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ANTIGENS OF *PSEUDOMONAS AERUGINOSA* AND THEIR USE IN IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY

(A REVIEW)

E. S. STANISLAVSKY, B. A. DMITRIEV, B. LÁNYI and I. JOÓ

Mechnikov Research Institute for Vaccines and Sera, Moscow, USSR, Zelinsky Institute of Organic Chemistry, USSR Academy of Sciences, Moscow, USSR, National Institute of Hygiene, Budapest, Hungary, and Human Institute for Serobacteriological Production and Research, Budapest, Hungary

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Introduction

In the last three decades nosocomial infections due to Gram-negative bacteria have played an important role in human pathology. One of the most important opportunistic pathogen in hospitals is *Pseudomonas aeruginosa*, which is widely distributed in nature, has a high degree of adaptability to the environment and readily acquires resistance to many antimicrobial agents. *P.*

EVGENY S. STANISLAVSKY
Mechnikov Research Institute for Vaccines and Sera
Per. Mechnikova 5a, 103064 Moscow, USSR

BORIS A. DMITRIEV
Zelinsky Institute of Organic Chemistry
Leninski Prospekt 47, 117913 Moscow, USSR

BÉLA LÁNYI
National Institute of Hygiene
Gyáli út 2–6, H-1966 Budapest, Hungary

ISTVÁN JOÓ
Human Institute for Serobacteriological Production and Research
Szállás u. 5, H-1107 Budapest, Hungary

aeruginosa is especially pathogenic for patients with decreased natural resistance to infections associated with age, burns, trauma, surgical operation, diabetes, tumour and other debilitating conditions. Predisposing therapy, as antibiotic treatment and administration of immunosuppressive and antimetabolite drugs, also play an important role in the development of pseudomonas infection.

Structure of the cell wall

P. aeruginosa produces an extracellular slime which covers the cell as a compact layer. Part of the strains produce slime in great amount; such "mucoid" strains are frequently isolated from the respiratory tract of patients with cystic fibrosis [1, 2]. The slime of *P. aeruginosa* differs from the true capsule of other bacteria which, in contrast to slime, adheres to the cell surface and does not separate from it in liquid medium [3]. The slime layer in Gram-negative bacteria including *P. aeruginosa*, contains highly organized complex patterns of polysaccharide fibres protruding radially from the cell wall [4, 5]. The polysaccharide fibres are attached to the outer membrane of the cell wall [6].

In ultrastructure, the cell wall of *P. aeruginosa* corresponds to that of other Gram-negative bacteria. It is composed of an outer membrane, peptidoglycan and a third electron-dense layer [7]. The outer membrane is convex, and consists of an external smooth layer and an internal layer of spherical protein-lipopolysaccharide aggregates 6–7 nm in thickness. The cell wall of *P. aeruginosa* contains 55–65% lipopolysaccharide and lipid, 30% protein and 5–15% peptidoglycan [8].

The peptidoglycan of *P. aeruginosa* is composed of a macromolecular network containing glutamic acid, 2,5-diaminopimelic acid, alanine, *N*-acetylglucosamine and *N*-acetylmuramic acid [9]. The cell wall of *P. aeruginosa* cultured in tryptose soy broth contains three kinds of protein: A (mol wt 43 000), B (mol wt 16 500) and C (mol wt 72 000). Proteins A and B are basic lipoprotein components of the outer membrane and are probably parts of the lipopolysaccharide–protein complex. Aqueous extraction of acetone-dried cells yielded two kinds of protein present probably in the outer membrane: A (mol wt 110 000–120 000) and B (mol wt 11 000–37 000) [10]. By EDTA–Tris treatment, 50% of the endotoxin aggregates can be extracted [11]. Extracts obtained in this manner were used for the preparation of *P. aeruginosa* vaccines consisting of 16 strains [12].

Virulence factors

Table I presents data for extracellular toxic substances which may be regarded as virulence factors of *P. aeruginosa*. Of the many kinds of extracellu-

lar toxic components synthesized by *P. aeruginosa* [13], exotoxin A is the one studied most extensively. This substance is produced by 90% of strains freshly isolated from clinical material [14]. Exotoxin A is an adenosine-diphospho-ribosyl transferase taking part in protein synthesis by catalizing the transfer of adenosine-diphosphate ribose from adenylnicotinamide dinucleotide to elongation factor 2 (EF-2) of the polypeptide chain. This reversible reaction is responsible for the inactivation of EF-2 needed for protein synthesis of the host cell [15]. Exotoxin A forms a single polypeptide chain of 71 000 mol wt. The native toxin is a proenzyme consisting of an active fragment (A, mol wt 26 000) and an inactive fragment (B, mol wt 45 000) [15, 16]. The native toxin molecule exhibits a minimum fermentative ability, but after denaturation, reduction or proteolysis, it is activated.

The biological activity of the purified exotoxin varies and depends on the presence of other extracellular products. Exotoxin A is a lethal agent to a wide variety of animals including primates; it exerts a cytotoxic effect in vitro and a necrotizing activity in vivo [16]. Exotoxin A obtained from *P. aeruginosa* strain PA-103 influences the respiration of mitochondria, reduces oxygen absorption stimulated by citrates, succinates and L-glycerophosphatase, but fails to act on oxygen absorption stimulated by pyruvic acid [17, 18]. Exotoxin A causes oedema of the mitochondria of liver cells [19]. In the opinion of Pavlovskis and Callahan [18], exotoxin A in the first step causes a change in the protoplasmic membrane and in the second step it inhibits intracellular protein and nucleic acid synthesis or acts directly upon extracellular processes. Exotoxin A is 2000 times more toxic to mice than the lipopolysaccharide of the same bacterial strain [20]. It is generally accepted that exotoxin A is sensitive to proteases; some data indicate that it is resistant to purified proteolytic enzymes of pseudomonas, e.g. to alkaline phosphatase and elastase [21].

The pathogenetic role of exotoxin is not perfectly clear. To produce this factor in vitro, defined conditions are needed. Some laboratory strains do not produce exotoxin but are still highly virulent for mice. It may be assumed that the synthesis of exotoxin A and protease is regulated mutually: in one phase of its life the bacterial cell synthesizes exotoxin and in the next phase protease, which destroys the exotoxin.

P. aeruginosa produces two or three kinds of protease [22] which probably play a role in the pathogenicity of the organism (Table I). According to Mull and Callahan [23] in septic conditions elastase is the main factor responsible for vasculitis. Proteases are assumed to be the main factors of tissue invasion by *P. aeruginosa* [24].

It has been described that *P. aeruginosa* may cause diarrhoea [30]. Kubota and Liu [32] have shown that, similarly to cholera exotoxin, virulent *P. aeruginosa* culture injected in ligated segments of the small intestine of

Table I

Virulence factors of P. aeruginosa

Substance	Chemical properties	Biological effect	References
Exotoxin A	Single polypeptide chain (mol wt 71 000); adenosine-diphosphate ribosyl transferase	Suppresses protein synthesis; lethal toxin	[16–18, 20, 25–30]
Haemolysin thermolabile	Protein (mol wt 70 000—90 000); phospholipase C	Lysis of animal erythrocytes; anti-leukocytic activity	[22, 31, 33]
thermostable	Acid glycolipid	Lysis of animal erythrocytes	
Elastase (protease P)	Dimerous protein (mol wt 23 000 or 39 000)	Suppresses complement activity, causes corneal keratolysis, hydrolyses casein, egg albumin, elastin, human and dog fibrin	[23, 34–36]
Proteases	Proteins	Cause corneal keratolysis, haemolytic inflammation, dilatation of ligated rabbit intestine, cytotoxic effect, cell proliferation	[22, 34, 37]
Enterotoxin	Heat-labile protein	Causes diarrhoea in course of intestinal infection	[30]
Collagenase	Protein, monomerous (mol wt 13 500) or dimerous (mol wt 34 000)	Hydrolyses collagen, congocol, azochyl and casein	[38]
Leukocidin	Protein (mol wt 27 000)	Cytotoxic on human, sheep and dog leukocytes	[39–42]
Lecithinase, lipase	Proteins	Toxic on HeLa cells, accelerate ulceration of mammal skin	[22, 43]
Extracellular slime	Glycoprotein (mol wt 100 000–300 000); possibly agglomeration of molecules	Exerts antiphagocytosis, causes leukopenia, toxic for mice, rabbits and rats	[44–49]

mice caused characteristic changes and accumulation of fluid. They have assumed that this was due to enterotoxin production by *P. aeruginosa*. However, the purified protease of *P. aeruginosa* has been shown to exert a similar effect [37], which makes it doubtful that the organism produces a true enterotoxin.

The role in virulence of extracellular slime has undoubtedly been demonstrated. The amount of slime produced by, and the virulence of, the strain showed a correlation in mouse experiments, although some strains which had less slime were as virulent as those producing it abundantly [50]. This finding

suggests that all these factors take part in the pathogenicity of *P. aeruginosa*. Strains which produce slime poorly may be virulent because of an abundant production of other substances, e.g. the highly mouse virulent strain PA-103, which is a good exotoxin producer, but has a very slight amount of extracellular slime.

By ultrafiltration the purified slime was fractionated into several components of different molecular weight: (1) > 300 000; (2) 100 000–300 000; (3) 30 000–100 000; (4) 10 000–30 000; (5) < 10 000. Compounds higher in molecular weight than 30 000 were the most toxic for mice [46]. The purified glycoprotein of the slime is toxic in a 30 μg/g dose for mice [47]; for this effect the lipid component is responsible [51]. The slime purified by phenol deproteinization contains galactose, glucose, rhamnose, mannose, xylose, ribose and uronic acid (Table II). The lipopolysaccharide also contains glucose and rhamnose, whereas galactose and probably mannose are characteristic of slime. Polyuronic acids, which are further characteristic components of the slime [44, 52] are responsible for the less ready solubility of slime in water.

Table II

Chemical composition of purified slime (glycolipoprotein) prepared from various strains of P. aeruginosa

Stanislavsky et al. [50]

Strain	Protein[1] %	Hexoses[2] %	Heptoses[3] %	Carbohydrate constituents						
				Uronic acids	Galactose	Glucose	Mannose	Xylose	Ribose	Rhamnose
170001	9.8	7.0	1.3	+	tr	+	tr	+	+	+
170002	12.6	8.0	nt	+	+	+	±	±	±	+
170005	10.0	8.0	0.9	+	±	+	tr	—	tr	+
170006	6.9	9.0	—	+	?	+	—	—	+	+
170007	10.0	8.0	—	+	+	+	tr	+	+	+
170009	16.6	6.0	—	+	+	+	tr	tr	+	+
170012	14.0	14.6	2.2	+	+	+	—	+	+	+
170014	10.9	10.0	nt	+	+	+	—	tr	tr	tr
170017	11.0	25.0	0.6	+	+	+	tr	—	tr	tr
170018	9.0	11.8	1.1	+	+	+	—	+	+	±
170019	20.2	10.0	—	+	+	+	—	—	+	tr
170021	nt	nt	nt	+	+	+	+	—	+	+
170022	11.7	21.0	—	+	±	+	+	tr	+	+
170023	nt	nt	nt	+	±	+	+	tr	+	+
O-II	8.6	7.0	—	+	+	+	+	+	+	—

[1] Protein assay with Folin reagent
[2] Assay with anthrone reagent
[3] Assay as described in [51]
\+ Considerable amount of sugar
± Small amount of sugar
tr Traces of sugar
— No sugar
nt Not tested

Some *P. aeruginosa* strains synthesize cell-bound substances that are toxic for human leukocytes. These factors, the leukocidins, are released from the bacteria after autolysis [39–42].

In the pathogenicity of *P. aeruginosa*, the virulence factors may be assumed to take the following schematic order. The first phase is characterized by adherence of, and invasion by, the agent in the host tissues. In this phase pili and extracellular slime play an important role. The latter ensures the resistance of the bacterial cell to phagocytosis and possibly to other immune mechanisms. In protecting the host against the first phase of infection, opsonins and probably anti-slime and anti-pilus antibodies play an important role.

When *P. aeruginosa* has gained access to the host tissue, an intensive multiplication phase ensues, in the course of which several extracellular factors are produced including exotoxin A, haemolysin, proteases, etc., which cause intoxication. In this phase of infection antitoxic immunity play the main part in protecting the host. Later, when the bacterial cells disintegrate, a more severe intoxication occurs as a result of endotoxin and leukocidin effects. A certain amount of exotoxin may be released from the intact bacteria, too.

Extracellular antigens

A. *Antigenic properties of proteases and exotoxin A*

In patients suffering from *P. aeruginosa* infection, antibodies have been shown to develop against *P. aeruginosa* proteases (more precisely, to elastase); as demonstrated by passive haemagglutination, the same antibodies occurred in healthy subjects, too, who probably had had a history of *P. aeruginosa* infection [53].

Exotoxin A is a potent antigen which in doses of low toxicity produces a fairly high immune response. Toxoid prepared from the exotoxin is also immunogenic in animals, but its use for vaccination of humans is problematic, as it may revert to toxin [16]. An anti-exotoxin serum prepared from strain PA-103 was found to protect animals against infection not only by homologous but also by heterologous strains. It has been assumed that different strains of *P. aeruginosa* produce the same, serologically identical exotoxin A [54]. An immunological cross-reaction has been shown between *P. aeruginosa* exotoxin A and diphtheria toxin A [55]. In the serum of patients with *P. aeruginosa* sepsis, antibodies for exotoxin A and lipopolysaccharide have been demonstrated [56].

B. *Antigenic properties of extracellular slime*

The capsular antigens of bacteria play an important, sometimes primary role in stimulating specific immunity to infection [57]. The extracellular slime

Table III

Active protection test using mice immunized with extracellular slime prepared from different strains of P. aeruginosa
Stanislavsky et al. [61]

Strains used for preparation of slime				Challenging strains									
Designation	Lányi serogroup [69]	Lányi–Bergan serogroup [67]	Fisher immunotype [80]	F1	F2	F3	F4	F5	F6	F7	170014	170019	PA-103
170001	1	3	—	++	++	+	+	++	+	—	+	+	+
170002	2a,2c	10a,10c	5	++	++	+	+	++	++	+	+	++	+
170005	3a,3d	2a,2d	7	++	++	+	+	++	+	++	+	++	+
170006	3a,3d,3e	2a,2d,2e	—	+	++	++	+	++	+	+	+	++	+
170007	(3a),3d,3f	(2a),2d,2f	—	++	++	+	nt	nt	+	++	+	++	+
170009	4a,4c	6a,6c	(1)	++	++	—	+	++	+	+	+	+	++
170012	5a,5b,5d	7a,7b,7d	(6)	++	++	—	+	++	++	+	+	++	+
170014	6	1	4	+	—	+	++	+	+	+	++	+	+
170017	8	—	—	+	++	—	+	++	+	+	+	++	++
170018	9	—	—	+	++	+	+	++	+	+	+	+	+
170019	10a	9a	—	++	++	+	+	++	++	+	+	++	++
170021	11	4a,4b	—	++	+	+	+	+	+	+	+	+	++
170022	12	15	—	++	+	+	+	+	+	+	+	++	++
170023	13	12	—	++	++	+	+	++	+	+	+	++	+
O-II	7a,7b	11a,11b	2	++	++	+	+	++	+	+	+	+	++

F1–F7 Fisher reference strains [80]
++ LD_{50} for heterologous strain corresponds to LD_{50} for homologous immunotype (serogroup) strain
+ LD_{50} for test is significantly higher than LD_{50} for control
— LD_{50} for test corresponds to LD_{50} for control
++ Results obtained with homologous strain or with strain regarded as homologous because of close antigenic relationship
nt Not tested

or slime layer of *P. aeruginosa* contains an antigen (or antigens) which produce immunity in experimental *P. aeruginosa* infection of animals [44, 49, 58–60].

It remains to be elucidated whether slime antigens of *P. aeruginosa* are species or subspecies specific factors. According to some authors [14, 20], the protective action of slime is O antigen specific. Other workers have shown that the purified slime induces immunity in animals not only against infection with the homologous serogroup (immunotype) but also against the heterologous serogroup [49, 61]. The presence in slime prepared from heterologous strains of common chemical compounds (e.g. uronic acids, galactose, glucose, rhamnose, see Table II) allows to conclude to the existence of common antigenic determinants. Active cross-mouse protection tests (Table III) seem to confirm that the extracellular slime is a species specific antigen.

Studies on slime components of different molecular weights have shown that large molecule fractions (30 000–300 000) are fairly similar or identical in immunogenicity. Fractions of lower molecular weight (10 000–30 000) have, in addition to common determinants with the large molecular weight ones, their own specific determinants. A serological cross-reaction has been demonstrated between the large molecular weight components and lipopolysaccharide [49]. In opinion of other authors [47], the purified slime differs in this respect from the lipopolysaccharide.

In active and passive mouse protection tests all slime components were active; activity was highest with the large molecular weight fractions. The non-toxic low molecular weight fractions exhibited a protective effect against strains with heterologous serogroup (immunotype) [49].

Active immunization with purified slime of strains belonging to 15 different serological units protected mice against infection (Table III). The protective effect in most cases was of the same level for strains with homologous and with heterologous O antigenic structure.

These experiments showed that fractions of extracellular slime exert a species specific protection. Accordingly, non-toxic low molecular weight fractions of the slime may be used together with other antigenic products of *P. aeruginosa*, for effective immunization against infection with the agent.

Cell wall antigens

The protein-lipopolysaccharide complex localized in the cell wall of *P. aeruginosa* represents two kinds of antigen: the lipopolysaccharide (LPS) part corresponds to the serogroup-specific O antigen, the protein component is the common protective antigen of pseudomonads [62–64]. There are other common protein antigens which elicit antibodies protective against pseudomonas infection [65, 66].

A. *Serological grouping of P. aeruginosa*

The history and the use in epidemiological tracing of the determination of *P. aeruginosa* O antigens were reviewed by Lányi and Bergan [67]. Since the beginning of this century, several serogrouping systems have been elaborated. Owing to a variety of designations for the O antigens, and to debates whether or not partial O antigens should be included in the antigenic schemes, the results of different authors are difficult to compare. In an international collaborative study, attempting to establish an international serogrouping scheme, Lányi and Bergan [67] recommended that antigens designated according to the Habs scheme [68] — the first system to have gained more widespread recognition — should be subdivided into subgroups as described by Lányi [69] and supplemented by antigens not included in these two systems (Sandvik [70], Verder and Evans [71], Meitert [72]). Later Akatova and Smirnova [73] and Homma [74] recommended for use the serogroup designation system of Lányi and Bergan. Akatova and Smirnova [73] supplemented the Lányi–Bergan scheme with new subgroups represented by strains chosen from the Wokatsch collection [75]. Liu et al. [76] preferred a scheme which designates the O antigens by continuous numbering, as opposed to the a, b–a, c system of Lányi and Bergan [67]. Table IV shows a comparison of antigenic symbols of the more widely used systems. It is advantageous that in the compiled schemes of Lányi and Bergan and of Liu et al. the numbering of O groups 1, 2, 3, 4, 6, 7, 9, 10, 11 and 12 corresponds to each other and to the original Habs system. In the Lányi–Bergan scheme the Habs groups O2 and O5 have been joined into one group, O2, on the basis that they share a well-defined partial antigen. Habs' groups O7 and O8, for the same reason, have also been united into the O7 complex. Strains Sandvik II and Verder–Evans 1M-1 are related in an a, b–a, c manner and were therefore classified into one group (O13) of the Lányi–Bergan scheme.

For the determination of *P. aeruginosa* O antigens, slide agglutination is the method of choice [67, 69]. Some isolates, especially those forming abundant slime, agglutinate less readily by the slide method. Such cultures become readily agglutinable after autoclaving at 120 °C for 1 h, or keeping at 100 °C for 2 ½ h followed by centrifugation and homogenization in glycerol and heating to 130 °C for 1 h. For agglutination, appropriate working dilutions of pooled O sera, unabsorbed group sera and absorbed subgroup sera are used [67, 69]. A simplified epidemiological tracing of *P. aeruginosa* may be performed by using one serum for each serogroup. Due to the major group antigen (e.g. O6a), strains belonging to different subgroups of the corresponding O group usually give ++ to ++++ agglutination reaction in a single group serum. For a more delicate method, determination of the partial antigens shown in Table IV is performed with absorbed O sera (e.g. O6b, O6c).

Table IV

O antigenic structure of reference strains of different Pseudomonas aeruginosa antigenic schemes

Compiled schemes			Original schemes					
O groups Lányi and Bergan, 1978 [67] Akatova and Smirnova, 1982 [73] Homma, 1982 [74]	Partial O antigens and reference strains Lányi and Bergan,1978 [67] * Supplemented by Akatova and Smirnova, 1982 [73]		Liu et al., 1983 [76]	Habs, 1957 [68]	Meitert, 1964, 1966, 1976, 1978 [72,77–79]	Lányi, 1966/67 [69]	Fisher et al., 1969 [80]	Homma, 1974, 1976 [81, 82]
1	1	170014 or Habs 1	1	1	XIII	6	4	10
2	2a,2b	170003	16	—	XVI	3a,3b	—	13
	*2a,2b,2e	Wokatsch 25	—	—	—	—	—	—
	(2a),2c	170004 or Habs 2	2	2	—	(3a),3c	3	7, 16
	2a,2d	170005	5	5	—	3a,3d	7	—
	2a,2d,2e	170006	—	—	II	3a,3d,3e	—	—
	(2a),2d,2f	170007	—	—	VI	(3a),3d,3f	—	2
3	*3a,3b	Wokatsch 14	—	—	—	—	—	—
	*3a,3b,3c	Habs 3 or 170001	3	3	V	1	—	1
	*3a,3d	Wokatsch 13	—	—	—	—	—	—
4	4a,4b	Habs 4 or 170021	4	4	VIII	11	—	6
	4a,4c	170040	—	—	—	—	—	—
6	6a	Habs 6	6	6	—	4a, . . .	1	—
	6a,6b	170008	—	—	IV	4a,4b	—	—
	6a,6c	170009	—	—	—	4a,4c	—	8
	6a,6d	170010	—	—	I	4a,4d	—	—
7	7a,7b,7c	170011	7	7	—	5a,5b,5c	—	—
	7a,7b,7d	170012	—	—	—	5a,5b,5d	—	—
	7a,7d	170013	8	8	III	5a,5d	6	3
9	*9a,9b,9d	170020	—	—	XIV	10a,10b	—	—
	*9a,9c	Wokatsch 16	—	—	—	—	—	—
	*9a,9d	170019	9	9	—	10a	—	4

10	10a,10b	Habs 10	10	10	XI	—	—	9
	10a,10c	170002	—	—	—	2	5	—
11	11a,11b	170015 or Habs 11	11	11	XV	7a,7b	2	5 (IID 1130)
	11a,11c	170016	—	—	—	7a,7c	—	—
12	12	170023 or Habs 12	12	12	VII	13	—	14
13	13a,13b	Sandvik II	13	—	XX	—	—	12
	13a,13c	Verder-Evans 1M-1	14	—	XXI	—	—	—
14	14	Meitert X	17	—	X	—	—	—
15	15	170022	15	—	XXII	12	—	11

The reference strains are maintained in Hungarian National Collection of Medical Bacteria, National Institute of Hygiene, Gyáli út 2–6, H-1966 Budapest Hungary; Czechoslovak National Collection of Type Cultures, Institute of Hygiene and Epidemiology, Šrobárova 48, 100 42 Prague 10, Czechoslovakia; Tarassevich State Research Institute for Standardization and Control of Medical Biological Preparations, 41, Sivtsev Vrazhek, 121002, Moscow, USSR

P. aeruginosa isolates frequently show polyagglutinability, i.e. smooth colony form bacteria react in a number of different O sera. In contrast, spontaneous agglutinability, i.e. clumping of cells in all sera of the set and also in physiological saline, is rarely encountered [67, 69]. Pitt and Erdman [83] presented evidence that the factor responsible for polyagglutinability is a heat-stable cell constituent distinct from the O antigen. In contrast, spontaneous agglutination is associated with mutation to serological R form [67].

P. aeruginosa O antigens may be grouped on the basis of electrophoretic mobility of their extracts. Van Eeden [84] showed that trichloracetic acid extracts of *P. aeruginosa* strains exhibited different immunoelectrophoretic patterns. On the basis of the behaviour of various extracts, Lányi et al. [85] classified *P. aeruginosa* O antigens into five immunoelectrophoretic groups; they divided groups I and III into 3 and 2 subgroups, respectively. O antigens identified by agglutination corresponded closely to immunoelectrophoretic patterns. Strains with identical O antigens or sharing major somatic components fell, with one exception, into the same immunoelectrophoretic group (Table V, Fig. 1). Immunoelectrophoresis has supported the assumption of Ádám et al. [86] that O antigens designated by Lányi in his original system [69] as O8 and O9 correspond to R factors. This was further proven by Ádám's (unpublished) results obtained with different preparations including phenol–chloroform–petrolether extracts of the reference strains (170017 and 170018). In the light of these studies, these antigens were omitted from the Lányi–Bergan scheme.

Table V

Immunoelectrophoretic groups of P. aeruginosa O antigens

IE group	O antigens	
	Lányi scheme [69]	Lányi–Bergan scheme [67]
Ia	2 3a,3b; (3a),3c; 3a,3d; 3a,3d,3e; (3a),3d,3f	10a,10c 2a,2b; (2a),2c; 2a,2d; 2a,2d,2e; (2a),2d,2f
Ib	1 6	3 1
Ic	4a,4c 5a,5b,5c; 5a,5b,5d; 5a,5d 10a; 10a,10b	6a,6c 7a,7b,7c; 7a,7b,7d; 7a,7d 9a; 9a,9b
II	4a,4b; 4a,4d	6a,6b; 6a,6d
IIIa	12	15
IIIb	13	12
IV	7a,7b; 7a,7c 11	11a,11b; 11a,11c 4a,4b
V	8; 9	—

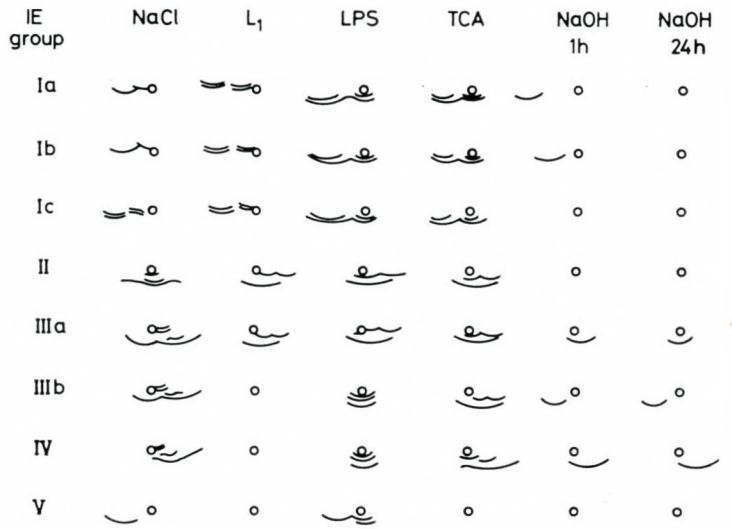

Fig. 1. Schematic diagram of precipitation arcs obtained in immunoelectrophoresis with different extracts of *P. aeruginosa* strains. Antigens in the wells, anode on left. IE = immunoelectrophoretic group; NaCl = supernatant of bacteria heated in physiological saline at 100 °C for $2^1/_2$ h; L_1 = supernatant of ultracentrifuged phenol–water extract; LPS = purified phenol–water extract; TCA = trichloracetic acid extract; NaOH 1 h = supernatant of bacteria extracted with 0.5 N NaOH at 80 °C for 1 h; NaOH 24 h = supernatant of bacteria extracted with 0.5 N NaOH at 80 °C for 24 h

P. aeruginosa and several other pseudomonads differ from most Gram-negative bacteria in that their living cells are readily agglutinable in the homologous O serum, but after exposure to 55–75 °C the suspension becomes slimy and the cells loose their agglutinability. Heating at 100 °C for 60 min renders the cells agglutinable in low titres. After boiling for 2 ½ h, the titre increases markedly. The highest O titres are obtained with bacteria heated at 120–130 °C. Treatment with formalin, ethanol and low concentrations of hydrochloric acid reduces agglutinability. The above procedures, however, do not influence the immunogenicity and agglutinin-binding capacity of the O antigen. As live *P. aeruginosa* cultures agglutinate readily in O serum, there is no reason to believe that they contain antigenic factors which would mask O agglutination like in the case of *Enterobacteriaceae* with K antigens. In *P. aeruginosa* and many other pseudomonads, evidently the same antigen is responsible for the agglutination of live and boiled cells. Exposure to mild heat and chemicals probably results in a change of the surface structure of *P. aeruginosa* rendering the cells inagglutinable, whereas heating at higher temperatures restores agglutinability by destroying the agglutination-inhibiting product. For further details of this problem see Lányi [69], Lányi and Bergan [67] and Lányi and Czirók [87].

B. *Structure and immunochemical specificity of LPS (O antigen)*

Tube precipitation, agar diffusion and passive haemagglutination experiments have proved that in *P. aeruginosa* the LPS corresponds to the O antigen [86, 88, 89]. Each O antigen is characterized by side-chain polysaccharides attached to a common core polysaccharide. Analysis of *P. aeruginosa* LPS for monosaccharide components and chemotyping of the species have been performed by several authors [89–97]. These studies were extended by systematic investigation into the immunochemical structure of *P. aeruginosa* antigens including the structure of complete monosaccharide composition of O-specific side chains and determination of components not identified by previous authors [98–102]. These experiments were aimed at establishing a complete immunochemical classification of *P. aeruginosa* O antigens, and to obtain data which may be useful in studies on LPS biosynthesis and its genetic control as well as in the production of less toxic artificial antigens.

Passive haemagglutination inhibition tests confirmed the O antigen specificity of LPS: in homologous serological systems LPS inhibited haemagglutination by anti-O serum (minimum inhibitory concentration of LPS 0.30–10 μg/ml), whereas in heterologous systems inhibition by LPS was nil or of a minimum degree. The only exceptions were subgroups Lányi O7a,7b and O7a,7c (Lányi and Bergan O11a,11b and O11a,11c) which were practically identical in LPS haemagglutination inhibition titres [98].

Data for the monosaccharide composition and structure of polysaccharide chains of O antigens [98–103] are summarized in Tables VI and VII. Neutral sugars, which are characteristic of polysaccharides of most Gram-negative bacteria [104] are infrequent in the LPS of *P. aeruginosa*. Among the demonstrated 15 monosaccharides, only two neutral sugars (L-rhamnose and D-glucose) were found and these occurred in some subgroups only. Amino sugars, which are the basic components of *P. aeruginosa* O specific polysaccharides, were present in all serogroups and subgroups. To the 13 amino sugars described in the literature, 2,4-diamino-2,4,6-trideoxy-D-glucose should be added; this component was demonstrated in strain 8505 [104] belonging to serogroup Lányi O1 (Lányi–Bergan O3). *P. aeruginosa* differs also in amino sugar composition from *Enterobacteriaceae* [104]. In the latter *N*-acetylglucosamine is a characteristic component, whereas in *P. aeruginosa* it has not been shown. Another amino sugar frequent in other Gram-negative bacteria, *N*-acetylgalactosamine, occurs only in one serogroup of *P. aeruginosa*. Characteristic *P. aeruginosa* polysaccharide components are *N*-acetylfucosamine and *N*-acetylquinosamine present in at least 10 and 8 subgroups, respectively. In serogroup Lányi O7 (Lányi–Bergan O11) both of these enanthiomeric forms of *N*-acetylfucosamine have been shown. Seven of the amino sugars represent uronic acids including 2-formamide-2-deoxy-D-galacturonic acid, three 2,3-

Table VI

Monosaccharide constituents of O-specific polysaccharides of P. aeruginosa
See references [98–102]

Serogroup Lányi [69]	Strain	LRha	DGlc	DGalNAc	DQuiNAc	DFucNAc	LFucNAc	DGalNAcA	LGalNAcA	DGalNFmA	DGlc(NAc)$_2$A	DMan(NAc)$_2$A	LGul(NAc)$_2$A	DManImA	Sia-1	Sia-2	OAc
2a,2b	170034*	+			+				+								+
2a,2c	170002	+			+				+								
3a,3b	170003					+						+		+			
(3a),3c	170004					+							+	+			
3a,3d	170005					+						+		+			
3a,3d,3e	170006					+							+	+			
(3a),3d,3f	170007					+								+			
4a,4b	170008	+			+			+		+							+
4a,4c	170009	+			+			+		+							
4a,4d	170010	+			+			+		+							+
6	170014			+	+	+					+						
7a,7b	170015		+			+	+										
7a,7c	170016		+			+	+										
10a	170019				+	+									+		
13	170023				+	+										+	

* 170034 = Habs 10

Abbreviations

LRha	L-rhamnose
DGlc	D-glucose
DGalNAc	*N*-acetyl-D-galactosamine
DQuiNAc	*N*-acetyl-D-quinovosamine
DFucNAc	*N*-acetyl-D-fucosamine
LFucNAc	*N*-acetyl-L-fucosamine
LGalNAcA	*N*-acetyl-L-galactosaminuronic acid
DGalNAcA	*N*-acetyl-D-galactosaminuronic acid
DGalNFmA	*N*-formamide-2-deoxy-D-galacturonic acid
DGlc(NAc)$_2$A	2,3-diacetamido-2,3-dideoxy-D-glucuronic acid
DMan(NAc)$_2$A	2,3-diacetamido-2,3-dideoxy-D-mannuronic acid
LGul(NAc)$_2$A	2,3-diacetamido-2,3-dideoxy-L-guluronic acid
DManIMA	2,3-(1-acetyl-2-methyl-2-imidazolino-5,4)-2,3-dideoxy-D-mannuronic acid
Sia	sialic acid
OAc	*O*-acetyl-group

-diacetamido-2,3-dideoxy compounds of D-glucuronic, D-mannuronic and L-guluronic acids, and a new bicyclic derivative, 2,3-(1-acetyl-2-methyl-2-imidazolino-5,4)-2,3-dideoxy-D-mannuronic acid. In addition, in serogroups Lányi O10 and O13 (Lányi–Bergan O9 and O12) sialic acids of unusual structure have been shown.

Repeating units of the O-specific polysaccharide are constituted by 3 or 4 monosaccharides. An exception is subgroup Lányi O(3a),3d,3f = Lányi–Bergan O(2a),2d,2f polysaccharide, in which the same components were detected as in other subgroups of the serogroup but not in the same stoichiometric

Table VII

Structure of repeating units of O-specific polysaccharide and serological relationship of LPS in different serogroups of P. aeruginosa

See references [99–103]

Serogroup Lányi [69]	Structure of repeating units	Serological relationship of LPS
2a,2b	→4)LGalNAcA(α1 → 3)DQuiNAc(α1→3)LRha(α1→ $^{2}\uparrow$ OAc	Intragroup cross-reaction between the two subgroups
2a,2c	→4)LGalNAcA(α1→3)DQuiNAc(α1→3)LRha(α1→	
3a,3b	→4)DManImA(β1→4)DMan(NAc)$_2$A(β1→3)DFucNAc(β1→	Intragroup cross-reactions between part of subgroups by precipitation
(3a),3c	→4)DManImA(β1→4)LGul(NAc)$_2$A(α1→3)DFucNAc(β1→	
3a,3d	→4)DManImA(β1→4)DMan(NAc)$_2$A(β1→3)DFucNAc(α1→	
3a,3d,3e	→4)DManImA(β1→4)LGul(NAc)$_2$A(α1→3)DFucNAc(α1→	
6	→4)DGalNAc(α1→4)DGlc(NAc)$_2$A(β1→3)DFucNAc(α1→3)DQuiNAc(α1→	No cross-reaction with other groups
7a,7b	→3)LFucNAc(α1→3)DFucNAc(β1→2)DGlc(β1→	Subgroups not distinguishable by precipitation
7a,7c	→3)LFucNAc(α1→3)DFucNAc(β1→2)DGlc(β1→	

For abbreviations see footnote to Table VI

ratio; this polysaccharide had no regular structure. Uronic acids are present in the repeating units of 11 different serogroups (subgroups), and 7 of them contain two different uronic acids. Acidity of the polysaccharides is associated with sialic acids in two serogroups. The polysaccharide of serogroup Lányi O7 (Lányi–Bergan O11) is neutral. *O*-Acetyl sugars are present in polysaccharides of three serogroups; each repeating unit of these has, as an average, 0.8 equivalent of *O*-acetyl group.

Analysis of monosaccharide composition of O-specific polysaccharides indicated an association between the structure of O antigens and immunochemical specificity (Table VI). Antigenic subgroups belonging to the same serogroup are either identical in monosaccharide components or differ from the others in a single monosaccharide (e.g. serogroup Lányi O3 = Lányi–Bergan O2 [103]) or a single acetyl group (e.g. serogroups Lányi O2 = Lányi–Bergan O10 and Lányi O4 = Lányi–Bergan O6).

These findings have been confirmed by structural analysis of the O-specific polysaccharides (Table VII), which have also proved the existence of an immunochemical relationship between subgroups belonging to the same serogroup. Structural analysis has also explained the lack of serological relationship between distinct serogroups: in the repeating units of O-specific polysaccharides of these, not a single structural element exists which could be responsible for a serological cross-reaction.

These results have confirmed that strains not being identical in antigenic structure but exhibiting a cross-reaction due to a common partial antigen, should be classified in one serogroup. It is, accordingly, not only on a serological basis, but also on an immunochemical one, justified to establish subgroups within the serogroup defined in this manner.

A comparison of strains Habs 10 and 170002 (Lányi O2a,2b and O2a, 2c = Lányi–Bergan O10a,10b and O10a,10c) revealed that both cultures have the same structure of the trisaccharide repeating unit; for the serological difference an *O*-acetyl group is responsible, which is present in strain Habs 10 on the rhamnose residues in position 2 [99]. The cross-reaction between the two subgroups, due to a partial antigen shared by both of them (Lányi O2a = = Lányi–Bergan O10a), is probably associated with the repeating unit fragments common to the two polysaccharides, the immunodominant sugar being *N*-acetyl-galactosaminuronic acid. The cross-reactivity of the two subgroups may be attributed to an incomplete *O*-acetylation of rhamnose in the O2a, 2b (= O10a,10b) polysaccharide, in consequence of which 20–30% of the repeating units of this subgroup have the structure of subgroup O2a,2c (= = O10a,10c). Thus, the factors responsible for the serological difference between subgroups of this serogroup, are necessarily related to the presence of the *O*-acetylated rhamnose residue in the O2a,2b polysaccharide (the factor O2b) and of the nonacetylated rhamnose in the O2a,2c polysaccharide (the

factor O2c), the latter being marked by the *O*-acetyl group in the O2a,2b polysaccharide. *O*-Deacetylation of the latter polysaccharide gives in fact rise to a different O-specificity and this is explained by the transition of factor O2b to O2c. This conclusion has been drawn from haemagglutination inhibition experiments, where O2a,2b polysaccharide had a high inhibitory titre in the homologous system (LPS O2a,2b vs. serum O2a,2b), but was inactive in the heterologous one; however, after *O*-deacetylation, it lost its activity in the homologous system, while became inhibitory in the heterologous system (LPS O2a,2b vs. serum O2a,2c).

In serogroup Lányi O3 (Lányi–Bergan O2) the subgroup specificity is associated with changes in the configuration of repeating units. For example, O3a,3b and O3a,3d on the one hand, and O(3a),3c and O3a,3d,3e on the other, differ in the anomeric configuration of the glycosidic bond of *N*-acetylfucosamine. Polysaccharides O3a,3b and O(3a),3c as well as O3a,3d and O3a,3d,3e differ from each other also in the configuration of the single asymmetric centre namely C-5 of 2,3-diacetamido-2,3-dideoxyuronic acid. It may, consequently, be assumed that the biosynthetic pathways are similar in these polysaccharides, involving a polymerization of the trisaccharide repeating unit containing the *N*-acetylfucosamine residue at the reducing end. Biosynthesis of O(3a),3c and O3a,3d,3e polysaccharides involves, in addition, isomerization of 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid into 2,3-diacetamido-2,3-dideoxy-L-guluronic acid. The reason for the aforementioned irregular structure of the O(3a), 3d,3f polysaccharide, where monosaccharides enter the polysaccharide in non-stoichiometric ratio, remains obscure [102, 103].

From these findings it may be concluded that the serological classification of serogroup O3 (Lányi–Bergan O2) elaborated by Lányi [69] is justified on the basis of the chemical structure of polysaccharides: there are valid reasons to include these cross-reacting strains into one serogroup and to distinguish subgroups within the serogroup. The specificity of an immunodominant sugar is usually dependent to some extent upon the structure of the neighbouring monosaccharide units in the polymeric chain [105]. One would expect, accordingly, that Lányi O3 (Lányi–Bergan O2) subgroups, the polysaccharide of which contain, besides the immunodominant 2-imidazoline derivative, another common structural element (the same diacetamidouronic acid or *N*-acetylfucosamine with an identical configuration of glycosidic bond), are in a closer antigenic relationship than that demonstrable serologically. In fact, the O antigenic factors determined by bacterial agglutination or LPS passive haemagglutination [69, 86, 102] do not quite correspond to those forecast on the basis of the structure of O-specific polysaccharides. The correlation is much more close between chemical structure and the degree of reaction obtained with the corresponding lipopolysaccharides in immuno-precipitation (Table VIII). Thus the interrelationships observed between O3a,3b and O(3a),3c or O3a,3d

and O3a,3d,3e are associated with the presence of common specific determinants DFucNAc(β1 → 4)DManImA(β1- or DFucNAc(α1 → 4)DManImA(β1-, while those between O3a,3b and O3a,3d or O(3a),3c and O3a,3d,3e with the presence of DManImA(β1 → 4)DMan(NAc)$_2$A(β1- or DManImA(β1 → 4)LGul(NAc)$_2$A(α1-, respectively. On the other hand, for lipopolysaccharides O3a,3b and O3a,3d,3e or O(3a),3c and O3a,3d, having no common specific determinants, cross-reaction in agar gel precipitation was not observed [102]. The antigenic insulation of O(3a),3d,3f, which reacted in precipitation test with none of the heterologous antisera, may be associated with a lack of common specific determinants or their low epitopic density in the polysaccharide chain, induced by structural irregularity. It should, however, be noted, that with saline extracts of heated bacteria and with less purified LPS preparations, more or less marked cross-reactions did appear between subgroups of O3, similarly to the results obtained with bacterial agglutination and LPS haemagglutination [88].

Table VIII

Cross-precipitation reactions of LPS prepared from subgroup reference strains for P. aeruginosa serogroup Lányi O3

Stanislavsky et al. [103]

Subgroup Lányi [69]	Serum				
	3a,3b	(3a),3c	3a,3d	3a,3d,3e	(3a),3d,3f
3a,3b	+	+	−	−	−
(3a),3c	±	+	−	−	−
3a,3d	+	−	+	+	+
3a,3d,3e	−	+	−	+	−
(3a),3d,3f	−	−	−	−	+

\+ Marked precipitation
± Weak precipitation
− No precipitation

In view of the structure of O-specific polysaccharides, the division of serogroup Lányi O7 (Lányi–Bergan O11) does not seem justified: the repeating units of subgroups O7a,7b and O7a,7c are identical. The practical identity of the two subgroups may be assumed from the result of haemagglutination tests [98]. Agar gel precipitation allows a similar conclusion: no well-defined O7b and O7c antigens could be demonstrated, since in serum O7a,7b absorbed by strain O7a,7c extracts of the homologous strain (O7a,7b) gave but a trace of precipitation, whereas in serum O7a,7c absorbed by strain O7a,7b, extracts of the homologous culture (O7a,7c) entirely failed to react [88]. Yet, the presence of factors named O7b and O7c, in addition to the group antigen O7a, is clearly demonstrable by bacterial agglutination and factor sera O7b and O7c have been used for nearly twenty years for valuable epidemiological tracing. The serological difference between the two subgroups of serogroup O7 may be due

to a surface factor other than the O antigen, e.g. to a heat-resistant envelope antigen. It should be noted that immunoelectrophoretic studies [85] also seem to indicate that O7 represents a peculiar kind of antigen (Table V).

Isolated O-specific polysaccharides of serogroups Lányi O3, O6 and O7 (Lányi–Bergan O2, O1 and O11), unlike purified LPS, fail to exert any activity in passive haemagglutination inhibition test. Comparison of O-specific polysaccharides and the corresponding LPS by ^{13}C nuclear magnetic resonance spectroscopy has shown that they are practically identical in the carbon signals for carbohydrate units. This means that polysaccharides obtained at the degradation of LPS are O-specific chains with unchanged structure. The loss of serological activity of polysaccharides detached from LPS has not been explained. This finding is characteristic not only of *P. aeruginosa*; polysaccharides obtained by degradation of *Escherichia coli* O8 and O9 LPS are likewise inactive in the haemagglutination inhibition test [106].

Unilateral cross-reactions in serogroup O3 shown in Table VIII [101–103] may be associated with a different degree of exposition of surface O-specific determinant groups [107]. The conception of conformation determinants [108] may offer another explanation. According to this conception, the presence of common structural elements do not necessarily mean a serological relationship, since differences in other parts of the structure cause such an alteration in the conformation of the polymeric chain that the active determinants are unaccessible for the antibodies.

It may be concluded that knowledge of the primary structure of the O-specific polysaccharide is very useful but not sufficient for stating the existence of common antigenic determinants and that, for elucidating the problem, data for the macromolecular structure of LPS are needed.

C. *Protein antigens*

The protein component of endotoxin was studied in detail by Homma et al. [62–64]. They extracted the endotoxin from the filtrate of autolysed culture ("original endotoxic protein", OEP). By electrophoresis two components were shown: (1) LPS protein and (2) nuclein polyribose complex. OEP was isolated from component 1. A protein, identical with OEP on the basis of electrophoresis, ultracentrifugation, nitrogen content, phosphorus content, sedimentation constant and antigenic specificity, was isolated from the cell wall.

OEP exerts pyocin and anti-tumour activity and is protective in mouse [63] and in mink [64] experiments. Homma termed OEP the common protective antigen of *P. aeruginosa*.

By aqueous extraction, two other proteins were isolated from *P. aeruginosa:* A (mol wt 120 000–140 000) and B (mol wt 11 000–37 000). Immunochemical analysis and active and passive mouse protection tests showed that

A and B were probably species-specific protective antigens [65, 66]. By immunoprecipitation and cross-immunoelectrophoresis it was demonstrated that the two proteins shared common antigenic determinants and each had its own specific determinant [66]. The ultrasonic lysate of *P. aeruginosa* yielded a common protein antigen [109].

There are probably other cellular substances which have antigenic properties. Leukocidin, a toxin bound to the bacterial cell, for example, is antigenic. Immunization of rabbits with purified leukocidin stimulates antibodies which inhibit the effect of leukocidin on bovine granulocytes [39].

Flagellar and pilus antigens

A. *Flagellar antigens; serotypes of P. aeruginosa*

Verder and Evans [71] were the first to subdivide *P. aeruginosa* serogroups into serotypes (serovars) by thermolabile antigens. Lányi in 1970 brought evidence [110] that thermolabile antigens detectable by agglutination and immobilization test in sera prepared with formalinized cultures are associated with flagella. He elaborated a scheme, distinguishing two serologically unrelated main H antigen complexes, H1 and H2. Strains with antigen H1 were in their majority characterized by formula H1a, while some of them had antigens H1a,1b, in which H1b represented a minor antigen. The H2 complex was divisible into different combinations of well-defined partial antigens: 2a,2b; 2a,2c; 2a,2b,2f; 2a,2c,2f and 2a,2d. Antigen combination 2a,2d,2e,2f was shown only in strains which later proved to be carrying R-like somatic antigens. On the basis of combination of O and H antigens, Lányi divided his strains into 53 serotypes [110].

In 1978 Ansorg [111], using indirect fluorescent antibody technique, devised a new scheme for the H antigens of *P. aeruginosa*. Ansorg distinguished a complex flagellar antigen „a" divisible by partial factors and a uniform H antigen "b". Strains with H antigen "a" shared a common factor (a_0) and fell into 15 different formulae by the combination of partial H antigens a_1, a_2, a_3 and a_4. Using the extended O antigenic scheme of Habs and the above classification of H antigens, Ansorg divided *P. aeruginosa* isolates into 99 serovars (serotypes). By the immunofluorescent technique, Ansorg confirmed Lányi's H antigen classification in respect to the differentiation of antigens H1 (= Ansorg Hb) and H2 (= Ansorg Ha). Between Lányi's subdivision of the H2 antigen complex and Ansorg's subdivision of the Ha complex there were discrepancies. These might be due to the use of sera absorbed in a different manner and with different strains, as well as to obvious technical differences between agglutination and the immunofluorescent method.

Instead of designating each partial H antigen with a symbol expressing relationship to the complex H antigens, Pitt in 1980 [112] devised a continuous numbering system of six factors (H1–H6). The corresponding H-specific sera were prepared by suitable absorptions. These sera sometimes agglutinated field strains without immobilizing them, because they may have contained antibodies to other heat labile factors. To make the sera more flagella-specific, Pitt elaborated a method for producing sera with purified flagella prepared from pilusless strains [112]. Pitt's antigen H3 was distinct and probably corresponded to the "uniform" antigen of Lányi H1 and Ansorg Hb. By cross-absorption Pitt distinguished complexes H1, H2, H5 and H4, H6; these antigens may be factors of the complex antigen of Lányi H2 and Ansorg Ha.

Living and formalinized cultures of motile *P. aeruginosa* strains produce H agglutinins in high titre. Immunogenicity and antibody-binding capacity remains unaffected after heating at 60 °C for 1 h, but is lost after ethanol and N HCl treatment or after heating at 75 °C or above. Agglutinability of H antigens remains intact after formalinization. After heating to 70 °C or above and after exposure to ethanol or N HCl, *P. aeruginosa* cells lose their H-agglutinability [110].

The flagellar antigen isolated by differential centrifugation by Montie et al. [113] showed in electrophoretic analysis a major protein band corresponding to flagellin of 53 000 molecular weight. The highly purified, single banded flagellin contained 16 amino acids. A non-flagellated strain exhibited no flagellin band.

It has been observed that motility and chemotaxis play an important part in the virulence of *P. aeruginosa* on burned rodents [114, 115]. Holder et al. [116] confirmed these findings by showing that mice immunized with *P. aeruginosa* flagellar antigen survived longer when they are subsequently burnt and infected at the burnt site. The protection is flagellar antigen specific and appears to be associated with the immobilization of bacteria in the burnt skin.

B. *Pilus antigens*

In *P. aeruginosa* two types of pili (fimbriae) have been shown: (1) thin, polar pili associated with heat-labile antigens [117, 118], and (2) thicker, non-polar pili with drug resistance plasmids [119]. The fimbrial antigen is a polymerized protein (pilin). It is demonstrable by agglutination of living bacteria in anti-pilus serum prepared from living cultures. The fimbrial antigen is heat-labile and under formaldehyde treatment or bacteriophage action, the pili apparently withdraw into the cell and the pilin is depolymerized [120]. The fimbrial antigens are serologically heterologous. To obtain a fimbria-specific reaction, the immune sera have to be absorbed with homologous O and H antigens. O agglutinins are removable with boiled cultures, H agglutinins are

absorbed with formalinized suspensions of flagellated strains, or preferably, with non-fimbriated flagellated mutants. The latter can be selected by their resistance to fimbria-specific phages [121].

Adherence of some bacteria to cells involves fimbria, so antibodies against fimbria may be protective to the host [122]. Woods et al. [123] have shown that adherence in vitro of *P. aeruginosa* to the upper respiratory epithelium of seriously ill patients is correlated with its subsequent colonization of the respiratory tract. Antisera to purified pili decreased the adherence of the homologous strain, whereas a heterologous antiserum failed to inhibit the adherence of the same strain. From these findings it is tempting to assume that adherence of, and possibly colonization, by, *P. aeruginosa* may be prevented by active or passive immunization against pilus antigens of the microorganism.

Immunoprophylaxis and immunotherapy of P. aeruginosa infections

Several comprehensive reviews have been published recently on this topic [124–132].

A. *Active immunization*

Active immunization may be effective if vaccine preparations exhibiting low toxicity, optimal immunization schedules and patients capable of sufficient immune response are chosen. Vaccine prophylaxis may be useful in burn patients and in some diseases caused by antibiotic resistant *P. aeruginosa* such as otitis, wound infections, osteomyelitis.

Table IX shows the characteristics of different *P. aeruginosa* vaccines (PV). Live or killed whole-cell (wc) vaccines produce immunity after 3–6 vaccinations [133–143], or even after a single dose of the antigen [140, 141]. Some authors state the wc PVs produce O-antigen specific immunity [135, 140], while according to others [142] they are able, in addition, to bring about a cross-immunity against heterologous *P. aeruginosa*. A polyvalent vaccine was applied in burn patients [137–139] during a period of 2 years with satisfactory results; in the vaccinees mortality was significantly reduced. In an other study [144], a polyvalent wc vaccine tested on 39 burn patients was well tolerated and stimulated antibody production.

In connection with the application of wc vaccines, several problems arise: (a) no experimental results are available on the chronic toxicity of vaccines for animals; (b) there are relatively few data on the reactivity of these vaccines containing a considerable amount of non-immunogenic ballast substances, which might contribute to their reactogenicity; (c) wc vaccines are fairly difficult to standardize.

Table IX

P. aeruginosa vaccines and their effectiveness

Product [reference]	Principle of preparation	Animal experiments		Human trials	
		effectiveness	toxicity	effectiveness	reactivity
Whole-cell vaccines					
I. Living [133]	Attenuated mutant	Protective for mice after 6 vaccinations	.	.	.
II. Killed					
1. Heated polyvalent [134]	Eight immunotypes + one untyped *P. aeruginosa* + *E. coli* O14	.	.	Production of O antibodies, bactericidal and protective antibodies	.
2. Polyvalent [135–139]	Eleven serogroups, phenolized; 5 serogroups, heated	Three vaccinations to mice: 30–100% survival after homologous, 0–20% survival after heterologous challenge	.	Effective in burn patients	Well tolerated
3. Polyvalent [140, 141]	Seven serogroups killed by chemicals heating	Single dose protects against homologous strain	.	Antibody production	.
4. Monovalent [142]	Killed by formalin or boiling	Single dose protects against homologous strain in 0–100%	.	.	
5. Mono- and polyvalent [143]	One or 6 serogroups, autolysis	Two doses protect against homologous or heterologous serogroup			

Cell-free vaccines

I. Polyvalent					
1. Pseudogen [126, 148]	LPS of 7 immunotypes	Two vaccinations protect guinea pigs and rats against homologous or heterologous immunotype	Toxic for mice	Seven doses protect burn patients	High
2. PEV-01 [12, 149–152]	Lipid-protein-polysaccharide cell wall complex of 16 serogroups	Single dose protects mice against homologous and heterologous serogroups. Protects guinea pigs against pneumonia	Low toxicity for mice and embryos	Antibody production, increased phagocytosis. Three doses protect burn patients	Low
3. Pioimmunogen [153–156]	Water-soluble slime and cell wall antigen complex of 3 or 5 strains	Single dose protects mice and rats against homologous and heterologous serogroups (immunotypes)	Low toxicity for mice, rats, guinea pigs and rabbits	Antibody production demonstrable by mouse protection test; improved recovery	Low
4. Cell-free vaccine [157]	Water-soluble and ultracentrifuged extract from 5 strains	Protection of mice against homologous and heterologous challenge	Low toxicity in experimental animals	.	.
II. Monovalent					
1. Slime [46–49] (a) Large mol wt complexes	Salt extraction, centrifugation, ultrafiltration	Single dose protects mice against homologous and heterologous serogroups	High	.	.
(b) Small mol wt complexes	Ditto	Ditto	Low for mice	.	.
2. High-molecular-weight polysaccharide [159–163]	Precipitation with ethanol and cetavlon gel filtration	Homologous and appreciable heterologous protection in mice	Very low toxicity in experimental animals	Low reactivity	Humoral immune response in man
3. Polysaccharide [164, 165]	Precipitation with ethanol	Homologous mouse protection	Very low toxicity in experimental animals	.	.

Table IX (continued)

Product [reference]	Principle of preparation	Animal experiments		Human trials	
		effectiveness	toxicity	effectiveness	reactivity
4. Cell-free vaccine (polysaccharide?) [166–167]	Supernatant heated to 70 °C precipitation with ethanol	Homologous mouse protection	Low toxicity in mice	·	·
5. Antigenic complex from slime [168, 169]	Precipitation with ethanol	Homologous mouse protection	Low toxicity in mice	·	·
6. Mono PV [170]	Water-soluble protein antigens	Single dose protects mice against homologous and heterologous serogroups	Low for mice	·	·
7. Polycomponent [62–64, 171]	Common protein antigen + toxoids	Protective after 2–6 vaccinations	Low for mice	·	·
8. Purified LPS [158]	Phenol–water extraction	Two vaccinations to mice: 50–90% survival	High for mice	·	·
9. Pilus polysaccharide [158]	Deacetylized LPS	Two vaccinations to mice: 50–90% survival	Not toxic for mice	·	·
10. Flagellar polysaccharide [158]	·	Survival: 30%	·	·	·
11. Flagellin protein [116]	Purified flagella antigen	Single dose protects burned mice	·	·	·
12. Ribosomal PV [172–174]	Ribosomal fraction	Single dose protects mice against homologous strain	·	·	·
13. Toxoid [16, 175]	Formalinized exotoxin A	Stimulates antitoxic immunity in mice	Possible reversion to toxicity	Antitoxin production	·
14. Culture filtrate [176]	Large molecular fraction separated by gel filtration	Two doses protect rats in 50–80% against *P. aeruginosa* sepsis	Low	·	·

· Not studied

In view of the above-mentioned problems of these vaccines [144], wide scale experiments have been started for the preparation of less toxic vaccines, which contain purified or partly purified cell wall antigen(s). Development of an effective PV should be based on the knowledge of pathogenesis of pseudomonas infections and of virulence factors of the agent (Table I). In the early stage of disease, antiinfectious immunity plays an important role in protecting the host. In the next stages antitoxic immunity takes over the leading role.

In opinion of Peterson [145] polymorphonuclear leukocytes are the main factors in the demarcation of *P. aeruginosa* invasion. Other workers [146] have agreed with this conception, and it has been shown that transfusion of leukocytes increases survival of neutropenic dogs infected with *P. aeruginosa* [147].

Capsular and cell wall antigens are significant stimulants of antiinfectious immunity [57]. For example, immunization with capsular antigens afford an effective protection against meningococcal or pneumococcal infections [21].

Cell-free PVs were effective in many experiments and in a few clinical trials (see Table IX). Polyvalent PVs, like Pseudogen [126, 148], PEV-01 [12, 149–152]; Pioimmunogen [153–156] and an other cell-free vaccine [157] were highly effective in active mouse and rat protection tests against a challenge with the homologous serogroup (immunotype). Pioimmunogen and PEV-01 were also protective against the heterologous serogroup (immunotype). Pseudogen was shown to be effective for the prophylaxis of pseudomonas infection in burn patients [148]; the serum of human vaccinees protected 60% of the rats against challenge with *P. aeruginosa* [148, 158]. However, this preparation was not sufficiently active in the prophylaxis of pseudomonas infection in patients with leukemia and cystic fibrosis [128]. To obtain an effective immunity, 6–7 doses of the vaccine were given; simultaneous administration of Pseudogen and hyperimmune human immune globulin yielded the best result [148]. Pseudogen, probably because of its LPS content, elicits several side effects involving local and systemic reactions [128].

Vaccine PEV-01 was effective upon trial on 20 burn patients in preventing infection by *P. aeruginosa* [151]. In volunteers the vaccine caused a short-term rise in body temperature (0.4–1 °C) 3–6 h after vaccination [150].

Pioimmunogen was tried on 20 healthy blood donors and 20 burn patients [154, 155]. The preparation stimulated the production of specific antibodies; locally a weak hyperaemia of the skin and a slight rise in body temperature (0.2–0.8 °C) were observed; the temperature decreased to normal in 6–12 h. Vaccination promoted the healing of burn wounds. The blood plasma of the donors exhibited a definite protective activity in mouse experiments and was beneficial in the treatment of patients with pseudomonas sepsis. At present Pioimmunogen is being further studied by vaccination of donors for effectiveness and reactivity.

In laboratory experiments some other poly- and monovalent preparations obtained from the slime were protective against homologous and partly heterologous serogroups (immunotypes) of *P. aeruginosa* [157–167, 169]; see Table IX. Another promising vaccine contains water-soluble protein antigen(s) [170].

Highly purified LPS and deacetylated LPS combined with pilus, flagella and modified exotoxin A antigens and also with tetanus toxoid were active in the O-specific inhibition of serological reactions. In mice these preparations except LPS and deacetylated LPS, elicited high titre O antibodies. A double immunizing dose in rats with purified LPS and LPS-pilus complex induced a definite protective effect, whereas the LPS-flagella complex stimulated specific immunity only in 30% of the animals [158]. Other authors [168] demonstrated that single immunization of mice and rats with purified LPS or O-specific polysaccharide combined with human or rabbit IgG elicited but slight immunity against pseudomonas infection. Considering the high toxicity of LPS, it seems obvious that LPS vaccines have little practical future.

The polycomponent vaccine developed by Homma et al. contains the original endotoxin-protein (OEP), protease and elastase toxoids [62, 64, 171]. This vaccine proved to be protective in mice and protected against haemorrhagic pneumonia in minks caused by *P. aeruginosa*.

The so called ribosomal vaccine has been debated now for 17 years, but no final agreement has been settled. Ribosomal vaccines of *P. aeruginosa* [172–174] were protective against challenge with the homologous strain, but not against the heterologous ones. It has been assumed that the protective action of *P. aeruginosa* ribosomal vaccine is associated with a high LPS content adsorbed on the ribosomes [174].

Toxoid prepared from exotoxin A elicits a definite antitoxic immunity in animals [16]. However, vaccination of the animals with toxoid failed to protect them against challenge with a non-toxin producing strain of *P. aeruginosa*. Although purified exotoxin A can be converted by formalinization and heating to toxoid, there are data showing that toxicity is restored when formalin is removed from the preparation [125]. One should be, therefore, cautious in using toxoid either per se or in combination with cell wall antigens of *P. aeruginosa* [16].

A mutant of *P. aeruginosa* synthesizes a non-toxic protein antigenically identical with exotoxin A. Such "natural" toxoids might be useful for human vaccination [125, 129, 175].

The use of toxoid prepared from exotoxin A in immunoprophylaxis is debated, as the role of exotoxin A in the pathogenesis of pseudomonas infection has not been sufficiently elucidated. Extracellular slime antigens [49, 61] and outer membrane protein antigens [170] produce immunity in animals against challenge by toxigenic strain PA-103. Incorporating toxoid in a polyvalent vaccine or vaccination with toxoid per se does not seem indicated. Toxoid may

be useful for the production of antitoxic immune plasma effective for the treatment of patients with *P. aeruginosa* sepsis.

Table X

Serum products for passive immunization against P. aeruginosa infection

Product [reference]	Principle of preparation	Effectiveness	
		animal experiments	clinical trials
Human			
1. Immune plasma [154]	Donors immunized with Pioimmunogen	Protects mice against homologous challenge	Effective in sepsis*
2. Immune plasma [177]	Donors immunized with toxoid	Protects mice against toxin; contains antibodies against toxoid	Effective in sepsis
3. Specific human immune globulin [178]	Prepared from immune plasma	Protects mice and rats	Prophylactic and therapeutic in burn patients
4. "Normal" human immune globulin [179]	Commercial preparation from placental blood	Contains antibodies against O antigen and slime; protects mice	Therapeutic in certain kinds of *P. aeruginosa* infection
5. "Normal" human immune globulin for intravenous use [180]	Commercial preparation from donor blood	Contains antibodies to LPS of 7 immunotypes and to exotoxin A; confers protection against burn-wound infection in mice	
6. Pseudomonas immunoglobulin [181]	Immunization of donors with vaccine PEV-01	Contains antibodies to the serogroups contained in vaccine PEV-01	Effective in burn patients
Animal			
1. Sheep immune serum and immune globulin [182–184]	Hyperimmunization with polyvalent formalized vaccine	Protects mice	Therapeutic in burn patients on parenteral application
2. Sheep immune serum and immune globulin to slime [185]	Hyperimmunization with slime	Protects mice against challenge with homologous and heterologous serogroups (immunotypes)	Therapeutic in certain kinds of *P. aeruginosa* infection after local application
3. Purified and concentrated horse serum [186]	Hyperimmunization with polyvalent wc vaccine	Protects mice against serogroups included in the vaccine	Applied parenterally in burn patients with good results
4. Rabbit immune serum [187]	Hyperimmunization with multistrain wc vaccine	Protects mice against challenge	Applied parenterally with good results in children

* Limited number of clinical trials (Stanislavsky, E. S., Krochina, M. A., Kolker, I. I., Grishina, I. A. and others; unpublished observations)

B. *Passive immunization*

Table X shows data for different preparations used in passive immunization experiments. Among homologous antibody preparations, immune plasma, especially specific immune globulin gave the most promising results. Commercial series of normal human immune globulin deserve a special attention: they have been proved to contain antibodies to slime and to O antigens of *P. aeruginosa* as well as antibodies protecting mice against challenge with this microorganism [179]. Accordingly, it may be expected that normal immune globulin is effective in certain kinds of pseudomonas infection, mainly in children.

Heterologous serum preparations may be effective in local treatment of pseudomonas infections. Sheep anti-pseudomonas serum and immune globulin prepared from it was recommended for medical practice [179–183]. Serum prepared against slime antigen or slime glycoprotein protected mice against challenge with homologous and heterologous serogroups of *P. aeruginosa* [182–184]. Such preparations may also be useful for local therapy of certain kinds of pseudomonas infection. Our preliminary clinical trials (unpublished data) have proved the effectiveness of serum of sheep immunized with purified slime in the treatment of wounds and abscesses.

Specific antibodies against slime glycoprotein, in the presence of complement, have an opsonizing effect on *P. aeruginosa* and in this manner decrease the resistance of the causative agent against phagocytosis.

It may be concluded that favourable experiences with purified homologous and heterologous anti-serum preparations make passive immunization a worthwile area for further investigation.

C. *Combined active-passive immunization*

The simultaneous application of combined immunotherapy seems logical, because the immune response of debilitated and immunodeficient patients to active immunization might be deficient and insufficient or often too late, while by passive immunization the patient receives immediately the specific antibodies. Cell-free vaccine (PEV-01) and specific hyperimmune human immunoglobulin were successfully applied in burn patients [181, 188]. A polyvalent wc vaccine and purified concentrated horse antiserum were applied simultaneously to severe burn patients with good results [137, 138, 186]. In patients with severe leukopenia, therapy might be completed with leukocyte transfusion [147, 189].

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IN VIVO MODEL FOR THE ACUTE, LATENT AND REACTIVATED PHASES OF CYTOMEGALOVIRUS INFECTION*

ÉVA GÖNCZÖL, ERZSÉBET DANCZIG, I. BOLDOGH, T. TÓTH and L. VÁCZI

Institute of Microbiology, University Medical School, Debrecen, and Szolnok Country Hospital, Szolnok

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Tissue samples from the salivory gland, lung spleen, liver and kidney of Balb/c nice infected with murine cytomegalovirus (MCMV) contained infective MCMV and virus-specific antigens for 6–7 weeks following the infection. After the 8th week no infective virus could be detected in any organ and antigens were found only in the spleen and the mesangial cells of the renal glomerules. As a result of cyclophosphamide treatment applied in the 22nd–24th weeks, the latent viral infection was reactivated in nearly all animals, infective virus production started again in the organs, and lasted for about 3 weeks. During the subsequent latent period the virus was again reactivated by repeated cyclophosphamide treatment. The experimental alternation of the latent and reactivated phases of viral infection renders the model suitable for study of the mechanism and consequences of viral latency and reactivation in vivo.

Experimental infection of mice with murine cytomegalovirus (MCMV), as a model of infection by human cytomegalovirus (HCMV), has been studied on many occasions [1–9]. According to these studies the pathogenesis of MCMV infection in mice shows a close similarity with the pathogenesis of human CMV infection.

On the other hand, study of the murine model has been restricted to the acute phase of the infection [1–6], and little attention has been paid to the elaboration of a model that might be helpful in studying the stage of viral latency and the possibility of reactivation from the latent phase [7–9]. This paper reports on the elaboration of such a model.

