid and tetrahydrocannabidiolic acid are effective in the inhibition of plasmid replication [6]. Several cannabispiro compounds have been discovered recently [8] and we have tested them for antibacterial and plasmid curing activity. Only one of them, cannabispirol, has been found to be active in the inhibition of plasmid replication.

The plasmid elimination by cannabispirol and tetrahydrocannabidiolic acid correlates with the inhibition of the transforming activity of plasmid DNA in *E. coli* strains. Acetylcannabispirol, cannabidiolic acid, cannabispirenone and cannabispirone were less effective than the two previous compounds. Moreover, cannabispirol and tetrahydrocannabidiolic acid had a weak antibacterial effect, at least on *E. coli*, which means that the two compounds affect the plasmid replication more specifically than replication of the bacterial chromosome. On the other hand plasmid carrying cells were more sensitive to antibacterial effect of cannabispirol than the plasmidless ones. Probably the curing effect of cannabispirol and tetrahydrocannabidiolic acid similar to the well known selection mechanism of SDS. The different sensitivity of plasmid carrying and plasmidless bacteria can explain the inhibition of intercellular plasmid transfer, however, some of the compounds (cannabispirol and tetrahydrocan-

nabidiolic acid) also can specifically change the permeability barrier of the bacterial membrane, inhibiting transformation. Because of the poor water-solutibility of cannabispiro compounds, it was supposed that they affect the membrane-dependent processes in such a way that the hydrophobic polycyclic structure of the derivatives interacts with the hydrophobic (proteins or lipids) region of the bacterial membrane. It is possible that the most active compounds have a similar charge distribution or conformation, which is important for binding or many cause similar physicochemical changes in the plasmid replication machinery. It seems possible that the hydroxy group in the paraposition on the cyclohexane ring of cannabispirol might be important for the plasmid elimination activity of the drug; a more negative acetyl or ketone substituent can diminish the biological effect. Since tetrahydrocannabidiolic acid causes plasmid elimination in the presence of magnesium ions [6] which also enhance the membrane effect of cannabis on mitochondria, it is possible that the actions of cannabis are similar to cannabispirol derivatives on bacteria and mitochondria [14]. Some of the cannabinoids are known to alter the structure and function of biological membranes [13, 14]. The most effective compound, tetrahydrocannabidiolic acid, apparently contained three different components and it is possible that only one of them is effective.

Besides the similarities in steric structure and charge distribution, the possibility cannot be ruled out that a common metabolically active compound is formed in the bacteria from the effective derivatives, and that this is responsible for plasmid elimination. At any rate, there is at least one common metabolite of many tetrahydrocannabinols, one epoxy-diol pathway [16] but we have no such data regarding the cannabispirols. Further common properties of the compounds active in plasmid replication are the rather high lipid solubility, together with some degree of water solubility, and the partial anaesthetic action on cell membranes. The cannabinoids reduce the order within the membrane bilayer and this effect is correlated with the psychotropic potency. In addition, some inactive cannabidiols increase the order parameters within the artificial membrane bilayers [14, 16]. The biochemical basis of plasmid curing is rather complex and still not clear in details.

Some tricyclic drugs are known to cause membrane damage such as lipid or protein oxidation and ATPase inhibition [5, 17–19]. In bacterial cells, oxidative phosphorylation is carried out in the cell membrane.

It is believed that the superhelicity of plasmid DNA in the cell is maintained by the equilibrium between the supercoiling activity of DNA-gyrase [20] and the relaxing activity of DNA topoisomerases [21]. ATP is required and hydrolysed in the supercoiling reaction of DNA-gyrase, but it is not required in the relaxation reactions of these DNA topoisomerases; it is therefore presumed that the ATP concentration in the cells considerably affects maintenance of the equilibrium of the activities of these enzymes. Thus, there are

some possibilities which can explain the inhibition of replication of plasmid DNA, but in case of cannabinoids the most possible explanation of plasmid curing effect is the selective action on pili or outer membrane of plasmid carrying bacteria.

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CHANGES OF ADENOVIRUS HEXON ASSOCIATED WITH DIFFERENT PASSAGE HISTORY OF Ad h 1

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Two descendants of the prototype strain AD71-Washington D. C. were obtained by independent passaging for at least 18 years in Kiev, and in Budapest (Ad h 1_K, and Ad h 1_B, respectively). By restriction endonuclease mapping, the DNA was identical corresponding to the patterns of human adenovirus type 1. In spite of this, SDS-polyacrylamide gel electrophoresis revealed that the purified hexon of Ad h 1_K was of lower M_r than the subunit of Ad h 1_B. In contrast to this, the native capsomer (hexon) of Ad h 1_K exhibited lower electrophoretic mobility in agarose gel electrophoresis than the native hexon of Ad h 1_B. Oligopeptide mapping of the main hexon bands from SDS-polyacrylamide gels revealed the presence of unique spots among the chymotryptic oligopeptides of Ad h 1_B, too. Thus, the differences in the sensivity to proteolytic cleavage during purification seem to have a structural basis. Antigenic analysis of the native hexon capsomers was performed using polyclonal antihexon immunsera. Immunodiffusion, immunoelectrophoresis, and competetive RIA were used for comparison. The results indicate that native hexon capsomers of Ad h 1_K and Ad ha 1_B possess antigenic differences within the type-specific regions, nevertheless, their genetic background could not be detected by the restriction endonucleases applied. It cannot be excluded that the differences were results of altered assembly of virions under different passage conditions.

Adenoviruses have been shown to be variable in nature even within one species [1]. Nevertheless, restiction endonuclease cleavage patterns of the viral DNA, and antigenic properties of the virion were found to be stable upon prolonged passage in permissive cells [1]. Minor genetic changes proved to be sufficient to cause dramatic alteration in the host range of adenovirus-Simian virus 40 hybrids [2, 3], and the adaptation of adenoviruses to growth in semi-, or non-permissive cells also produced very small differences in the genomes [3–8]. In contrast to a detailed examination of genetic differences, the consecutive or associated antigenic modifications have not yet been registered.

The prototype strain AD71 of human adenovirus type 1 (Ad h 1) was isolated in 1954, Washington, D. C. [8, 9]. Two descendant strains of AD71 became recently available; they had been passaged independently at least for

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25 years. Experimental analysis of purified hexon preparations revealed both antigenic and biochemical differences between them. A variation of similar nature has been published recently in association with large-plaque mutants of simian adenovirus type 7 [10].

Materials and methods

Viruses and tissue cultures. The "Ad h 1_B" descendant of strain AD71–Washington D. C was kindly supplied by U. H. Krech, Switzerland in 1967, and passaged continuously at the Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary. HeLa, Detroit-6 and primary human amniotic cells were used to support the growth of the virus. From the year 1960 HEp-2 monolayer cultures [11] and the "Cincinnati line" of HEp-2 cells adapted to growth in suspension culture [11] were used continuously. Parker's 199 supplemented with 0.1 part of adult bovine serum was used for monolayer cultures. Dulbecco's modification of Eagle's Ca-free MEM with penicillin or derivatives and streptomycin or other aminoglycoside additives, 100 mg/l each, supplemented with 0.09 part bovine serum was used for suspension cultures. During the last 10 years only suspension cultures were used at very high multiplicity of infection between 0.1 to 10.

The other descendant strain of AD71–Washington D. C. is specified as "Ad h 1_K" and was obtained from the collection of H. G. Pereira, UK in 1964. It was sustained in HeLa cells for 3 years in Budapest, but has not been used for experimental purposes. In 1967 it was forewarded to Kiev, USSR and passaged regularly in HeLa cells between 1974 and 1981 (about 20 passages). The monolayer cultures of HeLa cells were grown in the mixture of Parker's 199 and Eagle's MEM (1 : 1) supplemented with 0.1 part of adult bovine serum, penicillin and streptomycin (100 mg/l each). For large-scale virus production of Ad h 1_B suspension cultures were used in contrast to Ad h 1_K, which was propagated in serum-free Parker's 199 or Eagle's MEM maintenance medium. The m.o.i. was about 20 during production of viruses as measured by inclusion body-forming units [12].

All Ad h 1_B preparations, purified hexon and antisera were produced in Budapest. The corresponding samples of Ad h 1_K were prepared in Kiev in order to avoid cross-contaminations.

Purification of hexon, virion and viral DNA. Virus-infected cells were pelleted by low speed cntrifugation and disrupted by 6 cycles of freezing and thawing, or by treatment with 5 mg/cm³ Triton X100 after the addition of 0.4 M NaCl and 0.01 M Tris–HCl, pH 7.5 Cell debris was removed by low speed centrifugation at 5000 *g* at +4 °C for 20 min. Virus particles were purified by equilibrium gradient centrifugation using preformed CsCl gradients (1.1 to 1.4 g/cm³) in 0.01 M Tris–HCl, or Na-phosphate buffers of pH 7.5 and 7.0, respectively [11].

Hexon was purified from low-density fractions of the CsCl gradients free of particulated material by techniques described previously [12–14]. Crude extracts of simian adenovirus type 7 free of virus particles were kindly supplied by R. S. Dreizin, Moscow, USSR [10, 14]. Some hexon preparations were further purified by sucrose gradient centrifugation or by crystallization in 0.5 M sodium acetate buffer, pH 4.5 [13, 14].

The viral DNA was purified from dialysed fractions of CsCl gradients, containing virus particles. Samples were digested with predigested pronase (1.0 mg/cm³, Calbiochem, San Diego, California, USA; B grade, free of nucleases) in the presence of 10 mg/cm³ SDS and 20 mM Tris–HCl, pH 7.5 at 37 °C for 60 min. Deproteinization was done with water-saturated phenol and chlorophorm : isoamylalcohol according to standard protocols [10, 11].

Polyacrylamide gel electrophoresis. Hexon preparations were compared and further purified using SDS-polyacrylamide slab gels according to the procedure described by Laemmli [15]. Gels of 80 mg to 150 mg/cm³ concentrations were used at 1 : 40 ratio of bisacrylamide to acrylamide (5 times crystallized; Merck, Darmstadt, GFR). Standars from M_r determination have been purchased from Serva, Heidelberg, GFR.

Oligopeptide mapping of hexon preparations. Hexon bands were reisolated from SDS-polyacrylamide gels and processed for oligopeptide mapping according to the procedure described by Elder et al. [16]. Labelling of polypeptides was performed following the second reisolation of bands from the polyacrylamide gels with ^{125}I using chloramine T. Chymotrypsin (Worthington Biochemicals, Freehold, New Jersey, USA) was applied at 0.1 mg/cm³ concentration for digestion, and the samples were separated on thin layers of cellulose purchased from Merck, Darmstadt, GFR [14, 16].

Agarose slab gel electrophoresis. DNA was digested with restriction endonucleases *KpnI*, *PstI*, *SalI* and *Hin*dIII [17], and the fragments were separated in horizontal slab gels of 9 to 11 mg/cm³ agarose (Agarose-HGT(P), SeaKem, Marine Colloids, Inc., Rockland, Maine, USA) using the buffer system described by Helling et al. [18].

Serological techniques. Agar gel diffusion and immuno-electrophoresis were performed in 10 mg/cm³ agarose gels (Koch-Light or Sigma Chem., Co., St. Louis, Mo., USA) in sodium phosphate buffer of 0.05 M, pH 7.2 or sodium acetate buffer of 10 mM added to 50 mM Tris-acetate, pH 7.8 [12, 13, 19]. Electrophoresis was performed using the Labor MIM (Esztergom, Hungary) equipment with 60 × 90 mm plates at 4 V/cm for 5 h at room temperature. The immunoprecipitation was continued at 25 or 37 °C for 48 or 96 h. NaN_3 was used as bactericidal additive.

Radioimmunoassay and competitive RIA. Hexon preparations purified by ion exchange chromatography and gel filtration [13, 14] were labelled in vitro by ^{125}I using chloramine T [20]. Low molecular mass components were separated by filtration through Sephadex G-25 gel and the macromolecular fractions were used for radioimmunoassay (RIA) in a buffer system consisting of 0.02 M Tris–HCl, pH 7.4, 1.0 mM EDTA, 0.1 M NaCl, 2 mg/cm³ bovine serum albumine, and 2 mg/cm³ Triton X100 [20]. Homogeneity of the labelled material and antigenicity of the samples were tested in SDS-acrylamide gels and agar gel diffusion test prior to the use of 3×10^4 to 5×10^4 cpm activities for the individual serum dilutions tested [20].

In the case of competitive RIA unlabelled sera and hexon samples diluted accordingly were preincubated at room temperature for 30 min in the presence of 0.1 dilution of normal rabbit serum, and then competed with 10^4 to 2×10^4 cpm of ^{125}I-labelled hexon samples at 37 °C for 90 min. Serum dilutions selected for the tests precipitated about 0.5 part of the ^{125}I activity in the absence of competing antigen. The degree of competition C was calculated according to the equation

$$C = 1.0 - \frac{A - a}{B - a}$$

where A is the radioactivity precipitated in the presence of competing antigen, B is the amount of radioactivity precipitated in the absence of competing antigen, and a is the radioactivity precipitated by normal rabbit serum without the addition of specific components [20].

Preparation of antisera. Rabbits were immunized intramuscularly with 3 or 4 injections of purified hexon preparations, and virion suspensions homogenized with equal amounts of Freund's complete adjuvant (Difco Labs., Detroit, Michigan, USA). IgG was prepared from the sera against Ad h 1_K hexon, SA 7 hexon and Ad h 1_B complete virion antigens by DEAE sephadex chromatography (Pharmacia Technical Bulletin, Ion exchange chromatography).

Results

The first observation indicating differences in the properties of the two Ad h 1 substrains was made when comparing purified hexon preparations by SDS-polyacrylamide gel electrophoresis. Figure 1 shows that the majority of hexon purified from infected HEp-2 cells was of the same M_r (lanes *a*, and *b*) as that of the hexon of purified virions (lane *e*). In contrast to this, hexon preparation of Ad h 1_K from infected HeLa cells had an apparent M_r only of 10^5 (lane *c*, and *d*). Breakdown products of hexon subunits, or contaminants of M_r lower than 120 000 also were visible in the preparation from Ad h 1_B (lanes *a*, and *b*), however, these were comprizing usually only less than 0.1 part of the material.

Oligopeptide mapping of the main bands of hexon preparations. The possibility, whether the native hexon purified from Ad h 1_K-infected HeLa cells was impaired to a higher extent by proteases than the hexon from Ad h 1_B seemed to be easily detectable using oligopeptide mapping. The results are

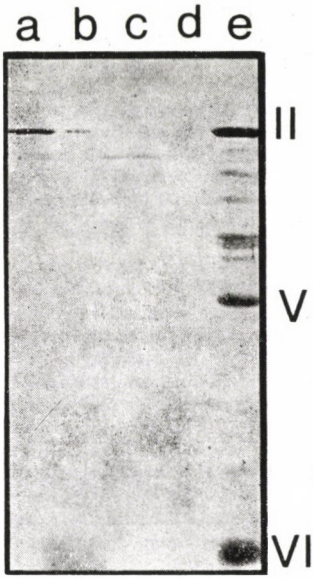

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified hexons of Ad h 1_B (lanes a and b), and Ad h 1_K (lanes c and d). Gel concentration, 0.16; acrylamide : bisacrylamide ratio, 40 : 1; Coomassie Brilliant Blue stain. Ad h 1_B preparations were used for M_K control (lane e). Roman numerals indicate the position of hexon (II), and the positions of core proteins (V; VII)

shown in Fig. 2. Six unique spots were detected in the chymotrypsin-map of Ad h 1_B hexon (Fig. 2a). Nevertheless, as indicated by arrowheads in Fig. 2b, 2 unique spots became visible also among the chymotryptic oligopeptides of the main band of Ad h 1_K of 10^5 M_r.

Attempts without success have been made to compare 92K and 100K minor bands from the preparation of Ad h 1_B hexon with the main band of Ad h 1_K preparation. Figure 2c shows the chymotryptic oligopeptides of the mixture of Ad ha 1_K and Ad ha 1_B hexons. It may be seen that spots labelled by arrow in Fig. 2a and 2b, and indicated in the insert are not amplified. The appearence of unique oligopeptides in the maps of both hexons shows that the differences are not caused simply by proteolytic cleavage during purification, but suggests the existence of true biochemical differences.

Comparison of native hexon by immunoelectrophoresis. Subunits of native hexon may be affected by proteases during storage or purification, but their tertiary structure remains unaffected. Therefore, both purified preparations of Ad h 1_B and Ad h 1_K hexon, crude extracts of virus infected HeLa (Ad h 1_K) and HEp-2 (Ad h 1_B) cells were compared by immunoelectrophoresis using a series of homologous and heterologous antisera.

The electrophoretic mobility of the two hexon preparations in the native form proved to be different, too (Fig. 3). Trench 1 contained Ad h 1_B-specific

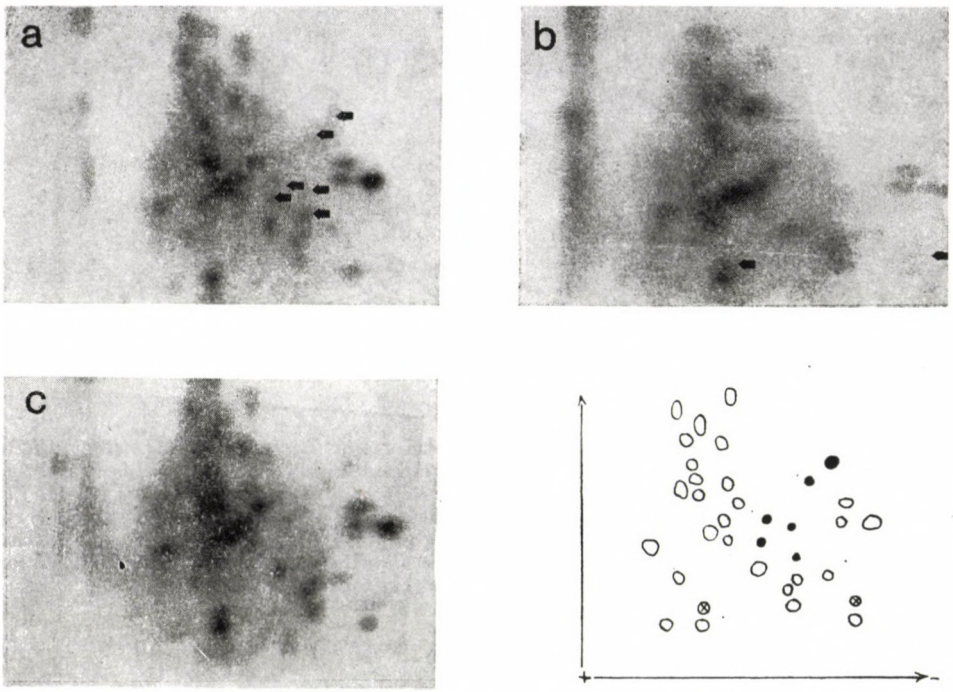

Fig. 2. Chymotryptic oligopeptides of hexon polypeptides purified from SDS-polyacrylamide gels. Main band of Ad h 1_B (a); main band of Ad h 1_K hexon (b); mixture of the two (c) hexon preparations digested as described in the text, and by Elder et al. [16]. Arrows indicate unique spots in the samples a and b, which are also shown in the insert (black spots and crossed circles). Direction of chromatography is indicated by the vertical arrow, + and — symbols show the location of the anode and cathode

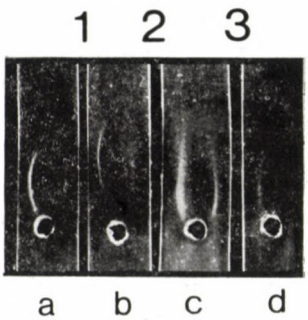

Fig. 3. Immunoelectrophoresis of purified hexons of Ad h 1_K (well a) and Ad h 1_B (well b) using antihexon sera prepared with Ad h 1_B hexon in rabbits (trenches 1 and 3) and with hexon of ad h 1_K (trench 2 and unlabelled trench to the left). The mixture of hexons (1 : 1) was pipetted into well c. Well d contained tissue culture supernatant from Ad h 1_K-infected HeLa cells. See text for other details. Under the conditions used the hexon runs toward the anode (upward)

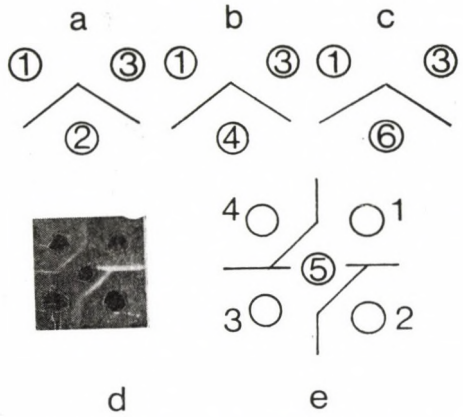

Fig. 4. Comparison of anti-hexon antibodies preparad against hexons of ad h 1_K (2), Ad h 1_B (4) and simian adenovirus type 16 (SA7, 6) in immunodiffusion tests. The wells containing purified hexons are labelled as follows: Ad h 1_K (1); Ad h 1_B (3); simian adenovirus type 16 (5). None of the antisera revealed differences between the purified hexons of Ad h 1_K, and Ad h 1_B, therefore the results are only schematically illustrated (a, b, and c). Insert d is the original photograph corresponding to schematic illustration e, showing results with the hexon of simian adenovirus type 16 (SA7, 5). See text for further details

anti-hexon serum. This reacted better with the homologous antigen, which migrated faster than the hexon of Ad h 1_K (well a). The neighbouring trenches (unlabelled, and 2) contained Ad h 1_K-specific anti-hexon IgG, and reacted better with the slow-migrating homologous hexon in well a than with the heterologous preparation in well b. Wells c and d were filled with the mixture of the two hexon preparation in order to see whether elongated precipitation arcs appear with Ad h 1_K-specific antihexon IgG and simian adenovirus specific antihexon IgG (trench 3). The results indicate that the mixture of the two hexon preparations formed precipitation arcs much longer than the controls in well a and b overlapping with both separate samples. The difference in electrophoretic mobility is not an artifact, since the antihexon IgG directed against Ad ha 1_K reacted with both kinds of the antigen. The antihexon IgG produced against SA7 (trench 3) reacted significantly better with the slower population of the hexon than with that more towards the anode. A subsequent series of experiments revealed, however, that antisera directed against Ad bos 2, Ad h 10 and complete virions of Ad h 1 do not react differently with the two hexon populations. Different dilutions of freshly prepared crude extracts of infected cells also were compared in immunoelectrophoresis using antisera even against guanidine-HCl-treated, and SDS-treated hexon of Ad h 1_B in addition to the antibodies mentioned before. The results indicate that the differences in the electrophoretic mobilities of Ad h 1_B and Ad h 1_K hexon populations may be observed in native crude extracts, too (experiments not shown).

Differences in antigenic composition of hexon preparations. It has been found that certain heterologous antibodies might reveal antigenic differences between Ad h 1_B and Ad h 1_K hexons (Fig. 3). Results of systematic screening by immunodiffusion tests for such differences are summarized in Fig. 4. Antihexon antibodies are labelled with even numbers. All three antibodies against Ad h 1_K (Fig. 4a; well 2), Ad h 1_B (Fig. 4b; well 4) and SA7 (Fig. 4c; well 6) gave complete homology if tested with purified hexons of Ad h 1_K (well 1) and Ad h 1_B (well 3). When the purified hexon of simian adenovirus 7 was also included (Fig. 4e; well 5) the homologous combinations were shown to reveal partial identity (spures photographed and drawn in Figs 4d and 4e). Any other combination of Ad h 1_K and Ad h 1_B failed to reveal differences in comparison to the hexon of SA7. These results show that Ad h 1_K-specific antihexon serum contains antibodies directed to epitopes absent in both other hexons. Ad h 1_B-specific antiserum also has epitopes absent from the two other preparations. Thus, both Ad h 1 hexons seem to carry unique epitopes, which are absent from the hexon of SA7, too.

Competitive radioimmunoassay (RIA) systems were composed in order to detect possible further antigenic differences of Ad h 1_K and Ad h 1_B. The hexon of Ad h 1_B was labelled in vitro with ^{125}I, and precipitated using antisera directed to Ad h 1_B, Ad h 1_K, Ad h 2, Ad h 10, and SA7. The results are shown in Fig. 5. Unlabelled hexons of Ad ha 1_B (continuous lines) and Ad h 1_K (dotted lines) were used as competing antigen. Hexon preparations of Ad ha 1_K competed with the labelled Ad h 1_B hexon at a level of 0.8 to 0.9 in the case of heterologous systems (Figs 5a, 5b, 5c; antihexon sera to adenovirus types 2, 10 and SA7, respectively). The same level of competition was obtained for the antibodies present in the serum specific for Ad h 1_K (Fig. 5e). From these results it may be concluded, that the genus- and subgenus-specific epitopes of Ad h 1_K and Ad h 1_B hexons are similar. The only significant difference in the level of competition was measured when the homologous system was used (Fig. 5d). In the presence of Ad h 1_B-specific antihexon serum, unlabelled Ad h 1_K antigen gave only a 0.5 level of competition. Thus, Ad ha 1_B hexon was able to induce antibodies against epitopes, which are absent or very weak antigens in Ad h 1_K.

Restriction endonuclease analysis of the DNA had to be included since agar gel diffusion, immunoelectrophoresis and RIA results might be due to a contamination of ad h 1_K or Ad h 1_B strains with other adenovirus species. The aim of these experiments was to exclude cross-contamination with other types, and reveal possible major genetic differences in the genome of the two descendant Ad h 1 strains. Partial and complete hydrolysates were prepared with *Bsp*I (more than 50 fragments), *Hin*dIII (13 fragments), *Kpn*I (11 fragments), *Pst*I (29 specific fragments), and *Sal*I (5 fragments). The electrophoretic pattern of each hydrolysate allows the differentiation of Ad ha 1 DNA

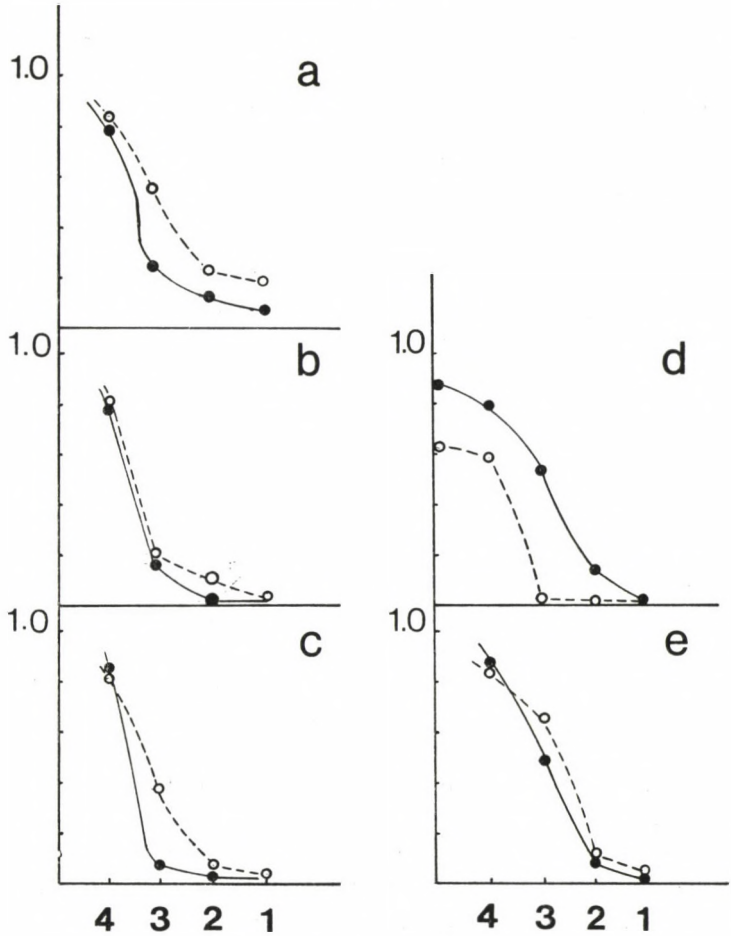

Fig. 5. Competition of purified hexons of Ad h 1_K (○ – – – ○) and of Ad h 1_B (●——●) for antihexon antibodies prepared against hexons of Ad h 2 (a), Ad h 10 (b); simian adenovirus type 16 (SA7, c); Ad h 1_B (d), and Ad h 1_K (e) with radioiodinated hexon of Ad h 1_B labelled in vitro, as described in the text, and by Tarassishin, et al. [20]. Arabic numerals (abscissa) indicate the log 10 ratios of labelled and unlabelled antigen (hexon). The level of competition is shown on the ordinate (from 1.0 to 0.0). The differences are only considered to be significant, if the unlabelled hexon can prevent the precipitation of more than 0.5 part of ^{125}I-labelled probes

from that of other adenovirus types. No difference could be found between the electrophoretic patterns of Ad h 1_K and Ad h 1_B. Figure 6 is included for demonstration. Partial digest of Ad h 1_K (a and c) and Ad h 1_B (b and d) with *Sal*I (a and b) and *Kpn*I (c and d) were electrophoresed in agarose slab gel of 7 mg/cm^3 concentration. Final fragments of the physical maps are labelled by capital letters, and shown in the inserts. Fragments *Sal*I-D, *Kpn*I-A and *Kpn*I-D are unique within the physical maps of Ad h 1 DNA in comparison to those of subgenus "C" adenoviruses [11].

Discussion

Two descendant viruses of the prototype strain AD71-Washington D. C. [8, 9] which had been passaged independently during 25 years were examined. Ad h 1_K (Kiev) may be characterized as passage material from HeLa cells, and Ad h 1_B (Budapest) as the product of suspension cultures of HEp-2 cells, maintained in this way since the year 1975. There was a 3-year period between 1964 and 1967, when both viruses were stored and maintained independently in the same laboratory, where probably identical serum lots and tissue cultures were used. Passage levels have been registered from time to time, but the present passage levels could not be followed up unequivocally.

Experiments using restriction endonucleases and immunodiffusion tests using purified hexon preparations and specific antisera provided evidence that both viruses are adenovirus type 1, without cross-contamination. Nevertheless, differences were revealed during purification and antigenic analysis of hexons. Hexon subunits were reproducibly found to be of lower M_r (probably degraded during purification) as measured by SDS-acrylamide gel electrophoresis of Ad h 1_K. Such degradation occurred probably also in the case of Ad h 1_B but affected only a small proportion of the subunits. Oligopeptide mapping of the hexon subunits provided evidence that the degradation does in fact occur, since at least six oligopeptides were found to be absent from the map of Ad h 1_K subunit.

In contrast to these a series of experimental results support the assumption that the above susceptibility of hexon of Ad h 1_K origin is the consequence of changes within the polypeptide composition or processing of the hexon. Immunodiffusion tests revealed that both preparations of native hexons possess epitopes absent from the other virus (Fig. 4d). The results indicate that the differences may be in the type specific epitopes, since certain epitopes unique forboth Ad h 1_K and Ad h 1_B were absent from the hexon of SA7. Unique oligopeptides (at least two; Fig. 2) could be identified within the chymotryptic map of Ad h 1_K hexon subunit. This is a strong indication to the fact that differences in hexon subunits are consequences of true structural or sequence-alterations, resulting in increased susceptibility to cellular proteases as well as conformational changes which caused reduction of the electrophoretic mobility of the native, undenatured hexon of Ad h 1_K (Fig. 3). Similar results have been obtained when the electrophoretic mobilities of unpurified hexon preparations were compared, using hexon-specific polyclonal sera for precipitation (experiments not shown). The experiments in RIA systems provided direct evidence, that native, undenatured hexon capsomers of Ad h 1_B are carrying unique epitopes in comparison to native Ad h 1_K (Fig. 5d).