Materials and methods

Virus. Smith strain of murine CMV (MCMV) was kindly supplied by Prof. C. A. Mims from Guy's Hospital, London. For the inoculation of 4–5 week old Balb/c mice, 0.1 ml (10^3 p.f.u./mouse) inoculum was applied intraperitoneally. The virus source was the salivary gland

ÉVA GÖNCZÖL, ERZSÉBET DANCZIG, ISTVÁN BOLDOGH, LAJOS VÁCZI
Institute of Microbiology, University Medical School,
H-4012 Debrecen, P.O.B. 17, Hungary

TIBOR TÓTH
Szolnok County Hospital,
Szolnok, Hungary

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of a MCMV-infected animal, removed 2–3 weeks after the infection. After mechanical homogenization in PBS and centrifugation at 2000 rpm for 10 min, the suspension was stored at –70 °C until use [10]. The virus produced in this way was suitable for elaboration of the model.

Reactivation of the latent virus by cyclophosphamide treatment. The mice that were in the phase of latent viral infection were injected intraperitoneally with 150 mg/kg cyclophosphamide dissolved in 0.5 ml PBS, on 4 occasions in 5-day intervals.

Virus isolation from the organs of infected animals. The organs were excised at the points of time given in "Results", mechanically homogenized and 10% suspensions (W/v) were prepared from them. After centrifugation at 2000 rpm for 10 min tissue cultures were inoculated with 0.1 ml and after 2 h adsorption the inoculum was removed and the cultures were washed once and covered by fresh culture medium. The appearance of cytophatogenic effect was followed for 2 weeks. For virus isolation syngenic Balb/c murine embryonic fibroblast cultures were used; and in the phase of latency, in addition to the Balb/c cultures, also fibroblast cultures from an allogenic DBA murine strain. Preparation of the murine fibroblast cultures was performed as described previously [11].

Detection of MCMV antigens in the organs of infected animals. The indirect immunofluorescence method (IF) was used. Quick-frozen sections prepared from the organs were fixed in a 1 : 1 mixture of acetone-methanol at –20 °C for 5 min. As serum the serum mixture of MCMV-immunized rabbits was used and as control serum, the mixture of the sera of the same rabbits taken before immunization. Immunization was performed with MCMV produced in rabbit kidney tissue in the presence of minimum essential medium (MEM) containing 10% rabbit serum. After the onset of diffuse cytopathic effect on the 8th–10th day after inoculation the supernatant of the cultures was purified from the cell debris by centrifugation at 8000 rpm for 10 min, then at 100000 *g* for 1 h. The sediment was suspended in PBS, then after division into several parts, stored at –70 °C. The animals were given 2 ml of virus suspension intravenously once a week six times. The titre of the virus was 9×10^6 p.f.u./ml titrated in murine fibroblast culture. In the 3rd week after the last dose the animals received further 5 ml virus suspension, then 20 days later they were exsanguinated. The titre of the immune serum was 1 : 160 according to the indirect IF method, on MCMV-infected murine fibroblast cultures, when the infected tissue was fixed 4 days after infection. The immune serum was applied in 1 : 60 dilution. The pre-immune rabbit serum was completely negative even at a dilution of 1 : 2 on MCMV-infected murine fibroblast cultures. As a conjugate, anti-rabbit IgG (Hyland) was used.

MCMV antibody determination in the sera of infected animals. The determinations were performed with the indirect immunofluorescence method. Coverslip cultures of Balb/c embryonic fibroblasts were fixed in 1 : 1 mixture of acetone and methanol at –20 °C for 3 min, 4 day after the infection. The sera used were mixed and diluted sera of 10 mice in each sample. Anti-mouse IgG (Hyland) was used as conjugate.

Reagents. Anti-rabbit IgG and anti-mouse IgG conjugates (Hyland Division Travelor Laboratories, Costa Mesa, Calif, USA). Cyclophosphamide (Jenapharm Ankerwerk, Rudolfstadt, GDR).

Results

Follow-up of the acute, latent and reactivated phases of infection through infective virus isolation from different organs. The results are summarized in Table I. It is clearly seen that infective virus could be recovered until the 7th week after infection from the salivary gland, lung, spleen, kidney and liver and longest from the salivary gland. The number of animals in whose organs infective MCMV was detected was identical with the number of infective MCMV-containing salivary glands. This means that there was no animal with negative salivary gland but positive other organ. This stage is called the acute phase of infection.

In the 8th postinfective week no infective virus could be detected in the organs any longer irrespective of whether syngenic Balb/c or allogenic DBA

Table I

Recovery of infective HCMV from infected mice in the acute (1st–7th weeks), latent (8th–22nd, 28th–30th and 37th weeks) and reactivated (25th–27th and 33rd–35th weeks) phase of the infection

Organs examined	Postinfective weeks																		
	1	2	3	4	5	6	7	8	12	15	22	25	26	27	28	30	33	34	35	37
Salivary gland	10	10	10	8	5	2	2	0	0	0	0	4	3	0	0	0	3	4	0	0
Lung	10	9	9	3	2	0	0	0	0	0	0	4	6	0	0	0	3	4	0	0
Spleen	10	10	8	8	4	0	0	0	0	0	0	5	6	1	0	0	4	6	2	0
Kidney	8	9	6	0	0	0	0	0	0	0	0	6	7	2	0	0	4	5	1	0
Liver	8	9	8	0	0	0	0	0	0	0	0	4	5	1	0	0	3	4	2	0
Skeletal muscle	0	0	0	0	0	0	0		NT			0	0	0	NT			NT		NT
Heart	0	0	0	0	0	0	0		NT			0	0	0	NT			NT		NT
Brain	0	0	0	0	0	0	0		NT			0	0	0	NT			NT		NT
Spinal cord	0	0	0	0	0	0	0		NT			0	0	0	NT			NT		NT
Total	10	10	10	8	5	2	2	0	0	0	0	9	8	2	0	0	8	6	4	0

Reactivation was performed by cyclophosphamide treatment (150 mg/kg, 4 times, on every fifth day) beginning with the 22nd and 30th postinfective weeks

Virus isolation was performed from the organs of 10 animals each time. The numerical values show the number of animals (out of 10) from whose particular organs infective MCMV was isolated

tissue cultures were exposed by the homogenized organs. However, the success of cyclophosphamide treatment started in the 22nd week, proves that the virus had been present in latent form in the cells of the organs prior to cyclophosphamide treatment. Therefore, the 14-week period between the 8th and 22nd weeks is called the latent phase of infection.

Out of the 10 animals exsanguinated 4 days after the last cyclophosphamide dose there were 9 in which infective virus was detected in the homogenisate of some organ. The reactivated virus could not in every case be detected in the salivary gland, despite the fact that in the acute phase of infection the salivary gland always contained infective virus. In most animals infective virus was present in the kidney and the spleen, these were followed by the lung, liver and salivary gland. Infective virus was only found in the course of 3 weeks; in the 4th week after the last cyclophosphamide dose the organs were negative again. The three-week period between the 25th and 27th weeks is called the reactivation phase. The second cyclophosphamide treatment started in the 30th week, after another latent period between the 28th–30th weeks, again resulted in reactivation of the virus. In the 33rd week infective virus was detected in the organs of 8 mice out of 10; by the 37th week the mice were again free of infective virus.

Follow-up of the acute, latent and reactivated phases by IF examination of quick-frozen sections from the organs. Quick-frozen sections were subjected to IF examination; four sections were prepared from each organ.

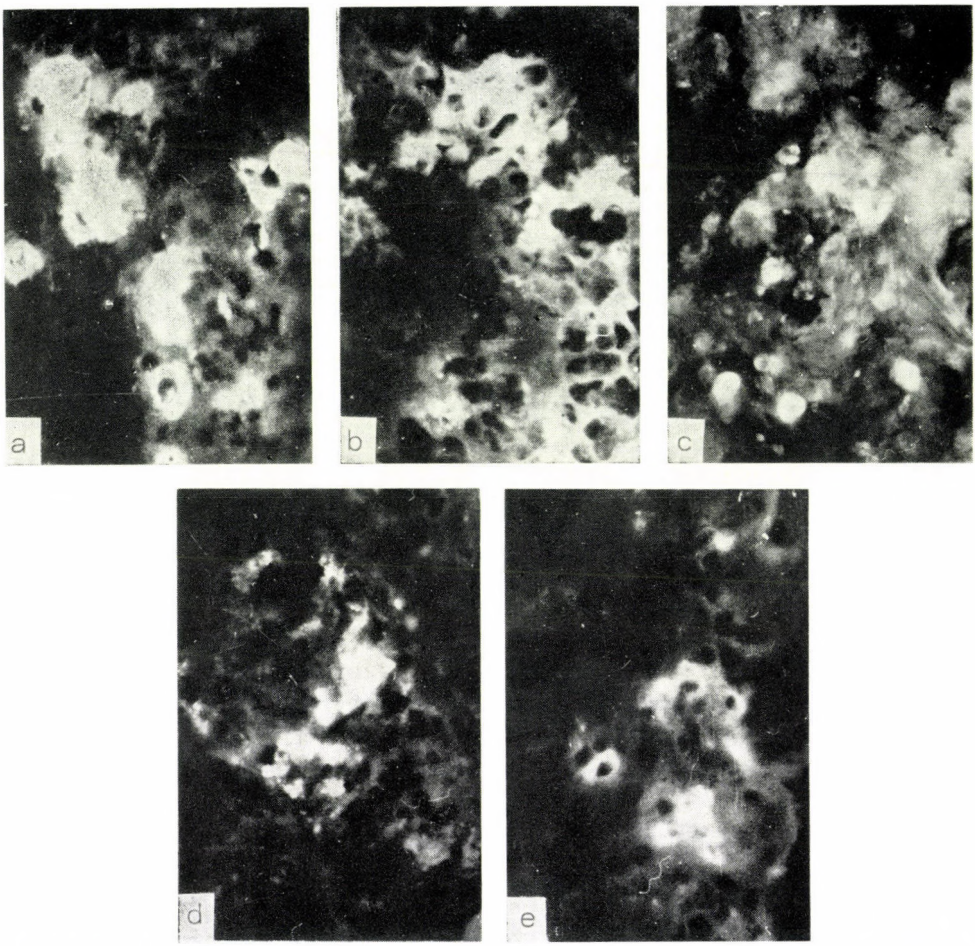

Fig. 1. MCMV-specific antigens in the salivary gland (a), lung (b), spleen (c), kidney (d) and liver (e) of animals in the acute phase of infection, determined by the IF method

In the acute phase MCMV specific antigens could be detected in nearly all organs from which infective viruses had been isolated (Figs 1a, b, c, d, e). Detectability of the antigens was in no correlation with virus isolation; it occurred that infective virus was isolated from an organ in which no MCMV-specific antigen was found. The possible cause was that for virus isolation the organs were homogenised so that there was a higher probability of the presence of virus-containing cells in the material, whereas the four sections subjected to IF examination may have originated in part of the organ in which there was no virus replication. Among the organs excised in the latent phase (8th, 12th, 15th and 22nd weeks after infection) only the spleen and the kidney were IF positive, 8 out of 40 spleens, and out of 40 13 kidneys. MCMV-specific antigens

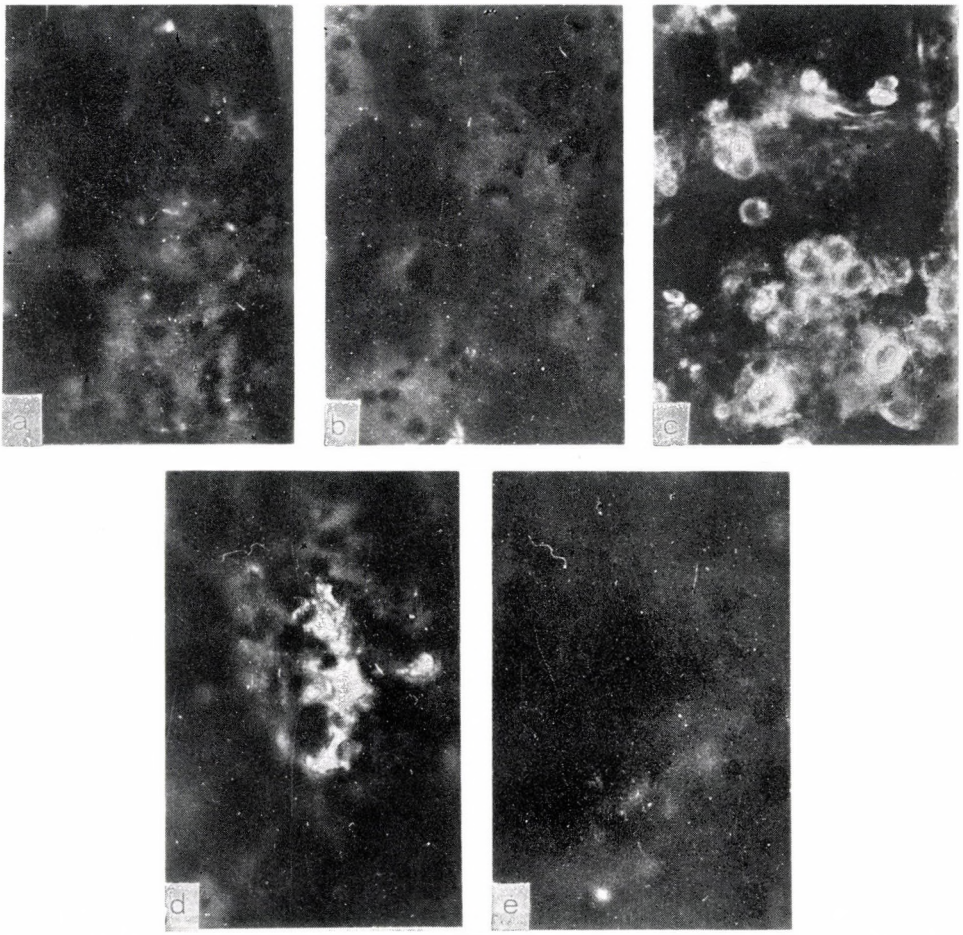

Fig. 2. MCMV-specific antigens in the spleen (c) and kidney (d) of animals in the latent phase of infection; no antigens could be detected in the cells of the salivary gland (a), lung (b) and liver (e). Examinations were performed with IF method

were found in the cells of the spleen and in the mesangial cells of the renal glomerules (Figs 2c, d).

In the phase of reactivation, IF antigens could be detected only in about 40–50% of sections prepared from infective virus containing organs. This may mean that the infection affected only some parts of the organs, and the foci of virus replication were missed when the sections were made. Figures 3a, b, c, d show the IF images of the sections made from these organs. The sections were prepared in the first week following the last cyclophosphamide dose.

Specificity of immunofluorescence. All sections proved to be completely negative when preimmune rabbit serum diluted 1 : 2 was applied.

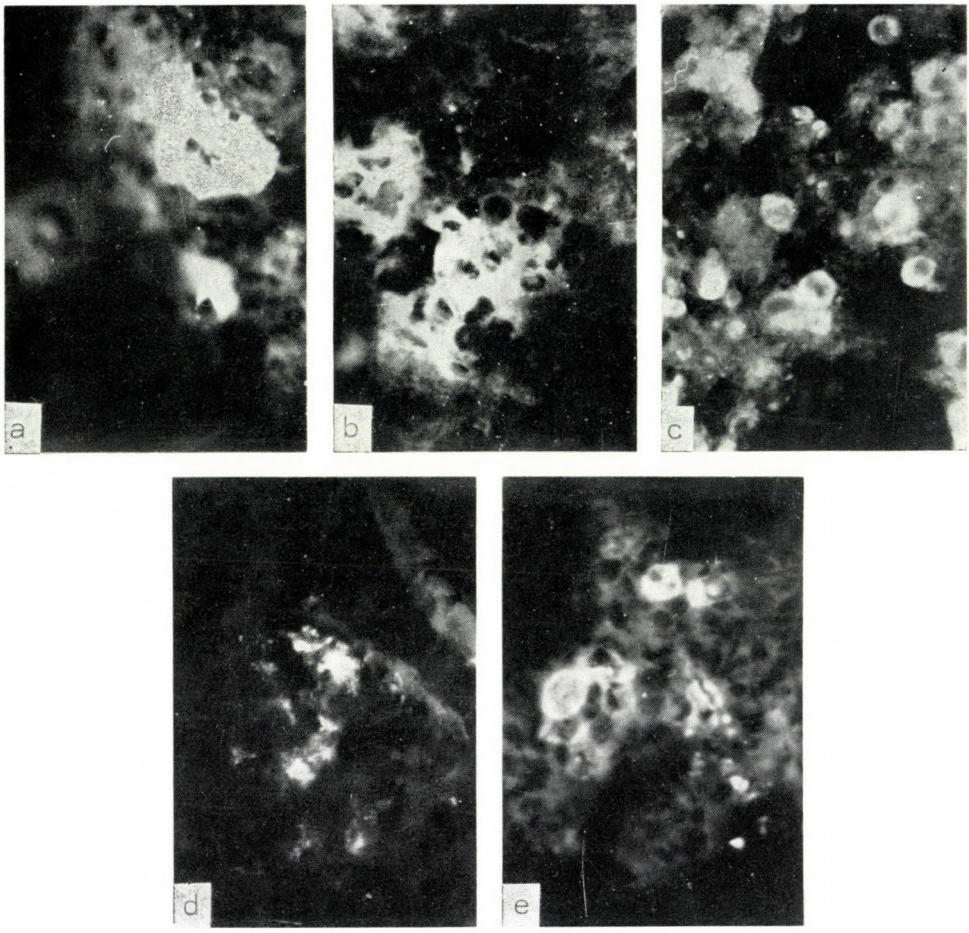

Fig. 3. MCMV-specific antigens in the salivary gland (a), lung (b), spleen (c), kidney (d) and liver (e) of animals in the reactivated phase of infection, determined by IF method

Serological response in MCMV-infected mice Results are given in Fig. 4. It is obvious that the maximum antibody level had developed by the 8th week, and remained practically unchanged during the latent and reactivated phases, although a non-significant increase of anti-MCMV IgG titre was detected after reactivation of the latent virus.

Effect of cyclophosphamide treatment in mice previously not inoculated with MCMV. Twenty Balb/c mice 6–7 months of age were treated with cyclophosphamide in a way similar to that of animals previously inoculated with MCMV. In the 1st and 2nd weeks after the last dose, 10 mice were used for the detection of infective virus and the other 10 for MCMV antigen separation. In the organs of these animals neither infective virus nor MCMV antigens were found.

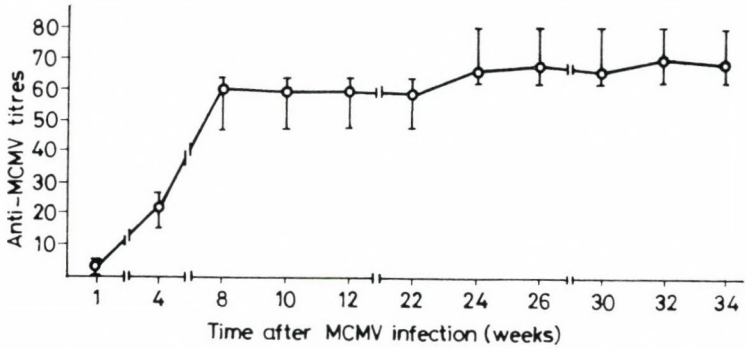

Fig. 4. Serological response of mice after intraperitoneal administration of MCMV. The figure shows the minimum, maximum and the average (○—○) IgG titre in each group of 10 animals

Discussion

The described model helps in investigation in vivo of a number of questions emerging in connection with virus latency and reactivation characteristics of herpesviruses. It allows to study how many times after primary infection and in what period of time CMV infection can be reactivated, what histological changes are characteristic of the reactivation, in what form and in which cells the latent virus persists, how it is expressed there, and what immune mechanisms influence the course of infection, etc. These questions were answered only partly in the present experiments, but it was shown that such an in vivo model can be developed.

In the experiments cyclophosphamide was used for the reactivation of latent virus, since it is widely used in human immunosuppressive therapy and has been reported to reactivate CMV infection in man [12] and mice [8]. The in vivo model developed by us may be considered appropriate, since cyclophosphamide treatment resulted in reactivation in almost every animal, i.e. nearly all of them were proven to be latent carriers of the virus prior to treatment, thus almost all could be utilized for the study of latency and reactivation.

When anti-mouse lymphocyte serum had been used as immunosuppressive agent, reactivation of latent virus was successful only in about half of the animals [7]. With more potent immunosuppressive treatment with anti-mouse lymphocyte serum together with cortisone, reactivation was more efficient, but because of the high death rate the experiment had to be terminated on the 16th day of reactivation.

In our experiments, deaths occurred only sporadically. After cyclophosphamide treatment the virus could be isolated from the liver, spleen, kidney, lung and salivary gland, i.e. it was either activated in an organ and dissemi-

nated from there, or it was activated in various organs at the same time. Since in the course of reactivation no organ was found positive on all occasions in all mice, the latter possibility is regarded as more likely.

It was remarkable that in the latent phase the kidneys contained virus antigens. Thus, a possible site of latency may be the mesangial cell system of the renal glomerules. This might explain the frequency of CMV infection in recipients of kidney grafts.

The cells of frozen spleen sections also contained MCMV specific antigens, detectable by IF method, in the latent phase.

Olding et al. [13] inoculated fetal and neonatal mice with MCMV attenuated by serial passages in tissue culture. The mice became latent carriers in the B lymphocytes of their spleen, and the latent virus could be activated by placing the lymphocytes in allogeneic fibroblasts. In our experiments no infective virus was detected in the spleen in the latent phase, using either allogenic or syngeneic fibroblasts. The difference between the results of the two experiments may have been due to the fact that the former authors used an attenuated virus strain, whereas our virus was virulent or perhaps to that in the former experiment the mice were inoculated at an earlier age. A proof of the spleen latently carrying MCMV was the observation of Mayo et al. [8] who produced MCMV infection in allogenic and syngenic mice with speen cells of mice in the latent stage. Infective virus could not be detected in these spleen cells either. It is not clear whether for the production of latency Mayo et al. used viruses attenuated in tissue culture or virulent ones from the salivary gland.

The problem of the specificity of IF positive cells in frozen sections needs discussion. In the IF method MCMV-immunized rabbit serum was applied, which gave a positive reaction with the cells. Preimmune serum gave a negative reaction in every case. This serum could detect non-MCMV-specific antigen only if the MCMV infection of rabbits activated some persisting microorganism which resulted in an immune reaction, and if this microorganism had some antigenic affinity to a microorganism persisting in mice. The probability of such a condition is not high, and it can completely be ruled out with the result that the frozen sections prepared from the organs of mice without MCMV infection and cyclophosphamide treatment and those without MCMV infection but subjected to cyclophosphamide treatment would give a negative result with rabbit immune serum.

The MCMV antibody level in the infected animals did not rise in the course of reactivation. The reason may be that immunosuppressive treatment inhibited the activity of the antibody-producing cells. It is, however, also possible that although the antibody level resulting from primary infection decreased with time and increased in consequence of reactivation, nevertheless the decrease and rise in antibody levels compensated each other and could not be

followed because of the quick alternation of the acute, latent and reactivated phases.

Our experiments are more informative than the previous in vivo murine models in that the immunosuppressive treatment was performed in the phase of either acute or chronic viral infection when infective virus was detectable in the organs, but reactivation of the virus took place from the state of real latency when no infective virus can be detected in the organs. Few similar works have been reported [7–9] but it should be noted that virus replication under the level of detectability may have occurred in the organs. Since we know of no more sensitive method for the isolation of the virus, this possibility cannot be ruled out and the definition can be considered acceptable. Moreover, our model is more informative than the previous ones since reactivation could be performed repeatedly, i.e. after a second latent period, too. We do not know of such attempts. It seems that the latent and reactivated phases may alternate periodically and be provoked experimentally by immunosuppression. Thus, the course of CMV infection reminds of that of herpes simplex, where the alternation of the active and latent phases of the virus is well-known, and it is not similar to varicella virus infection, where reactivation, to the best of our knowledge, appears only once or in very limited numbers, in the form of herpes zoster. We do not know how many times and in what form a reactivation of Epstein–Barr virus takes place during a lifetime.

Our model is useful and its study may open up new possibilities in the recognition of viral latencies and reactivations.

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IMMUNOLOGICAL STUDY OF *PSEUDOMONAS AERUGINOSA* EXTRACELLULAR SLIME

E. S. Stanislavsky, Nataly A. Yushkova, Liliya S. Edvabnaya, Nataly M. Landsman and Irena G. Zaidner

Laboratory of Protective Antigens, Mechnikov Research Institute for Vaccines and Sera, Moscow, USSR

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Crude extracellular slime (C-ES) was isolated from 15 laboratory strains of *Pseudomonas aeruginosa*. Glycolypoprotein (GLP) was prepared from partially purified C-ES by the phenol treatment method. Lipopolysaccharides (LPS) were isolated from *P. aeruginosa* and *Salmonella typhi-murium*. Some correlation has been found between the quantity of the produced C-ES and virulence of *P. aeruginosa* for mice. Toxicity (LD_{50} value) and the yield of GLP did not correlate with the strain virulence. C-ES and GLP contained hexose, protein and hexosamine. Heptoses (characteristic LPS components) were present in C-ES and practically absent from GLP. Paper chromatographic analysis of GLP showed the presence of uronic acids, galactose, glucose and ribose in all strains; GLP of most strains contained rhamnose and a low amount of mannose and several strains xylose. Uronic acids, galactose and, probably, ribose and mannose were characteristic ES components. C-ES and GLP were studied in active and passive mouse protection. ES isolated from *P. aeruginosa* strains of different O serogroups or immunotypes induced a marked cross protective response.

The control of *Pseudomonas aeruginosa* infection has become an urgent problem of practical medicine. Active and/or passive immunization can play an important role in solving this problem. Among the numerous *P. aeruginosa* virulence factors, extracellular slime (ES) is of substantial pathogenetic significance as a functional analogue of the capsule. ES of *P. aeruginosa*, like capsules of other microbes, exerts an antiphagocytic effect [1] which makes slime-producing *P. aeruginosa* virulent for the host [2–6]. Isolated ES is toxic [7–9] and immunogenic for laboratory animals [2, 3, 6, 7, 9–12]. Liu et al. [13] was the first to show that *P. aeruginosa* ES stimulates type specific immunity. However, some other authors reported cross-reactivity of ES in tests on mice [1, 14, 15].

We think that the question of the antigenic cross-activity of *P. aeruginosa* ES is not clear enough, and the problem of using ES as a protective immunogenic agent is not yet solved. In the present paper we give an account of cross-immunologic tests with various O-serogroups or immunotypes.

Evgeny S. Stanislavsky, Nataly A. Yushkova, Liliya S. Edvabnaya, Nataly M. Landsman, Irena G. Zaidner
Laboratory of Protective Antigens, Mechnikov Research
Institute for Vaccines and Sera
Per. Mechnikova 5a, 103064 Moscow, USSR

Materials and methods

Bacterial strains. We used *P. aeruginosa* strains 170001–170023 [16, 17] from the Hungarian National Collection of Medical Bacteria; immunotypes 1–7 of Fisher's scheme [18] obtained from Dr. B. Lányi [Hungary]; strain No. 8 isolated from burned patients in Vishnevsky Institute for Surgery of the USSR Academy of Sciences and received from Dr. I. A. Grishina; toxigenic strain PA-103 received from Dr. P. V. Liu (USA); *P. aeruginosa* strain O-11 and *Salmonella typhi-murium* strain No. 78 (O1, 4, 5, 12) from the Collection of the Tarasevich State Control Institute (Moscow, USSR).

Preparation of ES. P. aeruginosa strains were grown on MacConkey agar [19] covered with cellophane disks in Petri dishes at 37 °C for 16–18 h and then at room temperature for 4 days. The bacterial cells were washed off the cellophane disks with saline, shaken for 30–60 s with glass beads in a flask and centrifuged at 1000 *g* for 40–60 min. The cells were removed and the supernatant was centrifuged again under the same condition. Dry sodium chloride was added to the supernatant up to 10% concentration; after the salt dissolved 2–4 volumes of ethanol (96%) were added. The precipitate was separated by centrifugation (3000 *g* for 20–30 min), washed twice with cold ethanol, redissolved in saline and ES was precipitated again by 2–4 volumes of cold ethanol. The precipitate was then separated by centrifugation and redissolved in a minimum volume of distilled water by careful mixing. The aqueous solution of ES was centrifuged at 1000 *g* for 40–60 min, and the supernatant was lyophilized. The preparation was named crude ES (C-ES).

Glycoprotein (GLP) was isolated from ES by means of phenol deproteinization [5].

Preparation of LPS. LPS was isolated from *P. aeruginosa* and *S. typhi-murium* by the method of phenol–water extraction as described earlier [20].

Passive haemagglutination test (PHA) was performed as described by Zaidner et al. [9] using ES antigen. Formalinized sheep red blood cells were sensitized with ES by incubating a 5% suspension of cells in saline with an equal volume of ES (250 μg/ml of saline) at 37 °C for 2 h. To 0.5 ml serial dilutions of anti-serum was added 0.05 ml of the suspension of sensitized cells. Agglutination was read after 2 h at room temperature.

Virulence determination. The culture grown on meat–peptone agar (MPA) for 16–18 h at 37 °C was suspended in saline and adjusted according to the optical standard for enterobacteria of the Tarasevich State Control Institute (Moscow, USSR). Five gradient doses were prepared by twofold dilutions in saline. The dosage was checked by inoculation of the suspensions onto MPA plates. The culture was injected intraperitoneally, the animals were observed for 7 days, then LD_{50} was estimated [21]. Similar method of preparing culture was used in active and passive mouse protection.

Active mouse protection. Non-inbred mice (type Swiss) weighing 18 to 20 g were immunized intraperitoneally with 100 μg of C-ES, GLP or LPS. Seven days after vaccination the mice were challenged by intraperitoneal administration of graded doses of live *P. aeruginosa* culture in saline. Death of the animals was recorded during 7 days. LD_{50} and standard error were calculated for both immunized and non-immunized (control) mice [21] and then standard intervals were calculated at $p = 0.05$.

Passive mouse protection. Mice were inoculated intraperitoneally with 1 ml of anti-C-ES serum (1 : 10) and 2 h later were challenged intraperitoneally with graded doses of *P. aeruginosa* live culture. Death of the animals was recorded for 7 days. LD_{50} values were calculated for immunized mice and for controls (non-immunized mice immunized with pooled preimmune rabbit serum).

Immunization of rabbits. Chinchilla rabbits weighing 2–3 kg were used. Before immunization all rabbits were sampled and the sera of individual rabbits were pooled (preimmue sera). Rabbits were then immunized with C-ES according to two schemes. (1) As first dose, a subcutaneous injection of 1 mg antigen in Freund complete adjuvant (FCA), as a second and third one intravenous injections of 2 mg antigen each with an interval of 3 to 4 days, were given. Four weeks later 2 intravenous injections of the antigen (4 mg and 6 mg) were administered with an interval of 3 to 4 days; 15 days after the last immunization the rabbits were bled. (2) The first and second immunizations were subcutaneous injections of the antigen in FCA (0.1 and 0.2 mg, respectively), followed by a course of intravenous injections (one 0.5 mg and five 1 mg doses at intervals of 3 to 4 days). Before each immunization and also on the 5th day and on the 8th day after the last immunization, the rabbits blood samples were taken and 15 days later total bleeding was made. The antisera were stored at −30 °C to −40 °C or lyophilized.

Chemical analysis. Determination of protein was done by the Folin reagent [22], of hexose by the anthrone reagent [23], heptose [24] and of hexosamine as described by Lüderitz et al. [25]. Carbohydrate composition of C-ES and GLP was shown by the paper chromatography [20].

Results

1. *Correlation between C-ES yields, C-ES toxicity and virulence.* Comparison of C-ES yield with virulence of the strain (Table I), showed some correlation between these parameters.

The greatest yield of C-ES was observed, for example, in strains 170006 and 170007 which appeared to be the most virulent for mice. Strains like, for instance, 170002, 170014 or 170017 with small C-ES yield showed comparatively low virulence and strains 170001 and 170019 showed an average production of ES as well as an average value of LD_{50}.

C-ES toxicity (LD_{50} value) or yield of GLP had no correlation with virulence of the corresponding strain.

2. *Chemical analysis of C-ES and GLP.* Results of the chemical analysis (Table I) proved that protein is present in C-ES of various strains (average $20.2 \pm 3.8\%$), and that its content becomes about two times lower after phenol treatment (see GLP). The amount of heptose (characteristic component of LPS) is about 7 times lower and hexose is about twice higher in GLP than in C-ES. GLP of strains 170002, 170006, 170007, 170009, 170014, 170019, 170021–23 and O–11 contained no heptose. The hexosamine content (not shown in Table I) averaged at $3.7 \pm 2\%$; only 170022 GLP was shown to contain 11% of hexosamines.

Results of paper chromatographic analysis (Table II) proved the presence of uronic acids, galactose, glucose and ribose in GLP of all strains; rhamnose was shown in all but one strain (O–11), whereas mannose and xylose in several strains. Data for carbohydrate content of LPS [25] and GLP of corresponding strains indicate that galactose is found only in GLP and is evidently a characteristic component of ES. Mannose is present to a certain extent, in GLP of strains 170001, 170002, 170005, 170007, 170009, 170017, 170021, 170022, 170023, O–11 but has not been revealed in LPS of these strains. However, mannose is absent from GLP of 170012, 170014, 170018 and 170019, whereas it is present in LPS of these strains. Ribose is mainly revealed also in GLP and is hardly found in LPS. Glucose is revealed in GLP of all the studied strains and also found in LPS.

3. *Active mouse protection with C-ES and GLP.* C-ES immunization induced a good immunity in mice against intraperitoneal challenge with homologous and heterologous *P. aeruginosa* strains (Table III). Thus, we have revealed statistically valid (in comparison with the controls) protection of mice immunized with almost all C-ES except for C-ES 170022 when challenged with F1 and except for C-ES 170014 and 170021 when challenged with F2. Significant C-ES cross protective response was demonstrated when immunized mice were challenged with F4, F5 and F7.

Table I

Yields and chemical analysis of crude extracellular slime and glycoprotein, virulence and toxicity of slime of P. aeruginosa strains

Strain	Lányi's serogroup [16]	Fisher's immuno-type [18]	C-ES yield (mg/dish)	GLP yield (% of C-ES)	Protein %		Hexose %		Heptose %		LD_{50} of	
					C-ES	GLP	C-ES	GLP	C-ES	GLP	culture $\times 10^6$ cells	C-ES (mg)
170001	1	—	31.5	21.1	31.4	9.8	3.4	7.0	6.1	1.3	93 (66–132)	0.8 (0.5–1.2)
170002	2a,2c	5	16.0	25.0	28.0	ND	5.0	8.0	5.8	0	282 (205–382)	1.4 (0.7–2.7)
170005	3a,3d	7	20.0	8.0	16.2	12.6	6.0	8.0	5.6	0.9	182 (138–280)	0.7 (0.4–1.2)
170006	3a,3d,3e	—	40.0	10.8	9.5	6.9	3.4	9.0	11.2	0	41 (30–59)	1.7 (1.1–1.8)
170007	(3a),3d,3f	—	84.8	12.5	27.2	10.0	6.0	8.0	0.9	0	22 (17–30)	2.2 (1.3–3.6)
170009	4a,4c	1	14.0	35.0	28.6	16.6	5.0	6.0	1.3	0	178 (123–257)	3.2 (2.2–4.7)
170012	5a,5b,5d	6	21.0	28.6	12.1	14.0	3.4	14.6	1.3	2.2	62 (47–81)	3.2 (1.9–5.6)
170014	6	4	9.0	29.5	16.9	10.9	5	10.0	1.1	15.1	151 (107–214)	ND
170017	8	—	6.0	32.3	23.2	11.0	4.2	25.0	2.8	0.6	195 (123–324)	ND
170018	9	—	10.0	34.0	23.6	9.0	5.0	11.8	1.1	1.1	ND	2.8 (1.9–4.3)
170019	10a	—	20.0	18.6	11.0	ND	6.0	10.0	0.4	0	93 (58–75)	1.2 (0.6–1.9)
170021	11	—	11.4	ND	16.5	ND	5.0	ND	2.9	0	62 (49–78)	1.6 (1.1–2.3)
170022	12	—	10.0	20.6	ND	11.7	ND	21.0	ND	0	ND	ND
170023	13	—	15.4	12.5	23.8	ND	6.0	ND	2.2	ND	162 (107–246)	0.7 (0.4–1.2)
0–11	7a,7b	2	25.9	13.3	15.2	8.6	5.0	7.0	0.4	0	62 (47–81)	1.4 (0.6–2.4)

ND = not determined

Table II

Carbohydrate composition of P. aeruginosa GLP

Strain	Uronic acids	Galactose	Glucose	Mannose	Xylose	Ribose	Rhamnose
170001	+	traces	+	traces	+	+	+
170002	+	+	+	±	±	±	+
170005	+	±	+	traces	—	traces	+
170006	+	?	+	—	—	+	+
170007	+	+	+	traces	+	+	+
170009	+	+	+	traces	traces	+	+
170012	+	+	+	—	+	+	+
170014	+	+	+	—	traces	traces	traces
170017	+	+	+	traces	—	traces	traces
170018	+	+	+	—	+	+	±
170019	+	±	+	—	—	+	traces
170021	+	+	+	+	—	+	+
170022	+	±	+	+	traces	+	+
170023	+	±	+	+	traces	+	+
O-11	+	+	+	+	+	+	—

\+ Present in significant amounts
± Present in small amounts
— Absent

Having compared the results (Table III) of challenge by different strains of similar virulence (F1 and F2 or F4 and F7) we demonstrated a similar protective activity of C-ES. Some cross activity of C-ES was revealed when immunized mice were challenged with F3.

Data showing that C-ES of various strains is able to stimulate immunity in mice against infection with the toxigenic strain PA-103, (Table III) are of great importance. C-ES evidently induces synthesis of opsonins promoting the phagocytosis of the toxigenic bacteria.

Immunization by GLP and challenge by F3 gave similar results (Table IV). However, it should be noted that C-ES 170001 and 170007 protected mice well against challenge with F3 (Table III) and that the corresponding GLP (Table IV) had a low protective activity. It should be noted that strain 170007 and strain F3 belong to the same serogroup or immunotype [16, 17].

The type-specific and especially cross protection of LPS appeared to be much weaker than the corresponding activity of C-ES (Table IV). However, LPS 170005, 170006 and especially 170007 stimulated a marked cross protective response in mice. LPS 170009 showed practically no protective activity after challenge with the homologous strain F1.

4. *Production of anti-C-ES antibodies in rabbits.* After the first immunization (Scheme 1) a low antibody level was discovered in rabbits (according to PHA) and after the second immunization a sharp rise of antibody production was observed. In the course of subsequent immunization the antibody level did not change substantially.

Table III

Active cross protection of mice immunized with C-ES of P. aeruginosa of various O serogroups (immunotypes)

C-ES of strain	LD_{50} and standard interval ($\times 10^6$ cells) of the strain								
	F1	F2	F3	F4	F5	F6	F7	170019	PA-103
170001	490 (309–776)	646 (355–1175)	33 (28–40)	427 (309–589)	1202 (919–1585)	302 (240–380)	54 (39–74)	562 (427–741)	178 (132–209)
170002	692 (437–4096)	347 (182–661)	68 (57–89)	302 (219–417)	1862 (1549–2239)	794 (575–1096)	87 (60–126)	851 (617–1175)	331 (263–417)
170005	851 (490–1479)	497 (234–776)	155 (123–195)	372 (282–490)	1202 (955–1514)	380 (263–550)	741 (617–977)	562 (468–676)	251 (202–316)
170006	302 (174–525)	368 (209–575)	646 (513–812)	141 (112–178)	1380 (1047–1820)	186 (135–257)	246 (204–295)	794 (575–1096)	178 (135–234)
170007	977 (562–1778)	1047 (603–1820)	246 (229–363)	ND	ND	302 (219–417)	977 (813–1175)	851 (617–1175)	141 (112–1781)
170009	1202 (724–1995)	302 (200–457)	<25	603 (479–759)	1862 (1549–2235)	229 (116–316)	263 (191–363)	692 (550–871)	316 (251–398)
170012	603 (316–1148)	302 (191–479)	<25	186 (129–269)	1479 (1122–1950)	912 (794–1148)	151 (115–207)	794 (575–1096)	93 (78–112)
170014	427 (269–676)	200 (126–316)	35 (28–45)	1479 (1175–1862)	1047 (794–1380)	372 (295–468)	66 (48–91)	741 (562–876)	115 (87–151)
170017	347 (209–575)	646 (355–1175)	<25	457 (347–603)	1698 (1342–2138)	372 (269–513)	246 (170–355)	977 (692–1413)	219 (170–269)
170018	302 (200–457)	490 (282–851)	47 (35–62)	427 (309–589)	1380 (1096–1738)	246 (195–309)	141 (93–214)	427 (339–537)	191 (145–251)
170019	525 (347–794)	427 (269–676)	107 (78–148)	347 (271–479)	2089 (1820–2390)	562 (407–776)	263 (101–363)	1288 (977–1698)	347 (288–417)
170021	646 (372–1122)	282 (141–562)	71 (56–89)	372 (295–468)	912 (724–1096)	246 (204–295)	123 (93–162)	603 (457–794)	302 (240–380)
170022	246 (141–427)	457 (288–724)	31 (28–37)	692 (550–871)	1122 (955–1349)	372 (282–490)	87 (60–126)	1047 (794–1380)	282 (224–355)
170023	372 (204–676)	1122 (617–2042)	200 (151–263)	282 (204–389)	2089 (1820–2399)	372 (282–490)	115 (83–159)	1202 (870–1660)	178 (135–234)
O-11	490 (246–977)	603 (302–1202)	29 (25–33)	200 (138–295)	1380 (1096–1738)	398 (275–575)	107 (87–141)	741 (617–891)	372 (295–468)
Control (non-immunized mice)	107 (68–170)	100 (57–174)	6 (4–9)	50 (36–69)	246 (195–309)	<25	35 (26–49)	123 (98–155)	25 (17–36)

C-ES of every strain was injected to 50 mice each and these mice was challenged with 5 different doses of the live culture: 10 mice per 1 challenge dose. Non-immunized mice were challenged in the same way 5 mice 1 per challenge dose. ND = not determined

Table IV

Active protection of mice immunized with GLP or P. aeruginosa LPS

GLP of strain	LD_{50} and standard interval ($\times 10^6$ cells)	
	immunization with GLP and challenge with F3	immunization with LPS and challenge with F1
170001	<100	ND
170002	123(93–162)	<100
170005	562(389–843)	427(309–589)
170006	ND	246(162–372)
170007	<100	851(617–1175)
170009	162(118–224)	<100
170012	115(87–151)	ND
170014	245(170–355)	263(200–347)
170017	100	ND
170018	107(85–135)	ND
170019	214(148–309)	372(269–513)
170022	<100	ND
170023	ND	123(71–170)
0–11	<100	ND
S. typhi murium	—	<100
Challenge control	10(7–14)	<50

Results of 2 experiments are summed up. Preparation of every strains was injected to 50 mice and these mice were challenged with 5 different doses of the live culture: 10 mice per 1 challenge dose. Non-immunized mice were challenged in the same way.

ND = not determined

In rabbits immunized according to the Scheme 2, a slow increase of PHA-antibody titres was observed. The level of antibodies reached its peak after the third to fifth immunization, and after that antibody titres slowly decreased though immunization continued.

The results of passive mouse protection with antiserum to C-ES (Table V) proves that both immunization schemes gave more or less the same effect. It was found that antisera protective activity (protective antibody level) rose sharply after the first immunization: EI (efficacy index, the ratio between LD_{50} for test mice and LD_{50} for non-immunized mice) was 24.8 after the challenge with homologous strain No. 8, and 7.9 after the challenge with heterologous strain 170015. Subsequent immunizations result in a certain increase of protective activity as very clearly shown by the challenge with a homologous strain.

Discussion

Among the clinical isolates of *P. aeruginosa* there are many mucoid (slime-producing) strains which are of great importance in human pathology [26–28]. *P. aeruginosa* extracellular slime (ES) is known to be one of the virulence factors [2–6].

The present results have clearly demonstrated that there is some correlation between ES production (yield per one Petri dish) and virulence of *P. aeruginosa*. However, the toxicity of ES (LD_{50} value), and yield of GLP had no correlation with the mouse virulence of the strain. The virulence of *P. aeruginosa* mucoid strains depends probably on the quantity of ES produced and not on its chemical (including carbohydrate) composition (Tables I and II). Specific opsonins (anti-ES antibodies) play an important role in the induction of postinfectious immunity against *P. aeruginosa* infection [1, 14, 15]. Serum preparations [12, 29] should evidently contain anti-ES antibodies able to protect animals from *P. aeruginosa* infection (Table V).

Isolated ES is a biopolymer with molecular weight 100 000–350 000 [8, 12, 30], consisting, probably, of subunits with molecular weight 10 000–30 000 [8, 12]. During extraction and fractionation isolated ES can dissociate into subunits and/or aggregate into large conglomerates with molecular weight from 300 000–2 000 000 [8, 12]. According to immunodifussion and immunoelectrophoresis, isolated C-ES is also heterogenous [8, 12]. We showed previously that low molecular weight ES components, i.e., its subunits are not toxic for mice and rats and that high molecular weight components, corresponding to crude ES, are toxic for laboratory animals [8]. However, all the ES components have induced good protective response [7, 12]. We and other authors [3, 30, 31] detected uronic acids, glucose, galactose, ribose and rhamnose in isolated slime, and also mannose in most strains and xylose in some strains. According to the results of Linker and Jones. [31] ES of *P. aeruginosa* contains alginic acids. Having compared our results with the results of the analysis of LPS [32], uronic acids or alginic acids, galactose and, perhaps, mannose can be considered as characteristic carbohydrate components of ES. The presence of glucose, rhamnose and xylose in ES is still questionable because these carbohydrates are also found in the corresponding LPS [32].

The results of our present experiments (Tables III and V) and earlier data [12, 29] have shown that ES induced a good cross protective response not depending on serogroup (or immunotype), which contradicts to previously known facts that ES of *P. aeruginosa* is a type-specific antigen [4, 6, 13]. We believe that ES has the properties of species-specific (or group specific) protective antigen. Moreover, high molecular weight toxic components could be used for preparation of antitoxic heterologous serum preparations [12] and low molecular weight non-toxic components could be used as a protective immunogen. We showed earlier that low molecular weight components of *P. aeruginosa* ES have cross antigenic activity [12].

Antigenic relationship between O antigen and ES is another interesting aspect of the problem. We [12] and other authors [30] found cross antigenic activity between ES and LPS of the corresponding strain. Is this due to an impurity in the ES preparation or existence of common antigenic determi-

Table V

Passive protection of mice injected with anti-C-ES-770006 (serogroup-03) serum (Rabbit immunization scheme 1)

Challenge strains and EI	Serogroup (immunotype)	LD_{50} of the strains and standard interval ($\times 10^6$ cells) ($p = 0.05$)						
		samples of the rabbit serum after immunization					pool preimmune rabbit serum	nonimmunized mice
		1st	2nd	3rd	4th	5th		
No. 8	3 (3/7)	473 (381–659)	646 (475–877)	741 (576–954)	831 (716–1034)	851 (692–1043)	72 (59–90)	23 (21–26)
EI		24.8	34	34	46	45	2.7	
No. 170015	7 (2)	562 (408–775)	562 (436–725)	603 (444–818)	488 (329–665)	603 (444–818)	87 (79–96)	50 (43–59)
EI		7.9	7.9	8.5	6.6	8.5	1.7	

Results of two experiments are summed up. Each sample of the antiserum (1 : 10) and of the preimmune serum (1 : 10) was injected to 50 mice; groups of 10 mice were challenged with 5 gradual doses of the live culture; non-immunized mice were challenged in the same say

$$\text{EI (efficacy index)} = \frac{LD_{50} \text{ for test mice}}{LD_{50} \text{ for non-immunized mice}}$$

nants? Sensakovic and Bartell [4] showed that antigenic properties of these substances are different. In our earlier experiments we showed that purified ES components which do not practically contain KDO (component of LPS) reacted with the corresponding LPS in the in vitro tests [12]. Carbohydrate components which are common for ES and LPS (for example, glucose and rhamnose), perhaps, condition antigenic relationship between these substances. The data of Prier et al. [30] showed that there were antigenic relationships between LPS and purified ES polysaccharide.

In active mouse protection we have shown that ES cross activity does not depend on the O serogroup (immunotype) of the strain (Table III). From data of the serological analysis [16, 17], it is known that LPS activity of *P. aeruginosa* is strictly type-specific. In our experiments we have revealed certain cross protective activity of LPS in tests on mice (Table IV). In rat experiments (model of wound *P. aeruginosa* sepsis) we have shown that LPS has no protective activity [33] and that isolated ES and its fractions protected rats from *P. aeruginosa* infection [7, 9]. We believe that C-ES cross protective activity is not conditioned by impurity or by existence of common LPS antigenic determinants but by "true" ES protective antigens which can be used as a "species-specific" protective immunogen after purification.

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INDUCTION OF STABLE L-FORMS OF *SALMONELLA TYPHI* AND *LISTERIA MONOCYTOGENES*

P. J. Asnani and K. Gill

Department of Microbiology, Panjab University Chandigarh, India

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Stable L-forms of *Salmonella typhi* and *Listeria monocytogenes* were produced using penicillin (4500 units/ml) as inducer, and sucrose, normal horse serum and Mg^{++} as stabilizers. Stable L-forms were produced after 100 and 56 passages, then adapted to grow and multiply in a medium free of inducer and stabilizers so that they did not revert to parental forms even after 12 continuous passages.

Bacterial variants without cell wall, being able to grow and multiply independently were first observed at the Lister Institute and were named as L-forms in honour of Lister [1]. These organisms, morphologically indistinguishable from mycoplasmas, are being recognized as a distinct entity although they could be induced from bacteria, using different inducing agents including penicillin [2–6].

Earlier, we have induced stable L-forms of *Staphylococcus aureus* and *Streptococcus faecalis* and observed their biological properties [7, 8]. In the present study attempts were made to induce L-forms of *Salmonella typhi* and *Listeria monocytogenes* using penicillin as an inducer and sucrose, normal horse serum and Mg^{++} as stabilizing agents.

Materials and methods

Source of organisms. One strain each of *S. typhi* (B-34–6) and *L. monocytogenes* obtained from Central Research Institute, Kasauli, and Indian Veterinary Research Institute, Izatnagar, India, respectively, were tested for purity and used to produce stable L-forms.

Induction process. The medium for induction, varying concentration of penicillin, the stabilizing agents and the process of induction have been described earlier [7]. Induced L-forms were then tested for reversion, stability and adaptation to grow in the absence of penicillin, horse serum and sucrose [9]. Smears of broth cultures and their colonies, stained with Gram and Giemsa stains, were studied for their cell and colony morphology.

P. J. Asnani, K. Gill
Department of Microbiology, Panjab University
Chandigarh, India

Results

S. typhi was passaged 10 times in the presence of 1000 units/ml of penicillin and stained smears of L-forms showed Gram-negative filamentous forms and round bodies. Later these were passaged in 1500 units/ml of penicillin. They formed "fried egg" colonies, displaying long bacillary forms with large round bodies which reverted in penicillin-free medium, therefore the penicillin concentration was increased to 2000 units/ml. Then typical "fried egg" colonies were formed showing dark centres and light periphery, displaying predominantly bacillary forms with small round bodies. After a total of 47 passages in 2500 and 3000 units/ml penicillin concentration L-forms revealed only filamentous forms in the first 4 passages and reverted back completely in the fifth passage. After further 16 passages in 3500 units/ml, it was noticed that these reverted when sucrose was decreased to 25% of the original amount but not in the same amount of serum. These were still unstable as they reverted when grown in a medium free of sucrose, serum and penicillin. After another 16 passages in 4000 and 4500 units/ml (total, 100 passages) the "fried egg" colonies with round bodies did not revert in penicillin, sucrose and serum free medium and could adapt to grow for 12 passages continuously without reversion in nutrient broth, agar and blood agar media.

Similar were the observations with the L-forms of *L. monocytogenes*, when passaged 10 times in the presence of 1000 units/ml and 6 times in 1500 units/ml of penicillin. It also formed "fried egg" colonies, showing similar cell and colony morphology as described above. After 9 more passages (total 25) in 3500 units/ml, these reverted to parental forms step-wise in 5 passages, namely Gram-negative filaments, bacilli, Gram-positive cocci and finally Gram-positive rods on penicillin, sucrose and serum free medium. These still being unstable were further passaged 6 times in increased, 4000 units/ml of penicillin. These could grow in a medium containing 25% of the original amount of sucrose and serum but reverted in the 3rd or 4th passage in sucrose, serum and penicillin free medium. After additional 25 (total 56) passages in 4500 units/ml, L-forms not only grew in sucrose, serum and penicillin and Mg^{++} free medium but adapted to grow in nutrient broth, agar and nutrient blood agar media for 12 continuous passages without any reversion, so that these could already be considered stable L-forms.

Discussion

Different inducing agents including penicillin and sodium chloride were used to produce stable L-forms [9–15]. In the present study attempts were made to induce stable L-forms of *S. typhi* and *L. monocytogenes* using penicillin

as an inducer and sucrose, normal horse serum and Mg^{++} as stabilizing agents. It was observed that L-forms could not be induced using 500 units/ml of penicillin and therefore its concentration was gradually increased to 4500 units per ml until stable L-forms had formed, confirming the observations [16–22] that the optimum concentration of penicillin depended upon the bacterial species, while other [23] noticed variations within the species.

Although L-forms are initiated using 1000 units/ml of penicillin, their stability seemed to depend upon the concentration of inducers. L-forms produced with 1500 units/ml reverted back spontaneously to their parental form after the first passage in penicillin-free medium. As the concentration was increased to 3500 units/ml, reversion was noticed in 5th passage, like in an earlier study with *S. aureus* and *S. faecalis* which in penicillin-free medium reverted in 4th and 5th passages, respectively. The optimum concentration was found to be 4500 units/ml for induction. Stable L-forms produced in this manner did not revert back even in 12 continuous passages and not only on penicillin-free medium but also in nutrient broth, agar and blood agar media, free of penicillin, sucrose, serum and Mg^{++}.

It was further observed that the stability and adaptability of these forms depended not only on the concentration of penicillin but also on that of sucrose and serum as these facilitated growth when the serum was gradually decreased to 50, 25, and zero per cent keeping the other ingredients constant. Similar was the effect of sucrose, as reported also by other authors [9] who similarly produced and adapted L-forms of *Streptococcus* MG.

It was also noticed that stable L-forms could not be induced spontaneously in single passage, even if the inducers and stabilizing factors were present. The number of passages necessary for induction and stabilization [7, 15] varied from species to species. Earlier [7], stable L-forms of *S. aureus* and *S. faecalis* were produced after 49 and 52 passages, respectively. Likewise, L-forms of group A haemolytic streptococci needed 229–233 passages [15]. Similarly stable L-forms of *S. typhi* and *L. monocytogenes* needed 100 and 56 passages, respectively.

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PARTIAL PURIFICATION AND SOME PROPERTIES OF TRYPTOPHAN DECARBOXYLASE FROM A *BACILLUS* STRAIN

K. G. Büki, Dang Quang Vinh and I. Horváth

Second Institute of Biochemistry, Semmelweis University Medical School, Budapest

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Bacteria of different origin were screened for tryptophan decarboxylase activity. The best producer belonged to an unidentified taxonomic entity of the genus *Bacillus*. In complete medium it produced tryptamine from tryptophan. The decarboxylase could partially be purified from the cells by sonication and DEAE-cellulose chromatography. The enzyme had an M_r of 150 000 and a pH optimum of about 7, was stable up to 37 °C, and its K_m was about 0.3 mM for tryptophan. The enzyme needed pyridoxal phosphate for maximum activity.

In our search for tryptophan decarboxylase activity in bacteria of potential manufacturing interest, we succeeded in isolating a few strains with the required activity. One of these was studied in detail, and the results are presented below.

Amino acid decarboxylase activity is widespread among microorganisms [1]. The only amino acid not frequently found on the decarboxylase lists is tryptophan. Mitoma and Udenfriend [2] found tryptophan decarboxylation in *Streptococcus faecalis*, Perley and Stowe [3] showed this activity in *Bacillus cereus*, Sabelnikowa et al. [4] reported on tryptophan decarboxylation in *Rhizobium phaseolis*. Nakazawa et al. [5] found several micrococci having this activity. One of their strains, *Micrococcus percitreus*, was further studied and the tryptophan decarboxylase of this strain was purified to homogeneity from several kilograms of wet cells [6]. The enzyme had a pH optimum of 9, K_m of 2.4 mM and transformed phenylalanine, tyrosine, 5-OH-tryptophan as well as tryptophan to the corresponding amines. Unfortunately, the authors did not publish more details of their enzyme; our studies with inhibitors have no parallel data in their work.

Materials and methods

Medium. The following medium was used throughout except otherwise stated [7]. Trypcasin, 20 g; glucose, 10 g; L-tryptophan, 1 g; salt mix A, 100 ml; salt mix B, 100 ml; distilled water ad 1000 ml. Salt mix A contains KH_2PO_4, 8.75 g; K_2HPO_4, 1.25 g; NaCl, 1.0 g;

Kálmán G. Büki, Dang Quang Vinh, István Horváth
Second Institute of Biochemistry, Semmelweis University Medical School
Puskin u. 9, H-1444 Budapest, P.O.B. 262, Hungary

distilled water ad 1000 ml. Salt mix B contains $MgSO_4 \cdot 7H_2O$, 5.0 g; $CaCl_2 \cdot 2H_2O$ 1.0 g; distilled water ad 1000 ml. Trypcasin is a tryptic digest of casein manufactured by Human Institute for Serobacteriological Production and Research, Budapest. The medium was sterilized at 121 °C in 500 ml Erlenmeyer flasks containing 100 ml medium.

Inoculation, growth. The microorganism was maintained on nutrient agar slants. Inoculum was made by transferring a loopful of cells into an Erlenmeyer flask, which was then shaken at room temperature for 24 h. Fermentation was carried out in Erlenmeyer flasks inoculated with 10% inoculum, shaken at 35 ° C for 20 h if not indicated otherwise.

The cells were harvested by centrifugation (angle rotor, 6000 rpm, Janetzky K 26 centrifuge, GDR). The supernatant was saved for tryptamine (metabolite) determination and the cells were washed once with 0.15 M NaCl. The washed cells were used immediately for enzyme preparation or frozen for later use.

Optical density. The optical densities of growing cultures were determined at 600 nm in a Spectromom 195 (MOM Works, Budapest) spectrophotometer. The cells were diluted to densities that did not exceed 0.25 absorbancy units. Under these condition OD followed wet cell mass in linear fashion; 6 absorbancy units were equivalent to 2 g of wet cells per 100 ml of culture.

Tryptamine and tryptophan determination in medium. Tryptamine and tryptophan were separated by using ion exchange resin. Their concentration was determined by p-dimethylaminobenzaldehyde (pDAB) reagent [8]: Amberlite CG 50 resin (Rohm and Haas, USA) was washed with hydrochloric acid, then with sodium hydroxide followed by distilled water. The resin was equilibrated with 50 mM Tris–HCl buffer pH 7.5.

One ml of the fermentation medium was let through a 0.6×3 cm column of the resin and washed with the buffer. The wash contained tryptophan. Tryptamine was eluted with 30% formid acid.

Colour reagents. (1) 6% pDAB in 3 N sulphuric acid; (2) 30 N sulphuric acid; (3) 0.1% $NaNO_2$ in water.

Colour reaction. One ml of the eluate was incubated with 0.2 ml of reagent 1 and 0.6 ml of reagent 2 at room temperature. After 60 min, 20 μl of reagent 3 was added. Colour intensity was measured after 30 min at 580 nm and compared to that of a standard tryptamine or tryptophan solution.

Assay of enzyme activity. The enzyme solution (300 μl in 50 mM imidazole buffer, pH 6.5 and 12 μl of 50 mM L-tryptophan in 100 mM NaOH, and 3 μl of 33 mM pyridoxal phosphate in 33 mM NaOH) was incubated at 37 °C for 0.5 to 2 h. The protein was precipitated and the reaction stopped by boiling for 2 min. The precipitate was separated by centrifugation, the tryptamine content of the supernatant was determined as above. Enzyme unit: μmoles of tryptamine formed/hour.

When pH dependence was studied, the enzyme was dialysed against 0.1 M NaCl.

Transformation of other aromatic amino acids. Tyrosine, phenylalanine and histidine (2 mM final concentration) were incubated with partially purified enzyme in 50 mM imidazole buffer, pH 6.5 in the presence of 0.3 mM pyridoxal phosphate as above for 2 h. After-incubation, the reaction mixture was acidified with concentrated HCl and 10 μl was applied on a Fixion 50×8 ion exchange thin layer sheet (Chinoin, Budapest). The chromatogram was developed with the following solvent: citric acid, 14.1 g; NaOH, 8.0 g; HCl (conc.), 12.3 ml; NaCl, 11.7 g; distilled water to 1000 ml [9]. The spots were visualized by spraying the sheets with ninhydrine reagent.

Results and discussion

Isolation and taxonomy. Over 600 bacterial strains isolated from soil or food were screened for tryptophan decarboxylase activity. Only four isolates produced tryptamine from tryptophan. The best producer of the four, No. 230, was characterized by standard taxonomical methods [10].

In broth, isolate No. 230 forms 3–5 μm long motile Gram-negative rods arranged in long chains. On agar, it produces endospores after 6–8 days of incubation. The spores are central and deform the body slightly.

On nutrient agar, strain No. 230 forms easily emulsifyable, irregularly edged colonies with rough surface, 3 mm in diameter. Colonies on blood agar

are similar and after several days they show haemolysis. The bacterium does not grow on eosin methylene blue agar, brilliant green agar, bismuth sulphite agar or deoxycholate citrate agar. Broth cultures are uniformly turbid and have a pellicle on the surface. No pigment is produced. Good growth at 37 °C, moderate growth at 56 °C. Grows aerobically and anaerobically. No acid during 14 days incubation in adonitol, dulcitol, inositol, inulin, raffinose, rhamnose and starch. Acid production in one day in glucose, mannose, lactose, maltose, trehalose, salicin and sucrose and delayed acid production in galactose, glycerol, mannitol, sorbitol and xylose. The d-tartrate, malonate, ornithine, indole, H_2S (thiosulphate–iron), Voges–Proskauer, oxidase, lecithinase, lipase and tryptophan deaminase tests are negative. The aesculin, Simmons' ammonium citrate, methyl red and catalase tests are positive; nitrate is reduced to nitrite, gelatin is liquefied slowly.

The data show that strain No. 230 belongs to the genus *Bacillus* but is not identical with any of the species described. Its colonies resemble that of *Bacillus cereus* and *Bacillus subtilis* but it differs from the former in position of the spore and in biochemical reactions such as lecithinase, Voges–Proskauer and behaviour in mannitol, xylose and starch, and from the latter in tolerance to 7% NaCl, in growing anaerobically and in the Voges–Proskauer and starch tests. According to biochemical reactions it resembles *Bacillus circulans* and *Bacillus polymyxa* but differs remarkably in colony morphology.

Tryptamine production by growing cultures. Figure 1 presents a typical growth curve and tryptamine production in shaken Erlenmeyer flasks in a rich medium containing 5 mM (0.1%) tryptophan. It is remarkable that in the first 18–24 h, tryptophan loss appears quantitatively in tryptamine production. Tryptamine production reaches about 1 mM, irrespective of the tryptophan concentration above 2 mM (data not shown). After 20–24 h of fermentation, the produced tryptamine begins to transform to unidentified metabolites, the tryptamine curve turns downward.

Tryptamine production at different temperatures. Growth curves and tryptamine production were studied at different temperatures (Fig. 2). Growth and tryptamine production were optimal at 35 °C. The two functions seem to run parallel.

Is tryptophan decarboxylase inducible by exogenous tryptophan? In our rich medium, about 0.5 mM of tryptophan came off with Trypcasin. In order to answer the above question, we had to destroy tryptophan in the medium. Twenty per cent Trypcasin was dissolved in 2 M HCl and heat treated at 121 °C for 30 min then neutralized with NaOH and used for preparation of medium without tryptophan. Control medium was made with the same acidic solution of Trypcasin, without heating and neutralized immediately after dissolving. Further control flasks were shaken containing treated or not treated Trypcasin medium supplemented with 5mM tryptophan. Results are shown in Table I.

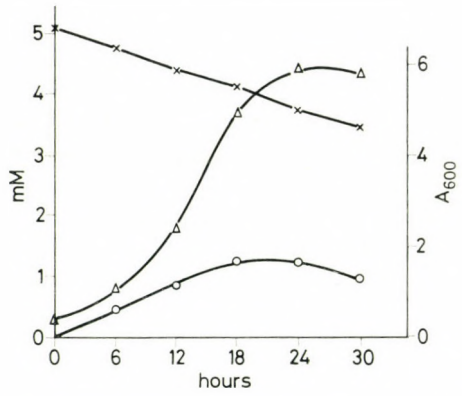

Fig. 1. Tryptamine production and growth curve of strain No. 230. Medium, absorbancy, tryptamine and tryptophan determination were as described in Methods. × — × tryptophan, ○ ——— ○ tryptamine, △ ——— △ growth

Tryptamine levels after 20 h growth reflect of course the initial tryptophan content, i.e. without tryptophan, and a very low level of tryptamine. Nevertheless, specific activities of cells grown under different conditions were essentially the same, the enzyme is not inductive.

Isolation of tryptophan decarboxylase enzyme. In preliminary experiments the enzyme proved to be very sensitive, it lost its activity rapidly upon purification. Therefore, we devised a rapid isolation method that allowed to obtain an enzyme preparation within a day. All the following steps were carried out at 4 °C.

First step. The harvested and washed wet cells were suspended in 50 mM imidazole buffer pH 6.5, containing tryptophan, EDTA, 2-mercaptoethanol,

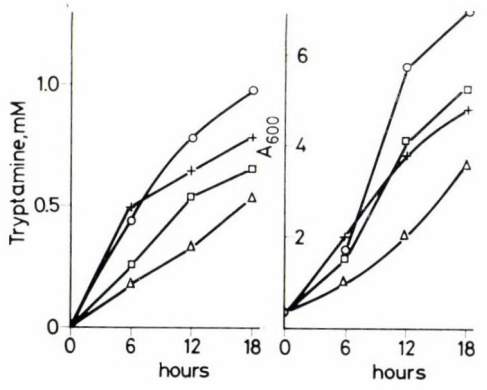

Fig. 2. Temperature dependence of tryptamine production and growth of strain No. 230. △ ——— △ 25 °C, □ ——— □ 30 °C, + ——— + 35 °C, ○ ——— ○ 40 °C

Table I

Tryptamine production and tryptophan decarboxylase levels in cells grown in the presence or absence of tryptophan, after 20 hours growth

Medium	Wet cell mass g/100 ml	Tryptamine in medium after 20 h, mM	Enzyme activity, U per mg wet cell
Acid treated Trypcasin	1.4	0.3	0.0058
Acid treated Trypcasin + tryptophan	2.1	1.07	0.0085
Untreated medium	2.0	0.8	0.0060
Untreated medium + tryptophan	2.1	1.1	0.0068

Incubation: 100 ml culture in 500 ml Erlenmeyer flasks at 37 °C. Wet cell mass was determined after centrifugation at 6000 rpm for 20 min. Enzyme assay: 20 mg of wet cells, 1 ml of 50 mM imidazole buffer pH 6.5, 40 μl of 50 mM tryptophan, 30 μl of 33 mM PLP; 37 °C, one hour.

Results of a representative experiment are shown. All experiments were done at least in triplicate. S.E.M. was 5%

5 mM each, and PLP 0.2 mM. To 1 g of wet cells, 2.5 ml buffer was used. The cell suspension was sonicated in a 100 W Ultrasound Disintegrator (MSE, London) 4 times for 5 min with appropriate cooling intervals.

Second step. The cell debris was centrifuged at 20 000 *g* for 20 min. The supernatant contained 60–70% of the total enzyme activity.

Third step. Precipitation of nucleic acid was made by 2% protamine sulphate solution. This solution was added as long as a threadlike precipitate was formed. Further addition precipitated the enzyme and caused considerable loss. Excess addition of protamine was signalled by homogeneous turbidity.

Fourth step. Centrifugation as in step 2.

Fifth step. DEAE-cellulose chromatography. DEAE-cellulose (Reanal, Budapest) equilibrated with 50 mM imidazole buffer pH 6.5 was stirred into the enzyme solution containing 0.2 mM PLP, just enough to adsorb the enzyme (2 ml per 10 g of original wet cell). After 30 min of stirring, the adsorbent was poured in a chromatography column and washed with the same buffer until the effluent contained negligible amount of protein (A_{280} less than 0.05). Elution was made with linear gradient of NaCl, reaching 0.4M. The enzyme came off as a broad peak, its maximum was at 0.25 M (Fig. 3).

Sixth step. Concentration of the protein solution. The fractions containing the peak enzyme activity were pooled, diluted and put on a small DEAE-cellulose column to adsorb the enzyme which was eluted with 50 mM imidazole buf-

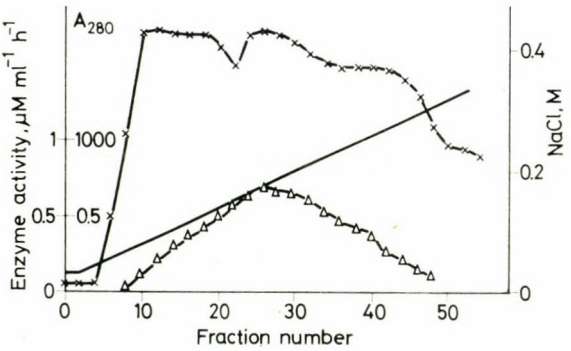

Fig. 3. DEAE-cellulose chromatography. The DEAE-cellulose column (2.3 × 7 cm) was equilibrated with 50 mM imidazole buffer containing 0.2 mM PLP. Linear gradient elution with 160 ml 0.0–0.4 M NaCl. × ——— × A_{280}, △ ——— △ enzyme activity, ——— NaCl concentration

fer containing 0.4 M NaCl. The enzyme solution obtained was about 10 times more concentrated (15–25 mg/ml) than the fractions from the 5th step. This concentration step was important because dilute enzyme lost its activity quickly. The enzyme solution was kept frozen. The yield of this procedure is shown in Table II

Properties of tryptophan decarboxylase isolated from Bacillus strain No. 230. The relative molecular mass was determined by gel filtration on a Sephadex G200 column. The result is shown in Fig. 4. The enzyme emerged in the same fractions as aldolase (M_r 148 000). Assuming that it is a globular protein, its M_r is about 150 000. The enzyme activity had its peak at pH 7–7.5 (Fig. 5) and was rapidly inactivated above 40 °C (Fig. 6).

Table II

Purification of tryptophan decarboxylase — a typical experiment

Steps	Protein		Enzyme activity			
	mg/ml	mg	U/ml	U	U/mg protein	yield %
Sonicated cell supernatant, 40 ml	22.5	900	0.54	22	0.024	100
Protamine sulphate supernatant, 41 ml	15	619	0.41	17	0.027	77
DEAE-cellulose chromatography, active fractions combined, 20.5 ml	3.5	72	0.46	9.4	0.130	43

Preparation was started with 18 g wet cells. Results of a representative experiment are shown. All experiments were done at least in triplicate. S.E.M. was 5%

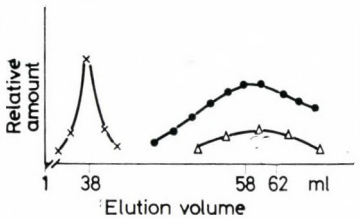

Fig. 4. Gel filtration of tryptophan decarboxylase on Sephadex G200. The reference compounds and the decarboxylase were gel-filtered on the same column (2×37 cm) after each other. Eluent: 50 mM imidazole buffer pH 6.5. × ——— × blue dextran, ● ——— ● aldolase (rabbit muscle, M_r 148 000), △ ——— △ tryptophan decarboxylase

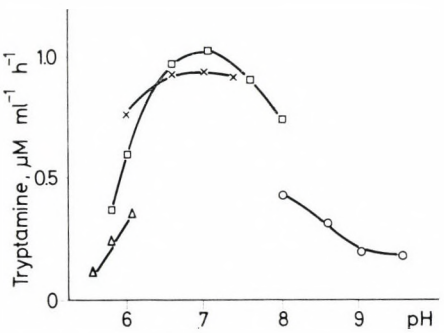

Fig. 5. pH dependence of enzyme activity. Incubation mixture contained 0.5 ml of enzyme (12 mg) dialysed against 0.1 M NaCl and 0.5 ml of 0.1 M buffer containing 10 mM tryptophan plus 0.4 mM PLP. Incubation time: one hour. Buffers: △ ——— △ sodium citrate, □ ——— □ sodium phosphate, × ——— × imidazole HCl, ○ ——— ○ ammonium chloride

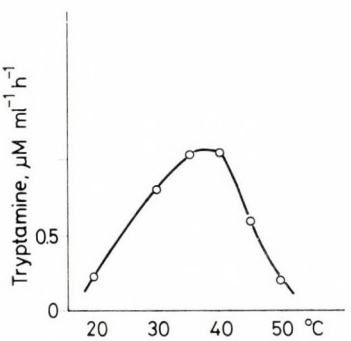

Fig. 6. Temperature dependence of enzyme activity. Incubation mixture contained in 1 ml: 12 mg of protein, 50 mM imidazole buffer pH 6.5, mM tryptophan and 0.2 mM PLP. Incubation time: one hour

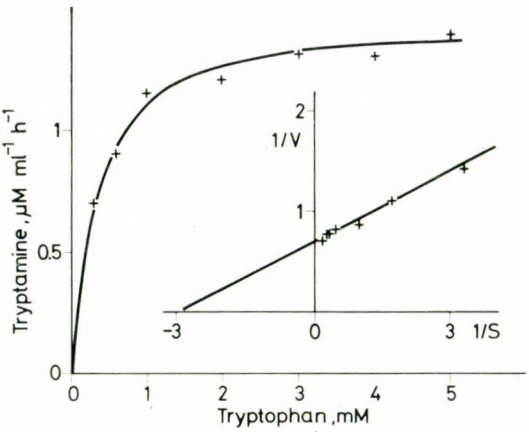

Fig. 7. Effect of substrate concentration on enzyme activity. The incubation mixture was the same as in legend to Fig. 6 except that tryptophan concentration varied from 0.3 to 5 mM

The substrate concentration versus reaction velocity is shown in Fig. 7. From the Lineweaver–Burk plot (insert) a K_m of about 0.3 mM is obtained for tryptophan.

The enzyme seems to be very specific for L-tryptophan. We could not detect any product form D-tryptophan or other aromatic amino acids like histidine, phenylalanine, tyrosine. (Detection limit was about 10% product formed.)

Pyridoxal phosphate requirement of the enzyme could be demonstrated on enzymes that were chromatographed on DEAE-cellulose several times in the absence of the cofactor. After the 3rd chromatography, the enzyme activity was enhanced 2.5-fold by 0.2 mM pyridoxal phosphate present in the enzyme assay.

Results of inhibitor experiments are listed in Table III. The effect of the carbonyl reagents KCN and phenylhydrazine indicated that there is a required carbonyl compound, most likely the pyridoxal phosphate cofactor itself. Up to now, there is only one amino acid decarboxylase known having no pyridoxal phosphate cofactor [11]; it contains covalently attached pyruvate instead.

Parachloromercury benzoate and iodoacetate inhibition pointed to (some) SH-group(s) that is (are) essential for activity. Among the pyridoxal phosphate enzymes studied, aspartate transaminase contains such a cysteinyl residue [12].

Phenylmethylsulphonyl fluoride indicates required OH-group(s) here. Glutamate-, arginine- and lysine-decarboxylases of *Escherichia coli* contain serine in the pyridoxal phosphate binding sequence of the enzyme; it is always the 4th residue from lysine towards the N-terminal [13, 14]. Our enzyme too, may contain such a sequence.

Table III

Inhibitors of tryptophan decarboxylase

	Inhibitor	Final concentration nM	Temperature °C	Residual activity %
1	KCN	5	22	18
2	Phenylhydrazine	1	22	20
3	*p*-Chloromercury benzoate	3	0	0
4	Iodoacetate	3.3	0	7
5	Phenylmethylsulphonyl fluoride	3×3	0	40
6	2,4,6-Trinitrobenzene sulphonic acid	1	22	31
7	The same in the presence of 0.2 mM PLP	1	22	50
8	Diethylpyrocarbonate	10	0	17
9	Ethanol as control	20	0	105
10	Sodium borohydride	10	22	0

Incubation of 5 mg enzyme was carried out in 300 µl of 50 nM buffer (1–6 in imidazole, pH 6.5; 7 and 8 in phosphate, pH 7) for 30 min. Gel filtration immediately on small Sephadex G25 column before enzyme assay. With each experiment, an untreated control was run to obtain the 100% value. Results of a representative experiment are shown. All experiments were done at least in triplicate. S.E.M. was 5%

Trinitrobenzene sulphonic acid may react, among other NH_2-groups, with that of the lysyl-residue responsible for pyridoxal phosphate binding; therefore, excess cofactor in the reaction mixture protects enzyme activity.

Diethylpyrocarbonate reacts exclusively with imidazole side chains at neutral pH [15]. This enzyme may contain such a functional histidyl residue as do the three mentioned *E. coli* enzymes [13, 14].

Finally, it is not surprising that sodium borohydride by reducing the Schiff base between resting cofactor-enzyme complex also effectively inhibits its activity.

Acknowledgement. We thank Mrs VERA PICHLER for skilful technical assistance.

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EPIDEMIOLOGICAL ANALYSIS OF *SALMONELLA TYPHI-MURIUM* INFECTIONS ON THE BASIS OF LABORATORY METHODS

I. DISTRIBUTION OF PHAGE TYPES AND BIOTYPES OF *SALMONELLA TYPHI-MURIUM* ISOLATED IN HUNGARY IN THE PERIOD 1960 TO 1981

HEDDA MILCH, VERA G. LÁSZLÓ and ERZSÉBET SZ. CSÓRIÁN

National Institute of Hygiene, Budapest

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Phage and biochemical types were determined of 34 937 *Salmonella typhi-murium* cultures including 31 708 human strains, 2732 animal strains and 497 strains isolated from water. Phage type 4, not typable strains (nt) and phage type 2b were predominant among the strains of human and animal origin, and nt, 4 and 2b among the strains isolated from water. The most frequent phage types and the nt strains were subdivided by biotyping and additional phages. The incidence of *S. typhi-murium* var. *copenhagen* strains was 12.4%, they belonged mainly to phage types 2b and nt. The number of *S. typhi-mirum* isolates of human origin showed a 2–4 year periodical fluctuation between 1960 and 1981. A connection was found between the incidence of the predominant phage types (4, nt, 2b) and the periodical changes in the total number of isolates. Phage type 4, which predominated among the strains of human and animal origin till 1976, was ousted gradually by nt ones. In the period when the change in predominance was observed the number of epidemics decreased and the number of sporadic cases increased. The change in the frequency of phage types took place at the same time when the frequency of phage types changed among the strains isolated from cattle and meat-products (4 → nt). The increased number of sporadic cases after 1976 refers to infections from cattle and not from poultry.

As the Hungarian National Centre of Enteric Phage Typing, participating in the International Salmonella Surveillance Programme coordinated by the World Health Organization, we determined phage and biotype of *S. typhi-murium* strains, their sensitivity to antibiotics and the R-plasmid carrier state. Results concerning phage and biotypes were reported periodically [1–5]. The present paper gives an account of phage and biotype results of 22 years (1960–1981).

Materials and methods

Bacterial strains. A total of 34 937 *S. typhi-murium* strains (31 708 human, 2 732 animal and 497 water strains) were examined for phage and biochemical types isolated between 1960 and 1981 in Hungary.

HEDDA MILCH, VERA G. LÁSZLÓ, ERZSÉBET SZ. CSÓRIÁN
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Phage typing was performed according to the Felix–Callow scheme [6] and 6 additional phages were also used: Anderson's phage 35, Lilleengen's phages L30, L31, L37 [7] and two phages, 284 and 183, isolated by us.

Biochemical typing was carried out according to Milch [3] on the basis of reactions in rhamnose and inositol. (Biochemical type, rhamnose/inositol:

$$1\ +/+^{2-5};\ 2\ +/-;\ 3\ +/+;\ 4\ -/+^{2-5};\ 5\ -/-;\ 6\ +^{8-10}/+;$$
$$7\ +^{5-8}/-;\ 8\ +^{5-8}/+.)$$

Media. Meat-extract broth and agar and Oxoid nutrient broth No. 2 were used.

Results

Table I shows the phage type distribution of *S. typhi-murium* strains isolated from patients and asymptomatic excreters in 1960–1981. Between 1960 and 1963 phage types 1a, la var. 1 and 2b were predominant and the not typable (nt) strains were also frequent. In the period 1964–1975, phage type 4 was the most common, in 1976 the ratio of phage type 4 and nt was identical and from 1977 onwards nt strains have become predominant.

Table I

Phage type distribution of S. typhi-murium strains of

Phage type \ Year	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969
1	1.8	4.3	5.3	6.5	2.0	0.9	2.1	3.0	3.6	2.6
1a	33.9	14.4	8.2	7.2	4.4	5.4	8.1	6.7	6.8	2.2
1a var. 1	14.3	22.6	19.9	20.3	8.6	12.1	1.6	8.7	3.6	4.2
1b	7.1	0.1	4.7	—	0.2	0.2	—	0.8	—	—
2	3.6	10.3	0.1	0.2	2.5	0.9	3.0	0.8	2.1	7.5
2a	—	0.3	1.0	—	0.2	0.3	1.0	0.1	—	—
2b and 2b + 5	9.0	13.7	19.7	12.4	14.0	30.9	25.1	4.5	—	—
2c	0.9	2.0	1.9	0.9	2.5	3.5	4.3	2.6	—	—
2d	—	—	—	—	—	—	0.4	0.2	—	—
3	—	0.1	—	—	—	—	—	0.6	—	—
3a	—	1.2	0.5	8.1	—	0.2	0.2	0.1	—	—
4	2.7	10.9	12.8	9.8	27.3	35.1	46.4	58.3	67.2	58.5
5	2.7	13.0	18.4	12.4	9.6	4.2	5.9	5.4	9.7	4.4
Not characteristic	—	1.6	1.4	0.5	—	—	0.7	3.4	5.4	3.2
Not typable	24.0	5.5	6.1	21.7	28.7	6.3	1.2	4.8	1.6	17.4
Number of examined strains	112	902	954	571	778	1108	1101	1086	1214	849

Among the strains isolated from animals and food-products of animal origin, phage types 1a var. 1, 1, 2b and nt were the most frequent between 1961 and 1964, similarly to human strains (Table II). Phage type 4 was predominant from 1965 to 1976, its predominance lasted one year longer than among the human strains. Phage types 4 and nt predominated alternatively between 1977 and 1981.

Strains originating from poultry belonged in the majority to phage types 4 and 2b from 1961 to 1975 and to phage types 2b, 4 and nt between 1976 and 1981. Among the strains isolated from cattle and meat products, the most frequent phage types were 1a var. 1 and 1 from 1961 to 1965, phage type 4 in 1966–1970 and nt between 1971 and 1981 (Table III).

Strains isolated from water were examined between 1973 and 1981 (Table IV). In this period, except in one year, nt strains predominated. Strains of phage type 2b, 1a, 4, 1a var. 1 were also frequent.

Figure 1 shows the total number of human *S. typhi-murium* isolates, the number of the phage typed strains and the frequency of the three most common phage types in the period between 1960 and 1981. The number of the *S. typhi-murium* isolates of human origin was observed to show a two to four year periodical fluctuation. During the ten year period between 1960 and 1969, phage type was determined from the isolations in 50–65% yearly and during the second period between 1970 and 1981 this ratio increased to 80–90%. A connection was found between the incidence of the predominant phage

human origin in percentage, in Hungary (1960–1981)

1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	Total
3.5	2.9	2.1	4.1	1.1	3.1	1.1	3.5	5.1	3.2	0.3	0.3	2.6
5.8	8.5	5.6	9.6	3.7	2.3	4.1	3.5	3.1	4.8	0.6	1.0	5.1
7.8	4.0	16.1	3.7	3.6	2.9	4.1	1.4	1.9	1.3	10.5	3.9	6.7
—	0.1	0.1	—	—	0.2	0.2	0.1	0.1	0.5	—	0.2	0.3
0.2	1.7	0.3	0.1	0.3	0.2	0.7	4.3	3.9	1.2	0.2	0.2	1.6
—	—	—	0.1	0.1	0.2	—	0.4	0.1	0.2	0.1	0.1	0.2
6.2	1.9	7.1	12.3	10.2	7.8	7.1	8.6	7.9	14.9	11.1	16.0	10.5
0.6	0.1	0.7	1.1	0.6	0.9	0.2	0.4	3.1	1.8	0.3	0.8	1.2
—	—	—	—	—	1.2	0.3	0.5	0.6	3.0	8.9	6.5	1.5
0.1	—	—	—	—	—	—	—	—	—	—	—	<0.1
—	—	0.4	0.2	—	—	0.1	0.1	0.1	0.7	1.7	1.4	0.5
54.5	67.0	52.1	44.7	57.7	49.9	37.0	29.6	16.8	26.1	19.4	29.6	39.3
11.4	6.9	3.0	2.6	2.2	2.6	3.5	8.5	3.3	4.4	1.1	2.2	5.4
6.3	3.4	5.3	7.9	5.2	1.6	4.7	3.9	1.3	0.8	0.2	—	2.9
3.6	3.5	7.2	13.6	15.3	27.1	36.9	35.2	52.7	37.1	45.6	37.8	22.1
1523	2080	1745	1923	1772	1992	1823	1921	1749	1664	2536	2305	31 708

types (4, nt, 2b) of the human and animal strains and the periodical changes in the total number of isolates (Figs 1 and 2). The incidence of the frequent phage types of strains isolated from water differed from that observed among the human and animal strains (Fig. 3).

Biochemical typing was used to subdivide phage types into 8 biotypes. Figure 4 shows the subdivision of the most frequent phage type 4 according to

Table II

Distribution of phage types of S. typhi-murium

Phage type \ Year	1961	1962	1963	1964	1965	1966	1967	1968	1969
1	3.3	40.6	47.6	—	—	—	5.0	1.5	—
1a	10.0	15.6	4.8	—	—	3.0	8.8	3.6	4.8
1a var. 1	46.7	21.9	4.8	21.1	24.5	—	10.0	—	1.6
1b	—	3.1	—	—	—	—	—	—	—
2	10.0	—	—	—	5.7	9.1	1.2	—	—
2a	—	—	—	—	—	—	—	—	—
2b and 2b + 5	—	6.3	—	36.8	22.6	45.5	2.5	0.7	—
2c	—	—	—	—	—	—	3.7	—	—
2d	—	—	—	—	—	—	—	—	—
3	3.3	—	—	—	—	—	—	—	—
3a	—	—	—	—	—	—	—	—	—
4	10.0	3.1	—	21.1	35.9	36.4	45.0	61.4	46.9
5	16.7	—	14.2	15.8	7.5	—	—	25.5	16.1
Not characteristic	—	—	—	—	1.9	6.0	8.8	4.4	—
Not typable	—	9.4	28.6	5.2	1.9	—	15.0	2.9	30.6
Number of examined strains	30	32	21	19	53	33	80	137	62

biotypes. Strains of biotype 1 were predominant from 1972 to 1974. They were ousted by biotype 3 from 1975. Strains of biotype 2 were common only between 1972 and 1974. Figure 5 shows the subdivision of not typable strains according to biotypes. The majority of nt strains were of biotype 2 in 1972, 1974, 1977; in the other years most of them fell into biotype 3.

For a better subdivision additional phages were used, because of the increasing number of nt strains in the last seven year period. Figure 6 shows the subdivision of nt biotypes by the help of six additional phages between

Table III

Phage type distribution of S. typhi-murium strains of animal origin in

Phage type	1961–1965				1966–1970			
	Poultry	Cattle	Meat-product	Hygienic examination, other animals	Poultry	Cattle	Meat-product	Hygienic examination, other animals
1	7.4	7.4	*40.0*	*33.3*	1.9	5.4	—	1.9
1a var. 1	13.6	*55.5*	—	*30.9*	3.3	4.1	—	5.7
2b	19.7	18.6	—	—	7.2	1.3	2.7	13.3
4	*29.6*	3.7	20.0	2.4	*69.3*	*44.6*	*56.0*	30.5
5	9.9	—	—	16.7	4.6	2.7	9.3	*36.2*
Not typable	6.2	—	—	14.3	1.3	28.4	8.0	7.6
Others	13.6	14.8	40.0	2.4	12.4	13.5	24.0	4.8
Number of examined strains	81	27	5	42	153	74	75	105

strains of animal origin in Hungary 1961–1981

1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	Total
4.2	2.2	6.2	3.1	4.2	—	—	—	1.6	8.1	—	—	3.6
1.1	2.2	6.7	4.6	4.2	4.6	—	2.4	1.6	3.5	—	1.6	3.2
5.3	8.5	9.5	4.6	3.9	1.5	0.9	4.0	1.3	3.5	8.5	3.2	5.3
—	—	—	—	—	—	—	—	—	0.4	—	—	0.1
—	—	1.1	—	0.3	—	—	1.6	3.1	—	—	—	1.0
—	—	—	—	—	—	—	—	—	0.4	—	—	<0.1
11.6	1.8	10.6	33.5	6.6	12.1	24.2	16.8	10.4	26.2	21.6	17.7	14.3
—	—	—	—	—	—	—	—	10.6	—	—	—	1.6
—	—	—	—	—	—	2.1	6.4	—	2.3	5.7	6.5	1.1
2.1	—	—	—	0.9	—	—	—	—	—	—	—	0.2
—	—	1.1	—	—	—	—	—	0.2	—	3.8	4.9	0.3
56.8	67.7	51.4	39.2	42.1	69.7	36.7	19.2	15.1	19.3	16.1	35.5	36.8
10.5	2.2	2.8	—	0.9	—	2.2	1.6	2.1	0.4	1.9	1.6	3.6
6.3	3.5	2.8	3.6	5.1	—	5.5	20.0	1.3	—	0.9	—	3.8
2.1	11.9	7.8	11.4	31.8	12.1	28.4	28.0	52.7	35.9	41.5	29.0	25.0
95	226	179	194	333	66	235	125	385	259	106	62	2732

1979 and 1981. The subtypes determined with additional phages provided valuable results for epidemiological examinations.

Out of the 9254 *S. typhi-murium* strains examined from 1978 to 1981, 1145 strains (i. e. 12.4%) belonged to *S. typhi-murium* var. *copenhagen* (human strains in 12.1%, animal strains in 14.2%, water strains in 14.9%). Tables V, VI and VII demonstrate the subdivision of *S. typhi-murium* var. *copenhagen* strains according to phage and biotypes (1978/1981). Strains of human and animal origin belonged in the majority to phage type 2b and nt, strains isolated

percentage according to the sources of isolation Hungary, 1961–1981

1971–1975				1976–1981			
Poultry	Cattle	Meatproduct	Hygienic examination, other animals	Poultry	Cattle	Meatproduct	Hygienic examination, other animals
0.7	11.1	7.5	3.7	0.2	2.5	10.2	0.8
4.4	5.5	13.1	6.4	2.8	5.0	3.2	1.7
13.7	3.5	6.5	16.4	*28.3*	9.2	5.7	13.2
71.4	29.8	25.2	27.7	*27.0*	13.4	7.6	23.4
0.7	2.1	0.9	1.4	2.0	2.5	1.9	0.6
4.4	*38.3*	*31.8*	29.4	23.3	*59.8*	*61.2*	47.1
4.7	9.7	15.0	15.0	16.4	7.6	10.2	13.2
527	144	107	220	541	119	157	355

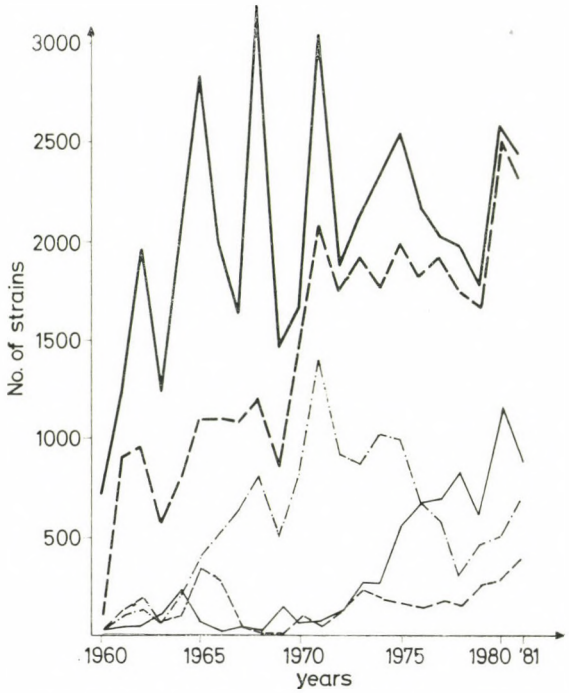

Fig. 1. Incidence of human *S. typhi-murium* strains and distribution of the frequent phage types (1960–1981). ——— total number of isolates; — — number of phage typed strains; — — — — — strains of phage type 2b; — . — . — . — strains of phage type 4; ——— not typable strains

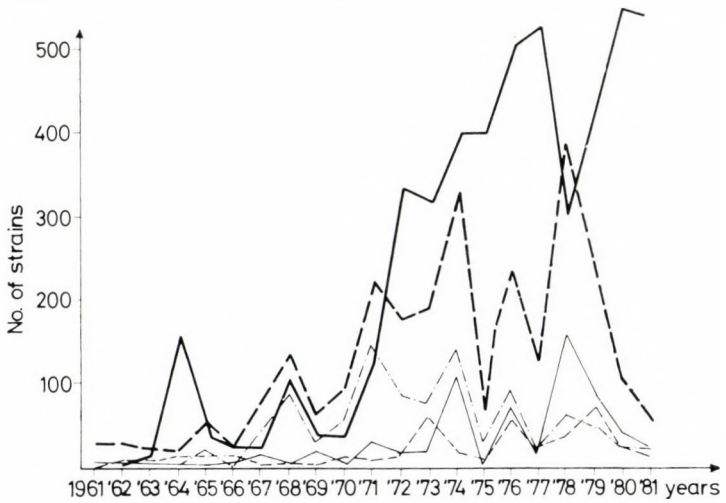

Fig. 2. Incidence of animal *S. typhi-murium* strains and distribution of the frequent phage types (1961–1981). ——— total number of isolates; — — number of phage typed strains; — — — — — strains of phage type 2b; — . — . — . — strains of phage type 4; ——— not typable strains

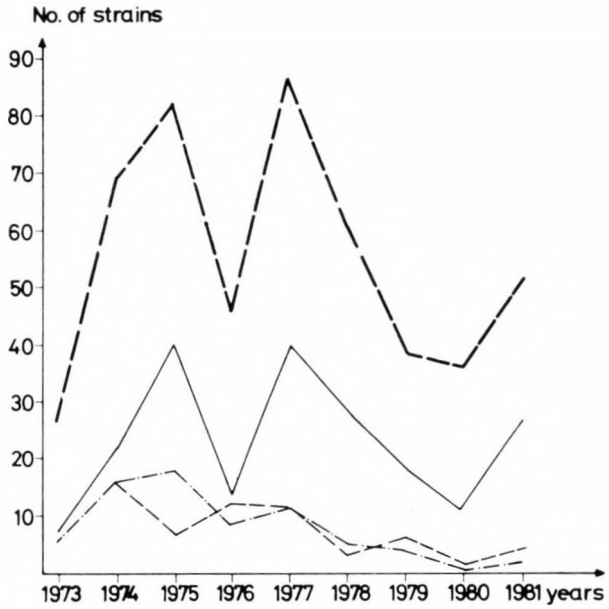

Fig. 3. Distri bution of frequent phage types of *S. typhi-murium* strains originating from water (1973–1981). ▬▬ number of phage typed strains; — — — — — strains of phage type 2b; — . — . — . — strains of phage type 4; ——— not typable strains

from water were nt. Biotype 5 was frequent among the *S. typhi-murium* var. *copenhagen* strains of phage type 2b, otherwise phage type distribution according to biotypes was similar to that observed among *S. typhi-murium* strains.

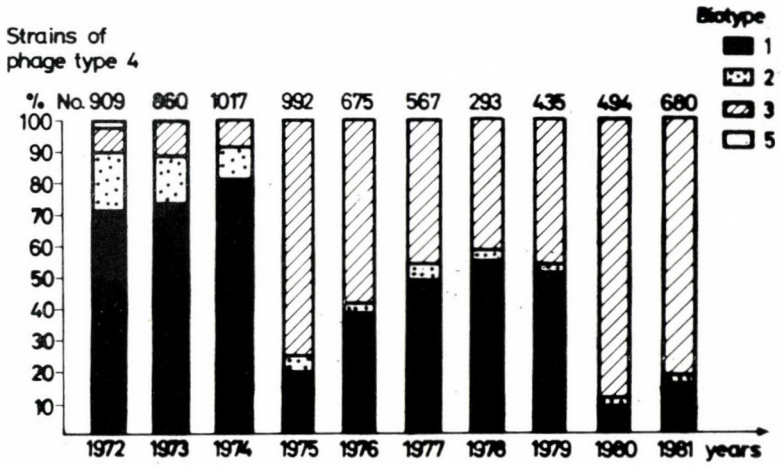

Fig. 4. Subdivision of human *S. typhi-murium* strains of phage type 4 according to biotypes (1972–1981)

Table IV

Phage type distribution of S. typhi-murium strains isolated from water in Hungary, 1973–1981

Phage type \ Year	1973	1974	1975	1976	1977	1978	1979	1980	1981	Total
1	—	2.9	6.2	2.2	3.5	4.9	5.1	—	2.0	3.4
1a	25.9	2.9	5.0	4.3	8.1	6.6	—	—	—	5.2
1a var. 1	—	5.8	2.4	4.3	—	8.2	7.7	19.4	7.7	5.4
1b	—	—	—	2.2	—	—	—	—	—	0.2
2	—	—	—	—	1.2	6.6	2.5	2.8	—	1.4
2a	—	—	—	—	3.5	—	—	—	—	0.6
2b	25.9	23.2	7.4	26.1	12.8	4.9	15.4	2.8	17.3	14.3
2c	—	—	—	—	—	11.4	—	2.8	—	1.6
2d	—	—	3.7	—	1.2	—	2.5	—	7.7	1.8
3a	—	—	—	—	—	—	—	11.1	5.6	1.4
4	22.3	23.2	22.3	17.4	12.7	8.2	10.3	2.8	7.7	14.7
5	—	5.8	—	13.0	3.5	—	—	—	—	2.6
Not characteristic	—	4.3	3.7	—	7.0	—	—	—	—	2.4
Not typable	25.9	31.9	49.3	30.5	46.5	49.2	56.5	58.3	52.0	45.0
Number of examined strains	27	69	81	46	86	61	39	36	52	497

Table V

Phage type distribution of human S. typhi-murium var. copenhagen strains in Hungary 1978–1981

Phage type \ Year	1978	1979	1980	1981	Total
1	5	2	—	1	8
1a	5	3	1	1	10
1a var. 1	2	4	7	12	25
2	7	—	—	—	7
2b	48	85	108	188	429
2c	—	—	—	1	1
2d	2	5	17	9	33
3a	1	10	13	20	44
4	9	9	9	12	39
5	1	30	3	1	35
Not characteristic	13	3	2	—	18
Not typable	67	115	57	113	352
Total	160	226	217	358	1001

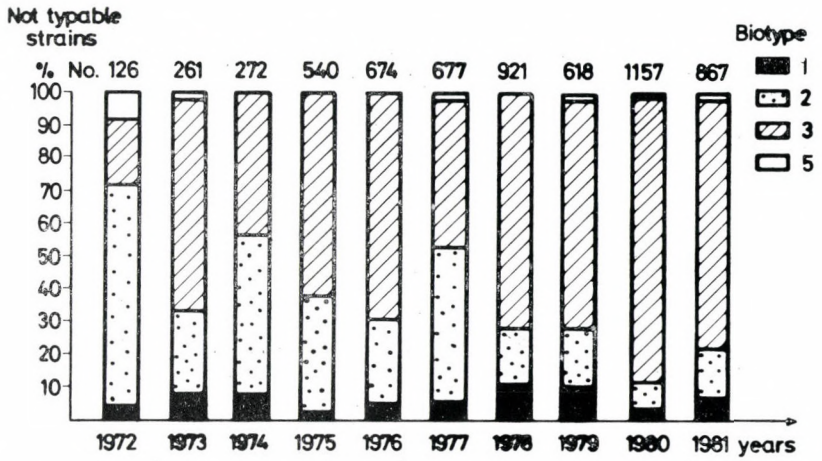

Fig. 5. Subdivision of human, not typable *S. typhi-murium* strains according to biotypes (1972–1981)

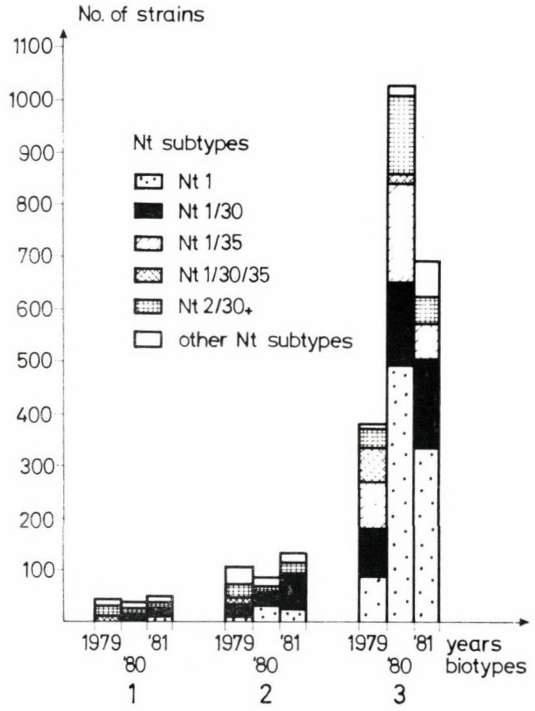

Fig. 6. Subdivision of human, not typable *S. typhi-murium* biotypes by additional phages (1979–1981)

Table VI

Phage type distribution of S. typhi-murium var. copenhagen strains of animal origin in Hungary, 1978–1981

Phage type \ Year	1978	1979	1980	1981	Total
1	1	3	—	—	4
1a var. 1	—	—	—	2	2
2b	4	18	12	5	39
2c	10	—	—	—	10
2d	—	—	—	1	1
3a	—	—	1	1	2
4	1	2	1	2	6
Not typable	38	9	4	1	52
Total	54	32	18	12	116

Table VII

Phage type distribution of S. typhi-murium var. copenhagen strains isolated from water in Hungary, 1978–1981

Phage type \ Year	1978	1979	1980	1981	Total
1a var. 1	—	—	2	—	2
2	1	1	—	—	2
2b	—	2	—	1	3
2d	—	—	1	1	2
3a	—	—	2	3	5
Not typable	1	5	6	2	14
Total	2	8	11	7	28

Discussion

On the basis of the data collected by the bacteriological laboratories of the Public Health Stations, since 1960 *S. typhi-murium* was the most frequent in each year but 1980. According to the report of the Hungarian National Salmonella Centre [4] the number of different kinds of serotypes isolated in one year was between 66 and 75 and *S. typhi-murium* was the most common between 1972 and 1976. Therefore, it was needed to subdivide first this serotype for epidemiological purposes by means of phage and biochemical typing. Yearly analysis of the isolates from epidemics and sporadic cases revealed that between 1960 and 1975 the highest points of the cycles were caused by epidemics and the lower periods were due to sporadic cases. From 1976 onwards spo-

radic cases amounted to 65–68% of the isolations. For this epidemiological observation an explanation was given by studying the changes in the predominance of phage types. The change in the predominance of phage types occurred in 1976 (phage type 4 was ousted by type nt); during this period the number of sporadic cases increased gradually without the development of significant epidemics.

Among the animal strains the isolates from poultry belonged mainly to phage type 4 between 1961 and 1981, those isolated from cattle were in the majority of phage type 1a var. 1, 1 and 4 between 1961 and 1970, and nt from 1971 to 1981. The change in the predominance of phage types observed among the human strains (4 → nt) probably refers to the contamination from cattle.

It is difficult to compare phage types found in Hungary and those reported from other countries, because different methods are used in different countries. The first *S. typhi-murium* phage typing scheme was elaborated by Felix and Callow [6]. Their method consisted first of 11 phages, later of 14 and 29 phages [7] and the strains were subdivided into 12, 17 and 34 phage types, respectively. Anderson et al. [8] reported a scheme according to which 207 phage types were distinguished with 34 phages. After this scheme another, completed variation was used, but it has not been published [9]. Lilleengen [10] described a method, which consists of 27 types using 12 phages. The method reported by Scholtens [11] and Guinée et al. [12] was used in the Netherlands. Kühn et al. [13] published their epidemiological observations and phage typing results using the same method as we did in the same period. Among human strains the frequent phage types were: 2d (1968/69), 4, 2 var. 1, 2b, 1a and nt. Among the animal strains isolated from poultry and cattle the common phage types were 2 var. 1 and 4. According to data from the German Democratic Republic, there in 1981 the nt strains were the most frequent, and using Felix–Callow's typing method, more than 80% of the strains belonged to this group. Therefore, Lilleengen's phage types were also used [14]. Lalko and Buczowski [15] also subdivided Felix–Callow's [6] phage types using Lilleengen's scheme [10]. Brandis et al. [16] found that the strains were not typable or not characteristic in 64.2%, using Anderson's type-phages in the German Federal Republic in 1971.

S. typhi-murium var. *copenhagen* strains examined in Hungary between 1978 and 1981 were isolated from poultry, cattle, pigeons and rabbits.

Phage and biotype results of *S. typhi-murium* strains were completed with determination of antibiotic resistance patterns and characterization of R-plasmids; this will be reported in another paper.

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EPIDEMIOLOGICAL ANALYSIS OF *SALMONELLA TYPHI-MURIUM* INFECTIONS ON THE BASIS OF LABORATORY METHODS

II. RESISTANCE TO ANTIBIOTICS AND R-PLASMID CARRIER STATE IN *SALMONELLA TYPHI-MURIUM* ISOLATED IN HUNGARY IN THE PERIOD 1974 TO 1981*

HEDDA MILCH, VERA G. LÁSZLÓ and S. NIKOLNIKOV

National Institute of Hygiene, Budapest

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Phage and biochemical typing of *Salmonella typhi-murium* strains performed in the course of the Salmonella Surveillance Programme were completed with examinations on resistance to antibiotics and R-plasmids. A total of 15 600 strains of human, animal and water origin were tested between 1975 and 1981 and most of the monoresistant strains were found among the animal strains (73.6%–94.9%), while double resistance was the most frequent among the human strains (5.5%–25.5%) and multiresistance occurred in the highest ratio (2.8%–25.6%) among the strains of water origin. Tetracycline resistance was the most frequent in all the three materials. The curves representing the incidence of tetracycline (Tc), chloramphenicol (Cm), streptomycin (Sm), kanamycin (Km) resistance were similar to the curve of multiple resistance and differed from the curve showing the incidence of ampicillin (Ap) resistance. Gentamicin resistance was found only among human strains (in 0.3 and 0.7%), strains resistant to nitrofurantoin and co-trimoxazole occurred among strains of human and water origin in low percentages. The most common antibiotic resistance patterns of the multiple resistant strains were Tc, Cm, Sm, Km, Ap, Su; Tc, Sm, Su; Sm, Km, Ap, Su. Multiple resistant strains belonged in the majority to phage type nt (not typable) and 2b. Out of the examined 512 *S. typhi-murium* strains resistant to antibiotics, the presence of R-plasmid was demonstrated in 408 strains (i.e. 79.7%). The R-plasmids, derived from strains of human, animal and water origin, of phage type nt, biotype 3 (nt/3) isolated in 1979, were characterized according to the resistance determinants, *fi*-character, incompatibility-group, phage-inhibition and molecular weight. Two kinds of R-plasmids were carried by three human strains (FI and H, FI and α). R-plasmids belonging to Inc P and Inc H were carried by one animal strain. Strains isolated from sewage carried R-plasmids of Inc groups H and Iα. Out of the examined 15 *S. typhi-murium* strains of phage type 2b, isolated in 1981, the molecular weight for 7 strains was 66 Md and four belonged to Inc Iα. The R-plasmids derived from 2b/2 strains, isolated in the same county, were identical according to antibiotic resistance determinants, phage inhibition and molecular weight. The molecular weights of R-plasmids derived from 10 strains out of the examined 30 nt strains were also 66 Md and the four examined plasmids belonged also to Inc Iα. Out of the examined 15 2b and 30 nt strains *S. typhi-murium* var. *copenhagen* were 12 and 9 strains, respectively. Plasmid characterization completed with determination of phage-inhibition effect gave a further possibility to trace plasmids epidemiologically.

The Salmonella Surveillance Programme coordinated by the World Health Organization started in Hungary in 1972 [1]. Besides phage and biotyp-

HEDDA MILCH, VERA G. LÁSZLÓ, SERGEI NIKOLNIKOV
National Institute of Hygiene,
H-1966 Budapest, P.O.B. 64, Hungary

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ing reported previously [2] it was important to determine the incidence of antibiotic resistance and R-plasmids and the characterization of R-plasmids. The present paper gives an account of our studies on antibiotic resistance of *S. typhi-murium* strains isolated in Hungary during 7 years, the incidence of R-plasmids and their characterization.

Materials and methods

Strains. A total of 15 600 *S. typhi-murium* strains, 13 974 human strains, 1 230 animal strains and 396 strains isolated from water, were tested for sensitivity to antibiotics. The strains were isolated in the epidemiological-bacteriological laboratories of 20 regional Public Health Stations and were sent to the Phage Research Department of the National Institute of Hygiene for phage typing.

Antibiotic sensitivity was determined to tetracycline (Tc), chloramphenicol (Cm), streptomycin (Sm), kanamycin (Km), neomycin (Nm), ampicillin (Ap), sulphadimidine (Su), nalidixic acid (Nal) by the use of "Resistest" disks (Human Institute for Serobacteriological Production and Research, Budapest); in the years 1980 and 1981 sulphadimidine was not used and additionally gentamicin (Gm), co-trimoxazole (Cot) and nitrofurantoin (Ft) were applied.

Media. To determine phage-restriction, meat-extract broth and agar (Oxoid nutrient broth No. 2) were used. For R-plasmid transfer antibiotic containing agar (Cm, Tc, Km: 20 μg/ml; Sm, Ap: 30 μg/ml; rifampicin (rif): 250 μg/ml; Nal: 50 μg/ml), and for antibiotic sensitivity test Mueller–Hinton agar was used.

R-plasmid transfer was carried out by the broth method: the 2 h broth cultures of the donor and recipient strains were mixed 1 : 1 and added to 4.5 ml broth, after overnight incubation at 37 °C diluted and 0.1 ml quantities plated on selective media and incubated at 37 °C for 17 h. The colonies were subcultured on selective agar plates.

Recipient strain. Escherichia coli J5-3 $F^-R^-rif^r$.

Determination of fi character. R-plasmid was transferred to *E. coli* K12 HfrH nal^r strain and the HfrH R^+ culture was tested for visible lysis on plating with male specific phages Ms2 and f2 by spot test.

Incompatibility groups were determined as described in [3–5].

To determine phage inhibition/restriction the following phages were used: T1–T7, Φ 2 [6] and the *E. coli* type phages 2, 3, 4, 6, 7, 12, 14, 15, 16, 17, 18, 23, 24 [7].

Plasmid molecular weight was determined according to Birnboim and Doly [8], in agarose-gel electrophoresis. As standard plasmids S-a (Inc W) 23 Md; RN3 (Inc N) 33 Md; R1 (Inc FII) 62 Md; R40a (Inc C) 96 Md; Tp116 (Inc H) 143.7 Md; R478 (Inc S) 166 Md were used.

Results

Sensitivity to antibiotics. Figure 1 shows the results of antibiotic sensitivity tests of *S. typhi-murium* strains of human, animal and water origin isolated between 1975 and 1981. Monoresistance varied among the human strains between 61.4% and 84.3%, among the animal strains between 73.6% and 94.9%, and among the strains isolated from water between 62.8% and 80.8%. The rate of double resistance was 5.5%–25.5%, 0.9%–20.0% and 2.8% and 19.7%, respectively. The incidence of resistance to three or more antibiotics was 4.4%–11.9% among human strains, 1.7%–9.7% among animal strains and 2.8%–25.6% among the strains of water origin. Predominance of monoresistance was observed among animal strains, of double resistance among human strains and of multiple resistance among the strains of water origin.

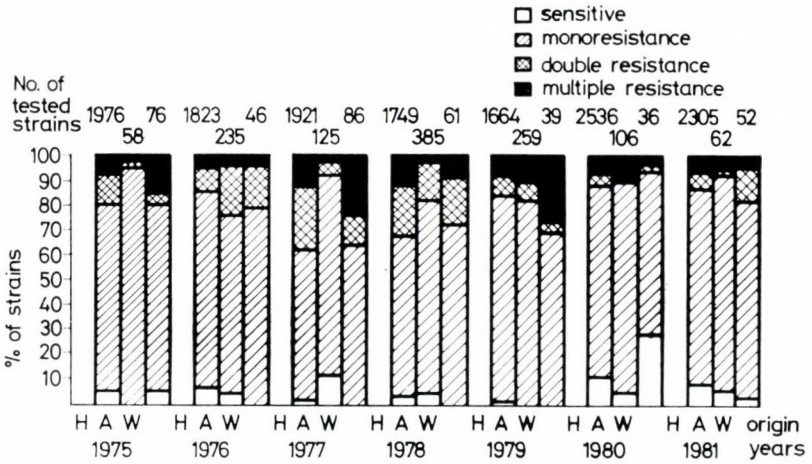

Fig. 1. Antibiotic resistance of *S. typhi-murium* strains isolated in the period 1975–1981. H = strains of human origin; A = strains of animal origin; W = strains of water origin

The incidence of resistance to single antibiotics among human, animal and water strains is given in percentages in Figs 2a–2e. Figure 2/a shows the curve of Tc, 2/b:Cm, 2/c:Sm, 2/d:Km and 2/e:Ap resistance. Tc resistance was the most frequent in all the three materials. Among the human strains it varied between 7.5% and 20.0% and was followed by Sm, Ap, Cm and Km (Nm) resistances. Among the animal strains Tc resistance varied between 0 and 9.6% and the order of frequency of the antibiotics was the following: Sm, Ap, Km (Nm) and Cm. The incidence of Tc resistance was the highest among the strains originating from water (3.8%–28.2%), Tc resistance was followed by Sm, Cm, Km (Nm) and Ap resistance. The curves representing the frequency of Tc, Cm, Sm and Km resistances are similar between 1975 and 1981, but differ from the Ap resistance curve. Among the human strains Tc, Cm, Sm, Km resistance showed the highest value in 1978, Ap resistance in 1975. Among the animal strains the frequency of Tc resistance was the highest in 1978, that of Cm resistance in 1977, 1979, 1980, of Sm resistance in 1978, of Km resistance in 1977 and 1979, and of Ap resistance in 1976. Among the strains isolated from water the peak frequency of resistance was reached in 1977 and 1979 with all of the examined antibiotics. Resistance to gentamicin, co-trimoxazole and nitrofurantoin occurred among human strains in 1980 (0.7%, 1.4%, 2.8%, respectively). In the year 1981, among human strains Gm resistance was found in 0.3%, Cot resistance in 0.4%, Ft resistance in 9.0%; among strains of water origin Cot and Ft resistance was found in 3.9% and 1.9%, respectively.

Figure 3 shows the incidence of multiple resistant strains according to their origin. The curve of multiple resistance and the curves of Tc, Cm, Sm, Km

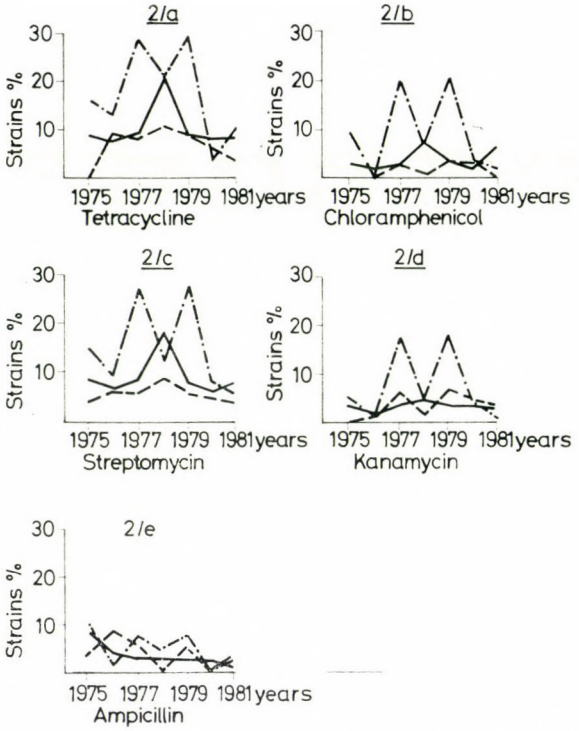

Fig. 2. Resistance to antibiotics of *S. typhi-murium* strains. ——— strains of human origin; — — — — strains of animal origin; —.—.—.— strains of water origin

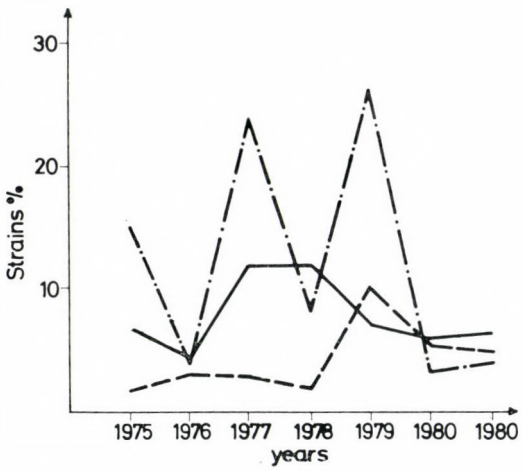

Fig. 3. Multiple resistant *S. typhi-murium* strains. ——— strains of human origin; — — — strains of animal origin; —.—.— strains of water origin

resistance are similar, but differ from the Ap-resistance curve. The rate of multiple resistance was the highest among the human strains in 1977 and 1978, among the animal strains in 1979 and among the water strains in 1977 and 1979. The most common antibiotic resistance patterns among the multiple resistant strains were, between 1975 and 1981: Tc, Cm, Sm, Km, Ap, Su; Tc, Sm, Su; Sm, Km, Ap, Su. The most common phage types among multiple resistant strains, during the same period were nt, 2b, 4 and 5. In the year 1981, multiple resistance was the most frequent among 2b and nt strains (11.7% and 8.9%, respectively), among the other phage types multiple resistance was found only in 2.5%.

Occurrence of R-plasmids. A total of 512 resistant *S. typhi-murium* strains isolated between 1976 and 1981 were examined for the presence of R-plasmids. R-plasmids were demonstrated from 408 strains (79.7%). All of the 120 resistant strains, examined in 1980 and 1981, were R-plasmid carriers. The crosses were carried out without mobilization. Out of the 408 R^+ strains 356 strains were of human, 36 of animal and 16 of water origin. The R^+ strains belonged in 78.9% to phage types nt, 2b and 5 (52.7%, 14.7% and 11.5%, respectively).

Characterization of R-plasmids. R-plasmids carried by nt and 2b strains were characterized, because most of the multiple resistant and R-plasmid carrier strains examined between 1979 and 1981 belonged to these phage types.

Table I

Characterization of R-plasmids carried by human S. typhi-murium strains of phage type nt/biotype 3, isolated in 1979

Host strain		R-plasmid					
Designation	Resistance determinant	Molecular weight, Md	Inc group	Fi-character	Phage inhibition of *E. coli* phages		
					Φ2	T-phages	type phages
1*	Tc, Cm	98	FI	+	+	1, 4, 5	14, 23, 24
2a*	Km	78	FI	+	+	1, 4, 5	12, 14, 16, 23, 24
2b*	Tc, Cm	98	H	—	—	1, 4	23
3a	Tc, Cm, Sm	.	FI	+	+	1, 4, 5	2, 3, 7, 12, 14, 23, 24
3b	Tc, Cm	98	H	+	+	1, 4	14, 23, 24
4a	Km	.	FI	+	+	1, 4, 5	2, 14, 23, 24
4b	Tc, Cm	92	Iα	—	+	1, 4, 5	2, 14, 23, 24
5	Km, Ap	68	Iδ	—	—	4	4, 14, 15, 16
6	Tc, Cm, Km	127	.	—	—	—	—
7	Tc, Cm, Sm, Km	133	H	—	—	—	—
8	Tc, Cm, Sm	141	H	—	—	—	—

* The host strain was *S. typhi-murium* var. *copenhagen*
. = not examined

R-plasmids, derived from antibiotic resistant strains of phage/biotype nt/3, isolated from sporadic human cases, examined and characterized in 1979 are listed in Table I. Out of the four human strains belonging to the same phage and biotype, two kinds of R-plasmid were carried by three strains. Plasmids of Inc FI and Inc H were carried by two strains and plasmids of Inc FI and I α were carried by one strain, and one of the strains was *S. typhi-murium* var. *copenhagen*. Plasmids belonging to Inc FI and Inc H differed in respect of phage inhibiting effect and molecular weight.

Four plasmids, derived from strains isolated from foodstuffs of animal origin were characterized. Two kinds of R-plasmid, Inc P (Km) and Inc H (Tc, Cm) were harboured by one strain isolated from sausage. Single plasmids belonging to Inc I α, Iω and W were carried by the other strains isolated from pork. Two R-plasmids, derived from two strains isolated from sewage, were of Inc H and I α, respectively (Table II).

R-plasmids, carried by 45 *S. typhi-murium* strains of phage/biotype nt/2, nt/3, 2b/2 isolated in 1981, were examined. Table III shows the characterization of the R-plasmids carried by the strains of phage type 2b and the data of the host strains. All plasmids were characterized on the basis of *fi*-property, phage-inhibition and molecular weight. Among the 15 examined phage type 2b strains, 7 carried R-plasmids with a molecular weight of 66 Mdal. By these R-plasmids Tc; Tc, Km; Tc, Ap and Ap resistances were determined and four belonged to Inc group I α. R-plasmids of 66 Mdal determining Tc and Ap resistances had no phage-inhibiting effect. The two R-plasmids carrying Tc, Km resistance determinants, had originated from the same county and inhib-

Table II

Characterization of R-plasmids carried by S. typhi-murium strains of phage type nt/biotype 3, of animal and water origin, isolated in 1979

Host strain		R-plasmid					
Designation	Resistance determinant	Molecular weight, Md	Inc group	Fi-character	Phage inhibition of *E. coli* phages		
					$\Phi 2$	T-phages	type phages
9 A	Tc	72	Iα	—	+	4	4, 14, 15, 16, 23
10 A	Tc, Cm, Km	112	Iω	—	+	—	—
11a A	Km	62	P	—	+	1, 3, 4, 5	23, 24
11b A	Tc, Km	115	H	—	—	—	—
12 A	Cm, Ap	.	W	—	+	1, 4, 5	14,16, 23
13 W	Tc, Cm, Km	118	H	—	+	—	—
14 W	Tc, Cm	90	Iα	—	+	1, 4, 5	14, 23, 24

A = strains of animal origin
W = strains of water origin
. = not examined

Table III

Characterization of R-plasmids carried by S. typhi-murium strains of phage type 2b, isolated in 1981

Host strain			R-plasmid					
Bio-type	Serovariant	Place of isolation	Desig-nation	Molecular weight, Md	Resistance determinant	Phage inhibition of *E. coli* phages		
						02	T-phages	type phages
1	—	County Pest	1	62	Tc	+	3	14, 15, 16
2	—	County Tolna	2	62	Tc, Cm	—	—	—
2	copenhagen	County Veszprém	3	56	Tc	—	—	—
2	copenhagen	County Heves	4	66	Tc	—	—	—
2	copenhagen	Budapest X.	5*	66	Ap	—	—	—
2	copenhagen	County Vas	6*	66	Ap	—	—	—
2	copenhagen	County Veszprém	7*	66	Ap	—	—	—
2	copenhagen	County Szolnok	8*	66	Tc, Km	+	3	14, 15, 16
2	copenhagen	County Szolnok	9	66	Tc, Km	+	3	14, 15, 16
2	copenhagen	County Zala	10	70	Tc	—	—	—
2	copenhagen	County Zala	11	70	Tc	—	—	—
2	copenhagen	County Baranya	12	100	Tc, Km	—	1, 5	14, 23, 24
2	copenhagen	County Baranya	13	100	Tc, Km	—	1, 5	14, 23, 24
2	copenhagen	County Somogy	14	126	Tc, Km	—	1, 5	14, 23, 24
3	—	Budapest X.	15	66	Tc, Ap	—	—	—

* Inc Iα

ited phages T3, 14, 15 and 16. The R-plasmids, harboured by 2b/2 strains, isolated from the same county (Nos 8, 9 and Nos 12, 13) were identical in respect of resistance determinants, phage-inhibiting property and molecular weight. The strains of phage type 2b carrying R-plasmids weighing 66 Mdal were *S. typhi-murium* var. *copenhagen*, except one.

Table IV shows the plasmids of nt strains, the majority of which belonged to biotype 3. Out of the examined 30 strains, 10 strains carried R-plasmids weighing 66 Mdal and their resistance determinants were Ap, Tc; Km, Ap; they were similar to those observed frequently among the 2b strains. According to incompatibility grouping, the four plasmids of 66 Mdal molecular weight also belonged to Inc I α. Out of the 10 nt strains carrying R-plasmids of 66 Mdal, only three were var. *copenhagen*. Out of the 10 R-plasmids weighing 66 Mdal (in four cases I α), 7 did not inhibit phages.

In four cases the R-plasmids carried by pairs of strains, originated from the same area, and were identical according to phage inhibition and molecular weight: 66 Mdal (Ap; Ap, Km) from county Veszprém (Nos 27, 28); 72 Mdal (Tc, Ap; Tc, Cm, Ap) from Budapest (Nos 30, 31); 86 Mdal (Sm, Ap; Tc, Cm,

Table IV

Characterization of R-plasmids carried by not typable S. typhi-murium strains isolated in 1981

Host strain			R-plasmid					
Bio-type	Serovariant	Place of isolation	Desig-nation	Mole-cular weight/ Md	Resistance determinant	Phage inhibition of *E. coli* phages		
						$\Phi 2$	T-phages	type-phages
1	—	County Szabolcs	16	66	Ap	—	—	—
1	copenhagen	County Hajdú-Bihar	17	90	Tc	—	5	14, 23
2	—	Budapest IX.	18*	66	Km, Ap	—	—	—
2	copenhagen	County Heves	19*	66	Ap	—	—	—
2	copenhagen	County Pest	20	72	Tc, Cm	—	1, 5	14,23
2	copenhagen	County Fejér	21	90	Ap	—	—	12
2	copenhagen	County Fejér	22	90	Tc, Ap	—	—	—
2	copenhagen	County Szabolcs	23	92	Ap	—	—	—
2	copenhagen	County Szabolcs	24	132	Ap	—	—	—
3	—	County Békés	25	66	Km, Ap	+	3	—
3	—	County Baranya	26	66	Tc	—	—	—
3	—	County Veszprém	27	66	Km, Ap	—	—	—
3	—	County Veszprém	28*	66	Ap	—	—	—
3	—	County Veszprém	29	66	Ap	+	1, 3, 5	16, 23
3	—	Budapest II.	30	72	Tc, Ap	+	1, 3, 5	14, 23, 24
3	—	Budapest V.	31	72	Tc, Cm, Ap	+	1, 3, 5	14, 18, 23 24
3	—	County Hajdú-Bihar	32	72	Tc, Cm, Km	—	—	—
3	—	County Veszprém	33	80	Tc, Ap	—	—	—
3	—	County Borsod	34	86	Tc, Cm, Ap	+	1, 3, 5	14, 23, 24
3	—	Budapest XIII.	35	86	Sm, Ap	—	1, 3, 5	14, 23, 24
3	—	Budapest XIII.	36*	86	Tc, Cm, Km, Ap	+	1, 3, 5	14, 23, 24
3	—	County Veszprém	37*	86	Km, Ap	—	—	—
3	—	County Komárom	38	90	Ap	—	1, 5	14, 15, 24
3	—	Budapest XV.	39	96	Tc, Cm	—	—	—
3	—	County Szabolcs	40	96	Tc, Cm, Km	—	—	—
3	—	County Békés	41	96	Tc	—	—	—
3	—	County Pest	42	138	Tc, Cm	+	3	14, 15, 16
3	—	County Pest	43	138	Tc, Cm	+	3	14, 15, 16
3	copenhagen	County Pest	44*	66	Ap	—	—	—
3	copenhagen	County Csongrád	45	66	Ap	—	—	12

* Inc Iα

Km, Ap) from Budapest (Nos 35, 36), and 138 Mdal (Tc, Cm) from county Pest (No. 42, 43).

Table V summarizes the distribution of the determined and characterized R-plasmids according to molecular weight and phage inhibition. On this basis, 63 R-plasmids were characterized and 27 according to incompatibility groups. Certain Inc groups could be subdivided into 1–8 groups by the help of phage inhibiting property.

Table V

Distribution of S. typhi-murium R-plasmids on the basis of Inc group, molecular weight and phage inhibition

Inc group	Md (No. of R-plasmids)	No. of groups, on basis of phage inhibition	Total number of R-plasmids
FI	78 (1), 98 (1), ne (2)	4	4
Iα	66 (8), 72 (1), 86 (2), 90 (1), 92 (1)	5	13
Iδ	68 (1)	1	1
Iω	112 (1)	1	1
H	98 (2), 115 (1), 118 (1), 133 (1), 141 (1)	3	6
P	62 (1)	1	1
W	ne (1)	1	1
ne	56 (1), 62 (2), 66 (9), 70 (2), 72 (4), 80 (1), 86 (2), 90 (4), 92 (1), 96 (3), 100 (2), 126 (1), 127 (1), 132 (1), 138 (2)	8	36
Total			63

ne = not examined

Discussion

Comparing the results of the frequency of antibiotic resistance to those reported from other countries, it was found that the rate of antibiotic resistant strains isolated in Hungary was lower than those isolated earlier (1959–1969) in England and the Netherlands. Anderson reported that the rate of resistant strains increased from 2.9% to 61% between 1961 and 1965 [9] and Guinée, too found [10] that the tetracycline resistance of *S. typhi-murium* strains isolated from livestock in the Netherlands between 1959 and 1969 increased from 6% to 37%. The rate of ampicillin resistant strains increased from 9.8% to 11%, while the number of chloramphenicol resistant strains decreased from 7% to 2% between 1966 and 1969. Among the animal strains isolated in Hungary from 1975 to 1981, tetracycline resistance varied between 5.9% and 9.6%, ampicillin resistance between 0.5% and 9.4% and chloramphenicol resis-

tance between 0.4% and 3.1%. Resistance to antibiotics was generally higher among human strains than among the animal strains, except ampicillin resistance (Tc resistance varied between 2.7% and 20.2%, Ap resistance between 2.7% and 8.8%, Cm resistance between 1.5% and 7.3%).

Anderson and Lewis demonstrated [11] the animal origin of the antibiotic resistant *S. typhi-murium* strains in 1964–1965. According to the observation of Rowe and Threlfall [12], the rate of multiple resistant strains was high in England in 1968 and 1978 (50% and 33%, respectively) and in the same period the rate of the human multiple resistant strains was also the highest (24% and 8%, respectively). In contrast, in Hungary the human multiple resistant strains were more frequent than those of animal origin. The antibiotic resistance of the Hungarian human strains originated probably also from the animal strains, but the spread of single resistance determinants was advanced by the therapeutical use of antibiotics in human therapy.

The most frequent antibiotic resistance patterns of *S. typhi-murium* strains in the period of examination were congruent with the predominant *S. typhi-murium* resistance patterns reported by the World Health Organization in 1976 [13], which summarized the data from 19 countries (Tc, Cm, Sm, Km, Ap, Su).

The incidence of R-plasmids among the antibiotic resistant strains (79.7%) was in agreement with the examinations of Neu et al. [14] carried out in New York State in 1975 and with the findings of Tanaka et al. [15] and Makino et al.[16] Japan in 1970–1979. Studies were published by Anderson [17] on the characteristics of R-plasmids derived from *S. typhi-murium* strains isolated from different parts of the world. It was established that plasmids of particular incompatibility groups showed a regional predominance. Outbreaks were caused by strains carrying group I α plasmids in North and West Europe, Greece and Mexico, and group N in South America (fi^+). The Inc I α plasmids which were common in Mexico determined Ap, Km resistances, similarly to the plasmids of 66 Mdal molecular weight frequent in Hungary in 1981. Inc Iα plasmids were frequent also in Japan [15]. Bezanson and Lior [18] published a study on the incidence and types of R-plasmids occurring in salmonellae in Canada where plasmids of Inc *group* Iα were most common among strains isolated from animals and feeds, and Inc H plasmids were encountered in bacteria from humans. In the Middle East plasmids of Inc FI were common while in Canada Inc H2 plasmids predominated in strains of both human and animal origin. Anderson's [17] analysis on the regional predominance of R-plasmids revealed that in recent years extensive human outbreaks of severe salmonellosis have been caused by strains carrying resistance plasmids in many parts of the world. Pohl et al. [19] examined *S. typhi-murium* strains in France; out of the isolated 165 R-plasmids 148 belonged to Inc group H, phages λ and T7 were restricted by the 14 examined R-plasmids. The carried resis-

tance determinants were Tc, Cm, Sm, Km, like with the Inc H R-plasmids isolated by us.

In different states of the USA identical, OXA-2-β-lactamase determining plasmids were carried by *S. typhi-murium* var. *copenhagen* strains [20].

Makino et al. [16] found two kinds of conjugative R-plasmids on the basis of heat sensitivity in 30% of 318 *S. typhi-murium* strains isolated from cattle. Two kinds of conjugative plasmid were also demonstrated from human and animal *S. typhi-murium* strains isolated in Hungary; they were separated on the basis of Inc group, phage-inhibition and molecular weight (Table I and II). R-plasmids of Inc FI—H, FI—Iα, P—H occurred together.

Anderson and Lewis [11] and Anderson [9] were the first to publish changes of *S. typhi-murium* phage types as a result of phage restriction caused by R-plasmids. The phage type of the recipient strain changed from 36 to 6 after R-plasmid acquisition; for the phage restriction, transfer factor Δ was responsible. Scholtens, too [21] found changes in phage types due to R-plasmid, using another phage set. Hérmán and Milch [22] tranferred R-plasmids into wild type *S.typhi-murium* strain of phage type 1 and the phage type changed to nt in 6 cases after the acquisition of R-plasmids and to 2b in the case of one R-plasmid. This seems to offer an explanation for the finding that most of the antibiotic resistant *S. typhi-murium* strains isolated in Hungary belonged to phage type nt and 2b. Rabsch et al. [23] also converted phage type la to nt by introducing an R-plasmid isolated from a multiple resistant nt *S. typhi--murium* strain.

The procedures used for characterization of plasmids are time consuming and costly so that much work has been spent on finding simpler methods suitable for epidemiological use. Eckhardt's [24] agarose gel electrophoresis method can be used for molecular weight estimation in a given molecular scale and Grant and Pittard [25] reported a replica-method to facilitate the incompatibility test.

Anderson in his review on the geographical distribution of salmonella R-plasmids [17] listed the phage restrictive types of plasmids and examined the restriction of salmonella type phages. In our phage-inhibition experiments where restriction has not been demonstrated in all cases, *E. coli* type phages were used after the salmonella R-plasmid had been transferred into *E. coli* K12 strain.

Phage-inhibition due to phage restriction proved useful for grouping certain R-plasmids and to complete other methods of subdivision. On the basis of phage-inhibition, examining R-plasmid acquired *E. coli* strains with T phages, Φ 2 and *E. coli* type phages, R-plasmids belonging to the same Inc group could further be subdivided. Different phage-inhibition patterns in different transconjugants have drawn attention to the presence of more than one R-plasmid, which then could be demonstrated by other methods. Though there was no

strict correlation between incompatibility groups and phage-inhibition patterns, some particular phage-inhibition patterns were observed, which were characteristic of given incompatibility groups.

Comparing plasmid characterization with the epidemiological data it was found that plasmid characterization completed with the determination of phage-inhibition provides further possibilities to prove or to exclude the common source of plasmids. To trace an epidemic process, it is not enough to type serologically the causative agent, to carry out phage and colicin typing, but it is essential to analyse the plasmid profile, too. Plasmid profile means, according to Tietze et al. [26] and Brunner et al. [27] the demonstration and characterization of the plasmids carried by the bacteria responsible for causing an outbreak.

The above results are the first steps toward such complex examinations.