Preliminary results indicate that the yield of Ad h 1_K is lower in HEp-2 cells than that of Ad h 1_B. Ad h 1_B was shown to possess two distinct popula-

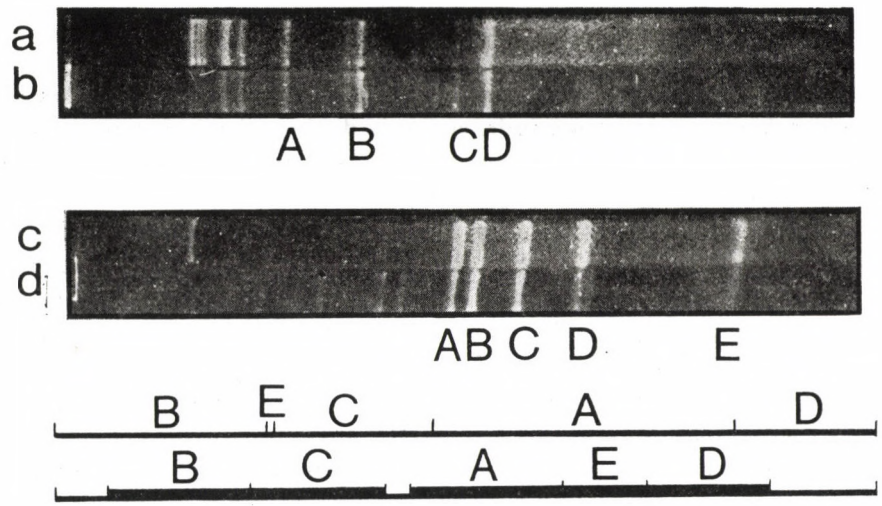

Fig. 6. Comparison of DNA fragments of Ad h 1_K (a, c) and Ad h 1_B (lanes b, and d) genomes prepared by use of *Sal*I, and *Kpn*I restriction endonucleases. Electrophoresis run from left to right at 1 V/cm for 36 h in agarose gel (7 mg/cm^3). Completely digested fragments are labelled by capital letters. Partial digestion with *Sal*I (lanes a, and b) allowes the demonstration of the smallest *E* fragment (see the physical map drawn below the photographs) since a faint band of *Sal*I-*B* + *E* may be visualized to the left of *Sal*I-*B* in both samples. *Kpn*I digestion was approximately complete, therefore smaller fragments (indicated by thin line the physical map) run out from the gel. The visible fragments *Kpn*I-A to E represent about 0.8 part of the unit length genome of 36 kb

tions of "empty" particles in CsCl gradients [19]. The particles in the light CsCl fractions of equilibrium gradients were found to be also different in the case of Ad h 1_K and Ad h 1_B preparations. Experiments are in progress to compare particle stability, and possible differences in the virus cycles.

Results presented here do not alow the quantitation of differences in primary structure of hexons. No data are available concerning possible differences of other structural proteins. Epitopes of hexons have been shown to be of different degree of specificity [21–24]. Results of agar gel diffusion, and competitive RIA experiments suggest that hexons of Ad h 1_K and Ad h 1_B differ only in the type-specific region of the capsomers, which are coded for by the right subterminal region of the structural gene [25–28]. There is evidence that such minor differences may be detected by the use of restriction endonucleases [10, 11, 29]. Unfortunately this did not account for the differences described above (Fig. 6).

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IMMUNOCHEMICAL AND IMMUNOLOGICAL STUDY OF CELL-WALL PROTEINS OF *PSEUDOMONAS AERUGINOSA*

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Crude aqueous extract was obtained from acetone-dried cells of *Pseudomonas aeruginosa* strain 868 (serogroup O2, Lányi and Bergan's schema) and subjected to ultracentrifugation (105 000 *g*, 3 h); the lipopolysaccharide (LPS)-containing precipitate was discarded and the supernatant containing water-soluble cell proteins was subjected to further fractionation. From a partially purified aqueous extract two fractions were obtained by step-wise precipitation with ammonium sulphate, namely, F1 (by 50% saturation), and F2 (by 80% saturation). By gel and ion-exchange chromatography from both fractions 9 subfractions were isolated differing in molecular weight, protein content, and LPS contamination. Subfractions 4 and 7 were practically free from LPS, and gave one precipitation line with antisera for strain 868. By immunoelectrophoresis subfraction 4 contained 2 cathodic and 1 anodic, whereas subraction 7 mainly 1 anodic component. These subfractions were antigenically identical. With ELISA these subfractions were less active as compared to other subfractions, in particular to those of high molecular weight. The anti-subfraction 4 and anti-subfraction 7 sera were found to protect passively mice against intraperitoneal challenge by *P. aeruginosa* strain 8 (serogroup O2). These data support the authors' opinion that subfraction SF-4 and SF-7 are protective protein antigens (mol wt about 40 000 and 30 000, respectively), that are localized in the outer membrane of *P. aeruginosa* cell envelope.

Antigens of the bacterial cell surface are known to be of extreme importance for the stimulation of specific and, perhaps, non-specific resistance of the host against infection [1]. Among the surface antigens particular attention is paid to some poorly studied cell proteins, especially the proteins of the outer membrane (OM) of the cell envelope [2–5]. These proteins are assumed to possess protective activity. Thus, experiments with *Salmonella typhi-murium* as a model [3] showed that a fraction of unpurified OM proteins (porins) were protective for mice. However, OM proteins purified from LPS, displayed no or very weak protective activity.

From *Pseudomonas aeruginosa* bacteria [4, 5] a protein component of endotoxin was isolated, a species-specific protective antigen (OEP) which was practically free from LPS. This antigen was proposed as one of the components of *P. aeruginosa* vaccine (PV) [6]. Other workers [8] also attempted to isolate protein antigens from *P. aeruginosa*.

Previously we have shown [9, 10] that from a cell lysate of *P. aeruginosa* two types of protective protein antigens can be isolated, protein A (mol wt

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120 000–140 000) and protein B (mol wt 37 000 or 11 000, depending on the strain). Cross-immunoelectrophoresis showed these components to be highly heterogeneous and to contain LPS [10].

In the present paper we report on the results of further investigations performed to isolate individual protein antigens from *P. aeruginosa*, and to determine their molecular weight and protective properties.

Materials and methods

Bacterial strains and cultivation. P. aeruginosa strains 868 and 8 were isolated from the wound of burn patients in the Vishnevsky Institute of Surgery, Academy of Medical Sciences, Moscow; they belonged to serogroup O2 (Lányi and Bergan's serogrouping system [11]). Earlier we used these organisms as experimental vaccine strains [12, 13].

Strain 868 was sultivated in agar medium containing tryptose casein, at 37 °C for 18 h in a Shesterenko device for cultivating microorganisms with aeration [14].

Strain 8 was used for the challenge of animals. It was initially cultivated in Hottinger broth (pH 7.2–7.4) at 37 °C for 3 h, and then it was transferred onto Hottinger agar medium (pH 7.2–7.4) in tubes. The tubes were incubated at 37 °C for 16–18 h, then used for the challenge of immunized mice (see passive protection of mice).

Preparation of bacterial extracts. Cells of strain 868 were washed off the agar surface with sterile isotonic saline and centrifuged at 1000 *g* for 1 h. The sedimented bacteria were washed three times with acetone and dried. A 10% cell suspension in distilled water was extracted three times in a homogenizer at 8000 rpm for 5 min as described elsewhere [13, 14]. The crude atueous extract (AE) was centrifuged at 105 000 *g* for 3 h (Beckman centrifuge, model 5–75, rotor 35). The LPS-containing precipitate was discarded. The supernatant containing water-soluble proteins (porins) is hereafter referred to as partially purified AE (PPAE).

Preparation of LPS. LPS was obtained from acetone-killed bacteria of *P. aeruginosa* strain 868 by phenol-water extraction [15], and purified by ultracentrifugation at 105 000 *g* for 3 h [16].

Salt fractionation. PPAE was subjected to salt fractionation [9]: 50% saturation with ammonium sulphate resulted in a precipitate which was separated by centrifugation (1000 *g*, 30 min) and redissolved in distilled water to give fraction 1 (Fl); to the supernatant the salt was added to a 80% saturation, and the precipitate formed was separated by centrifugation to give fraction 2 (F2).

Gel-chromatography. Gel-chromatography was performed on a column (85 × 2.8 cm) with Sephadex G-100 (Pharmacia, Sweden). The material (Fl and F2) was eluted with distilled water (pH 6.5–6.8); sampling was performed with an automatic collector HKOV-1 (Frunze, USSR), and optical density of samples at 280 nm was determined with a SF-26 spectrophotometer (LOMO, Leningrad, USSR).

The column was calibrated with blue dextran (mol wt 2×10^6, Pharmacia, Sweden), bovine serum albumin (mol wt 6.7×10^4, Koch–Light, Great Britain), pepsin (mol wt 3.6 ×, × 10^4, Serva, FRG), trypsin (mol wt 2.38×10^4, Serva, FRG), and lysozyme (mol wt 1.7×10^4 Reanal, Budapest, Hungary).

Desalting of fractions (Fl and F2) was carried out on a column (85 × 2.8 cm) with Sephadex G-25 (Pharmacia, Sweden); sampling was performed with an automatic collector, and optical density at 280 nm was determined with a spectrophotometer. The presence of ammonium sulphate in samples was determined qualitatively with 5% $CaCl_2$ solution.

Ion-exchange chromatography was carried out on a column (24 × 1.8 cm) with DEAE-cellulose (Merck, FRG). The column was equilibrated by a starting buffer (1/15 M phosphate, pH 7.3). A sample introduced into a column was dissolved in the same buffer. Step-wise elution was performed successively with the starting buffer and with saline added (from 0.05 to 0.5 M). Sampling was performed with an automatic collector. Elution rate was 20 ml/h.

Immunodiffusion in an agar gel (Difco agar) was carried out by the method of Ouchterlony [17].

Immunoelectrophoresis in an agar gel (Difco agar) was carried out by the macromethod [18].

Chemical analysis. Protein was assayed using the Folin reagent [19], carbohydrates were analyzed with the anthrone reagent [20], and LPS was determined by ELISA [21].

Analytical centrifugation was made in a Beckman ultracentrifuge model E, rotor AN-D. Sedimentation coefficients were calculated in Svedberg (S) units [22].

ELISA. Enzyme-linked immunosorbent assay of preparations was carried out by use of an anti-rabbit IgG goat serum conjugate labeled with peroxidase (Human Institute for Serobacteriological Production and Research, Budapest, Hungary). The procedure was as follows. (1) The studied subfractions SF, see Results) were adsorbed to a polystyrene plate at 100 μl volumes in concentrations from 1 mg to 0.1 μg in 0.5 M carbonate buffer (pH 9.6), and incubated at room temperature. (2) The plate was washed with a working solution (isotonic saline with 0.5% of Tween 20), and serum, diluted with a 1/15 M phosphate buffer (pH 7.3) with 0.5% of Tween 20, was applied on the plate, and then incubated at room temperature for 3 h. (3) The plate was washed with a working solution, and the conjugate diluted with phosphate buffer (1 : 1000) was applied on the plate, and then incubated at room temperature for 20 h. (4) The plate was washed with a working solution, and the substrate (0.04% o-phenylenediamine with 0.003% H_2O_2 in 0.05 M phosphate-citrate buffer, pH 5.8) was applied on the plate, and then incubated at room temperature for 30 min. (5) Spectrophotometric analysis was carried out at 410 nm using an ELISA reader spectrophotometer (Dynatech, FRG).

Animals. Experiments were carried out with outbred Swiss mice weighing 18–20 g, and rabbits (chinchilla) weighing 2–3 kg.

Immunization of rabbits. Prior to immunization blood samples were taken. Sera obtained from several rabbits were pooled and used as a preimmunization pool. Animals were immunized with a suspension of acetone-dried and live *P. aeruginosa* bacteria according to the following schedule: 1st immunization, 1 mg of dry bacteria in complete Freund adjuvant (Difco) subcutaneously; 2nd immunization, 2 mg; and 3rd immunization, 3 mg of dry bacteria intravenously. Intervals between immunizations were 3–4 days; after 4 weeks the animals were immunized twice with killed cells (4 and 6 mg of dry bacteria, respectively) and simultaneously with live bacteria (7×10^9 bacteria) intravenously with the same intervals between immunizations. Immunization with protein preparations was performed as follows: 1st immunization — 1 mg of lyophilized material, 2nd — 2 mg of protein in complete Freund adjuvant (Difco) subcutaneously with an one-week interval; after 2 weeks the animals were immunized intravenously three times (4, 4 and 6 mg) at the same intervals. Seven days after the last immunization the rabbits were exsanguinated, antisera obtained were distributed in ampoules and kept at —30 °C or lyophilized.

Passive protection of mice. The animals received antiserum intraperitoneally, and after 2 h they were challenged intraperitoneally with a 16–18-hour agar culture of strain 8 suspended in isotonic saline. Experiments were carried using two methods: (a) titration of the challenge dose (LD_{50}) of the culture, and (b) titration of the immunizing dose (ED_{50}) of serum.

(a) Titration of the challenge dose of the culture: mice (50 animals) were inoculated intraperitoneally with 1 ml of antiserum diluted 1 : 50 (i.e. 0.02 ml of the starting antiserum); control animals (50 mice) received a similar dose of preimmunization serum (control 1); in both immunized groups and a non-immunized group (50 animals) (control 2) determination of the LD_{50} of the *P. aeruginosa* live culture was performed (10 mice/dose). (b) Titration of the immunizing dose (ED_{50}) of antiserum: mice received intraperitoneally antisera and preimmunization serum (control 1) diluted 1 : 50, 1 : 250, 1 : 1250, or 1 : 6250 (10 mice per dilution). Immunized mice were infected with 3.6 or 7.1 LD_{50} of live culture. Simultaneously, LD_{50} of non-immunized mice was determined (control 2).

After challenge the animals were observed for 3–4 days, and the death of animals was recorded. LD_{50} and ED_{50} were calculated by Van der Varden's method [23], significance of the difference between mean values was determined by the Student's t criterion [23]. The viable germ count of the challenge dose was determined from colony counts (colony-forming units) obtained by the pour-plate technique.

Results

Chemical and physico-chemical characterization of isolated cell components. Gel-chromatography of Fl and F2 on a column with Sephadex G-100 (Fig. 1) yielded the following subfractions (SF):SF-1 and SF-2 from F1 (the third peak contained ammonium sulphate only, and hence was discarded), and SF-3 and SF-4 from F2. Rechromatography of SF-3 on a column with Sepha-

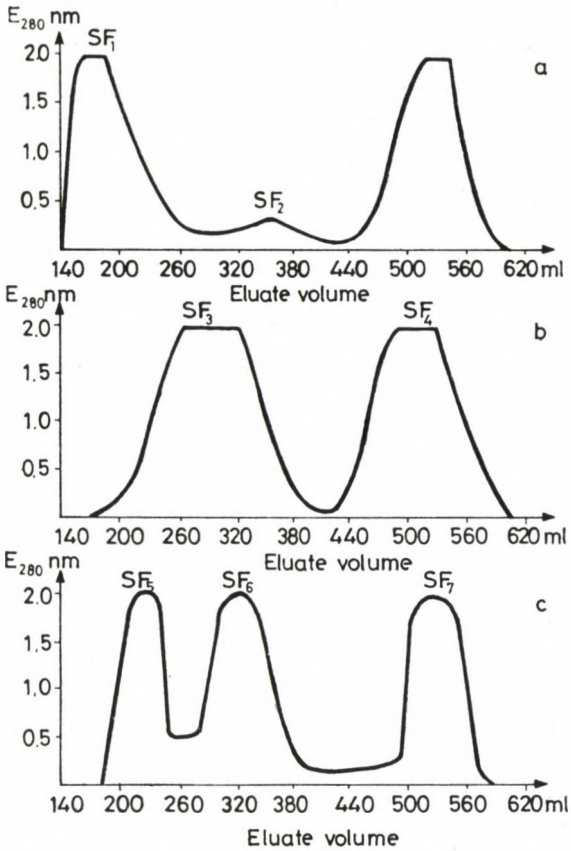

Fig. 1. Gel-chromatography on Sephadex G-100 column (85 × 2.8 cm). Elution with distilled water. A, elution profile for Fl; B, elution profile for F2; C, rechromatography of SF-3

dex G-100 gave SF-5, SF-6 and SF-7. SF-4 was desalted on a column with Sephadex G-25.

SF-1 was chromatographed on a column with DEAE-cellulose (Fig. 2) to give SF-8 and SF-9.

Table I shows the characteristics of the isolated SFs. The highest yield was obtained from SF-6 and SF-3, while the lowest one from SF-4, SF-7 and SF-8. The high molecular weight of SF-1, as well as the initial preparation of PPAE, proved to be heterogeneous as judged by the data on analytical centrifugation. SF-2 and SF-3 also were heterogenous. Accordingly, the sedimentation coefficient could not be calculated for these subfractions. Being more or less homogeneous, the sedimentation coefficients could be calculated for SF-1, SF-5, SF-6, and SF-7 (2–3 S). The relatively low molecular weight subfractions SF-4 and SF-7 (mol wt about 40.000 and 30 000, respectively), as well as

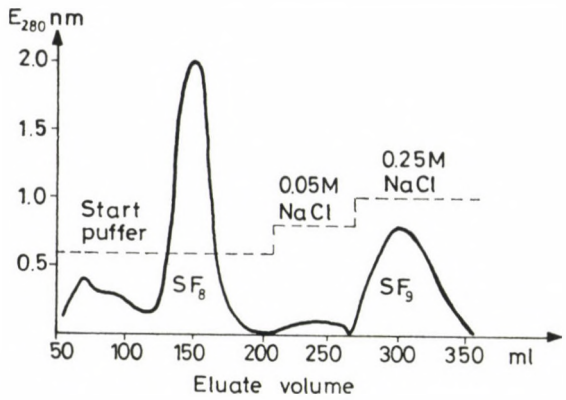

Fig. 2. Ion-exchange chromatography on DEAE-cellulose column (24 × 1.8 cm). Elution profile for SF-1 (obtained by gel-chromatography of Fl, see Fig. 1)

SF-5 and SF-6 (molwt 60 000 and 80 000, respectively) proved to be homogeneous as judged by the data on analytical centrifugation. The results of the determination of the LPS content in SFs by ELISA (Fig. 3) show that SF-4 and SF-7 are practically free from LPS contamination, the protein/LPS ratio for SF-4 is 560 while for SF-7 this ratio is 714, i.e. these SFs consist of almost pure protein.

Serological activity of components according to ELISA. The high molecular weight subfractions SF-1 and SF-2 (Fig. 4) were most active while the relatively low molecular weight SF-4 and SF-7 were the least active. Other SFs displayed intermediate activity by ELISA. The results of serological analysis

Table I

Physico-chemical, and chemical characterization, and yields of isolated proteins from P. aeruginosa

Preparation	Yield (% protein)	Mol wt	Sedimentation coefficient (Svedberg units)	Protein (%)	LPS (%)	Protein/LPS ratio
PPAE	100	?	heterogeneous	38	0.3	126
SF-1	5.6*	> 150 000	heterogeneous	39	8	4.8
SF-2	4.3	?	heterogeneous	41	4	10
SF-3	9.2	?	heterogeneous	37	0.1	370
SF-4	0.7	~ 40 000	~ 2	28	0.05	560
SF-5	1.62	~ 80 000	~ 3	55	2	27.5
SF-6	14.8	~ 60 000	3	75	1	75
SF-7	0.89	~ 30 000	2	50	0.07	714
SF-8	0.27	NS	NS	40	0.08	500
SF-9	0.86	NS	NS	74	1.2	61.6

* The yield of SF with respect to PPAE
NS = Not studied

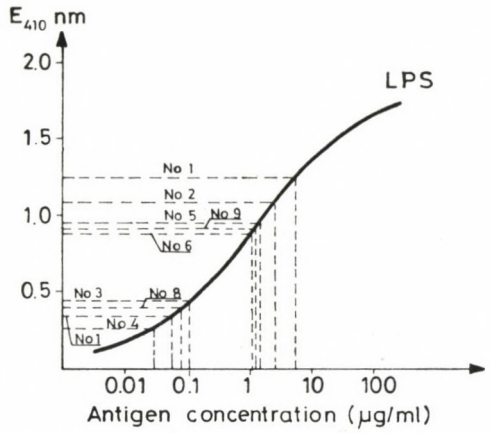

Fig. 3. Determination of the LPS content in protein preparations obtained from PPAE o-*P. aeruginosa*. Antiserum to *P. aeruginosa* LPS in 1 : 1000 dilution. Concentration of preparaf tions, 100 μg/ml. Nos 1—9, corresponding subfractions

show direct correlation between the value of the molecular weight of the isolated protein components and their activity in vitro.

Immunodiffusion. Immunodiffusion analysis (Fig. 5) showed that SF-4 and SF-7 gave one precipitation line each, and these subfractions proved to be antigenically identical. Other SFs. gave 2–4 precipitation lines each, i.e. these were heterogeneous. Thus, immunoprecipitation and analytical ultracentrifugation showed SF-4 and SF-7 to be homogeneous protein preparations.

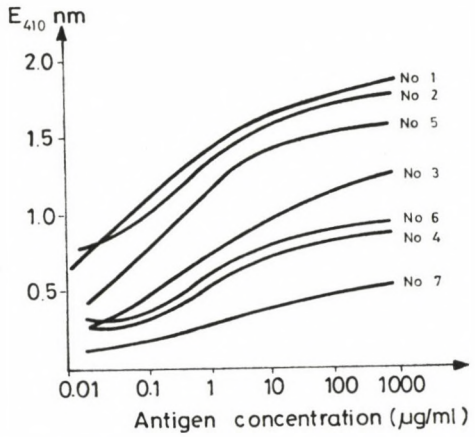

Fig. 4. Comparative characterization of protein preparation activity (different SFs) with antiserum to *P. aeruginosa* cells. Antiserum dilution 1 : 1000. Nos 1—9 are the corresponding SFs

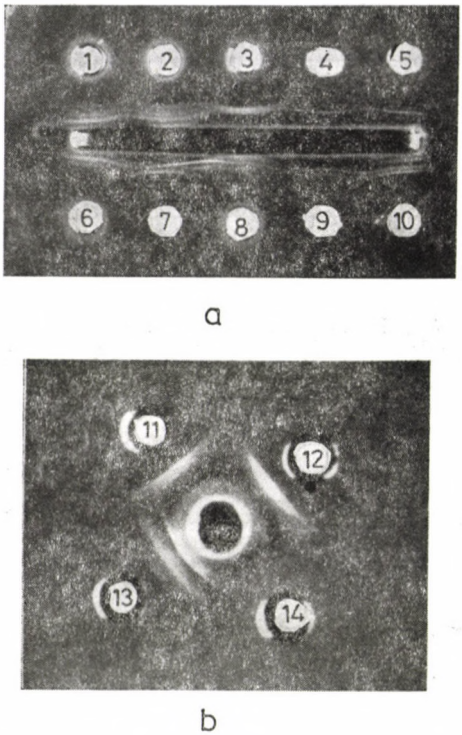

Fig. 5. Immunodiffusion in an agar gel. (A) Trough: antiserum to *P. aeruginosa* cells. Holes' 1 and 6, SF-6; 2 and 8, SF-3; 3, SF-5; 4, SF-4; 7 and 10, SF-2; 9, SF-1; 5, SF-7. (B) Central hole: antiserum to *P. aeruginosa* cells. Holes 11 and 12, Fl; 13, SF-8; 14, SF-9

SF-8 and SF-9, obtained from SF-1 by ion-exchange chromatography, turned out to be heterogeneous. (particularly SF-8). These subfractions are, perhaps, identical to one another in the antigenic sense. However, for SF-8 the precipitation line which is located nearer to the hole with serum is, perhaps, not identical to one or two precipitation lines of the initial SF-1.

Immunoelectrophoresis. Immunoelectrophoresis (Fig. 6) indicated that almost all subfractions had both cathodic and anodic components. For SF-7 the anodic components was quite pronounced, whereas the cathodic component very weak. A thoroughly purified SF-4 showed both anodic and cathodic components; in addition, a second cathodic component located nearer to the start was detected. Other subfractions proved to be rather heterogeneous.

Passive protection of mice. Since SF-4 and SF-7 were the most homogeneous and practically free from LPS contamination, rabbits were immunized with them to obtain antisera (see Materials and methods). Passive protection

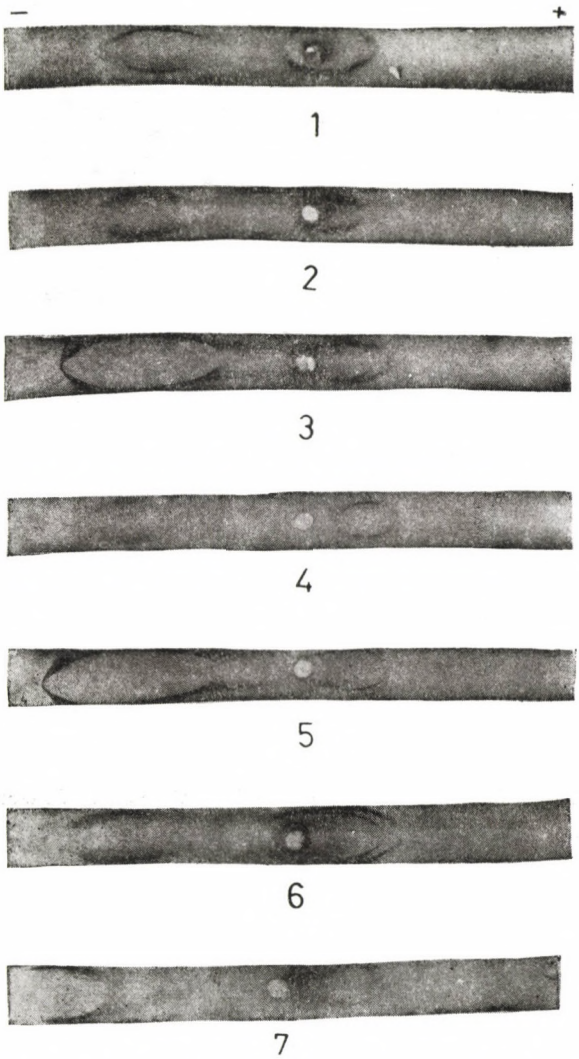

Fig. 6. Immunoelectrophoresis. Troughs: antiserum to *P. aeruginosa* bacteria. Holes: 1, SF-1; 2, SF-2; 3, SF-3; 4, SF-4; 5, SF-6; 6, SF-5; 7, PPAE

experiments with mice (Tables II and III) showed that anti-SF-4 and anti-SF-7 sera possessed well-defined protective properties. Their activity was practically identical, and considerably higher than that of a preimmune serum.

Discussion

Using step-wise salt fractionation, followed by gel and ion-exchange chromatography, nine subfractions (SF) were isolated from a partially purified aqueous extract (PPAE) obtained from acetone-treated *P. aeruginosa* bacteria.

Table II

Passive protection of mice immunized with anti-SF-4 and anti-SF-7 sera
Determination of LD values for the challenge strain
P. aeruginosa 8

Immunization with serum	Ratio of the number of killed mice to the total number of inoculated animals; challenge dose × 10^6 bacteria								LD_{50} and confidence intervals] (× 10^6 bacteria)	EI**
	800	400	200	100	50	25	12.5	6.25		
Anti-SF-4	30/30	30/30	14/30	3/30	1/30	1/30	—	—	182 (155–214)	7
Anti-SF-7	30/30	24/30	14/30	4/30	1/30	0/30	—	—	214 (177–259)	8.2
Normal (control 1)	30/30	30/30	29/30	28/30	17/30	10/30	—	—	41 (34–49)	1.6
Non-immunized mice (control 2)	—	—	29/30	30/30	24/30	14/30	7/30	0/30	26 (19–34)	1

* Confidence intervals for p = 0.05
EI (efficiency index) = $\frac{LD_{50} \text{ in experiment}}{LD_{50} \text{ in control 2}}$

Two of the subfractions, SF-4 and SF-7, contained but trace amounts of LPS (0.05 and 0.07%, respectively) and consisted almost exclusively of cell proteins, presumably those of the outer membrane (OM). The molecular weights of

Table III

Passive protection of mice immunized with anti-SF-4 and anti-SF-7 sera
Determination of the ED_{50} values for the sera

Immunization with sera	Ratio of the number of survived mice to the total number of challenged animals; challenge dose 200 × 10^6 bacteria (7.1 LD_{50})				ED_{50} and confidence intervals
Dilution of sera	1 : 50	1 : 250	1 : 1250	1 : 6250	
Anti-SF-4	17/20	12/20	3/20	2/20	1 : 377 (1 : 155–1 : 776)
Anti-SF-7	25/30	18/28	3/28	1/20	1 : 372 (1 : 309–1 : 448)
Normal	4/28	3/28	1/28	1/20	< 1 : 50

Immunization with sera	Ratio of the number of survived mice to the total number of challenged animals; challenge dose 100 × 10^6 bacteria (3.6 LD_{50})				ED_{50}
Dilution of sera	1 : 50	1 : 250	1 : 1250	1 : 6250	
Anti-SF-4	19/20	15/20	15/20	5/20	1 : 1738 (1 : 776–1 : 3890)
Anti-SF-7	28/28	24/28	19/20	10/20	1 : 4625 (1520–1 : 10 409)
Normal	5/28	2/28	3/28	6/28	< 1 : 50

Challenge control (nonimmunized mice)	Ratio of the number of killed mice to the total number of challenged animals; challenge dose ×10^6 bacteria						LD_{50} (× 10^6 bacteria)
	200	100	50	25	12.5	6.25	
	20/20	25/25	17/25	11/25	5/25	1/20	28 (22–35)

proteins in SF-4 were not higher than 30 000 ($S_{20m} = 2$), whereas in SF-5 they were in the range of 80 000–150 000. Thus, OM contains proteins of a molecular weight of at least 30 000 to 150 000 and higher. Previously we isolated proteins with molecular weights of 11 000 and 33 000 and also of 120 000–140 000 which displayed protective properties in direct and cross-protectiont experiments in mice [10]. Using a somewhat modified procedure, in the present work we isolated proteins of practically the same range of the molecular weight. However, the proteins studied earlier contained LPS contamination revealed by immunoelectrophoresis [10]. It may be assumed that the LPS contamination makes more specific or enhances as an adjuvant the immune protective response in mice.

From *P. aeruginosa* endotoxin an LPS-free protein was isolated, the common protective antigen (OEP) of which displayed the characteristics of a species-specific antigen [4–6]. In this work it was shown that isolated proteins with molecular weights of 30 000 and 40 000 stimulate rabbits to produce protective antibodies against *P. aeruginosa*. Thus, OM contains several proteins displaying protective activity.

Experiments with the *S. typhi-murium* model have shown that porins contaminated with LPS possessed pronounced protective activity, whereas IM proteins without LPS lost protective activity practically completely [3]. Our experiments with the *P.aeruginosa* model evidence, however, that LPS-free proteins of OM possess protective properties (Table II and III), although their serological activity (ELISA) is relatively low (Fig. 4).

It would appear that OM of Gram-negative bacteria contains, together with the type-specific 0 antigen (LPS), also species-specific antigens. Taking into account the data on the ability of OM proteins to stimulate protection against infection in mice with toxigenic strain PA-103 (9, 10, 13, 14), the OM proteins can be considered to be tentative candidates for a *P. aeruginosa* vaccine.

Our investigations on isolated extracellular slime [24] and cell proteins [9, 10] carried out previously, and the results of the present study show that several species- (or groups-) specific protective antigens are located on the surface of *P.aeruginosa*, namely, glycoprotein (capsule-like slime) and protein (OM proteins) antigens, as well as a type-specific O antigen (LPS). However, LPS exerted in some cases also a cross-protective activity [24].

Besides the antigens from the outer membrane described above, a protective antigen (or antigens) may, perhaps, be found within the cell, in particular in its cytoplasma. From the water-soluble extracts (cytoplasmic fraction of disintegrated bacteria) of *P. aeruginosa* the common protein antigen (CA) consisting of polypeptide subunits with a molecular weight of about 62 000 was isolated, while the initial protein in our opinion had a molecular weight ranging 665 000–900 000 [7, 8]. We believe that from these data [7]

one can hardly conclude that the isolated CA is a cytoplasmic antigen, since disintegration of bacteria seems to be accompanied with extraction of proteins of the cell enveloped. From the present experiments it may only be assumed that the isolated proteins are OM ones, since the cytoplasmic fraction of enterobacteria has been shown to possess a weak protective activity [1].

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EFFECT OF HEAT TREATMENT ON PHAGE SUSCEPTIBILITY OF A *MYCOBACTERIUM SMEGMATIS* STRAIN

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Plating efficiency of mycobacteriophage butyricum (By) proved to be 10^{-4}–10^{-3} on *Mycobacterium smegmatis* strain Rabinowitz (M. sm. R.) cells being in the logarithmic phase. This increased to 10^{-2}–10^{-1} when the cells were held at 50–57 °C for 2 h before infection. After replacing the cells to 37 °C, plating efficiency of the phages returned to the starting values. This phenomenon could be inhibited by nalidixic acid (150 μg/ml) and chloramphenicol (10 μg/ml) but not by mitomycin C (0.05 μg/ml). No return to the starting plating efficiency values were observed, if incubation of cells at 37 °C after heat treatment has been performed in buffer. The data suggest that By phage propagation in M. sm. R. cells is inhibited by a thermosensitive protein.

Phages of *Mycobacterium smegmatis* strain butyricum practically do not form plaques on cells of *Mycobacterium smegmatis* strain Rabinowitz, although their DNA penetrate into M. sm. R. cells [1].

It was published that incubation of *Mycobacterium avium* cells at 42 °C prior to exposure to phages affected their susceptibility to lysis: cells incubated at 37 °C were phage resistant, but after incubation at 42 °C six of nine strains proved to be phage-sensitive [2].

In this paper we describe experiments showing that there is a thermosensitive protein in M. sm. R. cells, which inhibits the lytic propagation cycle of mycobacteriophage butyricum in these cells.

Materials and methods

Bacterial and bacteriophage strains. The bacteria and bacteriophages used were *M. smegmatis* strain Rabinowitz (M. sm. R.), and its phage Rabinowitz (designated V72), and phage of the *M. smegmatis* strain butyricum (designated By).