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CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM DRINKING WATER BY SEROGROUPING, PHAGE SENSITIVITY AND PYOCIN PATTERN

PIROSKA KISS, JUDITH LANTOS, B. LÁNYI and ZSÓFIA FODRÉ

Public Health Station, Szeged, and National Institute of Hygiene, Budapest

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Pseudomonas aeruginosa strains isolated in the years 1977–1981 from drinking water samples fell into a large number of epidemiological units determined on the basis of serogroups, phage sensitivity and pyocin pattern. Strains isolated from water were, as a rule, sensitive to more phages than strains cultured from clinical material. The 427 water isolates fell into 8 serogroups and 31 pyocin patterns; 25.1% were untypable by the pyocin method. The frequency of isolation of different phage patterns varied annually. The most frequent epidemiological unit, comprising 9.1% of the isolates, was (serogroup: phage pattern: pyocin pattern) O1: 2/7/16/21/44/68/73/F7/F8/109/119x/352/1214/M4/C11/C18/C21:123567; 73.8% of the strains belonged to epidemiological units each represented by less than 4 strains. The large number of epidemiological units indicated that the distribution system had frequently been polluted with *P. aeruginosa* at different sites, but the organism was unable to invade the whole water supply system.

Serogrouping, phage typing and pyocin typing are widely used for the epidemiological tracing of *Pseudomonas aeruginosa* isolated from clinical samples. A few data are available for the serogroup distribution of *P. aeruginosa* cultured from water [1–3]. Recently we gave an account of the geographical occurrence of *P. aeruginosa* serogroups and the use of serotyping in checking the hygienic conditions of water plants [4, 5]. Serological, phage and pyocin typing was used for tracing *P. aeruginosa* associated with a mass pollution of a town water supply [6, 7]. The present work deals with the correlation between serogroup, phage sensitivity and pyocin type of *P. aeruginosa* isolated from deep well water of the Szeged plants.

Materials and methods

In the years 1977–1981, drinking water samples from the Szeged municipal water supply and peripheral sites yielded 427 *P. aeruginosa* strains. For comparison, 100 *P. aeruginosa* strains from clinical samples were examined. The organism was isolated and identified as

PIROSKA KISS, JUDITH LANTOS, ZSÓFIA FODRÉ
Public Health Station
Derkovits fasor 7–11, H-6726 Szeged, Hungary

BÉLA LÁNYI
National Institute of Hygiene
Gyáli út 2–6, H-1966 Budapest, Hungary

described in the Hungarian National Standard for the Examination of Water [8]. Serogrouping was performed as described by Lányi [9] and the serogroups were designated as in the scheme of Lányi and Bergan [10]. The phage pattern was determined by using the Colindale (London) phage set as described by Lindberg et al. [11]. Pyocin typing was performed according to Govan and Gillies [12] and Govan [13], and the result was recorded as a pattern of inhibition which the strain under investigation produced on indicator strains No. 1–8.

Results

Phage sensitivity of the isolates. Phage 1214 acted the most frequently on the isolates (89.2%); phages C18, 68 and C11 were next in order (86.6, 84.3 and 82.3% respectively). Phages M6, F10, 24 and 16 were the least active, lysing only 2.8, 4.1, 10.3 and 11.2% of the strains, respectively.

Figure 1 shows the width of phage spectrum for strains isolated from water and clinical samples. The isolates were divided into three arbitrary groups on the basis of the number of phages acting on them. It is seen that 62.5% of isolates from water (Fig. 1a) was lysed by 8 to 14 phages, whereas clinical strains (Fig. 1b) exhibited a narrower spectrum, being lysed in 65.0% by 1 to 7 phages and only 28.0% by 8 to 14 phages.

The phage sensitivity of the isolates varied yearly, although the incidence of strains sensitive to phages F10, M6, C18 and 24 was fairly uniform throughout the examination period. Strains sensitive to phage C188 became very frequent in 1980, whereas those lysed by phages C21, M4 and 31 showed a high peak in 1978, then decreased steeply. In 1979 there was a marked fall in sensitivity to phages 119× and 73.

Correlation between phage patterns and serogroups. Isolates belonging to the more frequent serogroups were classified according to the width of phage spectrum (Fig. 2). The serogroups varied in this respect, for example group O10 consisted mainly of narrow phage spectrum strains (82.8%) and wide spectrum strains were absent. In other serogroups, the incidence of wide spectrum strains was fairly uniform (8.5–12.5%). The serogroups differed also in sensitivity of the isolates to individual phages. For example, serogroup O9 strains were lysed by phages 2, 44, F8 and 109 much more frequently than O1 strains. In contrast, strains sensitive to phages 21 and 73 occurred more frequently in serogroup O1.

Table I shows the serogroups of strains belonging to the 16 most frequent phage patterns. A correlation is evident: phage patterns characteristic of one given serogroup were absent or occurred unfrequently in other serogroups. Two-thirds (66.1%) of the strains examined belonged to a wide variety of phage patterns summarized in Table I as "other phage patterns"; each of these was represented by less than 4 strains.

Correlation between pyocin patterns and serogroups. In Table II the isolates are classified according to serogroup and pyocin activity on indicator strains

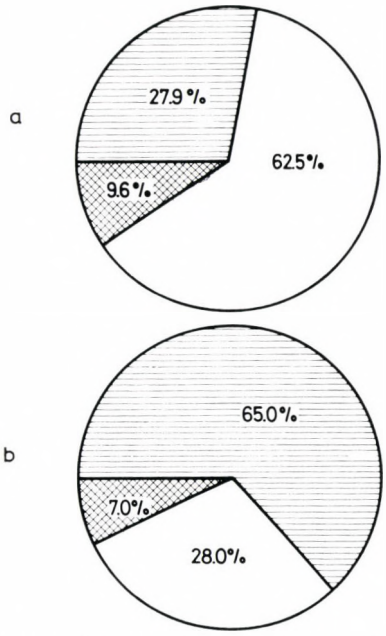

Fig. 1. Width of phage spectrum of *P. aeruginosa* strains. (a) 427 strains isolated from water; (b) 100 strains isolated from clinical samples. Number of phages causing lysis: shaded sectors, 1–7; open sectors, 8–14; hatched sectors, 15–22

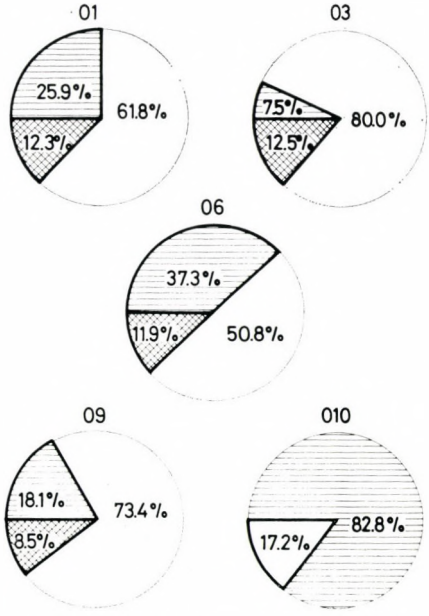

Fig. 2. Width of phage spectrum of frequent serogroups of *P. aeruginosa*. Number of phages causing lysis: shaded sectors, 1–7; open sectors, 8–14; hatched sectors, 15–22

Table I

Classification of P. aeruginosa strains isolated from water according to frequent phage patterns and serogroups

Phage pattern	Serogroup								Total	
	O1	O2	O3	O6	O7	O9	O10	O11	No.	%
2/7/44/68/F8/109/119x/352/1214/C11/C18/C188	—	—	17	—	—	6	—	—	23	5.4
7/21/68/73/119x/1214/M4/C11/C18	17	—	—	—	—	—	—	—	17	4.1
7/21/68/73/1214/C11/C18	15	—	—	—	—	—	—	—	15	3.5
7/21/68/73/352/1214/C11/C18/C21	6	—	—	—	—	—	—	8	14	3.3
21/68/73/109/119x/1214/C11/C18/C21	13	—	—	—	—	—	—	—	13	3.0
2/7/44/68/F8/109/1214/M4/C11/C18/C21	—	—	—	1	—	11	—	—	12	2.8
2/7/31/109/352/1214/C18	—	—	—	—	—	11	—	—	11	2.7
2/7/16/21/31/44/68/73/F7/F8/109/119x/352/1214/M4/C11/C18/C21	8	—	—	—	—	—	—	—	8	1.9
7/21/31/44/68/73/F7/109/119x/352/1214/M4/C11/C18/C21	4	—	—	—	—	—	—	—	4	0.9
2/7/44/68/73/109/119x/352/1214/C11/C18	—	4	—	—	—	—	—	—	4	0.9
2/7/24/44/68/73/F7/119x/1214/M4/C11/C18	—	—	—	4	—	—	—	—	4	0.9
68/F7/119x/1214/C18/C188	—	—	—	—	—	—	4	—	4	0.9
7/44/68/F8/109/119x/352/1214/C11/C18/C21	—	—	—	—	—	—	—	—	4	0.9
2/7/31/44/68/F8/109/1214/M4/C18	—	—	—	—	—	4	—	—	4	0.9
2/7/31/44/68/109/119x/352/1214/C11/C18/C188	—	—	—	—	—	4	—	—	4	0.9
21/68/F7/119x/1214/C11/C18/C188/C21	—	—	—	—	—	—	4	—	4	0.9
Other phage patterns	107	10	23	54	8	54	21	5	282	66.1
Number of strains	170	14	40	59	8	94	29	13	427	100.0

Table II

Classification of P. aeruginosa strains isolated from water according to pyocin pattern and serogroup

Pyocin pattern*	Serogroup								Total	
	O1	O2	O3	O6	O7	O9	O10	O11	No.	%
123456	3	—	—	—	—	7	2	2	14	3.3
12345	—	—	—	—	—	3	—	—	3	0.7
12356	71	—	1	5	1	1	—	—	79	18.5
1234	7	5	—	1	1	11	26	—	51	11.9
1235	2	—	—	—	—	—	—	—	2	0.5
1236	2	—	—	—	—	—	—	—	2	0.5
123	9	3	—	11	—	—	—	—	23	5.4
1256	—	—	1	—	1	—	—	—	2	0.5
125	4	2	9	1	—	5	—	—	21	4.9
1356	5	1	—	—	—	—	—	5	11	2.6
134	—	—	—	2	—	11	—	—	13	3.0
136	1	—	—	—	—	—	—	—	1	0.2
13	—	—	—	—	—	1	—	—	1	0.2
2346	1	—	—	—	—	—	—	—	1	0.2
234	—	1	—	—	—	1	—	—	2	0.5
2356	—	—	—	1	—	—	—	1	2	0.5
23	1	—		4	—	—	—	—	5	1.2
156	—	—	1	—	—	1	—	—	2	0.5
15	1	—	—	—	2	—	—	—	3	0.7
1	3	—	16	6	—	2	—	—	27	6.3
256	—	—	—	—	—	1	—	—	1	0.2
24	—	—	—	2	—	—	—	—	2	0.5
2	—	1	3	6	—	2	—	—	12	2.8
345	—	—	—	—	—	1	—	—	1	0.2
356	7	—	—	1	—	—	—	1	9	2.1
34	—	—	1	2	—	—	—	—	3	0.7
36	1	—	—	1	—	1	—	—	3	0.7
3	5	—	—	4	1	1	—	3	14	3.3
56	3	—	—	—	—	1	—	—	4	0.9
4	1	—	—	1	—	1	—	—	3	0.7
5	2	—	1	—	—	—	—	—	3	0.7
nt	41	1	7	11	2	43	1	1	107	25.1
Total	170	14	40	59	8	94	29	13	427	100.0

* Inhibition pattern of the strain on indicator strains No. 1–6

No. 1–6. Strains belonging to the most frequent serogroups O1 and O9 differed in the distribution of pyocin patterns. In serogroup O1 71 strains out of 170 fell into pyocin pattern 12356, whereas the majority of O9 strains were not typable, i.e. they exerted no activity on any of the six indicator strains.

Epidemiological units. Strains falling into the same serogroup, phage pattern and pyocin pattern were regarded to belong to one epidemiological unit. Table III characterizes on the basis of these features those epidemiological units which were represented by 4 or more strains. To a wide variety of epidemiological units each including less than four strains belonged the majority

Table III

Classification according to epidemiological unit of P. aeruginosa strains isolated from drinking water

Epidemiological unit			Strains	
Serogroup	Phage pattern	Pyocin pattern	No.	per cent
O1	7/21/68/73/119x/1214/N4/C11/C18	123567	14	3.3
	21/68/73/109/119x/1214/C11/C18/C21	123567	13	3.0
	2/7/16/21/44/68/73/F7/F8/109/119x/352/1214/M4/C11/C18/C21	123567	8	1.9
	7/21/68/73/1214/C11/C18	nt	6	1.4
	7/21/68/73/352/1214/C11/C18/C21	123567	4	0.9
	7/21/31/44/68/73/F7/109/119x/352/1214/M4/C11/C18/C21	3	4	0.9
O3	2/7/44/68/F8/109/119x/352/1214/C11/C18/C188	17	17	4.1
O6	2/7/24/44/68/73/F7/119x/1214/M4/C11/C18/C188	123567	4	0.8
O9	2/7/44/68/F8/109/1214/M4/C11/C18/C21	nt	10	2.3
	2/7/31/109/352/1214/C18	13478	7	1.6
	7/44/68/F8/109/119x/352/1214/C11/C18/C21	1237	4	0.9
	2/7/31/44/68/F8/109/1214/M4/C18/	nt	4	0.9
	2/7/31/68/109/119x/352/1214/C11/C18/C188	1257	4	0.9
O10	68/F7/119x/1214/C18/C188	123478	4	0.9
	21/68/F7/119x/1214/C11/C18/C188/C21	123478	4	0.9
O11	7/21/68/73/352/1214/C11/C18/C21	13567	5	
Other epidemiological unit			315	73.8
Total			427	

of isolates (73.8%). In serogroup O1 strains with pyocin pattern 123567 fell into 4 different phage patterns. In spite of differences in sensitivity to certain phages (e.g. resistance to 352/C21, 119x, M4 or 7/352/M4), these phage patterns are closely related. In view of literary data on the frequent instability of *P. aeruginosa* phage patterns, the corresponding strains may be regarded as members of one epidemiological unit. Accordingly, the most frequent *P. aeruginosa* epidemiological unit encountered in the present work had the formula (serogroup: phage pattern: pyocin pattern) O1:2/7/16/21/44/68/73/F7/F8/109/119x/352/1214/M4/C11/C18/C21:123567. This unit was represented by 39 strains (9.1% of all isolates).

Discussion

In Bergan's opinion [14, 15] there appears to be little correlation between serogroups and phage types of *P. aeruginosa* isolated from clinical samples. Ito and Kageyama [16] showed some association between pyocin sensitivity and phage sensitivity and concluded that pyocin and phage receptors were identical. Homma and Shionoya [17] supported this assumption by showing that antiphage serum inhibited pyocin activity and vice versa, antipyocin serum neutralized susceptibility to certain phages.

Our present study on isolates from drinking water showed some correlation between serogroups and phage sensitivity: the width of phage spectra and the lytic patterns were in many cases characteristic of individual serogroups. Isolates from water differed from our clinical strains in being sensitive to a wider spectrum of phages. A difference between environmental and clinical *P. aeruginosa* isolates was noted by Al-Dujaili and Harris [18] who observed that the former were less frequently typable by the pyocin method.

Comparison of our results of pyocin typing with that of Csiszár and Lányi [7] allow a similar conclusion: the Szeged drinking water strains were untypable in 25.1%, whereas the mainly clinical isolates of Csiszár and Lányi only in 9.2%. There appears to be little similarity in the incidence of pyocin patterns between our strains and those of Csiszár and Lányi. E.g. strains belonging to pyocin pattern 1234578 and 12357 (Gillies and Govan's pyocin types 1 and 3) occurred in Csiszár and Lányi's material in 28.7 and 20.1%, respectively, but were not represented among the 16 most frequent epidemiological units of our isolates.

Csiszár and Lányi [7] described a considerable correlation between serogroups and pyocin types. Some correlation is evident from the present findings, too (see Table II). It is worth mentioning that in our study pyocin pattern 123567 (Gillies and Govan's type 22) strains were the most frequent in serogroup O1 (Table III). Converting Lányi's original serogroup nomenclature to the Lányi–Bergan system, it turns out that this pyocin type was the most frequent in, and occurred in fact only among, serogroup O1 strains of Csiszár and Lányi.

Literary data that *P. aeruginosa* bacteria of a single ancestral origin may suffer frequent alterations in phage pattern either while living in their natural habitat or in the course of laboratory isolation, is supported by the finding that serogroup O1 pyocin pattern 123567 strains fell into four different, yet similar phage patterns. Pyocin typing may, accordingly, help in elucidating the source of isolates differing somewhat in phage sensitivity.

Although the constancy of some phage patterns of our strains may be questioned, it is evident that still a very wide variety of epidemiological units have been isolated. The high diversity in serogroups, phage sensitivity and

pyocin patterns undoubtedly shows that incidental contaminations were frequent, but none of the pollutant epidemiological units was able to invade the whole water supply system.

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BINDING OF LINCOMYCIN TO IMMUNOGLOBULINS HAS NO EFFECT ON THEIR ANTIGEN-BINDING CAPACITY

I. Barcs, Vera Bus and I. Dobronyi

László Central Hospital for Infectious Diseases, and Central Hospital of Hungarian Army, Budapest

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The interaction of lincomycin and immunoglobulins was examined in vitro. While lincomycin bound to the immunoglobulin molecules seemed to decrease the quantity of IgG and IgM detected by radial immunodiffusion and microzone electrophoresis, the level of specific antibodies could not be demonstrated by enzyme-linked immunosorbent assay (ELISA).

Unlike the majority of antimicrobial agents [1, 2], lincomycin is bound to the globulin component of serum [3, 4]. The antibiotic bound reversibly to proteins in serum, which is inactive microbiologically, represents 70 to 80% of the total [3, 5, 6]. The therapeutic effect of the drug is prolonged by the dissociation of lincomycin molecules from the drug–protein complex [2]. Its elimination half life ranges from 4 to 5 h and it is excreted in a biologically active form [3, 5, 6].

According to our preliminary studies, the alterations in serum proteins caused by lincomycin correspond to a serious hypogammaglobulinaemia [7]. In this report we have studied whether the antigen binding capacity of immunoglobulins was affected by lincomycin therapy.

Materials and methods

Human sera containing high level-antibodies to Rubella virus, Cytomegalovirus and *Toxoplasma gondii* were incubated in the presence of 10 mg/l and 20 mg/l lincomycin (Upjohn, Puurs) at 37 °C for 4 h under moderate shaking, and were assayed simultaneously with control sera incubated without drug. The sera were obtained from the Departments of Microbiology and of Clinical Chemistry of the Central Hospital for Infectious Diseases.

The level of antibodies was determined by ELISA based on alkaline phosphatase reaction by Cordia R, Cordia CMV and Cordia T diagnostic kits of Cordis Laboratories, Inc. (Miami, Fla., USA); the absorbance values were detected by Cordis Digital 405 Photometer II.

István Barcs, Vera Bus
László Central Hospital for Infectious Diseases
H-1450 Budapest, P.O.B. 29, Hungary

István Dobronyi
Central Hospital of Hungarian Army
H-1553 Budapest, P.O.B. 1, Hungary

Electrophoretic analysis of human plasma proteins [8] was carried out in 1% agar gel (Oxoid, London) stained with Coomassie Brilliant Blue R 250 (Sigma, St. Louis). Evaluation was performed by use of the Radelkis Densitometer.

Quantitative determination of immunoglobulins was performed by single radial diffusion according to Mancini et al. [9] using heavy- and light-chain specific anti IgG and heavy-chain specific anti IgM (Behring, Marburg).

Results

The densitograms of sera incubated in the presence and absence of lincomycin are shown in Fig. 1. There seemed to be a decrease in the level of alpha, beta and first of all gamma globulins in the presence of 20 mg/l lincomycin (Table I).

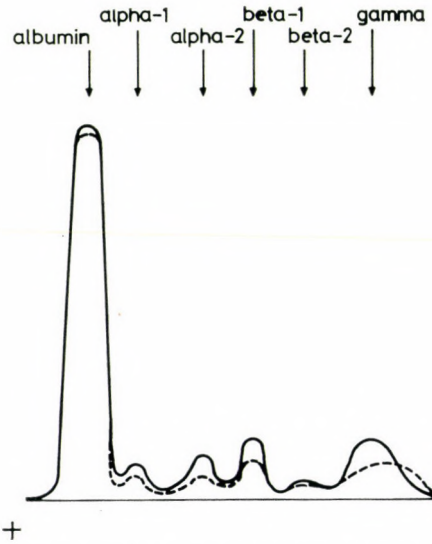

Fig. 1. Agar gel electrophoretic densitograms before and after lincomycin treatment. The solid line represents the electrophoretic separation of serum proteins in control serum. The broken line represents their altered separation after treatment with 20 mg/l of lincomycin

Table I

Change of the protein components of a serum treated with 20 mg/l of lincomycin

Protein component	Ratio of total (per cent)	
	control	treated
Albumin	63.22	72.00
Alpha-1 globulin	4.98	3.55
Alpha-2 globulin	4.74	3.55
Beta-1 globulin	5.81	4.49
Beta-2 globulin	2.96	1.58
Gamma globulin	18.27	14.81

Similar alterations were observed in clinical specimens. A 41-year-old woman with bacterial endocarditis and septicaemia was given lincomycin in combination with tobramycin, ampicillin and methicillin. The alterations in serum proteins are shown in Fig. 2. Quantitative changes of IgG and IgM in sera incubated in the presence of lincomycin are shown in Table II.

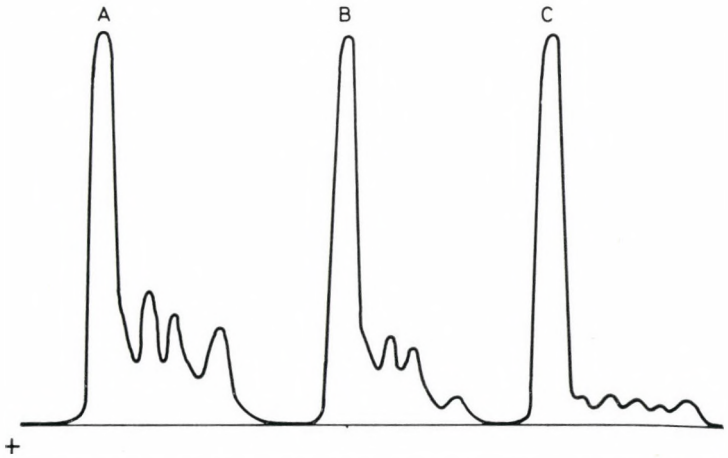

Fig. 2. Altered electrophoretic separation of serum proteins during lincomycin therapy. The densitograms demonstrate the change of the ratio of serum globulins in the serum of a patient given intravenously lincomycin. A: before administration of lincomycin; B: on 2nd day of lincomycin administration; C: on 6th day of lincomycin administration

Table II

Change of immunoglobulin level after lincomycin treatment

Type of immuno-globulin	Quantitity of immunoglobulins in grams per litre		
	1.	2.	3.
IgG	13.97	12.99	6.70
IgM	2.28	2.07	1.49

1. = control serum
2. = after treatment with 10 mg/l of lincomycin
3. = after treatment with 20 mg/l of lincomycin

The antibody levels of samples are presented in Table III. Lincomycin in therapeutic concentration caused no change in the specific antibody level as compared to control sera.

Table III

Level of the specific antibodies in sera incubated with 20 mg/l of lincomycin

(A) IgG type antibody to Cytomegalovirus

Sample	CORDIA CMV values*	
	control	treated
1.	192	198
2.	85	82
3.	96	97

(B) IgG type antibody to Rubella virus

Sample	Antibody-level (IU/ml)	
	control	treated
1.	2.4	2.3
2.	1.4	1.3
3.	2.4	2.8
4.	3.3	3.5

(C) IgG and IgM type antibody to *T. gondii*

Sample	Antibody-level (IU/ml)	
	control	treated
1.	239	264
2.	53	50

* The sera are negative when CMV values are less than 18, and positive when they are equal to or greater than 23.

Discussion

The fact that some antibiotics affect both cell-mediated and humoral immune responses is well known [10]. There are some data indicating an inhibition by lincomycin of the activity of phagocytes [11]. It was therefore of interest whether the humoral immune response i.e. the specific immunity of macroorganisms, was damaged during lincomycin therapy.

Our preliminary results [7] suggested a significant (20 to 30%) decrease in the level of gamma globulins both in humans and experimental animals during lincomycin administration. Nearly the initial quantities of IgG and IgM were recovered 48 h after lincomycin administration had been discontinued.

The observed reductions were caused not by a rapid decomposition or excretion of immunoglobulins: after eliminating the drug by active carbon column, the initial IgG and IgM values were detected.

The alteration of both the antigen structure and the electrophoretic mobility of immunoglobulin molecules was caused by their binding the drug. The altered immunoglobulin molecules failed to be precipitated by anti-human immunoglobulins. The initial physicochemical and antigen properties of proteins appeared to be restored after dissociation of the lincomycin molecule [4, 7].

A sensitive method was selected to clarify whether this change affected the antibody property of immunoglobulins. The sera were examined only in vitro since in a previous study we obtained similar results in vitro and in healthy volunteers. Our results showed no decrease in the level of IgG and IgM type antibodies to toxoplasma, Rubella virus and Cytomegalovirus. This fact speaks for the unchanged ability of the altered immunoglobulin molecule to bind to the antigen. In other words, lincomycin therapy did not reduce the level of specific antibodies.

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REACTIVITY OF MOUSE ASCITIC FLUIDS CONTAINING MONOCLONAL ANTIBODIES DIRECTED AGAINST ADENOVIRUS HEXON

ÉVA ÁDÁM, J. ERDEI, ANNA LENGYEL, ANNAMÁRIA KATONA, GY. BERENCSI, J. FACHET, I. NÁSZ

Institute of Microbiology, Semmelweis University Medical School, Budapest and Institute of Pathophysiology, University Medical School, Debrecen

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Thirty-two mouse hybridoma ascites were produced, containing hexon specific monoclonal antibodies, using crystallized hexons of human adenovirus type 1 as immunizing and selecting antigen. The reactivity of the ascitic fluids was tested with hexon preparations of 11 different human adenovirus species (serological types) belonging to 4 different subgenera by ELISA and passive haemagglutination (HA) methods. By these methods the 32 hybridoma antibodies showed 4 and 12 distinct reactivity patterns (type of cross-reactions) respectively. In the positive cases significant differences could be detected in the titres of the hybridoma antibodies in both ELISA and HA. It can be assumed that adenovirus type 1 hexon-related epitopes are present on a number of heterologous hexons in characteristic (interspecies) combinations for the given heterologous hexon and that a gradient of relationship may exist among the antigenic structures of hexons of the different adenovirus species.

In previous experiments the supernatants of hybridoma clones directed against crystallized hexon of adenovirus type 1 were used for the analysis of the antigenic relationship among heterologous hexons. The monoclonal antibodies in the supernatants were tested for their cross-reactivity with hexon preparations of nine different heterologous human adenovirus species (type) belonging to four subgenera. The diverse reactivity profiles of the different supernatants delineated a number of adenovirus type 1 hexon-related antigenic determinants present on the heterologous hexons in different interspecies combinations [1, 2]. In this paper, the characterization of ascitic fluids produced in mice injected with hybridoma cells is reported.

Materials and methods

Production of hybridomas was carried out as described earlier [1] using crystallized adenovirus type 1 hexons as immunizing and selecting antigen [3, 4]. The nonsecreting parental cell line, Sp2/0 originated from G. Köhler [5].

ÉVA ÁDÁM, ANNA LENGYEL, GYÖRGY BERENCSI, ISTVÁN NÁSZ
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

JÁNOS ERDEI, ANNAMÁRIA KATONA, JÓZSEF FACHET
Institute of Pathophysiology, University Medical School
Debrecen, H-4012 P.O.B. 23, Hungary

Production of ascites. Cultivation of antibody-producing hybrid cells was carried out in Petri dishes. Exponential growth phase cells ($1–5 \times 10^6$) were separated from the culture media by centrifugation at 200 *g*, then suspended in 0.5 ml PBS and injected in mice i.p. After 7 to 21 days the ascitic fluid was sucked off from the abdomen, centrifuged and stored at −20 °C.

Determination of hybridoma antibody isotype. Isotype of monoclonal antibodies was determined by indirect solid phase radioimmunoassay as described by Mongini et al. [6]. Briefly, 96-well PVC microtiter plates (Dynatech) were coated with 100 μg/ml antigen solution in PBS. After several washings the coated wells were incubated with the ascitic fluids diluted 1 : 100 in PBS-BSA (PBS containing 1% BSA and 0.1% sodium azide). Bound antibodies were detected by ^{125}I-labeled isotype specific antibodies. The specificity of antibodies was checked by the same method using monoclonal antibodies of known specificity and isotype.

Enzyme-linked Immunosorbent Assay (ELISA). Wells of 96-well flat-bottomed polystyrene plates (Falcon Micro Test II T. C. Plates) were coated with 50 μl hexon dilution of the different adenovirus types in PBS (pH 7.4) at 40 μg/ml final concentration. In order to produce purified hexon antigens, HEp-2 cells were infected with the following adenovirus species: types 1, 2, 5, and 6 (subgenus C), types 8, 9, 10 and 13 (subgenus D), types 7 and 35 (subgenus B) and type 12 (subgenus A). All the virus strains used were prototype strains except types 7, 8 and 35. Type 7 strain was the Pinkney [7], type 35 the Holdern isolate [8], while type 8 strain was isolated and characterized in detail by us [9, 10]. Maintenance and infection of the cells and purification of hexon proteins by chromatography were carried out according to the method described earlier [3, 10, 11]. The plates were incubated overnight at 37 °C and after washing two times with PBS containing 0,5% Tween 20, they were covered with 100 μl of PBS containing 0,5 M NaCl and 0,5% BSA. After 1 hour incubation at 37 °C, the wells were washed twice and then 50 μl of serial dilutions of ascitic fluids were added per well and incubated at 37 °C for 2 h. Antibodies in the ascitic fluids were tested from 1 : 100 dilution and titres were expressed as $\log_2 \times 10^{-2}$. Thereafter the washing procedure was repeated four times, and 50 μl of anti-mouse IgG peroxidase conjugate (Human) were added to each well in 1 : 500 dilution. Following incubation for 1 hour at 37 °C, the washing procedure was repeated and 50 μl of freshly prepared substrate (o-phenylene diamine) were added to each well and incubated for 30 to 35 min at room temperature. The colour reaction was stopped by the addition of 50 μl 4 M H_2SO_4. The absorbance was determined using a Titertek Multiscan photometer equipped with a 492 wavelength filter. Dilution of ascitic fluids displaying at least the double absorbance of negative controls was considered to be positive.

Haemagglutination (HA) was carried out according to the method of Ross and Ginsberg [12] modified as described [1].

Gel precipitation was carried out as described earlier [11] in 1% agar gel (Difco, Noble) containing 3% PEG 2000. The antigens were used in 1 to 2 mg/ml concentration, the ascitic fluids were diluted to 1 : 3, 1 : 6 and 1 : 20. Ten μl of antigens and diluted ascitic fluids were put into the wells. The experiments were evaluated after 24 h incubation at 37 °C.

Immunoosmophoresis was carried out according to our method for testing the antigenic structure of adenoviruses described earlier [13, 14]. The antigens and the antibodies were studied in the same concentration as described for gel precipitation experiments. The immunoosmophoresis was carried out with a current of 40 mA for 3 h. Antigens and the ascitic fluids were put into the wells towards the anode and the cathode, respectively.

Results

Reactivity patterns of ascitic fluids. In order to examine the antigenic relationship among different hexons, 32 ascitic fluids containing monoclonal antibodies were tested by both ELISA and HA methods with hexon preparations of 10 different heterologous human adenovirus species (serological types) belonging to 4 different subgenera. Subgenus A was represented by one, subgenus B by two and subgenus D by four virus types. As the antisera were produced against type 1 hexon, belonging to subgenus C, all four members of this subgenus were included in the experiments [15].

The number of reactive heterologous hexon types giving positive results varied between eight and ten in ELISA. These belonged mainly to members of subgenus C and of subgenus D (Table I). The number of reactive heterologous hexon types varied between five and ten in HA. These were also the members of subgenus C and types 9 and 13 of subgenus D. Four ascitic fluids reacted with all ten heterologous types in ELISA and sixteen in HA.

Table I

Reactivity patterns of 32 hybridoma ascitic fluids and their frequency tested by ELISA and haemagglutination (HA)

	Number of ascitic fluids showing identical reactivity pattern	Reactivity with human adenovirus types according to subgenera										
		A	B		C					D		
		12	7	35	1	2	5	6	8	9	10	13
ELISA	4	+	+	+	+	+	+	+	+	+	+	+
	1	−	+	+	+	+	+	+	+	+	+	+
	7	+	+	−	+	+	+	+	+	+	+	+
	20	−	+	−	+	+	+	+	+	+	+	+
Sum total of reactive ascitic fluids		11	32	5	32	32	32	32	32	32	32	32
HA	16	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	−	+	+	+	+	+	+	+	+
	1	+	−	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	−	+	−	+
	1	+	+	−	+	+	+	+	−	+	+	+
	1	−	+	+	+	+	+	+	−	+	+	+
	1	+	+	−	+	+	+	+	−	+	−	+
	1	+	−	−	+	+	+	+	+	+	−	+
	1	−	+	+	+	+	+	+	−	+	−	+
	1	−	+	−	+	+	+	+	−	+	+	+
	2	−	+	−	+	+	+	+	−	+	−	+
	1	+	−	−	+	+	+	+	−	+	−	+
Sum total of reactive ascitic fluids		27	29	23	32	32	32	32	20	32	22	32

The reactivity patterns (RP, i.e. type of cross-reaction of a given ascitic fluid with the panel of the heterologous hexons) of the 32 antibodies and their frequency in the two assay systems are demonstrated in Table I. Four RPs were observed with ELISA and twelve ones with HA. Two RPs were identical in the two assays. The most frequent pattern in ELISA was represented by 20 clones, whereas in HA an other one by 16 clones.

Table II shows the characterization of the individual ascitic fluids on the basis of the cumulative results obtained by ELISA and HA. Evaluating together the reactions of the ascitic fluids with the two methods, 17 different RPs have been distinguished. Three ascitic fluids reacted with all ten hetero-

Table II

Specificity of 32 hybridoma ascitic fluids characterized by the cumulative results obtained with ELISA (E) and haemagglutination (H)

Designation of ascitic fluids	Human adenovirus types according to subgenera										
	A	B		C					D		
	12 EH	7 EH	35 EH	1 EH	2 EH	5 EH	6 EH	8 EH	9 EH	10 EH	13 EH
H12, 1A3, 2C2	++	++	++	++	++	++	++	++	++	++	++
2A6	++	++	++	++	++	++	++	+−	++	+−	++
2A1	−+	++	++	++	++	++	++	++	++	++	++
1A5, 1D6, 2C5, 2D6	++	++	−+	++	++	++	++	++	++	++	++
1C5	++	++	−−	++	++	++	++	+−	++	++	++
2C1	++	+−	−−	++	++	++	++	++	++	+−	++
2A4	+−	++	−−	++	++	++	++	+−	++	+−	++
2D2, 1B5, 2C3, 2C6, 2B3, 2C4, 1A6, 1B6	−+	++	−+	++	++	++	++	++	++	++	++
2B5, 2D1	−+	++	−−	++	++	++	++	++	++	++	++
1A2	−+	+−	−+	++	++	++	++	++	++	++	++
2A2, 2B1, 2A3	−+	++	−+	++	++	++	++	+−	++	+−	++
1B2	−−	++	−+	++	++	++	++	+−	++	+−	++
1C4	−−	++	−−	++	++	++	++	+−	++	++	++
2A5	−−	++	−−	++	++	++	++	+−	++	+−	++
2B2	−+	++	−−	++	++	++	++	+−	++	+−	++
1D4	−−	++	−+	++	++	++	++	+−	++	++	++
1B1	−+	+−	−−	++	++	++	++	+−	++	+−	++

logous hexon types in both ELISA and HA. Sixteen RPs represented by 29 ascitic fluids were characterized with certain hexon types by divergent results between HA and ELISA. With four types 2 RPs (four ascitic fluids), with three types 4 RPs (four ascitic fluids), with two types 5 RPs (twelve ascitic fluids), with one type 5 RPs (nine ascitic fluids) exhibited positive ELISA and negative HA tests or vice versa. Considering the individual types of hexons, it may be summarized that each ascitic antibody reacted similarly by the two methods with types 1, 2, 5 and 6 of subgenus C and with types 9 and 13 of subgenus D. With the type 7 hexon of subgenus B, three antibodies reacted differently. Diverse results between the two methods were frequent for the other heterologous hexons examined. In about one third of the seventeen different interspecies specificities (RPs), each clone exhibited unique reactivity pattern; in nearly two third of the clones, however, 2 to 4 or even 8 clones displayed identical patterns. Monoclonal antibodies in all the 32 ascitic fluids belonged to IgG subclass IgG 2a.

Comparison of ELISA and HA titres of ascitic fluids. ELISA titres of the 32 hybridoma ascitic fluids are summarized in Table III. Mean geometric titres of ascitic fluids reactive with the different heterologous hexon types are also indicated. Mean titres in the cases of hexon types of subgenus C and of types 8 and 9 of subgenus D were identical or nearly identical with those obtained by the homologous hexon type 1 examined in ELISA (Table III). The

Table III

ELISA titres of 32 hybridoma ascitic fluids with adenovirus hexons*

Designation of ascitic fluids	Human adenovirus types according to subgenera										
	A	B		C					D		
	12	7	35	1	2	5	6	8	9	10	13
H12	9	12	9	12	12	12	12	11	12	11	11
1A3	5	11	6	11	11	11	11	11	10	11	11
2C2	5	9	4	8	9	9	9	9	9	9	8
2A6	0.3	11	0.3	11	11	11	12	11	11	10	8
2A1	—**	8	3	9	9	10	10	9	9	8	8
2D6	0.3	12	—	14	13	13	13	13	13	12	13
2C5	0.3	3	—	11	11	11	11	11	10	8	8
1A5	0.3	3	—	10	11	10	11	11	11	9	7
1D6	0.3	3	—	10	10	10	10	12	10	8	6
1C5	0.3	3	—	11	11	10	9	11	11	9	8
2C1	0.3	2	—	9	9	8	9	9	9	8	8
2A4	0.3	3	—	8	8	8	8	7	7	6	8
2D2	—	3	—	11	11	11	12	12	11	10	7
1B5	—	2	—	11	11	11	12	11	11	11	8
2C3	—	3	—	11	11	10	11	11	11	10	8
2C6	—	2	—	10	10	10	10	10	9	9	8
2B3	—	3	—	10	10	9	10	11	10	9	8
2C4	—	2	—	10	10	9	10	10	10	8	8
1A6	—	2	—	10	10	9	10	10	10	8	6
1B6	—	2		9	10	9	10	10	10	8	6
2B5	—	2	—	11	11	11	11	11	12	9	8
2D1	—	1	—	9	9	8	9	9	8	8	7
1A2	—	2	—	10	10	9	9	10	11	8	7
2A2	—	3	—	11	11	10	11	11	10	10	8
2B1	—	3	—	11	10	9	10	10	10	9	8
2A3	—	3		10	9	9	9	9	10	6	10
1B2	—	4	—	11	11	11	12	11	11	10	7
1C4	—	2	—	9	9	9	9	10	8	6	6
2A5	—	2	—	10	8	8	8	8	8	6	8
2B2	—	1	—	9	9	8	9	8	9	8	7
1D4	—	2	—	9	8	8	8	7	8	6	8
1B1	—	1	—	8	8	8	8	7	7	6	7
Mean titres of reactive ascitic fluids:	1.9	3.9	4.6	10.1	10	9.6	10.1	10	9.9	8.6	7.9

* $\log_2 \times 10^{-2}$. ** < 0.3

mean titres were lower in the cases of types 10 and 13 of subgenus D, the other examined types gave significantly lower mean titres than the values of homologous hexon type 1. With types 12 and 35, markedly fewer ascitic fluids were

reactive (Table I). When evaluating one by one the titres of the separate ascitic fluids, it could be seen that 13 out of the 32 ascitic fluids had higher titres with 1 to 7 heterologous hexons than with the homologous hexon type 1, but usually only by one dilution step. Only the titre of ascitic fluid 1D6 was two steps higher with the heterologous hexon type 8 than with the homologous type 1. Ascitic fluid 2C2 had higher titre with 7, ascitic fluids 1A5 and 1B6 with 4 each and the others only with 1 or 2 heterologous hexons than the values obtained with the homologous hexon. Out of the 13 ascitic fluids, eight ascites had higher titres with hexon type 6 and seven with hexon type 8 than the titres with the homologous hexon type 1. Only one or few ascitic fluids had one dilution step higher titres with hexon types 2, 5, 7, 9 and 10. In the case of hexon types 12, 13 and 35, there occurred no higher titre than the ELISA titre with the homologous hexon type 1.

In HA (Table IV) the difference of the mean titre values is much higher with the heterologous hexon types when compared with one another or with the homologous type 1 than in ELISA. The HA titres with the homologous type 1 were significantly higher than with any other types. There are also significant differences among the titres obtained with the hexon types belonging to subgenus C but in spite of this, these are much higher than the titre values in ELISA. The mean titres with hexon types 9, 13, 8 and 10 belonging to subgenus D in the given sequence decrease significantly and gradually and are much lower than the titres with the same types in ELISA. Mean titre values with the other heterologous hexons examined are even lower and are of about the same degree as the values in ELISA, except for the mean titre with hexon type 35, which is significantly lower than the value in ELISA; the number of reacting antibodies, however, is much higher (Table I). In accordance with the mean titre values, the HA titres of the individual ascitic fluids within subgenus C are usually much higher than the ELISA titres. Thus, each ascitic fluid gave higher titre with hexon type 2 in HA than in ELISA with one exception, with hexon type 5 nine ascitic fluids, with hexon type 6 three ascitic fluids had the same titres, the others had higher ones. One ascitic fluid (2C4) was found to have higher titre in HA with the heterologous hexon type 2 than with the homologous type 1.

The titres of all the other ascitic fluids were much higher in HA than in ELISA when tested with the homologous hexon type 1. The highest titre value with hexon type 1 in ELISA corresponded to more than 1.5×10^6-fold (2D6), and in HA it corresponded to more than 1.5×10^9-fold ascitic fluid dilution (2C2).

Gel-precipitation and immunoosmophoresis. For further examination of hybridoma ascitic fluids agar gel precipitation and also immunoosmophoresis were applied. In the immunoprecipitation test (carried out in 1% agar gel), about two third of the 32 ascites examined formed a precipitation line with

Table IV

Haemagglutination titres of 32 hybridoma ascitic fluids with adenovirus hexons*

Designation of ascitic fluids	Human adenovirus types according to subgenera										
	A	B		C					D		
	12	7	35	1	2	5	6	8	9	10	13
H12	4	14	14	16	11	12	12	7	11	7	5
1A3	6	11	6	16	13	12	12	9	11	7	14
2C2	5	9	4	24	18	12	12	8	11	5	12
2A6	1	9	0.3	17	13	14	13	—**	9	—	9
2A1	2	9	0.3	17	16	12	12	5	8	5	9
2D6	2	9	1	19	16	16	16	6	9	6	9
2C5	3	6	3	19	17	10	14	7	9	5	6
1A5	2	1	2	19	14	11	12	4	7	3	5
1D6	4	4	2	19	14	10	12	5	9	4	6
1C5	1	1	—	20	16	10	12	—	9	3	6
2C1	1	—	—	17	14	9	11	2	5	—	1
2A4	—	1	—	15	11	9	11	—	6	—	4
2D2	2	3	1	18	13	9	12	5	7	4	4
1B5	3	3	3	18	16	10	13	5	9	5	6
2C3	3	3	2	19	18	10	12	5	9	3	6
2C6	2	2	0.3	19	13	9	12	5	8	4	4
2B3	1	1	0.3	16	14	11	12	4	9	3	4
2C4	2	1	1	16	17	9	12	4	4	3	4
1A6	2	2	0.3	18	13	11	12	4	7	3	4
1B6	3	2	0.3	18	16	8	11	4	7	4	4
2B5	1	1	—	17	15	10	12	3	7	2	4
2D1	1	1	—	16	11	9	11	4	7	3	3
1A2	2	—	0.3	17	12	9	12	5	7	2	4
2A2	2	2	1	17	13	11	12	—	7	—	6
2B1	2	2	1	17	12	10	12	—	7	—	5
2A3	2	2	0.3	16	13	9	12	—	8	—	5
1B2	—	1	0.3	17	13	9	12	—	8	—	5
1C4	—	1	—	18	15	9	11	—	7	5	4
2A5	—	2	—	16	12	9	12	—	7	—	5
2B2	1	1	—	15	12	9	12	—	6	—	4
1D4	—	1	0.3	14	11	9	11	—	6	4	3
1B1	2	—	—	16	13	9	12	—	7	—	4
Mean titres of reactive ascitic fluids:	2.3	3.6	1.9	17.4	13.9	10.2	12.1	5	7.7	4.1	5.4

* $\log_2 \times 10^{-2}$. ** < 0.3

the homologous hexon type 1 and a considerable number reacted also with heterologous hexons. Some results of gel precipitation experiments are shown in Fig. 1. The reservoir in the middle (Fig. 1a) was filled with polyclonal anti-

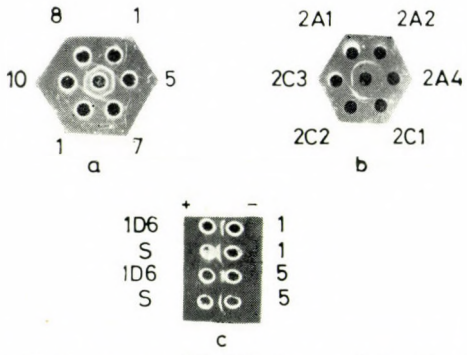

Fig. 1. Immunodiffusion in PEG agar. (a) Gel diffusion precipitation of polyclonal adenovirus type 1 hexon antiserum (central well) with purified adenovirus hexons of the indicated types. (b) Gel diffusion precipitation of the indicated ascitic fluids with adenovirus type 1 hexon (central well). (c) Immunoosmopherogram of polyclonal serum (S) and an ascitic fluid (1D6) with purified hexon of adenovirus type 1 and 5; anode on left with polyclonal antihexon control serum and ascitic fluid, cathode on right with hexons

hexon immune serum prepared against adenovirus type 1 and in the wells around it the indicated hexon types were pipetted. Between the homologous type 1 and the heterologous hexons the so-called spur-formation can clearly be seen.

The middle reservoir of Fig. 1b contains hexon type 1, and the peripheral wells the indicated ascitic fluids. It can be seen that with ascitic fluids 2C3, 2A1 and 2A2, the precipitation arc forms a confluent line, indicating that these antibodies were produced against the same determinants. Between ascitic fluids 2C2 and 2C3 as well as 2C2 and 2C1 a spur formation can be seen, which refers to the different nature of the determinants. In some ascites, precipitating antibodies were found with the bovine adenovirus type 2 (not shown).

In Fig. 1c the picture of micro-immunoosmophoresis (counter-electrophoresis) can be seen [14]. Precipitation appeared in 1 hour with both control sera and ascitic fluids. Under suitable conditions this method can also be applied for the examination of monoclonal antibodies.

Comparison of the reactivity patterns of hybridoma supernatants and ascitic fluids. Out of the examined ascitic fluids, 27 originated from hybridomas whose supernatants were studied in earlier examinations [1, 2]. The 27 different supernatants displayed 12 separate RPs, while the ascitic fluids originating from the same hybridoma clones fell into 10 different RPs (Fig. 2). Out of these, only one RP was the same, which is characterized by general positivity, i.e. reactivity with all heterologous hexons. This involves one supernatant and six ascitic fluids. In the case of the other RPs, the number of reactive heterologous hexon types with the given monoclonals varied between five and eight in ELISA and three and nine in HA for the supernatants, between eight and nine in ELISA and six and nine in HA for the ascitic fluids. It is generally

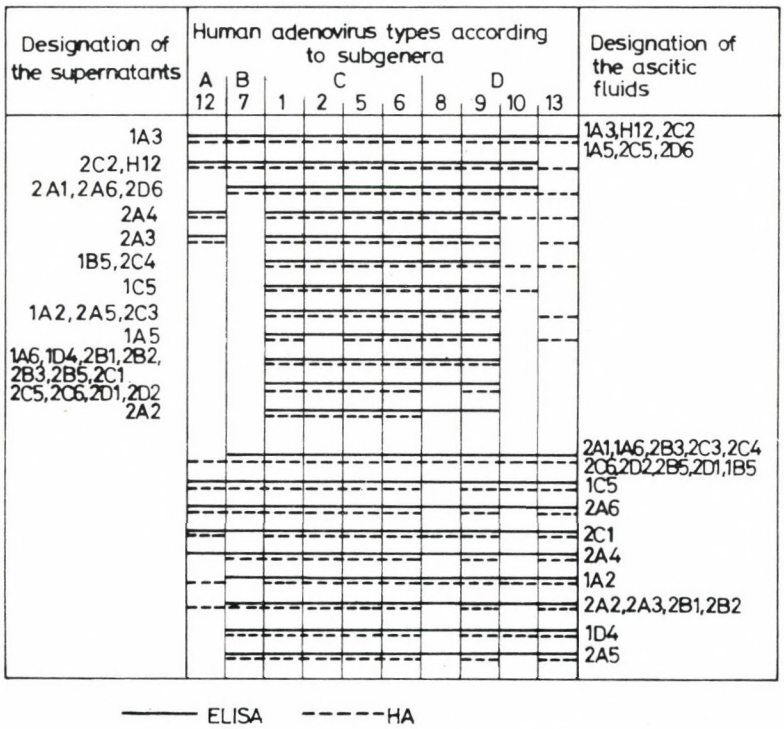

Fig. 2. Comparison of the supernatants and the ascitic fluids of 27 mouse hybridomas. Cumulative results of ELISA and HA experiments

characteristic of the ascitic fluids that they have wider cross-reactivity with heterologous hexons than the supernatants. The reactions of the ascitic fluids with the heterologous hexon type 35 is left out from the comparison because its testing was not carried out with the supernatants. The most frequent RP was characteristic of seven supernatants, whereas an other pattern of ten ascitic fluids.

Discussion

In the 32 ascitic fluids — similarly to the supernatants tested [1, 2] — we have not found any antibody that reacted only with hexon type 1, i.e. that would be type-specific. It cannot be excluded, however, that this specific determinant was damaged during the purification procedure. It may also be supposed that the hexon built into the virus capsid can display a reaction immunologically different from that of the soluble hexon, because these two kinds of hexons can be found in slightly different configurations and therefore different regions of polypeptides are accessible for the antibodies [16]. Among

the 32 ascitic fluids three hybridoma antibodies were found, which reacted with all heterologous human adenovirus hexons examined in both assay systems. One of these reacted with bovine adenovirus type 2 in gel precipitation. This means that this monoclonal antibody is able to recognize adenoviruses not only of human but also of bovine origin, which is important from the point of view of veterinary diagnosis. It is supposed that this antibody reacts with genus-specific (Mastadenovirus) epitopes. Reactivity patterns of all the other antibodies are characterized by interspecies specificities, which can be demonstrated among hexon types belonging either to the same or different subgenera. The number of antibodies giving positive or negative ELISA and HA results with distinct hexon types and the values of the ELISA and HA mean titres may indicate different degrees of relationship of the heterologous hexons to the homologous type 1 hexons (Fig. 3), provided that the concentration of the purified hexon preparations used as test antigen is the same. In the case of the species examined in this work, the closest antigenic relationship seems to exist among the members of subgenus C. Hexons of subgenus D join with this group in a somewhat looser degree of relationship. An even lower degree of antigenic relationship to type 1 hexon can be assumed for hexons of type 12 of subgenus A and of types 7 and 35 of subgenus B. On the basis of the 4 parameters summarized in Fig. 3, the heterologous hexons — according to the degree of the relationship compared to hexon type 1 — can be put into a tentative sequence, like 1, 2, 6, 5, 9, 8, 10, 13, 7, 12, 35. Thus the results obtained by the examination of the 32 ascites verified our results on testing the supernatants of hybridoma cells, which revealed the existence of a gradient of relationship among the examined hexon types. There may be found, however, some differences in the tentative sequence of the heterologous hexons.

On the basis of the several reactivity patterns and differences of the titres, it seems probable that on the hexon not only the species, genus, intersubgenus and intrasubgenus epitopes detected by polyclonal sera and the five epitopes detected by monoclonal antibodies (15, 17, 18) are present, but it may also be assumed that separate or overlapping, similar or structurally related epitopes occur on the whole surface or on a significant part of the different hexon proteins. These epitopes are present in great number on the surface of hexon proteins of different types, but in the intertype combination they are characteristic of the given hexon type. A map of all epitopes in the hexon might finally be achieved with monoclonal antibodies [19]. As the complete hexon is a trimer of three indentical polypeptide subunits [19–21], it can also be supposed that on all three subunits the same epitopes can be found.

The finding that immunoosmophoresis can be applied for the examination of monoclonal antibodies could be of importance because the method can be applied for quick diagnostic purposes.

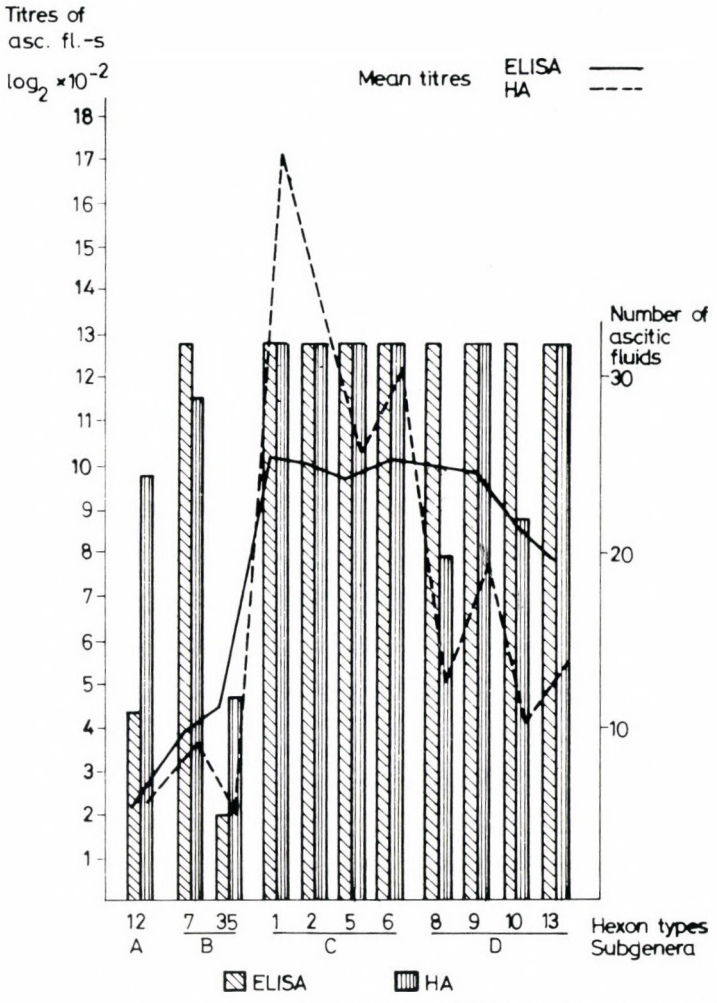

Fig. 3. Qualitative and quantitative evaluation of the ELISA and HA titres of 32 ascitic fluids with 11 different hexon types: number of reacting monoclonals (columns) and mean titre values (curves)

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CAMPYLOBACTER STRAINS ISOLATED FROM SLAUGHTERED CHICKENS: THEIR SENSITIVITY TO ANTIBIOTICS AND RESISTANCE TO ERYTHROMYCIN

ELISABETH MARJAI, MÁRIA M. ÁDÁM, ZSUZSANNA HORVÁTH, IRÉN KAJÁRY and ZSUZSANNA KOVÁTS

Public Health Station, Szeged, and National Institute of Hygiene, Budapest

(Received November 27, 1983)

Antibiograms for 304 *Campylobacter* strains isolated in Csongrád county from slaughtered chickens in the years 1981 to 1983 were determined with the disk-agar diffusion method. The isolates, originating from two poultry-processing plants and two canteen kitchens proved to be sensitive to nalidixic acid, chloramphenicol, gentamicin and nitrofurantoin and resistant to penicillin. The antibiogram depended on the source of samples and, to a higher degree, on the year of sampling. The frequency of strains resistant to erythromycin was 5.7% in 1981 and 39.8% in 1983; for the resistant strains the minimum inhibiting concentration was >250 μg erythromycin per ml. In the same periods 25.0% and 32.8% of the isolates were resistant to tetracycline and 23.3% and 49.2% to lincomycin, respectively. Simultaneous resistance to tetracycline, lincomycin and erythromycin occurred in 16.4% of the isolates.

A high-rate infectedness of slaughtered poultry by campylobacters has been shown both abroad [1–6] and in Hungary [7]. Poultry is considered the most important campylobacter reservoir and its raw meat seems to be one of the most frequent sources of human infection. As to the classification of *Campylobacter* strains causing enteritis, the use of procedures based upon biochemical reactions shows an upward tendency [8, 9] while serotyping, a more sensitive method of strain differentiation is used by few laboratories [10–14]. In everyday practice the antibiogram of isolates may be a useful tool for strain differentiation. For this purpose, above all, antibiotics to which campylobacter strains show variable resistance, e.g., tetracycline, ampicillin, lincomycin and metronidazole may come into account.

In the present work, we wished to obtain information on the antibiogram of campylobacters isolated in Hungary from slaughtered chickens, seeking an answer to the question whether there was any relation between breeding place of the chickens and the year of slaughtering on the one hand and the sensitivity of the isolates on the other.

ELISABETH MARJAI, ZSUZSANNA HORVÁTH, IRÉN KAJÁRY, ZSUZSANNA KOVÁTS
Public Health Station
Derkovits fasor 7-11, H-6726 Szeged, Hungary

MÁRIA M. ÁDÁM
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Materials and methods

Bacterial strains. The 304 strains of *Campylobacter jejuni* were isolated in January, February and March, 1981, and in March, April and June, 1983. The samples had been taken from two poultry-processing plants and two canteen kitchens in Csongrád county (South-Hungary). The plants bought the poultry from different breeders. The method of taking samples and isolation and identification of strains was the same as reported elsewhere [7, 15]. The thermotolerant, nalidixic acid-sensitive strains designated as *C. jejuni* were not differentiated with the sodium hippurate test.

Determination of resistance to antibiotics. Penicillin, nalidixic acid, erythromycin, tetracycline, ampicillin, lincomycin, gentamicin, chloramphenicol, nitrofurantoin and metronidazole disks of the "Resistest" series (Institute for Serobacterial Production and Research Human, Budapest) were used in the agar-diffusion test. The campylobacter agar preparation (Ca_0) was free from antibacterial substances [15]. Agar plates were touched with a cotton swab soaked in a 48 h bacterial culture and direct plating was made. Then, the disks were placed on the plates and the cultures were incubated at 42 °C in an atmosphere containing approx. 5% oxygen and 10% CO_2. The zones of inhibition were compared to zones obtained with control strains [16].

Minimum inhibiting concentration (MIC). The MIC values were determined for isolates that had proved resistant to erythromycin with the disk method. Serial dilutions of erythromycin (Marsin Co. Ltd, Copenhagen, Denmark), from 2.5 μg to 5000 μg/ml, were prepared in the campylobacter agar medium. The agar plate was divided into sectors and volumes as small as a poppy-seed of a 48 h culture were evenly smeared on each sector. The plates were incubated for 48 h at 42 °C in 10% CO_2 and 5% oxygen atmosphere. The lowest erythromycin concentration without visible growth was recorded as the MIC value.

Results

All the 304 strains were sensitive to nalidixic acid and resistant to penicillin. Apart from a few moderately sensitive strains all were sensitive to gentamicin, chloramphenicol and nitrofurantoin (Table I). The sensitivity to tetracycline, ampicillin, lincomycin and metronidazole was variable.

Table I

Sensitivity to antibiotics of Campylobacter strains isolated from slaughtered chickens

Antibiotic	No. of strains tested total	Sensitive strains		Moderately sensitive strains		Resistant strains	
		No.	%	No.	%	No.	%
Nalidixic acid	304	304	100.0	—	—	—	—
Penicillin	304	—	—	—	—	304	100.0
Tetracycline	304	109	35.9	109	35.9	86	28.2
Ampicillin	304	130	42.8	134	44.0	40	13.2
Lincomycin	304	52	17.1	148	48.7	104	34.2
Erythromycin	304	243	79.9	—	—	61	20.1
Gentamicin	304	290	95.4	14	4.6	—	—
Chloramphenicol	304	299	98.4	5	1.6	—	—
Nitrofurantoin	283	278	98.2	5	1.8	—	—
Metronidazole	27	1		8		18	

The resistance of the *C. jejuni* strains originating from the poultry-processing plants varied according to the poultry farm and the year of isolation (Table II).

In 1981, the majority of the strains resistant to tetracycline (30/44) were isolated from samples taken in plant II from poultry raised in farm SzT. In 1983, on the other hand, the tetracycline-resistant strains were isolated from samples taken in plant II from birds raised in farms SzT, BM and MU.

In 1981, 16 of the 17 strains resistant to ampicillin and the majority of the lincomycin-resistant strains (32/41) originated from farms supplying plant

Table II

Occurrence of Campylobacter isolates resistant to antibiotics. Distribution by place and year of isolation

Place of sampling	Date	Place of raising	No. of strains tested	Strains resistant to				
				tetra-cycline	ampi-cillin	linco-mycin	erythro-mycin	metro-nidazole
			1981					
II*	20/1	OB	4	—	—	—	—	—
	12/2	HV	7	1	1	1	—	.
	16/2	HH	9	1	6	7	8	1. .
	23/2	SM	27	5	6	19	—	1.
	04/3	MT	11	1	1	1	—	1.
	13/3**	MT	36	6	2	4	2	.
II	19/3	SzT	66	21	—	1	—	.
	01/4**	SzT	16	9	1	8	—	.
	Total 1981	No.	176	44	17	41	10	.
		%	100.0	25.3	9.7	23.3	5.7	.
			1983					
I	08/6	MT	21	—	1	—	—	
II	01/3	SzT	13	12	1	5	7	5
		BM	17	17	—	16	16	13
	26/4	MU	37	10	12	13	5	1.
		BM	40	3	9	29	23	.
	Total 1983	No.	128	42	23	63	51	18
		%	100.0	32.8	18.0	49.2	39.8	.

* Poultry-processing plant
** Samples taken in canteens

I, whereas in 1981 strains resistant to ampicillin and lincomycin were isolated from poultry raised in farms BM and MU and processed in plant II.

In 1981, all the 10 strains resistant to erythromycin were isolated from samples taken in plant I from birds raised in two farms. Eight of the strains were isolated from faecal samples of chickens processed in the plant, the remaining two from samples taken a month later from poultry in a canteen kitchen. In 1983, on the other hand, 51 of the 107 *C. jejuni* strains isolated in plant II were resistant to erythromycin. For the resistant isolates the MIC was >250 μg/ml.

The resistance patterns that occurred in 1981 and 1983 are shown in Table III. Strikingly, in 1981 58%, whereas in 1983 only 36%, of the isolates were sensitive to all the four antibiotics shown in Table III (pattern 1); resistance to erythromycin and lincomycin combined with sensitivity to the other two drugs (pattern 10) occurred in 0.6 and 21.1%, respectively. Strains resistant to each of tetracycline, lincomycin and erythromycin (pattern 12) were not isolated in 1981, but 16.4% of the 128 strains tested in 1983 showed this pattern. Combined resistance to tetracycline and lincomycin (pattern 7) and combined resistance to ampicillin, lincomycin and erythromycin (pat-

Table III

Distribution of isolates by resistance pattern

Resistance pattern	Tetracycline	Ampicillin	Lincomycin	Erythromycin	No. of isolates 1981		1983	
					No.	%	No.	%
1	[S]	[S]	[S]	S	102	58.0	46	35.9
2	R	[S]	[S]	S	28	15.9	6	4.7
3	[S]	R	[S]	S	2	1.1	4	3.1
4	[S]	[S]	R	S	16	9.1	2	1.6
5	R	R	[S]	S	2	1.1	6	4.7
6	[S]	lR	R	S	2	1.1	7	5.5
7	R	[S]	R	S	11	6.3	—	—
8	R	R	R	S	3	1.7	6	4.7
9	[S]	[S]	[S]	R	1	0.6	—	—
10	[S]	[S]	R	R	1	0.6	27	21.1
11	[S]	R	R	R	8	4.5	—	—
12	R	[S]	R	R	—	—	21	16.4
13	R	[S]	[S]	R	—	—	3	2.3
			Total		176	100.0	128	100.0

[S] = sensitive or moderately sensitive
S = sensitive
R = resistant

tern 11) occurred in 6.3% and 4.5%, respectively, in 1981 and in no case in 1983. Among the resistant strains isolated in 1981, strains of pattern 2 occurred most frequently, followed by the pattern 4 strains, both patterns indicating resistance to a single drug; among the strains of 1983, on the other hand, the patterns occurring most frequently (patterns 10 and 12) indicated double (lincomycin–erythromycin) and triple (tetracycline–lincomycin–erythromycin) resistance, respectively.

Discussion

The sensitivity to antibiotics of campylobacters isolated from human enteritis have been thorougly investigated by several groups (15, 17–25). Swedhem et al. [24] failed to find any difference in sensitivity to antibiotics between strains isolated from human enteritis and those isolated from the intestines of healthy chickens.

In the 304 *C. jejuni* isolates examined by us, in accordance with literary data [15, 17–25], sensitivity to nalidixic acid, gentamicin, chloramphenicol and nitrofurantoin occurred most frequently; to the antibiotics to which campylobacters showed variable sensitivity, the frequency of resistant strains increased considerably between 1981 and 1983.

Examination of sensitivity to erythromycin is of importance because this drug is used most frequently in the therapy of human enteritis caused by campylobacter [17], though numerous authors [24, 26–28] have reported on the appearance in human material of strains resistant to erythromycin. In Hungary, the first campylobacter culture resistant to erythromycin was isolated from slaughtered poultry in February, 1981. Since then, isolation of two, one and two erythromycin-resistant strains have been reported from countries Pest, Nógrád and Csongrád, respectively, but there is no central documentation of the resistance of campylobacters isolated in Hungary.

The fact that of the 61 erythromycin-resistant strains isolated from slaughtered chickens only four were sensitive to lincomycin points to a possible cross-resistance between erythromycin and lincomycin. In the literature, erythromycin–lincomycin–clindamycin cross-resistance has been reported by Walder [20] and erythromycin–clindamycin cross-resistance by Karmali et al. [22].

Resistance to lincomycin reached a high level in our material (34.2%). Nevertheless, other authors [15, 19, 24] have reported still higher frequencies of resistance (51–70%) for strains isolated from humans or poultry.

The resistance to tetracycline shows a considerable geographical variation. For strains of human origin, frequencies of 5.3, 8.1, 20 and 26% have been reported [19, 21, 22, 24]. In Hungary, 10.9% was reported from Pest county [15] and 16% from Csongrád county [25]. For strains isolated from

chickens higher percentages have been reported, e.g., 34% [24] and 37.9% [23]. The 32.8% resistance to tetracycline for strains isolated by us in 1983 agrees well with the latter data.

In the frequency of resistance to ampicillin, no striking changes could be observed in 1983. The literary data in this respect [17–23] show great variation. In Hungary, 56% of the strains isolated from humans in Csongrád county in 1980 and 27% of those isolated in Pest county in 1981 proved to be resistant to ampicillin [15, 25].

The appointment of the days of sampling was arbitrary in both years. It is of interest that for slaughtered poultry raised in farm MT, the resistance to antibiotics was found higher in 1983 than in 1981, whereas for those raised in farm SzT the resistance was higher in 1981. It may be assumed that the degree of resistance may depend on the period of raising of broiler chickens. Removal of slaughtered chickens is followed by a big cleaning before new chickens are introduced.

It may happen that even healthy chickens are fed with a premix containing oxytetracycline. In addition, if justified by the occurrence of disease, commercial preparations containing erythromycin, neomycin, oxytetracycline and/or tylosine are also administered. E.g., in farms SzT and BM, during the raising period in 1983 the chicken examined by us were given neomycin, oxytetracycline and tylosine, whereas in farm MU only a premix containing oxytetracycline was given. It cannot be excluded that the high level of tetracycline-resistance in farm SzT was due to the intensive administration of antibiotics. As erythromycin had not been administered, the increased occurrence of isolates resistant to this antibiotic could be attributed to tylosine treatment. Our preliminary experiments suggest that tylosine, an antibiotic widely used by veterinarians, may cause cross-resistance with erythromycin.