Media, buffer and chemicals. Bacteria were maintained on nutrient agar slants, and YRP medium was used for the experiments [3]. To store and dilute phages 0.05 M trishydroxymethylaminomethane buffer (pH 7.4) containing 0.004 M $CaCl_2$ was applied. Nalidixic acid (Chinoin), mitomycin C (Sigma) and chloramphenicol (Sigma) were used to inhibit the metabolism of heat-treated cells.

Assay of phage titres. Phage titres were assayed by the double agar layer method of Adams [4]. Plates and soft agar overlayer containing 1.5 and 0.6% agar (Difco), respectively, were prepared from YRP medium.

Experimental conditions. In the first series of experiments bacteria grown overnight were centrifuged, resuspended in fresh medium to reach an absorbancy of 0.2–0.4 measured at

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660 nm. After incubating at 37 °C for 2 h the cells were centrifuged and resuspended in medium or in Tris buffer to reach again an absorbancy of 0.2–0.4 at 660 nm. Aliquots of the suspension were incubated at 50, 53, 55 and 57 °C, for 120 min. Samples were taken at 10–15 min intervals, and infected with phages By or V72. Plates prepared were incubated at 37 °C for 24 h and the plaques developed were counted.

In the second series of experiments M. sm. R. cells were incubated at 50 °C for 120 min, then quickly cooled to 37 °C and incubated for 5 h. Samples were taken at 30 min intervals an after infection with phages By or V72 plaque-formation was assayed as in the first series of experiments.

In the third series of experiments different quantities of nalidixic acid, mitomycin C or chloramphenicol were added to the cells cooled from 50 to 37 °C, then the same procedure was carried out as in the second series of experiments. Nalidixic acid, mitomycin C or chloramphenicol were removed by centrifugation before phage infection.

Results and discussion

The effect of heat treatment on the plaque forming ability of phage By on M. sm. R. cells is shown in Fig. 1. The number of plaques increased with the time of heat treatment and the increase proved to be the quicker the higher

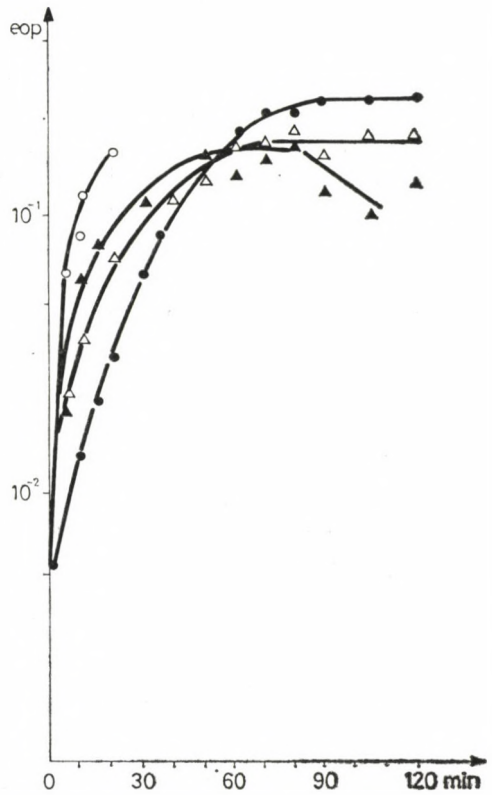

Fig. 1. Effect of heat treatment of M. sm. R. cells on the plating efficiency (eop) of phage By. Titre on the original host (M. sm. b) cells was taken as the unit. Temperature of heat treatment: ● 50 °C; △ 53 °C; ▲ 55 °C; ○ 57 °C

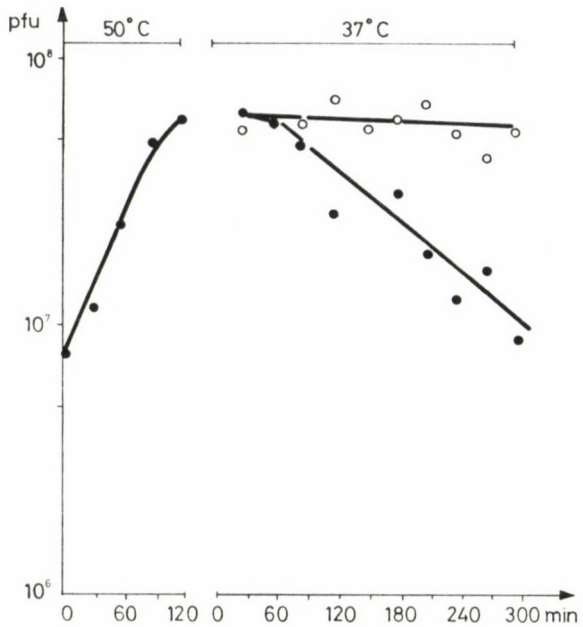

Fig. 2. Plaque formation (pfu) of phage By on M. sm. R. cells held at 50 °C for 2 h and then replaced to 37 °C. ● M. sm. R. cells in YRP medium; ○ M. sm. R. cells in Tris buffer

temperature had been applied. Heat treatment of cells in medium or in buffer resulted in similar increase of plating efficiency. Control titration of phage V72 on heat-treated M. sm. R. cells revealed no influence of heat treatment on plating efficiency.

In the second series of experiments heat treatment was carried out at 50 °C which is not lethal to the cells. All M. sm. R. cells survived a two-hour heat treatment. Figure 2 shows that if plaque-forming ability of By phages was assayed on M. sm. R. cells treated first at 50 °C then incubated further at 37 °C, the number of plaques approached the same values as on M. sm. R. cells not exposed to heat. This phenomenon could not be observed when Tris buffer was used instead of culture medium.

On the basis of these results we suggested the presence of a heat labile factor in M. sm. R. cells inhibiting lytic propagation of phage By.

To study this hypothesized thermosensitive factor, in the third series of experiments the effect of nalidixic acid, an inhibitor of DNA gyrase [5–7], mitomycin C, an inhibitor of DNA synthesis, or chloramphenicol, an inhibitor of protein synthesis, was analysed. Subbacteriolytic concentrations of these compounds determined in preliminary experiments were used.

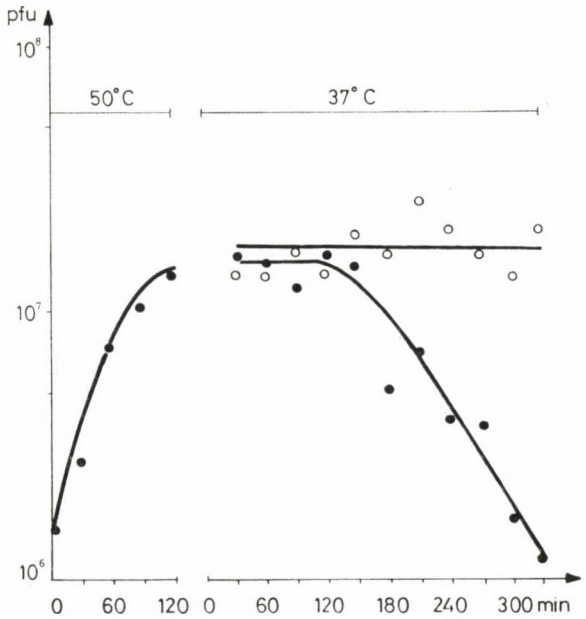

Fig. 3. Effect of nalidixic acid on the plaque formation (pfu) of By phages on M. sm. R. cells held at 50 °C and then replaced to 37 °C; ● incubation at 37 °C in the absence of, and ○ in the presence of nalidixic acid (150 μg/ml)

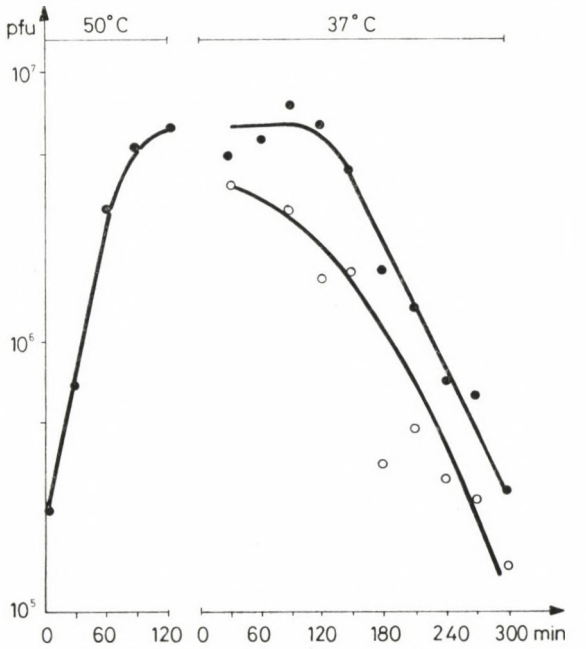

Fig. 4. Effect of mitomycin C on the plaque formation (pfu) of By phages on M. sm. R. cells held at 50 °C and replaced to 37 °C; ● incubation at 37 °C in the absence of, and ○ in the presence of mitomycin C (0.05 μg/ml)

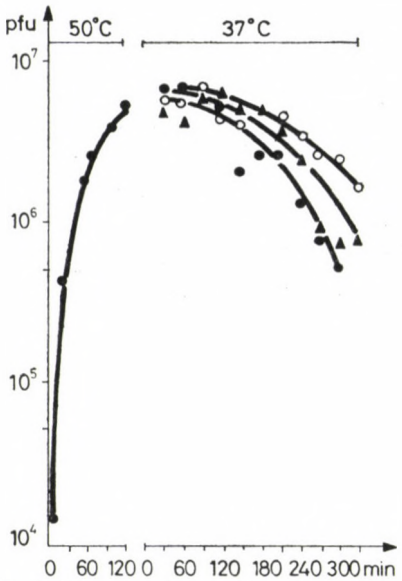

Fig. 5. Effect of chloramphenicol on the plaque formation (pfu) of By phages on M. sm. R. cells held at 50 °C and replaced to 37 °C; incubation at 37 °C in the absence (●), and in the presence of 5.0 μg/ml. (▲) and 10 μg/ml (○) chloramphenicol

Nalidixic acid (150 μg/ml) inhibited "regeneration" of the thermosensitive factor, as the number of plaques remained unchanged, when titrated on M. sm. R. cells replaced from 50 °C to 37 °C (Fig. 3).

Figure 4 shows that mitomycin C had no effect, as after adding of 0.005–0.05 μg/ml mitomycin C, the decrease in the number of plaques at 37 °C was similar as in the absence of this DNA synthesis inhibitor. Accordingly, DNA synthesis is not required for re-establishing the phage restricting ability of M. sm. R. cells.

To inhibit protein synthesis, 2.5–10 μg/ml chloramphenicol was added to the medium. Figure 5 shows that the higher the chloramphenicol concentrations were, the decrease in the number of plaques was the slower, indicating that for "regeneration" of the hypothesized thermosensitive factor protein synthesis de novo is necessary. These data prove that the heat labile factor inhibiting lytic propagation of phage By is of protein nature.

It would be only too natural to say that this protein is a restriction endonuclease, as data in the literature show for different bacterium-phage systems that heat-treatment of bacteria destroys this enzyme thus allowing propagation of phages otherwise restricted [8–10]. Our earlier results, however, contradict to this explanation: we isolated an M. sm. R. strain lysogenized by phage By and phages liberated from this strain proved to be not modified [1]. Thus, further experiments are needed for studying the nature and biological role of the heat labil protein described.

Acknowledgement. Our thanks are due to Mrs Ágnes Zathurecky for skilled technical assistance.

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EFFECT OF MANNOZYM ON THE CHEMILUMINESCENCE OF PHAGOCYTES

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Mannozym (zymosan) induces chemiluminescence (CL) in human neutrophils, monocytes and in the cells of C4Mφ murine macrophage cell line. The CL enhancing effect of Mannozym opsonized in human serum is higher than that of non-opsonized material. This may be due to the capacity of Mannozym to bind complement components and immunoglobulins from serum and to activate the phagocytes via their C3b and Fc receptors. Besides, Mannozym can be phagocytozed both in opsonized and non-opsonized forms.

Mannozym^R is a glucomannan cell wall derivative of *Saccharomyces cerevisiae* and a "zymosan-type" product of the Human Institute for Serobacteriological Production and Research (Hungary). This material is indicated for enhancement of non-specific immunity and to prevent radiodermatitis in X-ray therapy of tumours and to decrease tumour cell dissemination [1–3]. Fachet and his coworkers have demonstrated that Mannozym increases both the cellular and humoral immune responses against a synthetic antigen and modulates the defence against tumour in inbred mice [4].

It is known that zymosan particles, e.g. Zymosan-A (Sigma) can induce chemiluminescence in both neutrophils and macrophages [5–7] and can activate the alternative pathway of complement activation [8].

The present study was designed to investigate the effects of Mannozym on the chemiluminescence induced in human neutrophils, monocytes and in the cells of the C4Mφ macrophage cell line. A particular attention was paid to study the difference in the CL inducing effect of Mannozym in opsonized and non-opsonized forms.

Materials and methods

Materials. Zymosan-A (Sigma) and superoxide dismutase (SOD, Sigma) were used. The original ampoules contain 1 mg of Mannozym suspended in 1 ml of physiological saline. In the experiments we used Mannozym in phenol red-free Hanks balanced salt solution (HBSS).

Measurement of complement consumption by Mannozym. Mannozym was suspended at different concentrations in HBSS and were mixed with equal volumes of human sera. After

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an incubation for 30 min at 37 °C, the mixtures were centrifuged for 10 min at 1000 *g*. Mannozym sedimented to the bottom of the tubes and the supernatants were collected. The pellets of Mannozym were dissolved in HBSS and were used as "opsonized Mannozym" (OM).

The supernatants were tested for their remaining total complement activities using equal volumes of the 2% suspensions of sheep red blood cells sensitized with specific rabbit antibody (haemolysin). The extent of complement consumption during the opsonization of Mannozym was calculated as a difference between the total and residual complement activities, expressed in per cent. The effect of Mannozym of the alternative complement pathway was measured according to Riches and Stanworth [9].

Preparation of human neutrophil granulocytes. Heparinized human blood was diluted in 6% dextran in saline (Macrodex/ m.w., Pharmacia, Sweden). The cells were allowed to sediment for 45 min at room temperature. The leukocyte-rich plasma was removed, centrifuged, and the pellet was treated with 0.83% ammonium chloride in order to lyse red cells. The remaining leukocytes were neutrophils in 65–83 and viable in 99–100%. The cells were washed and resuspended in HBSS [6].

To prepare human monocytes, 8×10^6 mononuclear cells (lymphocytes, monocytes) derived from heparinized human blood after a gradient centrifugation on Ficoll-Uromiro, were allowed to adhere in Parker's 199 medium in Petri dishes at 37 °C for 4 h. The non-adhering cells were removed by washing three times with HBSS. The monocytes were collected from the glass surface by rubber. The ratio of monocytes was above 80% [10].

Murine macrophage cell line (C4Mφ). The establishment of a continuous murine macrophage cell line coded as C4Mφ has been reported previously by Zákány et al. [11]. These cells were routinely grown as monolayers in RPMI 1640 with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland). The cells were removed from the glass surface with rubber. Trypsinization and the use of chelating agents were avoided throughout the procedure. The cells were washed three times with HBSS and the density was adjusted to 4×10^6/ml. The viability was above 95% tested by trypan-blue exclusion.

Measurement of chemiluminescence. In a final volume of 2 ml, 2×10^6 cells of neutrophils, monocytes and C4Mφ macrophages were incubated with 1 mg/ml dose of Mannozym or opsonized Mannozym in plastic tubes at 37 °C for 10 min. Then the CL was measured in glass vials (preheated at 37 °C) using the Nuclear Chicago Isocop/300 liquid scintillation counter (Searle Industries, Des Plains, USA) in the off coincidence mode [12, 13]. The total number of photons in cpm, measured five times repeatedly at 5 min intervals was regarded as a characteristic value for a sample. The inherent, non amplified (luminol-free) CL was measured.

Results

Opsonization of Mannozym at various concentrations in human sera results in a dose-dependent decrease of the residual total complement activities. Namely, a dose-dependent increase in the total complement consumption and in the consumption of the factors of alternative complement activation can be observed, as it is demonstrated in Table I.

Table I

Complement consumption by Mannozym in human serum

Concentration of Mannozym (mg/ml)	Complement consumption (%)	
	in alternative pathway	in total activity
0	0	0
0.25	45	24
0.50	82	62
1.00	85	90

Comparing the time dependence of CL induced by 1 mg/ml of opsonized Mannozym in human neutrophils, monocytes and in C4Mφ macrophages, the highest peak of CL curve can be measured in the neutrophils, the smallest one in the C4Mφ cells. It is common in all the three systems that the peaks of CL are found within the 10th and 30th min of the stimulation. These data are shown in Fig. 1.

Opsonized Mannozym induces a significant CL in all the three types of cells already at the quantity of 0.25 mg/ml. Increasing the dose of opsonized Mannozym up to 1 mg/ml. a plateau can be observed in the CL. Therefore, we used this dose of opsonized Mannozym in the further studies. Zymosan-A (Sigma) opsonized in human sera increases the CL of neutrophils more intensively than the same quantity of opsonized Mannozym. Inactivation of the complement system at 56 °C for 45 min in human sera used for opsonization of Mannozym expressively decreases its CL inducing effect in neutrophils. These data are shown in Fig .2.

Both opsonized and non-opsonized forms of Mannozym induce CL in all the three types of cells. However, CL provoked by non-opsonized Mannozym is much less than that elicited by the opsonized one (Fig. 3). Opsonization of Mannozym with human IgG dose-dependently increases the CL of human neutrophils (Fig. 4).

Human IgG added simultaneously with Mannozym opsonized with human IgG, dose-dependently inhibits the production of CL by neutrophils. Human IgG at the dose of 50 μg/ml induces a slight increase in CL of neutrophils. These data are shown in Fig. 5.

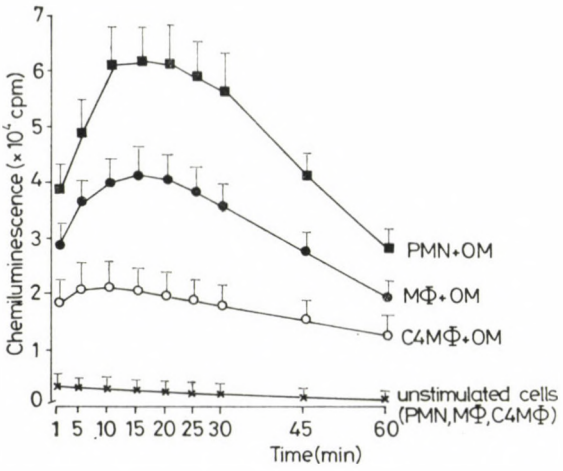

Fig. 1. Time dependence of chemiluminescence of human neutrophils (PMN), monocytes (Mφ) and murine macrophage cell line (C4Mφ) by Mannozym opsonized in human serum (OM). Means $\pm$ SD, n = 4

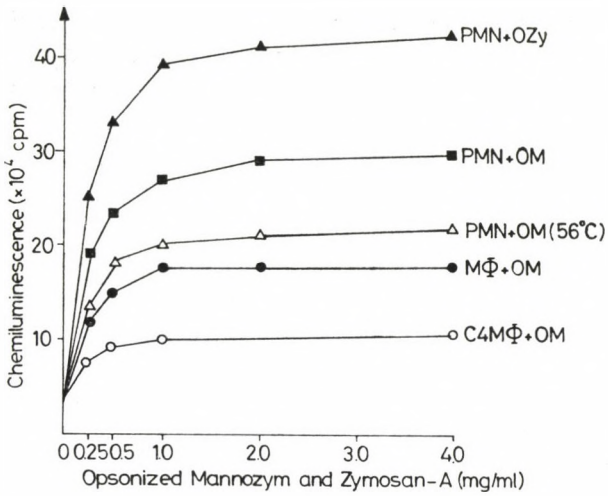

Fig. 2. **Dose dependence of chemiluminescence of human neutrophils (PMN), monocytes (Mφ) and murine macrophage cell line (C4Mφ) by Mannozym opsonized in human serum (OM) and Zymosan-A opsonized in human serum (OZy). Means ± SD, n = 5**

Chemiluminescence produced by human neutrophils can be partly inhibited by superoxide dismutase (SOD). These data are demonstrated in Fig. 6.

Mannozym either opsonized in serum (Fig. 7) or in non-opsonized form (Fig. 8) can be phagocytized by human neutrophils. The particles of Mannozym have a diameter of 2–4 μm.

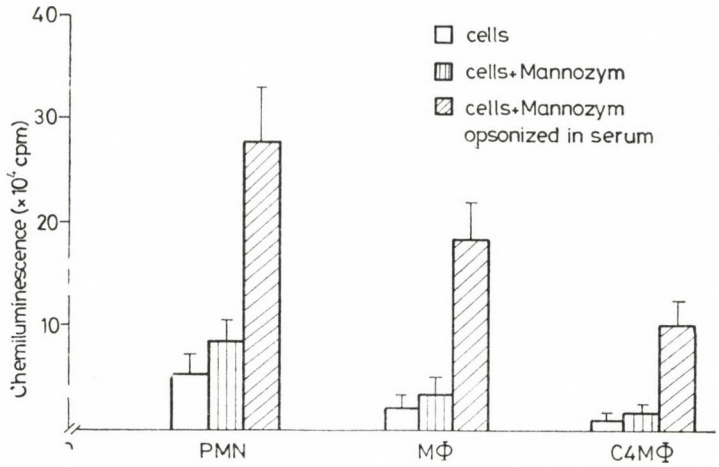

Fig. 3. **Effect of Mannozym opsonized and non-opsonized in human serum on the chemiluminescence of human neutrophils (PMN), monocytes (Mφ) and murine macrophage cell line (C4Mφ). Means ± SD, n = 6**

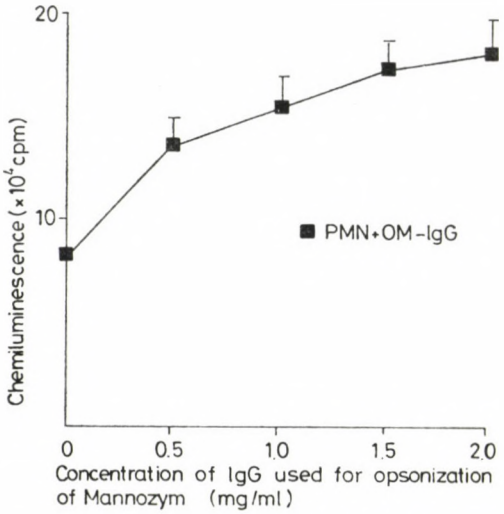

Fig. 4. **Effect of Mannozym opsonized with human IgG (OM-IgG) on the chemiluminescenc of human neutrophils (PMN). Means ± SD, n = 5**

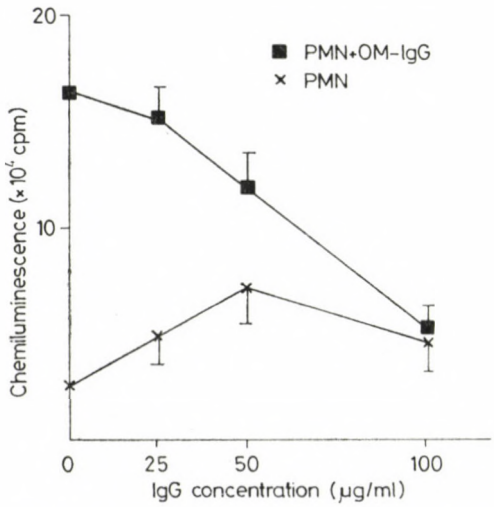

Fig. 5. **Effect of human IgG on the chemiluminescence induced by Mannozym opsonized with human IgG (OM-IgG) in human neutrophils (PMN). Means ± SD, n = 3**

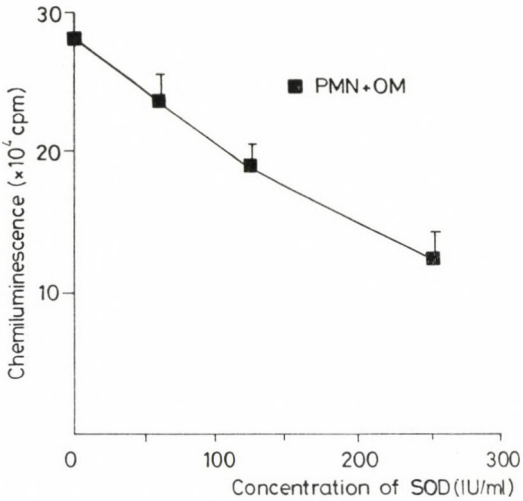

Fig. 6. Effect of superoxide dismutase (SOD) on the chemiluminescence induced by Mannozym opsonized with human serum in human neutrophils (PMN). Means ± SD, n = 3

Fig. 7. Phagocytosis of the particles of Mannozym (empty spherules) opsonized with human serum by a human neutrophil granulocyte

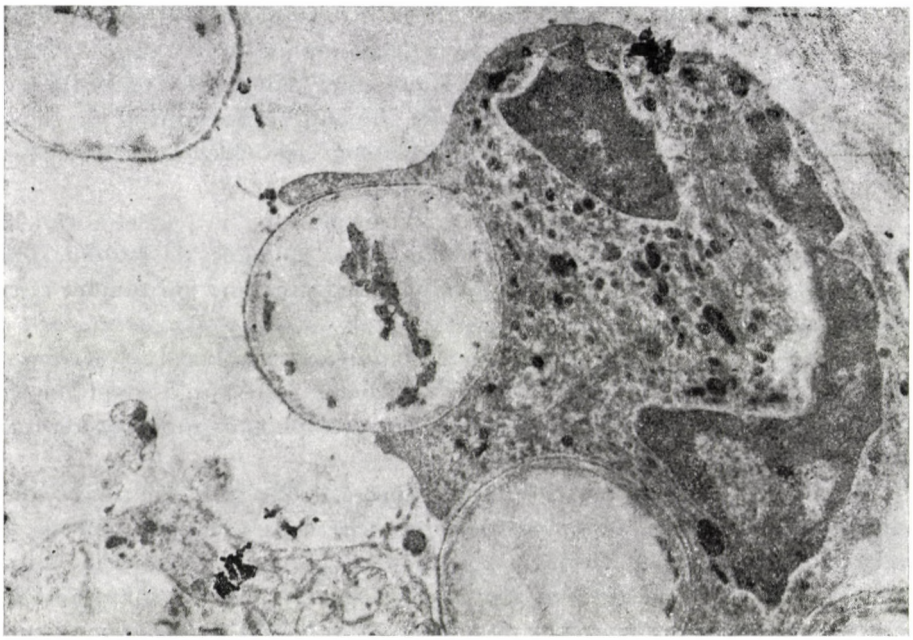

Fig. 8. Phagocytosis of the particles of non-opsonized Mannozym (empty spherules) by a human neutrophil granulocyte

Discussion

It is already known that opsonized zymosan stimulates the production of CL in neutrophils and macrophages [7], activates the alternative pathway of the complement activation [14], and binds complement factors and IgG [5].

Our results show that Mannozym, this zymosan product represents all these characteristic features: stimulates the CL of human neutrophils, monocytes and C4Mφ cells in both opsonized and non-opsonized forms (the production of CL by the cells is much higher in opsonized form than in non-opsonized one); activates the alternative pathway of complement activation; binds complement factors and IgG. In contrast to Zymosan-A (Sigma) which has a diameter of 0.1 μm, the diameter of Mannozym is about 3 μm. Besides, the CL inducing effect of Zymosan-A opsonized in human sera is higher than that of Mannozym respresenting the same quantity (1 mg/ml). The CL decreasing effect of SOD suggests that superoxide anions are generated during the stimulation of neutrophils and macrophages by opsonized Mannozym. However, other oxygen derivatives, e.g. singlet molecular oxygen and hydroxyl radicals can also be produced, because SOD — even at high concentrations — is not able to abolish completely the increase of CL in the neutrophils stimulated by opsonized Mannozym.

The phagocytosis of particles — both in opsonized and non-opsonized forms — can be very important factor in the complex in vivo effects of Mannozym. It seems likely that opsonized Mannozym (binding C3b and IgG) can be phagocytozed very easily and fast by the C3b and Fc receptors of phagocytes inducing an intensive CL, too; meanwhile the non-opsonized particles also can be endocytozed by these cells but at a less extent.

Finally, both ways of the phagocytosis of Mannozym particles lead to a rather permanent loading and activation of the phagocytic system. These possibilities may be involved in the immunomodulating and antitumour effects of Mannozym [1–4].

Besides, the measurement of chemiluminescence induced by Mannozym opsonized with human IgG seems to be a rather sensitive and simple method for the detection of Fc receptor activity of neutrophils or other Fc receptor-bearing phagocytes.

We tested the C4Mφ murine macrophage cell line from the aspect of the CL-inducing effect of Mannozym because these cells are homogeneous and standardized in contrast to the suspensions of neutrophils and monocytes prepared from different donors. However, it is also obvious that the C4Mφ macrophages do not produce such an intensively inherent CL as human neutrophils and monocytes. Therefore, when C4Mφ macrophages are used for CL studies, the application of luminol is also advised.

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Acta Microbiologica Hungarica 33 (4), pp. 271—283 (1986)

DESERTOMYCIN: A POTENTIALLY INTERESTING ANTIBIOTIC

(A REVIEW)

J. V. URI

Research and Development Division Smith Kline and French Laboratories, Philadelphia, PA, USA

(Received February 3, 1986)

CONTENTS

Introduction

Desertomycin is a stable, crystalline non-polyene macrolide antibiotic of *Streptomyces* origin with an apparent molecular formula of $C_{61}H_{109}NO_{21}$. It has in vitro broad antibacterial and selective antifungal activity as well as considerable cytotoxic/cytolytic effect. It induces morphological changes on filamentous fungi. Presently available experimental data suggest that the mechanism of action of desertomycin, primarily involves alteration of membrane permeability or direct damage to the cell membranes. The acute toxicity of desertomycin in mice is dependent on the route of administration. This review will present, organize and interpret the published data related to the chemistry and biology of desertomycin, and speculates on further studies and possibilities.

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Acta Microbiologica Hungarica 33, 1986

Producing microorganisms

Desertomycin was first described in 1959 as a coproduct of flavofungin produced by *Streptomyces flavofungini* [1] which was isolated from a soil sample collected in the Sahara desert [2–5]. I coined the name "desertomycin". The first half of the name, "desert" denotes the origin of the producing strain, while the second half of the word "mycin" expresses that the antibiotic is biosynthesized by a *Streptomyces* strain. *S. flavofungini* was classified as a novum species, not known before, on the basis of its morphological, physiological and biochemical properties [3–5]. The culture appearance of *S. flavofungini* is highly characteristic. On Czapek-Dox agar, containing an inorganic nitrogen source and sucrose, *S. flavofungini* produces snow-white aerial mycelia and spores. On complex-organic media especially with high (up to 4%) glucose content, it develops greenish-yellow, wrinkled, crumbly, asporogenic colonies similar to those of *Mycobacterium* and *Nocardia* [6, 7]. These dual culture forms of *S. flavofungini* are shown in Fig. 1.

On the basis of these characteristic cultural-morphological features I reisolated the organism in 1970 from a soil sample collected in the cactus section of the Desert Garden in Huntington Park, Pasadena, California. This strain is identical to the original *S. flavofungini* strain based not only on appearance in culture but also on antibiotic production. It was deposited in the SK&F Culture Collection under the designation of BC-2031 *(S. flavofungini)*. It is an interesting coincidence that both cultures were isolated from desert soil. Today these are the two strains known to have the ability to co-produce flavofungin and desertomycin. The other described *Streptomyces* strains produce either solely flavofungin [8], or only desertomycin [9].

Recently a new *Streptomyces* strain was reported to produce desertomycin [9]. The isolation of this strain resulted from a systematic soil screening study for antibiotic producing microorganisms. The strain produced an antibiotic identical to desertomycin by direct comparison of data and was designated as *Streptomyces macronensis* Dietz sp. nov. UC 8271 (NRRL 12566).

Isolation and purification

Desertomycin can be isolated either from the mycelial mass or from the fermentation medium. In both cases the fermentation medium must be a rich, complex organic culture medium. In the case of *S. flavofungini* fermentation more antibiotics (flavofungin and desertomycin) were contained in the mycelia than in the fermentation broth; therefore, it was more practical to isolate them from the mycelial mass obtained in laboratory and/or pilot plant fermentors and separated from the broth by conventional techniques. The isolation method

Fig. 1. The two culture forms of *S. flavofungini*. On Czapek-Dox agar containing inorganic nitrogen and sucrose, it forms smooth growth covered with snow-white aerial mycelia and spores (left) and produces only desertomycin. On complex-organic media with 4% glucose it develops greenish-yellow, wrinkled, crumbly asporogenic colonies (right) and produces both flavofungin and desertomycin

was, in summarized form, as follows [1, 8, 10, 11]. After extraction of flavofungin from the biomass with hot ethyl acetate, desertomycin was extracted by boiling the wet mycelial mass under reflux with n-butanol. Evaporation of the butanol resulted in a dark brownish powder. The coloured impurities were gradually removed by repeated treatment with activated charcoal in boiling butanol. From the purified yellowish product, the desertomycin was crystallized from boiling water-saturated n-butanol containing 10–30% methanol. After cooling at room temperature, desertomycin separates out in glittering snow-white hexagonal crystals (Fig. 2). It can also be obtained in quadratic,

Fig. 2. Hexagonal crystals of desertomycin

columnar and even needle-like crystals (with identical physicochemical data) depending on the degree of purity of the starting amorphous materials, the concentration of the methanol in the crystallization mixture, and also the presence of possible isomer(s).

S. macronensis, in contrast to *S. flavofungini*, does not produce flavofungin and it secretes the desertomycin into the culture media under submerged conditions in shaken flasks [9]. Desertomycin was preferably isolated from the filtered culture broth at the peak antibiotic titre (1–2 days shaken incubation at 28 °C) by adsorption to XAD-2 resin in column. After washing the column, the desertomycin was eluted with water-acetone (1 : 1), and lyophilized. The dark solid thus obtained was dissolved in methanol-water (2 : 1), and percolated over an LH-20 Sephadex bed. Methanol was evaporated and the aqueous solution was treated with DEAE cellulose to remove the coloured impurity. The colourless solution obtained was lyophilized and yielded virtually pure desertomycin. Several methods were used for further purification to remove traces of coloured impurities. The best product was a white, fluffy, amorphous solid which did not crystallize.

During the isolation and purification desertomycin was detected either by microbiological agar diffusion assay (*Bacillus subtilis* ATCC 6633 or *Micrococcus luteus* UC 130 as indicator bacteria) or by chromatography (paper or TLC on silicagel plates; microbiological or chemical visualization).

Chemical and physical reactions

Before the chemical structure of desertomycin has been published, considerable amounts of information have been accumulated related to its characteristic chemical and physical reactions as well as identification of its hydrolysis products [9–11].

Desertomycin is only sparingly soluble in distilled water at room temperature, but warming increases the solubility. The aqueous solution is very stable and shows capillar activity. It is more soluble in water-containing polar solvents, in aqueous acetone and acetic acid. It is almost completely insoluble in ethyl acetate. The melting point of crystalline desertomycin is 185–186 °C with decomposition. It has characteristic infrared and ^{13}C NMR spectra and an UV absorption maximum at 225 nm in water and also in pH 7.2 phosphate buffer (Fig. 3). Desertomycin behaves like a week base (potentiometric titration); it also exhibits amphoteric behavior which is reflected in its pH-paper chromatogram (Fig. 4). Using water as the developing solvent it runs far at low pH values (2.2, 3) on the buffered (McIlvaine [12] and phosphate buffers) paper strips with high R_f values (1.0 and 0.5, respectively), does not move at pH 6 and 7, and starts moving again with increasing (8, 9, 10) pH values. Figure 4 is

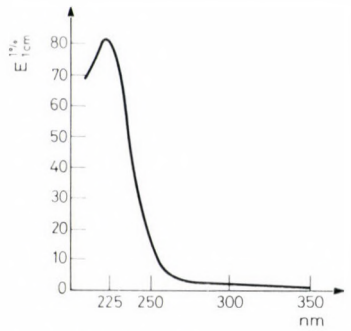

Fig. 3. UV absorption spectrum of desertomycin

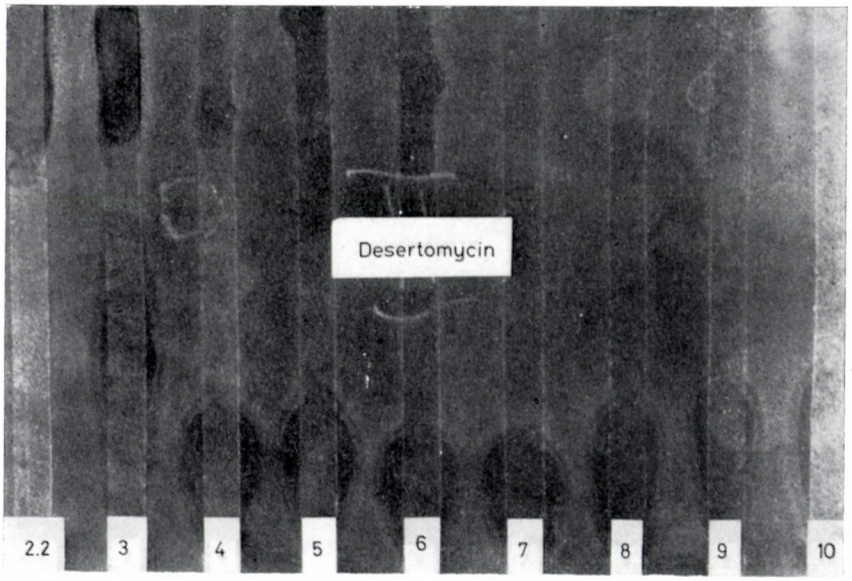

Fig. 4. pH-Paper-chromatogram of desertomycin. The Whatman No. 1 paper strips were impregnated with the buffer solutions. The numbers on the paper strips indicate the pH values. Paper strips were developed in distilled water. Desertomycin activity (inhibition zones) was indicated on agar plates seeded with *B. subtilis* ATCC 6633 spores

a bioautography using an agar plate seeded with spores of *B. subtilis* ATCC 6633 as the indicator microbe for detection of desertomycin activity (darker inhibition spots, no bacterial growth).

Desertomycin gives positive Ehrlich, Molisch and Malaprade tests. The violet-blue Ehrlich colour reaction is specific, as is its colour reaction with phenol. These reactions can be used for its quantitative estimation. With vanillin, desertomycin in concentrated hydrochloric acid develops a violet colour which darkens on standing at room temperature.

Structural investigations/considerations

Mild acid hydrolysis of desertomycin yields mannose, but not glutamic acid, as reported earlier. Desertomycin is the first known antibiotic which contains D-mannose as the only carbohydrate moiety, probably bound *O*-glycosidically.

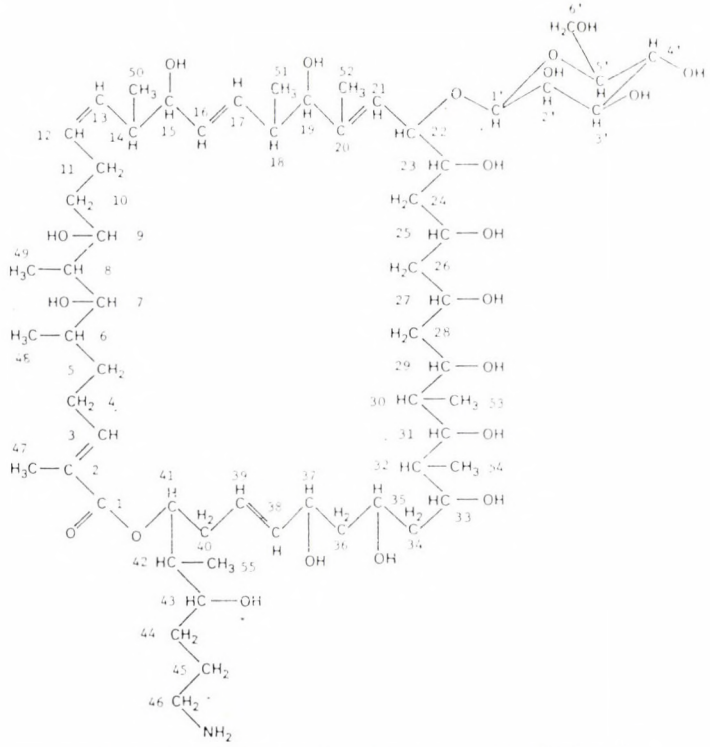

Fig. 5. Chemical formula of desertomycin (Ref. 13)

The molecular composition of desertomycin was determined recently with the conclusion that the molecular formula is $C_{61}H_{109}NO_{21}$ (mol wt 1192). In the same paper the structural formula of desertomycin was published [13]. It is a large ring macrolide (Fig. 5) similar to azalomycin F_{4a}, monazomycin, niphithricins A and B, primycin and scopafungin [9].

The uniqueness of desertomycin was also demonstrated by salting-out paper chromatography [14], the method first published in 1953 [15], and accepted as a useful technique for classification of antibiotics [16]. In this method antibiotics are examined by ascending paper chromatography using aqueous solutions of ammonium chloride at seven increasing concentrations (0.5–20% and saturated solution) and distilled water, as developing solvents. In this system desertomycin was the only antibiotic which produced a unique picture [17].

Its R_f values are O in distilled water and saturated ammonium chloride solution. The R_f value is maximal (approximately 1) in 5% salt solution and, the R_f values gradually decrease both directions to form a complete paraboloid curve [14], as shown in Fig. 6. This type of paper chromatogram is typical for the macrolides such as oleandomycin, erythromycin and carbomycin, but their curves can be considered as truncated parabolas. The descending part of the curves, as expressed by the R_f values in saturated ammonium chloride solution, are proportional to the molecular weight of these large cyclic macrolide antibiotics. Fig. 7 demonstrates that the R_f value in saturated ammonium chloride

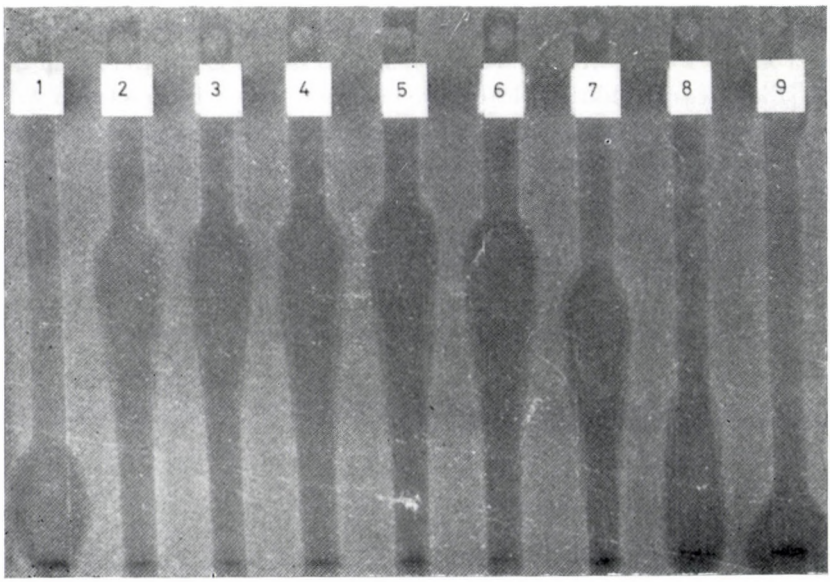

Fig. 6. Salting-out paper chromatogram of desertomycin. Developing solvents are from left to right: 1. distilled water; 2. through 8 ammonium chloride solutions as follows: 0.5, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0%; and 9. saturated ammonium chloride solutions. Desertomycin activity was detected as in Fig. 4

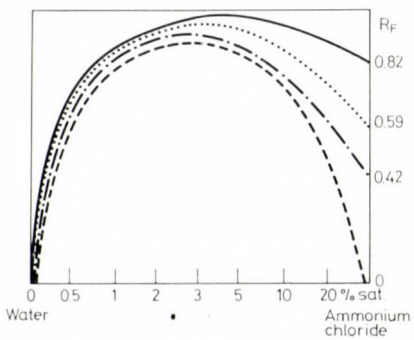

Fig. 7. Curves of salting-out paper chromatograms of four macrolide antibiotics. The R_f values in saturated ammonium chloride solution are inversely related to the molecular weight of the antibiotics. —— oleandomycin; · · · erythromycin; — · — carbomycin; — — desertomycin

solution for oleandomycin is 0.82 and its molecular weight is 689; and that these values (R_{fs} and mol wts) are for erythromycin 0.59, 733; for carbomycin 0.42, 844 and for desertomycin 0, 1191, respectively. The greater the molecular weight, the lower is the corresponding R_f value.

Additional data on the chemistry of desertomycin can be found in the individual relevant publications (see references).

Antibacterial activity

The antibacterial activity of the crystalline desertomycin was examined against a number of bacterial isolates in liquid media using the serial dilution method [1]. It was found that desertomycin has a broad antibacterial spectrum with minimum inhibitory concentrations of 1–25 μg/ml against most strains. Some strains, including *Pseudomonas aeruginosa*, needed higher concentrations than 25 μg/ml desertomycin. Table I demonstrates these findings.

Table I

Antibacterial activity of desertomycin

Strains	Inhibitory concentrations (μg/ml)
Bacillus subtilis (ATCC 6633)	1–5
Bacillus subtilis (streptomycin resistant)	1–5
Bacillus subtilis (penicillinase producing)	1–5
Bacillus megatherium	1–5
Staphylococcus aureus (Duncan)	10–25
Staphylococcus aureus (penicillin resistant)	10
Bacillus cereus 569	10
Escherichia coli 111	10–25
Escherichia coli S. (mouse pathogen)	10–25
Staphylococcus aureus (mouse pathogen)	25
Staphylococcus aureus	25
Staphylococcus aureus (polyresistant)	25
Micrococcus ureae	25
Streptococcus D group	>25
Salmonella typhi	>25
Escherichia coli	>25
Klebsiella pneumoniae	>25
Pseudomonas aeruginosa	>50

Antifungal spectrum

The in vitro antifungal activity of desertomycin was studied against a large number of filamentous (phytopathogenic and saprophytic) fungi, dermatophytes and strains producing systemic deep mycoses in man, as well as against yeasts and yeast-like fungi using broth [6] and agar dilution [7] methodology conventionally employed in the mycology.

Desertomycin was found to have a selective antifungal activity [6, 7]. It inhibited the growth (MIC = 10–50 μg/ml) of the overwhelming majority of the saprophytic and phytopathogenic fungi (Table II). However, with the exception of *Cryptococcus neoformans* (10 μg/ml), *Nocardia asteroides* (25 μg/ml) and *Achorion quinckeanum* (50 μg/ml), desertomycin does not influence the growth of the human-pathogenic fungi, yeasts and yeast-like fungi. In this respect it resembles the activity of actidione (cyclohexamide [18]), and can be used, similarly, for the selective isolation of human-pathogenic fungi and yeasts from clinical specimens [6, 19]. Desertomycin has several advantages over actidione: (a) it is heat stable and can be autoclaved within culture media without any loss of activity; (b) it remains stable in media for long periods of time; (c) it has additional antibacterial activity to suppress overgrowth by contaminating bacterial strains (actidione does not have any antibacterial activity); (d) it is more active against potential contaminating fungi than actidione and therefore can be added to the medium at about one-tenth of the concentration needed to obtain the same antifungal activity as actidione; and (e) unlike actidione it does not irritate or damage the skin and therefore does not require special precautions during handling. Table III illustrates the success rates which are attainable in the isolation of the pathogenic dermatophytes and/or yeasts in pure culture from human specimens sent to the laboratory on desertomycin containing Sabouraud-agar medium in comparison with control medium.

It is interesting, although not unexpected, that desertomycin strongly inhibits the growth of *Streptomyces* strains (which are classified as bacteria). Table IV shows that most of the *Streptomyces* strains tested are inhibited by 1 μg/ml (a few by 10 μg/ml) of desertomycin. The outstanding exception is *S. flavofungini*, the desertomycin-producing strain which is not inhibited even by 500 μg/ml concentrations [6]. This is an important phenomenon by which the antibiotic-producing organisms, by poorly understood mechanisms, remain insensitive or resistant to their own metabolite(s) and thus avoid "suicide" or "autotoxicity" [20–22]. *S. flavofungini* is such a strain. It is a useful mechanism by which the yield of antibiotic (desertomycin in this case) can increase during the producing (biosynthetic) period.

Table II

In vitro activity of desertomycin against saprophytic and phytopathogenic fungi

Strains	Inhibitory concentrations (μg/ml)
Penicillium notatum	10
Penicillium chrysogenum	25
Penicillium expansum	25
Penicillium glaucum	25
Penicillium novum hybrid	50
Penicillium janczewskii	50
Penicillium roqueforti	50
Penicillium funiculosum	25
Penicillium monoverticillatum	25
Botrytis allii	10
Botrytis cinerea	25
Helminthosporum sativum	10
Rhisoctonia solani	10–25
Fusarium monoliforme	25
Fusarium oxysporum	25
Fusarium culmorum	50
Alternaria solani	25
Alternaria dianthy	25
Alternaria cincinata	>100
Scopulariopsis brevicaulis	10–25
Cladosporium sp.	10
Colletotrichum lini	25
Phyllosticta betae	50
Monilia stiophyla	10–25
Trichotecium roseum	50
Aspergillus niger	50
Aspergillus flavus	>100
Aspergillus fumigatus	100
Aspergillus oryzae	>100
Aspergillus clavatus	100
Actinomucor repens	100
Synchitrium racemosum	100
Gibberella fujikuroi	>100
Neurospora sitophyla	>100
Rhizomucor sp.	>100
Rhizopus nigricans	>100
Circinella minor	>100
Cunninghamella echinulata	100
Stemphyllium spp.	25–50

Table III

The effect of the addition of desertomycin to culture medium in obtaining pure (positive) cultures from clinical specimens

Total No. of specimens	Total No. of isolates	Positive on both agars	Positive on desertomycin agar, negative on Sabouraud agar
308	172	57	115

Table IV

Anti-streptomycetes activity of desertomycin

Strains	Inhibitory concentrations (μg/ml)
Antinomyces hominis	1
Streptomyces ipomoe	1
Streptomyces gelaticus	1
Streptomyces gibsoni	1
Streptomyces griseus	1
Streptomyces griseus	10
Streptomyces erythreus	10
Streptomyces coelicolor	10
Streptomyces 1854	1
Streptomyces 1483	1
Streptomyces AF–2	1
Streptomyces BA–50	1
Streptomyces BA–64	<1
Streptomyces BA–65	<1
Streptomyces flavofungini	>500

Cytotoxic activity

In preliminary experiments desertomycin was found to exhibit considerable cytotoxic activity [1]. In the methylene blue-cell dehydrogenase inhibition system [23] to detect cytostatic and cytotoxic effects, desertomycin in low concentrations (10–30 μg/10^6 cells) inhibited the life-activity of white blood cells of healthy humans and rats as well as those obtained from human acute and chronic leukaemias as well as Ehrlich ascites tumour cells of mice [24, 25]. Some cytotoxicity against KB cells in agar has also been demonstrated [26]. In tissue cultures, desertomycin was found to be cytostatic (10 μg/ml) and in higher concentrations (50–100 μg/ml) cytolytic on fibroblast, HeLa and Crocker

Table V

Acute toxicity of desertomycin in mice

Route of administration	LD_{50} (mg/kg)
Intravenous	1.35
Intraperitoneal	2.6
Subcutaneous	5.3
Oral	12.5

cells [27]. Additional detailed studies are currently being carried out using the most developed battery of anti-neoplastic tests, to evaluate this kind of activity of desertomycin [28].

Toxicity data

At present only acute toxicity data in the mouse are available [1]. Table V shows that the LD_{50} values in mg/kg are dependent on the route of administration. Desertomycin is about ten times more toxic when injected intravenously than when given orally. Once the chemical structure is known, chemical modifications may be employed to increase the desired effect and decrease the toxicity.

Possible mode of action

On the basis of the currently available experimental data the primary site of action of desertomycin is the cell membrane. Like polyene antibiotics, desertomycin induces branching of the hyphae of *Botrytis cinerea* [29] and like griseofulvin it produces stunted forms of the young hyphae as well as branching and curling of the mycelia of *Botrytis allii* [30]. These phenomena reflect drug-induced alterations of the cell membrane. Desertomycin, like primycin, influences the electric properties of the membrane of cells and that of the T-tubules of the striated muscles of the frog [31].

It has also been demonstrated [29] that desertomycin, like amphothericin B, exerts direct damage in low concentrations (2–20 μg/ml) on the cell membranes of human erythrocytes, red beet and mycelia of *Eremothecium ashbyi*. The damage caused by desertomycin on cell membranes resulted in lysis of the cells and leakage of cytoplasmic materials; haemoglobin, anthocyanin and riboflavin, respectively. In addition to the cell membrane permeability changes induced by desertomycin, other yet unknown mechanisms may also be involved in the mode of action of desertomycin.

Although after the original publications in the late 50s and early 60s most of the major textbooks of antibiotics [17, 32–35] included a short account on the properties of desertomycin, only recently, after a "period of latency" this antibiotic started to enjoy a resurgence of interest.

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PHAGE TYPES, ANTIBIOGRAMS AND R-PLASMIDS OF *KLEBSIELLA* AND *ENTEROBACTER* ISOLATED FROM HOSPITAL ENVIRONMENT AND FOOD

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Fourhundred and twenty-two *Klebsiella* strains and 294 *Enterobacter* strains were isolated (i) from direct or indirect environment of hospitalized patients, (ii) from foodstuffs, foods, culinary utensils and staff in hospital and (iii) in catering establishments. Of *Klebsiella*, the species *K. aerogenes* (76.5%) of *Enterobacter*, the species *E. cloacae* (77.6%) occurred the most frequently in all specimens. *Klebsiella* strains were typable in 68.5%; 53.1% of the *Enterobacter* strains were sensitive to phage. Most of the untypable *Klebsiella* and *Enterobacter* strains and the multiresistant strains originated from screening in hospitals. Sensitive bacteria as well as those resistant to one or two antibiotics may be potentially dangerous for the patient consuming them, since they may become multiresistant due to R-plasmid transfer.

Antibiotic-resistant strains belonging to the *Enterobacteriaceae* family and carrying R plasmid have proved to be the most dangerous among nosocomial bacteria. The special environment in hospitals, namely, the constant presence and inadequate administration of antibiotics [1], carriers among the hospital personnel [2–4], instrumentation, and transference of the bacteria from patient to patient [5–9] play an important role in the wide spread of causative agents resistant to antibiotics. More recently the possibility of spreading through the alimentary route has come into prominence.

Results obtained in the last few decades have been reviewed by Linton [10]. It is well-known that in animal breeding antibiotics have been administered not only for therapeutic purposes but also, in low concentrations in the feed, for stimulating weight gain. For the latter reason, the frequency of resistant bacteria in foodstuffs of animal origin has suddenly increased. From studies performed in England, it became clear that, owing to the alimentary chain, resistant bacteria may threaten man. Therefore, the use of antibiotics in animal feed has been reduced first in England and subsequently in countries of the European Common Market. However, a striking selection of resistant strains had already occurred by that time in factories dealing with slaughtered animals and even in the workers of those factories. Hartley et al. [11] compared the distribution of the R plasmid in *Escherichia coli* isolated from healthy people and those isolated from various animals (poultry, swine and cattle). The fre-

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quency of strains carrying R plasmid was 0.02% among strains of human origin in contrast to the 60% among those isolated from animals. The same authors demonstrated that *E. coli* strains present in meat preparations may colonize in man within 10 days after consumption.

In Hungary, Milch et al. [12] compared strains isolated from patients, surface waters and foodstuffs as regards sensitivity to antibiotics, serogroup and phage sensitivity. They, too, showed that strains carrying the R factor were most frequent in meat products.

Considering these results it occurred that *Klebsiella* and *Enterobacter* often isolated in the course of hospital screening investigations, among other pathogens, might be conveyed into hospital wards with food. In this paper we describe the properties of 422 *Klebsiella* and 294 *Enterobacter* strains isolated in this study.

Materials and methods

Strains and sources. Part of the samples (Ho) were taken in hospital wards, from the surface of hospital equipment (beds, textiles, bed tables, medicine boxes, medicine, nailbrushes) or in the more remote environment (corridor, wound-dressing room, operating theatre). The remaining samples originated from food-stuffs, food, culinary utensils and from the staff; part of these (HoF samples) were taken in hospital kitchens, the others (F samples) from foodstuff taken in catering establishments (outside hospital). The sampling lasted from January 1983 to February 1984. The Ho strains were cultured and identified as described in [13], the strains of foodstuff origin according to the Hungarian Standard for Food Microbiology.

The biochemical tests recommended by Ke and Milch [14] were carried out to distinguish *Klebsiella* and *Enterobacter* strains by species.

In the test for R plasmid an *E. coli* Hfr lac$^-$ nal^r strain was used as recipient.

Resistance to antibiotics and plasmid transfer were determined as published earlier [15]. The following antibiotics were used: ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), tetracycline (Tc), kanamycin (Km) and nalidixic acid (Nal).

In phage typing the *Klebsiella* phages I–XV described by Slopek et al. [16] and three accessory phages [4; 7; 35] as well as the *Enterobacter* phage C_{14} [17] were used.

Results

Distribution and incidence of Klebsiella and Enterobacter species. Out of the 716 isolates 422 (58.9%) belonged to *Klebsiella* and 294 (41.1%) to *Enterobacter* Table I shows the species distribution of the strains according to source. *K. aerogenes* strains occurred the most frequently, irrespective of origin. In hospital screening samples *K. oxytoca* showed a relatively high frequency (6.7%), almost ten time as high as shown by Ke and Milch [14] in patients. The same species occurred frequently also in food samples of either hospital or other origin. Among *Enterobacter* strains, *E. cloacae* was the most frequent, independently of their origin.

Phage type distribution. Table II presents the phage type distribution of *Klebsiella* and *Enterobacter* strains according to the source of isolation. Of the

Table I

Distribution of Klebsiella and Enterobacter strains according to source and species

Source	*Klebsiella* species, %									*Enterobacter* species, %					*Klebsiella* and *Enterobacter*	
	Total		*K. aerogenes*	*K. atlantae*	*K. edwardsii*	*K. oxytoca*	*K. ozaenae*	*K. pneumoniae*	*K. rhinoscleromatis*	Total		*E. aerogenes*	*E. cloacae*	*E. liquefaciens*	Total	
	No.	%								No.	%				No.	%
Ho	164	38.9	89.6	0.6	—	6.7	1.3	1.2	0.6	159	54.1	14.5	82.4	3.1	323	45.1
HoF	78	18.5	67.9	—	1.3	28.2	—	—	2.6	76	25.8	30.3	68.4	1.3	154	21.5
F	180	42.6	68.3	0.6	0.6	27.2	3.3	—	—	59	20.1	20.3	76.3	3.4	239	33.4
Total	422	58.9	76.5	0.5	0.5	19.4	1.9	0.5	0.7	294	41.1	19.7	77.6	2.7	716	100.0

Ho = strains isolated from direct or indirect environment of hospitalized patients; HoF = strains isolated from food in hospital kitchens
F = strains isolated from public catering establishments

Table II

Distribution of Klebsiella and Enterobacter strains according to source and phage type

Strains	Source	Total	Typable		Not typable		Phage type, %														
		No.	No.	%	No.	%	IA_1	IB_1	IIA_1	IIB_2	$IIIA_1$	IVA_1	$VIIA_1$	$VIIIA_1$	XA_1	$XIIIA_1$	Kl_4	Kl_7	Kl_{35}	Other	C_{14}
Klebsiella	Ho	164	99	60.4	65	39.6	3.7	6.7	12.2	1.2	1.8	2.4	4.3	2.4	3.1	3.7	4.3	5.5	1.2	7.9	—
	HoF	78	52	66.7	26	33.3	5.1	7.7	10.3	9.0	5.1	—	—	2.6	—	6.4	3.8	3.8	1.3	11.5	—
	F	180	139	77.2	41	22.8	10.5	10.0	19.4	1.1	3.3	4.4	0.6	2.2	1.1	1.1	3.9	1.7	1.7	16.1	—
Total		422	289	68.5	133	31.5	6.9	8.3	14.9	2.6	3.1	2.8	1.9	2.4	1.6	3.1	4.0	3.5	1.4	12.0	—
Enterobacter	Ho	159	80	50.3	79	49.7	—	—	—	—	—	—	—	—	5.7	—	—	—	3.1	—	44.0
	HoF	76	44	57.9	32	42.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	57.9
	F	59	32	54.2	27	45.8	—	—	—	—	—	—	—	—	—	—	—	—	10.2	1.7	52.5
Total		294	156	53.1	138	46.9	—	—	—	—	—	—	—	—	3.1	—	—	—	3.7	0.3	49.3

For abbreviation see Table I

Table IIIa

Phage type distribution of the two

Species	Source	Total		Typable		Not typable	
		No.	%	No.	%	No.	%
K. aerogenes	Ho	147	45.5	89	60.5	58	39.5
	HoF	53	16.4	27	50.9	26	49.1
	F	123	38.1	87	70.7	36	29.3
Total		323	100.0	203	62.8	120	37.2
K. oxytoca	Ho	11	13.4	9	81.8	2	18.2
	HoF	22	26.8	22	100.0	—	—
	F	49	59.8	46	93.9	3	6.1
Total		82	100.0	77	93.9	5	6.1

For abbreviations see Table I

Enterobacter strains 53.1% showed lysis, 7.1% of which occurred with *Klebsiella* phages. The greatest part of both the nontypable *Enterobacter* and *Klebsiella* strains were Ho isolates.

Typable *Klebsiella* strains fell into 38 phage types; in Table II detailed data are presented for those with more than 1% of strains; the incidence of the less frequent phage types is summarized as "other phage types". Phage type IIA_1 occurred the most frequently, independently of the origin of the isolate. This phage type is followed by IB_1 in Ho isolates, by IIB_2 in HoF isolates and by IA_1 in F isolates. Phage C_{14} lysed 49.3% of the *Enterobacter* strains, but none of the *Klebsiella* strains.

Tables IIIa and IIIb show the distribution of the most frequently occurring phage types of the *Klebsiella* and *Enterobacter* species, respectively. The predominant phage type for *K. aerogenes* was IIA_1, irrespective of the origin of the isolate. Among *K. oxytoca* strains, phage type IA_1 was the commonest among Ho isolates, whereas IB_1 among HoF and F isolates. Parallel lysis of *E. cloacae* strains by both the diagnostic phage C_{14} and phage K35 occurred frequently, whereas phage types XA_1 and the $VIIIB_1$ were isolated less frequently. *E. aerogenes* strains uniformly belonged to phage type XA_1.

Antibiotic sensitivity. The distribution of *Klebsiella* and *Enterobacter* strains by resistance to antibiotics and origin is shown in Fig. 1. On the average 12.7% of the *Klebsiella* and *Enterobacter* strains of Ho origin (n = 323), 38.5% of the HoF strains (n = 154) and 24.3% of the F strains (n = 239) proved to be sensitive. The resistance to Ap was the most frequent in all groups especially among Ho strains. As to the other antibiotics, HoF and F strains were considerably less frequently resistant than Ho isolates. The incidence of resistance to certain antibiotics (Tc, Km, Nal) was higher in the F than in the HoF group.

most frequent Klebsiella species

Species	Source	Most frequent phage types						Other phage types
		type	%	type	%	type	%	%
K. aerogenes	Ho	II A_1	12.9	KL_7	6.1	IB_1	5.4	36.1
	HoF	II A_1	9.4	II B_2	9.4	XIII A_1	9.4	22.7
	F	II A_1	21.1	I A_1	7.3	I B_1	5.7	30.9
Total								
K. oxytoca	Ho	I A_1	45.4	I B_1	18.2	II A_1	9.1	—
	HoF	I B_1	27.3	I A_1	13.6	III A_1 II A_1	13.6	45.5
	F	I B_1	22.4	I A_1	16.3	II A_1	14.3	40.9
Total								

In Table IV the percent distribution of antibiotic resistance of the most frequently isolated *Klebsiella* and *Enterobacter* species is summarized. Since 96% of the *Klebsiella* strains proved to be *K. aerogens* or *K. oxytoca*, and 97% of the *Enterobacter* strains were *E. cloacae* or *E. aerogenes*, the percent distributions were approximately the same as shown by the column diagrams in Fig. 1. The percentage of multiresistant isolates was the highest among *K. aerogenes* and *E. cloacae* strains isolated from hospital environment (44.2 and 33.6%, respectively).