Taking into account the role of rodents in the transmission of campylobacter [29], we cannot exclude their role in the maintenance of resistant strains.

In spite of the high incidence of campylobacter in slaughtered poultry, the risk for humans due to the low contagiosity, is low. Moreover, owing to the usually mild course of human enteritis, only few cases are seen by physicians. Furthermore, since in campylobacter enteritis the source of infection may be other than chicken, the resistance of the strains isolated from human patients may not reflect the resistance of the strains occurring in slaughtered poultry in the same period of time.

At last, we call attention to the variation in the resistance to antibiotics of the strains wide-spread in poultry farms and often present in raw poultry meat. It is therefore justified to follow up, mainly for epidemiological and therapeutical reasons, the changes in resistance.

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DETECTION OF BETA-LACTAMASE ACTIVITY WITH NITROCEFIN OF MULTIPLE STRAINS OF VARIOUS MICROBIAL GENERA

J. V. URI

Department of Clinical Research and Development, Smith Kline and French Laboratories, Philadelphia, PA. USA

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The production or presence of beta-lactamase(s) was studied by the rapid method utilizing the chromogenic cephalosporin compound nitrocefin in cultures of multiple strains belonging to the same genus as well as groups of microorganisms. The genera were: *Staphylococcus* spp., *Streptococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, indole-positive *Proteus* spp., *Enterobacter* spp., *Serratia marcescens*, rare *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. With this sensitive and rapid assay for beta-lactamase, it was possible to verify and separate the beta-lactamase producing cultures from the non-producers and include the useful strains to ongoing research, such as beta-lactam screen, beta-lactamase inhibitory study and lytic properties of beta-lactams. The data also provide evidence for the possible role of beta-lactamase(s) in the physiology, biochemistry and pathogenicity of bacterial strains. The nitrocefin method was found a very specific and extremely useful procedure for the detection and estimation of beta-lactamase activity.

Bacterial beta-lactamase(s) or penicillin and cephalosporin amido-beta-lactam hydrolase(s) (EC 3.5.2.6) are active proteins or group of enzymes capable of catalysing with variable specificity the hydrolysis of the beta-lactam ring of many members of the family of the penicillin and cephalosporin antibiotics [1]. The beta-lactamases (penicillinases and cephalosporinases) produced by pathogenic bacteria are rather specific and highly effective catalysts in opening the beta-lactam ring by which the antibiotics lose completely their antibacterial activities. This process is of paramount importance by which pathogenic bacteria develop resistance to these antibiotics with important therapeutic and economic consequences. No wonder the study of these enzymes generated extensive theoretical and practical worldwide research. Since the discovery and first description of the penicillin-destroying enzyme named penicillinase by Abraham and Chain [2], the number of publications on the beta-lactamases continues to increase exponentially. Fortunately, review papers on the enzyme(s) appeared quite early and have been followed continuously by newer surveys. Instead of the numerous individual papers only some selected comprehensive review articles, monographs and books will be listed here which deal with the origin, function, terminology, bio-

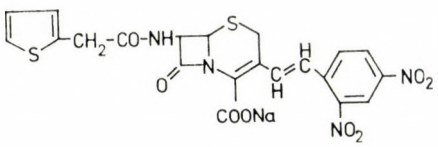
JOSEPH V. URI
Department of Clinical Research and Development, Smith Kline and French Laboratories
P.O. Box 7929, 1500 Spring Garden Street, Philadelphia, PA 19101, USA

chemistry, chemical and biological properties, distribution, classification and therapeutic relevance of the beta-lactamases [3–19].

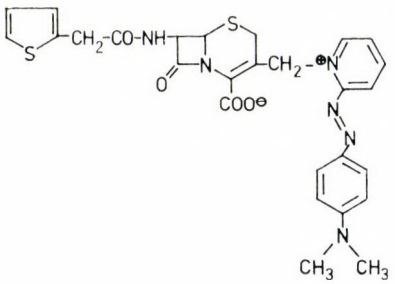
Because of the extreme clinical importance of the beta-lactamases, in addition to their fascinating scientific interest, several methods have been developed to their qualitative detection and quantitative estimation. The earlier assay methods (microbiological, manometric, acidometric, hydroxylamine, spectrophotometric, analytical isoelectric focussing) are either much involved or not completely satisfactory [20–24]. The new colorimetric methods using chromogenic cephalosporin substrates (Fig. 1) are claimed to be very sensitive, reliable and usually quick [25–30]. Of these chromogenic substrates nitrocefin (chromogenic cephalosporin 87/312) was used the same way as first described by O'Callaghan et al. [25] for a detailed screening program including multiple strains of various genera. The results of this study are described in this paper.

For completeness it should be mentioned that in addition to beta-lactamases of microbial origin a beta-lactam-ring hydrolysing enzyme was detected in animal and human kidney tissues [31]. A dipeptidase, dehydropepitdase I (DHP-I) found in the brush border of the proximal renal tubular

Niortcefin
Cephalosporin 87/312)



Cephacetrile
(36278-Ba)



PADAC
(Pyridine-2-Azo-
p-Dimethyl
aniline Cephalosporin)

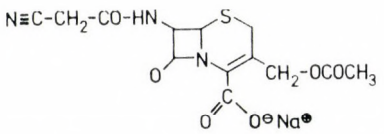


Fig. 1. Chemical structures of chromogenic cephalosporins used for detection and determination of beta-lactamase activity

epithelium is of broad substrate specificity, has practical importance because it hydrolyses the beta-lactam ring, with consequent elimination of the activity of thienamycin and other carbapenems, compounds otherwise highly resistant to bacterial beta-lactamases [32].

Materials and methods

The production of beta-lactamase(s) was monitored using nitrocefin, also known as chromogenic cephalosporin 87/312 as the substrate of the enzyme. These two names will be used interchangeably throughout the text. The working solution was prepared by dissolving 2.5 mg of the compound in 5 ml deionized water. Solutions of later batches of nitrocefin were prepared by dissolving 2.5 mg of the powder in 0.25 ml dimethyl-sulphoxide followed by the addition of 4.75 ml 0.1 M phosphate buffer pH 7.0, and shaken thoroughly (producer specification). Of this solution, 0.05 ml was added either to 2 ml of overnight grown broth cultures, or cell suspensions, or colonies on solid media. In the presence of beta-lactamase the colour of the cultures or the colonies (and often the surrounding solid media) turns red or purple usually immediately or after a few minutes in the case of weak enzyme producing strains (Fig. 2 and Fig. 3). The colour change is the result of an electron shift, when the beta-lactam ring is opened by the beta-lactamase along the molecule containing at the 3-position the conjugated 2.4-dinitrostyryl moiety (Fig. 1). In the Tables, the empty places indicate no colour development, and + through ++++ indicate in increasing order the onset and intensity of the developing red (purple) colour, whereas d means that a longer waiting time was needed for the colour development.

The strains tested were cultured in or on synthetic, semi-synthetic and complex organic media (liquid and/or solid). The synthetic media contained ammonium sulphate, glucose and inorganic salts. The composition of the semi-synthetic medium that we developed for studying the lytic properties of beta-lactam antibiotics is as follows: 0.5% peptone, 0.1% glucose with and without McIlvaine buffer or agar [33]. This medium is colourless, pH-balanced and nutritive for all strains studied [34]. The complex media employed were of commercial origin. The legends to each Table identify the media used.

Results

The production and activity of beta-lactamase(s) of multiple strains of the same genus of bacterial strains, employed in our laboratory for expanded spectrum evaluations of cephalosporins detected by the chromogenic cephalosporin 87/312 (nitrocefin) method, are summarized in Table I. Tables II, III and IV present similar data for special strains used for primary assay of new compounds and fermentation broths and/or products. These Tables usually contain the name, designation and/or number of strains, media used for growth of the microbes, indication and short description of onset and degree of the purple colour.

Staphylococcus aureus and *Staphylococcus epidermidis*. Of the 25 *Staphylococcus* strains, 18 were haemolytic *S. aureus*, and 7 non-pigmented, coagulase-negative *S. epidermidis*. Neither pigment or coagulase production nor haemolytic property seemed to have any correlation with the beta-lactamase production. Twelve strains gave no colour reaction with nitrocefin (a few delayed

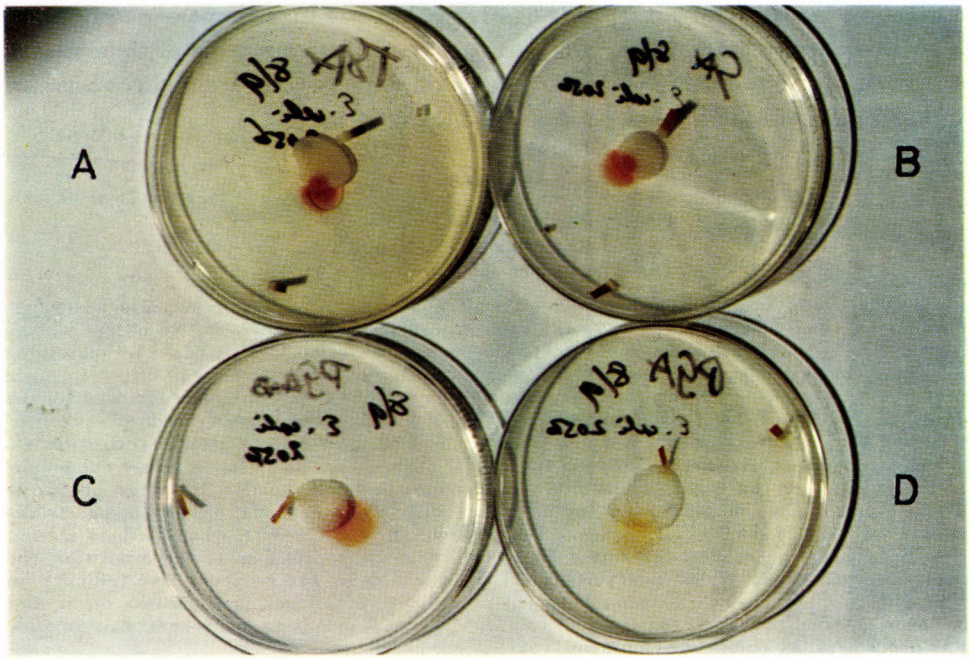


Fig. 2. Demonstration of beta-lactamase production of *E. coli* on solid media with nitrocefin. Enzyme diffuses into the medium around the colony on the following media: A = Trypticase–soy agar; B = Synthetic agar; C = Pepton–glucose agar buffered; D = Pepton–glucose agar. The non-buffered agar becomes acidic (colony D), the colour is paler, in contrast to the others, where the pH is either neutral or weakly alkaline

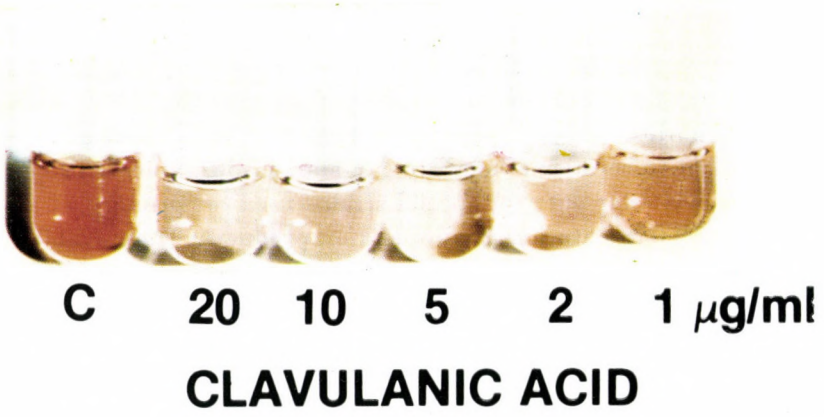


Fig. 3. Inhibition of *E. coli* strain 804 beta-lactamase in solution, obtained by lysis of the cells with cephamycins and ×100 diluted. In the presence of beta-lactamase nitrocefin changes colour from yellow to red as in C (control) tube. Clavulanic acid inhibits enzyme activity and colour will not develop. Clavulanic acid is inhibitory at 1 μg/ml concentration

reactions are exceptions) and these strains were found to be sensitive to ampicillin and penicillin G. Thirteen strains were strong beta-lactamase producers and also were found, in earlier experiments, to be resistant to ampicillin and benzylpenicillin. Note that strain 127 (670) which is our regular in vitro and in vivo test strain, appeared to be a weak beta-lactamase producer. We know that it is an inducible beta-lactamase producer strain and here it was not induced. On solid media, especially on peptone–glucose agar (PGA) and buffered peptone–glucose agar (PGA-B), the colour developed not only in the colonies but also in their surroundings, indicating diffusible (extracellular) enzyme(s) expected with staphylococci. Strain No. 671 used in earlier beta-lactamase inhibition studies [35] was a very fast and strong beta-lactamase producer (immediate and dark purple colour). The unique specifically mouse-pathogenic strain (No. 674) was the original *S. aureus* Tour (souche T) strain widely used in mouse infection-protection assays known never to produce beta-lactamase [36, 37]. Synthetic media were not included in this study because typical staphylococci do not grow in/on these media.

Escherichia coli. Of the 31 *E. coli* strains, 13 were sensitive to ampicillin to various degrees and they were at least very weak beta-lactamase producers (the colour took many hours to develop), depending on the medium. The only exception was strain No. 1538. Synthetic agar appeared to be the best for enzyme production of these strains. The rest of the strains were ampicillin resistant and strong beta-lactamase producers (deep purple colour immediately) on almost all media used. Strains No. 897 and No. 915 are of interest because they were highly resistant to ampicillin but very weak colour producers with nitrocefin. This kind of behaviour was characteristic of the TEM-type beta-lactamase producing *E. coli* strains. *E. coli* No. 12140, the strain-regularly used in our in vitro and in vivo evaluation of beta-lactams, is a very weak beta-lactamase producer and so are the ATCC 25922 strains used for quality controls (Fig. 2). On solid media (especially on PGA-B) the enzyme diffuses into the agar and the red colour develops not only in the colonies but also in the surrounding agar. This phenomenon was found to be pH-dependent. When the medium was not buffered (Fig. 2D) the pH of the agar around the colony turned acidic (pH 5.0) and only a yellowish colour developed, but if the same medium was buffered (PGA-B), the pH of the agar remained about 7.0 and red colour developed immediately in the agar neighbourhood of the colonies (Fig. 2C).

On the basis of these experiments, *E. coli* No. 211 was selected for lytic studies using cephamycins [38]. Strain No. 804 was selected for inclusion into the regular screen for beta-lactams [35], and beta-lactamase inhibitors (Fig. 3). Beta-lactamase production of these strains was so abundant that colour developed even at a 10 000 times dilution of the culture in PGB-B medium.

Klebsiella pneumoniae. Data of Table I show that basically all *Klebsiella pneumoniae* strains are beta-lactamase producers and form purple colour with nitrocefin on and in all media. Our regular assay strain, No. 4200 is a rare exception, because it failed to give the colour-reaction characteristic of the presence of beta-lactamase. It was repeated many times under various conditions. It was undoubtedly a *Klebsiella* but with unusual characteristics. Strain No. 1200 is a moderate producer in PGB-B medium and its culture was selected for studies of beta-lactamase inhibitors [35]. *K. pneumoniae* No. 982 was found to be a strong enzyme-producer and recommended for inclusion into the assay strains aimed at searching for beta-lactamase resistant beta-lactams.

Proteus mirabilis. Altogether 25 *P. mirabilis* strains were examined for the presence of beta-lactamase (Table I). Only one strain, No. 346 developed a definite colour reaction. Its full-grown culture was included in our beta-lactamase inhibitory study [35]. Strain No. 444 was included some years ago as a very sensitive strain. Some but not all strains produced a delayed, probably non-specific light pinkish-yellowish colour on synthetic agar and an orange-like colour in TSB, but never a red or purple one. The beta-lactamase of strain No. 346 operated also at acidic pH and did not diffuse into the agar. Swarming of these strains was minimal on the solid media used.

Indole-positive Proteus spp. All of the indole-positive *Proteus* strains shown in Table I, i.e., 10 *P. morganii (Morganella morganii)*, nine *P. vulgaris* and six *P. rettgeri* were found to produce the purple colour easily with the beta-lactamase substrate, nitrocefin. Our regular *P. morganii* test strain No. 179 (a non-swarming one) produced a dark purple colour on TSA media. Some of the other strains swarmed mostly on TSA and less on SA. Enzyme diffused into the neighbourhood of the colony (or was detectable) only if the pH of the medium was neutral or slightly alkaline but not at acidic pH. The strongest colour-(beta-lactamase-) producing strains were Nos 415 and 625, but all strains produced enough enzyme to destroy beta-lactam antibiotics, being not absolutely beta-lactamase stable. *P. morganii* No. 179 was selected for routine assay purposes, because it was not swarming, and highly pathogenic to mice.

Enterobacter spp. Data of Table I demonstrate that, without exception, all 25 *Enterobacter* strains (twenty *E. cloacae* and five *E. aerogenes*) produced beta-lactamase with definite colour formation in most of the media used. The strongest enzyme (colour) producers were strains Nos 212, 1181, and 1222. The pH of the solid medium influenced the diffusion of beta-lactamase into the agar around the colonies; alkalinity increased it but the enzyme of strain No. 212 diffused and worked at acidic pH as well. The colour developed on the agar medium (PGA-B) persisted for weeks at room temperature with the exception of strain No. 212 which was decolourized overnight. This ample beta-lactamase production (colour development) by *Enterobacter* strains pro-

Table I

Summary presentation of the results of beta-lactamase production by multiple bacterial strains using nitrocefin, the chromogenic cephalosporin substrate

Bacteria	Total No. of strains	No. of beta-lactamase producers	No. of non-beta-lactamase producers	Remarks
Staphylococcus aureus	18	9	9	
Staphylococcus epidermidis	7	3	4	
Streptococcus faecalis	14	0	14	
Escherichia coli	31	30 (12)	1	(12) weak producers
Klebsiella pneumoniae	28	27	1	
Proteus mirabilis	25	13 (12)	12	(12) weak producers
Proteus morganii	10	10	0	
Proteus vulgaris	9	9	0	
Proteus rettgeri	6	6	0	
Enterobacter cloacae	20	20	0	
Enterobacter aerogenes	5	5	0	
Serratia marcescens	25	25 (2)	0	(2) weak producers
Other bacteria	25	25 (2)	0	(2) weak producers
Pseudomonas aeruginosa	10	10	0	
Neisseria gonorrhoeae	10	5	5	The beta-lactamase producing strains are resistant to penicillin G (MIC's 3.1–25 μg/ml)
Haemophilus influenzae	18	11	7	The enzyme-producing strains are resistant to ampicillin (MIC's 6.2–50 μg/ml)

Weak producer means that the colour develops slowly, over many hours, and never becomes deep purple

Table II

Colour reaction for beta-lactamase of various bacterial assay strains with nitrocefin

Strains	PGB-B	PGA-B	SB	SA	PSA
S. aureus 910	++++	++++	+++	+++	++++
S. aureus 209			No growth	+++	++
E. coli KN	d	d	d	++	+++
E. coli STN	d	d	d	++	+++
E. coli R_6	d	d	d	++	+++
E. coli 214	d	d	d	++	+++
E. coli SS	d	d	d	++	+++
Sarcina lutea (wild)	d	d	d	++	+++
B. subtilis (ATCC 6633)	d	d	d	++	+++.

PGB-B = Peptone–glucose broth buffered; PGA-B = Peptone–glucose agar buffered; SB = Synthetic broth; SA = Synthetic agar; PSA = Penassay seed agar. ++++ = Immediate and strong red colour; +++ = Colour within 5 min; ++ = Colour within 30 min; d = Delayed (hours or overnight) colour development

vides an acceptable explanation for their overall resistance to earlier beta-lactam antibiotics.

Serratia marcescens. All the 25 *S. marcescens* strains studied were beta-lactamase producers (Table I). There were minor differences in the onset and intensity of colour development. The very strong beta-lactamase producer strains had high (>200 μg/ml) MIC values even against the cephamycins. The majority of these strains displayed variable sensitivity to the third generation cephalosporins. The beta-lactamases of *Serratia* strains diffused better at neutral and alkaline pH of the media. Strains Nos 572, 584, 586, and 587 were red pigment producers which in most media made evaluation difficult and less realistic.

Other Enterobacteriaceae and Acinetobacter. The so-called "rare" *Enterobacteriaceae* (like *Citrobacter*, *Providencia* and *Arizona*) as well as *Acinetobacter* are strong beta-lactamase producers. Table I shows the results obtained. The strongest colour-producing strains were Nos 317, 276, 364, and 600. Strain No. 439 secreted a viscous material, similar to dextran. The enzymes were equally active and diffusible at acidic, neutral and alkaline pH values, explaining their high degree of resistance to the available beta-lactam antibiotics, including most of the third generation compounds.

Pseudomonas aeruginosa. All the ten *P. aeruginosa* strains included in this study and selected according to their varying (4–500 μg/ml) MIC values to carbenicillin, turned the colour of the chromogenic cephalosporin 87/312 red or pink in almost all media (Table I). This colour change, reflecting the presence of beta-lactamase, was independent of the MIC's of these strains to carbenicillin. In the case of *P. aeruginosa* strains, the presence and expression of beta-lactamase activity appeared to be not entirely a beta-lactam susceptibility-related phenomenon. The strains produced pigment materials characteristic of the *Pseudomonas* genus. It was an interesting observation that even the green or blue colour was overshaded by the very deep purple colour developed with nitrocefin. Many pseudomonas tended to grow in pellets in liquid media. These pellets usually became red first, indicating the predominantly cell-bound location of the beta-lactamase (and also the participation of beta-lactamase of initial cell wall synthesis); and the liquid medium turned pink or red only later. The known insensitivity of *P. aeruginosa* to most beta-lactam antibiotics is a much more complex phenomenon and cannot be reduced only to beta-lactamase production of the strain.

Streptococcus faecalis. None of the 14 *S. faecalis* (enterococcus, group D streptococcus) strains changed the colour of the chromogenic cephalosporin compound 87/312 (nitrocefin) in any media. The disturbing insensitivity of strains of this genus to most beta-lactams esp. cephalosporins rests on mechanisms other than beta-lactamase production.

Neisseria gonorrhoeae and *Haemophilus influenzae* strains need blood constituents for growth. Such medium is the chocolate (or any other fortified) agar. It is too coloured to be used for direct test with nitrocefin. For these organisms, the so-called "cell suspension" method was used as specified by the supplier of nitrocefin (Glaxo). These cell-suspensions are colourless and the colour-change with nitrocefin is conspicuous.

The *N. gonorrhoeae* strains which are penicillin G sensitive (MIC data provided by Dr. I. Zajac) did not give the colour reaction with nitrocefin. The resistant strains with high MIC values (3.1–25 μg/ml) immediately produced a deep purple colour with nitrocefin, indicating the presence of large amounts of beta-lactamase. These resistant strains were designated as plasmid component containing strains (Dr. S. Falkow). This finding had therapeutic consequences for treatment with beta-lactams of infections caused by beta-lactamase producing *N. gonorrhoeae* strains. They appeared as a threatening public health problem. Fortunately most of the third generation cephalosporins (cefotaxime, ceftizoxime, ceftriaxone) were highly effective against these strains.

In the case of the eighteen *H. influenzae* strains studied, the MIC values for ampicillin were in good correlation with the colour-production of nitrocefin (Table I). The sensitive strains (MIC's 0.2–0.8 μg/ml) did not produce the colour reaction whereas the resistant strains (MIC's 6.2–50 μg/ml) developed an immediate and deep purple colour with nitrocefin, indicating the beta-lactamase origin of resistance.

Selected assay strains used for screening. Table II demonstrates the results of the beta-lactamase test with nitrocefin (chromogenic cephalosporin 87/312) with strains we used to apply for specific bioassay purposes. They included Gram-positive and Gram-negative strains, both wild and genetically manipulated ones. All these strains produced a certain degree of colour. *S. aureus* 910 was a strong beta-lactamase producer and an unusual staphylococcus since it grew in/on synthetic media, unlike the typical staphylococci. The presence of beta-lactamase in the extensively used assay strain, *B. subtilis* ATCC 6633, was a surprise and has therefore been studied in considerable detail [39].

Assay strains for new beta-lactam antibiotics. Table III shows the results of the colour reaction for beta-lactamase activity of 15 bacterial strains which we used for primary assay of beta-lactam antibiotics. The list of these test strains was changed somewhat on the basis of the experiments reported in this paper. The results are those expected from other similar experiments including these strains and they positively corroborated the findings. *S. aureus* Villaluze is a methicillin-resistant strain with strong beta-lactamase activity. The methicillin-resistant strains may or may not be beta-lactamase producers. This type of resistance is independent from the enzyme production.

Table III

Colour reaction for beta-lactamase of the 15 selected bacterial strains regularly used for testing beta-lactam antibiotics using nitrocefin

Strains	TSB	TSA	SB	SA	PGA	PGA-B	PGB-B
S. aureus 127	++	+++	d	No growth	++	++	++
S. aureus 23390							
S. villaluze (meth. res.)	++++	++++	++	++		+++	+++
S. faecalis 34358							
E. coli 12140	+	+	d	+		d	
E. coli 33779	++++	++++	+++	+		+	+++
K. pneumoniae 4200							
K. pneumoniae 1200	++++	++++	+++	+++		+++	++++
S. paratyphi 12176							
P. mirabilis 444							
P. aeruginosa 63	+	++	++	++	++	++	++
S. marcescens 13880	++++	++++	+++	++++		++	+++
P. morganii 179	++	+++	d			++	++
E. aerogenes 13048	++	++	++	++	+	+	++
E. cloacae 31254	++++	++++	++++	++++	+	++	+++

TSB = Trypticase–soy broth; TSA = Trypticase–soy agar; SB = Synthetic broth; SA = Synthetic agar; PGA = Peptone–glucose agar; PGA-B = Peptone–glucose agar buffered; PGB-B = Peptone–glucose broth buffered. ++++ = Immediate and strong red colour; +++ = Colour within 5 min; ++ = Colour within 30 min; + = Colour within 1–3 h; d = Delayed (hours or overnight) colour development

Assay strains for new antibiotics. Table IV demonstrates the results of the beta-lactamase test by the colour reaction with compound 87/312 using the 15 microbial strains employed at that time for the primary assay of fermentation specimens. The bacterial strains which had not been included in any of the genera used for the study of expanded spectra were, *Mycobacterium phlei* 1228 (strong colour, i.e., beta-lactamase producer) and *Salmonella gallinarum* 9184 (no colour, not a beta-lactamase producer). The other bacterial strains elicited reactions as expected. An interesting finding was that *Candida albicans* 759 and also *Trichophyton mentagrophytes* 1258 strains were weak colour producers. For *C. albicans* No. 759, this observation has been corroborated [40]. The meaning and significance of colour-production and the corresponding beta-lactamase function in these and other fungal (yeast) strains remain to be established. It is known for streptomycetes but they are classified as bacteria.

Discussion

In the classification and characterization of beta-lactam antibiotics, their stability to bacterial beta-lactamases plays a paramount importance in addition to their spectrum, pharmacokinetic and pharmacodynamic prop-

Table IV

Colour reaction for beta-lactamase of the 15 selected microbial strains regularly used for testing fermentation specimens with nitrocefin

Strains	PSA	TSB	TSA	SA	PGA	PGA-B	PGB-B
S. aureus 127	++	++	++	No growth	++	++	++
S. aureus 910	++++	++++	++++	+++	++++	++++	++++
S. faecalis 34358							
M. phlei 1228	++++	++++	No growth	++++	+	+++	+++
P. mirabilis 444							
E. coli 12140	d	d		d			+
K. pneumoniae 4200							
S. gallinarum 9184							
P. aeruginosa 63	++	++	++	++		d	++
S. marcescens 13089	++++	++++	++++	++++		++++	++++
P. morganii 179	++	+++	++++	d		++	++
Providencia 276	+++	+++	++++	+++	+++	+++	++++
E. cloacae 31254	+++	++++	++++	+++	+++	+++	++++
C. albicans 759						d	d
T. mentagrophytes 1258					d	d	d

PSA = Penassay seed agar; TSB = Trypticase–soy broth; TSA = Trypticase–soy agar; SA = Synthetic agar; PGA = Peptone–glucose agar; PGA-B = Peptone–glucose agar buffered; PGB-B = Peptone–glucose broth buffered. ++++ = Immediate and strong red colour; +++ = Colour within 5 min; ++ = Colour within 30 min; + = Colour within 1–3 h; d = Delayed (hours or overnight) colour development

erties [12]. Therefore, the production of or the presence in the microbial growth of beta-lactamase(s) is an important parameter in the search for new beta-lactam antibiotics as well as in the clinical microbiology laboratory and rational therapy of infections. There are many methods for the detection and estimation of bacterial beta-lactamases. The chromogenic cephalosporin compound 87/312 (Glaxo) allows a rapid detection and quantitation of beta-lactamase activity. The highly purified preparation is available under the name of Nitrocefin. Since the original description of this compound and method [25], many publications confirmed the usefulness and reliability of this technique [41–46]. Using this novel method, we examined the beta-lactamase activity of a host of bacterial and a few fungal strains and found that the colour produced by the presence of beta-lactamase(s) was in good agreement with previous resistance studies. It is also true that the presence of beta-lactamase(s) not always and not arbitrarily signify beta-lactam resistance of a given microbial strain but provides a good indication for this possibility.

There are strains (or genera) the resistance of which is not beta-lactamase related (*S. faecalis* and many methicillin resistant *S. aureus*). There is a balance between the amount of available enzyme (rate of antibiotic inactivation) and survival of the microbial population in the continuous presence and sufficient amount of a beta-lactam antibiotic under in vitro conditions. In vivo the problem is usually even more complex. Nevertheless, the data of these experiments were instrumental in selecting appropriate strains (strong and weak beta-lactamase producers and non-producers in the same genera) for inclusion in the primary assay of beta-lactam compounds, for detailed studies of compounds and also for the evaluation of lytic properties of beta-lactams as well as for searching for and testing of beta-lactamase inhibitors, as shown in Fig. 3 [35, 47]. In addition to the utilitarian (selection of proper beta-lactams for therapy) applications, the present results appear to provide some experimental evidence to the basic question concerning the classification and role of beta-lactamase in the biology, physiology and biochemistry of microbes [10, 48–52].

The data presented here, together with others' observations, are convincing that nitrocefin (chromogenic cephalosporin substrate 87/312) [25] provides an elegant, rapid, selective and reliable tool for detecting beta-lactamase activity of bacteria in serum, urine or body fluid-free environment. [53]. It is available in diagnostic disk form.

We have only limited experience with the use of cephacetrile (Celaspor®, C 36278-Ba) for the same purpose [26–28]. The violet-reddish colour formed is striking, but its slow and often spontaneous development raises the suspicion that the reaction may not be specific. It offers no real advantage over nitrocefin. We have no personal experience with pyridium-2-azo-p-dimethylaniline cephalosporin (PADAC) as a chromogenic reagent for rapid beta-lactamase testing [29, 30]. When the side chain at 3-position is released upon the opening of the beta-lactam ring (hydrolysis by beta-lactamase) the colour changes from purple to yellow. It has been reported that the colour change takes time, the reaction is specific and not influenced by the protein content of the specimens. Now it is available in the form of filter paper strip as a convenient laboratory diagnostic tool to detect beta-lactamase production of microorganisms.

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VIRAL DNA SEQUENCES IN HUMAN CYTOMEGALOVIRUS TRANSFORMED HAMSTER CELL LINE AT LOW PASSAGE LEVELS

I. Boldogh, B. Brichacek, Éva Gönczöl, I. Hirsch and L. Váczi

Institute of Microbiology, University Medical School, Debrecen, Hungary, and Department of Experimental Virology, Institute of Sera and Vaccines, Prague, Czechoslovakia

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The characteristics of a human cytomegalovirus (HCMV) transformed syrian hamster cell line (87-TRH-5) were examined. The cytomegalovirus DNA (HCMV-DNA) labelled in vitro by nick translation was used as a probe to detect viral DNA sequences in the 87-TRH-5 line. Cytomegalovirus specified DNA sequences were detected in cells examined up to passage 58, but were undetectable at higher passage levels. Six clones were derived from the 28th passage of 87-TRH-5 cells and examined for HCMV-DNA sequences. Various amounts of HCMV-DNA were found. A tumour induced by passage 28 cells contained no detectable HCMV-DNA sequences.

Human cytomegalovirus is an ubiquitous pathogen with a variety of clinical manifestations [1]. Recent evidence suggests that HCMV may be oncogenic. HCMV infected human fibroblasts will grow in agar but eventually undergo lysis [2]. HCMV has been isolated from a paraganglioma [3] and from Kaposi's sarcoma [4]. A culture of human prostate cells derived from a HCMV infected child released a strain of HCMV spontaneously [5]. It is difficult to establish a correlation between HCMV and tumour development, although an association has been discovered between HCMV and various types of human neoplastic disease [6, 7]. The oncogenicity of HCMV is supported by several studies which establish the ability of the virus to stimulate host cell DNA synthesis [8–10]. It has been demonstrated that HCMV can transform cells in vitro [11–13]. It was previously shown that infection of hamster embryo fibroblast cells with UV irradiated HCMV resulted in the appearance of morphologically transformed cells. The multilayered foci were isolated and, after cloning, a continuous cell line (87-TRH-5) was developed [12]. In the present paper, we describe experiments based on detection of the HCMV genome in the 87-TRH-5 cell line by DNA-DNA reassociation kinetics. It is shown that the amount of HCMV-genome in these transformed cells gradually decreases to undetectable levels.

István Boldogh, Éva Gönczöl, Lajos Váczi
Institute of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

Beda Brichacek, Ivan Hirsch
Department of Experimental Virology, Institute of Sera and Vaccines
103 Wilhelm-Pieck Street, 101 03 Prague 10, Czechoslovakia

Materials and methods

Cell culture. The 87-TRH-5 transformed cell line has already been described [12]. The growth medium consisted of Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.125% $NaHCO_3$.

The hamster embryonic fibroblast cells were prepared from 14-day-old hamster embryos and propagated in the medium described above.

Human embryonic lung (HEL) cells were obtained from aborted human embryos, and cultivated in MEM described above.

Virus and virus propagation. The HEL cell monolayers were infected with human CMV strain AD-169 at a multiple of 0.2 PFU per cell. The virus was absorbed for 2 h at 37 °C and the medium was changed. Twenty hours after infection 5 μCi/ml of $(H)^3$ TdR (spec. act. 17.5 Ci/mM, UVVVR) was added to the medium to label the viral DNA. The virus was harvested from the extracellular fluid 12 days after infection.

Preparation of extracellular virus. The virus from the extracellular fluid was concentrated by ultracentrifugation and purified by sedimentation through linear sucrose and CsCl gradients as described by Huang et al. [14].

Preparation of viral DNA. The resuspended virus was treated with self-digested nuclease-free pronase (Calbiochem, EP grade), 1 mg/ml, in the presence of 1% sodium lauryl sulphate (BDH, specially pure) at 37 °C for 3 h. The DNA was purified by centrifugation to equilibrium in CsCl in MSE (8 × 14 rotor) at 30 000 rpm at 20 °C for 65 h. The radioactive fractions were collected and rerun in a second CsCl gradient. The DNA was dialysed against TNE, precipitated with ethanol and after centrifugation dissolved in 0.1 × SSC. This viral DNA was used for the hybridization tests.

Radioisotope labelling of HCMV-DNA. Purified HCMV-DNA was labelled with α^{32}P-triphosphate by nick translation as described by Rigby et al. [15]. Briefly, the labelling of HCMV-DNA with thymidine 5'α^{32}P-triphosphate (Radiochemical Centre, Amersham, England, spec. activity: 350 Ci/mmol) was done by repair synthesis of the virus DNA with *Escherichia coli* polymerase I. (Boehringer, Mannheim GmbH, West Germany) in the presence of DNase (Sigma, EP grade). The reaction mixture contained 50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol (DBH) and 0.1 mM cold d-ATP, d-CTP, d-GTP (Sigma Chemical Co.). The labelled HCMV-DNA was then separated by chromatography on a 14 ml column of Sephadex G-50/Fine (Pharmacia). The specific activity of ^{32}P-labelled HCMV-DNA was calculated to be 5.4×10^7 cts/min per μg.

Extraction and preparation of cellular DNA. The cell monolayers were scraped and washed in TNE (0.01 M Tris–HCl, pH 7.9, 0.01 M EDTA, 0.15 M NaCl). The cell suspensions were treated with selfdigested nuclease-free pronase (Calbiochem), 1 mg/ml, in the presence of 1% sodium dodecyl sulphate (SDS, BDH specially pure) at 37 °C for 10 h and the cell DNA was purified further according to Frenkel et al. [16].

DNA-DNA reassociation kinetics. The hybridization test was carried out as previously described by Frenkel et al. [16]. The sheared ^{32}P-labelled HCMV-DNA was mixed with control DNA's in the presence of known amounts of non-labelled HCMV-DNA. The DNA concentrations are given in the Figures.

The mixture in volumes of 25 μl were sealed in glass microcaps, denatured at 115 °C for 7 min and then incubated at 63 °C for various lengths of time. The hybridizations were done in solution containing 0.1 M Tris-HCl, pH 8.1, 0.025 M EDTA, 1 M NaCl and 20% (vol/vol) formamide (Sigma Chemical Co.).

The fractions of double stranded DNA's were determined by using single-strand-specific nuclease of *Aspergillus oryzae* as described by Frenkel et al. [16].

Calculations. Reassociation data were evaluated by using the equation

$$\frac{1}{\text{fss}} = \text{k} \times \text{Co}_\text{t} + 1,$$

derived by Britten and Kohne [17] and Biegeleisen and Rush [18], which describes the reassociation kinetics of randomly sheared DNA when duplex formation is measured by S_1 nuclease resistance. In this equation, fss is the fraction of probe DNA sequences remaining single stranded at time t, k is the second order rate constant and Co is the initial concentration of DNA nucleotides. The N value was defined as the ratio of genome equivalent of cell test DNA to genome equivalent of viral probe DNA in the hybridization mixture [16]. The molecular weight of the HCMV genome was assumed to be 1.5×10^8 [19, 20].

Results

History of the 87-TRH-5 cell line. In an earlier study, we reported that transformation of HEF cells by HCMV produced a tumourogenic cell line [12]; its history is shown in Fig. 1. The original cell line was examined at passages 28, 58, 76 and 141. Six subclones derived from passage 28 cells were examined at passage 45. Tumours produced by passage 28 cells were analysed also by DNA-DNA reassociation kinetics. HCMV specific antigens could be detected

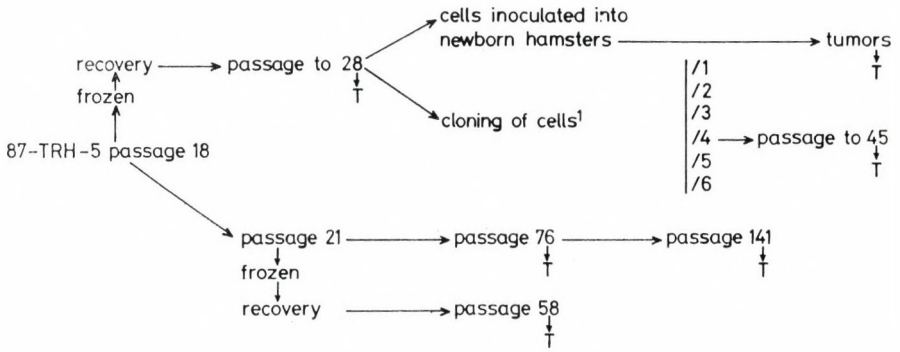

Fig. 1. In vitro history of the 87-TRH-5 cell line originally derived from hamster embryo fibroblasts transformed by HCMV. Six clones were isolated at passage 28 (87-TRH-5 per 1, 2, 3, 4, 5, 6) and analysed at passage 45. The passage 28 cells were injected into newborn hamsters. The DNA extracted from developed tumours and clones of 87-TRH-5 cells were analysed by DNA-DNA reassociation kinetics. T: Tested for detection of HCMV-DNA

in the cells by immunofluorescence studies using anti HCMV IgM antibodies even in passage 58 cells [12]. The cells were also positive for membrane antigen by antibody mediated cytotoxicity detected by a ^{51}Cr release assay (Boldogh and Kutinova, unpublished data).

Properties of ^{32}P-labelled probe DNA. In the present study, the viral DNA probe was prepared from culture fluid of cells infected with HCMV (AD-169). Low multiplicities of infection were used (about 0.2 PFU/cell) to avoid accumulation of defective DNA molecules. Before using the ^{32}P-labelled HCMV-DNA as a probe, we checked it for contamination by host DNA sequences. The probe DNA was mixed with human embryonic lung (HEL), hamster embryonic fibroblast (HEF) and calf thymus (CT) DNA. For calibration, the probe was allowed to reassociate in the presence of 7.2 mg/ml host DNA; plus 10^{-4} mg/ml (0.4 HCMV genome per diploid cell) or 10^{-3} mg/ml (4 HCMV genome per diploid cell) unlabelled HCMV-DNA. The mixture was denatured at 115 °C for 7 min into single strands and the rate of conversion of the label into double-stranded form was followed, using single strand specific S_1 nuclease.

The resulting control and calibration curves are shown in Fig. 2. In the absence of added cold viral DNA, the renaturation of probe sequences proceeded at a very slow rate, indicating no contamination by host cell DNA sequences (Fig. 2, open symbols). In calibration experiments, the rate of annealing of probe into double-stranded form was proportional to the con-

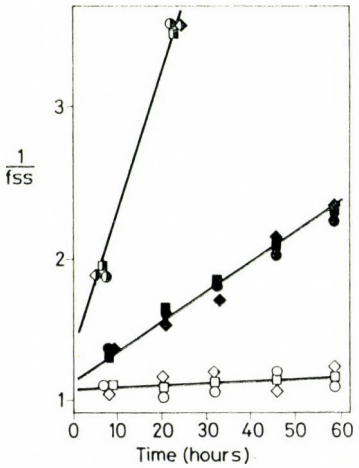

Fig. 2. Renaturation kinetics of probe DNA in the presence of cell DNA and various concentrations of unlabelled HCMV-DNA. Reassociation kinetics of the ^{32}P-labelled HCMV-DNA probe (1.620×10^{-6} mg/ml) were measured in the presence of 7.3 mg/ml HEL DNA alone (□), with 10^{-4} mg/ml (■) and 10^{-3} mg/ml (◨) unlabelled HCMV-DNA; 7.2 mg/ml HEF-DNA alone (○), with 10^{-4} mg/ml (●) and 10^{-3} mg/ml (◑) unlabelled HCMV-DNA; 7.2 mg/ml CT-DNA alone (◇), with 10^{-4} mg/ml (◆) and 10^{-3} mg/ml (◈) unlabelled HCMV-DNA. N value = 29.6 (7.2 mg/ml host cell DNA plus 10^{-4} mg/ml unlabelled HCMV-DNA = 0.4 HCMV genome per diploid cell)

centration of unlabelled HCMV-DNA added to the hybridization mixture. A plot of 1/fss (t) versus Co_t resulted in a straight line with slope k, indicating that the entire viral genome was represented in the probe.

Detection of HCMV-DNA in low passage 87-TRH-5 cells. The 87-TRH-5 cells were frozen at passages 18 and 21. After some months they were resuscitated and grown until passages 28th and 58th, respectively. DNA extracts of these cells were examined by DNA-DNA reassociation kinetics as shown in Fig. 3. Passage 28 cells contained the equivalent of 35×10^{-6} mg HCMV-DNA per mg of cell DNA, corresponding to about 1 HCMV genome equivalent per diploid cell. Passage 58 cells, however, contained only 6×10^{-6} mg HCMV-DNA per mg of cell DNA (about 0.18 genome equivalent per diploid cell). If a single passage corresponded to three cell generations, the 87-TRH-5 cells had carried at least one complete HCMV genome for 84 generations. After a further 90 cell generations the amount of HCMV-DNA sequences had

decreased to 0.18 genome equivalent per cell. The observed decrease might represent an actual loss of HCMV DNA sequences from the cell or it might be simply due to a decrease in the proportion of HCMV DNA containing cells in the culture at late passages. It was, therefore, decided to test individual clones of early passage cells to see whether HCMV-DNA sequences occurred in every clone. Clones were isolated from passage 28 of the 87-TRH-5 cell line (Fig. 1) and propagated until passage 45. DNA isolated from each clone was analysed by DNA-DNA reassociation kinetics. Results are summarized in Table I. The amount of HCMV-DNA varied from 9.7×10^{-6} to 19.1×10^{-6} mg

Table I

Analysis of Cloned Derivations of 87-TRH-5 Cell for HCMV-DNA Sequences by DNA-DNA Reassociation Kinetics[1]

Clones	Passage number	Amount of HCMV-DNA per mg 87-TRH-5 DNA[2]	HCMV genome per diploid cell
87-TRH-5/1	45	19.1×10^{-6} mg	0.54
87-TRH-5/2	45	9.7×10^{-6} mg	0.27
87-TRH-5/3	45	12.5×10^{-6} mg	0.35
87-TRH-5/4	45	18.3×10^{-6} mg	0.52
87-TRH-5/5	45	12.7×10^{-6} mg	0.36
87-TRH-5/6	45	14.9×10^{-6} mg	0.42
HEF[3]	12	14.0×10^{-6} mg	0.40

[1] The reassociation was allowed to proceed for 158 h

[2] The reassociation of ^{32}P-labelled probe (0.81×10^{-6} mg/ml) was measured in the presence of 3.6 mg/ml DNA from individual clones of cell

[3] Probe 0.81×10^{-6} mg/ml was reassociated in the presence of a mixture containing 3.6 mg/ml HEF cell DNA plus 0.5×10^{-4} mg/ml unlabelled HCMV-DNA

HCMV-DNA per 1 mg 87-TRH-5 cellular DNA. We have no evidence to suggest that only some HCMV-DNA sequences had been selected since there was no deviation from the theoretical straight line plots in the case of passage 58 cells (Fig. 3). We have no explanation for the ability of the entire virus genome to persist in the cells up to 84 generations and to be lost to an undetectable level only in later generations.

Failure of detection of HCMV-DNA sequences in the high passages of 87-TRH-5 cells and tumours. The 87-TRH-5 cells were cultivated until 141 passages. At various passage levels (passage 76, 141) cell DNA was isolated and tested for presence of HCMV-DNA sequences. Neonatal hamsters were inoculated with 10^6 87-TRH-5 cell (passage 28) per animal. After six weeks, tumour developed. The tumour cell DNA was extracted and tested. Results

are shown in Fig. 4. No HCMV-DNA sequences were detected. Even the use of higher N values (41.2) and longer reassociation time (1–218 h) revealed no viral sequences. We could not for the time being discriminate between two alternatives: that the entire HCMV genome was lost from the cells, or, the cells had retained a set of HCMV sequences, which was insufficient for detection using the entire DNA as a probe.

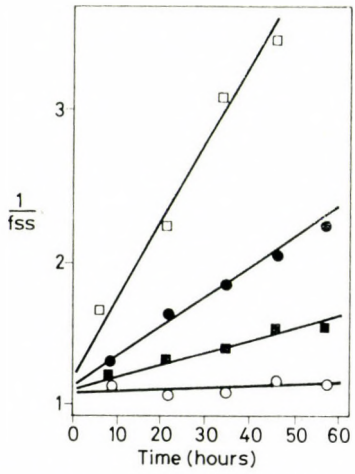

Fig. 3. Renaturation of probe DNA in the presence of DNA extracted from 87-TRH-5 cells at low passage levels. The reassociation of ^{32}P-labelled HCMV-DNA (1.620×10^{-6} mg/ml) was followed in the presence of 7.2 mg/ml 87-TRH-5 DNA at passage 28 (□), and 58 (■), and in the presence of 7.2 mg/ml HEF-DNA alone (○), or with 10^{-4} mg/ml unlabelled HCMV-DNA (●). N value = 29.6

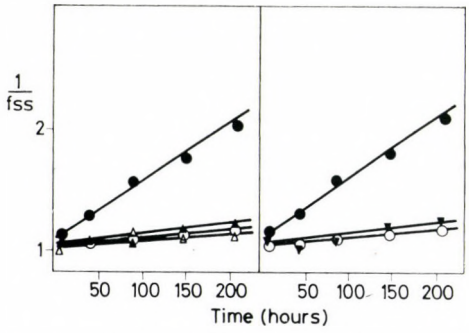

Fig. 4. Renaturation of probe DNA in the presence of DNA extracted from 87-TRH-5 cells at high passage levels (A) and from tumour (B). Reassociation of probe DNA (1.620×10^{-6} mg/ml) was measured in the presence of DNA extracted from 87-TRH-5 cells at passages 76 (△), 141 (▲) and from tumour (▼). Probe DNA at a concentration of 1.620×10^{-6} mg/ml was allowed to renature in the presence of HEF-DNA alone (○) or a mixture containing HEF-DNA and unlabelled HCMV-DNA (0.4 HCMV genome per diploid cell) (●). 87-TRH-5 and tumour DNA as well as HEF-DNA were present at a concentration of 10 mg/ml. N value = 41.2

Discussion

The results presented indicate that hamster embryo fibroblast cell transformed by UV-irradiated HCMV retain viral DNA sequences up to passage 58 but later passage cells contain little or no detectable virus-related DNA sequences. No viral DNA sequences could be demonstrated in a tumour produced in a hamster by passage 28 cells. Passage 28 cells contained about one complete viral genome per cell. Until passage 58, however, the amount of viral DNA had been reduced to 0.18 genome per cell. Whether these fractional values were due to some passage 58 cells containing a complete genome or to every cell containing only part of the viral DNA, is not clear. Since the clones derived from passage 28 cells contained viral DNA when examined at passage 45, it seemed likely that the original cell population consisted uniformly of cells with viral genome. This implies that viral DNA was lost from cells during later passages and suggests that passage 58 cells are a heterogeneous population containing an average 0.18 genome per cell.

The question is why and how the cells retained the HCMV-DNA for prolonged periods and what role the viral DNA sequences have in the induction and maintenance of the transformation. First, with the other herpesviruses there are evidences of the HCMV being able to persist in a latent form within the infected cells in vivo and in vitro [5, 21–26]. The latent infection led to the production of certain viral antigens but there was no viral DNA synthesis or detectable virus replication [21, 24, 25]. Indeed, a high percentage of the 87-TRH-5 cells exhibited viral antigens in low passage levels [12]. The detectability of this (these) specific antigen(s) decreased during serial passages but it was possible to produce human cytomegalovirus–vesicular stomatitis virus (VSV) pseudotypes by VSV infection of 87-TRH-5 cells in passage 135 [26]. The form of the latent viral DNA is probably episomal, since we could not detect covalent interaction between cellular and viral DNA by using density centrifugation and blot hybridization technique at passage 36 (unpublished data).

Transformation of NIH 3T3 and primary rat embryo cells with DNA fragment of HCMV (strain AD-169), identified about 2.9 kilobases [27], has been demonstrated recently. Furthermore, the HCMV-DNA (strains Towne and AD-169) is representing sequence homology to the 5' half of viral and 5' half of human c-myc [28]. The role of HCMV-DNA in transformation of normal cells is unknown, since viral-DNA homologous sequences were not detected in the HCMV-transformed cells [27]. The virus might have been involved in the initial transformation step, but the continued presence of viral sequences is not essential for the maintenance of the transformed state.

The 87-TRH-5 cell line produced a tumour when inoculated into a newborn hamster. When this tumour was tested for HCMV-DNA sequences, the

result was negative: no virus-specific sequences could be detected by renaturation kinetics. It is most likely that the viral DNA content of the tumour cells decreased to undetectable levels during tumour development. It is conceivable that cells having some HCMV antigens were selected in the hamster as a consequence of an immune response. The serum of the tumour bearing hamster showed specific neutralization activity to HCMV (unpublished data).

The results suggest that the role of HCMV in oncogenic cell transformation may be different from that of other DNA tumour viruses [29, 30], where a continued presence of the viral DNA is required.

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PATHOGENICITY AND VIRULENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*: SLIME LAYER PRODUCTION*

F. Rozgonyi and G. Seltmann

Institute of Microbiology, University Medical School, Debrecen, Hungary, and Institute for Experimental Epidemiology, Wernigerode, GDR

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Nine methicillin-resistant (MR) mutants and three methicillin-sensitive (MS) substrains, all derived from naturally occurring heteroresistant isolates of *Staphylococcus aureus* were examined for slime production. All strains showed an increased mucoid character when cultured on a modified Staphylococcus Medium No. 110. The uranic acid content of the slime layer ranged from 2% to 6% in the MR mutants and from 1.3% to 5.1% in the MS substrains. The amount of uronic acid per g of dry bacteria ranged from 82.5 mg to 143.8 mg in the MR mutants, and between 51.4 mg and 98.8 mg in the MS substrains. In 3 pairs of MR mutants and MS substrains originating from the same parents, the MR cells possessed more of uronic acid than their MS counterparts. The partially purified polysaccharide part of the slime contained D-galactose, D-mannose, D-xylose, D-galacturonic acid, D-galactosamine and D-glucuronic acid in all strains studied. Its quantitative composition was identical in each pair of the MR mutant and MS substrain; there were, however, considerable differences among the strains and between the pairs. Methicillin resistance and slime formation seem to be independent properties in *S. aureus*. The presumable significance of the readiness of slime production by MR cocci during infections is discussed.

Methicillin resistant (MR) strains of *Staphylococcus aureus* have been shown to consist of mixed populations in which only one out of 10^4–10^5 cocci is highly MR and stable in resistance. The MR mutants grow significantly slower both on solid [1–7] and in liquid media [8] than the wild-type strains or the congenic methicillin sensitive (MS) counterparts. From a part of these data it has been hypothesized that MR *S. aureus* strains appear to be clinically unimportant because most of these cocci may be destroyed by methicillin (MET) in vivo, and that any surviving cocci may be avirulent because of their slow growth-rate [9]. However, treatment of MR *S. aureus* infections with MET or other penicillinase-stable beta-lactams failed to give satisfactory results in each case [10–17], claiming against the prediction mentioned. The fact that the MR *S. aureus* has become wide-spread in many hospitals of numerous countries [14–19] including our university hospitals with 10–14% incidence of MR *S. aureus* isolates, indicates the MR *S. aureus* strains to

Ferenc Rozgonyi
Institute of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

Guntram Seltmann
Institute for Experimental Epidemiology
Burgstrasse 37, DDR-3700 Wernigerode (Harz), GDR

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possess such pathogenic properties that can overcome the slow growth rate as their disadvantageous virulence factor.

It is now generally accepted that slime layer production of *S. aureus* contributes to its virulence [20–26]. The purpose of the present communication is to show the ability of highly MR *S. aureus* mutants to produce slime layer as one of the virulence factors. According to Caputy and Costerton [27] slime induced by in vivo passage appeared similar to that induced by modified Staphylococcus Medium 110.

Materials and methods

Organism. Twelve strains of *S. aureus*, 9 highly MR mutants and 3 MS substrains, were examined. Both the MR mutants and the MS substrains were derived from naturally occurring *S. aureus* strains consisting of heteroresistant populations. The selection of MR mutants and the separation of MS substrains from the parents were carried out as described [28]. The strains were maintained by monthly transfer on nutrient agar slants free of MET stored at room temperature between transfers. The strains used are listed in Table I.

Antibiotic susceptibility tests. All strains were tested by the disk method as described in reference [29] against 3 IU of benzyl-penicillin (PEN), 20 μg ampicillin (AMP), 20 μg methicillin (MET), 10 μg oxacillin (OXA), 50 μg carbenicillin (CAR), 10 μg cephaloridine (CFR), 30 μg streptomycin (STR), 30 μg kanamycin (KAN), 100 μg neomycin (NEO), 20 μg gentamicin (GEN), 20 μg tobramycin (TOB), 30 μg oxytetracycline (TET), 30 μg nalidixic acid (NAL), 30 μg chloramphenicol (CMP), 10 μg erythromycin (ERY), 30 μg oleandomycin (OLE), 30 μg spiramycin (SPI), 50 μg vancomycin (VAN), 30 μg novobiocin (NOV), 10 μg lincomycin (LIN), and 300 μg nitrofurantoin (NIT). All but two disks were purchased from Human, Budapest, Hungary. GEN and TOB disks were made by us, from the substances of Biogal, Debrecen, Hungary.

Tube susceptibility tests were carried out using twofold dilutions of MET (Chinoin, Budapest, Hungary) from two stock solutions of different concentrations in Difco Casitone broth in the presence and in the absence of 1% glucose and in modified Staphyloccus 110 broth [20] as described previously [28]. Difco Casitone broth consisted of 10 g Bacto-Casitone (Difco), 5 g NaCl, 5 g $Na_2HPO_4 \cdot 12\ H_2O$, 0.03 g $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 0.01 g $MgSO_4 \cdot 4\ H_2O$, 0.01 g $MnSO_4 \cdot 7\ H_2O$ and distilled water up to 1000 ml, pH 7.2.

Phage typing. This work was kindly done by Dr. L. Ér in the Hajdú-Bihar County Public Health Station, Debrecen, using the method of Blair and Williams [30] according to the instructions of the National Institute of Hygiene, Budapest, Hungary.

Production of slime. The organisms were grown in 150 ml volumes of modified Staphylococcus 110 broth [20] at 37 °C for 18 h without shaking. Roux or Sial bottles of 1 litre containing 200 ml of modified Staphylococcus 110 agar [20] were then inoculated with 40 ml of the broth culture and incubated again at 37 °C for 20 h. For each strain 3 bottles each from 2 batches of the medium were used.

Demonstration of slime. For this purpose the following procedure was developed. A loopful of growth was smeared on a coverslip which had previously been cleaned with detergent and then methanol. After allowing the smear to air-dry, a slight heat-fixing was done. One or two drops of patent blue V (Guerbet, France, 2.5% solution for lymphography) were dropped on the smear, then it was mounted with a concave glass slide, and sealed with a heated paraffin–vaseline mixture. After quick reversal, the bacteria were examined by a light microscope through the opposite side of the slip. The slime appeared as a clear wide halo that surrounded the dark blue bacterial cell or lay between the bacterium and the surrounding homogeneous light blue solution of patent blue.

Preparation of slime layer. Following incubation, the cells and slime layer materials were loosened from the surface of the agar by gently agitating the bottles, and removed with a pipette. The surface of the agar was washed off with 40 ml of 0.85% NaCl solution pH 7.2, and the cell suspensions were combined.

The crude slime layer was prepared as described previously [31, 32]. The yields of slime material from the 2 batches of agar were combined.

Partial purification of the crude slime was carried out as follows. One g of the crude material was dissolved in 50 ml of ice-cold distilled water and then 25 ml of a 5 : 1 (v : v) mixture of chloroform and n-butanol was added. The solution was stirred with an Ultra-Turrax (Janke and Kunkel KG, GFR) at full speed for 5 min, while the vessel was kept in an ice bath. Stirring was once repeated after an interruption of 5 min. The phases were separated by centrifugation at 7000 $\times g$ for 1 h at 4 °C, and the organic phase was discarded. To the water phase 5.0 ml of a saturated aqueous solution of sodium acetate was added. After chilling the solution in ice bath, the polysaccharide was precipitated by dropping 3–4 volumes of cold ethanol into the solution. The precipitate was collected by centrifugation at 12 000 $\times g$ for 10 min at 4 °C. The sediment was dissolved in about 1/4 the original volume, and precipitation was repeated. The sediment was dissolved in a small volume of water and then lyophilized [32, 33]. The final ethanol precipitate was designated partially purified slime [31].

Hydrolysis. Acidic hydrolysis of the samples was carried out in 0.1% solution in 4 N HCl, boiled for 4 h. Acid was removed in vacuo above NaOH. The residue was dissolved in pyridine [33].

Thin-layer chromatography. For one-dimensional TLC, 18 $\times$ 18 plates were covered with a 0.5 mm layer of cellulose powder MN 300 (Schleicher and Schuell, Düren, GFR) and dried. Ten μl of each hydrolysed sample was put on the plate and after evaporating the solvent development was done in pyridine–n-butanol–water (4 : 6 : 3). The carbohydrate compounds were stained with alkaline silver nitrate solution as described [34].

Thin-layer electrophoresis. This procedure was performed on plates prepared as described above, following one-dimensional TLC or without TLC or before TLC, in Desage Thin Layer Electrophoresis apparatus (Ref. No. 12 1200) using a buffer system (pH 5.2) of pyridine–acetic acid–water (20 : 9.5 : 970) at 20 V/cm for 30 min [33].

In order to identify the spots on the chromatograms the following standards were run: D-galacturonic acid and D-glucuronic acid (BDH, Laboratory Chemicals Division, England), D-galactosamine HCl and D-glucosamine HCl (GMA), galactose, glucose, mannose and xylose (Reanal, Hungary).

Estimation of uronic acids. Quantitative determination of total uronic acid content of the partially purified slime material was carried out by the carbazole method [35] using glucuronic acid (BDH) as standard.

Gas chromatographic analysis. The hydrolysed partially purified slime material was reduced by $NaBH_4$ and afterwards peracetylated by acetic anhydride [36]. The alditol acetates obtained in this manner were separated on 2 m columns of 1.5% OV 225 on Gas-Chrom Q 100–120 mesh as described earlier [33].

Results

Characterization of the strains. When someone wants to study some aspects of MET resistance in *S. aureus*, he meets two difficulties which have to be overcome. Firstly, almost all the naturally occurring MR isolates of *S. aureus* consist of mixed populations in which the frequency of the highly MR mutants is 1 to 10^4 or 10^5 cells. Therefore, the data of experiments with such isolates concern the susceptible majority of the population rather than the really MR ones. Secondly, many MR isolates seem to be in vivo subcultures of a single MR strain of *S. aureus*, and in such cases the results of the studies reflect only one heterogeneous MR strain although many have been examined.

In order to avoid both ambiguities we have used such highly MR mutants that had been selected from the parents and proved to be relatively stable in MET resistance after transfers on MET-free agar. Also they had to differ from one another in phage type and in antibiotic resistance pattern. As Table I indicates the 9 MR mutants seem to be really independent from each other.

Table I

Characterization of methicillin-resistant mutants and of methicillin-sensitive substrains

Strain	Sources			Selected at μg/ml of methicillin
	Hospital	Disease	Specimen	
1188R	Medicine I	chronic bronchitis	sputum	800
1969R	Paediatrics	acute nasopharingitis	cough discharge	400
2842R	Medicine II	chronic bronchitis	sputum	400
9965R	Paediatrics	pemphigoid	pus	800
10391R	Surgery I	purulent cholelithiasis	content of cholecyst	800
14929R	Medicine II	chronic bronchitis	sputum	800
2100R	Medicine II	chronic bronchitis	sputum	800
2100S				0
5814R	Paediatrics	pemphigoid	pus	500
5814S				0
13137R	See Jevons, M. P.: Br Med J *I*: 124 (1961)			12.5
13137S				0

Note. R = methicillin-resistant, S = methicillin-sensitive, RTD = routine test dilution of phages, N.t. = non-typable. Abbreviations of the antibiotics are listed in Materials and methods. All strains produced coagulase and yellow pigment, fermented mannitol, liquefied gelatin, showed type A haemolysis pattern, were crystal-violet negative and did not hydrolyse urea

On the contrary, each pair of the MR and MS substrains derived from three independent parents appears to possess an identical susceptibility to phages and to antimicrobials except beta-lactam antibiotics stable for lactamase (compare mutant 2100R with 2100S substrain, and 5814R with 5814S and 13137R with 13137S).

deriving from heteroresistant S. aureus strains tested for slime layer production

Antibiotic resistance pattern	Phage pattern	
	at RTD	at RTD × 100
PEN AMP MET OXA CAR CFR STR TET NAL CMP ERY OLE	83A+	77/83A/85/90/42B/89/92/Bovine 107
PEN AMP MET OXA CAR CFR STR TET NAL ERY SPI	89/90	42B/89/90
PEN AMP MET OXA CAR CFR TET NAL CMP ERY OLE	N.t.	90
PEN AMP MET OXA CAR CFR STR KAN NEO NAL CMP TET ERY OLE	90/92	90/92
PEN AMP MET OXA CAR CFR STR NAL ERY	N.t..	54/83A
PEN AMP MET OXA CAR CFR TET NAL ERY OLE	N.t.	77/83A
PEN AMP MET OXA CAR CFR STR TET NAL CMP ERY OLE	N.t.	54/77/83A/42B/89
PEN AMP STR TET NAL CMP ERY OLE		
PEN AMP MET OXA CAR CFR STR TET NAL CMP ERY	77	77/92/Bovine 117
PEN STR TET NAL CMP ERY		
PEN AMP MET OXA STR TET NAL	42E/47/53/54/75/83A/84/85+	42E/47/53/54/75/77/83A/84/85/42D/81/47C/47B/950/759/69/
PEN AMP STR TET NAL	47/53/54/75/77/83A/84/85+	42C/42B/89/377/92/Bovine 107/117

Production of slime. All the studied organisms showed a strikingly viscous type of growth on modified Staphylococcus 110 agar as compared with organisms grown on nutrient agar. When smears were made, a marked slime layer could be observed around the bacteria in all cases. It must be mentioned that a slight slime formation both on nutrient agar and in Difco Casitone broth supplemented with 1% glucose could also be demonstrated either by the method of Hiss or by our procedure.

The amount of slime produced by the strains was measured after partial purification and lyophilization of the slime layer, and was expressed as g of slime material per g of dry bacteria. As can be seen from Table II, the amount

Table II

Slime layer material and its uronic acid content of methicillin-resistant mutants and methicillin-sensitive substrains of heteroresistant S. aureus strains

Strain	g of slime material per g of dry bacteria	Percent of uronic acid in slime material	mg of uronic acid per g of dry bacteria
1188R	3.8	3.1	118.9
1969R	nm	4.5	nm
2842R	3.1	4.6	143.8
9965R	o.8	4.9	87.7
10391R	nm	4.2	nm
14929R	2.4	6.0	143.3
2100R	6.6	2.0	131.1
2100S	7.6	1.3	98.8
5814R	3.0	2.8	82.5
5814S	2.7	2.9	77.5
13137R	1.6	5.8	92.2
13137S	1.0	5.1	51.4

Note. Slime layer materials were partially purified and then lyophilised before measurement. nm = Dry weight of bacteria was not measured

of the partially purified slime varied considerably from strain to strain. Between members of the pairs of the MR mutants and the MS substrains there was, however, no significant difference in the amount of slime.

Since the uronic acid content reflects more exactly the real amount of slime, both the percentage of uronic acid in the slime and the amount of uronic acid per g of dry bacteria were estimated. The results are also shown in Table II. The percentage of uronic acid in the slime varied from 2.0% to 6.0% in the MR mutants and from 1.3% to 5.1% in the MS substrains. Comparing the MR organisms with the MS ones, in 2 pairs the MR mutants contained more uronic acid than their MS counterparts, while one pair contained almost the same percentage. No correlation could be detected between the percentage of uronic acid and the age of the strains, or the level of the basic MET resistance of the mutants. When the amount of uronic acid per g of dry bacteria was calculated, it appeared that 2 of the 3 MR mutants produced more uronic acid than their MS counterparts.

Although at least 7 of the 9 MR mutants had a higher amount of uronic acid than did 2 of 3 MS substrains, the rule that the highly MR mutants of *S. aureus* were stronger slime producers than the MS substrains tested could

not be stated, since only 3 of 7 MR mutants yielded less uronic acid than 1 of 3 MS substrains. It was also observed that the uronic acid content in the bacteria did not correlate either with the youth or with the MET resistance level of the mutants.

Composition of slime. One-dimensional TLC revealed that the qualitative composition of the polysaccharide moiety of the slime material was identical in the MR mutants and the MS substrains of *S. aureus*. The partially purified slime appeared to consist of at least 6 components. From the start to the front the following components were visualized: galacturonic acid, $R_F = 0.17$; glucuronic acid, $R_F = 0.21$; galactosamine, $R_F = 0.28$; galactose, $R_F = 0.40$; mannose, $R_F = 0.48$; xylose, $R_F = 0.53$. The slime content of charged carbohydrate material was confirmed by thin-layer electrophoresis. No more components could be separated and detected under the conditions used.