Table IIIb

Phage type distribution of the two most frequent Enterobacter species

Species	Source	Total		Typable		Not typable		Phage types							
								VIII B_1		X A_1		Kl 35		C 14	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
E. aerogenes	Ho	23	39.7	11	47.8	12	52.2	—	—	2	8.7	—	—	9	39.1
	HoF	23	39.7	15	65.2	8	34.8	—	—	—	—	—	—	15	65.2
	F	12	20.6	6	50.0	6	50.0	—	—	—	—	—	—	6	50.0
Total		58	100.0	32	55.2	26	44.8	—	—	2	3.5	—	—	30	51.7
E. cloacae	Ho	131	57.5	67	51.1	64	48.9	—	—	7	5.3	5	3.8	59	45.0
	HoF	52	22.8	29	55.8	23	44.2	—	—	—	—	—	—	29	55.8
	F	45	19.7	26	57.8	19	42.2	1	2.2	—	—	6	13.3	25	55.6
Total		228	100.0	122	53.5	106	46.5	1	0.4	7	3.1	11	4.8	113	49.6

For abbreviations see Table I

Table IV

Percent distribution of the most frequently isolated Klebsiella and Enterobacter species by antibiotic resistance

Species	Source	No.	Sensitive %	Resistant, %						Multiple resistant	
				Ap	Cm	Sm	Tc	Km	Nal	No.	%
K. aerogenes	Ho	147	5.4	91.2	46.3	40.8	44.2	25.1	6.1	65	44.2
	HoF	53	9.4	92.1	15.1	11.3	3.8	1.9	—	2	3.8
	F	123	15.4	85.4	8.9	4.1	12.2	—	2.4	8	6.5
Total		323								74	
K. oxytoca	Ho	11	—	81.8	18.2	18.2	9.1	18.2	9.1	2	18.2
	HoF	22	54.5	36.4	9.1	4.5	—	9.1	—	—	—
	F	49	28.6	67.3	2.0	—	6.1	14.3	—	1	2.0
Total		82								3	
E. aerogenes	Ho	23	21.7	73.9	8.7	13.0	8.7	8.7	—	2	8.7
	HoF	23	73.9	30.4	—	—	—	—	—	—	—
	F	12	75.0	25.0	8.3	—	8.3	—	8.3	1	8.3
Total		58								3	
E. cloacae	Ho	131	18.3	75.6	37.4	35.9	33.6	12.2	—	44	33.6
	HoF	52	42.3	55.8	3.8	1.9	—	—	—	—	—
	F	45	26.7	64.4	2.2	2.2	4.4	—	6.6	1	2.2
Total		228								45	

For abbreviations see Table I

Table V

The most common antibiotic resistance patterns

Ap	Cm	Sm	Tc	Km	Nal	Klebsiella							
						Ho		HoF		F		Total	
						No.	%	No.	%	No.	%	No.	%
−	−	−	−	−	−	11	6.7	19	24.4	37	20.5	67	15.9
+	−	−	−	−	−	66	40.2	42	53.8	102	56.7	210	49.8
+	+	−	−	−	−	5	3.0	6	7.7	5	2.8	16	3.8
+	−	+	−	−	−	3	1.8	6	7.7	3	1.7	12	2.8
+	−	−	+	−	−	4	2.4	—	—	9	5.0	13	3.1
+	−	−	−	+	−	1	0.6	2	2.5	8	4.4	11	2.6
+	+	−	+	−	−	9	5.4	—	—	4	2.2	13	3.1
+	+	+	+	−	−	14	8.5	1	1.3	3	1.7	18	4.2
+	+	+	+	+	−	31	18.9	1	1.3	—	—	32	7.6
Other						20	12.1	1	1.3	9	5.0	30	7.1
Total						164	100.0	78	100.0	180	100.0	422	100.0

For abbreviations see Table I

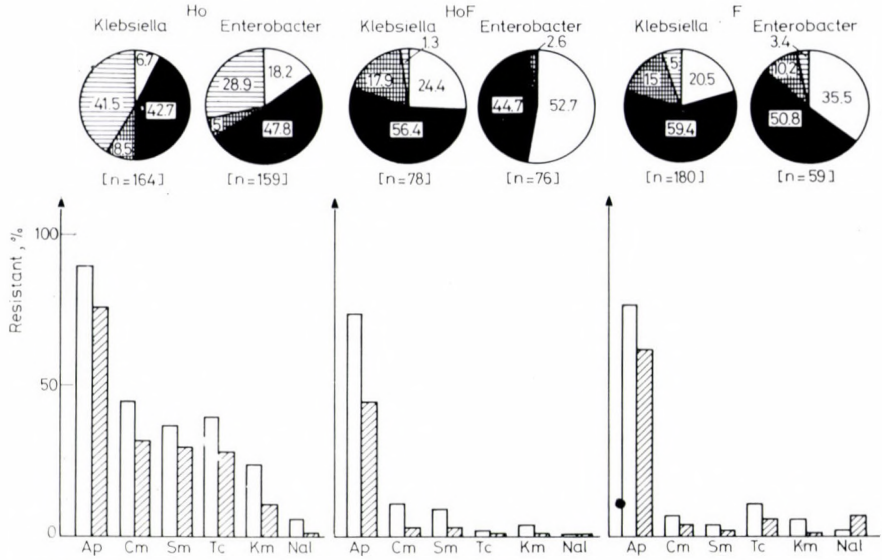

Fig. 1. Distribution of *Klebsiella* and *Enterobacter* strains by resistance to six antibiotics. Ho = strains isolated from direct or indirect environment of hospitalized patients; HoF = strains isolated from food in hospital kitchens; F = strains isolated from public catering establishments. Percentage of strains in circle diagrams: sensitive (open sectors), resistant to one (solid sectors); two (hatched sectors); three or more (shaded sectors) antibiotics. Percentage of strains resistant to individual antibiotics: *Klebsiella* (open columns) and *Enterobacter* (shaded columns)

The most frequent resistance patterns are shown in Table V. Resistance only to Ap characterized most *Klebsiella* and *Enterobacter* strains from all sources. This was followed in order by the simultaneous presence of resistance to Ap, Cm, Sm, Tc, and Km in Ho *Klebsiella* strains (18.9%) and to Ap, Cm,

of Klebsiella and Enterobacter strains

Ap	Cm	Sm	Tc	Km	Nal	Enterobacter							
						Ho		HoF		F		Total	
						No.	%	No.	%	No.	%	No.	%
−	−	−	−	−	−	29	18.2	40	52.7	21	35.5	90	30.6
+	−	−	−	−	−	69	43.4	32	42.1	28	47.5	129	43.9
+	+	−	−	−	−	3	1.9	1	1.3	1	1.7	5	1.7
+	−	+	−	−	−	5	3.1	1	1.3	—	—	6	2.0
+	−	−	+	−	−	—	—	—	—	—	—	—	—
+	−	−	−	+	−	—	—	—	—	2	3.4	2	0.7
+	+	−	+	−	−	—	—	—	—	1	1.7	1	0.3
+	+	+	+	−	−	26	16.4	—	—	—	—	26	8.9
+	+	+	+	+	−	13	8.2	—	—	—	—	13	4.4
Other						14	8.8	2	2.6	6	10.2	22	7.5
Total						159	100.0	76	100.0	59	100.0	294	100.0

Sm, and Tc in Ho *Enterobacter* strains (16.4%). The strains from food (F isolates) showed resistance to two antibiotics (Ap and Cm; Ap and Sm; Ap and Tc; Ap and Km) at a relatively high frequency.

Correlation between antibiotic resistance and phage type. No specific antibiotic pattern was characteristic for any definite phage type of *Klebsiella*. The multiresistant strains were, in general untypable.

Frequency of R plasmid in Klebsiella and Enterobacter strains. Table VI demonstrates the relationship between typability, multiresistance and R plasmid transfer of *Klebsiella* and *Enterobacter* strains. R plasmid was demonstrable in 66.7% of the Ho isolates of multiple resistant *Klebsiella* strains. Of the typable *Klebsiella* strains 45.7%, of the untypable ones 90.3% contained plasmid and 96% of these strains were multiresistant. The presence of R plasmid in the HoF and F isolates of *Klebsiella* was considerably lower and plasmid transfer failed between such *Enterobacter* strains. The Ho strains of *Enterobacter* carried transferable R plasmid in 76.5% (46.2% and 86.8% of the typable strains, respectively).

Table VI

Relationship between typability, multiresistance and R-plasmic transfer of Klebsiella and Enterobacter strains

Origin of strains		Klebsiella			Enterobacter		
		typable	not typable	total	typable	not typable	total
Ho	No. of strains tested for phage type	99	65	164	80	79	159
	No. of multiple resistant strains	32	36	68	8	38	46
	No. of strains tested for plasmid transfer	35	31	66	13	38	51
	No. of R^+ strains	16	28	44	6	33	39
	R^+ strains in per cent of strains tested for transfer	45.7	90.3	66.7	46.2	86.8	76.5
HoF	No. of strains tested for phage type	52	26	78	44	32	76
	No. of multiple resistant strains	1	1	2	—	—	—
	No. of strains tested for plasmid transfer	13	4	17	—	1	1
	No. of R^+ strains	1	—	1	—	—	—
	R^+ strains in per cent of strains tested for transfer	7.7	0	5.9	0	0	0
F	No. of strains tested for phage type	139	41	180	32	27	59
	No. of multiple resistant strains	6	3	9	—	2	2
	No. of strains tested for plasmid transfer	33	13	46	1	5	6
	No. of R^+ strains	4	1	5	—	—	—
	R^+ strains in per cent of strains tested for transfer	12.1	7.7	10.9	0	0	0

The resistance to Ap, Sm and Km was transferred by R plasmid in 32% of the multiresistant (Ap, Cm, Sm, Tc, Km) Ho *Klebsiella* isolates tested for plasmid transfer. Among Ap, Cm, Sm and Tc resistant and Ap, Cm, Sm, Tc and Km resistant Ho isolates of *Enterobacter* resistance could be transferred by R plasmid in 31.4 and 11.8%, respectively.

Discussion

We have shown that in all groups of samples examined, *K. aerogenes* (76.5%) and *E. cloacae* (77.6%) were the predominating *Klebsiella* and *Enterobacter* species. The same species distribution was observed by Ke and Milch [14] among strains isolated in Hungary from patients, except *K. oxytoca*, which was ten times frequent in our Ho samples. In food samples originating either from hospital or elsewhere *K. oxytoca* was still more frequent (about 28%). According to recent literary data *K. oxytoca* is of special importance in hospital infections, for this species has proved to be more virulent than *K. aerogenes*, the most common *Klebsiella* species [18].

In agreement with literary data [14, 19], 68.5% of the *Klebsiella* strains could be typed. Most of the untypable strains, either *Klebsiella* or *Enterobacter*, derive from studies performed in hospital environment. Due to the selective effect of the antibiotics used in hospitals, usually the same strains are the most reistant [20], often multiresistant. Our *Klebsiella* and *Enterobacter* strains isolated from food samples taken outside hospital were more frequently resistant to antibiotics than the isolates of hospital origin. This is explanable by the fact that of the latter samples 4%, whereas of the former ones 27% were taken from raw foodstuff, raw meal and dairy products. The abundance of raw products of animal origin in resistant bacteria might be due to feeding the animals with antibiotics.

Two-thirds of the resistant Ho isolates of *Klebsiella* strains carried R plasmid in agreement with the finding of Milch and Ke [21].

It has been shown that bacteria introduced in raw foodstuffs into the kitchen frequently gain access via contaminated kitchen utensils into ready meals because of hygienic irregularities. Cooke et al. [22] described intestinal colonization in two patients by *Klebsiella* of the same phage type and bacteriocin type cultured from salads and cold meals. Casewell and Phillips [23] showed that raw, unwashed salad was contaminated by *Klebsiella* in only 8%, while the organism was isolated from 45% of the samples taken after washing. Cooke et al. [24] isolated *Klebsiella* strains of the same serological and bacteriocin type from wound and intestine in 50% of their patients.

We failed to unequivocally demonstrate a chain of contamination or infection from food to patient. Nevertheless, sensitive bacteria consumed by the

patients may be dangerous because, taking into account that R plasmid was carried by 75% of our multiresistant isolates, they may acquire multiresistance by R plasmid transfer.

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AGE OF THE CELL CULTURE: A FACTOR INFLUENCING HORMONAL IMPRINTING OF *TETRAHYMENA*

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Insulin imprinting of *Tetrahymena pyriformis* in different growth phases had been investigated. Cells formed in the early logarithmic phase (18-hour culture) showed enhanced hormone binding at the second encounter with the hormone proving that imprinting had developed. This phenomenon was not observed in cells formed in the late logarithmic phase (42-hour culture) or in the stationary phase (66-hour culture). Lipid transformation processes, alteration of the guanyl cyclase activity and enhanced cell division may be responsible for this effect. Cell-growth phase G_1 was especially favourable for development of imprinting.

Tetrahymena is a wild living ciliated unicellular organism greatly dependent on its environment. The shape of the growth curve of its cell culture is influenced by changes of the environment, some of which being determined by the unicellular itself.

The multiplication curve of *Tetrahymena* can be divided into several phases, similarly to the growth curve of bacteria or other unicellular mass cultures. There is an initial or lag phase showing no increase in cell number and occasionally even a decrease can be observed. Subsequently the acceleration and exponential (logarithmic) phases follow: the increase of the cell number can be delineated logarithmically by a steep straight line that indicates a rather quick multiplication. A moderate cell growth prevails in the next period followed by the stationary phase with a relative constancy of the maximal excessible cell number. In this phase multiplication and cell death are in balance. Finally the culture reaches a declining period where cell lysis exceeds the growth rate of new cells [1].

This process causes alterations in the cell as well in the medium. As the cell number increases, the amount of the nutrient materials decreases and degradation products accumulate parallel with the aging of the culture. The amount of the accumulated "alien" materials is further increased by the degradation material of the lysing cells in course of the life cycle, and a shift in the proportion of the respiratory bases ensues in the logarithmic phase. These changes may also be responsible for the rise of the pH [2] and for the

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growing amount of neutral lipids [1]. The quantity of the free fatty acids deriving from the triglyceride deposits redoubles in the stationary phase compared to that in the logarithmic phase [3]. Subsequently it falls sharply, indicated by the gradually accumulating lipid drops. These changes are accompanied by simultaneous alterations of the intracellular enzyme activity: the acid phosphatase activity decreases in the logarithmic phase and rises to the maximum in the stationary phase [4]. A reversed change is shown in the activity of guanyl cyclase exerting the highest activity in the logarithmic phase and a gradually decreasing activity in the stationary phase [5].

These changes may be accompanied by alterations in the membrane level. An enzyme system is known to transform phospholipids to fatty acids. Its activity is more intense in the stationary phase than in the preceding logarithmic phase [6, 7]. At the same time production of phospholipids undergoes a change, too. A large amount of palmitic acid is incorporated into the phospholipid in the logarithmic phase, while in the stationary phase it is involved in production of glycerides and non-phospholipids [8]. These observations suggest that membrane structure and intracellular parameters are different in each growth phase of these cell cultures.

Hormonal imprinting is a phenomenon developing at the first encounter with the hormone: the cell shows an altered, usually enhanced responsiveness at the next encounter, as a consequence, and is capable of an increased hormone-binding [9–11]. Evolution of an imprinting is a complicated process influenced by several factors, involving momentary structural and functional state of the membrane [12, 13], as well as the activity of associated intracellular systems and enzyme groups [14]. The phases of the growth cycle potentiate the rise of an imprinting differently. Phase G_1, a period after the "birth" of the cell, yields a much easier development of imprinting than phase S or G_2 [15]. This is due to changes of the membrane structure in the different growth phases. In view of these data it may be assumed that since the cells of different growth phases differ in several aspects from each other, they can not be regarded equivalent concerning the development of hormonal imprinting either.

In the present experiment hormonal imprintability was investigated in the different growth phases of *Tetrahymena pyriformis* cultures.

Materials and methods

Cells of *T. pyriformis* GL strain were investigated in three different growth phases in a medium containing 0.1% yeast extract and 1% Bacto Tryptone (Difco) at 28 °C: (i) early logarithmic phase (18-hour culture); (ii) late logarithmic phase (42-hour culture); (iii) stationary phase (66-hour culture).

All cultures were divided into two parts: one was left untreated for control, the other was treated with 10^{-6} M insulin (Semilente Novo, Copenhagen Denmark) for 1 h. A representative sample of cells was incubated in normal medium for 24 h. Samples of the six groups were fixed in 4% formalin and washed in PBS. After one-hour incubation with fluorescein-

isothiocyanate (FITC-BHD Chemicals Ltd., Poole, England) labelled insulin (Semilente Novo, Copenhagen, Denmark) the cells were washed in PBS twice and dropped on slide. Binding of labelled insulin was determined cytofluorimetrically in a Zeiss Fluoval cytofluorimeter combined with a HP 41C computer used also for analysis of variance. Insulin-binding of 20 cells from each group was measured. Since the experiment was repeated 5 times the results of each experimental group represented the binding capacity of 100 cells. At the time of sampling the cells were examined with Sudan red staining for the amount of lipid drops accumulated in the cytoplasm [16].

Results and discussion

Hormonal imprinting varied in the different growth phases of *Tetrahymena*. The growth phases were distinguished on the basis of two indices: the cell number of the cultures (Fig. 1) and the accumulation of cytoplasmic lipid drops (Fig. 2). The amount of lipid increased parallel with the aging of the culture [17].

The assay of insulin imprinting revealed a striking difference between the imprintability of the 18-hour and the two older (42- and 66-hour) cultures (Fig. 3). While cells in the early logarithmic phase developed a strong imprinting (120%), those in the later phases showed no significant difference of imprintability as compared to the control.

The development of hormonal imprinting is a complicated process [18]. Reactions on the occasion of the first encounter with a polypeptide hormone capable of binding to the cell membrane, such as insulin, induce the cell in different ways to "remember" the previous encounter at the next one [19], and the cell reacts by an enhanced response, e.g. by hormone binding. The state of the cell membrane plays an important role in the development of this process line. Membrane receptors are the first to meet the hormone and bind it. Subsequent processes may diverge. The membrane itself is capable of preserving such informations by its plasticity [20], but certain enzymes of the membrane may also be active in the development of the membrane-bound memory [14]. An important transferring function is attributed to the phenomenon of mem-

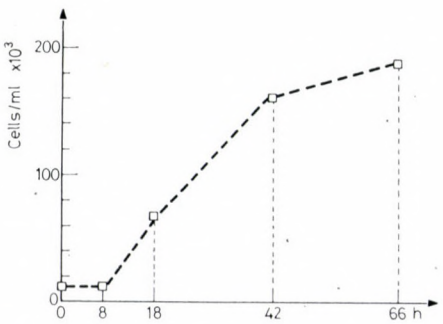

Fig. 1. Numbers of *T. pyriformis* cells in different growth phases

brane-flow, that establishes a continuous connection between the membrane, covering the cell surface and the intracellular membrane system [13, 21]. It also permits hormone-induced biochemical structural alterations in the outer membrane to be transferred as an information carrier to the nucleus, or facilitates the intracellular, endocytotic processes associated activity of the hormone itself. Thus, state of the cell-covering membrane may be of primary importance

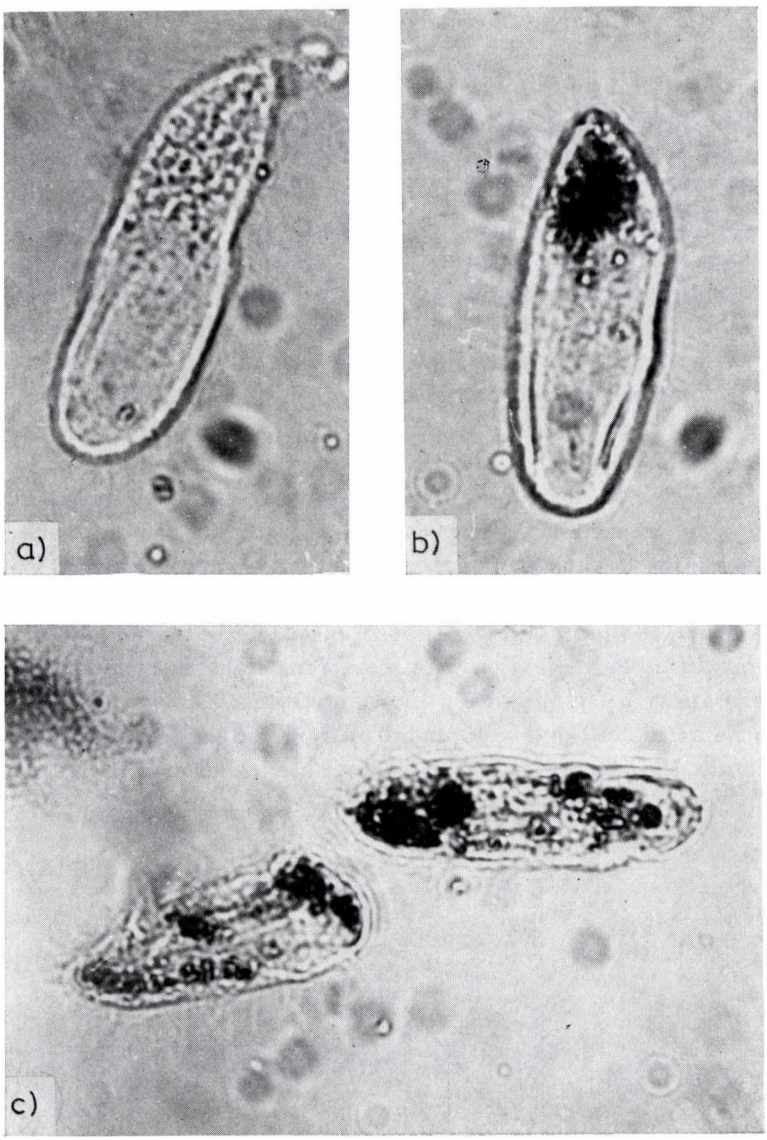

Fig. 2. Change of lipid contents of *T. pyriformis* cells stained with Sudan red. (a) 18-hour, (b) 42-hour, (c) 66-hour culture

for the development and effectiveness of the imprinting. The variable responsiveness of the different growth phases found in this study may also be partly attributed to these changes in the membrane structure.

Changes in the membrane structure may be reflected by the two-fold increase of the amount of the free fatty acids in the stationary phase compared to that of the logarithmic phase [3]. Other data indicate an 8-fold increase of

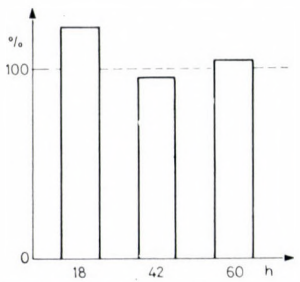

Fig. 3. Imprintabiiity of *T. pyriformis* cells by insulin (control = 100% intensity of fluorescence)

the triglyceride level in 24–72-hour cultures [22]. These changes may be partly due to active transformation processes of the membrane. The enzyme system capable of degradation of phospholipids to fatty acid, functions with a much greater activity in the stationary phase than in the preceding logarithmic phase [6, 7]. These changes may indicate an essential structural transformation of the membrane and, as a conseuqnce, a modification of the hormone binding capacity may ensue.

An important determinant of the membrane's structure and function is its state of fluidity. It has been demonstrated that the amount of saturated fatty acid grows and the lipid composition changes parallel with the aging of the cultures: the consequence on membrane level is the formation of a more rigid membrane structure [1].

After the establishment of the hormone-receptor connection, adenylate-cyclase is the enzyme that plays an essential role in transferring information to the intracellular space. Guanylate cyclase is another, similar enzyme detected in *Tetrahymena* in insoluble and in soluble form [23]. Its membrane associated form together with the regulatory calmodulin-Ca^{++} complex is an important factor in transformation of the membrane associated processes [24]. The activity of the guanyl cyclase varies in the different growth phases of the cultures [5]. It is the greatest in the early logarithmic phase, in a period when the cells seemed to be the most sensitive to insulin. This finding suggests the presumption that this enzyme also plays a role in the development of imprinting.

Finally a type of insulin effect should be mentioned in which insulin is bound to the membrane and permeates into the cell by endocytosis and evokes

there specific alterations. The endocytotic processes in the model cells are at their maximum in the pre-division period [25]. In tissue culture the greatest number of subsequent divisions takes place in the early logarithmic phase. Should insulin have an imprinting effect in this phase, imprintability of these cells is expected to be the greatest. Considering, however, the results of our earlier studies [15] giving evidence for the greatest imprintability of the "newborn", just divided cells, it may be assumed that cell-formation is a favourable state for hormonal imprinting, for the development of receptor memory, but in view of the particular role of phase G_1, not necessarily by endocytosis.

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THE EFFECT OF BACTERIAL ENDOTOXIN ON PHAGOCYTOSIS OF *TETRAHYMENA* AND SEROTONIN INDUCED IMPRINTING

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Endotoxin inhibited the phagocytosis of *Tetrahymena pyriformis* after a short exposure and, to a lesser degree, after repeated treatments during one week (about 35 generations). Endotoxin also prevented the development of serotonin imprinting. Detoxified endotoxin (Tolerin®) affected the phagocytosis of *Tetrahymena* much less, indicating that the lipid-A part of the molecule may account for the membrane-toxic effect.

Endotoxins are heterogenic lipopolysaccharides containing lipid-A as a part responsible for toxicity [1–4]. The basic damaging action of the endotoxin results probably in membrane destruction, affecting the plasma membrane as well as the lysosomal and microsomal membranes [5, 6].

As we demonstrated in vivo and in tissue culture [7–9], the endotoxin destroys also the membrane receptors; its presence interferes with the normal hormonal imprinting in a crucial period of the development of hormonal receptors [10, 11].

Tetrahymena, a ciliated unicellular organism, proved to be an excellent model for investigation of receptors [12] and suitable for hormonal imprinting. Hormonal imprinting means a longlasting modification in the cell function after the first encounter with a hormone, persisting also in subsequent generations, providing enhanced response to the same hormone at a repeated encounter [12, 13]. This fact suggested an investigation of the endotoxic effect on the membrane of *Tetrahymena* as a model, and to study how it influences the effect of serotonin, a phagocytosis stimulating membrane effective hormone. It also had to be cleared whether this effect was due to the endotoxin as a macromolecule or it was associated with its toxicity.

Materials and methods

Organism. The experiments were carried out with *Tetrahymena pyriformis* GL strain cultured in a medium containing 0.1% yeast extract and 1% Bacto Tryptone (Difco) at 28 °C.

Materials. Endotoxin was prepared from *Escherichia coli* serogroup O89 strain with heating in aqueous phenol solution (LD_{50} = 1.9 mg/kg rat). Radio-detoxified endotoxin (Tole-

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rin®, Human Institute for Serobacteriological Production and Research, Budapest) was obtained from this product after exposure to ionizing radiation (Co-60 gamma, 150 kGy); LD_{50} = = 21 mg/kg rat [14]. Serotonin (Fluka, Buchs, Switzerland) was used for stimulation of phagocytosis.

Assay of phagocytosis. Changes in the rate of phagocytosis were investigated as an indicator of the membrane function. After a 3-hour starvation in Losina solution, the tetrahymenae were fed with Indian-ink and incubated either with different concentrations of endotoxin or with serotonin + endotoxin or with serotonin alone for 10 min at 22 °C. The control group remained untreated. Phagocytosis was terminated by 4% formalin in PBS. Subsequently the cells were centrifuged, dropped on slide and dried. The Indian-ink contaning phagosomes were counted in 100 cells of each cohort. Phagocytosis index was determined as the mean value of the treated samples related to that of the control group. This experiment was repeated with radio-detoxified endotoxin (Tolerin). Finally the effect of the endotoxin on the successive generations was investigated. After a four-hour exposure to endotoxin or serotonin or to both, the materials were eliminated by centrifugation and the tetrahymenae were inoculated into normal medium. Serotonin treatment lasting for 10 min was repeated after 1, 3 and 6 days.

Results and discussion

Phagocytosis decreased in a direct proportion to endotoxin concentration after a short exposure. To terminate the phagocytosis stimulating effect of serotonin 2 μg/ml endotoxin was sufficient (Fig. 1). Having submitted *Tetrahymena* to repeated treatments, the phagocytosis reducing effect of the endotoxin was still observed after a week (about 35 generations), though to a lesser degree (Fig. 2). A simultaneous treatment with endotoxin and serotonin, however, did not reduce phagocytosis or reduced it less than the endotoxin treatment alone. The radio-detoxified endotoxin (Tolerin) did not decrease the phagocytosis or induced only a minimal reduction, and inhibited the phagocytosis stimulating effect of serotonin less than the not detoxified endotoxin (Fig. 1). Phagocytosis index was >1 in case of a simultaneous application of Tolerin and serotonin.

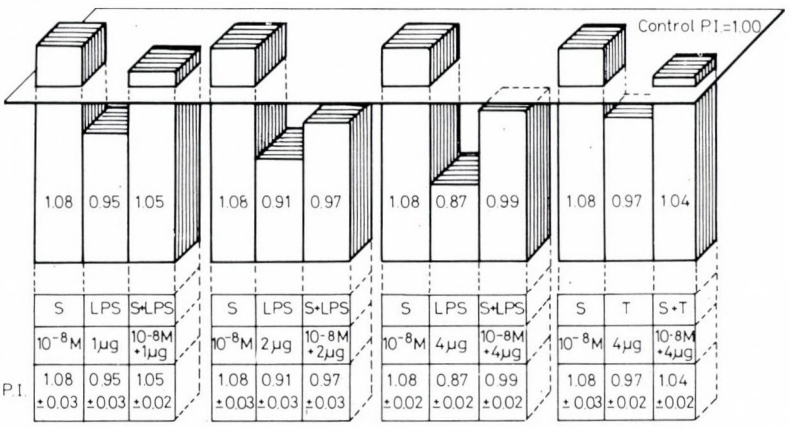

Fig. 1. The effect of endotoxin and radio-detoxified endotoxin (Tolerin) on the phagocytosis of *Tetrahymena* (short treatment). LPS = endotoxin, T = Tolerin, S = serotonin, P.I. = phagocytosis index. The mean value of the treated cohorts related to that of the control groups (n = 5 treated with 1 μg/ml and 2 μg/ml, respectively, n = 7 treated with 4 μg/ml LPS, n = 4 exposed to Tolerin). All differences are significant ($p < 0.01$) except the Tolerin-effect

These results led us to investigate whether endotoxin tolerance, characteristic of higher organisms was inducible in unicellulars by pretreatment with subtoxic amount of endotoxin [1, 15]. Our results have shown that the phagocytosis reducing effect of the endotoxin could be prevented by a 10 min pretreatment with 0.25 μg/ml endotoxin as well as with 4 μg/ml Tolerin. After

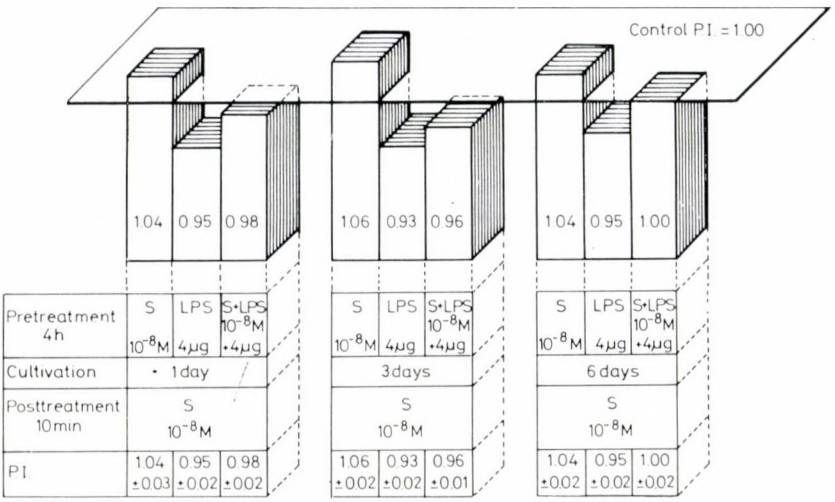

Fig. 2. The effect of endotoxin on the phagocytosis of successive generations of *Tetrahymena*. A 10 min posttreatment 1, 3 and 6 days after a 4-hour pretreatment either with serotonin, or endotoxin, or endotoxin + serotonin (n = 3). LPS = endotoxin, S = serotonin, P.I. = phagocytosis index. The differences are significant ($p < 0.01$) except S + LPS-effect related to LPS-effect and S + 4 μg LPS-effect related to S-effect

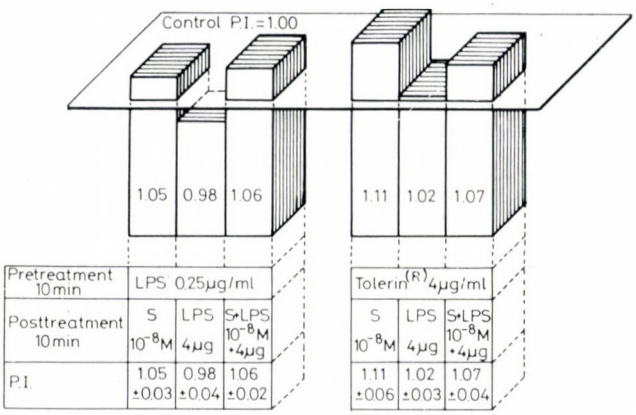

Fig. 3. Membrane tolerance induced in *Tetrahymena* by a subtoxic concentration of endotoxin and Tolerin. 10 min pretreatment with endotoxin (0.25 μg/ml) and Tolerin (4 μg/ml) and subsequently a 10 min posttreatment either with serotonin, or endotoxin or serotonin + endotoxin (n = 4). LPS = endotoxin, S = serotonin, T = Tolerin, P.I. = phagocytosis index. The differences are significant ($p < 0.01$) except for treatment with LPS alone after the pretreatments

these pretreatments a concentration normally efficient for reduction of phagocytosis (4 μg/ml) failed to produce any decrease. The 10 min posttreatment with endotoxin + serotonin did not influence the phagocytosis-stimulating effect of serotonin, while Tolerin^R had a moderate effect (Fig. 3).

These experiments provided convincing evidence for the membrane function damaging effect of the endotoxin. This is probably due to the toxic lipid-A part of the molecule, since the radio-detoxified endotoxin, which is deprived of most of its toxic activity but is still similar to the original molecule, failed to induce any reduction in phagocytosis. Our results that the so-called endotoxin-tolerance can also be developed in unicellulars, indicate a general membrane effect. The results support presumptive evidence for the major role of the membranes in the mechanism of developing endotoxin tolerance in higher organisms. Perturbation of the membrane's normal state in *Tetrahymena* inhibits development of hormonal imprinting similarly to mammalian cell culture [7].

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NOVEL FERMENTATION PROCEDURES FOR THE PRODUCTION AND THE ISOLATION OF ANTIBIOTICS

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Novel procedures for the production, in shaken and/or air-agitated fermentations, and the isolation of antibiotics were developed by which active crystalline flavofungin by *Streptomyces flavofungini* and an antibiotic produced by *Streptomyces* SK&F, BC-1652 were obtained.

The experimental or industrial (i.e. non-synthetic) production of antibiotics involves cultivation of microbial strains producing the antibiotic in various synthetic, semisynthetic or complex organic culture media. The technique used may be surface culture, shaken-flask fermentation or deep-agitated-aerated fermentation. Whichever technique is used, the quantity of biosynthetized antibiotic is usually small relative to the large volume of liquid medium, and must be separated from the medium and isolated in solid form. The isolation procedure represents a tremendous reduction in volume. The most widely used isolation methods are precipitation, adsorption and extraction. These are, among others, described in detail in text books [1–3].

New methods of production and concentration inside the fermentation liquid, resulting in quick isolation of flavofungin and an antibiotic produced by a *Streptomyces* sp., SK&F, BC-1652 are described in this paper.

Materials and methods

Streptomyces flavofungini and its antibiotic, flavofungin. Flavofungin is produced by a new species named *Streptomyces flavofungini* [4] isolated from a soil sample collected in the Sahara desert. Originally it was designated and published as *Streptomyces* SA-IX nov.sp. [5]. A stable natural variant SA-IX/3, now known as *S. flavofungini* produces enhanced amounts of flavofungin [6] and smaller amounts of its second antibiotic, desertomycin [7]. Flavofungin is biosynthesized by SA-IX/3 in any complex organic medium, especially if the sugar content is increased to 4%. *S. flavofungini* can be grown in shaker-flasks as well as in laboratory and pilot plant fermenters with best antibiotic production at 28 °C. The fermentation broth gradually turns greenish-yellow with luminescence in ultraviolet (UV)-light. This vivid yellow luminescence can be used for qualitative estimation [8] and quantitative determination [9] of flavofungin during fermentation and also for quality control, similar to mycoticin [10], roseofungin [11], flavomycoin [12, 13] and a flavofungin-like antibiotic [14].

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When production of flavofungin was maximal, as determined by the above-mentioned UV-luminescence method, the fermenters were placed in the cold-room (4 °C) overnight. Unexpectedly, the sunflower-seed oil used as an antifoam agent formed a solid layer on the surface of the fermentation broth containing (encompassing) all the flavofungin produced (dark greenish-yellow colour). This solid phase can easily be separated from the rest of the fermentation fluid and mycelia, representing a tremendous reduction in volume. Flavofungin is isolated from the oily phase in crystalline form by two methods. In the first method, the solid superlayer is shaken vigorously with acetone which solubilize the sunflower oil, and the flavofungin separates out in needle-like, yellow crystals in one step. The other procedure consists of boiling the solid oil phase under reflux in ethyl acetate for about 30 min followed by immediate filtration. On cooling at room temperature, flavofungin crystallizes as greenish-yellow hair-like crystals in sheaf-like formation (Fig. 1). I have coined the name "flavofungin" which reflects the colour of this antibiotic; flavus is the Latin equivalent of yellow, and fungin represents the antifungal nature of its antimicrobial spectrum.

Streptomyces sp., SK&F, BC-1652, and its antibiotic product(s). This *Streptomyces* strain was discovered as an air-contaminant on a *Trichophyton mentagrophytes* culture-plate with antagonistic property (Fig. 2). The strain was isolated, subcultured and further tested for inhibitory properties on various solid media. It was found antagonistic against many bacterial and fungal strains, but the activity was medium dependent and not always reproducible. The antagonistic effect was the broadest and most reproducible on nutrient agar. The inhibitory zone around the *Streptomyces* BC-1652 colony became pinkish-yellow, suggesting that the active principle might be a coloured material.

Production of the antibiotic in nutrient broth, in shaken cultures by this *Streptomyces* strain was just as erratic as that observed on solid media. Antibiotic effect was found at a certain stage of the shaken fermentation, but disappeared later. Based on this observation, it was hypothesized that the active principle would be a volatile material with basic character, and on the basis of the experience gained with the production of flavofungin, it was hoped that adding vegetable oil to the fermentation broth would "trap" the active antibiotic from the culture medium. After many failures, one of our routine media (Medium 19) led to positive results. The composition of this medium is: soy peptone, 2%; cerelose, 3%; starch, 1%; $CaCO_3$, 0.1%; and $CoCl_2$, 0.0001% in tap water; starting pH 7.0. To each 100 ml of culture medium in flask, 4 ml sterile peanut oil was added. During shaken fermentation at 28 °C the oil, which was originally dispersed in the medium, started to agglomerate and by the 4th day or so had formed a clump with a light yellow/pinkish colour (Fig. 3). The clumps contained the antibiotic activity while the fermentation broth had no or only traces of activity. During agglomeration of oil phase, the pH of the medium rose from 7.0 to 8–8.5. This clump-formation was not observed in other routine media or with other vegetable oils.

The active principle was isolated from clumps after chilling, by vigorously shaking with ether or ethyl acetate which extracted the coloured (pinkish yellow) active principle from the oil phase. This organic solution was dried with anhydrous Na_2SO_4, and then treated with an organic acid (citric or fumaric) dissolved in acetone, which led to the precipitation of the active antibiotic. Further purification of the formate salt was obtained in crystalline form (Fig. 4) which is highly water soluble and hygroscopic.

Results and discussion

Flavofungin and an antibiotic from the *Streptomyces* strain SK&F, BC-1652 can be produced in and isolated from shaken cultures and/or deep fermenturs by a new procedure utilizing the high lipid-solubility of these antibiotics.

In the case of flavofungin its high solubility in the sunflower-seed oil is the critical factor. The oil dissolves flavofungin and continuously extracts it from the fermentation fluid. Flavofungin then can easily be isolated from the oily concentrate in crystalline form in one step. Since flavofungin is a neutral compound the ambient pH does not seem to play any significant role in the process.

Fig. 1. Crystals of flavofungin from ethyl acetate

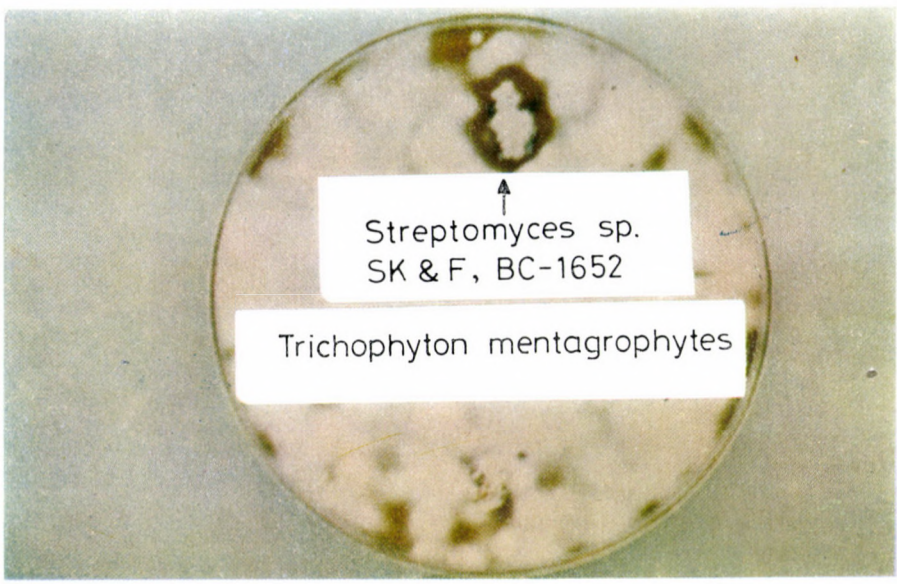

Fig. 2. Antagonistic effect of *Streptomyces* sp. BC-1652 against *Trichophyton mentagrophytes*

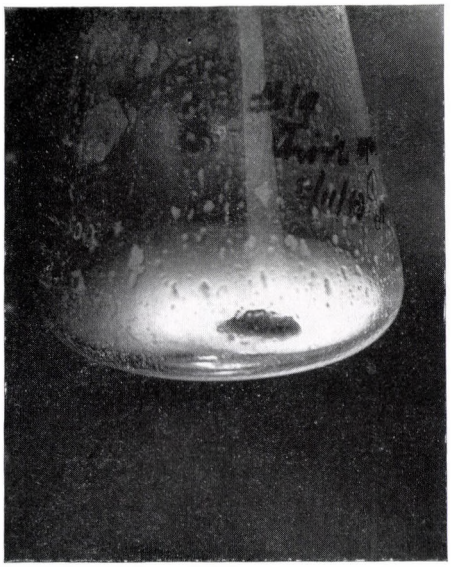

Fig. 3. During shaker fermentation of BC-1652 the added peanut oil forms a clump which traps the antibiotic

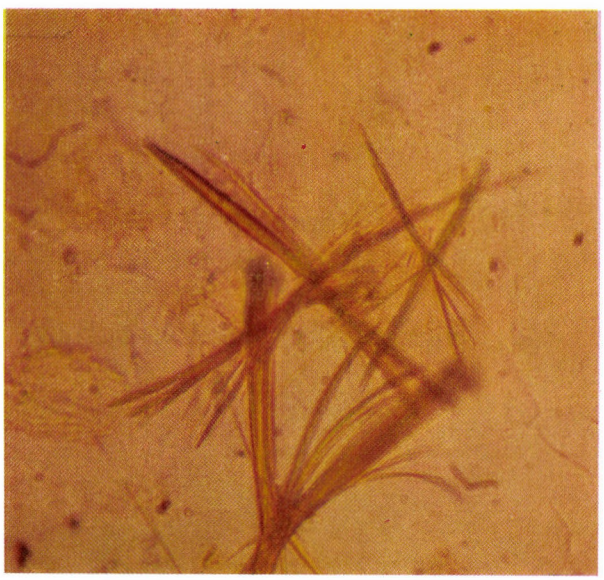

Fig. 4. Crystals of the formate of the product of *Streptomyces* SK&F, BC-1652

In the case of BC-1652, other as yet unknown factors (e.g., esterification), may also be important for extraction of the antibiotic out of the fermentation medium into the added peanut oil. Here, however, pH plays a definite role, since the antibiotic(s) is/are of basic character and the clump formation requires a pH of 8–8.5 of the fermentation liquid. The isolation from the clump of the active antibiotic(s) utilizes its basic character as described in the methods section.

Flavofungin has been produced at industrial scale using the novel methods for production and isolation described above. Bognár et al. [15–18] have identified flavofungin as a "lactone-conjugated" pentaenic antifungal polyene antibiotic (Fig. 5).

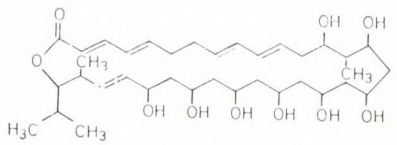

Fig. 5. Chemical formula of flavofungin

Flavofungin has a broad-spectrum antifungal activity, including activity against dermatophytes, yeasts and yeast-like as well as phytopathogenic fungi [4, 6, 19]. Its therapeutic application is similar to that of nystatin [20].

Flavofungin, like filipin, also possesses larvicidal activity (death or stunted forms) when added in tiny amounts (1 : 1000) to the food of *Musca*

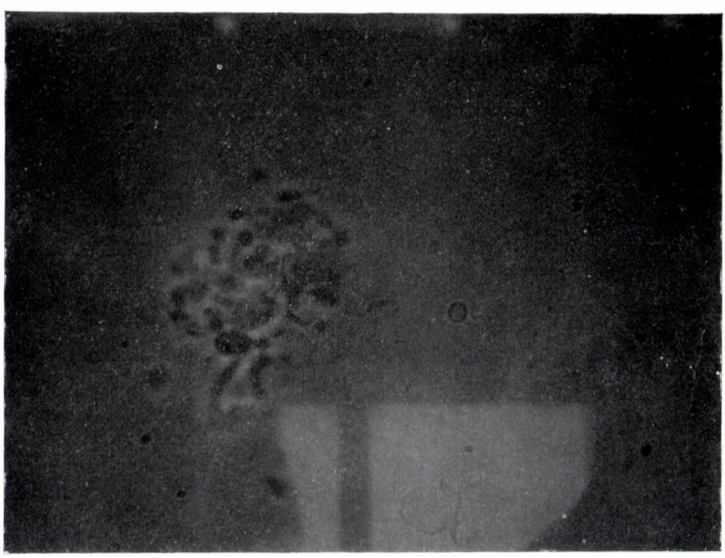

Fig. 6. Protoplast formation of *B. subtilis* by the antibiotic produced by SK&F, BC-1652 (Dr. Y. Oh)

domestica larvae, apparently by blocking the uptake of vital cholesterol which insects are unable to produce [21].

Presently, only very small amounts of purified BC-1652 are available limiting the study of its chemical and biological properties. Although in some preliminary tests it appears to have a broad spectrum, the activity seems to be of low order and is influenced by many test conditions such as pH, composition of medium, incubation temperature and inoculum level. Y. Oh [22] found that it produces protoplasts of *Bacillus subtilis* like many cell-wall-active antibiotics (Fig. 6). It is not even known whether it is a new antibiotic being a single compound or a mixture of active entities.

It is possible that the novel methods and procedures described in this paper for the isolation of flavofungin and the product of *Streptomyces* strain SK&F, BC-1652 could be applied for the production and isolation of other antibiotics as well. Comprehensive methodology is to be found in a recently published excellent handbook [23].

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COMPARISON OF ADJUVANTICITY AND AUTOANTIBODY INDUCING CAPACITY OF ENDOTOXIN AND RADIO-DETOXIFIED ENDOTOXIN IN MICE

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Endotoxin (LPS) and radio-detoxified endotoxin (RD-LPS: 150 kGy ^{60}Co-gamma irradiated) preparations were compared in mice (C57Bl × CBA: F_1/Rapo) for capacity to enhance the immune response against sheep red blood cells and to induce the production of antibody against autologous (bromelain-treated) erythrocytes. As RD-LPS retains its capacity to stimulate immune response against heterologous antigen, it may be used as an immuno-adjuvant. The LPS preparation gave rise to a significant increase of autoreactive cells. However, RD-LPS activated the autoantibody forming cells only to a very small degree.

There are extensive data for the action of endotoxins of Gram-negative bacteria upon practically all organs of the whole organism including the immune system [1, 2]. Treatment with endotoxins or purified lipopolysaccharides (LPS) results in the destruction of a large number of lymphoid cells [2]. In a time- and dose-dependent manner, LPS may inhibit or stimulate humoral immune responses [2, 3]. This feature may be regarded as a cause of the ability of LPS to act as adjuvant [4]. On the other hand, LPS may provoke several noxious effects such as autoimmune reactions, complement decrease, blood coagulation and lethal shock [4, 5]. These effects greatly restrict the applicability of LPS as immunomodulator and nonspecific resistance enhancer [5, 6]. In order to solve this problem it was proposed to modify LPS so as to decrease its toxic features but retain the beneficial ones [6, 7]. Several techniques have been used for detoxification with varying results. Perhaps the best result is obtained with ionizing radiation [6, 7]. The radio-detoxified endotoxin (RD-LPS) is less active than the parent (toxic) LPS inducing various other undesirable responses, however, preserves its beneficial effects e.g. shock-preventing, radio-protective, nonspecific resistance-enhancing, infection-preventing, immunoadjuvant, inter-

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feron-inducing and pluripotent stem-cell-mobilizing capacities [6–10]. The aim of this study was to compare the capacity of parent LPS and RD-LP S to enhance the immune response against xenogenic red blood cells and in parallel to induce the production of autoantibodies to autologous erythrocytes.

Materials and methods

Mice. Male C57Bl/6 × CBA: F_1/Rapo mice obtained from the breeding farm "Rappolovo" were used in all experiments. The animals weighing 22–24 g were 3 to 5 months old.

Endotoxin preparation. LPS from *Escherichia coli* 7573 (LPS-1) was extracted according to Boivin's method and purified by ultracentrifugation at 100 000 *g* [11]. LPS from *E. coli* O89 was extracted by the phenol-water method and purified by ultracentrifugation (LPS-2). RD-LPS was prepared from LPS-2 by ^{60}Co-gamma (150 kGy) irradiation [6, 7]. RD-LPS, Tolerin® will be manufactured by the Human Institute for Serobacteriological Production and Research, Budapest, Hungary.

Assay of the adjuvant effect of LPS and RD-LPS. Sheep red blood cells (SRBC) were stored in Alsever's solution until use. SRBC were loaded with LPS or RD-LPS by incubation for 30 min at 37 °C in phosphate-buffered saline (PBS) containing 50 μg/ml of either preparation. The mixture was washed 3 times in PBS. For the investigation of the adjuvant activity of LPS and RD-LPS, they were inoculated intravenously into mice together with 5×10^8 SRBC. Control mice received only SRBC. The number of plaque-forming cells (PFC) in the spleens of immunized mice was determined 4 days after immunization. The test was carried out according to Cunningham's method [12]. Local haemolysis in the liquid monolayer indicated PFC. Repeatedly used capillary chambers formed by means of two microscopic slides separated by one-sided adhesive plastic strip were applied instead of Cunningham-type disposable chambers [13]. The chambers were filled with a suspension consisting of SRBC, splenocytes and with mouse splenocytes absorbed guinea pig serum diluted 1 : 9 which served as the source of complement. Haemolytic zones were formed in the course of one-hour incubation in a humid chamber, and scored with a 5-fold magnification lens.

Investigation of the autoantibody production. For induction of auto-antibodies, LPS, and RD-LPS preparations suspended in PBS, were inoculated intraperitoneally into mice in doses ranging from 5 μg to 200 μg. The number of plaque-forming spleen cells was enumerated 3 days after the autoantibody induction. The test was performed according to Cunningham's method [12]. Fresh mouse erythrocytes were washed and incubated as a 50% suspension with Bromelain (Serva) at a final concentration of 10 mg/ml in PBS for 40 min at 37 °C. The bromelain-treated erythrocytes (BMEs) were washed 5 times in PBS containing 0.5% of bovine serum albumin, then incubated for one hour at 37 °C with spleen cells as in the adjuvant assay. Rabbit serum absorbed by splenocytes and diluted 1 : 2 served as the source of complement. Further on the test was performed as in the adjuvant assay.

Results

Comparison of the adjuvancity of LPS and RD-LPS. The LPS-1 when injected simultaneously with antigen elevated the number of anti-SRBC PFC. In this respect LPS-1 and LPS-2 did not differ one from another. RD-LPS had the same capacity to enhance the immune response as the negative one (Fig. 1). The parent LPS was capable to increase the antibody formation when adsorbed on SRBC. The same level of enhancement was achieved when LPS-2 was used instead of LPS-1. RD-LPS completely retained its ability to stimulate immune response against heterologous erythrocytes. In conclusion, RD-LPS did not differ from the parent LPS in respect of its capacity to act as adjuvant on the immune response against thymus-dependent heterologus antigen.

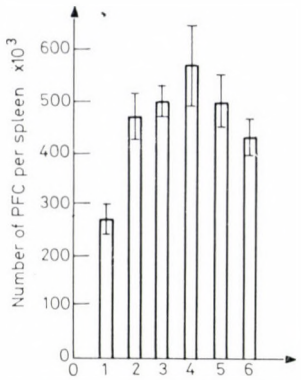

Fig. 1. The number of antibody-forming cells in the spleen of mice immunized with SRBC alone or in combination with LPS or RD-LPS (mean ± SD). 1 = SRBC control; 2 = SRBC loaded with LPS-1; 3 = SRBC and LPS-1; 4 = SRBC and LPS-2; 5 = SRBC and RD-LPS; 6 = = SRBC loaded with RD-LPS

Induction of auto-antibodies by LPS and RD-LPS. There is a large number of cells producing autoantibodies in the spleen of intact animals without any treatment by antigens or mitogens. In mice 3 to 5 months old the background level of anti-BME was about 7000 per spleen (Fig. 2). A single injection of LPS-1 to these mice resulted in a significant increase in the number of autoreactive cells (3-fold after 5 μg and 6–8 fold after 15–100 μg). Higher doses of LPS-1 caused the death of mice. LPS-2 had a lower capacity to induce autoantibodies compared with the former. Only a 4-fold increase of background level of anti-BME PFC was revealed after injection of 15–100 μg LPS-2. RD-LPS injection activated tha autoantibody forming cells only to a very slight

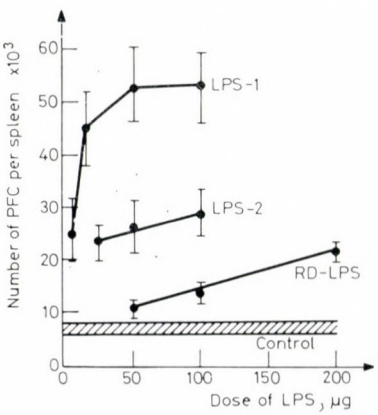

Fig. 2. The number of autoantibody-forming cells in the spleen of mice treated by single injection of LPS or RD-LPS (mean ± SD). The shaded area indicates background level of PFC in intact mice

degree. Even at 200 μg dose there was only some elevation in the number of these cells. This dose was not lethal for mice.

The increase in number of autoantibody-forming cells after a single injection of LPS was maximum and could not be further elevated by repeated injections. Moreover, two successive injections of LPS given at weekly intervals induced a lower number of autoantibody-forming cells than the single dose.

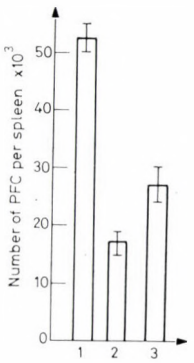

Fig. 3. The number of autoantibody-forming cells in the spleen of mice treated by one or two successive injections of LPS and RD-LPS (mean $\pm$ SD). 1 = 50 μg LPS-1; 2 = 50 μg LPS-1 + + 50 μg LPS-1; 3 = 100 μg RD-LPS + 50 μg LPS-1

In these experiments the first group of mice was treated with 50 μg of LPS-1, sacrificed three days later and served as a control. The second and third groups were injected with 50 μg of LPS-1 or 100 μg of RD-LPS, respectively. On day 6, animals of the second and third groups were injected as controls with 50 μg of LPS-1 and sacrificed three days later. Challenge with LPS-1 of mice pretreated with the same preparation revealed three-fold lower level of autoantibody-forming cells as compared to the control. Thus the first injection of native LPS results in sui generis "desensitization" of the organism against further stimulation of autoimmunity by endotoxin. RD-LPS caused only a two-fold but significant depression of autoimmune response on challenge with LPS-1 (Fig. 3).

Comparison of two different variants of native LPS revealed their different autoantibody-inducing capacity. LPS-1 (Boivin-type) is a stronger stimulator of autoantibody-forming cells than LPS-2 (Westphal-type). Detoxified form of LPS-2, RD-LPS practically did not stimulate autoantibody production when administered at the same doses as the two native preparations. At the same time RD-LPS did not lost the capacity to modulate the autoimmune response, as pretreatment of animals with it caused a decrease in the number of autoantibody-forming cells generated during the response induced by a subsequent challenge with LPS-1.

Discussion

From the data reported here we may conclude that it is possible to use this preparation as an adjuvant in doses larger than that of the native substance. RD-LPS may be useful in cases when the organism's resistance to toxic action of LPS decreases as a sequence of tumour growth [14], radiation injury or cytostatic therapy [5, 7, 15]. On the other hand, it has been shown in this study that the detoxified preparation has a reduced ability to induce autoantibody production. This indicates that administration of LPS might stimulate the immune reactivity of the organism suffering simultaneously from autoimmune disease and immune deficiency. In addition, the data presented here suggest that there may be some connections between toxicity of native LPS and its capacity to induce autoimmune reactions.

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DETECTION OF ADENOVIRUS HEXON USING MONOCLONAL ANTIBODIES FOR ANTIGEN CAPTURE IN ELISA

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The feasibility of using monoclonal antibodies to capture specific viral polypeptides in double monoclonal antibody (MAb) sandwich ELISA has been demonstrated. Ten MAbs with different reactivity patterns were used for antigen capture and four of them were labelled with horse radish peroxidase and used for the detection of specifically bound hexon antigen and for the determination of the lowest reacting hexon concentrations. Differences have been found in the effectiveness of the different MAbs used for capturing or detecting the antigen. Two assay systems (MAbs 1A3/1A3 and H12/1A3), which were the most sensitive for the hexon types studied could be useful for detection of the adenovirus hexons. Assay system MAb 2D1/2A1 and several combinations of MAb 2B2 proved to be specific for subgenus C.

The adenovirus capsid is built up of 252 capsomers designated as penton, fiber and hexon. The major coat protein, the hexon consists of three identical polypeptide subunits, and contains different antigenic determinants [1–5]. With the help of MAbs to adenovirus type 1 (Ad h 1) hexon data were presented [6] that a large number of Ad h 1 related epitopes exist on different heterologous hexon types. These epitopes are present on different antigenic sites in the form of partially overlapping epitope clusters on the surface of hexon molecule [7] in more than one copies [8]. The feasibility of using MAbs as antigen capture and detector reagents in double MAb sandwich ELISA was investigated in this study.

Materials and methods

Antigens. Human adenovirus types of subgenera C (1, 2, 5, and 6), A (12), B (7 and 35) and D (8, 9, 10 and 13) were propagated in HEp-2 cells and the hexon protein was purified as described earlier [9, 10]. In the case of Ad h 1 hexon the purification procedures were completed with crystallization [11]. Ad sim 16 (SA 7) hexon and Ad bos 3 were kindly supplied by Dr. Khilko and Dr. A. Bartha, respectively. The protein concentration of the hexon preparations was measured by the method of Lowry et al. [12].

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Monoclonal antibodies. BALB/c mice were injected with redissolved crystals of Ad h 1 hexon [13]. The Sp2/O nonsecreting parental myeloma cell line was used for fusion [14]. The specific antibody secreting hybridoma cell lines were injected into mice and the developped ascitic fluids were studied. Labelling of the Mabs with horse radish peroxidase was carried out by the method of Nakane and Kawaoi [15].

Indirect ELISA. All the ELISA experiments were carried our on polystyrene plates (Novogen) using PBS (pH 7.2) as coating buffer. For the determination of the antibody titres and reactivity patterns of the ascitic fluids, the wells were sensitized overnight with hexon preparations of different hexon types in 40 μg/ml concentration. Serial dilutions of MAb containing ascitic fluids were examined. The enzyme substrate was o-phenylene diamine in every ELISA experiments and the colour change was assessed at 492 nm in a Titertek Multiskan spectrophotometer. The antibody titres were expressed as $\log_2$ (reciprocal dilution $\times 10^{-2}$).

Double MAb sandwich ELISA. Ten MAb containing ascitic fluids showing different reactivity patterns [16] were used for antigen capture in different dilutions depending on the titres. Wells were coated with the diluted ascitic fluids [17] overnight. Serial dilutions of different hexon types were adsorbed to the wells coated with MAbs. As detector antibodies four peroxidase labelled MAbs with different reactivity patterns were used, and the lowest detectable hexon concentrations were determined.

Results

Selection of ascitic fluids for double MAb sandwich ELISA. Several different criteria were established for the selection of MAbs in double MAb sandwich ELISA. These criteria included (1) the antibody titre of the ascitic fluids with homologous type 1 hexon, (2) reactivity pattern with different hexon types studied, (3) recognition of different epitopes on different antigenic sites on the surface of Ad h 1 hexon, (4) recognition of different epitope clusters on the same antigenic site. On the basis of these criteria ten MAbs were selected for antigen capture in double MAb sandwich ELISA.

Ad (1). The titres of MAbs with Ad h 1 hexon varied between 1 : 25 000 to 1 : 1 638 400 (Table I).

Ad (2). MAbs represented four different reactivity patterns by the results with different heterologous hexon types studied. Two of the MAbs reacted with all the hexon types investigated (MAbs 1A3 and 2C2). Three MAbs (H12, 2A6 and 2D6) failed to react with Ad bos 3 hexon. MAb 2A1 did not react with Ad bos 3 and Ad h 12. Four of the MAbs (1B2, 2D1, 1C5 and 2B2) failed to react either with human types 12 and 35 or with animal hexon types studied. Four of the MAbs representing four different reactivity patterns were chosen to be conjugated with horse radish peroxidase.

Ad (3) and (4). Third and fourth criteria were the recognition of different antigenic sites and epitope clusters. Two of the MAbs (1A3 and 2C2) were reactive with an epitope on antigenic site I, the remaining eight MAbs were specific for epitopes on antigenic site II and recognized three different epitope clusters [7].

Four variations of double MAb sandwich ELISA were studied for the detection of the lowest reacting hexon concentrations of different adenovirus types: (i) identical or different capture and detector MAbs recognizing identical

Table I

Reactivity patterns and titres of the MAbs in indirect ELISA

MAbs	Human adnovirus types according to subgenera											Simian	Bovine
	A	B		C				D				SA 7	bos 3
	12	7	35	1	2	5	6	8	9	10	13		
1A3	5	11	6	11	11	11	11	11	10	11	11	7	0
2C2	5	9	4	8	9	9	9	9	9	9	8	3	0
H12	9	12	9	12	12	12	12	11	12	11	11	10	<0
2A6	0	11	<0	11	11	11	12	11	11	10	8	10	<0
2D6	0	12	<0	14	13	13	13	13	13	12	12	12	<0
2A1	<0	8	3	9	9	10	10	9	9	8	8	10	<0
1B2	<0	4	<0	11	11	11	12	11	11	10	7	<0	<0
1C5	<0	3	<0	11	11	10	9	11	11	9	8	<0	<0
2D1	<0	1	<0	9	9	8	9	9	9	8	7	<0	<0
2B2	<0	1	<0	9	9	8	9	8	9	8	7	<0	<0

Titres are expressed as $\log_2$ (reciprocal dilution $\times 10^{-2}$); 0, positive reaction in 1 : 100 dilution; <0, no positive reaction in 1 : 100 dilution

epitopes (1A3/1A3, 2A1/2A1, 2A6/2A6, 2B2/2B2 and 2C2/1A3, 2D6/2A6, 1B2/2A6 systems); (ii) capture and detector MAbs directed against epitopes on different antigenic sites (1A3 or 2C2/2A1, 2A6 and 2B2, as well as 2A1, 2A6, 2D6, H12, 2D1, 2B2, 1B2 and 1C5/1A3 systems); (iii) capture and detector MAbs recognizing the same epitope cluster but the competition ELISA pattern showing differences in complete and partial inhibition (1B2/2A6, H12/2A1, 2D1/2A1 and 1C5/2A1 systems); (iv) capture and detector MAbs reacting with different epitope clusters (2A6, 2D6, 1B2/2A1, 2B2/2A1, 2A1, H12, 2D1, 1C5/2A6, and 2A6, 2D6, 1B2 or 2A1, H12, 1C5, 2D1/2B2 systems).

Double MAb sandwich ELISA with MAbs reacting with identical epitopes. Four of the ten MAbs were used in unlabelled form for antigen capture and in labelled form for the detection of the bound antigen. In a few cases, the capture and the detector MAbs were not the same but they recognized the identical epitope (Tables I and II). MAbs 1A3 and 2C2 showed the same reactivity patterns in indirect ELISA. Using them for antigen capture and detecting the bound antigen by labelled MAb 1A3, differences were detected between the lowest reacting hexon concentrations of heterologous hexon types of subgenera A, B and D, while no such differences could be detected with the members of subgenus C. These differences were shown in other three sandwich systems, too. Using the 2B2/2B2 system for the detection of different hexon antigens, there was no reaction with types of subgenus D and with type 7 of subgenus B even in 51.2 μg/ml concentration. It was surprising in the case of types of subgenus D, because MAb 2B2 had a high titre with these heterologous types in indirect ELISA (Table I).