The results of gas liquid chromatography of the partially purified slime material shown in Table III were in accordance with the results of thin layer analyses. As can be seen, the main component of the slime was galactose ranging between 13.0% and 31.2%, which is in agreement with earlier observations on wild strains of *S. aureus* [31, 33]. Besides, large quantities (7.3% to

Table III

Percentage distribution of sugars in the partially purified slime material of methicillin-resistant mutants and methicillin-sensitive substrains of heteroresistant S. aureus strains

Strain	Percent of			
	Gal	Man	Xyl	Glc
1188R	17.2	7.5	<1	<1
1969R	25.6	13.2	<1	<1
2842R	28.1	12.0	<1	<1
9965R	31.2	13.6	2.1	<1
10391R	29.0	15.2	<1	<1
14929R	29.0	14.6	1.9	<1
2100R	13.9	7.3	0.8	<1
2100S	13.0	7.7	0.8	<1
5814R	16.8	7.7	0.9	<1
5814S	16.6	7.7	0.9	<1
13137R	28.4	14.1	2.0	<1
13137S	30.6	14.8	2.2	<1

Gal = galactose; Man = mannose; Xyl = xylose; Glc = glucose

15.2%) of mannose were present, while the other monosaccharides were found in minor quantities. Uronic acids and amino sugars could not be estimated by the method used. Although the percentage of galactose and mannose was twice as much in one strain than in another one, concerning both the MR mutants and the MS substrains, however, the distribution of sugars in the slime of the MR mutant and the corresponding MS substrain was always the same.

It should be noted that in mutants 1969R, 2842R and 10391R an additional, sometimes very strong peak was found near the xylose-peak in the gas liquid chromatogram. This peak has not been identified. As no trace of an additional sugar was found in the thin layer chromatogram, the peak is believed not to be caused by a sugar material.

Stability of methicillin resistance during slime production. In order to answer this question, MIC of methicillin for the strains was determined in Difco Casitone broth in the absence and in the presence of 1% glucose, as well as in the modified Staphylococcus Medium 110 (containing 0.2% lactose and 1% mannitol) at 37 °C for 48 h. The results are given in Table IV. As can be seen, although the strains behaved very individually, the degrees of MET

Table IV

Susceptibility to methicillin of methicillin-resistant mutants and of methicillin-sensitive substrains of heteroresistant S. aureus strains determined in different Difco media at 37 °C for 48 h

Strain	MIC of methicillin, μg/ml, in		
	Casitone broth without glucose	Casitone broth with 1% glucose	modified 110 broth
1188R	3200	2400	3200
1969R	3200	2400	3200
2842R	2400	3200	3200
9965R	1600	1200	1200
10391R	1600	3200	3200
14929R	1600	1600	1600
2100R	800	800	600
2100S	3.12	3.12	3.12
5814R	2400	1600	800
5814S	1.6	3.12	3.12
13137R	200	200	100
13137S	1.6	3.12	1.6

susceptibility of either the MR mutants or the MS substrains failed to increase or to decrease by 2 tubes in either medium, the minimum change that would have been significant. So, slime formation, in essence, neither lowered nor elevated significantly the resistance of the MR mutants to MET. In other words, MET resistance seemed to be absolutely independent from slime production in *S. aureus*.

Discussion

The results of the present study clearly indicate that mutation to MET resistance in *S. aureus* is not accompanied either by a loss or by a suppression of the ability to produce slime. In general, the opposite was also true, i.e. slime production of MR *S. aureus* did not diminish the resistance to MET. The slime layer of the highly MR mutants originating from hospital strains of *S. aureus* proved to be a complex polysaccharide similar to that found in other strains of *S. aureus* [22, 23, 31–33, 37]. The sugar portion of the partially purified slime material of both the MR mutants and the MS substrains contained galacturonic acid, glucuronic acid, galactosamine, galactose, mannose and xylose. The fact that most of the MR *S. aureus* strains examined seemed to possess the ability to produce a large amount of slime layer may, induced many consequences [20–27] and explained why the MR cocci are hardly phagetypable [37–39] and colonize easily in the human body [40].

From the point of view of pathogenicity and virulence, slime layer production during infection may enhance penetration, colonization and invasiveness [25] and in this manner may antagonize the disadvantages of the slow growth-rate.

Slime produced in vivo [38] may delay or inhibit phagocytosis [20, 41–43] of MR cocci. Furthermore, after the cocci being phagocyted, the insensitivity of the MR cells to the lytic action of lysozyme and/or trypsin [44], moreover, the thicker side walls of MR cocci at normal human body-temperature in the presence of subinhibitory concentrations of MET [45] unattainable in a patient's serum on normal dosage, may result in a long survival of the MR bacteria inside the phagocytes.

In other words, in the course of natural infections due to MR staphylococci, the minority of MR cocci will not be adequately overcome by the patients' own defence mechanisms, once the majority of MS cocci have been eliminated by MET. On the contrary, treatment with MET under such conditions may result in selection of stable mutants with high resistance to MET and these bacteria will be able to persist and multiply, although at a slow rate. This assumption was supported by Vaudaux and Waldvogel [46] who showed that the MR strains of *S. aureus* as well as other virulent but MS strains survived after incubation in a modified phagocytic assay for 1, 3, 5 or 24 h.

In the presence of 50 μg MET/ml, however, only the MS strains and the MS population derived from one resistant parent strain were eliminated after incubation for 24 h. On the other hand, Siboni and Krogh [10] demonstrated that these more resistant cocci could persist and multiply during nosocomial staphylococcal septicaemias in spite of treatment with high doses of MET. Furthermore, Bulger et al. [12] found that treatment with MET or saline of rats infected with MR staphylococci did not show any statistically significant difference in recovery. Finally, MET and cephaloridine added before and during infections did not significantly protect mice against MR strains of *S. aureus* [13].

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HCMV SPECIFIC EXPRESSION IN HEL CELLS TRANSFORMED BY Xba I ENDONUCLEASE FRAGMENTED HCMV-DNA*

I. Boldogh, E-S. Huang, J. F. Baskar and L. Váczi

Institute of Microbiology, University Medical School, Debrecen, Hungary, and The Cancer Research Center, School of Medicine, University of North Carolina, Chapel Hill, NC, USA

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The transfection of subconfluent monolayers of HEL cells with Xba I endonuclease fragmented HCMV-DNA resulted in a large number of morphologically transformed foci. The frequency of focus formation of Xba I fragmented DNA increased after TPA treatment of the cells. The morphologically altered cells showed specific reactivity with anti-HCMV serum pool in the nuclear and perinuclear region and in the cytoplasm. The HCMV-^{32}P-labelled probe DNA has been specifically hybridized to the cells of morphologically altered foci. The autoradiographic grains were localized in both nucleic and cytoplasm of the cells showing a specific hybridization between the probe DNA and nuclear as well as polyribosomal RNA.

It has been assumed that the human cytomegalovirus (HCMV) was related to several kinds of human diseases and might play some role in induction of human cancer. These observations were based on experiments of in vitro oncogenic cell transformation of hamster [1, 2] or human cells [3]. HCMV is able to induce the synthesis of macromolecules of infected cells [4], which properties are closely associated with the known DNA tumour viruses. The nucleic acids of the virus were determined in different human tumours using the molecular hybridization technique [5–7].

Recently, however, evidence has been published supporting the theory that the oncogenicity of herpesviruses may be a multistep process and the virus is involved in the initial step of cell transformation and may play a role as a cocarcinogenic agent [8]. The purpose of the present work was to study the presence of HCMV specific antigen(s) and RNS(s) in morphologically transformed HEL cells by endonuclease fragmented HCMV-DNA.

István Boldogh, Lajos Váczi
Institute of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

E-S. Huang, J. F. Baskar
The Cancer Research Center, School of Medicine, University of North Carolina
Chapel Hill, 27514 NC USA

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Materials and methods

Human embryonic lung (HEL) cells propagated in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS) were infected with Towne strain of human cytomegalovirus (HCMV) using low multiplicity infection (0.1 PFU/cells).

The sedimented virions were purified and the viral DNA was prepared as described [9]. HSV-2 was propagated in HEp-2 cells. Virus DNA purification was carried out as described earlier [10].

Transfection with entire and variously treated viral DNA was carried out by using the calcium phosphate precipitation technique [11].

The endonucleases were applied to the viral DNA according to an earlier method [12]. Digestion of HCMV-DNA (1 μg DNA per 4 units enzyme) was performed at 37 °C for 6 h and terminated by heating at 68 °C for 15 min. Digestion was tested by agarose (Sigma) electrophoresis [13] and the DNA fragments were visualized by ethidium bromide. The restriction enzyme were obtained from Boehringer Mannheim GmbH, West Germany.

Ultrasonic disintegration of DNAs was carried out at 4 °C for 30 sec. The average size of randomly sheared DNA was determined by agarose electrophoresis [13].

The anti-complement immunofluorescent (ACIF) test was carried out as published earlier [14]. The fluorescent conjugate for anti-complement staining was purchased from Cappel Laboratories, USA. Guinea-pig complement was used.

Viral-DNA was labelled in vitro with α-^{32}P-dATP (spec. activity 450 Ci/mmol, ICN Chemical and Radioisotope Division, Irwin, CA, USA) by nick translation technique [15, 16]. The specific activity of HCMV and HSV-2 DNA were 1.32×10^8 and 1.14×10^8 cpm per 1 μg DNA, respectively.

In situ cytohybridization was carried out as published previously [15, 17]. The cellular nucleic acids were denaturated by using glyoxal [18] and S_1 nuclease was applied as described earlier [19].

Results and discussion

Transfection with entire HCMV-DNA molecules resulted in a complete virus replication cycle and death of the cells. Results obtained after transfection with variously treated viral DNA are summarized in Table I. The infectivity of viral DNA was very sensitive to treatment with DNase, endonucleases (Xba I and Eco RI) and sonication (for 30 sec) but was insensitive to treatment with proteinase K or RNase.

The subconfluent monolayers of HEL cells (in 17th tissue culture passage) were transfected with HCMV-DNA (0.32 μg DNA per 1 cm^2 monolayer), sheared by endonuclease (Xba I and Eco RI) or sonication for 30 s. Two days later the cells were reseeded at low density and kept thereafter in MEM enriched with 3% FCS. To increase the number of morphologically altered foci after transfection with fragmented HCMV-DNA, on the 2nd, 19th, and 29th days the cells were treated with the tumour promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), 25 μM/ml, for 24 h. After exposure to TPA the cultures were washed 3 times with PBS and feeded with MEM supplemented with 3% FCS. Fortytwo $\pm$6 days after transfection, independently of treatment, morphologically altered foci appeared in monolayers transfected with Xby I fragmented DNA. There were no foci visible in tissue cultures transfected with Eco RI cleaved and sonicated HCMV-DNA or non-transfected cultures of HEL cells. TPA treatment of the transfected cells increased the

frequency of focus formation 5–6 fold (Table I). The foci were isolated and the cell suspensions were divided into two parts. The fewer cells were transferred to microslides (0.5 × 1 cm), the higher number of cells was placed into tissue cultures and both of them were fed with MEM conditional media enriched with 10% FCS. After one day incubation of the cells on microslides they were

Table I

Comparison of infectivity and transforming activity of intact and fragmented HCMV-DNA

Treatment	Infectivity PFU/1 μg HCMV-DNA	Transforming ability of HCMV-DNA foci/1 μg			
		not treated	TPA treated on		
			2nd	19th	29th
			day		
—	327	0 (CPE)	NT	NT	NT
DNase[a]	0	0	NT	NT	NT
RNase[b]	297	0 (CPE)	NT	NT	NT
Proteinase K[c]	302	0 (CPE)	NT	NT	NT
Eco RI[d]	0	<0.01	<0.01	<0.01	<0.01
Xba I[e]	0	0.12	0.11	0.63	0.56
Sonication[f]	0	<0.01	<0.01	<0.01	<0.01

NT: not tested; [a, b, c] 50 μg/ml enzyme incubated at 37 °C for 60 min; [d, e] 4 units/1 μg enzyme incubated at 37 °C for 6 h; [f] sonicated for 30 s at 4 °C

tested for the presence of HCMV specific antigen(s) by anti-complement immunofluorescent (ACIF) test. The human anti-CMV and seronegative serum pool used in these experiments were characterized by ACIF test using Towne infected (multiplicity of infection, 0.5 PFU per cell) and uninfected HEL cultures. After infection for 2 h, in the presence of 100 μg/ml cycloheximide, and after a subsequent treatment with actinomycin D for 3 h, the anti-CMV serum pool reacted specifically with the cell nuclei (IEA: immediate early antigen). When the cells were infected for 36 h in the presence of phosphonoacetic acid (PAA, 100 μg/ml) or incubated for 96 h without any drug, the virus antigens were considered to be early (EA) and late antigens (LA), respectively. The titres of the serum pool against the IEA, EA and LA of HCMV are shown in Table II.

The rabbit anti-HSV-2 positive and negative sera were characterized in the same way as above, using HSV-2 (strain 333) infected HEL cells (Table II).

The results of experiments on morphologically transformed and control cell obtained by ACIF test are summarized in Table III.

With the exception of a single one, all cultures of morphologically altered cells, were positive for viral specific antigen(s). Their fluorescence was

Table II

Characterization of immune serum pools and ^{32}P-labelled viral probe DNA using ACIF test and in situ cytohybridization

Cell culture	Antibody titre of serum pools						In situ cytohybridization with ^{32}P-labelled viral DNA	
	HCMV			HSV-2				
	IEA[a]	EA[b]	LA[c]	IEA[a]	EA[b]	LA[c]	HCMV	HSV-2
HEL	0	0	0	0	0	0	—	—
HEL + HCMV	1 : 32	1 : 128	1 : 512	<1 : 2	<1 : 2	<1 : 2	++++	—
HEL + HSV-2	<1 : 2	<1 : 2	<1 : 2	1 : 16	1 : 512	1 : 1824	—	++++

[a] immediate early antigen; [b] early antigen; [c] late antigen

Table III

Reactivity of morphologically altered cells to anti-HCMV serum and ^{32}P-labelled HCMV-DNA

Time of TPA treatment, day	Number of tested foci	Reactivity of serum pool						In situ cytohybridization with ^{32}P-labelled viral DNA of	
		anti-HCMV				anti-HSV-2	negative[c]		
		1 : 10[a]			1 : 64[a]	1 : 10[a]	1 : 10[a]		
		N[b]	PN[b]	C[b]				HCMV	HSV-2
NT	5	4/5	2/5	1/5	0/5	0/5	0/5	4/5	0/5
2nd	6	4/6	6/6	2/6	0/6	0/6	0/6	6/6	0/6
18th	5	5/5	2/5	3/5	0/5	0/5	0/5	5/5	0/5
29th	6	3/6	6/6	3/6	0/6	0/6	0/6	6/6	0/6
Controll cell cultures:									
HEL[d]		—	—	—	—	—	—	—	—
HEL[e]		—	—	—	—	—	—	—	—
CX-90-3B		—	—	—	—	—	—	—	—
CMV-MJ-HEL-1		—	—	—	—	—	—	—	—

NT: not treated; N: nucleus; PN: perinuclear region; C: cytoplasm; [a] dilution of serum pool; [b] localization of fluorescence; [c] serum pool, negative for HCMV and HSV-2 antibodies; [d] nineteen coverslip cultures of morphologically unchanged HEL cells were tested; the cultures were picked from the same monolayers from which the transformed foci had been isolated; [e] cultures of HEL cells treated with TPA

bright in the nuclei and/or the perinuclear region and also in the cytoplasm (Fig. 1a). With CMV negative human sera or HSV-2 immune sera no fluorescence could be seen. There was a correlation between the antigen detectability by immune serum and the serum dilution. A serum dilution of 1 : 10 was used for detecting viral specific antigens in transformed cells. When the serum was diluted 1 : 64 there was no fluorescence while a 1 : 128 serum dilution showed bright fluorescence with HCMV infected HEL cells for 36 h in

the presence of PAA. Whether the viral antigen(s) belonged to immediate early, early or late products of the viral genome, could not be known exactly, but the detectability of antigens at low (1 : 10) serum dilution suggested that IE non-cytotoxic viral proteins were probably present in the transformed cells. Presumably, the viral antigen(s) originally localized in the cell nuclei

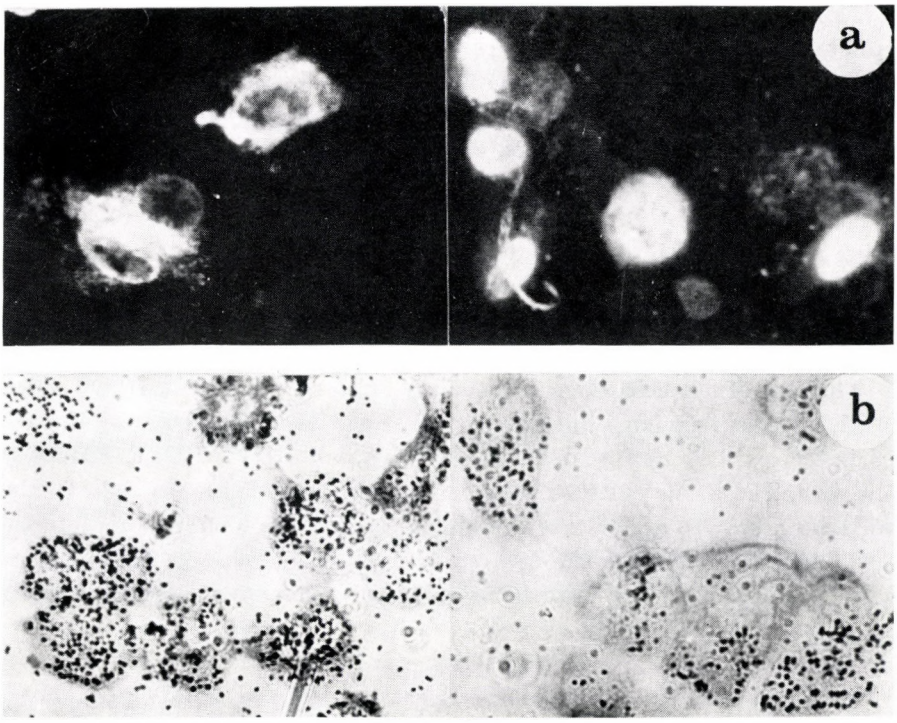

Fig. 1. HCMV-specific macromolecules in morphologically transformed HEL cells. (a) Localization of HCMV specific antigen(s) in the cells of foci. (b) In situ cytohybridization visualized by autoradiographic grains. ×320

and during the maintenance of the cells this (these) protein(s) could migrate between the nucleus and the cytoplasm.

The anti-HCMV serum reacted neither with the cells of CX-90-3B [1] and CMV-MJ-HEL-1 [3] cell lines, nor with the HSV-2 infected and non-infected HEL cells.

After ACIF test, the cells were extensively washed with PBS at 4 °C for 12 h and the presence of virus specific nucleic acids were investigated by cytohybridization in situ. In the control experiments the HCMV-^{32}P-labelled probe DNA was specifically hybridized to cells infected with HCMV. No hybridization was observed with uninfected or HSV-2 infected HEL cells.

When the morphologically altered cells of foci have been used as target cells, the ^{32}P-DNA of HCMV specifically hybridized to their nuclei and cytoplasms, except one clone derived from TPA untreated foci. Results are summarized in Table III while hybridization in situ can be seen in Fig. 1b.

In all cases there was a close correlation between the results obtained by ACIF test and the in situ cytohybridization technique.

From the monolayers with transformed foci 19 coverslip cultures of morphologically unchanged HEL cells were isolated. In these cultures we could not detect any cells having HCMV specific antigens or nucleic acids by ACIF and the in situ cytohybridization technique (Table III). The cells of CX-90-3B hamster [1] and CMV-MJ-HEL-1 human [3] cell lines, transformed by UV-irradiated and living HCMV, respectively, have been shown to lack reactivity to ^{32}P-probe DNA. In these experiments the results were considered positive when the grain number of the test cells was at least ten times that of uninfected or HSV-2 infected cells using HCMV probe DNA.

In parallel experiments, ^{32}P-labelled HSV-2-probe DNA did not hybridize to HCMV infected or uninfected or to morphologically transformed cells.

The rest of the cells of each focus was maintained and cultured. Most of the cell cultures from foci did not survive more than 10 passages. However, three independently derived cell lines, designated BH19, BH21, BH47 were established. The tissue cultures were cloned and passaged more than 90 times in our laboratory, to investigate the biological and biochemical characteristics of CMV transformed cells. The cell cultures consisted of short fibroepitheloid and epitheloid cells. They were transferred to coverslips in passages Nos 24, 57 and 72 and the ACIF test as well as in situ hybridization were carried out. No fluorescence and in situ hybridization were observed.

In conclusion, the results showed that the DNA of HCMV probably has a specific region for the transformation of normal cells. This region of HCMV-DNA is supposed to have one or more cleavage sites to Eco RI restriction enzyme. The Xba I endonuclease fragmented HCMV-DNA is able to induce morphologically altered foci in HEL cell monolayers. The morphologically transformed cells contain HCMV-specific antigen(s) and nucleic acid(s), independently of TPA treatment. The results obtained suggest that the immediate early product(s) of HCMV-DNA is/are involved in the initial step of cell transformation. It is also presumable that HCMV induced cell transformation might be a multistep process. It needs an initiator (fragment of HCMV-DNA), a still unknown genetic background of normal cells and/or some environmental factor such as TPA, since for example the focus formation of Xba I fragmented HCMV-DNA arose 4–5 fold after TPA treatment.

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BINDING OF FIBRONECTIN TO DNA: NEW APPLICATION OF THE *CRITHIDIA LUCILIAE* IMMUNOFLUORESCENCE TEST*

K. Cseh, L. Jakab, Judit Török, L. Kalabay and Terezia Pozsonyi

Third Department of Medicine, Semmelweis University Medical School, Budapest, and Institute of Enzymology, Biological Research Centre of the Hungarian Academy of Sciences, Budapest

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Binding of purified plasma fibronection to *Crithidia luciliae* kinetoplast DNA was demonstrated by fluorescence microscopy. The method was suitable for the detection of fibronectin in human plasma diluted 1:256 and 1 μg/ml concentration of isolated fibronectin. Purified human Clq, monoclonal human myeloma proteins of IgG1 and IgG3 subclasses and calf thymus DNA inhibited the binding of fibronectin to kinetoplasts. The method can be used as a functional assay for fibronectin and for various materials containing fibronectin.

Fibronectin (FN) in a high molecular mass glycoprotein (440 000 d), which is present in insoluble form on the cell surfaces and in connective tissue, and in soluble form in plasma and other body fluids. FN on cell surfaces apparently mediates adhesion of cells to extracellular matrix. Cell lines during malignant transformation tend to lack cell surface FN and this may contribute to their capacity for invasive and metastatic growth [1–5]. Plasma FN is one of the main non-specific opsonin proteins in blood plasma; it takes part in clot formation, wound healing, the function of platelets and in formation of cryoglobulins [6–8]. The involvement of FN in a variety of seemingly unrelated phenomena is attributed to the many binding domains or sites on the protein to various important biological molecules. FN can bind to cell surfaces, to collagen and gelatin, fibrinogen and fibrin, amino acids, complement components, Clq and C3, actin, heparin and other glycosaminoglycans, various gangliosides, staphylococcus protein A and other Gram-positive and -negative bacteria, polyamines, certain immunoglobulins, myeloma immunoglobulins and cryoglobulins and to DNA [9–24]. Recently, the DNA-binding properties of FN have been described and DNA binding domains of the protein have been isolated [14, 25–28]. The *Crithidia luciliae* test has proved to be a rapid and appropriate method for detection of anti-native double stranded desoxyribonucleic acid (ds-DNA) antibodies [29—34]. *C. luciliae* kinetoplast

Károly Cseh, Lajos, Jakab, László Kalabay, Terezia Pozsonyi
Third Department of Medicine, Semmelweis University Medical School
Eötvös út 12, H-1121 Budapest, Hungary

Judit Török
Institute of Enzymology, Biological Research Centre of the Hungarian Academy of Sciences
Karolina út 29, H-1113 Budapest, Hungary

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DNA has also been used as a substrate for detection of binding of other molecules to native ds-DNA [35, 36]. The present study describes a sensitive immunofluorescence assay for detection of binding of fibronectin to ds-DNA present in the *C. luciliae* kinetoplasts.

Materials and methods

Isolation of human plasma FN. FN was isolated from fresh normal human plasma by affinity chromatography on gelatin-Sepharose 4B and L-arginine-Sepharose 4B columns and on Sephacryl S 300 gel (Pharmacia, Sweden) [24]. Ten ml of human plasma passed through an equal volume of gelatin-Sepharose 4B column was used as FN-depleted human plasma for control purposes. Sepharose 4B (Pharmacia) was activated by cyanogen bromide [37]. Gelatin (pig skin, type I, Sigma) or L-arginine (Reanal, Hungary) of purified FN were coupled to the gel [38]. The purity and indentity of the isolated FN was determined on basis of the molecular mass (440 000 b) and the sedimentation rate (15 S) in the analytical ultracentrifuge (MOM 3170, Hungary) and by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis [39]. The purity of isolated FN was at least 99%. There was no contaminating Clq, IgG, IgM and fibrinogen in the FN preparations detectable by immune diffusion (antisera against human Clq, IgG, IgM and fibrinogen were purchased from Behringwerke).

Anti-FN antibodies. Antiserum against FN was produced by immunizing rabbits with purified FN. Anti-FN rabbit immunoglobulins were isolated on FN-Sepharose 4B immunoabsorbent [38]. The monospecificity of isolated immunoglobulins was checked by immune diffusion (anti-FN immunoglobulins did not react with purified Clq, fibrinogen, IgG or IgM), or by crossed immunoelectrophoresis with human plasma, purified FN and FN depleted human plasma [40] (Fig. 1a, b).

Conjugation of purified FN and rabbit anti-FN immunoglobulins with fluorescein isothiocyanate (FITC). Purified FN and rabbit anti-FN immunoglobulines were conjugated with FITC (Serva) and the unreacted fluorochrome was removed by gel filtration of Sephadex G 50 column [41]. For the isolation of optimally conjugated protein molecules, the FITC-labelled proteins were applied to DEAE-cellulose columns (Whatman) [42]. The labelling ratios of the conjugates were 0.98 for FITC—FN and 0.99 for FITC-anti-FN immunoglobulins, as evaluated by the optical densities at 495 and 280 μm.

Isolation of human Clq. Clq was isolated from human serum by affinity chromatography on IgG-Sepharose 4B column [43]. Purity and identity of isolated Clq were checked by the methods used for FN and also by double immune diffusion tests (anti-human Clq antiserum was purchased from Behringwerke). The Clq-depleted human serum was used for demonstration of binding of FN to the *C. luciliae* kinetoplasts without the presence of Clq (no Clq was detected in the Clq-depleted serum by a double immune diffusion test).

Human plasma and sera. Fresh human plasma was obtained from the National Institute of Haematology and Blood Transfusion, Budapest for the separation of FN and Clq Sera from 10 healthy blood donors and from 10 hospitalized patients with active systemic lupus erythematosus (SLE; ARA criteria) were obtained and subjected immediately to *C. luciliae* test. Sera of patients with active SLE, which gave strong reactions even at dilutions of 1:1024 in *C. luciliae* immunofluorescence test with FITC conjugated anti-human immunoglobulin (Behringwerke, working dilution 1:64), contained antibodies against ds-DNA and were used as markes [29] in the inhibition experiments. Plasma FN concentration of the 10 patients with SLE was 215—270 μg/ml ($\bar{x} = 240$ μg/ml $\pm$ 25) as determined by electroimmunoassay [40]. The normal value was 200–400 μg/ml. FN depletion of patient sera was performed as mentioned earlier. Calf thymus DNA was purchased from Worthington Biochemical Corporation (Freehold, USA).

Preparation of substrate. C. luciliae were kindly supplied by Institute of Microbiology, University Medical School, Szeged, and the haemoflagellates were grown in Bacto tryptose medium at room temperature [44]. A suspension was washed in phosphate buffered saline (PBS, pH 7.2) three times by low speed centrifugation (1500$\times$g, 5 min). A suspension of approximately 20×10^6 organisms/ml was prepared in distilled water containing 0.1% bovine serum albumin, and after standing for 1—2 min, 10 μl drops were applied on microscope slides. After air drying, the organisms were fixed in 96% ethanol for 10 min, followed by washing in PBS.

Immunofluorescence studies. Dried and fixed monolayers of *C. luciliae* were incubated with purified FITC—FN (concentration 100–0.1 μg/ml) for 30 min in moist chamber at room

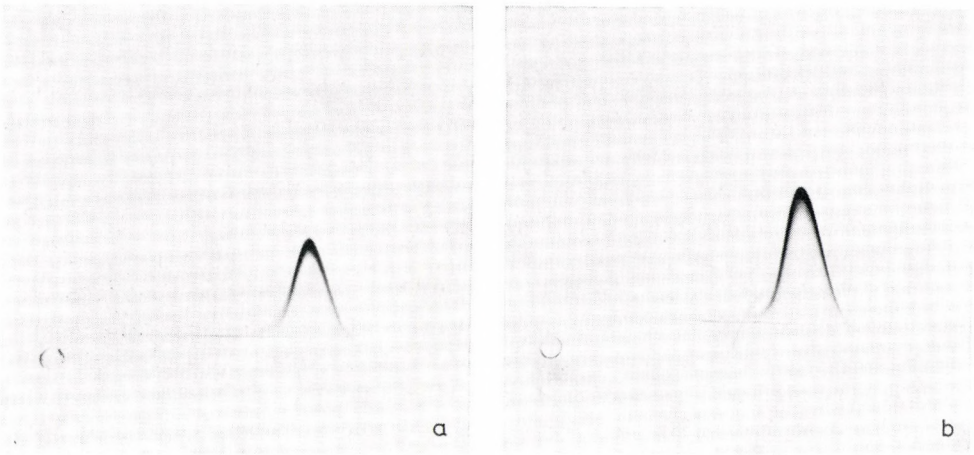

Fig. **1.** Demonstration of monospecificity of purified rabbit anti-FN immunoglobulins by crossed immunoelectrophoresis. (a) 2 μl of human plasma (undiluted) served as antigen; (b) 2 μl of purified FN (300 μg/ml) served as antigen. Electrophoresis was done in 0.8% agarose gel (Litex) in veronal-Na–veronal buffer (pH 8.6), first direction: 10 V/cm, 1.5 h, second direction: 1.5 V/cm, 16 h. 10 μl of rabbit antifibronectin immunoglobulins were applied. Stained with amido black

temperature. After washing in PBS three times for 5 min the slides were mounted with PBS–glycerol and examined in a Fluoval 2 fluorescence microscope (Zeiss, Jena, GDR). Orwochrom UT 18 film (Umkehr-Farbfilm, GDR) was used for photography.

For determination of binding of unlabelled purified FN, plasma or serum FN, *C. luciliae* monolayers were incubated with purified FN (concentration 1000–0.1 μg/ml) or plasma or sera (diluted 1:1—1:1024) for 30 min in moist chamber at room temperature. After washing in PBS three times for 5 min, slides were inculated for 30 min with FITS–anti-FN antibodies (concentration 8 mg/ml) diluted 1:80 and washed three times in PBS, mounted and studied as above.

Clq depleted serum was used in the same procedure to demonstrate the binding of FN to kinetoplast DNA in the absence of Clq.

FN depleted human plasma, which did not contain any FN detectable by crossed immunoelectrophoresis was used as a negative control in the *C. luciliae* test.

In the inhibition experiments, various concentrations of purified FN (1000–5 μg/ml) were preincubated with the *C. luciliae* monolayers for 30 min at room temperature, then washed three times in PBS. Subsequently, the slides were incubated with sera of patients with active SLE, at various dilutions (1:1–1:1024). After incubation for 30 min and washing three times in PBS, the slides were incubated with FITC-conjugated anti-human immunoglobulin. Slides without FN-preincubation were used as positive controls.

Preincubation with human plasma (diluted 1:10) and sera (diluted 1:5) and purified FN (30 μg/ml) was performed in order to study the inhibitory effect on the binding of FN to kinetoplasts with the following substances: calf thymus DNA (0.01–100 μg/ml), purified Clq (10–100 μg/ml), purified human monoclonal myeloma proteins from IgG1 and IgG3 subsclasses [5] (10–100 μg/ml). Incubation was done for 1 h at 37 °C before subjecting samples to the *C. luciliae* test.

Results

Binding of FN to C. luciliae kinetoplasts. Purified FITC—FN was bound to the kinetoplast of *C. luciliae* in the immunofluorescence test as was the purified unlabelled FN (Fig. 2a, b). We detected the binding of FN to native

DNA at very low concentrations (1 μg/ml). Preincubation of the haemoflagellates with purified unlabelled FN abolished the binding of FITC–FN to the kinetoplasts. The binding of human plasma and serum FN to *C. luciliae* kinetoplasts was also detectable using FITC–anti-FN antibodies at a plasma dilution of 1:256 and serum dilution of 1:128 (Fig. 2c, d). FN in Clq-depleted human serum was also bound to the kinetoplasts at a serum dilution of 1:128. There was, however, no detectable FN binding in *C. luciliae* immunofluorescence test in FN-depleted human plasma.

Inhibition of binding of anti-native ds-DNA antibodies to C.luciliae *kinetoplasts by FN.* Sera from 10 patients with active SLE gave intense fluorescence in the *C. luciliae* test at serum dilution of 1:1024. Depletion of FN in the patient sera treated with gelatin–Sepharose 4B gel did not affect their titre in the *C. luciliae* test, indicating that serum FN does not interfere significantly with reactions of anti-native ds-DNA antibodies in diluted sera. Preincubation of *C. luciliae* monolayers with high concentrations of purified FN (500 μg/ml or more) inhibited the fluorescence of the patient sera with SLE at serum dilutions of 1:32 or more. Lower FN concentrations (250 μg/ml or less) had inhibitory activity only at high serum dilutions (1:512 or above; Table I).

Table I

Inhibition of binding of native ds-DNA antibodies to C. luciliae kinetoplasts by FN

Applied FN μg/ml	Serum dilution	Inhibition of kinetoplast fluorescence wits ds-DNA antibodies of serum of SLE patients
50	1:1024	—
100	1:1024	—
150	1:1024 only	+
200	1:1024 only	+
250	1:512 or more	+
500	1:32 or more	+
600	1:16 or more	+
700	1:8 or more	+
1000	1:8 or more	+

Inhibition of binding of plasma, serum or purified FN to C. luciliae *kinetoplasts.* Preincubation of diluted human plasma or sera or purified FN with calf thymus DNA, purified human Clq and with purified human monoclonal myeloma proteins of IgG1 and IgG3 subclasses abolished the binding of FN to the kinetoplast DNA. This was presumably due to the binding of the molecules to FN and probably also to their blocking effect on DNA-binding sites of FN (Table II).

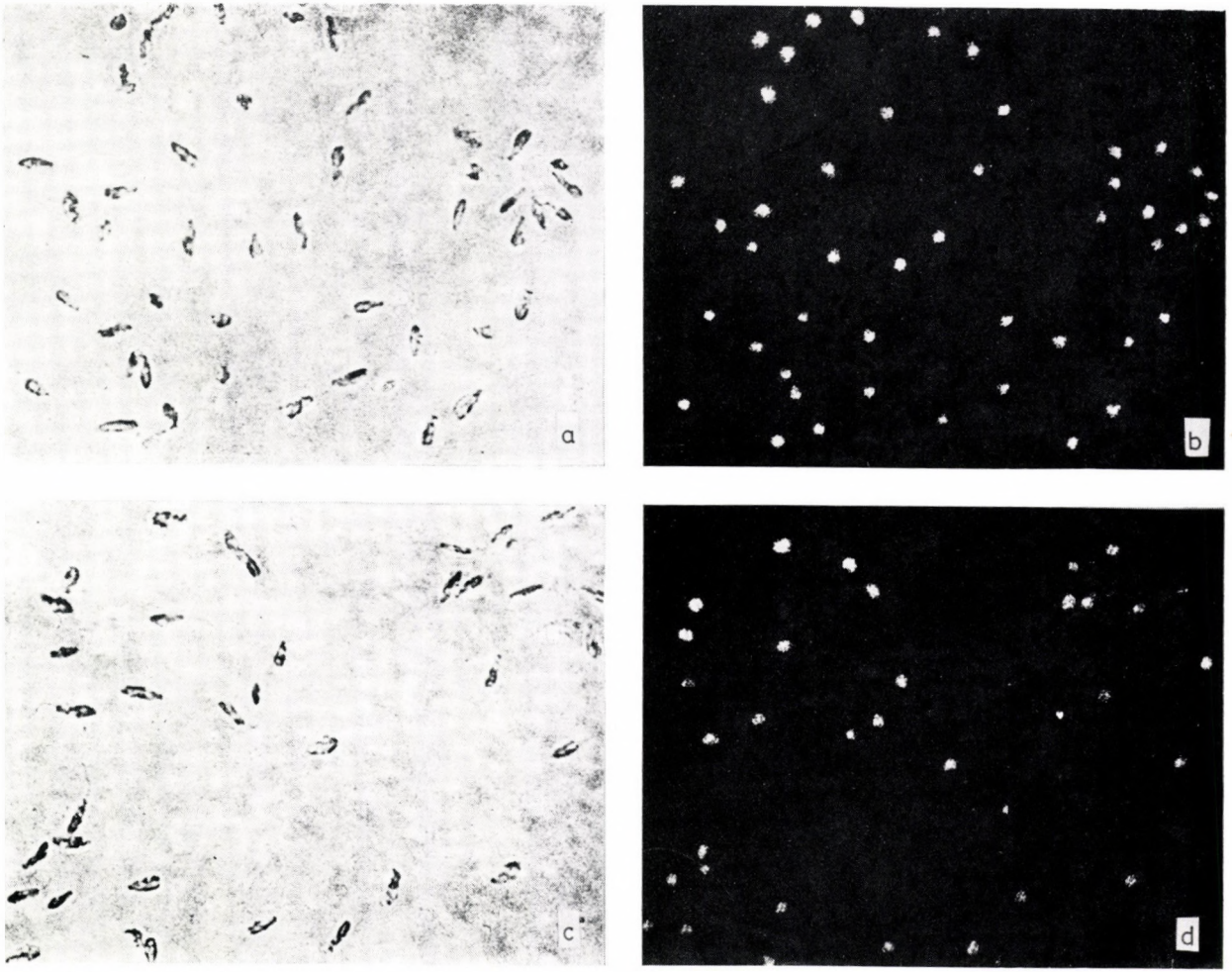

Fig. 2. Binding of FN to *C. luciliae* kinetoplast DNA. Binding of FITC-conjugated FN to the organisms (native, ×750); (b) fluorescence of kinetoplasts in UV light (×750). Binding of plasma FN to *C. luciliae* kinetoplasts: (c) monolayer of *C. luciliae* (native, ×750); (d) fluorescence of kinetoplasts in UV light (×750). (Antibodies: FITC–anti-FN immunoglobulins, applied as described in Materials and methods)

Table II

Inhibition of binding of plasma, serum or purified FN to C. luciliae kinetoplasts after preincubation with Clq, DNA, IgG1 kappa and IgG3 lambda monoclonal human myeloma proteins

Substances added to plasma, serum or purified FN	Inhibitory concentration, μg/ml
Human Clq	20
Calf thymus DNA	1
IgG1	30
IgG3	25
IgG2, IgG4 or normal human IgG	no inhibition at 1000 μg/ml concentration

Discussion

The kinetoplasts of *C. luciliae* proved to be an appropriate substrate for studying the binding of various substances to native ds-DNA [29, 35, 36]. FN can also bind to DNA. In this assay the binding of purified FITC–FN to DNA was studied by the *C. luciliae* test. The method allows rapid and simple detection of binding of FN to DNA even at low FN concentrations. The assay is suitable for studying the DNA-binding properties of varoius FN samples, for semiquantitative extimation of FN concentration and for the study of various substances interfering with FN–DNA binding. Previously, *C. luciliae* kinetoplast fluorescence was used for detection of binding of human Clq to DNA and it was suggested that this interaction may interfere with the binding of anti-DNA-antibodies to the kinetoplast DNA [36]. Binding of serum or plasma FN to the kinetoplasts was not dependent on the presence of Clq and occurred with Clq-depleted serum. There was no difference in the binding of anti-DNA immunoglobulins to the kinetoplast DNA in the sera of patients with SLE with or without FN depletion. FN at concentrations considerably higher than physiological interfered, however, with the interaction of anti-DNA immunoglobulins and the kinetoplast DNA. In the patients' diluted sera, the low FN level did not inhibit the high avidity binding of anti-DNA immunoglobulins. Therefore, FN-depletion of patient sera is not necessary before *C. luciliae* tests are performed for ds-DNA antibodies. In contrast, the specificity of the FITC-conjugated anti-human immunoglobulins is essential because their reactivity with FN or Clq could give false positive results in the *C. luciliae* test. DNA, Clq and myeloma IgG1 and IgG3 immunoglobulins interfered with the FN–DNA interaction. Clq and certain monoclonal myeloma proteins can bind to FN [10, 13]. Clq interacts with FN by its collagen-like moiety [15, 16, 19, 21], but the possible interaction with the globular region of Clq was also suggested [11]. It has been emphasised that FN is involved in the Clq binding not only by its collagen binding sites but also by its heparin or DNA binding sites [11]. There-

fore it seems possible that the Clq, DNA and immunoglobulin binding sites on FN are common or at least partly common. FN is one of the main opsonic proteins in human plasma and can bind to bacteria. The opsonisation mechanism could thus play an important role in the host defence against various microbal infections. The binding of FN to immunoglobulins, cryoimmunoglobulins, Clq and C3 may be involved in the metabolism of these proteins within the organism. Binding of FN to DNA may be involved in the elimination of DNA from plasma by opsonisation. On the other hand, FN associated with basement membranes might contribute to the deposition of DNA-containing antigens or immune complexes in the vessel walls or the renal glomeruli.

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VIRULENCE FACTORS OF *ESCHERICHIA COLI*

II. ANTIGENS O4, O6 AND O18, HAEMOLYSIN PRODUCTION AND MANNOSE RESISTANT HAEMAGGLUTINATING CAPACITY ARE CLOSELY ASSOCIATED

ÉVA CZIRÓK

National Institute of Hygiene, Budapest

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Escherichia coli strains isolated from a variety of human samples were examined for alpha haemolysin (Hly) production. A total of 1156 strains was compared for incidence of Hly positivity, and the capacity of mannose resistant haemagglutinating activity of human erythrocytes (MRHA). Incidence of Hly production in serogroups O4, O6 and O18ac was significantly higher than in other ones (70.1% vs. 18.7%), independently of the origin of strains; 78% of Hly^+ strains belonging to serogroups O4, O6, O18 was $MRHA^+$, too. The marked correlation between Hly positivity, MRHA activity and these serogroups suggested that in serogroup O4, O18 and in a lesser degree O6, genetic informations concerning O antigen, MRHA and Hly are linked in the chromosome.

Escherichia coli is the most common opportunistic pathogen associated with extraintestinal diseases [1, 2] and at the same time the leading cause of community acquired infections [3]. There are certain characteristics of *E. coli* strains that contribute in their enhanced virulence [4]. In an earlier study [5] an analysis for mannose resistant haemagglutinating capacity of *E. coli* strains revealed that these bacteria are capable of colonizing the bowel and outside the gut they cause extraintestinal infections.

Several studies have shown that haemolysin production is another factor indicative of pathogenicity, since strains with this behaviour predominated among those isolated from urinary tract infections, appendicitis, peritonitis and other extraintestinal infections [1, 4, 6–13]. The purpose of the present investigation was to study the frequency of haemolysin producing isolates in faecal, urinary and other extraintestinal specimens, and their coincidence with the other presumptive virulence factors namely with O antigens and mannose resistant haemagglutinating activity [5].

Materials and methods

E. 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 or lochia, 22).

Diagnostic criteria have been described previously [5].

Serological examination of O antigens was carried out by the agglutination method of Ørskov and Ørskov [14] with a slight modification as described earlier [5].

ÉVA CZIRÓK
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Haemolysin production. Two different methods were used and compared for testing haemolytic activity of strains: (i) blood agar base medium containing 5% defibrinated bovine blood; (ii) 10 ml blood agar base medium overlayered by 10 ml blood agar base medium containing 5% defibrinated bovine blood. In both cases the plates were incubated overnight at 37 °C and observed for haemolysin production. Haemolytic strains were further tested for alpha type haemolysis [11].

Alpha haemolytic activity was determined as described by Walton and Smith [15].

Haemagglutination test was performed as described previously [5].

Statistical analysis was carried out in 2×2 contingency tables and examined for significance by the chi^2 test [16].

Results

A comparison was made between the two methods for detection of haemolytic activity of isolates. Method (ii) proved to be more sensitive than method (i) as it yielded 210 haemolysin positive (Hly^+) strains as opposed to 156 Hly^+ isolates detected by method (i). For this reason results of method (ii) were taken into consideration in further analysis.

The incidence of Hly^+ strains in faecal samples from patients and healthy subjects of different age groups is presented in Table I. Faecal strains showed

Table I

Incidence of Hly positive E. coli strains in faecal samples of patients and healthy persons

Test groups	Age groups (years)	No. of strains	Strains with Hly activity	
			No.	%
Patients with enteritis	<1	107	38	35.5
	1–2	29	4	13.8
	3–5	24	1	4.2
	6–14	24	2	8.3
	>14	72	13	18.1.
Sub total		256	58	22.6
Healthy subjects	<1	69	7	10.1
	1–2	27	1	3.7
	3–5	41	1	2.4
	6–14	24	0	0
	>14	72	4	5.5
Sub total		233	13	5.6
Total		489	71	14.5

Hly = haemolysin

Hly$^+$ in 14.5% (22.6% for enteritis and 5.6% for healthy subjects). The difference in Hly$^+$ strains between patients and healthy subjects was significant statistically ($p < 0.001$). Significant differences were found in every age group between patients with enteritis and healthy subjects (in age group under one year, $p < 0.001$; between 1–14 years, $p < 0.05$; above 14 years, $p < 0.01$). Significant differences were found also in Hly production between different age groups within the group of enteric patients (between age groups under one year and above 14 years, $p < 0.05$; between age groups under one year and 1–14 years, $p < 0.001$; between age groups 1–14 years and above 14 years, $p < 0.05$). In healthy subjects the only difference existed between infants under one year and subjects above 14 years of age ($p < 0.05$).

The distribution of *E. coli* strains according to serogroups and Hly positivity is demonstrated in Table II. Faecal isolates belonged to 86 sero-

Table II

Serogroup distribution of MRHA and Hly positive E. coli strains isolated from faecal samples

Serogroup	Strains isolated from				Total	
	Patients with enteritis		Healthy subjects		MRHA	Hly
	MRHA	Hly	MRHA	Hly		
O1	3/9[1]	0/9	4/7	0/7	7/16	0/16
O2	1/5	2/5	2/8	0/8	3/13	2/13
O4	10/14	11/14	1/4	1/4	11/18	12/18
O6	9/13	11/13	5/6	3/6	14/19	14/19
O7	2/3	0/3	1/7	0/7	3/10	0/10
O15	3/6	0/6	5/9	1/9	8/15	1/15
O18ac	18/20	17/20	4/6	1/6	22/26	18/26
O75	1/7	3/7	1/7	1/7	2/14	4/14
Other[2]	7/85	7/85	1/90	2/90	8/175	9/175
Sp. aggl.[3]	0/10	0/10	1/6	2/6	1/16	2/16
NT[4]	8/84	7/84	2/83	2/83	10/167	9/167
Tosal	62/256	58/256	27/233	13/233	89/489	71/489

MRHA = mannose resistant haemagglutination with human erythrocytes; Hly = haemolysin

[1] No. of strains haemagglutinating or haemolysing/No. of strains examined

[2] O3, O5, O8, O9, O10, O11, O12, O13, O16, O17, O17,77, O18ab, O19,133, O20, O21, O22, O23, 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, O147, O148, O149, O151, O152, O154, O156, O158, O159, O164

[3] Spontaneous agglutination

[4] Not typable

groups, but 26.7% of the strains were associated with 8 serogroups. Hly$^+$ strains belonged in 71.8% to these most frequently occurring serogroups except strains falling in serogroups O1, O7, O15 which showed hardly any haemolysis. Hly positivity was significantly more frequent in serogroups O2, O4, O6, O18ac, O75 than in others ($p < 0.001$). There was a significant difference in Hly activity of these serogroups between enteritis patients and healthy subjects.

From urine 384 strains were isolated (Table III); Hly positivity occurred in 14.6% (16.7% for pyelonephritis, 14.7% for cystitis, 8.5% for asymptomatic bacteriuria strains). There was no significant difference in Hly positivity between strains isolated from pyelonephritis and cystitis ($p > 0.8$), between those from pyelonephritis and asymptomatic bacteriuria ($p > 0.05$), between strains isolated from cystitis and asymptomatic bacteriuria ($p > 0.2$), or between faecal and urinary strains ($p = 0.99$).

Table III

Serogroup distribution of MRHA positive and Hly positive E. coli strains isolated from urinary tract infections

Serogroup	Strains isolated from						Total	
	Pyelonephritis		Cystitis		ABU[1]		MRHA	Hly
	MRHA	Hly	MRHA	Hly	MRHA	Hly		
O1	6/10[2]	0/10	5/6	0/6	0/0	0/0	11/16	0/16
O2	10/13	3/13	8/13	3/13	0/6	0/6	18/32	6/32
O4	2/5	3/5	1/4	1/4	4/5	2/5	7/14	6/14
O6	11/12	9/12	9/18	12/18	1/2	0/2	21/32	21/32
O7	0/3	0/3	1/2	0/2	0/5	0/5	1/10	1/10
O8	0/7	0/7	1/8	1/8	0/1	0/1	1/16	1/16
O18ac	2/5	1/5	1/3	0/3	2/3	1/3	5/11	2/11
O75	2/5	4/5	1/3	0/3	0/1	0/1	3/9	4/9
Other[3]	6/40	0/40	0/35	0/35	1/11	1/11	7/86	1/86
Sp. aggl.[4]	0/8	0/8	3/12	2/12	2/3	1/3	5/23	3/23
NT[5]	6/47	6/47	5/66	6/66	1/22	0/22	12/135	12/135
Total	45/155	26/155	35/170	25/170	11/59	5/59	91/384	56/384

[1] Asymptomatic bacteriuria
[2] No. of strains haemagglutinating or haemolysing/No. of strains examined
[3] O3, O5, O7,16, O9, O10, O11, O12, O15, 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

Urinary isolates belonged to 51 serogroups (Table III). A total of 140 strains belonged to the most frequent 8 serogroups (O1, O2, O4, O6, O7, O8, O18ac, O75), 40 of them proved to be Hly^+. The haemolytic capacity in serogroups O2, O4, O6, O18ac, O75 was significantly higher than in other serogroups ($p < 0.001$).

Hly^+ isolates occurred most frequently (29.3%) in different extraintestinal samples (Table IV), with an especially high incidence in blood, autopsy material, wound swabs and upper respiratory tract specimens. Strains isolated from extraintestinal materials belonged to 38 serogroups. Six serogroups were the most frequent (O1, O2, O4, O6, O18ac, O78) — to these serogroups belonged 155 strains, 69 with Hly activity. The difference in incidence of haemolytic activity in these 6 serogroups and the remaining ones was highly significant ($p < 0.001$).

Out of 210 Hly^+ strains 197 had alpha type haemolysin.

There was a marked correlation between the presence of haemagglutinins, haemolytic activity and the serogroups of *E. coli* isolates (Table II, III, IV). In the most frequently occurring serogroups both haemolytic activity and mannose resistant haemagglutinating capacity of human erythrocytes (MRHA) were significantly more frequent ($p < 0.001$) than in the others. There was no significant difference in the incidence of either Hly positivity or MRHA positivity of O1, O2, O4, O6, O18ac, O75 serogroups between faecal and urinary tract infection (UTI), faecal and other extraintestinal, UTI and other extraintestinal groups of isolates ($p > 0.05$—$p > 0.99$). No significant difference existed between Hly and MRHA positivity of serogroups O4, O6, O18ac ($p > 0.7$). A significant difference was shown between Hly and MRHA activity in serogroups O1, O2 ($p < 0.001$).

Next we analysed the correlations of MRHA, Hly positivity and O antigen of our strains. The results of these studies are summarized in Table V. Out of 210 Hly^+ strains Hly^+ and $MRHA^+$ were associated in 144. Simultaneous Hly and MRHA properties were found more frequently among strains belonging to serogroups O4, O6 and O18ac (45.8%), than among those belonging to the other serogroups (7.3%) ($p < 0.001$).

Discussion

Data of different authors indicate that haemolysin production should be regarded as a virulence factor playing a role in the pathogenesis of extraintestinal infections. *E. coli* strains isolated from extraintestinal diseases had haemolytic activity more frequently than those isolated from the faeces of normal individuals [1, 4, 10, 11]. The reported frequency of haemolytic strains in the faecal flora was similar to ours (14.5%) as it ranged between 2% [17]

Table IV

Serogroup distribution of MRHA and Hly positive

Strains

Serogroup	Blood		CSF		Autopsy material		Wound	
	MRHA	Hly	MRHA	Hly	MRHA	Hly	MRHA	Hly
O1	1/1	0/1	—	—	—	—	2/3	1/3
O2	1/1	0/1	—	—	0/4	0/4	1/4	1/4
O4	2/2	2/2	2/2	2/2	6/8	8/8	6/6	6/6
O6	6/6	5/6	0/1	1/1	3/7	7/7	9/13	10/13
O18ac	3/10	4/10	0/19	0/19	1/4	0/4	1/2	2/2
O78	2/2	0/2	1/2	0/2	—	—	—	—
O75	—	—	—	—	—	—	—	—
Other[3]	3/7	0/7	1/6	0/6	0/10	0/10	1/16	2/16
Sp. aggl.[4]	1/3	1/3	1/4	1/4	—	—	0/4	0/4
NT[5]	2/8	3/8	1/1	0/1	0/8	0/8	1/15	2/15
Total	21/40	15/40	6/35	4/35	10/41	15/41	21/63	24/63

[1] Throat, nose, sputum and ear swab
[2] Placenta, lochia
[3] O5, O6,9, O7, O8, O12, O15, O17, O18ab, O19, O20, O21, O22, O23, O33,56, O40, O45, O82, O83, O84, O96, O105ac, O106, O107, O108, O110, O114, O118,160, O120, O131, O134, O162
[4] Spontaneous agglutination
[5] Not typable

and 18% [18]. Cooke [10] and Hacker et al. [12] isolated Hly positive strains less frequently among normal individuals (8–10%) than among patients with enteritis (10–14%), thus our data fit into the trend (5.6% and 22.6%, respectively).

The results concerning our UTI strains differed from those given by several authors. In studies made by Hacker et al. [12], the incidence of Hly positive isolates was three times more frequent among UTI strains than among faecal ones. Similar results were obtained by Green and Thomas [4]. In contrast, in agreement with van den Bosch et al. [19], we failed to find any difference between these two groups of strains.

In studies carried out by Minshew et al. [1] the frequency of strains with haemolysis among extraintestinal isolates was higher (35–59%) than among faecal isolates (3%). Arai et al. [17] also stated that the incidence of alpha Hly producing strains was most frequent among respiratory isolates (23%). Our material corresponds to literary data as Hly^+ isolates occurred more frequently (29.3%) in extraintestinal samples than among faecal isolates (14.5%).

E. coli strains isolated from extraintestinal sources

isolated from								Total	
Umbilical cord		Vagina		Upper respiratory tract[1]		Miscellaneous[2] sources		MRHA	Hly
MRHA	Hly	MRHA	Hly	MRHA	Hly	MRHA	Hly		
1/1	0/1	4/4	1/4	2/4	0/4	1/1	1/1	11/14	3/14
1/4	0/4	3/5	0/5	2/7	1/7	0/1	1/1	8/26	3/26
1/1	1/1	—	—	0/1	1/1	1/1	1/1	18/21	21/21
—	—	1/5	4/5	4/4	2/4	1/4	2/4	24/40	31/40
0/1	0/1	0/5	0/5	5/6	5/6	0/3	0/3	10/50	11/50
—	—	—	—	—	—	—	—	3/4	0/4
—	—	—	—	0/2	2/2	—	—	0/2	2/2
0/1	0/1	2/11	1/11	1/9	0/9	1/7	1/7	9/67	4/67
—	—	0/1	0/1	—	—	0/1	0/1	2/13	2/13
—	—	0/7	0/7	0/3	1/3	0/4	0/4	4/46	6/46
3/8	1/8	10/38	6/38	14/36	12/36	4/22	6/22	89/283	83/283

As shown in Tables II, III and IV, 62% of faecal, 52% of urinary and 76% of other extraintestinal haemolysin producing isolates belonged to serogroups O4, O6 and O18. The incidence of haemolytic capacity in these serogroups was significantly higher than in others, independently of the origin of strains. These data corresponded to some earlier findings. Cooke [10] e.g. found that serogroups O2 and O4 occurred in patients with serious illnesses. Kauffmann's [20] haemolytic strains were extremely frequent in serogroups O2, O4, O6. Van den Bosch et al. [19] observed a frequent association of haemolysis with certain serogroups e.g. O6 and O18ac. The majority of the haemolytic strains of Waalvijk et al. [21] also belonged to serogroups O4, O6, O18 and O75. Among Emődy's [13] 115 Hly^+ strains, 58 (50%) had antigens O4, O6 and O18.

On the basis of these and the present data it may be concluded that the extreme haemolysing capacity of serogroups O4, O6 and O18 was independent of the site of infection.

The most striking finding in the course of these examinations was that 144 (68%) out of 210 Hly^+ *E. coli* strains agglutinated human erythrocytes as well. Moreover, 106 (78%) out of 136 Hly^+ strains belonging to serogroups O4, O6 and O18 were at the same time MRHA positive. On the other hand, 3 out of 79 isolates of serogroup O1 and O7 were only Hly^+ and another 33 gave mannose resistant haemagglutination with human erythrocytes.

Table V

MRHA and Hly positivity in certain serogroups of E. coli strains of different origin

Serogroup	Strains isolated from									Total No.	
	Faeces			Urine			Other extraintestinal sources				
	MRHA+ Hly+	MRHA− Hly+	MRHA+ Hly−	MRHA+ Hly+	MRHA− Hly+	MRHA+ Hly−	MRHA+ Hly+	MRHA− Hly+	MRHA+ HlyV	MRHA− Hly−	Examined
O1	—	—	7	—	—	11	3	—	8	17	46
O2	—	2	3	4	2	14	1	2	7	36	71
O4	11	1	—	3	3	4	18	3	—	10	53
O6	12	2	2	14	7	7	20	11	4	12	91
O18ac	17	1	5	2	—	3	9	2	1	47	87
O75	2	2	—	2	2	1	—	1	—	15	25
Others[1]	12	9	18	7	10	19	7	6	11	684	783
Total of O4, O6, O18ac	40	4	7	19	10	14	47	16	5	69	231
Total of others	14	13	28	13	14	45	11	9	26	752	925

[1] See Tables II, III and IV

In their papers Evans et al. [22], Green and Thomas [4], and Hacker et al. [12] reported on results very similar to ours. Green and Thomas [4] found a significant correlation between haemolysin production and HA positivity of strains, as 67% of their haemolysin producing isolates were agglutinating human type O erythrocytes. Similar results were reported by Emődy [13]; 32 out of 56 Hly^+ strains were $MRHA^+$.

There was, on the other hand, a marked correlation between Hly positivity and certain serogroups. Evans et al. [22] observed that Hly^+ and HA type VI were very frequent in serogroups O6 and O18, and that serogroups O1 and O7 had no haemolytic activity. Green and Thomas [4] reported that particular combinations of O4 and MRHA type VI occur very frequently while Hacker et al. [12] found that among Hly^+ faecal strains the occurrence of HA positivity and serogroups O1, O2, O4, O6, O7, O8, O18 was similar to that observed among *E. coli* urinary isolates (see Table VI). In this respect their results were similar to ours. Emődy [13] also found a significant correlation between Hly positivity and serogroups O4, O6 and O18 on the one hand, and a correlation between Hly positivity and MRHA positivity on the other. Van den Bosch [19] could not show any association between the origin of strains and the presence of haemagglutinins.

Table VI

Incidence of MRHA and Hly positivity in serogroups O1, O2, O4, O6, O18, according to Hacker et al [12] and the present study

Origin of strains	Haemolyzing activity	Percent of strains with MRHA		Percent of strains belonging to serogroups O1, O2, O4, O6, O18	
		Hacker et al.	Present data	Hacker et al.	Present data
Faecal	+	41	76	50	56
	—	8	8	12	10
UTI	+	60	57	55	62
	—	40	18	25	21

URI = urinary tract infection

Thus, serogroups O4, O6 and O18, Hly production and mannose resistant haemagglutinating capacity are closely associated in *E. coli*. That is, MRHA positive strains belonging to serogroups O4, O6 and O18 produce, as a rule, alpha haemolysin, in contrast to strains of serogroup O1, which are usually MRHA positive but non-haemolytic.

Minshew et al. [2] supposed that haemolysin production might be determined by particular plasmids in strains from UTI in contrast to strains from the enteric flora. According to van den Bosch et al. [11] alpha haemolysin production in most haemolytic strains of *E. coli* is mediated by a Hly plasmid. However, Minshew et al. [1] and Hull et al. [23] suggested that haemolysin production was of chromosomal origin. Recently, Hacker et al. [12] demonstrated that Hly determinants of urinary strains were located in the chromosome.

It may be assumed that Hly producing capacity in serogroups O4 and O18 — and sometimes in O6 — is associated with O, K, H and F antigens (i.e. genetic informations are linked in the chromosome). This conception has also been emphasized by Hacker et al. [24] who stated that "genes encoding Hly and MRHA actually lie adjacent to each other on the chromosome". This suggestion was supported by our earlier study [25] according to which such strains had a common ancestral origin.

According to the theory advanced by van den Bosch et al. [19], both Hly and MRHA properties render *E. coli* especially pathogenic. In other words the special pathogenicity of the O4, O6 and O18 strains should be attributed to special virulence factors associated with these O antigens. In our opinion their importance is that by means of these properties *E. coli* is able to colonize not only extraintestinal sites but also the bowel, thus becoming a permanent potential source of extraintestinal infections.

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GROUP AND TYPE DISTRIBUTION OF BETA-HAEMOLYTIC STREPTOCOCCI IN SCARLET FEVER, BELGRADE, YUGOSLAVIA, 1973–1982

(A NOTE)

B. ADANJA and HRISTINA VLAJINAC

Institute of Epidemiology, School of Medicine, Belgrade, Yugoslavia

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The majority of 2033 beta-haemolytic streptococcus strains isolated from patients with scarlet fever belonged to group A (82.3%). Among group A streptococci the most frequent were types T1, T4 and T12, followed by types T2, T13 and T6.

Group and type identification was performed on 2033 beta-haemolytic streptococcus strains isolated from patients with scarlet fever. All patients seen during the period 1973–1982 at the Clinic for Infectious Diseases, School of Medicine in Belgrade, were included in the investigation.

Materials and methods

For both group and type identification, Griffith's method of agglutination [1] was used. Grouping sera (from A to S) and T typing sera — polyvalent (T, U, W, X, Y) and monovalent (1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 22, 23, 25, 27, 28, 47, Imp.19, B_{3264}) — were provided by the Institute of Sera and Vaccines, Prague, Czechoslovakia.

Results and discussion

As could be expected from data in the literature [2–4], the majority of 2033 beta haemolytic streptococcus strains belonged to group A (82.3%). The percentage of group A strains varied from 67.9% to 91.4% over the years. Among other groups, groups B and C were the most frequent. Group C was found in 6.9%, and B in 3.4%, of patients. In sporadic cases groups D, E, F, G and M were identified. An insignificant number of strains (0.9%) could not be classified into any of the groups, and 3.6% of the strains were auto-agglutinable.

Among group A haemolytic streptococci the most frequent were types T1, T4 and T12, followed by types T2, T13 and T6 (Table I). With the excep-

B. ADANJA, HRISTINA VLAJINAC
Institute of Epidemiology, School of Medicine
Visegradska 26, 11000 Belgrade, Yugoslavia

Table I

T-type distribution of group A haemolytic streptococci isolated from scarlet fever patients, Belgrade, 1973–1982

T-type	No. of strains	%
T 1	497	29.6
T 2	123	7.3
T 3	34	2.0
T 4	333	19.8
T 5	17	1.0
T 6	78	4.6
T 8	12	0.7
T 11	20	1.2
T 12	203	12.1
T 13	96	5.7
T 23	12	0.7
T 25	10	0.6
T 28	59	3.5
T Imp19	42	2.5
T B3264	10	0.6
Other*	38	2.5
Untypable	94	5.6
Total	1678	100.0

* Types: 9, 14, 22
Type patterns: 3/13, 1/3/13, 2/28, 4/28, 11/12, 5/11/12, 8/25/Imp19

tion of type T6, the frequency of which oscillated considerably, all these types were among the most prevalent in every year under observation, changes only occurred in the order of their frequency.

The type distribution of group A haemolytic streptococci isolated from scarlet fever patients in Belgrade, was in agreement with the findings in other countries. In the earliest period of typing, 1937–38, in Edinburgh, types T1, T4 and T12 were found to be the most frequent [5]. The same types, accompanied by T6 were the most frequent in Ireland [6]. During the period 1949–59, in Rumania, Cuica et al. [2] found type T1 the most frequent "epidemic" type, and type pattern T4/24/29, the dominant "endemic" type. In the international study of type distribution of group A haemolytic streptococci in scarlet fever patients, carried out in the years 1964–65 [7] and 1968–69 [8],

types T1 and T4 were the most frequent. In Hungary, types and type patterns T13, T19, T5/12/27/44, T12 and T1 dominated in 1964–65, and types and type patterns T19, T4/24/26, T12 and T3, in 1968–69 [9]. In Japan, during the period 1964–70, again types T4 and T12 were dominant among streptococci isolated from patients with scarlet fever [10].

Our findings as well as those of other authors have confirmed that several types are predominant and that they circulate in a certain area for long periods. It is also evident that those dominant types are almost the same in different and distant territories.

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ENOXACIN: A POTENT INDUCER OF FILAMENTOUS ESCHERICHIA COLI CELLS

(A NOTE)

J. V. URI and P. ACTOR

Research and Development Division, Smith Kline and French Laboratories, Philadelphia, PA (USA)

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Enoxacin (CI-919; AT-2266), a new naphthyridine derivative was found to induce morphologic changes at very low concentrations in *Escherichia coli* but not in *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells. The development of the long filamentous forms observed with nalidixic acid is most probably a consequence of inhibition of DNA synthesis. The phenomenon may, however, not be the sole mechanism of the broad-spectrum antimicrobial activity of enoxacin.

Since the introduction of nalidixic acid in therapy in 1963, several analogues were synthesized in the hope of overcoming its therapeutic shortcomings (narrow bacterial spectrum and adverse effects). Compounds containing the naphthyridine, quinoline, cinnoline, pyridopyrimidine and other less studied ring-systems have been synthesized and evaluated [1, 2]. Some of the recent compounds have improved activities over nalidixic acid. One of the most promising of these is enoxacin, 1-ethyl-6-fluoro-1,4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid. Unlike nalidixic acid, it possesses favourable pharmacokinetic properties as well as excellent broad-spectrum activities against a series of bacteria [3–11], including *Serratia marcescens*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Since nalidixic acid is known to produce morphologic changes in *E. coli* cells [12, 13], it seemed interesting to study the phenomenon with this new analogue; the results of the study are reported here. The chemical structures of the two compounds are shown in Fig. 1.

Materials and methods

Nalidixic acid and enoxacin (kindly supplied as a research sample only by Dainippon Pharmaceutical Company) were dissolved as sodium salts in deionized water. After sterile filtration through Millipore filters (0.45 μm), the solutions were added in doubling dilutions to buffered (pH 7.3) peptone–glucose broth [14]. The minimum inhibitory concentration (MIC) values were determined and the observed morphologic changes were studied in *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853, strains recommended for quality control of antimicrobial agents. Overnight cultures (37 °C) were stained with gentian violet and examined microscopically for morphologic changes.

JOSEPH V. URI, P. ACTOR
Research and Development Division, Smith Kline and French Laboratories
P.O.B. 7927 1500 Spring Garden Street, Philadelphia, PA 19101, USA

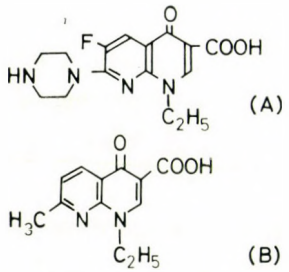

Fig. 1. Chemical structures of enoxacin (A) and nalidixic acid (B)

Results and discussion

The MIC values for enoxacin are seen in Table I. Enoxacin was highly active against *E. coli* with MIC of 0.08 μg/ml. In addition, unlike nalidixic acid, it was active against *S. aureus* and *P. aeruginosa*. Although the MIC value for *E. coli* remained the same after two additional days of incubation, these values for the other two strains shifted 1–2 dilutions.

Table I

Minimum inhibitory concentrations of enoxacin against three representative bacterial strains in peptone-glucose broth (pH 7.3) [14]

Strain	μg/ml
E. coli ATCC 25922	0.08*
S. aureus ATCC 25923	0.6
P. aeruginosa ATCC 27853	1.25

* Morphological changes observed

As shown in Fig. 2, enoxacin produced morphologic changes in *E. coli* ATCC 25922 cells, similar to those observed with nalidixic acid, but at much lower concentrations. Some elongated cells were seen at as low as 0.01 μg/ml level and ot 0.04 μg/ml long filamentous cells were formed. Nalidixic acid induced similar elongated cells at 3 μg/ml concentration [12, 13]. No morphologic alterations were observed in *S. aureus* and *P. aeruginosa* in the same system at these low concentrations of enoxacin.

Nalidixic acid is a powerful inhibitor of DNA replication (little effect on RNA and protein synthesis) [12, 15, 16]. It is not known, however, whether its antibacterial effect depended solely on this action [17]. The formation of elongated *E. coli* cells is most probably a consequence of the inhibition of DNA

synthesis. The relationships between DNA synthesis inhibition, morphologic changes and bacterial cell death cannot be studied fully with nalidixic acid, since it has activity only on a limited number of *Enterobacteriaceae*. Enoxacin is of broad antibacterial spectrum and presumably has the same mechanism of action as nalidixic acid, with similar induction of long filamentous cells of

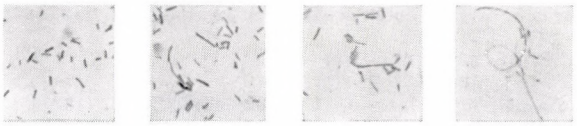

Fig. 2. Morphologic changes induced in *E. coli* ATCC 25922 cells by enoxacin at various concentrations

E. coli. Both compounds contain the 1-ethyl-1,4-dihydro-4-oxo-3-pyridine-carboxylic acid as part of their molecule. Filamentation was not observed in *S. aureus* and *P. aeruginosa* cells, despite the strong inhibition of their growth. This finding would suggest that the inhibition of DNA replication in these species may not alone be responsible for the antibacterial action of the naphthyridines, although the selective and reversible blocking by nalidixic acid and oxolinic acid of a component of DNA gyrase activity and DNA replication in *E. coli* is well documented [18–26].

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STAPHYLOCOCCUS AUREUS TOUR, A SELECTIVELY MOUSE-PATHOGENIC STRAIN FOR EXPERIMENTAL CHEMOTHERAPEUTIC STUDY

(A NOTE)

J. V. URI and LILLIAN PHILLIPS

Research and Development Division, Smith Kline and French Laboratories, Philadelphia, PA, USA

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Staphylococcus aureus Tour is a unique strain. It is highly pathogenic to mice but not to other laboratory animals and primates. This selective pathogenicity makes it useful for experimental chemotherapeutic studies. Since it is highly specialized for mice, it imitates the natural course of infection and produces death after intraperitoneal infection of a relatively few cells even when suspended in isotonic saline. This strain has been found to be very sensitive (MIC's) to various beta-lactams and gentamicin as well as useful for infection-protection studies in mice (ED_{50}'s) with a series of cephalosporins.

Staphylococcus aureus T, — souche T — (T stands for Tour, the first letter of the name of the city in France where this strain originated), was originally isolated, described and used in mouse infection-protection experiments while studying the effects of new long-acting penicillins by Levaditi and co-workers [1, 2] in the Institut Pasteur, Paris, France. Uri et al. [3] receiving a subculture of the strain, used it for comparing the chemotherapeutic effects of benzylpenicillins of diverse origin. A subculture of this strain was sent from Uri's laboratory to Gruppo Lepetit, Milan, Italy, where Fürész and co-workers utilized this strain's unique characteristic for their initial experimental chemotherapeutic evaluation of rifamycins and it was instrumental in selecting rifampicin, an analogue with well-established, therapeutic Gram-positive activity [4, 5].

Materials and methods

The same *S. aureus* T was included in the SK&F strain collection under the designation of No. 674 and is being kept in broth culture at —80 °C. It is coagulase positive, beta-haemolytic and non-beta-lactamase producer [6]. The strain retains its exceptionally high degree of selective pathogenicity for mice for decades although we have strengthened its virulence by mouse-passage years ago.

The minimal inhibitory concentration (MIC's) were determined by the conventional broth-dilution assay using the serial doubling concentrations of the antibiotics. These MIC's were obtained in media buffered to pH 7.3 [7].

JOSEPH V. URI, LILLIAN PHILLIPS
Research and Development Division, Smith Kline and French Laboratories
P.O. Box 7929, Spring Garden Street, Philadelphia, PA 19101, USA

The comparative i.p. infective potential in mice of *S. aureus* T in saline or in mucin, and the protective value of cephalosporins injected subcutaneously immediately and 5 h after inoculation were studied with the conventional method.

Results and discussion

Data of Table I demonstrate the MIC's of 19 older and newer semi-synthetic cephalosporins, 3 penicillins and gentamicin against *S. aureus* T. It can be seen that *S. aureus* T is very sensitive in vitro to most of the beta-lactam antibiotics studied and also to gentamicin with good MIC values. With other pH values the MIC's may change slightly or occasionally dras-

Table I

Sensitivity of S. aureus T (SK&F 674) in vitro to many cephalosporins, some penicillins and gentamicin

Antibiotic	MIC (μg/ml)*	Antibiotic	MIC (μg/ml)*
Cephaloridine	0.05	Cefotaxime	1.5
Cephalothin	0.2	Ceftizoxime	1.5
Cefazolin	0.2	Ceftazidime	12.0
Cephalexin	1.5	Ceftriaxone	1.5
Cefatrizine	1.5	Cefmenoxime	0.8
Cefamandole	0.8	Cefoperazone	1.5
Ceforanide	6.0	Moxalactam	12.0
Cefonicid	3.0	Penicillin G	0.05
SK&F 80303	1.5	Ampicillin	0.1
Cefoxitin	3.0	Methicillin	1.5
Cefotiam	0.8	Gentamicin	0.05
Cefsulodin	3.0		

* MIC values (μg/ml) obtained in liquid medium at pH 7.3

tically. The comparative effective values (ED_{50} mg/kg) of 3 cephalosporins as the sum total of 2 subcutaneous doses are presented in Table II. The data clearly demonstrate that *S. aureus* T produces infection in mice even when suspended in isotonic saline. It can also be seen that suspension of the bacterial cells in mucin, the same way as in saline, still increases the protective values of the same cephalosporins as expressed in lower ED_{50} values. Although the role of mucin is not clearly delineated, it can probably alter the kinetic parameters of the antibiotics in the blood of the mouse [8].

Table III demonstrates the subcutaneous protective values of 10 parenteral and 2 oral cephalosporins in mice experimentally infected intraperitoneally with a suspension in 5% gastric mucin of 10^4 cells of *S. aureus* T. The data show the highly pathogenic nature of *S. aureus* T to mice and the varying but strong ED_{50}'s of the cephalosporins studied.

Table II

Experimental chemotherapeutic studies in mice, infected with S. aureus T (SK&F 674) with and without mucin, using three selected cephalosporins

Cephalosporin	Subcutaneous ED_{50} (mg/kg)*; infection in saline suspension	Subcutaneous ED_{50} (mg/kg)*; infection in mucin suspension
Cephaloridine	0.7	0.2
Cefazolin	6.0	0.6
Cephalexin	25.0	10.0

* ED_{50} values are expressed in mg/kg body weight

Table III

Efficacy (ED_{50}) of various cephalosporins in experimental infection of mice with S. aureus T (SK&F 674)

Cephalosporin	Route of dose	LD_{50}*	ED_{50} (mg/kg)*
Cephaloridine	s.c.	66	0.195
Cefoxitin	s.c.		4.2
Cephanone	s.c.		0.63
Cefuroxime	s.c.		2.1
Cephalothin	s.c.	7.4	1.36
Cephapirin	s.c.		0.78
Cefazaflur	s.c.		1.28
Cephacetrile	s.c.		1.76
Cefazolin	s.c.		0.78
Cefamandole	s.c.		1.56
Cefatrizine	s.c.	6	1.95
Cefatrizine	oral		0.24
Cephaloglycin	s.c.		0.52
Cephaloglycin	oral		4.1

* Inoculation of mice (i.p.) with approximately 10^4 bacterial cells in 5% mucin

Most of the microorganisms are highly adapted to and specialized for their host. It is especially true for human pathogenic bacteria. But experimental chemotherapy has always been looking for animal models which more or less imitate the human infections. In the bacterial infections this goal is not yet completely achieved [9]. *S. aureus* T seems to be such a strain. In the literature another staphylococcus strain, *S. aureus* Smith, is described to be an exceptionally virulent strain for mice [10, 11]. In this respect it is similar to *S. aureus* T; however, it is not known whether or not it has the same specificity for mice as the Tour strain has. Like most bacterial strains used in the mouse for infection-protection studies, the Smith strain is usually injected in mucin suspension. The action of mucin is not yet clear, it should be virulence (pathogenicity) enhancing or resistance-lowering of the mice [12–18]. Whichever may be the case, the mucin-induced infections are sufficiently artificial, usually of the nature of septicaemia and not imitating the human pathologic events.

S. aureus T appears to be a unique strain for animal (mouse) model; it is highly pathogenic, selective and sensitive to almost all beta-lactams and many other antibiotics. This strain, adapted to mice, serves a useful purpose in mouse infection-protection chemotherapeutic (and other) studies as an acceptable animal model for human infections with *S. aureus*.

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STRUCTURE-RELATED EFFECT OF pH ON THE BIOASSAY SENSITIVITY OF FIVE THIADIAZOLE CEPHALOSPORINS

(A NOTE)

J. V. URI

Research and Development Division, Smith Kline and French Laboratories, Philadelphia, PA, USA

(Received June 11, 1984)

Using the buffered disc agar-diffusion method with *Bacillus subtilis* as test organism, a direct relationship was found between the pH, incubation temperature and the structure-dependent activity of five thiadiazole cephalosporins. This appears to be related to the ionizable group(s) of the molecules.

The influence of pH on the activity and efficacy of antibiotics have been known from the early studies on penicillin [1–4], streptomycin, and other aminoglycosides [5–12], as well as erythromycin [13–16] and rosamicin [17, 18]. Some of the studies included other classes of antibiotics as well. It has also been reported that the pH can influence the methicillin resistance of *Staphylococcus aureus* and *S. epidermidis* [19] and also the activity of certain beta-lactamases [20, 21].

Penicillins and cephalosporins are weak (a few even strong) acids (aminocyclitols are weak bases) and as such are prone to ionization. The ionized (polar) form is more water soluble, and the un-ionized (molecular) counterpart is more lipid soluble. It is well established that the degree of ionization influences the activity of any drug, in this case that of the beta-lactams. The changes in the degree of ionization are brought about by the ambient pH. It was expected that small changes in the molecular structure of the cephalosporins will modify their tendency to ionize with consequent changes in activity.

Materials and methods

In this experiment, the effect of ionization was tested by examining the activity of five variably substituted thiadiazole cephalosporins. Their antibacterial activity was assayed by using the pH-ed disc agar-diffusion method as described earlier [22, 23]. Antibiotic sensitivity discs (6.35 mm diameter, Schleicher–Schuell Inc.) were pretreated with McIlvaine's citric acid–phosphate buffers (pH's between 2.3 and 8) or with phosphate buffers (pH's 9 and 10), and stored in dry condition. Prior to assay discs were individually saturated with

JOSEPH V. URI
Research and Development Division, Smith Kline and French Laboratories
P.O. Box 7929, 1500 Spring Garden Street, Philadelphia, PA 19101, USA

the freshly prepared aqueous solutions of the appropriate thiadiazole cephalosporins so that each disc contained 1 μg of the antibiotic. Discs were placed on the surface of Penassay agar plates seeded with spore suspension of *Bacillus subtilis* ATCC 6633 and incubated overnight simultaneously at 30 °C and 37 °C. The diameters of the inhibition zones were measured and plotted. Duplicate experiments with 3 discs were carried out with each compound and the inhibition zones presented in Fig. 1 are the mean values of the diameters of the zones.

Of the five thiadiazole cephalosporins two (#1 and #3) contain the mandelic acid side chain at the 7-position and a C-methyl (#1) or C-amino (#3) group at the thiadiazole ring. Three compounds (#'s 2, 4 and 5) contain the phenylglycine side chain at the 7-position and a C-methyl (#2), a C-amino (#4) and no substituent (#5) on the thiadiazole ring. These analogues were selected because they contain various ionizable groups and it turned out that this is reflected in their activities measured by the diameters of the inhibition zones at the various pH values.

Results and discussion

Figure 1 shows the structures of the five thiadiazole cephalosporins and depicts the results of their relevant bioassay results. It can be seen in graph A that the least ionizable compound (#1) basically has the same level of activity at all pH values. The rest of the compounds contain, in addition to the carboxyl group, one or two $-NH_2$ group(s) for ionization and they show decreasing activity with the increasing alkalinity. It is most prominent with analogue #4 which contains two $-NH_2$ groups. The decrease of activities is negligible between pH's 3 and 5 or 6 but more pronounced between pH's 6 and 9. On the basis of this study we selected pH 6 for the assay of all classical beta-lactams, with increased sensitivity and satisfactory results. The explanation of the decreasing activity with increasing alkalinity (and neutrality) may be that the ionization is increased at these pH values, consequently most part of the compound is in the water soluble ionized form which penetrates the bacterial cell less than the non-ionized more lipid soluble molecular form which easily penetrates the cell envelop. In other words, the nonionized molecule has been found to be far more active than the ionized counterpart. The best known thiadiazole-containing cephalosporin, cefazolin behaves like cound #1 since it contains only one carboxyl group for ionization [24–26]. Its activity is basically not influenced by the pH. Ceftezole, another thiadiazole cephalosporin can be considered as demethylcefazolin [27, 28]. They do not contain additional functional ionizable (—OH, $-NH_2$, —SH) groups.

In addition to the state of ionization, the stability of the compounds seems to contribute to their activity as can be seen in graph B, where the incubation temperature was 37 °C, in contrast to graph A, where the assay plates were incubated at 30 °C. It is known that at lower incubation temperatures certain phenylglycine-type cephalosporins are more stable than at 37 °C.

The activity of a beta-lactam (and probably most non-neutral antimicrobial agents) is determined in addition to the ionization of the compound, by the ionization of the receptor molecules, i.e., the corresponding penicillin-binding proteins, which are also pH-dependent. They contribute to the ultimate

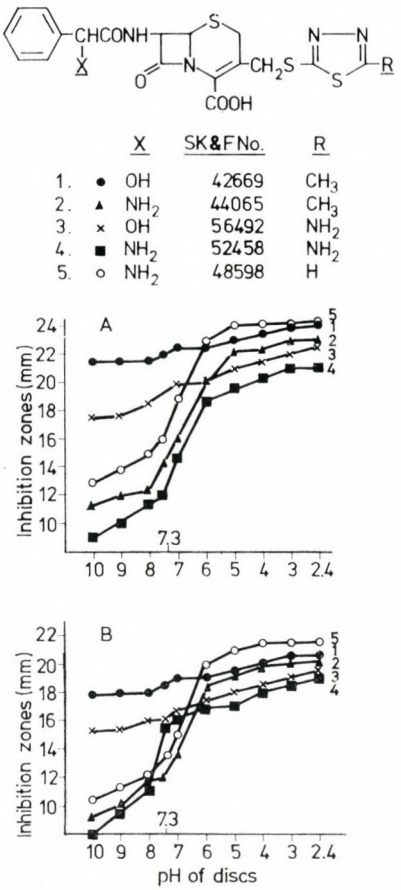

Fig. 1. Influence of pH and incubation temperature (A = 30 °C and B = 37 °C) on the activity of five thiadiazole cephalosporins

action of the compound. It can explain why the influence of pH varies according to the bacterial genera on the activity of ceftizoxime [23], cefoxitin [29], carbenicillin [30], CGP 81, CGP 3940 and cephalexin [31], pirbenicillin, carbenicillin and ticarcillin [32, 33].

The activity of most beta-lactam antibiotics (and many other drugs) is definitely pH-dependent. The relative role of the outstandingly significant ionization of the drug molecule and/or receptor or both, its pH-dependency and diffusibility may change from structure to structure. The pH often dramatically influences the bioassay sensitivity. In the case of pyrazinamide it produces an almost all or nothing effect [34]. It has in vitro tuberculostatic activity at about pH 5.0 and no activity at pH around neutrality. Gentamicin was found to be in vitro 100 or more times as active at pH 8.5 as at pH 5.0

against most Gram-negative bacilli [35] and ketoconazole is more than 1000 times more active at pH 7.0 than at pH 3.0 against *Candida albicans* [36].

The role of the pH is clear. Buffered experimental conditions provide full meaning of the bioassay by maintaining a standard, uniformly optimal condition for the study.

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A TUBE ASSAY FOR CONTAMINATION CAUSED BY PECTOLYTIC ASPERGILLI

J. O. OFFEM and R. K. DART

Microbiology Unit, Department of Chemistry, University of Technology, Loughborough, Leicestershire, U.K.

(Received December 6, 1983)

Spoilage fungi of the genus *Aspergillus* were detected and estimated in 24 h by a modification of the Most Probable Number method. The method is based on the production of pectolytic enzymes in a medium supplemented with pectin. These enzymes hydrolyse the bonds of pectin to produce galacturonic acid, causing a fall in pH and an indicator present in the medium changes colour, thus allowing tubes to be scored as positive. Results obtained by this method were generally higher than those obtained by traditional plate counts.

The Most Probable Number (MPN) method of estimating bacterial numbers is widely used. It was described by McCrady [1] and has since been reviewed on a number of occasions [2–6]. It has been used mainly for estimating bacterial contamination of water and milk, but has also been applied to food [7] and soil [8]. The traditional criteria for detecting growth involves a visual determination of gas bubbles and a colour change of the pH indicator incorporated into the medium [9].

Very little has been published about the application of the MPN method to the detection and estimation of fungi, although Koburger and Norden [10] applied it to the estimation of yeasts in food and compared the results with surface and pour plate methods. Their method required 5 days due to a reliance on the visual detection of yeast colonies or the use of the Gram stain when the medium was turbid.

Dormant spores on the genus *Aspergillus* are found widely on agricultural products. The threat to quality is minimal during cultivation and harvesting [11, 12], but there is deterioration frequently during long periods of storage in tropical conditions of high humidity and temperature. Deterioration has been reported in peanuts [13], cocoa beans [14, 15], soybeans [16], palm produce [17–19] and maize [20].

Several types of deterioration have been attributed to fungi, the most important of which is toxin production. However, non-toxigenic strains can also cause deterioration by changing the flavour components, causing a loss

J. O. OFFEM
Department of Chemistry, University of Calabar
Calabar, Nigeria

R. K. DART
Microbiology Unit, Department of Chemistry, University of Technology
Loughborough LE11 3TU, Leicestershire, U.K.

of seed viability and hydrolyzing triglycerides causing a rise in free fatty acids leading to autoxidation and rancidity.

This deterioration causes severe economic losses, and from the criterion of produce inspection it is desirable that dormant spores capable of causing such deterioration should be detected as rapidly as possible and appropriate corrective measures taken.

This paper reports an attempt to establish a MPN method for the rapid detection and estimation of low numbers of spoilage fungi. It is based on the observation by Verbina and Tsytsura [21] that fungi producing pectolytic enzymes can be rapidly detected by observing the colour change of methyl red caused when such fungi hydrolyse pectin to galacturonic acid.

Materials and methods

Organisms. The six organisms used were *Aspergillus flavus* (CMI 15959), *A. flavus* (CMI 39178a), *A. flavus* (CMI 86769), *A. nidulans* (CMI 16643), *A. niger* (CMI 31821) and *A. luchuensis* Inui. This last is an illegitimate species and is probably a synonym for *A. awamari* Nakazawa. All were obtained from the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, U.K.

Media and cultivation. Cultures were grown on the following medium to obtain conidia: K_3PO_4, 2.0 g; KNO_3, 2.0 g; NH_4NO_3, 2.0 g; $CaCl_2$, 0.25 g; yeast extract (Oxoid), 5.0 g; glucose, 10 g; Bacto casamino acids (Difco), 5.0 g; metals solution, 0.1 ml; water 1 litre. The metals solution consisted of the following: $MgSO_4$, 5 mg; $ZnSO_4$, 0.2 mg; $FeSO_4$, 0.2 mg; $MnSO_4$, 0.1 mg; $CuSO_4$, 0.5 mg; water, 100 ml. The medium was adjusted to pH 5.4 and dispensed as 250 ml volumes into Roux bottles.

Each Roux bottle was inoculated with the appropriate culture and incubated on its side at 30 °C for 3 weeks. At the end of this period each culture was covered with a thick layer of conidia which were purified aseptically as described by Dart [22]. Conidia were stored as stock suspensions at 0 °C until required.

The medium used for plate counts was made as follows: maltose, 38 g; neutralized soya peptone, 8 g; yeast extract, 2.5 g; malt extract, 2.0 g; agar, 20 g; distilled water to 1 litre. The pH of the medium was 5.4.

Surface plates were prepared by placing 10 ml of medium into Petri dishes and allowing them to solidify. After drying 1.0 ml of conidium suspension was spread over the surface of the plate, which was then incubated at 30 °C for 48 h.

Pour plates were prepared by placing 1.0 ml of conidium suspension in a Petri dish and adding 10 ml of molten agar which had been allowed to cool to just above the solidification point. These plates were rapidly mixed and when set, they were incubated at 30 °C for 48 h.

The tube assay medium used consisted of 4% glucose, 1% neutralized soya peptone, 1% pectin (Citrus pectin, rapid set type, 104 Bulmer Ltd., Hereford, U.K.), chloramphenicol and chlortetracycline (0.01% each). The medium was sterilized by autoclaving, three drops of sterile 0.1% bromocresol purple in 2% ethanol were added and sufficient sterile 0.1 N NaOH to just give an alkaline colour.

The detection and counting was carried out by a modification of the MPN method of Halvorson and Ziegler [4]. This consisted of a series (A) of eleven test tubes each containing 9 ml of medium. Ten tubes were inoculated with 1 ml of an appropriate dilution of a conidium suspension (A) and the eleventh tube was inoculated with 1 ml of sterile water as control. A second series (B) and a third series (C) of eleven tubes each were similarly inoculated with the appropriate dilutions of conidium suspensions and sterile water. The conidium suspensions were diluted with sterile distilled water such that the ratio of dilutions of A : B : C was 100 : 10 : 1.

Before using the conidium suspensions for both tube and plate counts, they underwent heat shock activation in a water bath at 50 °C for 25 min [23], and were then agitated vigorously to prevent clumping. The tubes were incubated in an orbital incubator operating at 150 rpm for 24 h at 30 °C.

After the appropriate time of incubation the tubes were examined and those tubes showing the yellow acid colour of the indicator at the surface of the culture were scored as positive (Plate I).

Results and discussion

Plate I shows typical series of tubes containing control, positive and negative results. The MPN table of Halvorson and Ziegler [2] was then consulted to find the most probable number of spores present in the original suspension. Results obtained by this method are shown in Table I.

The presence of conidia of spoilage fungi in cereals is a major problem, especially in many developing countries where the ambient temperature and humidity are high and storage facilities are poor. Rapid methods of quality control are obviously needed because of the toxic nature of many fungal metabolites.

Chitin determination [24, 25] may be used for rapid analysis of fungi, but this method suffers from several serious drawbacks. Although it is rapid (4–5 h), it does not distinguish viable from non-viable propagules, it requires high levels of fungal contamination to provide sufficient chitin for analysis, and it is subject to interference from insect contamination.

The estimation of the fungal sterol, ergosterol, has also been used [26], but this also suffers from the fact that it measures total numbers and not viable numbers of fungi.

Dart and Offem [27] and Offem and Dart [28, 29] used gas chromatography to determine methanol released from pectin by the enzyme pectin-

Table I

Comparison of fungal spore counts obtained by an MPN tube method and plate counts

Organism	Number of organisms/ml. Mean ± SD		
	MPN method	Pour plate	Surface plate
A. flavus 15959	122 000 ± 55 000	104 000 ± 38 000	112 000 ± 46 000
A. flavus 39178a	3 780 ± 1 450	3 400 ± 1 400	3 500 ± 1 380
A. flavus 86769	11 000 ± 2 400	11 000 ± 3 500	11 400 ± 3 900
A. nidulans	86 000 ± 3 200	80 400 ± 2 860	83 000 ± 3 200
A. niger	850 ± 130	800 ± 110	820 ± 120
A. luchuensis lnul	3 200 ± 370	3 030 ± 360	3 040 ± 380
Composite suspension of all 6 strains	13 500 ± 1 800	13 000 ± 1 700	13 000 ± 280
Time	24 h	48 h	48 h

Each result is the mean of three suspensions with 2 determinations being carried out on each suspension.

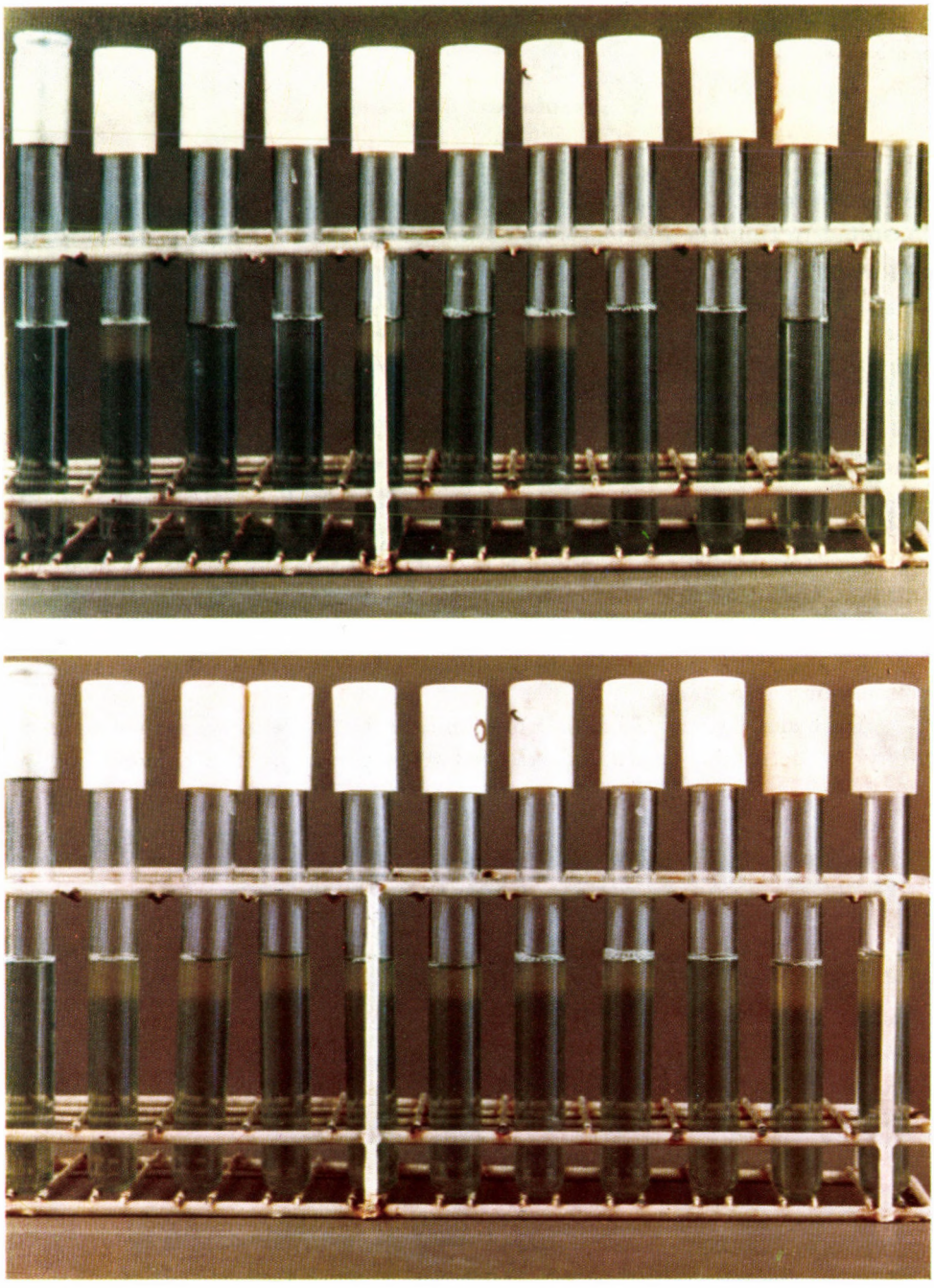

Plate I. Tube method for fungal spore counts. Uninoculated control: first tube on left; positive tubes: yellow at the surface; negative tubes: no colour change

esterase. The methanol could be related to fungal numbers and distinguished viable from non-viable cells, and therefore had potential use for predicting viable fungal spores. A major advantage of this method is that it is linear over a wide range, but major disadvantages are that it requires expensive, fairly sophisticated equipment (GLC), and a reasonable level of technical expertise which may not be appropriate to a developing country.

The method described in this paper is much cheaper to carry out, only requires a relatively low level of technical training and gives results in 24 h compatible with those obtained by plate counts at 48 or more hours where the plate count is relying on visible growth. Its major disadvantage is that it requires sterile serial dilutions which may be difficult to carry out in field testing, although sterile serial dilutions are also a requirement for plate counts.

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INFLAMMATORY REACTION IN GERMFREE MICE

I. Budavári, Piroska Anderlik, Zsuzsanna Bános, J. Fürész and Ilona Szeri

Institute of Pathophysiology and Institute of Microbiology, Semmelweis University Medical School, Budapest, and Hungarian Army Medical Corps, Budapest

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In intact germfree animals, the total leukocyte count, the number of polymorphonuclear (PMN) granulocytes, the phagocytic index of peritoneal PMN granulocytes, the serum seromucoid and coeruloplasmin levels are significantly lower than in conventional controls. As a result of treatment with turpentine, the total leukocyte count and the number of PMN granulocytes increased significantly in both groups, but the phagocytic index and the level of the two examined acute phase proteins rose significantly less in germfree animals than in conventionals. The decreased phagocytic function of PMN granulocytes in germfree animals has been explained by the lack of the stimulating effect of the normal bacterial flora.

It was proved in the last decade that some reactions of the specific immune response are different both quantitatively and qualitatively in germfree mice from those in conventional ones. Several authors demonstrated that the cellular type immune response was much less marked in germfree than in conventional animals [1, 2]. The humoural immune response also shows divergences. It was shown that the immunoglobulin level was about 50% lower in germfree than in conventional animals of the same age [3]. Other authors showed that the turnover of immunoglobulins was significantly slower in gremfree mice than in conventional controls [4]. Still, a lower immunoglobulin level following the germfree condition and the consequentially weaker humoural immune reaction are not necessarily valid for all immonoglobulin classes [5].

Concerning the non-specific resistance to infection, i.e. phagocytosis, the results are ambiguous. According to some data, the macrophage activity of germfree animals differs considerably from that of conventional animals. Adherence and chemotactic activity of the former are significantly less intense and lysosomal enzyme activity and phagocytosis mediated by C3b receptors are also smaller in the cells mentioned above [6–8]. Other experiments showed

István Budavári
Institute of Pathophysiology, Semmelweis University Medical School
Nagyvárad tér 4, H-1089 Budapest, Hungary

Piroska Anderlik, Zsuzsanna Bános, Ilona Szeri
Institute of Microbiology, Semmelweis University Medical Schoo
H-1445 Budapest, P.O.B. 370, Hungary

József Fürész
Hungarian Army Medical Corps
Budapest, Hungary

a decreased phagocytic activity of the polymorphonuclear (PMN) granulocytes of germfree animals [9]. Other authors, however, have not found such difference between germfree and conventional animals [10, 11].

It has been shown that the acute phase proteins (coeruloplasmin, α_1-acid glycoproted, C-reactive protein, etc.) migrating in the α_1 and α_2 globulin fraction of plasma proteins have an important modulatory role in the specific immune response and also in phagocytosis [12, 13] and that besides immunoglobulins the amount of α_1 and α_2 globulin fractions was also less in germfree animals [14–16].

In the present experiments we have examined some parameters of the non-specific host resistance in germfree and conventional mice to experimental inflammation induced by turpentine.

Materials and methods

Experimental animals. Experiments were carried out on 20 conventional and 20 germfree C3H He (LATI, Gödöllő, Hungary) male mice. Germfree environment was provided by a special plastic isolator (Velaz-PO-I, Czechoslovakia). The animals in isolators were offered radiation-sterilized pellets and autoclaved water *ad libitum*. The germfree state was maintained as described earlier [17].

Induction of experimental inflammation and collection of peritoneal PMN granulocytes. Inflammation was induced on the hind limbs of 10 conventional and 10 germfree mice by 0.2 ml/100 g body weight of sterile turpentine oil injected intramuscularly. Ten conventional and ten germfree mice served as untreated controls. The animals were sacrificed 3 days after turpentine administration. Before sacrifice, the animals were injected intraperitoneally with 1.5 ml 6% dextran (mol wt 200–300 000), then after a three-hour interval, peritoneal fluid was sucked off by a plastic canula. From this fluid, the PMN granulocytes were isolated by centrifugation (1000 rpm). The cell suspension contained about 98% of PMN granulocytes.

Examination of phagocytosis. The yeast-cell phagocytosis method of Kotulova [18] was applied. A mixture of 0.9 ml PMN granulocyte suspension (10^6 cells/ml) and 0.1 ml yeast-cell suspension (PMN : yeast cell ratio, 1 : 7) was incubated at 37 °C for 90 min, then smears were prepared, stained with Giemsa solution and phagocytic activity (percentage of phagocyting PMN granulocytes) and phagocytic index (average number of yeast cells per phagocyting PMN granulocyte) were determined.

Blood analysis. Before sacrifice, the total leukocyte count and the number and ratio of PMN granulocytes in the peripheral blood were determined. After bleeding, serum was obtained and from among the acute phase proteins the seromucoid (α_1 acid glycoproteid) level, based on the hexose content bound to protein, was determined by the method of Winzler [19], and the coeruloplasmin content, based on its property to oxidize paraphenylendiamine, was determined by the method of Ravin [20].

Statistical evalution. For evaluation of the results, Student's two-sample t test was applied.

Results

The total leukocyte count and the number and ratio of PMN granulocytes in conventional and germfree mice are shown in Fig. 1 and Table I.

The leukocyte count was about 35% less in germfree animals than in conventional ones ($p < 0.05$). As a result of the inflammation caused by turpentine, the leukocyte count increased by about 70% in conventional mice

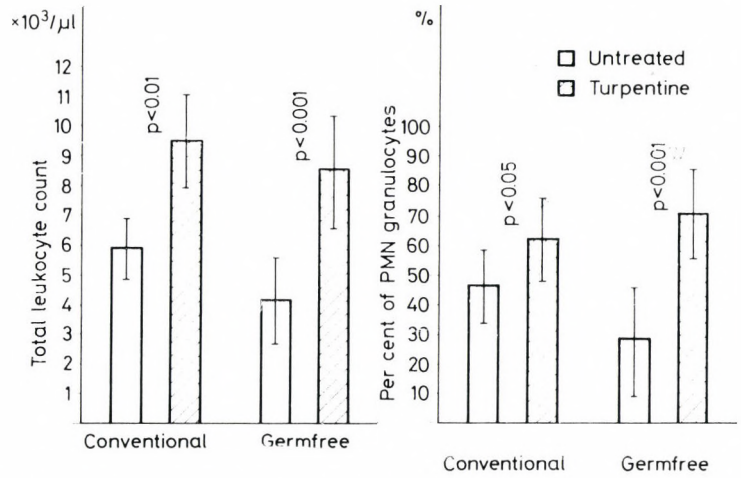

Fig. 1. Total leukocyte count and the ratio of PMN granulocytes in the peripheral blood of conventional and germfree mice in inflammation induced by turpentine

and about 100% in germfree animals ($p < 0.01$; $p < 0.001$) as compared to untreated controls. The proportion of PMN granulocytes in conventional animals amounted to 45% while in germfree animals to only 25% of the total leukocyte count. On the third day of inflammation the percentage of PMN granulocytes was about 50% higher in the conventional animals, but in the germfree ones it was more than 100% higher than in untreated controls ($p < 0.05$; $p < 0.001$). The difference between the two groups was significant statistically ($p < 0.02$).

Figure 2 shows the phagocytic capacity of peritoneal PMN granulocytes in conventional and germfree animals with inflammation caused by turpentine.

The phagocytic activity did not differ in the two groups of untreated mice. As a result of the inflammation, the percentage of phagocyting PMN granulocytes increased by 15% in conventional animals and by about 20% in

Table I

Number of PMN granulocytes in the peripheral blood

Mice	PMN granulocytes pr µg	
	untreated	turpentine
Germfree	1200 ± 680	5950 ± 1450
Conventional	2760 ± 610	5890 ± 1200

germfree ones; the changes were not significant statistically. The phagocytic index of untreated germfree animals was about 25% lower than that of the conventional animals ($p < 0.01$). The phagocytic index of conventional animals with turpentine-induced inflammation was about 70%, while that of the germfree animals was about 30% higher than that of untreated mice. The degree

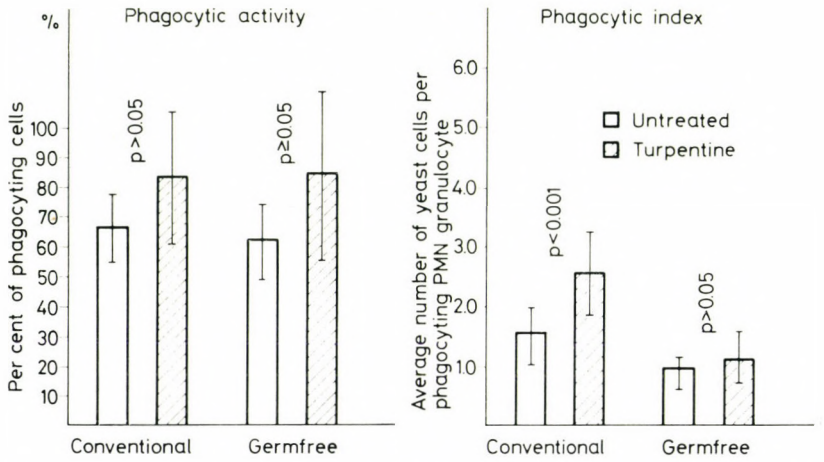

Fig 2. Phagocytosis of peritoneal PMN granulocytes in conventional and germfree mice in inflammation induced by turpentine

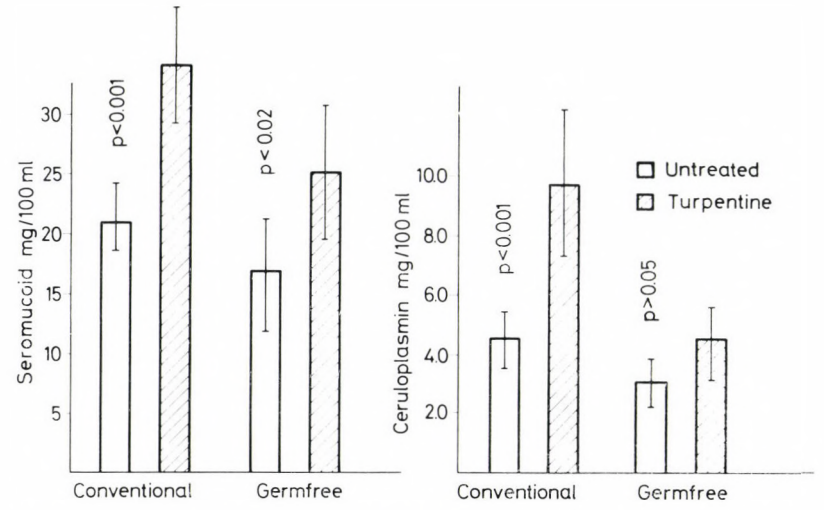

Fig. 3. Seromucoid and coeruloplasmin levels in conventional and germfree mice in inflammation induced by turpentine

of alteration was significant only in the conventional animals ($p < 0.001$; $p > 0.05$).

The serum acute phase proteins are shown in Fig. 3. The seromucoid (α_1-acid glycoproteid) level of intact germfree animals was about 20%, the coeruloplasmin level 33% lower than that of conventional mice; the difference was significant only in the case of coeruloplasmin ($p < 0.02$). As a result of the inflammation induced by turpentine, the seromucoid level in conventional animals was 60%, while in germfree mice 40% higher than in the untreated ones; the difference was significant in both groups ($p < 0.001$; $p < 0.02$). The coeruloplasmin level showed a 30% rise in germfree animals, while more than 100% in conventional ones as compared to untreated controls; the change was significant only in the case of conventional animals ($p < 0.001$).

Discussion

The present results showed that the total leukocyte count and the number of PMN granulocytes in peripheral blood, the phagocytic function of PMN granulocytes collected from the peritoneal cavity and the serum acute phase proteins participating in the regulation of phagocytosis are less in germfree animals than in intact conventional ones. As regards the level of acute phase proteins, our results supported those data [14, 15] according to which the level of some proteins of the α_1 and α_2 globulin fraction is lower in germfree animals than in conventional ones. Concerning the phagocytic function, our results agreed with the data of Morland et al. [8] on macrophage phagocytosis.

According to Kenworthy and Allen [21], in conventional animals the level and activity of the defence against bacteria is evolving both phylogenetically and ontogenetically during symbiosis of the macroorganism and the normal bacterial flora, as a result of the inducing effect of the permanently existing so-called "background" inflammation due to continuous bacterial irritation. The lack of this effect of the normal bacterial flora might explain the lower levels of the leukocyte count, of the phagocytic function and of the acute phase proteins in germfree animals. This would also explain the contradictory literary data [6], according to which in the case of the plasma-proteins having no direct role in non-specific resistance (like the α_2M, haptoglobin) there is no difference between germfree and conventional animals.

In our experiment after turpentine treatment the phagocytic index did not rise in germfree animals and the level of acute phase proteins also rose less than in conventional controls.

It is known that α_1-acid glycoproteid inhibits chemotaxis, opsonization and ingestion [12, 13], and that coeruloplasmin has a role in the elimination of the superactive free oxygen radicals evolving during phagocytosis [21].

The serum level of these two proteins changes in parallel with the change of phagocytic function. Therefore our finding that the seromucoid and coeruloplasmin levels are rising as a result of the inflammation induced by turpentine, and this rise is less in germfree than in conventional animals, can be ascribed to the decreased phagocytosis of germfree animals.

After turpentine treatment, the total leukocyte count and the number of PMN granulocytes showed a similar change in both groups, and the rise was even higher in germfree than in conventional animals. A possible explanation for this could be that besides an ancient humoural regulation (leucopoietin effect), a phylogenetically younger and more mobile neuroendocrine regulation, too plays an important role in the inflammatory reaction of peripheral leukocytes, and this reaction depends less on the presence or absence of the bacterial flora than the ancient one [12]. It therefore seems that besides the specific immune response the normal bacterial flora plays an important role in the development of non-specific resistance (phagocytosis).

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SALT TOLERANCE OF *AZOSPIRILLUM BRASILENSE*

A. V. Rao and B. Venkateswarlu

Division of Soil–Water–Plant Relationship, Central Arid Zone Research Institute, Jodhpur, India

(Received March 15, 1984)

The effect of various salts on the growth and N_2-ase activity of *Azospirillum brasilense* was tested. Bicarbonate was found to be the most toxic, followed by chlorides and sulphate. Tolerance of *A. brasilense* to these salts was comparable to that of many species of *Rhizobium*. SO_4^{--} was stimulatory to growth and N_2-ase activity up to 40 meq. The process of N_2-fixation (N_2-ase activity) was found to be more sensitive to all the salts tested as compared to growth.

Azospirillum has been receiving much attention recently in view of its nitrogen fixing property associated with a large number of grasses and grain crops [1, 2]. Studies on its taxonomy, physiology and inoculation effects on several plants have been reported by Tarrand et al. [3], Döbereiner and Depolli [4] and Venkateswarlu and Rao [5]. There is, however, no information available on the influence of various environmental factors like salinity, desiccation and temperature on this organism, which would be essential for understanding its survival and establishment in the soils and rhizosphere. The present paper reports on a study of the effect of different salts on the growth and N_2-ase activity of *Azospirillum brasilense* under laboratory conditions.

Materials and methods

The *A. brasilense* culture used in these experiments was isolated by the authors from the roots of *Pennisetum americanum* (L.) Leeke, and identified according to the classification suggested by Tarrand et al. [3].

To study the effect of various salts on growth (OD), nitrogen free malate liquid medium of Döbereiner and Day [1] (Composition g/l: malic acid, 5.0; K_2HPO_4, 0.5; $FeSO_4 \cdot 7\ H_2O$, 0.01; $MgSO_4 \cdot 7\ H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2\ H_2O$, 0.02; $Na_2MoO_4 \cdot 2\ H_2O$, 0.002; KOH, 4.7; pH adjusted to 7.0) supplemented with 250 ppm ammonium sulphate was used as the basal medium. Five salts (NaCl, KCl, $MgCl_2$, Na_2SO_4 and $NaHCO_3$) were added to this medium before sterilization so as to give a final concentrations of 0 to 300 meq of Cl^- and SO_4^{--} and of 0 to 200 meq of HCO_3^-. Each concentration was replicated thrice. The medium was later distributed in 20 ml tubes of 6 ml each. Inoculation was done with 0.2 ml pure washed cell suspension (0.6 OD) of *A. brasilense* grown in nutrient broth for 4 days. The tubes were incubated at 30 °C for 72 h and the OD was measured at 520 nm by a Systronics Spectrophotometer.

A. V. Rao, B. Venkateswarlu
Division of Soil-Water-Plant Relationship, Central Arid Zone Research Institute
Jodhpur 342 003 India

For studying the effect of salts on N_2-ase activity, N-free semi-solid malate medium with 0.175% agar was used. Cultures were grown in 7 ml serum vials containing 3 ml medium with salts at different concentrations. Inoculation was done with 0.1 ml of pure cell suspension (OD 0.6). After incubation at 30 °C for 2 days the cotton plugs were replaced with suba seals and 10% air from each tube was replaced with acetylene. The ethylene formed after incubation at 30 °C for 24 h was estimated by an AIMIL-Nucon gas chromatograph employing a poropak-T-column (2×0.003 m) with N_2 carrier gas at a flow rate of 30 ml min^{-1}. The activity was expressed as nmoles of C_2H_4 produced per hour.

Results and discussion

Among the salts tested, bicarbonate was found to be the most toxic to *A. brasilense*, followed by chlorides and sulphate (Fig. 1). HCO_3^- at 60 meq completely inhibited N_2-ase activity although traces of growth could be found up to 80 meq. There was a 95% reduction in growth and N_2-ase activity at

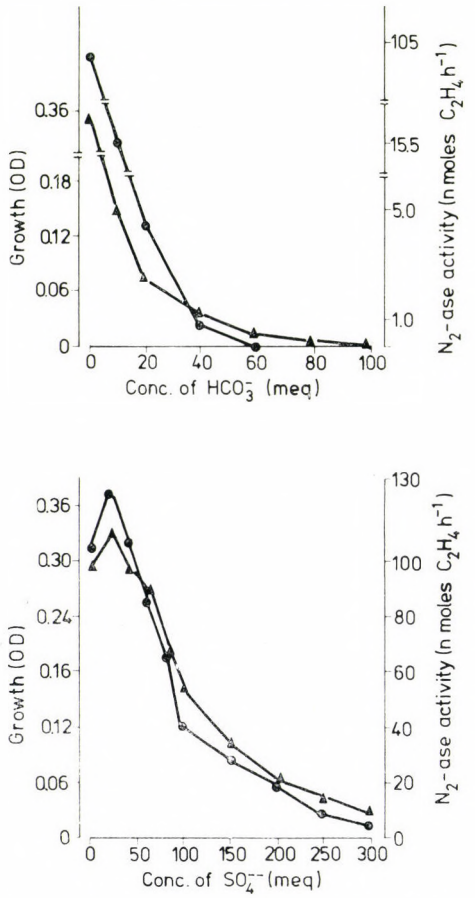

Fig. 1. Effect of HCO_3^- and SO_4^{--} on growth (▲—▲) and N_2-ase activity (●—●) of *A. brasilense*

40 meq HCO_3^-. Studies on salt tolerance of several rhizobial strains indicated that most of the strains from lucerne, black gram and pea were inhibited by 0.2 to 0.4% $NaHCO_3$, equivalent to 25 to 50 meq HCO_3^- [6, 7]. It appears that, like most of the strains of *Rhizobium*, *A. brasilense* is also sensitive to low concentrations of HCO_3^-.

Among chlorides, NaCl was the most inhibitory for growth, followed by KCl and $MgCl_2$ (Table I), but the maximum reduction in N_2-ase activity was observed with KCl. There was a gradual decline in N_2-ase activity with all the salts up to 300 meq but growth was stimulated at low concentrations (up to 100 meq) of $MgCl_2$. Stimulation of growth of *Rhizobium* by $MgCl_2$ and $MgSO_4$ was reported also by Yadav and Vyas [6] who attributed the phenomenon to the importance of magnesium for *Rhizobium* [8] and *A. brasilense* might also be responding to this element in a similar way.

Although *A. brasilense* could grow even with 300 meq chloride as NaCl and KCl, the N_2-ase activity was completely inhibited by 250 meq (Table II). Similarly, $MgCl_2$ did not affect growth up to 100 meq but at this concentration caused a significant reduction in N_2-ase activity. These results suggest that in this bacterium the process of N_2-fixation is more sensitive to salts than growth.

The results of earlier studies on salt tolerance of microorganisms like *Rhizobium* were far from uniform [6, 9, 10]. Different strains from the same host showed varying tolerance (0.2 to 3.0%) to NaCl. However, Graham and Parker [11] reported that except *Rhizobium meliloti* all other rhizobia were inhibited by 2.0% NaCl (340 meq Cl^-). Strains of *R. meliloti* showed 30–40% growth even with 3.0% NaCl [6]. *A. brasilense* showed a 60% inhibition of growth at 300 meq Cl^- (1.75% NaCl), which indicates that the salt tolerance of this organism is comparable to most of the rhizobial strains except *R. meliloti*.

Table I

Influence of chlorides on the growth of A. brasilense

Concentration of Cl (meq)	Growth (optical density)		
	NaCl	KCl	$MgCl_2$
0	0.304	—	—
50	0.281	0.298	0.312
75	0.272	0.280	0.385
100	0.251	0.265	0.320
150	0.225	0.236	0.295
200	0.201	0.192	0.248
250	0.158	0.163	0.201
300	0.129	0.141	0.182

LSD (P = 0.05); salts: 0.027; concentrations: 0.059; interaction: 0.089

Table II

Effect of chlorides on N_2-ase activity of A. brasilense

Concentration of Cl^- (meq)	N_2-ase activity n moles C_2H_4 $tube^{-1}h^{-1}$		
	NaCl	KCl	$MgCl_2$
0	105.3	—	—
50	80.7	71.4	102.3
75	74.3	52.2	98.6
100	59.1	36.4	86.4
150	32.2	25.16	52.9
200	20.8	13.26	40.0
250	7.2	1.49	18.7
300	0	0	2.8

LSD (P = 0.05); salts: 2.45; concentrations: 4.47; interaction: 12.2

There was a stimulation of growth and N_2-ase activity up to 20 meq of SO_4^{--} added as Na_2SO_4 (Fig. 1), but when the concentration exceeded 100 meq it was found to be even more inhibitory than were the chlorides. This was in contrast with the results of Yadav and Vyas [6] who did not find any stimulation of *R. meliloti* by sulphates. Thus SO_4^{--} appears to be more critical than chlorides because SO_4^{--} became highly toxic beyond 200 meq.

From these results it may be concluded that the N_2-fixation process (N_2-ase activity) in *A. brasilense* is more sensitive than its growth to all the salts and this bacterium has a comparable degree of salt tolerance to that of many species of *Rhizobium*. Further studies on the effect of salts on nitrogen fixation are, however, necessary to understand the mechanism of salt action on the enzyme, i.e. the inhibition of enzyme synthesis or the activity of the enzyme already formed.

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INTERFERON PRODUCTION BY AND RADIOPROTECTIVE EFFECT OF POLY I : C AND TILORONE IN MICE EXPOSED TO HELIUM ALPHA IRRADIATION

MARGARITA TÁLAS, BORIS FEDORENKO, LÁSZLÓ BÁTKAI and IVANA STÖGER

National Institute of Hygiene, Budapest, Hungary, and Institute of Medicobiological Problems, Moscow, USSR

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Serum interferon production induced by poly I : C and tilorone and their radioprotective effect in mice exposed to He-alpha particles was studied. The results were compared to those observed after exposure of mice to acute ^{60}Co-gamma irradiation. Interferon production induced by poly I : C was depressed by He-alpha irradiation less than that induced by tilorone. Treatment of mice with poly I : C or tilorone before He-alpha irradiation had no effect on the mortality of animals in contrast to the radioprotective activity of these compounds against ^{60}Co-gamma irradiation.

Interferon (IFN) production in mice exposed to X-irradiation was studied previously. Jullien and de Maeyer [1] and de Maeyer et al. [2] examined the effect of acute X-irradiation on virus-induced IFN production and observed that radiosensitivity of the process depended on the viral inducer applied. Our experiments showed that acute and fractional X-irradiation diminished IFN production induced by Newcastle Disease Virus (NDV), but had no influence on Semliki Forest Virus, poly I : C and *Escherichia coli* lipopolysaccharide-induced mouse interferon synthesis [3, 4]. Further, it was found that similar to X-irradiation IFN response to poly I : C was not reduced by ^{60}Co-gamma irradiation, but NDV-induced serum IFN titres were depressed [5].

The possible radioprotective activity of IFN inducers was also studied. Semina et al. [6] observed that poly I : C pretreatment prolonged the survival of acutely X-irradiated mice and activated their haemopoietic stem-cells. Pretreatment of mice with poly I : C, NDV and *E. coli* endotoxin resulted in a 2–2.5 fold increase in the number of karyocytes in mice irradiated with high energy protons [7]. Moreover, the number of aberrant mitoses decreased. While in proton-irradiated mice the number of aberrant mitoses was about 37 %, in animals treated with IFN inducers it was 2–4 times less [8]. The

MARGARITA TÁLAS, LÁSZLÓ BÁTKAI, IVANA STÖGER
Microbiological Research Group of the National Institute of Hygiene
Pihenő út 1, H-1529 Budapest, Hungary

BORIS FEDORENKO
Institute of Medicobiological Problems
Horoshevskoe Shosse 76a, Moscow, 123007, USSR

radioprotective activity of poly I : C, tilorone and poly G : C in mice exposed to acute X-ray and prolonged ^{60}Co-gamma irradiation was also shown [9, 10].

In this report results referring to IFN production induced by poly I : C and tilorone and the radioprotective activity of these compounds in mice irradiated with high energy He-alpha particles have been compared to those observed after exposition of mice to acute Co-gamma irradiation.

Materials and methods

Experimental animals. Inbred, 8–10 weeks old CBA$\times$C$_{57}$Bl$_6$(F$_1$) mice of both sexes weighing 18–20 g, were used. Each group consisted of 20–25 mice.

Irradiation. Mice were exposed to heavy ions of helium (alpha-particles) in doses of 2, 4, 5, 6, 7 and 8 Gy with energy of 1.8, 3.3 and 4.6 GeV/nuclon. Linear energy transmission (LET) was 8.3 and 8.8 MeV $\cdot$ g^{-1} $\cdot$ cm^{-2}, the intensity of irradiation 0.055 Gy/sec. The source of He-alpha particles was the synchrophasotron of the United Institute of Nuclear Investigations (Dubna, USSR). ^{60}Co-gamma irradiation was administered by means of a PX-gamma-30 apparatus. Doses applied ranged from 2 to 8 Gy. Intensity of irradiation was 0.004 Gy/s.

IFN induction. Polyriboinosinic–polyribocytidylic acid (poly I : C) (Calbiochem), and tilorone dihydrochloride (kindly donated by Dr. R. F. Krueger, The Merrell National Laboratories, Cincinatti, USA) were used as IFN inducers. Poly I : C was injected intraperitoneally in a dose of 2.5 mg/kg, 1 day, 3 and 5 days after irradiation. Tilorone was applied intragastrically (200 mg/kg) at 10 and 48 h, and 7 days after irradiation. Mice were exsanguinated by puncture of the orbital plexus 2 h after injection of poly I : C and 18 h after administration of tilorone.

IFN assay. Mouse serum IFN was assayed in microtest (Falcon TC plates) by means of the CPE inhibition method [11] in L-929 permanent mouse fibroblast cultures using encephalomyocarditis (EMC) virus for challenge.

Laboratory mouse IFN standard was compared to the WHO International Reference Preparation (Mouse interferon 12 000 IU/ml NIAID-NIH, Bethesda, USA). IFN titres were expressed in international units (IU).

Prophylactic treatment. Poly I : C in a dose of 2.5 mg/kg was injected intraperitoneally into mice 2 h before exposition to ^{60}Co-gamma rays and He-alpha particles. Tilorone (200 mg/kg) was administered to mice intragastrically 18 h before ^{60}Co-gamma and He-alpha irradiation. Mortality of mice was registered on the 30th day after irradiation.

Standard error of percentage was calculated according to the equation

$$S_p = \sqrt{\frac{p\,(100 - p)}{n}}$$

where p = mortality in per cent, n = number of animals.

Results

Interferon production induced by poly I : C and tilorone in He-alpha and ^{60}Co-gamma irradiated mice. The effect of ^{60}Co-gamma and He-alpha irradiation on poly I : C-induced serum IFN production in mice is presented in Table I. ^{60}Co-gamma irradiation had no effect on IFN production of mice. Exposure of mice to the lowest dose (2 Gy) of He-alpha particles was without effect on IFN synthesis. Higher doses (4 Gy and 6 Gy) depressed IFN production on the 3rd day after irradiation to one third of the control value. On the

Table I

Production of poly I : C-induced interferon in mice exposed to ^{60}Co gamma and He-alpha irradiation

Dose of irradiation (Gy)	Time of poly I : C administration (days after irradiation)	Interferon titre (IU) in 0.1 ml of serum* of mice exposed to irradiation	
		^{60}Co-gamma	He-alpha
–	1	280 ± 40	280 ± 40
2		290 ± 62	305 ± 25
4		303 ± 53	292 ± 57
6		289 ± 69	105 ± 28
–	3	300 ± 25	300 ± 25
2		292 ± 31	308 ± 42
4		304 ± 28	92 ± 18
6		320 ± 22	52 ± 15
–	5	260 ± 28	260 ± 28
2		279 ± 35	256 ± 17
4		301 ± 37	262 ± 21
6		295 ± 31	71 ± 15

Polyriboinosinic-polyribocytidylic acid (poly I : C), 2.5 mg/kg, was injected intraperitoneally. Blood was taken 2 hours after administration of inducer. Energy of He-ions was 4.6 GeV/nuclon

* Mean ± SD

5th day after irradiation IFN production decreased only when the highest dose (6 Gy) was used.

In Table II the effect of Co-gamma and He-alpha irradiation on tilorone-induced IFN production is shown. Ten hours after 2 Gy Co-gamma-rays IFN production was unaltered; its notable depression was observed only after higher (4 and 6 Gy) doses. Two days after gamma-irradiation the depression was more pronounced and a significant decrease of the serum IFN levels (5–10% of the non-irradiated controls) was seen at exposure to doses of 4 Gy and 6 Gy. Seven days after exposition to 2 Gy Co-gamma-rays IFN production reached already the control level. In the groups exposed to 4 Gy and 6 Gy, IFN production began to approach the normal values. After He-alpha irradiation a strong decline of tilorone-induced IFN synthesis was observed as soon as 10 h after exposition. In mice irradiated with the lowest dose (2 Gy), the IFN titre was about 8 times less than in non-irradiated controls and when higher doses (4 Gy and 6 Gy) were applied, it did not reach assayable values. Two days after alpha particle irradiation the depressing effect was similar as at 10 h. One week after exposition to alpha-ions, the tilorone-induced IFN titre was still significantly lower than those of controls, but a tendency to

Table II

Production of tilorone-induced interferon in mice exposed to ^{60}Co-gamma and He-alpha irradiation

Dose of irradiation (Gy)	Time of tilorone administration (after irradiation)	Interferon titre (IU) in 0.1 ml of serum* of mice exposed to irradiation	
		^{60}Co-gamma	He-alpha
–	10 h	504 ± 43	504 ± 43
2		495 ± 50	60 ± 12
4		250 ± 23	<10
6		160 ± 21	<10
–	48 h	520 ± 32	520 ± 32
2		301 ± 24	124 ± 25
4		68 ± 13	<10
6		25 ± 8	<10
–	7 days	480 ± 52	480 ± 52
2		505 ± 32	250 ± 30
4		320 ± 25	40 ± 13
6		250 ± 27	<10

Tilorone (200 mg/kg) was given intragastrically. Mice were exsanguinated 18 hours after tilorone administration. Energy of He-ions was 3.3 GeV/nuclon

* Mean ± SD

Table III

Effect of prophylactic administration of poly I : C on mortality of mice exposed to ^{60}Co-gamma and He-alpha irradiation

Treatment	Mortality of mice (%) on the 30th day after exposure to		
	5 Gy	6 Gy	7 Gy
^{60}Co-gamma	4.2 ± 1.3	10.5 ± 1.6	32.0 ± 2.5
poly I : C + ^{60}Co-gamma	0	0	5.2 ± 1.1
He-alpha	7.2 ± 6.9	50.0 ± 13.4	96.7 ± 3.3
poly I : C + He-alpha	7.1 ± 6.7	35.7 ± 12.8	85.7 ± 9.3

Polyriboinosinic-polyribocytidylic acid (poly I : C) was administered intraperitoneally (2.5 mg/kg) 2 hours before irradiation. Energy of He-ions was 4.6 GeV/nuclon

Table IV

Effect of prophylactic administration of tilorone on mortality of mice exposed to ^{60}Co-gamma and He-alpha irradiation

Treatment	Mortality of mice (%) on 30th day after exposition to			
	5 Gy	6 Gy	7 Gy	8 Gy
^{60}Co-gamma	5.1 ± 1.8	12.5 ± 1.7	25.0 ± 2.5	49.5 ± 5.4
Tilorone + ^{60}Co-gamma	0.2 ± 0.1	1.5 ± 1.2	5.2 ± 1.1	10.4 ± 2.5
He-alpha	12.5 ± 8.3	31.3 ± 11.6	96.7 ± 0.3	100
Tilorone + He-alpha	n.d.*	37.5 ± 12.1	100	100

Tilorone was administered intragastrically (200 mg/kg) 18 hours before irradiation. Energy of He-ions was 1.8 GeV/nuclon (doses 5 and 6 Gy) and 4.6 GeV/nuclon (doses 7 and 8 Gy)
* Not done

normalization was observed. After irradiation with 2 Gy, the IFN level approached 50% of the control, after 4 Gy the IFN titre was low and after 6 Gy it was under the assayable level.

Radioprotective activity of poly I : C and tilorone. The results concerning the effect of prophylactic administration of poly I : C on the mortality of mice exposed to Co-gamma and He-alpha irradiation are presented in Table III. When Co-gamma irradiation was preceded by poly I : C treatment, no mortality was observed in the groups exposed to 5 Gy and 6 Gy. In mice irradiated with 7 Gy a notable depression of mortality was observed. Pretreatment of mice with poly I : C before He-alpha irradiation was, in contrast to Co-gamma irradiation, without effect on mortality, regardless of the dose applied.

The effect of tilorone pretreatment on the mortality of mice irradiated with ^{60}Co-gamma rays and He-alpha particles is shown in Table IV. Tilorone applied 18 h before Co-gamma irradiation strongly influenced the mortality, whatever the dose of irradiation. On the contrary, prophylactic administration of tilorone before He-alpha irradiation was without effect on the mortality of mice.

Discussion

Since 1961, when the first cosmonaut was sent to Space, a number of problems have arisen concerning the effect of cosmic radiation on the human organism. Irradiation by protons and heavy ions of different energies as part of cosmic radiation could endanger man during long-term spaceflight, especially in the periods of protuberances.

Nevzgodina and Maksimova [12] studied the influence of heavy components of galactic cosmic radiation on lettuce *(Lactuca sativa)* cells flown on board of "Cosmos 1129". They found significant cytogenetic differences between seedlings which were hit by heavy charged particles and those that remained intact.

In laboratory experiments the effect of heavy ions on various cells was more intense than that of standard (X and gamma) irradiations. Govorun et al. [13] investigated the cytogenetic effects of protons, deuterons and He-ions on bone marrow and corneal epithelial cells of mice, V 79-4 Chinese hamster cells and human leukocytes. They observed that the relative biological effect (RBE) coefficients of high energy particles varied from 1.3 to 2.7 depending on the cell type and the parameters measured.

The increase in the number of chromosome aberrations and the depression of bone marrow proliferation was in good correlation with the irradiation dose. Moreover, it was found that ^{12}C ions had the highest and ^{22}Na ions the lowest RBE coefficients [14, 15].

The results showed that production of IFN in mice is highly sensitive to He-alpha particles when poly I : C and tilorone are used as inducers. Poly I : C induced IFN synthesis was depressed by He-alpha irradiation with the maximum impairment seen on the 3rd day after exposure to 4 Gy and 6 Gy. When tilorone was administered, serum IFN production decreased as early as 10 h after irradiation even after a dose of 2 Gy. After 4 Gy and 6 Gy, serum IFN could not be assayed 10 and 48 h after exposition to alpha-ions.

In both cases of IFN induction, the IFN producing system showed a tendency to normalization 1 week after irradiation. Recovery was more pronounced after Co-gamma than after He-alpha irradiation.

The results confirmed the observation of Govorun et al. [13] concerning the intensity of the biological effect of heavy ions, and indicate that the IFN-producing system of the organism represents a suitable object for further investigations. It has to be taken into consideration that the effect of He-alpha irradiation manifests in another way when different inducers are administered: poly I : C-induced IFN production is more resistant to this kind of irradiation than that induced by tilorone.

It is interesting that Vorobyov et al. [16], comparing the protection by beta-alkylamine against electromagnetic radiations (X- and gamma-rays) and high energy proton radiations found no significant difference in protection during proton and standard irradiations. Our previous results showed that poly I : C and tilorone acted as effective radioprotectors against Co-gamma irradiation [9, 10]. In contrast, similar treatment of mice irradiated with He-alpha particles had no influence on their mortality, indicating the possibility of different mechanisms of radioprotection in the case of alpha and gamma irradiation.

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APPLICATION OF RADIO-DETOXIFIED ENDOTOXIN AS ADJUVANT FOR EXPERIMENTAL FOOT-AND-MOUTH DISEASE VACCINE

F. Sólyom and L. Bertók

Phylaxia Veterinary Biologicals Co., Budapest, and "Frédéric Joliot Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

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The immunity enhancing adjuvant activity of radiodetoxified endotoxin (RD-LPS) on the potency of "C" type foot-and-mouth (FMD) vaccine was tested in different animal species. The suitable quantity of RD-LPS (20 μg per mouse) adjuvated a small than a high dose of FMD antigen. In cattle and sheep the adjuvant effect of oil + RD + LPS surpassed only slightly that of oil alone. The effect of RD-LPS in the pig was very pronounced when applied in small doses but further studies in larger animal populations have to confirm this result.

The enhancement of the immunogenic potency of antigen by means of different adjuvants (mineral carriers, lanolin, saponin, bacterial endotoxins, Freund's adjuvant, quaternary ammonium compounds, etc.) has been tested. The role and significance of these substances was especially important in the case of certain inactivated vaccines of low immunogenic potency.

Although remarkable success has been achieved by the application of attenuated virus vaccines, the use of inactivated ones has been preferred recently. Thus, it is more and more essential to find substances able to improve or to enhance the immunogenicity of the inactivated vaccines.

It has been known since long that bacterial endotoxins are excellent adjuvants, but they could not be used because even in small doses they had a toxic effect. Therefore, it has been tried to produce detoxified products from endotoxin, and we have succeeded in producing by irradiation such a detoxified endotoxic product which maintained its immunostimulant and adjuvant activities [1–6].

The object of our studies was to investigate the probable improvement of the immunogenicity of inactivated foot-and-mouth disease (FMD) virus in various animal species by means of addition of radio-detoxified endotoxin.

Ferenc Sólyom
Phylaxia Veterinary Biologicals Co.
H-1486 Budapest, P.O.B. 23, Hungary

Lóránd Bertók
"Frédéric Joliot Curie" National Research Institute for Radiobiology and Radiohygiene
H-1775 Budapest, P.O.B. 101, Hungary

Materials and methods

Radio-detoxified endotoxin (RD-LPS). Preparation produced from the endotoxin of the *Escherichia coli* O89 strain processed with heated aqueous phenol solution [7] by radio-detoxification of 5 Mrad gamma radiation according to the method of Bertók et al. [1].