Table II

Double MAb sandwich ELISA with MAbs directed to the same epitope

Capture MAb	Detector MAb	Human adenovirus types according to subgenera										
		A	B		C				D			
		12	7	35	1	2	5	6	8	9	10	13
1A3	1A3	0.1	0.1	12.8	0.1	0.2	0.1	0.1	0.8	0.8	0.8	0.1
2C2	1A3	25.6	51.2	51.2	0.1	0.2	0.2	0.2	51.2	51.2	51.2	51.2
2A1	2A1	n. r.	1.6	—	0.025	0.025	0.025	0.025	3.2	3.2	6.4	0.4
2A6	2A6	n. r.	0.2	—	0.006	0.0125	0.0125	0.0125	3.2	3.2	1.6	0.8
2D6	2A6	n. r.	0.2	—	0.025	0.1	0.05	0.1	3.2	1.6	0.4	0.2
1B2	2A6	n. r.	—	n. r.	0.05	0.5	0.4	0.4	51.2	25.6	—	6.4
2B2	2B2	n. r.	—	n. r.	0.05	0.2	6.4	0.1	—	—	—	—

Figures mean the lowest reacting hexon concentration in μg/ml; —, not reacting in 51.2 μg/ml concentration; n. r., no positive reaction in indirect ELISA

Table III

Double MAb sandwich ELISA using MAbs directed against epitopes on different antigenic sites

Capture MAb	Detector MAb	Human adenovirus types according to subgenera										
		A	B		C				D			
		12	7	35	1	2	5	6	8	9	10	13
1A3	2A1	0.1	0.1	—	0.006	0.0125	0.05	0.006	0.4	0.4	0.2	0.1
1A3	2A6	0.05	0.1	—	0.006	0.0125	0.006	0.006	0.8	0.8	0.2	0.4
1A3	2B2	n. r.	—	—	0.025	0.1	0.2	0.2	—	—	—	—
2C2	2A1	25.6	51.2	—	0.006	0.025	0.025	0.05	51.2	51.2	51.2	51.2
2C2	2A6	25.6	51.2	—	0.05	0.05	0.4	0.1	51.2	51.2	51.2	51.2
2C2	2B2	n. r.	—	n. r.	0.025	0.1	0.2	0.2	—	—	—	—
H12	1A3	0.1	0.1	12.8	0.05	0.2	0.05	0.1	1.6	3.2	0.4	0.8
2A1	1A3	n. r.	0.2	—	0.05	0.1	0.05	0.05	12.8	12.8	6.4	25.6
1C5	1A3	n. r.	—	n. r.	0.05	0.1	0.1	0.1	12.8	—	—	6.4
2D1	1A3	n. r.	—	n. r.	0.1	0.2	12.8	0.8	—	—	—	25.6
2A6	1A3	n. r.	0.4	—	0.025	0.05	0.05	0.05	3.2	6.4	3.2	0.8
2D6	1A3	n. r.	0.1	—	0.025	0.05	0.05	0.1	0.2	3.2	0.4	0.4
1B2	1A3	n. r.	—	n. r.	0.05	0.1	0.8	0.4	51.2	51.2	51.2	6.4
2B2	1A3	n. r.	—	n. r.	0.1	0.2	12.8	0.1	—	—	—	—

For explanation see Table II

Double MAb sandwich ELISA with MAbs reacting with epitopes on different antigenic sites. MAbs used for antigen capture as well as for the detection of the captured antigens were reactive with epitopes on two sterically distinct antigenic sites. Two of the MAbs were specific for antigenic site I and eight other MAbs for antigenic site II. Using the MAbs directed against antigenic site I for antigen capture and labelled MAbs specific to antigenic site II (2A1 and 2A6) all the hexon types studied were detectable with the exception of type 35 hexon. With the help of the third labelled MAb (2B2) recognizing epitopes on antigenic site II, only the members of subgenus C were detectable (Table III). The differences in the effectivity of antigen capture of MAbs 1A3 and 2C2 were similar to the results showed in Table II for 1A3/1A3 and 2C2/1A3 systems. For the detection of hexon antigens of adenovirus types belonging to subgenus C, MAb combinations of 1A3/2A1 or 2A6 were the most effective with a lowest detectable hexon concentration of 0.006 μg/ml for Ad h 1 and 6, as well as for Ad h 1, 5 and 6, respectively. Using MAb H12 for antigen capture (directed against antigenic site II) and labelled MAb 1A3 to detect the bound hexon (specific to antigenic site I), all the adenovirus types could be detected, and the lowest reacting hexon concentration was in a range of 0.05 to 0.2 μg/ml with the members of subgenus C and with types 7 (subgenus B) and 12 (subgenus A). The other hexon types studied were reactive only in higher concentration compared to the members of subgenus C, or types 12 and 7. MAbs 2A6 and 2D6 used for antigen capture showed similar results to each other in the case of subgenus C, but there were differences within hexon types belonging to other subgenera. With capture MAb 1B2 differences could be detected within the members of subgenus C, and types of subgenus D were detected with labelled MAb 1A3 in significantly higher concentration. In 1C5/1A3 system similar results were obtained with the members of subgenus C, with types 8 and 13 (subgenus D), however, reaction was observed only in high concentrations. The other members of subgenus D and type 7 (subgenus B) did not react even in 51.2 μg/ml concentrations.

Using MAb 2D1 for antigen capture and MAb 1A3 as detector MAb, only the members of subgenus C and type 13 of subgenus D gave positive reaction but the latter one in significantly higher concentration than the types of subgenus C. In this assay system, the members of subgenus C reacted differently, types 1 and 2 reacted in much more lower concentration than types 5 and 6. Using the MAb system 2B2/1A3, only the members of subgenus C were detectable, but the lowest reacting hexon concentration of type 5 compared to the other members of subgenus C was high. Types of subgenus D and type 7 (subgenus B) did not react even in 51.2 μg/ml concentration.

Double MAb sandwich ELISA with MAbs specific for the same epitope cluster. Table IV shows the results when MAbs used for antigen capture and detection of the bound hexon were not identical, but they recognized the same

Table IV

Double MAb sandwich ELISA with MAbs directed against the same epitope cluster

Capture MAb	Detector MAb	Human adenovirus types according to subgenera										
		A	B		C				D			
		12	7	35	1	2	5	6	8	9	10	13
H12	2A1	0.2	0.1	—	0.0125	0.1	0.0125	0.025	3.2	1.6	0.4	0.8
1C5	2A1	n. r.	—	n. r.	0.025	0.05	0.4	0.05	25.6	—	—	12.8
2D1	2A1	n. r.	—	n. r.	0.05	0.05	0.4	0.1	—	—	—	—

For explanation see Table II

or closely related epitopes based on the results of competition binding ELISA [7]. In H12/2A1 system all the hexon types were detectable with the exception of type 35, in the case of capture MAb 1C5 and detector MAb 2A1, types 9 and 10 (subgenus D) and type 7 (subgenus B) did not react in the highest concentration studied. With MAbs 2D1/2A1 only the members of subgenus C were detectable.

Double MAb sandwich ELISA with MAbs specific for different epitope clusters. In these experiments eight MAbs reactive with three different epitope clusters on antigenic site II were used for antigen capture and three different

Table V

Double MAb sandwich ELISA with MAbs directed against different epitope clusters

Capture MAb	Detector MAb	Human adenovirus types according to subgenera										
		A	B		C				D			
		12	7	35	1	2	5	6	8	9	10	13
2A6	2A1	n. r.	0.8	—	0.0125	0.0125	0.0125	0.05	3.2	3.2	3.2	0.8
2D6	2A1	n. r.	0.8	—	0.0125	0.05	0.1	0.05	3.2	1.6	0.4	0.2
1B2	2A1	n. r.	—	n. r.	0.025	0.05	0.4	0.4	51.2	51.2	51.2	6.4
2B2	2A1	n. r.	—	n. r.	0.025	0.05	0.4	0.1	—	—	—	—
2A1	2A6	n. r.	—	—	0.05	0.05	0.4	0.4	—	—	—	25.6
H12	2A6	0.2	0.1	—	0.0125	0.05	0.0125	0.05	3.2	6.4	0.4	0.4
1C5	2A6	n. r.	—	n. r.	0.05	0.05	0.1	0.4	—	—	—	1.6
2D1	2A6	n. r.	—	n. r.	0.05	0.1	12.8	0.4	—	—	—	25.6
2B2	2A6	n. r.	—	n. r.	0.05	0.2	6.4	0.1	—	—	—	—
2A6	2B2	n. r.	—	n. r.	0.025	0.1	0.2	0.2	—	—	—	—
2D6	2B2	n. r.	—	n. r.	0.025	0.1	0.2	0.2	—	—	—	—
1B2	2B2	n. r.	—	n. r.	0.025	0.1	0.2	0.2	—	—	—	—
2A1	2B2	n. r.	—	n. r.	0.0125	0.05	0.1	0.0125	—	—	—	—
H12	2B2	n. r.	—	n. r.	0.025	0.1	0.2	0.1	—	—	—	—
1C5	2B2	n. r.	—	n. r.	0.05	0.1	12.8	0.2	—	—	—	—
2D1	2B2	n. r.	—	n. r.	0.1	0.1	12.8	0.4	—	—	—	—

For explanation see Table II

MAbs specific for the given epitope clusters were used as detector antibodies. According to the results shown in Table V, only a few combination of the MAbs could be used for detection of hexon types belonging to subgenera A, B and D, and in significantly higher concentration compared to the results of subgenus C. No variation could detect type 35 of subgenus B. There are a lot of variations in which only the members of subgenus C could be detected, and the hexon types of other subgenera did not react even in 51.2 μg/ml concentration.

Discussion

A double MAb sandwich ELISA was developed and used for the detection of adenovirus antigens. Ten MAbs with different reactivity patterns were used as antigen capture antibodies and four of them in peroxidase labelled form as detector MAbs. Although all the MAbs used in this study had high titres in indirect ELISA, there were obvious differences in their ability for antigen capture. The efficiency of capturing ability of a given MAb possibly reflects to the relative affinity of the MAb to an epitope. Using the same MAb for antigen capture and for detecting of the specifically bound hexon antigen, positive reaction could be only found if the epitope specific for the given MAb was present in more than one copies on the hexon molecule. The situation is the same, when the two MAbs used in this assay system are not the same, but directed against identical epitope. The range of reactivity of any given capture antibody, however, is dependent on the relatedness of the reactive epitopes [18].

Using these ten MAbs for antigen capture and the labelled ones as detectors, there are some variations, which could be useful for detection several different hexon types (1A3/1A3, or H12/1A3). In these assay systems, all the hexon types studied could be detected, and the method is simpler than the test systems used by Anderson et al. [19]. On the other hand, with the help of double MAb sandwich ELISA, differences could be observed among the subgenera. Variations of assay systems, in which MAb 2B2 was the detector or the capture MAb seemed to be subgenus specific, i.e. only the members of subgenus C reacted in lower concentration than 51.2 μg/ml, in spite of the fact that such differences could not be found in indirect ELISA. The reason for this discrepancy is not yet clear. It could be caused by the sterical inhibition of binding of MAb 2B2 or it could be due to the difference of its avidity.

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EFFECT OF ATTENUATED VIRAL VACCINES ON SUCKLING MICE INFECTED WITH LCMV

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A single intraperitoneal treatment with live Newcastle Disease Virus (NDV) containing attenuated NDV vaccine, and with live infectious bursal disease virus (IBDV) containing attenuated IBDV vaccine, one day before intracerebral infection with lymphocytic choriomeninginis virus (LCMV) increased, whereas a similar treatment with inactivated NDV or IBDV vaccine did not influence the death rate of suckling mice from experimental lymphocytic choriomeningitis. Thus the attenuated live vaccine stimulated, whereas the inactivated ones failed to affect the cell-mediated immune response to LCMV. Control studies set up with the supernatant of plain tissue culture routinely used for the propagation of IBDV have shown that unlike the attenuated NDV vaccine, the immunostimulatory action is associated not so much with the virus itself, as with an as yet unidentified component of the tissue culture supernatant.

Intracerebral inoculation of mice with lymphocytyc choriomeningitis virus (LCMV) can lead to a lethal choriomeningitis, which is a sequal to a cellular immune response against soft meningeal cells. The immune response against the soft meningeal cells results from a change in the antigenicity of the cells as a consequence of the viral infection. It has been shown that T-lymphocytes react with the meningeal cells and play an important role in this process [1–3]. The course of LCMV infection in mice depends on the state of the host's cellular immune system. Healthy mice with a mature immune system upon infection with LCMV develop a lethal lymphocytic choriomeningitis within 6 to 8 days after infection. The death rate in these mice is 100%. In contrast, mice with an inadequate immune system, for example newborn mice with an immature immune system do not develop meningitis and survive the infection as symptomfree carriers [4–7]. It has been shown that the survival rate and the period of time between inoculation and death depends on the host's age at infection; that is, it tends to decrease with progressing age and maturation of immune system [4]. In suckling mice it has been shown that immunostimulants

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enhance the development of lethal meningitis, following intracerebral LCMV infection, whereas immunodepressants hamper the development of this disease.

In this study, we evaluated the effects of Infectious Bursal Disease Virus (IBDV) and Newcastle Disease Virus (NDV) on the outcome of LCMV infection in mice. The reason for choosing these viruses is that NDV is known to effect the cellular immune system. More importantly, mice infected with NDV show a decrease in contact sensitivity [8, 9]. This inhibition results from the fact that NDV infection damages the T-suppressor-afferent cells, and results in an inhibit of cell proliferation in regional lymph nodes. It has been further shown that the virus induced this effect only in its infective state, since virus inactivated by ultraviolet irradiation did not cause the same effect [10]. IBDV also known as the agent for Gumboro disease or infectious avian bursitis is known to cause a "viral bursectomy" by damaging the lymphoid cells of the bursa of Fabricius [11]. Young birds infected by IBVD show atrophy of the bursa; histologically they demonstrate lymphocyte destruction and necrosis, and reticuloendothelial cell proliferation not only in the bursa [12, 13], but also in the thymus and spleen [14, 15,]. The humoral immune response of IBDV-infected birds to heterologous antigens is low [16–18], owing to the suppression of B lymphocytes by the virus [9]. Electron microscopic examination of tissue from infected animals reduced the presence of virus particles in the lymphocytes and in macrophages [20, 21]. These results support the notion that IBDV depresses the cellular immune response. In contrast to the effect of IBDV in chickens, it has been further shown that IBDV in other animals tends to be immunostimulatory. Marmosets vaccinated with IBDV were protected from developing hepatitis upon challenge with hepatitis A virus [22]. Our interest in the immunostimulatory effect of these viruses is prompted by unpublished observation (Csatáry et al.] that cancer patients treated with attenuated viral vaccines show clinical improvement, such as tumor regression.

Taking into consideration the immunomodulatory property of IBDV and NDV, we investigated in the present study the influence of pretreatment with IBDV vaccines or with NDV vaccines on the course of i.cer. LCMV infection in suckling mice.

Materials and methods

Experimental animals. CFLP mice (LATI, Gödöllő) of both sexes were used, on a total 60 litters of 7–12 suckling mice aged 2 weeks. The mice used for titration and reisolation were 6 weeks old.

IBDV vaccines. Attenuated IBDV vaccine was prepared from IBDV virus of reduced virulence, propagated in chick embryo fibroblast (CEF) culture. Mice were treated with 0.03 ml of vaccine intraperitoneally one day before intracerebral infection with LCMV. The vaccine contained $10^{6.5}$ $TCID_{50}$/ml.

To prepare *inactivated IBDV vaccine*, the attenuated IBDV vaccine was treated with 2000 μg/ml ethylenimide, which was removed after the completion of inactivation and the preparation was applied exactly in the same schedule as the live attenuated vaccine.

Tissue culture supernatants were used as controls. The supernatant of noninfected CEF cultures, used otherwise for the production of the IBDV vaccine strain, and the inactivated supernatant of noninfected CEF cultures which was treated like inactivated IBDV vaccine, were used for pretreatment of the control mice in the same schedule as the vaccines.

NDV vaccines. Attenuated NDV vaccine: The attenuated, mezogene variety (H strain) of the fowl plague virus (NDV) propagated in embryonated chicken egg was used. Mice were treated intraperitoneally with 0.1 ml of vaccine (HA titre: $\log 2^{16}$/ml) one day before LCM infection.

Inactivated NDV vaccine. The inactivation of attenuated NDV vaccine was carried out with 100 μg/ml ehtylenimin, which was removed from the medium after inactivation. Pretreatment of mice was carried out in the same way, time and quantities, both with the inactivated and the attenuated vaccines.

Allantoamnion fluids were applied as control. The allantoamnion fluid of uninfected embryonated chicken egg serving otherwise for the production of NDV vaccine strain, or allantoamnion fluid obtained in the process for virus inactivation were used. Pretreatment of mice was carried out in the same way, time and quantities, both with the two allantoamnion fluids and the virus vaccines.

The live attenuated and the inactivated IBDV vaccine, the live attenuated and the inactivated NDV vaccine were equally produced by the Phylaxia Veterinary Biologicals and Feedstuffs Co. Ltd., Budapest for the active immunization of poultry. The vaccines, tissue culture supernatants and allantoamnion fluids were made available for the experiments by the producer.

LCMV. The strain WE maintained in this laboratory by serial intracerebral passages in mice was used throughout. For titration it was administered intracerebrally to 6 weeks old mice. For experimental infection, a single dose containing 100 LD_{50} virus was administered intracerebrally.

Recovery of LCMV. Brain suspensions obtained from mice surviving the infection by 30 days were diluted 1 : 10 and inoculated intracerebrally to groups of several mice. Virus recovery was established on the basis of typical nervous symptoms and deaths.

Results

In the experiment with IBDV vaccines six litters each were pretreated with live attenuated IBDV vaccine, inactivated IBDV vaccine, tissue culture supernatant, inactivated supernatant or PBS, by the same route and at the same time. Next day, half of the animals of each lot were infected intracerebrally with LCMV, and the remaining animals were treated by the same route with plain (virus-free) brain suspension (Table I).

In the experiment with NDV vaccines six litters each were pretreated with live attenuated NDV vaccine, inactivated NDV vaccine, with allantoamnion fluid, inactivated allantoamnion fluid or PBS. The following day half of the animals were infected intracerebrally with LCMV. The remaining mice were given normal brain suspension in the same route (Table II).

The experiments lasted 30 days. The mice infected with LCMV were observed for typical symptoms, and the deaths were recorded. The survivors were killed on the 30th day, and attempt was made to reisolate the virus from the brain of each. Reisolation of LCMV was positive in each case indicated that all survivors had become latent carriers of LCMV.

The effect of IBDV vaccines on the course of LCMV infection. The control mice treated with the virus-free brain suspension showed no symptoms and all survived. The time and the cumulative incidence of deaths in groups treated

with LCMV is shown in Fig. 1. In every case, the typical symptoms of LCM were responsible for the fatal outcome.

Forty-five per cent of the mice infected with LCMV died between days 7 and 22. Deaths characteristically occurred in the same period also in the groups differently pretreated before LCMV infection, but their cumulative incidence differed markedly between the groups, having been 82 and 79% in groups

Table I

Scheme of the experiment with IBDV vaccines

Group	No. of mice	Treatment	
		intraperitoneal	intracerebral
IBDV–LCM	30	attenuated IBDV vaccine	LCM virus
iIBDV–LCM	30	inactivated IBDV vaccine	LCM virus
S–LCM	30	tissue culture supernatant	LCM virus
iS–LCM	28	inactivated supernatant	LCM virus
LCM	30	PBS	LCM virus
IBDV	28	attenuated IBDV vaccine	×
iIBDV	28	inactivated IBDV vaccine	×
S	27	tissue culture supernatant	×
iS	26	inactivated supernatant	×
C	27	PBS	×

× = virus-free mouse brain suspension

Table II

Scheme of the experiment with NDV vaccines

Group	No. of mice	Treatment	
		intraperitoneal	intracerebral
NDV–LCM	35	attenuated NDV vaccine	LCM virus
iNDV–LCM	34	inactivated NDV vaccine	LCM virus
A–LCM	23	allantoamnion fluid	LCM virus
iA–LCM	31	inactivated allantoamnion fluid	LCM virus
LCM	29	PBS	LCM virus
NDV	24	attenuated NDV vaccine	×
iNDV	23	inactivated NDV vaccine	×
A	25	allantoamnion fluid	×
iA	24	inactivated allantoamnion fluid	×
C	23	PBS	×

× = virus free mouse brain suspension

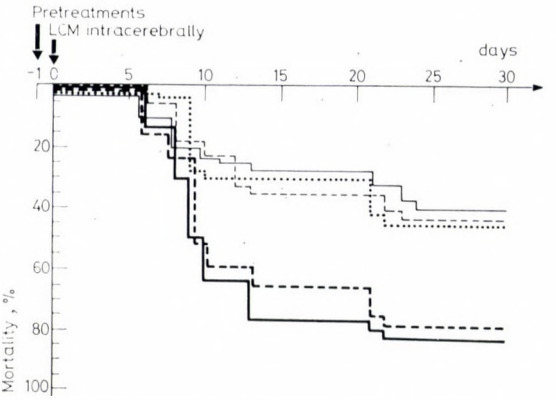

Fig. 1. Rate and time of deaths in experiment with IBDV vaccines. —— iIBDV-LCM; ····· iS-LCM; ▬▬ IBDV-LCM; ------ S-LCM; ····· LCM

IBDV-LCM and S-LCM, respectively. In the other two groups the ratio of deaths was in fact similar to the one observed in the LCM group, as out of the mice of the iIBDV-LCM group 40%, and in the iS-LCM group 43% died.

The effect of NDV vaccines on the course of LCMV infection. The control mice treated with virus-free brain suspension showed no symptoms and all survived. The time and the cumulative incidence of deaths in groups treated with LCMV is shown in Fig. 2. In every case, the typical symptoms of LCM were responsible for the fatal outcome. In this experiment, 41% of the mice of the LCM group died on the 10–20th day following virus infection. In the following three experimental groups death occurred in the same time and ratio: 35% in the iNDV-LCM group, 39% in the A-LCM group and 45% in the iA-LCM group. The ratio and time of deaths in the NDV-LCM group was significantly different, where 70% of the animals died between the 8–13th day.

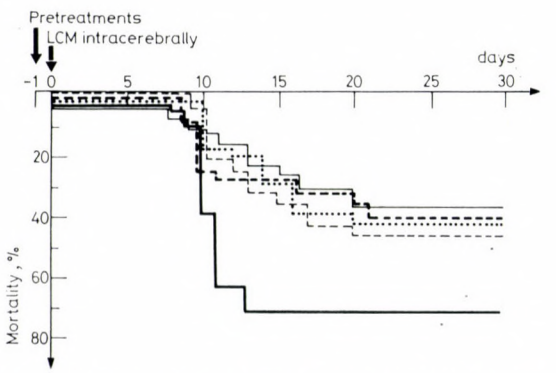

Fig. 2. Rate and time of deaths in experiment with NDV vaccines. —— iNDV-LCM; ····· iA-LCM; ▬▬ NDV-LCM; ------ A-LCM; ····· LCM

Discussion

Attenuated NDV and IBDV vaccines signicfiantly increased the death rate in mice infected with LCMV. It appears that the two vaccines had an immunostimulatory effect. In contrast, the inactivated NDV vaccine and the inactivated IBDV vaccine did not influence the course of LCMV infection. It was further shown that tissue culture supernatant fluid from cells that are used to propagate IBDV virus also were immunostimulatory and increased the death rate in mice infected with LCMV. The results indicate that an other component, separate from IBDV was responsible for the effect seen. Allantoamniotic fluids from embryonated eggs used to propagate the NDV did not have an effect on the outcome of LCMV infection. The difference observed between attenuated and killed NDV virus is consistent with other reports which have shown that NDV inactivated by ultraviolet radiation no longer was able to effect the T-suppressor-afferent cells [10]. In our studies it was further shown that LCMV could be isolated from the brain tissue of the surviving animals in each case. Therefore, the pretreatment had not inhibited the propagation of LCMV in mice. The immunostimulatory action of the attenuated vaccines and of the tissue culture supernatant fluid was similar to that reported from phytohaemagglutinin [23], *Bordetella pertussis* vaccine [24, 25] and LPS [26]. The stimulation of the immune system which follows treatment of suckling mice by inoculation with attenuated viral vaccine should be carefully evaluated as a model for generally modulating the immune system. Although the immunostimulatory effect of these attenuated viral vaccines on LCMV-infected mice leads to an undesirable effect. i.e. the death of the animals, in different circumstances the effect would be beneficial. For example we have shown that IBDV prevented the development of hepatitis [22]. In fact, it has been reported that tumour regressions occur in cancer patients inoculated with NDV [27, 28]. Further studies are warranted to evaluate the effect.

The stimulation of the development of cellular immune response was observed in suckling mice as a result of a single treatment with two attenuated, living virus vaccines which are widely used in veterinary practice for active immunization in prevention. The aspecific, immunomodulatory effect of vaccines observed in experiments can be effective in practical application as well, and this ability — besides their specific antigen effect — can widen their practical value.

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GROWTH OF SPONTANEOUS BALB/C TUMOURS EXCISED FROM AND RETRANSPLANTED TO AUTOCHTONOUS HOSTS

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Tumours of aged Balb/c mice developed without any conscious experimental interference were excised and retransplanted to the autochtonous hosts. The autotransplantation resulted in tumour take after a prolonged period of latency or in no take for an extended period of observation (more than 80 days) in 6 out of 19 cases. This can be regarded as a sign of antitumoural resistance, although it seems to be ineffective against development of recidives and metastases or second tumours. The sensitivity of the autotransplantation method in detecting antitumoural resistance was compared to that of the transplantation–excision–retransplantation assay using a benzpyrene induced Balb/c fibrosarcoma; the autotransplantation method proved to be less sensitive. According to these data the existence of some kind of resistance against spontaneous tumour cells cannot be excluded.

As a consequence of difficulties in the recognition of early, in situ neoplasms in vivo, existence of immunological [1], paraimmunological [2], or other [3] forms of surveillance capable to eliminate neoplastic cells cannot be proved or dismissed at present by direct experiments. According to indirect evidence of transplantation studies, one of the basic assumptions of the concept of immunological surveillance — i.e. the existence of tumour cell antigens potentially recognisable as foreign for the immune system of the host — seems to be valid in most cases of virus-, chemical carcinogen- and irradiation-induced tumours. Autochtonous or syngeneic hosts pretreated with such tumour cells become resistant to subsequent grafts of the same tumour [4–12]. On the contrary, similar methods fail to detect tumour rejection antigens on the surface of the majority of spontaneous tumours [7, 9, 13].

We report here the results of autochtonous transplantation of 19 spontaneous Balb/c mouse tumours. No take for an extended period of observation (81–187 days) or a prolonged latency period (longer than 82 days) was recorded in 6 cases. According to these data the existence of some kind of resistance against spontaneous tumour cells cannot be excluded.

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Materials and methods

Mice and tumours. Inbred Balb/c mice were used. They were fed a standard laboratory chow and water ad libitum.

Aged (more than one-year-old) mice bearing apparent tumours detected during routine changing of cages were separated and operated on under aseptic conditions in aether anaesthesia. Tumours were excised and the wounds were sealed by metal clips. The removed material was trimmed of extraneous tissues and central necrotic or haemorrhagic areas, minced into small pieces and homogenized in PBS. Enzymatic treatment of the tissues was avoided in order to preserve putative cell surface antigens. The number of trypan blue excluding cells was determined in Bürker chamber. Autochtonous and syngeneic transplantation was performed inoculating defined numbers of tumour cells intramuscularly into the rear leg(s) or subcutaneously under the dorsal skin. As a control, a benzpyrene induced Balb/c fibrosarcoma (BaFl), a gift of. J. Fachet, was used, which was serially transplanted to 8 weeks old syngeneic mice (10^6 cells).

Latency period, take and growth of tumours. Tumour development was monitored by palpation twice weekly. The time taken for palpable growth to appear determined the latency period. The tumours were measured in two dimensions with a caliper.

Post mortem examinations. Mice were examined post mortem for overt metastases. Suspected metastatic foci were further examined histologically. Tumour samples were fixed in 10% neutral buffered formalin, embedded in paraffin. Five μm sections were prepared and stained with hematoxylin-eosin.

Table I

Localization and mean diameter of autotransplanted spontaneous Balb/c mouse adenocarcinomas

Designation	Localization	Mean diameter at time of excision (mm)
	Neck	
SP 2		7
SP 7		8
SP 9		19
SP 20		13,5
SP 26/II		8
	Thoracal region	
SP 15		14
SP 16		23
SP 26/I		8
SP 36		13
	Abdominal region	
SP 11		13
SP 12		8
SP 17		25
SP 21		11
SP 33		9
SP 35		14.5
SP 38		22
SP 40		10
	Dorsal region	
SP 8		15.5
SP 42		11.5

Mean tumour diameter, 13.3 mm

Results

Table I shows the localization and size of successfully excised and autotransplanted spontaneous tumours of aged female Balb/c mice. All of them were malignant both by their biological behaviour (recidivation after excision, capability to give metastases) and by their histological picture (adenocarcinomas). The mean tumour diameter was 13 mm at time of excision (range, 7—25 mm).

Table II shows the results of autotransplantations performed by intramuscular inoculation of 6×10^5 tumour cells. In case of mouse bearing SP26 tumour two distinct tumours were detected and both of them were transplanted into the left and right thigh, respectively. The mean period of latency was 51 days (range, 29–82 days). Recidives developed at the site of excision in 6 cases. No take of the autotransplant was recorded in 5 mice, but out of these 2 succumbed within 45 days in consequence of the original tumour. In one case (SP2) take of the autotransplant could be registered only after 82 days.

Table III shows the results after autotransplanting of 10^6 cells intramuscularly (with the exception of SP11 which was injected subcutaneously). The autotransplant was taken in 5 out of 8 cases. The mean period of latency was 62 days (range, 30–84 days). Recidives developed in 6 mice. The latency period in case of tumour SP 35 was more than 80 days and the autotransplant of tumour SP42 was not taken during 187 days of observation.

In control experiments the autotransplantations of spontaneous tumours were simulated using a transplantable benzpyrene induced Balb/c fibrosarcoma

Table II

Take of spontaneous Balb/c mouse tumours autotransplanted by intramuscular inoculation of 6×10^5 tumour cells

Designation	Period of latency (days) till the		Survival time (days)
	take of autotransplant	appearance of recidive of the original tumour	
SP 2	82	—	99
SP 7	69	60	138
SP 8	No take	—	45
SP 9	41	56	99
SP 12	No take	—	125
SP 16	29	29	74
SP 17	No take	35	44
SP 20	40	24	70
SP 21	No take	91	117
SP 26/I[a]	No take	—	81
SP 26/II	43	—	
	Mean: 51 days Take: 6/11	Mean: 49 days	Mean: 89 days

[a] In case of SP 26 two distinct tumours were detected and both of them were transplanted into the left and right tight, respectively

Table III

Take of spontaneous Balb/c mouse tumours autotransplanted by intramuscular inoculation of 10^6 tumour cells

Designation	Period of latency (days) till the		Survival time (days)
	take of autotransplant	appearance of recidive	
SP 11[a]	No take	43	73
SP 15	46	23	54
SP 33	No take	—	75
SP 35	84	13	163
SP 36	79	—	85
SP 38	30	30	53
SP 40	71	71	77
SP 42	No take	58	187
	Mean: 62 days	Mean: 40 days Take: 5/8	Mean: 96 days

[a] SP 11 was inoculated subcutaneously

Table IV

Comparison of the sensitivity of transplantation-excision-retransplantation assay with that of autotransplantation in detecting antitumoral resistance

	Source of im inoculated cells	Period between tumorexcision and challenge	No. of tumuor cells inoculated im	Take of im injected BaFl[a] cells in	
				mice underwent excision of a transplanted BaFl tumor	controls
Transplantation — excision — retransplantation assay	Tumour cells from BaFl bearing (donor) mice	Exp. 1: 3 days	10^6	1/8	8/8
		Exp. 2: 14 days	10^6	3/9	9/10
Autotransplantation	Cells from sc growing BaFl tumours were reinoculated im into the same hosts after excision	challenge immediately after excision	6×10^5	3/8	14/23[b]
			10^6	5/8	19/23

[a] A benzpyrene-induced Balb/c fibrosarcoma

[b] Three untreated Balb/c mice were inoculated with BaFl cells from each tumour used for autotransplantation (1 mouse in each group died from intercurrent disease)

(BaFl) capable to induce transplantation resistance in syngeneic hosts (Table IV). Subcutaneously growing BaFl tumours were excised and the mice were retransplanted with BaFl tumour grown on syngeneic host 3 or 14 days after excision (transplantation-excision-retransplantation assay); alternatively, the excised tumours were used for autotransplantation immediately after excision. Non-immunized Balb/c mice were inoculated with the same tumours as well. There are marked differences in the take of BaFl tumour between the preimmunized and control group using the transplantation-excision-retransplanta-

tion assay; the proportion of resistant mice decreased when the method of autotransplantation was applied, thus the latter seems to be less sensitive in detecting antitumoural resistance.

Discussion

Various experimentally induced tumours possess antigens capable of inducing resistance to the same tumour [4–12]. On the contrary, in the majority of cases no trace of resistance could be demonstrated in syngeneic hosts to spontaneous tumours [7–9, 13–15]. The basis of this fundamental difference is unexplored at present. The absence of an effective immune response could be explained, disregarding the absence of tumour specific antigens, by missing Ir genes, by the inhibition of afferent, central or efferent phases of the response as well as by central or peripheric tolerance [16–24]. Another possible explanation for the lack of detectable tumour rejection antigens on the surface of spontaneously transformed cells could be that these antigens are less immunogenic than tumour rejection antigens of chemical-carcinogen or virus-induced tumours, and the methods avaiable were not sensitive enough to demonstrate the immunological reaction. Early works of Foley [7], and Prehn and Main [8] revealed the immunogenicity of methylcholanthrene-induced sarcomas and the non-immunogenicity of C3H mammary carcinomas and spontaneous sarcomas in isologous mice using tumour fragments implanted by trocar as challenge. Subtle antigenic differences, however, cannot be expected to detect after transplantation of large inocula [25]. Révész [9] and Klein et al. [10] confirmed the existence of antigenic differences between MCA-induced sarcomas and their isologous and autochtonous hosts. Preimmunization by heavily irradiated tumour cells induced a relative resistance to subsequent grafts of these tumours, and Révész [9] was also unable to detect resistance to C3H mammary carcinomas and spontaneous lymphomas of early passage generations. Hewitt et al. [13] used heavily irradiated cells as well in an attempt to immunize isogeneic mice against seven spontaneous tumours of high (19–289) serial passage number. However, Gross and Dreyfuss [26] reported successful immunization of guinea pigs against L_2C spontaneous leukaemia cells by intradermal inoculation of live leukaemic cells; irradiated cells did not induce immunity. Moreover, the antigen dose and timing profoundly influence the immune response to malignant ascites cells in C3H mice [27]. Woodruff et al. [2] tested various immunization procedures and observed a significant reduction in growth rate of a spontaneous murine adenocarcinoma after pretreatment of mice with viable cells or with mitomycin-treated cells; pretreatment by irradiated carcinoma cells was ineffective. A similar result was reported by Wrathmell and Alexander [28]: mitomycin-treated spontaneous rat leukaemia cells proved to be immunogenic, but irradiated cells could not immunize the animals.

According to Hewitt [29], a tumour can be regarded as "spontaneous" if it arises in an animal not deliberately exposed to any carcinogenic agent and which is not known to harbor any vertically or horizontally transmitted oncogenic viruses. Since the majority of spontaneous Balb/c mouse tumours investigated in this study seems to be of mammary origin, the possibility of expression of murine mammary tumour virus (MuMTV) antigens cannot be excluded. Balb/c mice as well as other inbred mouse strains and wild mice investigated so far carry in the genome MuMTV-specific sequences most likely acquired by the genus Mus 10–15 million years ago (see 30 for review). In the Balb/c mouse strain, there is a considerable inhibition of the expression of endogenous MuMTV and these mice do not carry the exogenous, milk-borne MuMTV. According to the results of the Netherlands Cancer Institute, Balb/c mammary tumours do not contain type B particles, though small amounts of viral antigen can be found in these tumours [30]. However, the expression of MuMTV structural antigens in mammary hyperplastic outgrowth lines of Balb/cfC_3H mice showed no correlation with the risk of tumour development after transplantation to Balb/c hosts [31]. Besides MuMTV, murine leukaemia virus [MuLV] can be also present in spontaneous Balb/c mammary tumours [32]. MuLV antigens, however, do not influence the tumour rejection activity of methylcholanthrene-induced sarcomas of Balb/c mice [33].

In conclusion, autotransplantation of spontaneous Balb/c adenocarcinomas resulted in take after a prolonged latency period or in no take for an extended period of observation (more than 80 days) in 6 out of 19 cases (31.5%). This could be a sign of antitumoural resistance, although it seems to be ineffective against the development of recidives and metastases or second tumours. In comparison, Hager et al. [34] observed a 9.5–65 days mean latency period after subcutaneous inoculation of 7 independently arising serially transplanted Balb/cfC_3H mouse mammary tumours into syngeneic hosts. In addition, the mean period of latency of SP_4 — a transplantable spontaneous Balb/c adenocarcinoma developed in our laboratory — is 14 days after intramuscular inoculation of 10^6 tumour cells.

The mechanism of this resistance is unexplored at present. Development of "concomitant immunity" [35, 36] cannot be excluded in mice with early recidive or second tumour (e.g. SP35, SP26). "Sinecomitant immunity" and other potential antitumoural effector mechanisms (e.g. natural killer cells, macrophages) may play a role in other cases [37–39]. In addition, the autotransplantation method seems to be less sensitive in detecting antitumoural resistance than the transplantation-excision-retransplantation assay. Thus, according to these data the existence of some kind of resistance against spontaneous tumour cells cannot be excluded.

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ELISA DETECTABLE VIRULENCE MARKER ANTIGEN OF ENTEROINVASIVE *ESCHERICHIA COLI* IS CODED BY A 140 MEGADALTON PLASMID

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The ELISA reactive virulence marker antigen, characteristic of enteroinvasive *Escherichia coli* and virulent *Shigella*, expressed in an *E. coli* K-12 recipient after the 140 Md plasmid of an enteroinvasive *E. coli* strain had been introduced into it. Similarly to the epithelial cell penetration and Congo red binding capacities — known to be coded by the above plasmid — the virulence marker antigen expressed also only at 37 °C but not at 30 °C. These data suggest the plasmid coded nature of the virulence marker antigen.

It is known that the 140 Md plasmid is responsible for the epithelial cell invasiveness of enteroinvasive *Escherichia coli* (EIEC) and *Shigella* strains [1–4]. This plasmid codes also for the capability to bind Congo red [5]. Both properties express at 37 °C but not at 30 °C [6].

Recently we have demonstrated by ELISA an antigenic relationship among EIEC and virulent *Shigella* strains [7]. The presence of this tentatively called virulence marker antigen (VMA) shows good correlation with the Serény test [8] regardless of the serogroup of isolates tested [9].

Although the presence of the 140 Md plasmid is necessary for the virulence, the plasmid may physically be present in noninvasive, avirulent and VMA negative strains, too [4, 9]. To investigate whether the non-conjugative virulence plasmid [2] of enteroinvasive strains is responsible for the expression of VMA we transferred it into a suitable recipient in order to examine its behaviour in ELISA test.

Materials and methods

Bacteria and culture conditions. Strains used are listed in Table I. *E. coli* strain 34 (O124: NM) was isolated from a patient with enteritis. J53 (K-12) was obtained from N. Datta (London England), UB281 harbouring plasmid pMR5 [10] was provided by M. Robinson (Bristol, England).

For ELISA test bacteria were grown on nutrient agar (Bacto Peptone 5 g, Bacto Beef Extract 3 g, NaCl 5 g, Bacto Agar 20 g, distilled water up to 1000 ml, pH 7.2). Tryptic Soy agar containing 100 μg/ml Congo red was used in the Congo red binding assay [11]. In mating experi-

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ments UB281(pMR5) was counterselected on minimal medium M9 [12]. Antibiotics were used when necessary in concentrations as follows: carbenicillin 250 μg/ml, kanamycin 30 μg/ml and nalidixic acid 50 μg/ml.

Transfer of the 140 Md plasmid was carried out by transposon mediated conduction using the Tnl harbouring temperature sensitive plasmid pMR5 [10] according the method of Watanabe and Nakamura [13] as detailed in the text.

Detection of the plasmids. Plasmid DNA was prepared and electrophorized by the method of Kado and Liu [14].

Epithelial cell penetration test. The capability of strains to invade epithelial cells was examined according to Hale and Formal [15].

Keratoconjunctivitis test. The virulence of strains was tested as described by Serény [8].

Production of VMA specific antibodies and ELISA test were carried out as previously described [9].

Results

As the virulence plasmid of enteroinvasive strains is not conjugative but can be mobilized by transposon mediated conduction [2] we followed the method of Watanabe and Nakamura [13] when plasmid pMR5 (Fig. 1, lane B) was introduced into the virulence plasmid pSP1 of strain No. 34 (Fig. 1, lane A). The proline and methionine dependent donor UB281(pMR5) was counterselected on minimal medium containing carbenicillin and kanamycin.

Forty-five transconjugants were checked for the presence of VMA by ELISA. All of them were VMA positive. One of them, 34(pSP1-pMR5), (Fig. 1, lane C) was chosen for further experiments.

In order to select donor cells harbouring pSP1-pMR5 cointegrates, 34(pSP1-pMR5) was cultivated overnight in nutrient broth containing carbeni-

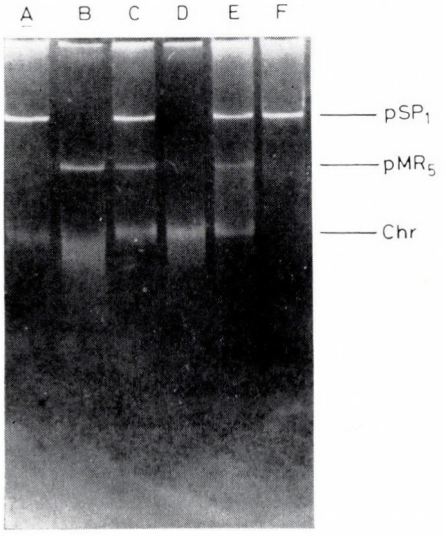

Fig. 1. Plasmid profile of bacterial strains used (A) No.34(pSP1), (B) UB281(pMR5), (C) 34(pSP1-pMR5), (D) J53, (E) J53(pSP1-pMR5), (F) J53(pSP1)

cillin and kanamycin at 42 °C, which is a nonpermissive temperature for pMR5 [10]. Next day this culture was mated with the recipient J53 (Fig. 1, lane D). Since Congo red binding is an easily detectable marker for the presence of the 140 Md plasmid [5] transconjugants were selected on Congo red agar [11] containing nalidixic acid to prevent the growth of the donor cells, and carbenicillin and kanamycin to counterselect non-transconjugant recipients. Among 3000 clones 6 Congo red positive (CR^+) ones were found. All the six clones gave positive reaction for VMA; harboured both pSP1 and pMR5; and metabolically proved to be the derivatives of J53. One of them, J53(pSP1-pMR5), is listed in Table I and its plasmid profile is shown in Fig. 1, lane E.

Table I

Properties of bacterial strains and their derivatives used in the study

Strain	Antibiotic resistance[1] Km	Carb	Nal	Lactose[2]	Auxotrophy[3] pro	met	Congo red Binding 30°	37°	Penetration of HeLa cells	Serény test	VMA[4] 30°	37°
No. 34 (pSP1)	S	S	S	−	+	+	−	+	+	+	−	+
UB281 (pMR5)	R	R	R	+	−	−	−	−	−	−	−	−
34 (pSP1-pMR5)	R	R	S	−	+	+	−	+	+	+	−	+
J53	S	S	R	+	−	−	−	−	−	−	−	−
J53 (pSP1-pMR5)	R	R	R	+	−	−	−	+	+	−	−	+
J53 (pSP1)	S	R	R	+	−	−	−	+	+	−	−	+

[1] Km: kanamycin, Carb: carbenicillin, Nal: nalidixic acid, S: sensitive, R: resistant. Km resistance is coded by pMR5, Carb resistance is coded by Tn1 harboured by pMR5 [10]
[2] Lactose fermentation
[3] pro: proline, met: methionine
[4] VMA: virulence marker antigen

Seventy CR^- clones were also tested by ELISA and none of them gave positive reaction for VMA. The plasmid content of three representatives of them were also examined. They harboured only pMR5 but not pSP1 (data not shown).

One of the CR^+, VMA^+ transconjugants, J53(pSP1-pMR5), was used to select clones harbouring Tn1 tagged pSP1 only. This strain was grown in nutrient broth free of antibiotics at 42 °C overnight and then plated onto nutrient agar. By replicating onto media containing carbenicillin and kanamycin and onto those containing carbenicillin only, from 200 clones tested six was selected being resistant to carbenicillin but sensitive to kanamycin. These clones were investigated by plasmid electrophoresis, by ELISA and for Congo red binding capability. All the six clones were CR^+, VMA^+ and harboured only pSP1 (Fig. 1, lane F).

The parent strains and transconjugants were tested for their invasiveness on HeLa cells and in Serény test [8]. While all the clones harbouring pSP1

penetrated the epithelial cells, were CR^+ and VMA^+, only 34(pSP1) and 34(pSP1-pMR5) gave positive reaction in the keratoconjunctivitis test (see Table I).

The temperature dependence of the expression of VMA was similar to that of CR positivity. As it can be seen in Table I, strains containing pSP1 were VMA positive only when cultured at 37 °C but not at 30 °C.

Discussion

Since the presence of the 140 Md plasmid is necessary for the virulence of enteroinvasive strains [1–4], and the existence of a virulence associated antigen (VMA) was also described [7, 9], it seemed to be worth to investigate the possible origin of VMA.

After transferring the 140 Md plasmid into strain J53 it became positive both in the Congo red binding assay and epithelial cell penetration test, properties known to be coded by the plasmid. In addition, the transconjugants expressed VMA while the original recipient did not. Similarly to the above mentioned plasmid coded markers, VMA also expressed only at 37 °C but not at 30 °C.

These data strongly suggest that VMA is coded by the 140 Md plasmid of EIEC. Further investigations are needed to clarify whether CR positivity and VMA positivity are alternative phenotypical expressions of the same genetic function or they are coded by separate DNA segments of the same plasmid.

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SEROTYPING OF *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED IN BULGARIA USING THE LÁNYI–BERGAN COMBINED SCHEME

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Two hundred *Pseudomonas aeruginosa* strains isolated in hospitals in Bulgaria were serotyped according to the combined scheme of Lányi and Bergan, supplemented by Akatova and Smirnova and Homma, using agglutinating O-antisera prepared in the National Institute of Hygiene, Budapest. The most frequently encountered serogroup is O2 (29%) followed by O11 (28.5%), O6, O3, O10 etc. The results were compared with those obtained by using Difco antisera prepared according to Liu et al. [4], and showed 96.5% coincidence. The strains were phage typed according to the scheme of Meitert and tested for antibiotic resistance to aminoglycosides (gentamicin, carbenicillin, tobramycin and amikacin). Phage groups 3 (3a and 3_3) and 1 (1a) predominated. The strains exhibited sensitivity to amikacin (99%) and frequent resistance to gentamicin (45.8%, carbenicillin (40%) and tobramycin (28%). Subdivision of the serogroups into phage and resisto-types contributes to analysis of nosocomial infections.

Proceeding from the necessity of unifying the methods for serotyping of *Pseudomonas aeruginosa* in COMECON countries, the coordination meeting of experts from socialist countries held in Bratislava (1983) recommended serotyping of *P. aeruginosa* to be carried out according to the scheme of Lányi and Bergan [1] supplemented by Akatova and Smirnova [2] and Homma [3]. In connection with this, serotyping of *P. aeruginosa* strains in Bulgaria was performed according to the combined scheme of Lányi and Bergan. The data were compared with those obtained by applying the serotyping scheme recommended by Liu et al. [4]. Phage typing and antibiotic typing were also employed for additional subdivision of strains in order to establish the prevalent sero, phage and antibiogram types occurring in Bulgaria.

Materials and methods

Slide agglutination was used for serotyping of 200 *P. aeruginosa* strains isolated from patients with *P. aeruginosa* infections (burn wound, nose, ear, throat, eye, urine, blood, sputum and faeces specimens) and hospital environment.

Agglutinating antisera for serotyping of *P. aeruginosa* by the combined scheme of Lányi and Bergan consisting of 15 serogroups were obtained from the National Institute of Hygiene in Budapest (23 antisera for partial O antigens).

Serotyping according to Liu et al. [4] was done with agglutinating antisera purchased from Difco (USA).

Phage typing was performed using the scheme of E. Meitert [5].

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Antibiotic resistance was assayed by the diffusion method of Bauer et al. [6, 7] using Mueller–Hinton medium and dry antibacterial discs (Institute of Infectious and Parasitic Diseases, Sofia).

Results

The results of serotyping of *P. aeruginosa* strains isolated in different hospitals in this country are summarized in Table I. From it becomes clear that all strains examined could be serotyped. Strains belonging to serogroup O2 ranked first (29%), followed by groups O11 (28.5%), O6 (12%), O3 (9%), O10 (7.5%), O4 (6.5%), O1 (4.5%), O9 (1.5%), O7 (1%), O12 (0.5%). No strains were identified in serogroups O13 and O15.

Table I

Serotyping of pseudomonas aeruginosa strains isolated from clinical specimens

Clinical specimens		Serogroups											
		1	2	3	4	6	7	9	10	11	12	13	15
Burn wound	82	4	20	12	3	8	1	0	2	22	1	—	—
Blood	9	—	4	4	—	—	1	—	—	—	—	—	—
Urine	45	2	7	—	3	8	—	1	3	21	—	—	—
Bile	1	—	—	—	—	—	—	—	1	—	—	—	—
Eye	8	2	1	—	—	2	—	—	2	1	—	—	—
Ear	2	—	1	—	—	—	—	—	—	1	—	—	—
Nose	9	—	3	—	1	1	—	1	2	1	—	—	—
Throat	3	—	—	—	1	—	—	—	1	1	—	—	—
Sputum	5	—	3	—	—	—	—	—	2	—	—	—	—
Faeces	10	1	2	1	3	2	—	1	—	—	—	—	—
Post mortem	2	—	1	—	—	—	—	—	—	1	—	—	—
Hospital environment	24	—	7	1	2	3	—	—	2	9	—	—	—
Total number	200	1	58	18	13	24	2	3	15	57	1	—	—
%	1	4,5	29	9	6,5	12	1	1,5	7,5	28,5	0,5	—	—

The data showed 96.5% coincidence with those obtained by the Difco sera. A discrepancy was noted in the case of 5 strains classified in serogroup 10 by the Difco sera, which were then included in the Lányi and Bergan group 2 (2 strains), 11 (2 strains) and 9 (1 strain). Two strains agglutinating in Difco serum group 6, had to be classified in group 3 of Lányi and Bergan. None of the strains reacted in Difco sera 8, 14, 16 and 17.

Examination of the clinical isolates in relation to the prevalent serogroups O2, O11 and O6, revealed that 48.3% of serogroup O2 strains originated from burn wounds, 11.7% from urine and 6.4% from blood cultures. Of the serogroup

O11 strains, 38.6% were isolated from burn wounds, 36.9% from urine and 15.8% from hospital environment. Serogroup O6 strains were cultured in 36.4% from urine and burn wounds and in 13.7% from hospital environment.

Table I shows that the strains isolated from burn wounds belonged mainly to serogroups O2 (35.3%) and O11 (24.8%). Isolates from blood cultures fell into serogroups O11 (46.7%), O6 (17.7%) and O2 (15.5%). The strains isolated from ear and eye specimens belonged mainly to serogroups O2, O10, O6 and O11. Isolates from faeces and hospital environment were distributed in most serogroups.

Table II presents data on phage-typing of 100 typable strains and their serogroup distribution. Phage group 3 strains predominated (52%), particularly

Table II

Phage typing of pseudomonas aeruginosa strains in different serogroups

Phage group	Number of strains	Phage type	Serogroup											Number of strains
			1	2	3	4	6	7	9	10	11	12	15	
1	11	1 a	—	3	1	—	2	—	—	1	4	—	—	11
		2 a	—	2	—	—	—	—	—	—	—	—	—	2
2	4	2 m	—	—	1	—	—	—	—	—	—	—	—	1
		2 n	—	—	—	1	—	—	—	—	—	—	—	1
		3 a	2	6	1	1	—	—	1	—	2	—	—	13
3		3 a_3	1	11	3	1	1	—	—	1	—	—	—	18
		3 a_4	1	1	1	—	—	—	—	—	—	—	—	3
	52	3 i	—	—	—	—	—	—	1	—	—	—	—	1
		3 i_3	—	—	3	1	—	—	1	—	—	—	—	5
		3 i_4	—	—	1	—	—	—	—	—	—	—	—	1
		3 m	—	2	—	—	1	1	—	—	—	—	—	4
		3 m_3	—	2	—	—	—	—	—	—	—	—	—	2
		3 l	—	5	—	—	—	—	—	—	—	—	—	5
4	6	4 j_3	—	6	—	—	—	—	—	—	—	—	—	6
6	4	6 a_3	—	2	2	—	—	—	—	—	—	—	—	4
		11 a_3	—	1	1	—	—	—	—	1	8	—	—	11
11	23	11 $_4$	—	—	—	2	1	—	—	1	7	1	—	12
Total number %	100		4	41	14	6	5	1	3	4	21	1	—	100

phage types $3a_3$ (18%) and 3a (11%). Phage group 11 was next in order (23%). Strains in this group were lysed only by phages 11 and 13 (the latter define only subserotypes in the original scheme of Meitert [8]. This was followed by phage groups 1 (11%), 4 (6%), 2 and 6 (4% each). There were no strains typable in other groups. A large percentage of the strains proved to be nontypable.

Phage typing revealed that strains present in the same serogroup belonged to different phage types. Strains possessing the same sero- and phage-type occur predominantly in cases associated with epidemics. Thus serogroups O2 and O11 included phage group 1 (1a) strains, isolated from wash-basins, baths and burn wounds. Strains of serogroup O11 were isolated from urine and a wash-basin in a dressing room and were shown to belong to phage-type 11 (11_3 and 11_4) [9].

The results of antibiotic typing of *P. aeruginosa* strains are given in Table III. They exhibited increased resistance to gentamicin (Gm) (45.5%), carbenicillin (Cb) (40%) and tobramycin (Tb) (28%). In serogroup O2 strains with increased sensitivity to Tb and amikacin (Ak) (65 and 100%, respectively)

Table III

Sensitivity of pseudomonas aeruginosa to aminoglycosides

Antibiotics	S%	I%	R%	Resistotypes							
				1	2	3	4	5	6	7	8
Carbenicillin	48	12	40	R	R	R	R	R	S	S	S
Gentamicin	47.2	7	45.8	R	R	S	S	R	R	S	S
Tobramycin	55.5	16.5	28	S	S	S	R	R	S	S	S
Amikacin	99	0	2	R	S	R	S	R	R	R	S

S = sensitive
I = intermediate
R = resistant

predominated; sensitivity to Cb and Gm was less frequent (27% and 31%), Group O11 strains were more frequently sensitive to Cb, Gm and Tb (37%. 47%, 63%) but two showed resistance to Ak. Serogroup 10 and 6 strains were characterized by high sensitivity to aminoglycosides (80–100%) whereas serogroup 3 strains exhibited a marked resistance to Gm and Tb (70%). Eight resistotypes were encountered only in epidemic-associated cases.

Discussion

The results of serotyping of 200 *P. aeruginosa* strains isolated in hospitals indicated that serogroup O2 occurred most frequently (29%), followed by serogroup O11 (28.5%) and serogroup O6 (12%). Data by Lányi and Bergan [1] on the distribution of serogroups in Europe, India, Japan and the Unted States show clearly the prevalence of serogroups O2 (18.6–37.1) and O6 (7.8–32.2%) in Europe, whereas serogroup O11 has been found more frequently in India (23–24.1%), in Japan (16.3%) and in the USA (8.9–17.7%). Recent data

by McManus and Mason [10] confirm the prevalence of serogroup O11 in the USA. The high incidence of serogroup O11 strains in Bulgaria may be accounted for by the expanded contacts between Bulgaria and many countries in Asia.

Phage typing data emphasize the leading role of phage group 3 (52%) ($3a_3$ 18%), followed by phage groups 11 (23%) and 1 (11%). Our data on the distribution of phage group 1 (1a) are close to those reported by Meitert [5] (13.6%) but differ considerably from those for phage group 4 which has been most frequently encountered in Rumania (4a, 34.4%) [5, 8] but ranks fourth in Bulgaria. Phage typing of most serogroup O2 and O11 strains (41% and 21%) provide the possibility for additional subdivision and reveal their involvement in nosocomial infections.

From the results of antibiotic resistance of *P. aeruginosa* to aminoglycosides it becomes evident that strains resistant to Gm (45%) and Cb (40%) predominate. In contrast to other authors [10] our isolates exhibited fully retained sensitivity to Ak (99%) and fairly frequent sensitivity to Tb (55.5%).

It is important from clinical and epidemiological point of view that serogroup O6 strains isolated from burn wounds of adult patients with surface burns showed relatively high sensitivities to the antibiotics tested, whereas serogroup O2 strains isolated mainly from children with deep burns and from blood cultures were highly resistant. The formulation of resistotypes contributes to epidemiological analysis.

It can be stated in conclusion that the agglutinating O antisera (National Institute of Hygiene, Budapest) for serotyping of *P. aeruginosa* according to the combined typing scheme of Lányi and Bergan allowed serotyping of all the strains isolated in Bulgaria. The antisera are highly specific and suitable for routine use. The experimental data show 96.5% correlation with those obtained by using Difco sera prepared according to Liu et al. [4].

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INTERPRETATION OF THE PRESENCE OF *PLESIOMONAS SHIGELLOIDES* IN FAECAL SAMPLES FROM PATIENTS WITH ENTERIC DISEASE

(A NOTE)

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Isolation of 18 strains of *Plesiomonas shigelloides* indicated that the organism is responsible for some of enteric infections in Hungary.

Ferguson and Henderson [1], in 1947, were the first to report on the isolation of this species. They described their isolate as a Gram-negative rod bearing the phase I somatic antigen of *Shigella sonnei*, and named it strain C27. At present, *Plesiomonas shigelloides* is classified in the family *Vibrionaceae* and in the genus *Plesiomonas*.

A number of papers, including review articles [1–12], are available on the cultivation, ecology and epidemiology of *P. shigelloides*.

The enteropathogenicity of the species is not quite clear yet. Its enterotoxin production is variously interpreted by different authors [12]. Recently, Sanyal et al. [2] passaged strains until their sufficient dose caused fluid accumulation in the rabbit intestinal loop and the cell-free filtrate of the strains changed the weight of the intestines of suckling mice.

In cell cultures CHO and Y_1, cytolysis and rounding off of cells was observed [2, etc.]. More recently, it has been claimed that *P. shigelloides* strains isolated from faecal samples obtained from children with diarrhoea were invasive in HeLa cells [3].

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In Hungary, the etiology of a great number of enteric illnesses have remained unknown even after the diagnosis of *Yersinia enterocolitica* and enteropathogenic *Campylobacter* species had been widely introduced. According to the Annual Reports of the National Institute of Hygiene, yearly 800 to 2000 cases of enteritis remained bacteriologically unknown in the years from 1979 to 1983.

We have made efforts to clear up the etiology of the obscure cases. In our best knowledge, we were the first to isolate *P. shigelloides* strains from human faeces in Hungary, namely in Szolnok county.

Materials and methods

Strains were cultivated and identified and their antibiograms were determined as usual in Hungary [4]. The biochemical features of the isolates agreed well with those given in the literature [1, 4, 12]. In each case, *P. shigelloides* was isolated from deoxycholate citrate agar medium, on which, with the exception of one strain, it appeared as pure culture or, at least, produced great numbers of shigella-like colonies.

Results and discussion

In the approximately four-year period from 1981 to 1985, 266 000 faecal samples were examined. *P. shigelloides* was isolated from 17 out of 86 000 patients with enteritis (21 000 infants and 65 000 patients above one year of age); positive results were obtained for one infant and 16 other patients. Of the 54 000 samples collected in the same period of time from contacts of patients suffering from diarrhoea, one strain of *P. shigelloides* was isolated.

We failed to isolate *P. shigelloides* in the course of screening of 126 000 healthy persons. According to literary data [5, 6], only few symptomless carriers of this bacterium have been found even in tropical countries.

Of the 18 positive cases 16 were revealed in the months from June to September, the remaining two were detected in other months, namely the one in March and the other in October. The relatively high incidence in the warm season may be consistent with Schubert's observation indicating that *P. shigelloides* fails to grow under 8 °C.

The 18 positive subjects were living in 12 communities of Szolnok county. We failed to demonstrate any contact or temporal relationship between them. The strains, in accordance with other reports from areas of the temperate climate [8–10], were isolated from sporadic cases.

The excreters varied in age between 8 months and 70 years. Seventeen of the 18 excreters showed enteric symptoms (Table I). Only one, a kindergarten teacher, stated that she had been healthy. Sixteen patients complained of frequent or very frequent episodes of diarrhoea, which was accompanied by

Table I

Isolation of P. shigelloides in Szolnok county in the years 1981—1985

Symptoms		No. of patients positive for *P. shigelloides*	Other pathogens isolated
Diarrhoea	frequent	16	*S. london*, *S. typhi-murium* and *C. jejuni* (1 patient each)
	rare	1	
	no	1	
Abdominal pain		10	*S. london* and *S. typhi-murium* (1 patient each)
Nausea		3	*S. london* and *S. typhi-murium* (1 patient each)
Headache		1	
Faeces	thin	12	*S. london* and *typhi-murium* (1 patient each)
	mucous	3	
	bloody	1	
	greenish	4	*C. jejuni* (1 patient)
Temperature, °C	≤36.9	14	*C. jejuni* (1 patient)
	37.0–38.9	3	*S. london* and *S. typhi-murium* (1 patient each)
	≥39.0	1	

abdominal pain in 10 cases. The diarrhoea was of abrupt (hyperacute) onset and lasted three or four days. The majority of the patients had thin, watery or mucous faeces. Fever was mentioned rarely. From faecal samples obtained from three of the positive patients an additional pathogen was isolated from each, namely, a *Salmonella london*, a *S. typhi-murium* and a *C. jejuni* strain.

Of the *P. shigelloides* isolates, 5 showed antigenic relationship with the phase I antigen of *Shigella sonnei*. All the 18 isolates proved to be sensitive to nalidixic acid, chloramphenicol and polymyxin-B and resistant to ampicillin and carbenicillin.

Extraintestinal *P. shigelloides* infection have also been reported [10], but the literature is much more abundant in reports, especially those from tropical countries, dealing with the gastro-intestinal appearance and the pathogenic role of this bacterium. In conclusion, *P. shigelloides* is present in Hungary in part of enteric infections.

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BOOKS RECEIVED

Sinkovics, J. G.: Medical Oncology. Second Edition. M. Dekker, Inc., New York 1986. Two volumes, pp. 2062. Price U. S. $ 174.

In a review about a scientific book, mention is rarely made of the Preface. In this book, however, the Preface forms a remarkable part, giving a short survey on the different trends — both success and disappointment — in cancer treatment in the last decades. In this Preface there is a frank and balanced view admitting honestly the shortcomings of cancer therapy today. The author is advancing his opinion about the possible — scientifically founded — treatment trends of the future and he is unfolding utterly impressing thoughts on medical ethics and scientific research.

The book differs from all other textbooks of oncology in having no separate part devoted to basic science and research, which are usually dealt with in the first half of other volumes. Instead, basis science and research are included in disease-oriented chapters. In the chapters on haematologic malignancies (I–IV), new data of basic research in virology and immunology are abundantly supplied prior to the presentation of clinical material. In chapters V–VI sarcomas are described in great detail and again research findings pertaining to these tumours are incorporated before diagnosis and treatment are dealt with. Neuroectodermal tumours and tumours of the central nervous system are presented in chapters VII—X integrated into complex sections with research and clinical material tabulated and sorted out under almost innumerable headings. Thereafter carcinomas are overviewed in chapters XI–XVIII according to organ systems (head and neck, lung, breast, endocrine glands, GI and GU tracts, skin and gynecological tumours), following the same principle of presentation in highly integrated up-to-date tabulations and concise accompanying text.

Chapter XIX is a most remarkable condensation of chemotherapy (drugs and their combinations into regimens, their modes of administration, up to the latest results). In this chapter there is an overview of cancer immunotherapy, that presents a critical and well-balanced view of bacterial products, tumour cell vaccines, cytotoxic lymphocytes, lympho- and monokines and monoclonal antibodies. Brief essays on supportive care (reconstructive surgery, pain medications; venous access) conclude this chapter.

Chapter XX is of great interest to medical microbiologists and infectologists. Infectious complications of cancer and their treatment are described condensed but in sufficient detail. An immense amount of new material (especially newer antibiotics) is included in this chapter.

The last part of this book consists of multiple choice, true and false and matching questions which have been referenced from the 1984–85 literature. Working through these problems contributes to an abundance of new knowledge, as they cover the entire field of medical oncology, integrating material still in the research phase with treatment modalities already in use.

This book is written in a highly condensed, almost telegraphic style, using lots of abbreviations. It may be regarded as a teaching experiment of the author to enable dissemination of an enormous amount of well-organised new material quickly and effectively to readers dealing with different branches of medical science. Other textbooks of this size are usually coauthored by 25 or more contributors with frequent overlappings and repetitions in the text. Most remarkably, *Medical Oncology* is written by one single author, thus overlapping areas among different chapters are avoided as much as possible. Citations of references are limited to journal, volume and page, serving as a guide to the library for details. The book is based on

over 25 000 original references. Apparently there was no other way but the author's unique arrangement for the presentation of this encyclopedic material in 2000 pages.

On the whole, not only practicing oncologists-haematologists but also basic scientists engaged in cancer research, virology and immunology and related research can greatly benefit from this book. Thus this book should be made available in hospitals and research institutions alike.

István Nász

Viesturs, U., Kuznetsov, A., Savenkov, S.: Fermentation Systems (in Russian). „Zinatne" Publishing House Riga, 1986. pp. 368. Price Rb 1,80.

INSTRUCTIONS TO AUTHORS

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Acknowledgement of grants and technical help.

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