Oily adjuvant. Freund's oily, incomplete adjuvant has been used to adjuvate the experimental vaccine.

FMD vaccine. Monovalent vaccines of "C" type produced by Phylaxia (Budapest) by Waldmann's method [8]. The virus antigen was inactivated by formalin in the vaccine, the dose of the vaccine was 2 ml/cattle.

Complement-fixation test. The antigen content of the vaccine was determined before inactivation by means of the quantitative complement-fixation test elaborated by Czelleng and Sólyom [9], following fluorocarbon (Arcton 113) treatment of the virus.

The standardized complement consumption of the virus antigen (1 $C'HD_{50} = 7$ μl) was determined in the presence of antiserum promising maximum reaction. The complement binding activity of the virus antigen treated with fluorocarbon, the quantity of complement fixed by unit quantity, has been expressed in terms of F and G values.

Laboratory animals. Mouse. In the course of the examination subjects weighing 20 to 22 g of the F_1 generation issued from the cross-breeding of BALB/c and CBA inbred mouse strains were used. The vaccine was applied subcutaneously in doses of 0.2 ml/mouse. *Sheep.* One- to two-year-old merino sheep weighing 30 to 40 kg were used. A dose of 1 ml vaccine/sheep was applied subcutaneously. *Cattle.* Four- to five-month-old calves weighing 60 to 80 kg and 2- to 3-year-old cattle of 300 to 400 kg weight were used. A dose of 2 ml/cattle was applied subcutaneously. *Pigs* weighing 30 to 40 kg were used; they were inoculated intramuscularly with 5 ml and 2 ml vaccine per pig.

Potency tests. (a) *K-index method.* The virus homologous to the vaccine was titrated by injecting it into the lingual mucosa of vaccinated and non-vaccinated animals according to the method of Lucam et al. [10]. The quotient of the two titres gave the index value. Altogether 8 cattle were used. The vaccine proved efficacious if its index value was ≥ 1.2 according to the official French control standard.

(b) *E-index method.* The virus of FMD homologous to the vaccine was titrated in vaccinated and non-vaccinated mice [11]. Altogether 100 mice were used. The quotient of the two titres gave the index value. The vaccine met the requirements if its index value was ≥ 2.39 according to the potency standard of Phylaxia.

(c) *M-index method.* The virus homologous to the vaccine was titrated as above in vaccinated and non-vaccinated sheep [12]. Altogether 8 sheep were used. The quotient of the two titres gave the index value. The vaccine proved appropriate if its index value was ≥ 1.0 according to the official French control standard.

(d) *Pig protection test.* Two pigs infected with the virus homologous to the vaccine were kept together with 3 pigs vaccinated 21 days earlier. Each of the pigs was considered positive if even a single aphtha was found on any foot.

(e) *S-index method.* Determination of the blood antibody content of vaccinated animals was carried out by the S-index method [13]. The vaccine met the requirements if its index value was ≥ 1.5 according to the official French control standard.

Results

In the first study the "C" type monovalent vaccine contained 228.8 F and G FMD antigen in 1 cattle dose (2 ml). Dilution 1 : 8 was also prepared from this vaccine, in such a manner that the antigen content varied while the quantity of its other constituents remained constant. This vaccine dilution 1 : 8 contained 28.6 F and G FMD virus antigen in 1 cattle dose. From both vaccines of different antigen content samples have been prepared which in 2 ml contained 200, 400 or 800/μg RD-LPS. The efficacy of the samples was tested by the E-index method in adults mice. Results of the test are summarized in Table I.

Table I

Comparative analysis by the E-index method in mice, of the efficacy of FMD vaccine samples containing different antigen quantity adjuvated by radio-detoxified endotoxin (RD-LPS)

RD-LPS μg quantity/mouse dose	Antigen content of 0.2 ml vaccine dose expressed in F&G	
	22.9	2.3
	E-index	
—	3.40	2.00
20	5.00	3.50
40	4.00	3.20
80	3.60	2.75

The results showed that the FMD antigen had successfully been adjuvated by RD-LPS. Although RD-LPS remarkably enhanced the immunogenicity of the vaccines of higher antigen content (especially with the 20 μg dose), but its effect on the vaccines of lower antigen content was more marked. Vaccines of low antigen content failed to meet the requirements of efficacy as their index value did not attain the threshold value of 2.39, but by RD-LPS complementation the mice were immunized with proper efficacy. By mean of other adjuvants (saponin, DEAE-dextran, Freund's adjuvant) the activity of inferior quality antigen could not be improved to such an extent.

In the second study, the efficacy of FMD vaccine of "C" type diluted 1 : 8 and complemented by 20 μg RD-LPS has been tested. The RD-LPS was either directly mixed into the vaccine or inoculated separately into the vaccinated mice. The potency test of the vaccine samples was carried out by the E-index method. Results are presented in Table II.

Potency testing of FMD vaccine in adult mice (20 μg/mouse) by the E-index method (a) RD-LPS was directly mixed into the vaccine; (b) RD-LPS was inoculated separately into simultaneously vaccinated mice. In the course of potency testing it was found that the potency of vaccines complemented by RD-LPS was higher than that of vaccines without any adjuvant, while RD-LPS applied separately suppressed the immune response.

In the third study, FMD vaccine of "C" type was applied in undiluted form (228.8 F and G antigen per dose) with different adjuvants. The potency of the vaccines was tested in cattle and calves by the K- and S-index. Results are summarized in Table III.

The protection index in cattle treated with oil and oil + RD + LPS adjuvated vaccines was greater than that afforded by aluminium hydroxide gel vaccines, and the efficacy index of all these vaccines was above the threshold-value of the K-index (1,2) and the S-index (1,5).

Table II

Comparative analysis by the E-index method of the efficacy of FMD vaccine of "C" type diluted 1 : 8 and complemented by 20 μg RD-LPS

Antigen content of vaccine expressed in F&G/mouse dose	Quantity of RD-LPS μg per mouse	Manner of inoculation	E-index
2.9	—	VO	2.20
2.9	20	VST	1.69
2.9	20	VTM	3.72

VO = Vaccine applied without RD-LPS complementation
VST = RD-LPS inoculated separately into simultaneously vaccinated mice
VTM = RD-LPS mixed directly into vaccine

Table III

Comparative analysis by the K- and S-index methods of the efficacy of FMD vaccine of "C" type complemented by different adjuvants

Adjuvant	Cattle		Calf K-index
	K-index	S-index	
$Al(OH)_3$ gel	1.37	2.61	0.98
$Al(OH)_3$ + oil	1.50	3.46	0.38
Oil	2.43	3.43	0.00
Oil + 1 mg RD-LPS/animal	2.45	3.71	0.91

The antigen content of each vaccine was 228.8 F&G/dose

Immunity could not be elicited in calves by oil vaccines, but this may have been due to some error. The adjuvant activity of oil was not further augmented by RD-LPS supplementation.

In the fourth experiment, the efficacy of the vaccine of "C" type diluted 1 : 8 with a steady antigen content of 28.6 F and G/dose was studied. The potency of the vaccine was tested in cattle by the K-index method. Results are presented in Table IV.

The "C" type vaccine diluted 1 : 8 failed to immunize cattle suitably even with the usual $Al(OH)_3$ + saponin adjuvant. Oil proved to be an efficacious adjuvant in this case, too. The complementary RD-LPS just boosted the immunity.

In the fifth experiment the potency of the same vaccines was tested by the M- and S-index method. Table V contains the results.

The results showed that the vaccines given by different types of adjuvant provoked in sheep about the same immunity, but the best average immunity was obtained when oil + RD + LPS was applied as adjuvant.

Table IV

Comparative analysis by the K-index method of the efficacy of FMD vaccine of "C" type diluted 1 : 8 and that complemented by different adjuvants

Adjuvant	Cattle K-index
$Al(OH)_3$ gel	0.56
$Al(OH)_3$ gel + 4 mg saponin	0.65
Oil	2.12
Oil + 1 g RD-LPS	2.41

The antigen content of each vaccine was 28.6 F&G/dose

Table V

Comparative analysis by the M- and S-index methods of the efficacy of FMD vaccine of "C" type complemented by different adjuvants

Adjuvant	Sheep	
	M-index	S-index
$Al(OH)_3$ gel	1.01	1.50
$Al(OH)_3$ gel + 4 mg saponin	0.94	1.30
Oil	0.81	1.40
Oil + 1 g RD-LPS	1.06	1.55

The antigen content of each vaccine was 114.4 F&G/dose

In the course of the sixth experiment, the "C" type vaccine was tested in pigs. The vaccine of undiluted antigen content was applied in 2 different doses and on basis of the response obtained on challenge in pigs, the number of non-immune (+) and immune (—) animals is presented in Table VI.

The results also verified that immunization of pigs against FMD by vaccination is a most difficult task. The traditional vaccines containing $Al(OH)_3$ proved completely ineffective for pigs under laboratory conditions. Nowadays, vaccines with oil or DEAE-dextran can be used for immunization; these evoke the relative highest protection in pig, but usually the rate of the pigs protected with vaccines of this type does not attain that obtained by other animal species.

In the course of our experiments the RD-LPS in 5 ml vaccine did not augment the immunity, but supplementation of the smaller dose (2 ml) increased the immunogenic activity of the vaccine.

Table VI

Comparative analysis of the efficacy of FMD vaccine of "C" type containing 228.8 F&G antigen/2 ml and complemented by different adjuvants

Adjuvant	Pig vaccine doses	
	5 ml 572 F&G	2 ml 228.8 F&G
$Al(OH)_3$ gel	+++	++
$Al(OH)_3$ gel + oil	+++	++
Oil	++—	++
Oil + 1 g RD-LPS/2 ml	++—	+—
DEAE-dextran	++—	++

+ = Number of non-immune animals after challenge
— = Number of immune animals after challenge

Discussion

The immunogenic activity of inactivated FMD antigen can be enhanced by means of adjuvant supplementation. The role of $Al(OH)_3$ gel, saponin, oil and DEAE-dextran in the improvement of immunity in some animal species is well known and our aim was to clarify the adjuvant role of RD-LPS. The results obtained in various animal species proved that FMD antigen activity was remarkably be enhanced by a proper dose of RD-LPS.

The augmentation of antigenicity was especially pronounced in mice, generally when applying a small dose of antigen. A vaccine not complemented by RD-LPS may not comply with the quality requirements. The immunogenicity of the same vaccine was enhanced to a degree higher than the 2.39 index value if complemented by RD-LPS.

In cattle and sheep the immunogenic activity enhancing effect of RD-LPS was not so expressed, due to the fact that it was always applied together with oil adjuvant, although the best immune response was obtained with the oil + RD + LPS combination. In pigs, representing the species most important for FMD vaccination, the effect of RD-LPS + oil adjuvated vaccines applied in higher antigen doses was not marked; the best results were obtained when the above vaccine was applied in a small dose, although the small number of animals did not allow to draw final conclusions.

Determination of the convenient quantity of RD-LPS proved to be an important point, because it was demonstrated in mice that augmentation of the RD-LPS dose influenced disadvantageously the immune response obtained with both high and small antigen doses. Mixing of RD-LPS to the vaccine also seemed essential because RD-LPS applied separately exerted an immuno-

suppressive activity. The cause of this phenomenon is not known and is in contradiction with our previous experiments [14] with saponin; saponin namely elicited the same immunostimulating activity when it was applied separately or mixed to the vaccine.

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RESISTANCE PLASMIDS FOR INDUCIBLE MACROLIDE–LINCOSAMIDE RESISTANCE IN *STAPHYLOCOCCUS SIMULANS* AND *STAPHYLOCOCCUS EPIDERMIDIS*

I. Barcs

László Central Hospital for Infectious Diseases, Budapest

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Localization and genetic nature of inducible macrolide resistance determinants in some coagulase negative staphylococci (CNS) were studied and compared with those in *Staphylococcus aureus*. An 1.7-megadalton R-plasmid, pEI 1107, mediating inducible resistance to macrolides and lincosamides was present in part of *Staphylococcus simulans* and *Staphylococcus epidermidis* strains isolated in the Central Hospital for Infectious Diseases. Two other plasmids were harboured by *S. simulans* LK 6108 strain. One of these plasmids, pEI 6108 (molecular mass 5.2 Md) was encoded for inducible macrolide–lincosamide resistance, and the second one, pE 6108 (molecular mass 4.9 Md) for constitutive marcolide resistance. The similarity in size and phenotype of small plasmids in CNS and *S. aureus* suggest a common origin of these resistance determinants.

The ability of some coagulase negative staphylococcal (CNS) strains to cause different human infections such as bacteriaemia, endocarditis, otitis media, conjunctivitis, urinary tract or wound infections, etc., is well documented [1–5]. On the other hand, these bacteria are common inhabitants of the human skin and mucous membranes. There are data that CNS might serve as a potential reservoir for resistance genes in the clinically more significant *S. aureus* [6, 7]. In vitro and in vivo studies have shown that staphylococci can exchange DNA by conjugation [8–11].

The division into eleven species of CNS is based on differences in cell wall structure, lactic acid metabolism, and DNA-homology [12]. This system seems to be too complicated for routine laboratories. According to our identification scheme based on easily performable biochemical tests and sensitivity to novobiocin [13, 14] 208 strains obtained from clinical specimens were classified during a 8-month period in 1983 [13]. About 20% of these strains harboured a resistance determinant (R-determinant) encoding for inducible macrolide–lincosamide resistance [15].

Although the R-determinants mediating resistance to macrolide, lincosamide, and group B streptogramins (MLS antibiotics) of *S. aureus* have been well characterized [16–27], little is known about these in CNS. The plasmid

István Barcs
László Central Hospital for Infectious Diseases
H-1450 Budapest, P.O.B. 29, Hungary

nature of MLS resistance determinants in 6 CNS strains were demonstrated, and similarities of phenotypic and physicochemical characters of *S. aureus*, *S. epidermidis*, and *S. simulans* plasmids were studied in the present paper.

Materials and methods

Bacterial strains and nomenclature. The staphylococcal strains examined in this study are listed in Table I along with their sources and phenotypic markers. Bacteria isolated from clinical specimens were identified according to the Approved List of Bacterial Names [29] by biochemical tests [13, 14]. Strains harbouring staphylococcal plasmids and used for determining molecular size and MIC values are presented in Table II.

The plasmids designate phenotypic abbreviation of the resistance marker followed by the strain number. Thus, pEI 1107 designates plasmid of the strain LK 1107 encoding for inducible erythromycin and lincomycin resistance.

Antibiotic sensitivity tests. In agar diffusion tests [30] the following set of Resistest (Human, Budapest) disks was used: penicillin (3 IU), chloramphenicol (30 μg), tetracycline (30 μg), erythromycin (10 μg), oleandomycin (30 μg), lincomycin (10 μg), novobiocin (5 μg). Macrolide–lincosamide cross-resistance of the strains was tested by the method of Weisblum and Demohn [31].

MIC values of antibiotics were determined by the agar dilution method [30]. The test was carried out on Nutrient agar (Oxoid, London) plates containing decreasing concentrations of erythromycin (Sigma, St. Louis), oleandomycin phosphate (Sigma, St. Louis), and lincomycin (Lincocin; Upjohn, Puurs).

Induction of resistance. The macrolide–lincosamide resistance of strains was induced by a modified method of Malke et al. [32]. Strains were cultivated on agar plate with 0.3 mg/l of erythromycin. After 18 h incubation at 37 °C the cultures were inoculated onto agar plates containing concentration ranges of erythromycin, oleandomycin or lincomycin.

Curing procedures. Curing of antibiotic resistance was accomplished according to Schaefler [33].

Preparation of DNA. The crude cell-lysates for detection of small staphylococcal plasmids were obtained by digestion with lysostaphin (Schwarz/Mann, Orangeburg) and ribonuclease (Reanal, Budapest) in a tris–EDTA–sucrose–saline buffer according to Iordănescu and Surdeanu [26].

Agarose gel electrophoresis. DNA was visualized on 3 mm horizontal gels of 0.7% agarose (Reanal, Budapest) by the method described by Meyers et al. [34].

The molecular mass of plasmids was determined by electrophoresis on 1% agarose gel by use of molecular mass standards [34].

Results

Curing of antibiotic resistance. Six strains were selected to determine the genetic localization of determinants encoding for inducible lincomycin resistance. Erythromycin-sensitive derivatives of all strains were isolated by the curing method. The strains *S. simulans* LK 6104 lost its chloramphenicol resistance simultaneously with erythromycin resistance; this derivative is BI 1101. Two type of derivates were isolated from *S. simulans* LK 6108 strain. One of these, BI 1501 lost the inducibility of lincomycin resistance but showed resistance to erythromycin; the other type represented by BI 1108 lost resistance to all MLS antibiotics (see Table I).

DNA electrophoresis. For a better understanding the alterations in antibiotic susceptibility we examined the molecular nature of antibiotic

Table I

Characterization of coagulase negative staphylococcal strains used for plasmid determination

Strain*	Species	Source	Resistance pattern**	Reference
LK 1107	*S. simulans*	nose	Em-i Om-i Lm-i Pc Tc	[28]
BI 1115	ditto	LK 1107	Pc Tc	[28]
LK 1108	*S. simulans*	nose	Em-i Om-i Lm-i Pc Tc	[28]
BI 1102	ditto	LK 1108	Pc Tc	[28]
LK 2101	*S. epidermidis*	throat	Em-i Om-i Lm-i Pc Tc	[28]
BI 1117	ditto	LK 2101	Pc Tc	[28]
LK 6104	*S. simulans*	blood culture	Em-i Om-i Lm-i Pc Tc Cm	[28]
BI 1101	ditto	LK 6104	Pc Tc	[28]
LK 9105	*S. epidermidis*	sinus	Em-i Om-i Lm-i Pc Cm	[28]
BI 1103	ditto	LK 9105	Pc Cm	[28]
LK 6108	*S. simulans*	blood culture	Em-i Om-i Lm-i Pc Tc Cm	this paper
BI 1501	ditto	LK 6108	Em-c Om-c Pc Tc Cm	this paper
BI 1108	ditto	LK 6108	Pc Tc Cm	this paper

* All strains were isolated in the Central Hospital for Infectious Diseases

** Abbreviations of phenotypes: Em-i = erythromycin (inducible); Em-c = erythromycin (constitutive); Om-i = oleandomycin (inducible); Om-c = oleandomycin (constitutive); Lm-i = lincomycin (inducible); Pc = penicillin; Tc = tetracycline; Cm = chloramphenicol

Table II

Staphylococcus plasmids used for determination of molecular mass and MIC values

Plasmid	Host strain*	Relative molecular mass (Md)	Phenotype***	References
pTU 512	NCTC 8325	29.8	Em-c Om-c Lm-c Pc	[18]
pI 258	RN 11	18.7	Em-c Om-c Lm-c Pc	[19, 20]
pEP 2104	BI 2101**	14.5	Em-c Om-c Pc	[21]
pSA 5700	SA 932	4.4	Em-i Om-i Lm-i Cm	[22]
pT 181	SA 389	3.0	Tc	[22, 23]
pE 194	SA 484	2.5	Em-i Om-i Lm-i	[22, 24]
pC 194	SA 25	2.0	Cm	[22, 25]
pE 1764	SA 1104	1.8	Em-i Om-i Lm-i	[26]
pE 1151	JL 1151-113-8325E5	1.8	Em-i Om-i Lm-i	[27]
pE 1007	JL 1007-113-8325E3	1.7	Em-i Om-i Lm-i	[27]
pE 2222	SA 1105	1.7	Em-c Om-c Lm-c	[26]

* All strains (except BI 2101) originated from Dr. L. Jánosi (National Institute of Hygiene, Budapest)

** Origin: PM 2104 $\xrightarrow[\text{Em}]{\text{Ø80}}$ NCTC 8325, transductant [28]

*** See Table I; abbreviation of further phenotype: Lm-c = lincomycin (constitutive)

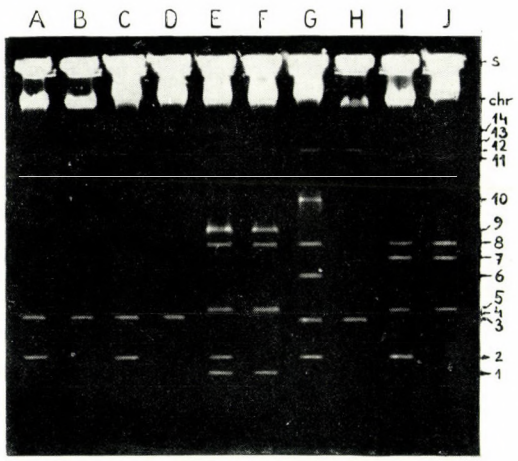

Fig. 1. Gel electrophoretograms of DNAs from wild type *S. simulans* and *S. epidermidis* strains and their erythromycin sensitive derivates in 0.7% agarose gel: (A) LK1107, (B) BI 1115, (C) LK 1108, (D), BI 1102, (E) LK 2101, (F) BI 1117, (G) LK 6104, (H) BI 1101, (I) LK 9105, (J) BI 1103. An "s" mark indicates the start of electrophoretic separation and a "chr" mark the linear fragments (chromosome). For further details, see text

resistance in CNS isolates. The high loss of resistance (frequency 10^{-3}–10^{-4}) suggested an extrachromosomal location of the genetic determinants for this antibiotic resistance. DNA patterns from five CNS strains and their derivates are shown in Fig. 1. All wild strains with antibiogram Em-i Lm-i showed common plasmid bands designated *2*; their derivates did not contain a plasmid of this size. Strains harboured other extrachromosomal DNA elements, too. The BI 1101 strain, a derivate of LK 6104, lost also bands *6*, *8* and *10*.

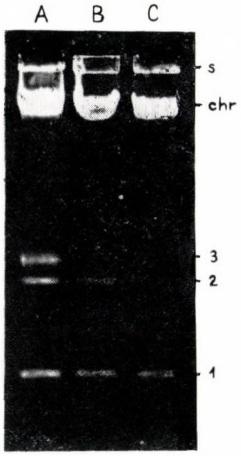

Fig. 2. DNA patterns of *S. simulans* LK 6108 strain and its derivates in 0.7% agarose gel: (A) LK 6108 wild type strain; (B) BI 1501, a derivate of the wild strain with erythromycin but no lincomycin resistance; (C) BI 1108 erythromycin sensitive derivate of the wild strain. For further details, see text

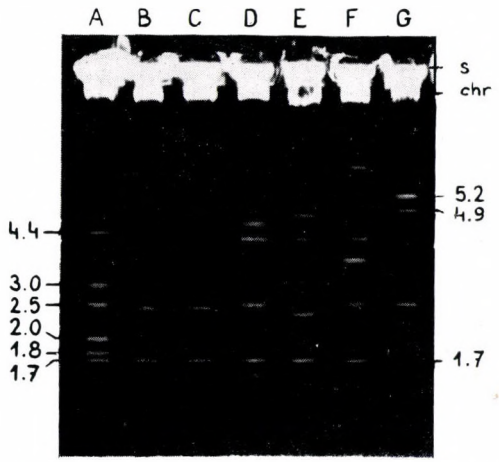

Fig. 3. Migration of DNAs from *S. simulans* and *S. epidermidis* strains harbouring plasmids for inducible macrolide–lincosamide resistance in 1% agarose gel: (A) molecular mass standards, (B) LK 1107, (C) LK 1108, (D) LK 2101, (E) LK 6104, (F) LK 9105, (G) LK 6108. The numbers on the left signify the molecular masses of the standards and on the right of the macrolide resistance plasmids of wild type coagulase negative staphylococcus strains. B, C, D, E and F isolates contain a common 1.7 Md plasmid

Table III

MIC values of Staphylococcus strains harbouring macrolide–lincosamide resistance determinants

Strain	MIC (mg/l)					
	Em		Om		Lm	
	A	B	A	B	A	B
NCTC 8325 (pTU 512)	1000	>1000	2000	3000	1000	>1000
RN 11	>1000	>1000	2000	3000	>1000	>1000
BI 2101	6.25	50	<7.8	31.5	0.5	6.5
SA 1105	>1000	>1000	3000	4000	800	>1000
SA 484	>1000	>1000	3000	4000	3.0	1000
SA 1104	>1000	>1000	2000	3000	1.0	>1000
JL 1151-113-8325E5	>1000	>1000	2000	3000	1.0	>1000
JL 1007-113-8325E3	>1000	>1000	2000	3000	1.0	>1000
LK 6104	>1000	>1000	2000	4000	1.0	>1000
LK 1108	>1000	>1000	2000	4000	1.0	>1000
LK 9105	>1000	>1000	2000	3000	1.0	>1000
LK 1107	>1000	>1000	2000	3000	1.0	>1000
LK 2101	>1000	>1000	2000	3000	1.0	>1000
LK 6108	<3.1	200	<7.8	62.5	<0.1	3.1
BI 1501	25	25	15.6	31.5	<0.1	0.1

A = without induction
B = after induction by 0.3 mg/l of erythromycin

The plasmid profiles of the *S. simulans* LK 6108 strain and its derivates showed two plasmid bands related to erythromycin resistance (Fig. 2). The strain BI 1501 with a single erythromycin resistance had only bands *1* and *2* from the pattern of the wild type strain, the erythromycin-sensitive derivate BI 1108 lost also plasmid band *2*.

Determination of molecular size of macrolide-lincosamide plasmids. Figure 3 shows DNA preparates of wild type CNS strains and molecular size-controls. Five isolates with wild antibiogram shared a common 1.7-Md plasmid: B, C, D, E and F. The relative molecular mass of erythromycin plasmids of isolate G were 4.9 and 5.2 Md.

Phenotypic testing of Staphylococcus strains harbouring MLS resistance determinants. The MIC values for two macrolide antibiotics and lincomycin of strains harbouring some staphylococcal plasmids to either inducible or constitutive MLS resistance are presented in Table III. The test was carried out both with and without previous induction by erythromycin.

Discussion

R determinants encoding for inducible MLS resistance were examined in CNS strains. This resistance marker was plasmid mediated as evidenced by the curing experiments and the electrophoretic analysis of crude DNA preparations. The size of pEI 1107, pEI 1108, pEI 2101, pEI 6104 and pEI 9105 plasmids were equal, their relative molecular mass proved to be 1.7 Md.

The *S. simulans* LK 6108 strain harboured two different erythromycin plasmids. One of these, pEI 6108 mediated also inducible macrolide–lincosamide resistance, its relative molecular mass was 5.2 Md. The second plasmid — pE 6108 (molecular mass 4.9 Md) — conferred constitutive macrolide but no lincomycin resistance.

The comparison of MIC values for strains harbouring staphylococcal MLS determinants showed that phenotypes determined by pEI 1107 and the other 1.7 Md CNS plasmids are considerably similar to those determined by pE 1151, pE 1007 and pE 1764 *S. aureus* plasmids originating from two staphylococcosis epidemics in Hungary [21, 35]. Since these plasmids had a similar molecular size and differed significantly from other R plasmids, their common origin was supposed.

The similarity of some R determinants of Gram-positive cocci is well-known. The presence of common R plasmids has previously been demonstrated for strains of resistant *S. aureus* and *S. epidermidis*. Numerous strains of CNS harbour plasmids indistinguishable from *S. aureus* tetracycline, penicillin or gentamicin plasmids as judged by endonuclease fingerpriting [11, 36–39]. Furthermore, the examination of MLS determinants of different genera showed

homology among them in Gram-positive cocci [16, 17, 40]. The intra- and interspecific DNA exchange occurred in both in vitro and in vivo experiments [8–11, 39, 41, 42].

The distribution of homologous plasmids in different species and genera suggests efficient mechanisms of gene transfer, perhaps promoted by intensive selective pressures.

The central role of antibiotic therapy in the spread of resistance is well-known [6, 43]. The studies of bacteria lyophilized before the advent of antibiotics showed that resistance genes and plasmids had been present before antibiotics were used. In consequence of frequent and/or inadequate administration of antibiotics the inhabitants of indigenous flora (e.g. *Escherichia coli*, enterococci, oral streptococci, CNS) have become reservoirs of R plasmids and transposons which may be passed on to neighbouring nonpathogenic and pathogenic strains [6, 7].

This theory has been supported by some clinical observations. Naidoo and Noble [44] gave account of the acquisition of gentamicin resistance by *S. aureus* strains. Strains were isolated from a patient with skin infection and had been observed to become resistant during gentamicin therapy. The acquired R determinants might have originated from *S. hominis* strains co-isolated with pathogenic strains at the beginning of disease. The transfer of resistance between these strains could be brought about in vitro and also on the surface of human or animal skin [9].

All these findings support the hypothesis that the transfer of plasmids between *S. aureus* and CNS may occur in nature. Further studies of staphylococcal R determinants may help to understand the spread of antibiotic resistance.

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INDUCTION OF HUMAN RHEUMATOID FACTOR AND OTHER AUTOANTIBODIES BY BACTERIAL LIPOPOLYSACCHARIDE

KLÁRA NÉMETH, A. FALUS, ERZSÉBET ELEKES, UTE BÖHM and KATALIN MERÉTEY

National Institute of Rheumatology and Physiotherapy, Budapest, and National Institute of Radiobiology and Radiohygiene Frédéric Joliot-Curie, Budapest

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In vitro autoantibody production induced by lipopolysaccharide (LPS) was studied using peripheral blood mononuclear (PBM) cell suspensions from patients with rheumatoid arthritis (RA) and healthy subjects. PBMs from both groups could be induced by LPS to secrete IgM and IgA rheumatoid factors (RF), antinuclear and anti-beta-2-microglobulin autoantibodies. Spontaneous production of IgM-RF was considerabe higher in RA than in controls. The rate of IgM-RF and IgA-RF secretion detected by ELISA increased with the dose of LPS in cultures of both groups. In RA, differences were found between the kinetics of IgM- and IgA-RFs secretion. LPS augmented the relative avidity of IgM-RF produced by PBMs from RA patients and this value was significantly higher than that of healthy persons. In some cases RFs cross-reacting with nuclear antigens and beta-2-microglobulin were detected.

Rheumatoid factors (RF) are anti-Ig autoantibodies appearing in large amounts in sera and synovial fluids of RA patients. Antinuclear antibodies (ANA) with specificity to nuclear antigens and anti-beta-2-microglobulin (anti-beta-2m) antibodies, too, are frequently present in RA patients [1–4]. Stimuli for maintenance of the high level of these autoantibodies (the exogenous inductive and endogenous regulative factors) are not fully known in RA and other autoimmune diseases.

Several compounds derived from different microorganisms are capable of inducing autoantibodies via their effect on B cell proliferation and polyclonal B cell activation [5]. In vitro Epstein–Barr virus infection can induce RF secretion of human lymphocytes in healthy subjects [6]. *Escherichia coli* injected into rabbits produced RF-like autoantibodies and polyarthritis [1]. Lipopolysaccharides induced RF production in vivo and in vitro in mice [7, 8]. It is suggested that polyclonal activation by exogenous infective agents may also take part in autoimmune processes.

KATALIN MERÉTEY, KLÁRA NÉMETH, ANDRÁS FALUS, UTE BÖHM
National Institute of Rheumatology and Physiotherapy
H-1525 Budapest, P.O.B. 54, Hungary

ERZSÉBET ELEKES
National Institute of Radiobiology and Radiohygiene Frédéric Joliot-Curie
Pentz K. u. 5, H-1221 Budapest, Hungary

Lipopolysaccharide (LPS), a component of the cell-wall of Gram-negative bacteria is one of the best studied polyclonal B cell activators and B cell mitogens in mice [9, 10]. Studying B cell mitogen response and polyclonal B cell activation in human peripheral B lymphocytes, LPS was, however, often ineffective [11, 12].

A comparative investigation of the in vitro induction of RF, ANA and anti-beta-2m production by LPS was done in peripheral blood mononuclear cell cultures from RA patients and healthy subjects. We have characterized the kinetics of the formation of LPS induced autoantibodies and immunoglobulins and measured the relative average avidity of IgM-RF as well as the RF cross-reactions with nuclear antigens and beta-2-microglobulin.

Materials and methods

Cell cultures. Venous blood was obtained from 6 patients with RA and 5 helthy subjects. Mononuclear cells were separated on Ficoll-Uromiro gradient (1200 *g*, 30 min) [13]. Cells were washed with culture medium containing Parker's TC 199, 10% fetal calf serum (FCS), 20 mM glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and resuspended in medium. Lymphocytes were counte and their viability controlled by eosin Y dye exclusion test. (Viability was approximately 98%.) LPS purified from *Escherichia coli* O89 (Human, Hungary) was diluted to 200 μg/ml and 1000 μg/ml in medium. Cell suspensions were distributed into round culture plates (Libro TM): 2.10^6 viable lymphocytes (1 ml medium) well. Cultures were incubated with LPS solutions (20 μg/ml and 100 μg/ml LPS, respectively) and in medium only (control groups) at 37 °C, with 5% CO_2 in humid air. Cultures were harvested on the 5th, 7th, 10th and 14th days without previous change of medium. Supernatants were stored at —20 °C until tested.

Indirect ELISA for detection of IgM- and IgA-RFs. ELISA micro-titre plates (Sterilin, England) were coated with 5 μg/ml human IgG in 100 μl aliquots incubated overnight at 4 °C. After washing with 0.1% Tween 20 in phosphate buffered saline (PBS, pH 7.3), 5% horse serum (100 μl in PBS) was added and incubated at 37 °C for 2 h. After washing, the plates were incubated with 100 μl culture supernatants overnight at +4 °C in duplicate. After three washings, 100 μl alkalinephosphatase-labelled anti-human-IgM or -IgA (Orion Diagnostic, Finland) were added to the wells (1 : 100 dilutions in PBS-0.1% Tween 20, 0.5% horse serum) and incubated for 1 h at room temperature and for 1 h at 37 °C. After three washings paranitrophenyl phosphate substrate (Merck, FRG) was added and the enzyme reaction was stopped with 0.7 M NaOH. An RF-pool from RA sera in 1 : 40 and 1 : 20 dilutions was used as positive control. OD was measured at 405 nm. Calculation:

$$\frac{\text{OD sample} - \text{OD background}}{\text{OD RF-pool} - \text{OD background}} = \text{OD relative [14].}$$

Determination of relative mean avidity of IgM-RF. IgM-RF of the supernatants was titrated by indirect ELISA (see above) using geometric dilutions on 3 plates with different IgG coating (25.5 and 1 μg/ml). A dilution (1 : 10) on the linear part of the titration curves was selected from every titration curve. We calculated ratios of OD relatives belonging to 1 : 10 dilution (OD25, OD5, OD1). Finally we used the OD1/OD25 ratio which seemed to be in direct proportion to the relative average avidity [15].

Indirect ELISA for detection of IgG anti-beta-2-microglobulin autoantibodies. Human beta-2m (100 μg/100 μl) isolated from urine of patients with nephropathy was used as antigen coating. Supernatants of cultures from the 6 RA patients and from 4 healthy subjects were added to the wells and incubated at 4 °C overnight. After washings, the plates were incubated with 1 : 500 diluted (PBS–0.1% Tween 20, and 0.5% horse serum) horse-radish peroxidase-labelled staphylococcal protein A (Human, Hungary) at room temperature for 2 h. After washings, the enzyme reaction was developed by ortho-phenylene-diamine (Human, Hun-

gary) substrate in the presence of H_2O_2 and stopped by addition of 4 N H_2SO_4. A human serum containing anti-beta-2m activity (diluted 1 : 100) was used as positive control. OD was determined at 500 nm [16].

Indirect immunofluorescent assay for measurement of antinuclear antibodies (IgG, IgA, IgM). Supernatants from each RA patient and from healthy donors of 10th and 14th days were incubated on acetone-fixed smears of rat liver nuclei at room temperature for 45 min. After washing with PBS the smears were incubated with FITC-conjugated anti-human Ig (1 : 20) (Hyland, USA) at room temperature for 45 min. Fluorescent staining was detected by epiluminescence under a fluorescent microscope (Fluoval, Zeiss, GDR). The degree of staining was indicated in arbitrary units $1 \rightarrow 4$ [17].

Detection of cross-reactive RF-s. ANA and anti-beta-2m positive supernatants were adsorbed on heat-aggregated IgG-Sepharose and bovine serum albumin (BSA)-Sepharose as a control. The ligands (heat aggregated human IgG and BSA) were coupled to CNBr activated Sepharose (Pharmacia, Sweden). Fifty μl of the supernatants were incubated with 50 μl IgG- and BSA-Sepharose at $+4$ °C overnight. ANA and anti-beta-2m antibodies of supernatants were detected after adsorption on immunosorbents. Decrease of ANA and anti-beta-2m activity compared with BSA-Sepharose indicated cross-reactive RFs [18].

Detection of IgG, IgA and IgM in culture supernatants. Immunoglobulins secreted during the culture period were measured by the linear immunodiffusion method of Mancini et al. [19].

Statistical analysis. Student's two-tailed *t*-test was used.

Results

IgM-RF production by PBMs of RA and healthy subjects. Kinetics of IgM-RF secretion is shown in Fig. 1. In the healthy group IgM-RF levels increased dose dependently and were significantly different at 14th day ($p < 0.01$) between the 0 and 100 μg/ml LPS groups. In the 100 μg/ml LPS group by 20 days ($p < 0.05$) and 14 days ($p < 0.01$) there was a significant difference compared to the supernatants on the 5th day of the cultures of healthy subjects. In RA groups, IgM-RF secretion increased slightly and significant differences were found only at 10 days ($p < 0.05$) between the values of 0 and the 20 μg/ml as well as 100 μg/ml LPS groups. IgM-RF levels produced by PBMs of RA patients were significantly higher than those of healthy persons ($p < 0.001$).

IgA-RF production in the cultures of PBMs from RA and healthy subjects. Changes of IgA-RF levels are demonstrated in Fig. 2. In the cultures of PBMs of healthy persons secretion of IgA-RF increased dose dependently as compared to the 7th day and significantly in the 20 μg/ml and 100 μg/ml LPS groups ($p < 0.05$) on the 14th day. Significant differences were found between the 0 and 100 μg/ml LPS groups ($p < 0.05$) on day 14, too. IgA-RF production was augmented dose dependently and differed significantly in the 100 μg/ml LPS group ($p < 0.05$) from the 5 day values and between the 0 and 100 μg/ml LPS groups on day 14 ($p < 0.05$) in the RA groups. IgA-RF levels of the culture supernatants of PBMs of RA patients were significantly higher than those of the healthy group between the 20 μg/ml LPS groups at 10 and 14 days and the 100 μg/ml LPS groups ($p < 0.05$).

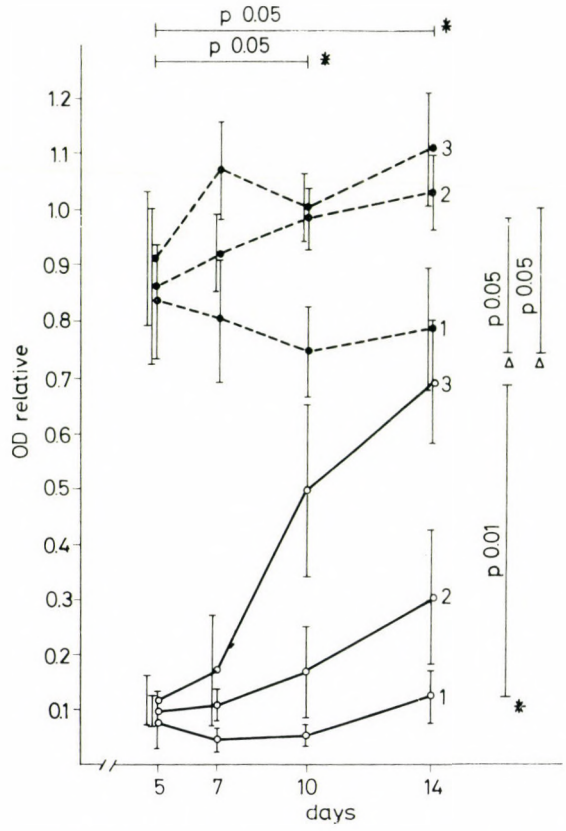

Fig. 1. Spontaneous and LPS induced IgM-RF secretion in peripheral blood mononuclear cell cultures of RA patients and healthy subjects. IgM-RF levels were detected by ELISA from supernatants. Means ±SEM are indicated. P values by Student's *t*-test are indicated by vertical bars between the different LPS concentrations and by horizontal bars between different days of the culture using the same LPS concentration. ●---● healthy subjects (n = 5), ○----○ RA patients △ (n = 6), 1 = medium; 2 = 20 μg/ml PLS; 3 = 100 μg/ml LPS

Relative mean avidity of in vitro secreted IgM-RF. Kinetics of relative average avidity of IgM-RF is shown in Fig. 3. The OD1/OD25 ratios increased significantly in the 100 μg/ml LPS group of "RA PBM-cultures" and showed the maximum value on the 7th day ($p < 0.05$). Significant differences were obtained between the 0 and the 100 μg/ml LPS groups from day 7 ($p < 0.05$). Mean OD1/OD25 values calculated from IgM-RF titration-curves of the healthy subjects was as low as in the unstimulated cultures of the RA patients.

Anti-beta-2-microglobulin IgG secretion by PBMs of RA and healthy persons. In vitro anti-beta-2m secretion is shown in Fig. 4. Using PBMs from RA patients, the change of the anti-beta-2m level was not significant between the different LPS concentrations and between different intervals of the cul-

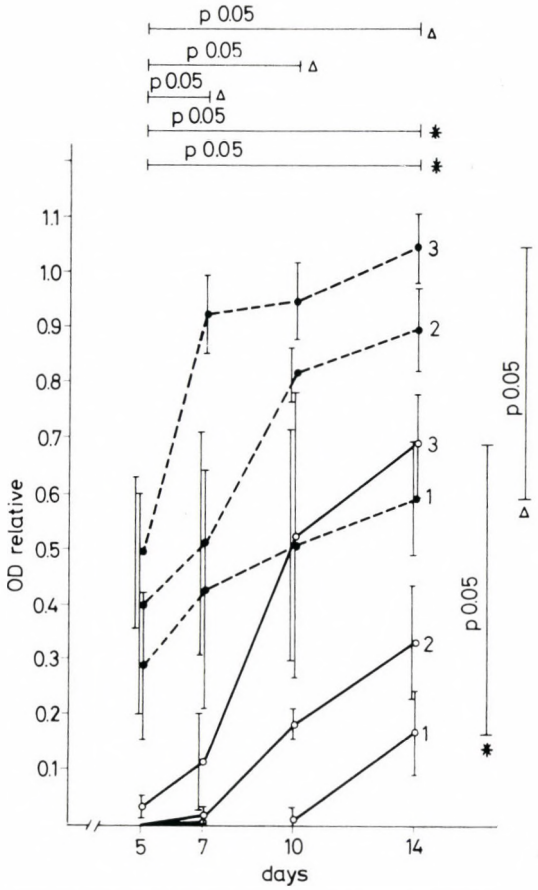

Fig. 2. Spontaneous and LPS induced IgA-RF secretion in peripheral blood mononuclear cell cultures of RA patients and controls. For explanation see Fig. 1

tures with the same LPS dose. When cultures of PBMs of healthy persons were tested, higher anti-beta-2m levels were measured in the 100 μg/ml LPS group than in the 20 μg/ml LPS group, but the difference was not significant.

Antinuclear antibodies in culture supernatants. Six culture supernatants of the 20 μg/ml LPS group, 2 of the 100 μg/ml LPS groups and 1 of the "no LPS" groups, showed ANA activity (out of the 72 samples of RA patients). Among the samples from cultures of healthy subjects only 1 ANA positivity was observed in the 20 μg/ml LPS group (Table I).

In vitro produced cross-reactive RFs. Nine ANA positive samples were adsorbed on IgG-Sepharose and RFs with ANA activity were detected in 5 samples of the "RA cultures". No detectable cross-reactivity was found in the only ANA positive samples from the "healthy cultures". Anti-beta-2m activity

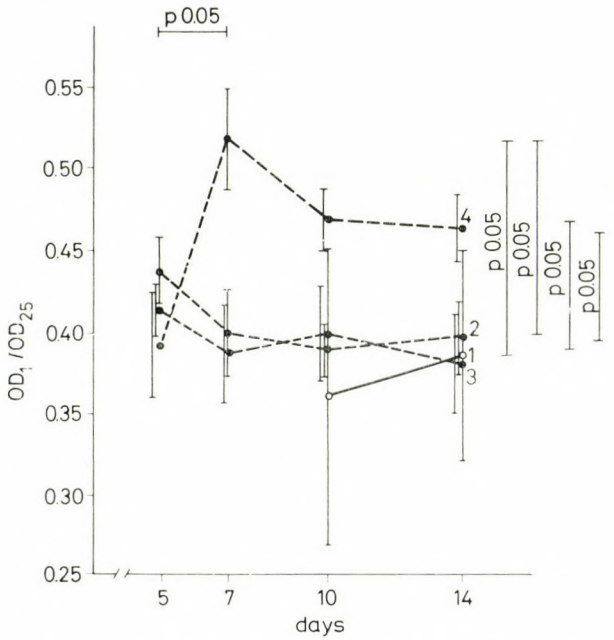

Fig. 3. Kinetics and LPS dose dependence of the relative avidity values of in vitro secreted IgM-RFs by healthy subjects and RA patients. OD1/OD25 values were calculated from titre-curves measured by ELISA. Means ±SEM are indicated. P values are shown. ○——○ healthy subjects (n = 3 and 4), *1* = 100 μg/ml LPS, ●---● RA patients (n = 6). *2* = medium; *3* = 20 μg/ml LPS; *4* = 100 μg/ml LPS

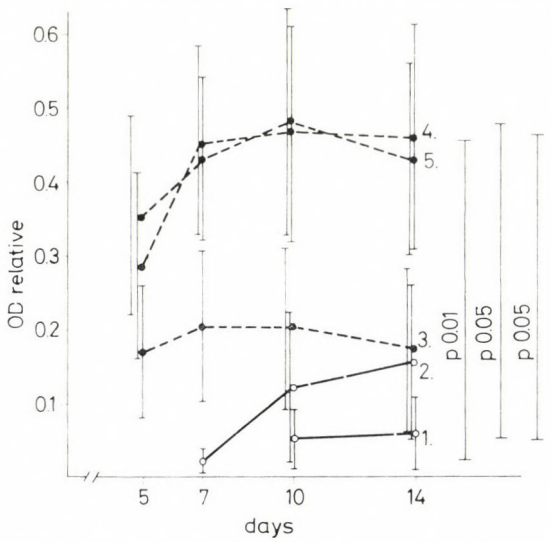

Fig. 4. Anti-beta-2-microglobulin autoantibody production in peripheral blood mononuclear cell cultures. Anti-beta-2m was measured by ELISA. Significant differences are shown between the healthy persons and RA patients. Means ±SEM are indicated. ○——○ healthy subjects (n = 4), 1 = 20 μg/ml LPS; 2 = 100 μg/ml LPS; ●---● RA patients (n = 6); 3 = medium; 4 = 20 μg/ml LPS; 5 = 100 μg/ml LPS

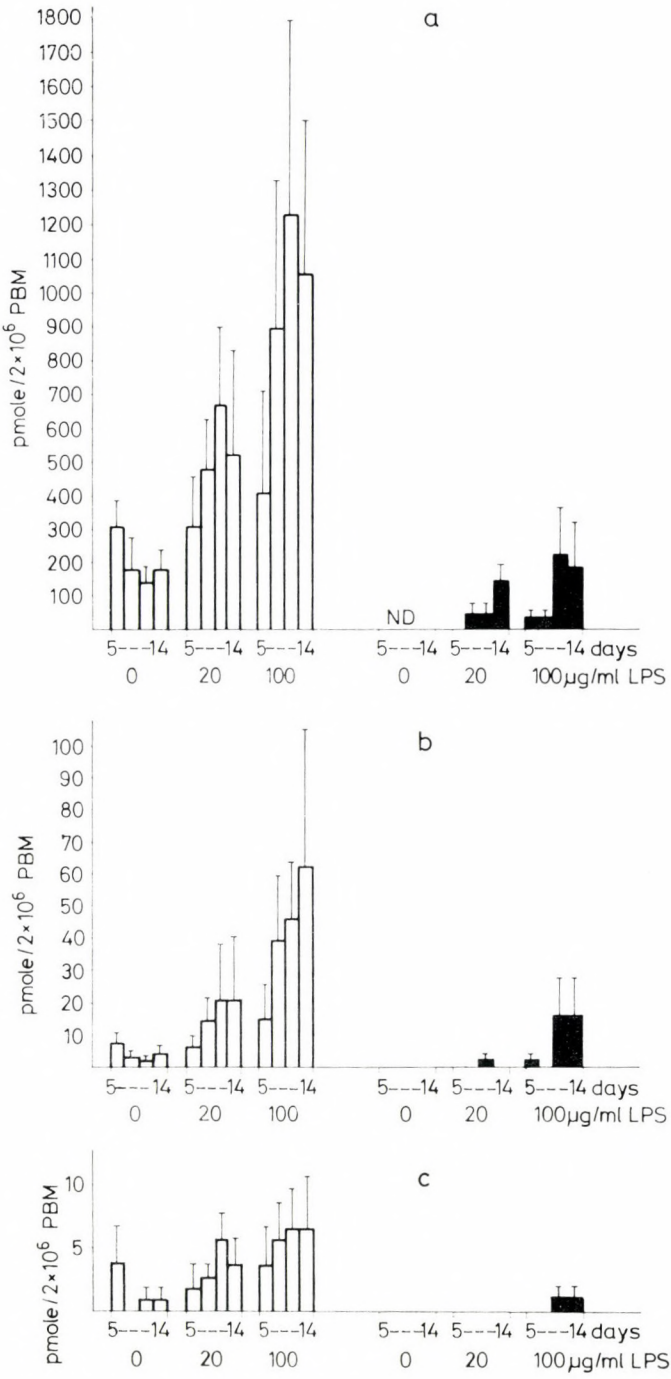

Fig. 5. IgG (a), IgA (b) and IgM (c) levels of culture supernatants of RA patients and healthy persons. LPS doses and the days of cultures are indicated. Means ±SEM are shown. RA patients: empty columns (n = 3), healthy subjects: full columns (n = 4). ND = not detectable

Table I

Antinuclear antibodies in culture supernatants (individual samples)

Sample*	LPS (μg/ml)	Day	ANA**	Cross-reacting ANA*** (ANA-RF)
$Healthy_2$	20	14	+++	—
RA_1	20	5	++	—
	20	7	++++	—
	20	14	++++	—
	100	10	++	+
RA_2	0	5	+++	++
	20	7	+++	—
	20	10	+++	++
RA_6	20	10	+	+
	100	10	+	+

* $Healthy_2$, RA_1, RA_2, RA_6 represent individual samples from four persons
** ANA results are given in arbitrary units
*** ANA-s binding to human IgG, are also called cross-reactive ANA

decreased by more than 50% in 10 of 35 anti-beta-2m positive (OD relative >0.300) samples adsorbed on IgG-Sepharose compared to the activity after absorption on BSA-Sepharose. We observed an apparent increase of the anti-beta-2m activity in adsorbed samples (data not shown). Occurrence of cross-reactive RFs was more frequent in the LPS treated groups.

In vitro produced IgG, IgA and IgM. Changes of immunoglobulin levels are shown in Fig. 5. No significant increase was demonstrated in cultures set up by PBMs of healthy persons. In "RA cultures", spontaneous production of IgG was enhanced by LPS but no significant dose dependent change was detected.

Discussion

A significantly higher spontaneous IgM-RF secretion was measured by ELISA in the cultures of PBMs from RA patients than in the "healthy" group. A further increase of the IgM-RF levels in the RA group was less easily achieved by LPS than in the healthy group in which the stimulation was dose-dependent and significant. Our data demonstrated that a certain population of B lymphocytes secreting IgM-RF may be activated in vivo in RA patients. Data of other authors also support this observation: B lymphocytes of some RA patients secrete detectable IgM-RF in vitro without mitogen and help of T cells [20].

Low spontaneous RF levels were detected by ELISA in PBMs also from healthy persons which documents the presence of resting autoreactive B cell clones in healthy subjects.

Differences between the IgA-RF and IgM-RF kinetics were apparent in the RA group. In RA patients, IgA-RF secretion induced by LPS was similar to IgM-RF secretion of healthy subjects and the spontaneous level of IgA-RF was lower than that of IgM-RF. This observation may be explained by the heterogeneous sensitivity to T helper and T suppressor signals of B cell clones secreting RFs of different isotypes. We have not investigated the ratio of T cell subpopulations in vitro, but the high spontaneous production of IgM-RF and IgA-RF may reflect the abnormal T cell regulation. In the blood and synovial fluid of RA patients and increase of ratios of T helper/T suppressor cells has been demonstrated [21].

Spontaneous anti-beta-2m production measured by ELISA was also demonstrated. Levels of anti-beta-2m were increased by LPS but the change was not significant. RA patients could be divided into "high and low" spontaneous secretion groups on the basis of spontaneous and provoked anti-beta-2m secretion. The in vitro heterogeneity of the production of anti-beta-2m of RA patients might reflect some differences in the clinical course of the disease. In the sera of RA patients, particularly of those with extraarticular symptoms, anti-beta-2m activity was significantly higher than in healthy subjects and RA patients with only joint involvement [4]. Moreover, anti-beta-2m autoantibodies were induced by LPS in PBMs from healthy persons, too.

The frequency of ANA positive samples was relatively high in cultures with LPS. It has been mentioned that, in mice, LPS induced the appearance of anti-DNA antibodies and DNA release into the circulation [22]. Other authors failed to detect DNA release but could demonstrate anti-DNA, anti-DNP and anti-Ig antibody responses [23].

Cross-reactive RFs were tested in culture supernatants. We could detect RFs with antinuclear activity and probably with anti-beta-2m activity. We could observe a decrease of anti-beta-2m activity in 10 samples adsorbed on Ig-Sepharose, but anti-beta-2m activity was increased in 7 samples after adsorption on IgG as compared with BSA-Sepharose. Cross-reactions have been shown by other authors [24] between heterologous antibodies against human Ig and beta-2m. Identity of the beta-2m binding materials with RFs in sera were found in few cases [25], since the domain-like structure of immunoglobulins and beta-2m shows considerable similarity [26]. RFs cross-reacting with nuclear antigens were detected in 5 of 92 samples, but in vivo the cross-reactive binding sites may have been blocked by excess IgG [1]. Cross-reactive RFs may also appear within the autoantibody population, as part of polyclonal antibody response.

In RA patients the in vitro secreted high level of spontaneous IgM-RF with low relative average avidity may show the in vivo activated state of B cells carrying receptors of low avidity. Additional B cell stimulation by LPS leads to the secretion of higher avidity IgM-RFs in RA. IgG as antigen for RF antibodies is present in high concentration in the blood and maintains tolerance on specific B cell carrying high avidity receptors. B cells with low avidity receptos can escape tolerance but the large amount of self antigen is unable of activating these cells. It is presumed that the polyclonal activators might stimulate these cells to secrete antibodies of low avidity [5]. This would explain the lower avidity of IgM-RF in the cells of healthy persons. Other authors [6] also found that EBV induced IgM-RF had a higher avidity in cell cultures of RA patients than in those of healthy subjects.

According to our data, a bacterial cell wall extract, LPS as polyclonal B cell activator is capable to stimulate the autoreactive B cell clones with different specificities present normally in vivo, and to induce RFs and other autoantibody production in vitro in healthy subjects, too. The higher spontaneous in vitro autoantibody secretion in RA patients may be related to an in vivo abnormal cell regulation of the autoreactive clones.

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CHANGES IN THE TENDENCY OF LYMPHOCYTES TO UNDERGO BLASTIC TRANSFORMATION IN THE POSTOPERATIVE PERIOD, IN IMMUNE-STIMULATED AND UNTREATED COLONIC TUMOUR PATIENTS

A. Nagy, Ildikó Petri, Z. Csizér, G. Baradnay and T. Kováts

First Department of Surgery, Blood Transfusion Centre, and Institute of Experimental Surgery, University Medical School, Szeged, and Human Institute for Serobacteriological Production and Research, Budapest

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A study was made of the blastic transformation of lymphocytes in the postoperative period in colonic tumour patients: 23 who had been immune-stimulated by vaccination, and 28 who had not been vaccinated. The immune reactivity decreased significantly in the majority of the cases; however, the deterioration of the immune status affected 68% of the non-vaccinated group, but only 48% of the vaccinated group. The data indicate that the vaccination primarily averted the deterioration of the defence ability in the early postoperative period in patients with a normal preoperative immune activity. An evaluable change in the immune reactivity did not occur in the 6–10 preoperative days following vaccination. Three months postoperatively, the number of patients giving a normal immune reaction was threefold in the vaccinated than in the non-vaccinated group.

From the research results attained in the past few years it has become increasingly clear that the prevailing immune status of surgical patients has a considerable influence on the incidence of septic complications and the course of existing complications [1–4]. Our earlier survey of colonic tumour patients confirmed that there is a close correlation between the presurgical cellular immune defence predisposition and the occurrence of wound infections [5]. The question arose, however, whether a single preoperative parameter provided an appropriate picture of the course of the immune status in the entire postoperative period. The immune reactivity of the patient is known to be affected by the surgical stress, by some of the antibiotics applied for preventive or therapeutic purposes, and even by the anaesthesia [6–8]. Accord-

Ildikó Petri
Blood Transfusion Centre, University Medical School
H-6701 Szeged, P.O.B. 464, Hungary

Attila Nagy, Gyula Baradnay
First Department of Surgery, University Medical School
H-6701 Szeged, P.O.B. 464, Hungary

Tibor Kováts
Institute of Experimental Surgery, University Medical School
H-6701 Szeged, P.O.B. 464, Hungary

Zoltán Csizér
Human Institute for Serobacteriological Production and Research
Szállás u. 5–7, H-1107 Budapest, Hungary

ingly, it was decided to extend investigations to the changes occurring in the cellular immune defence ability in the postoperative period. A related study was made to clarify whether a vaccine produced from Gram-negative bacteria isolated from local infections was capable of stimulating the patient's immune defence in the postoperative period, and whether a difference could be observed in the immune reactivity of immune-stimulated and untreated patients 3 months postoperatively [9–12].

Materials and methods

Patients. A randomized study was made on 51 colorectal cancer patients in whom elective, radical colonic surgery was performed. Table I provides details on the sex and age of the patients, the type of surgery, their pretreatment and the nature of antibiotic prevention. On the day following admission, 23 patients received into the deltoid 1 ml of a vaccine containing in each ml 10^9 phenol-killed bacteria of *Escherichia coli*, *Klebsiella*, *Proteus* and *Pseudomonas aeruginosa;* 28 patients did not participate in immune stimulation. Blood samples were taken on the day of admission (before vaccination in the treated group), on the day of surgery (6–10 days after the first blood sampling), and on the 10th postoperative day. The fourth sampling took place 3 months postoperatively. The tendency of the lymphocytes to undergo blast cell transformation was determined in blood samples, in order to characterize the activity of cellular immune defence.

Preparation of peripheral lymphocyte cultures. Heparinized venous blood was added to an equal amount of salt solution. The mononuclear cells were isolated by Ficoll-Uromiro (Pharmacia Fine Chemicals, Uppsala, and Bracco Ind. Chimica S.P.A., Milano) density

Table I

Age, sex, type of surgery and surgical preparation in vaccinated and non-vaccinated patients

Patients and treatment	Vaccinated	Non-vaccinated
No. of cases	23	28
Average age (years)	58.7	59.3
Male	11	15
Female	12	13
Type of surgery		
Right hemicolectomy	4	4
Left colon resection	10	13
Rectal extirpation	9	11
Surgical preparation + antibiotic prophylaxis	Puring and irrigation 4×500 mg metronidazole orally for 5 days 4×500 mg neomycin sulphate orally for 2 days 4×500 mg cephalothin parenterally for 7 days 2×80 mg gentamicin parenterally for 2 days 2×40 mg gentamicin parenterally for 5 days	

Note: parenteral antibiotic administration was begun 12 h preoperatively

gradient centrifugation, washed three times and resuspended in medium RPMI 1640 (Serva) containing 10% fetal calf serum, L-glutamine (20 M), penicillin (200 IU/ml) and streptomycin (100 μg/ml). Triplicates of peripheral lymphocytes from controls and patients were cultured in microtitre plates (Greiner) containing 2×10^5 viable cells/well. Mitogen-stimulated triplicates contained and optimum mitogen concentration: 5, 10 or 20 μl of a 1 : 10 dilution of a commercial stock solution of phytohaemagglutinin (PHA, Difco) and 2.5, 5 or 10 μl of Concanavalin A (Con A, Serva) were added to 200 μl culture. The cultures were incubated for 72 h in a humidified atmosphere with 5% carbon dioxide at 37 °C before the addition of ^{3}H-thymidine 1 μCi/well. The cultures were harvested and counted (Packard, Tri-Carb, Prague, CSSR) 5 h after the addition of thymidine. Mean values of the samples resulting in maximum stimulation were calculated and applied [13, 14].

The control group contained 50 healthy subjects. Their mean value ±2 SD was considered to represent the normal blast cell transformation activity in the colonic tumour patients. Data above the mean were considered typical of a strong reaction, those below the mean, typical of a weak reaction. The mean blastic transformation provoked with phytohaemagglutinin was 33 515 ± 14 440 cpm/2×10^5 lymphocytes, while that with Con A was 24 291 ± 12 151 cpm/2×10^5 lymphocytes. The changes in the immune activities of the patients were examined subsequently by a self-control method, with reference to the personal starting values. Differences from the control means were taken as real changes.

Evaluation of results The patients were divided into 4 groups.

(1) The blastic transformation activity of the lymphocytes was initially normal or strong, and it remained normal or strong in the postoperative period.

(2) The blastic transformation activity of the lymphocytes was initially normal or strong, but it became weak in the postoperative period.

(3) The blastic transformation activity of the lymphocytes was initially weak, but it became normal or strong in the postoperative period.

(4) The blastic transformation activity of the lymphocytes was initially weak, and it remained weak in the postoperative period.

Groups 1 and 3 were regarded as positive, and groups 2 and 4 as negative, as concerns the effectiveness of the immune defence activity.

To assess the effect of vaccination, the changes in blastic transformation activity in samples taken at admission and on the day of surgery were compared separately in the vaccinated and the non-vaccinated subjects. During this period, there was no difference between the members of the two groups apart from the vaccination. For technical reasons certain examinations could not be performed, and accordingly this comparison was made only for 16 vaccinated and 17 non-vaccinated cases. Nineteen vaccinated and 17 untreated patients presented for the 3-month control.

Statistical analysis was performed with the χ^2 test.

Results

Table II contains the distribution data of the blastic transformation capacity of the lymphocytes at the beginning of the examination in the vaccinated and the non-vaccinated groups. Table II reveals that the two groups immunologically equivalent, since the ratio of the number of cases with normal or strong and with weak immune activities was almost the same in the two groups at the beginning of the examination: 52–48% and 57–43%, respectively. The 40–50% incidence of anergic cases among the patients operated on for colonic tumour corresponded to the average given in the literature [5, 15, 16].

Table III presents the changes in blastic transformation activity of lymphocytes from the day of admission until the 10th postoperative day in the vaccinated and non-vaccinated groups. On the 10th postoperative day a positive change in the blastic transformation activity of the lymphocytes could be observed in 52% of the cases in the vaccinated group, compared with 32% of the non-vaccinated patients (the difference was significant:

$p < 0.05$). From the aspect of a deteriorating immune activity, this means that the immune defence ability worsened during the postoperative period in half of the vaccinated cases and in two-thirds of the untreated ones.

It is noteworthy that the difference between the vaccinated and the non-vaccinated groups was in fact that 35% of vaccinated cases giving a normal or strong reaction retained their initial immune activity, while 14% of the non-vaccinated cases did so. At the same time, only 17% of the vaccinated cases initially giving a normal or strong immune activity displayed a weaker activity in the postoperative period, whereas 43% of the non-vaccinated cases became anergic (the difference was significant: $p < 0.05$).

The proportions of the cases in which the originally weak immune activity became normal or strong, or remained weak throughout, were practically identical in the vaccinated and the non-vaccinated groups.

Table IV compares the blastic transformation activities on the day of clinical admission and on the day of surgery in 16 vaccinated and 17 non-

Table II

Distribution of blastic transformation capacity of lymphocytes in the vaccinated and non-vaccinated groups at the beginning of the examination, compared to normal control cases

Blastic transformation	Vaccinated		Non-vaccinated	
	PHA	Con A	PHA	Con A
Normal or strong	12	12	16	16
Weak	11	11	12	12
Total	23	23	28	28

Table III

Types of lymphocyte blastic transformation in vaccinated and non-vaccinated groups from the day of admission until the 10th postoperative day

Blastic transformation	Vaccinated (23 patients)		Non-vaccinated (28 patients)	
	PHA	Con A	PHA	Con A
1. Initially normal or strong value remained normal or strong	8*	7	4*	6
2. Initially weak value became normal or strong	4	5	5	3
Total	12	12	9	9
3. Initially normal or strong value became weak	4*	5	12*	10
4. Initially weak value remained weak	7	6	7	9
Total	11	11	19	19

* $p < 0.05$

vaccinated patients. Two sub-groups were performed, depending on whether the immune activity of the patients at the beginning of the study was normal or strong, or whether it was weak. The tabulated data clearly demonstrate that both in the vaccinated and non-vaccinated groups initially with a normal or strong activity the blastic transformation activity fell in half, and rose in half of the cases. In contrast, no change was observed in the majority of patients originally exhibiting a weak immune activity; however, in some cases a remarkable activity increase occurred (no significant difference in any of the compared groups).

Table V contains the corresponding data relating to the 3-months control. Three months postoperatively, a significant difference ($p < 0.05$) in immune reactivity was found between the vaccinated and non-vaccinated cases. Whereas the defence activity remained strong or normal, or became strong or normal, in 14 of the 19 vaccinated patients, a similar tendency was displayed by only 7 of the 17 non-vaccinated patients.

Table IV

Blastic transformation of lymphocytes from day of admission until day of surgery (assessment of effect of vaccination)

Blastic transformation	Vaccinated (16 patients)	Non-vaccinated (17 patients)
A. Cases with initially normal or strong immune activity	8	9
— No change or activity increase up to day of surgery	4	5
— Activity decrease	4	4
B. Cases with initially weak immune activity	8	8
— Activity increase up to day of surgery	2	3
— No change*	6	5

* The group classification method allowed no significant decrease of the activity

Table V

Blastic transformation of lymphocytes from day of admission until 3 months postoperatively

Blastic transformation	Vaccinated (19 patients)		Non-vaccinated (17 patients)	
A. Cases with initially normal or strong immune reactivity	10		9	
— No change or activity increase	7		4	
— Activity decrease	3		5	
B. Cases with initially weak immune reactivity	9	14**	8	7**
— Activity increase	7		3	
— No change*	2		5	

* The group classification method allowed no significant decrease of activity
** $p < 0.05$

Discussion

Our results supported the earlier finding that the cellular immune reactivity decreases considerably in a large proportion of the patients as a consequence of the events during surgery and the postoperative period [1–3, 6]. A negative change was observed in 68% of our non-vaccinated cases. It must be noted, however, that there was a definite improvement in blastic transformation activity of the lymphocytes in certain subjects.

We found that after the vaccination significantly more patients, compared to the untreated ones (see Table III), retained their strong preoperative immune reactivity level in the early postoperative period. Similarly to the non-vaccinated cases, patients with an initially weak cellular immune reactivity responded only weakly to vaccination, according to our previous indirect evidence [5]. In some of the patients with initially normal or strong reactivity, the vaccination prevented or shortened the effects of factors unfavourably influencing the immune defence. Changes in the opposite direction may in all probability be ascribed to reasons which, because of the low number of the patients, could not be taken into consideration in the present group classification. Examples of such possible factors are the state of nutrition [14, 16–18], the metabolic equilibrium, the stage of the tumorous disease, the quantity of blood transfusions [19], and, perhaps the most important factors are the septic complications [14, 20–22]. It has been shown that lymphoid cells originating from the human spleen, lymph nodes and tonsils are stimulated by endotoxin, which at the same time does not act on peripheral lymphocytes. In vitro experiments, however, showed that it decreases the blast cell transformation activity induced by phytohaemagglutinin [23]. It has also been observed that a slight slow infection stimulates to a certain extent the immune apparatus, whereas a severe one exhausts it [21, 24, 25]. Other authors interpreted the connection between the severity of infections and the immune status in that the course of the infection is determined by the immune status [14, 20, 22, 26]. We intend to carry out further investigations in an attempt to clarify this question.

Animal and clinical experimental studies have been reported in which vaccination led to immune depression for some time [26–29]. This observation could be controlled by comparison of blastic transformation activity in blood samples taken on the day of surgery with the data obtained on the day of admission in vaccinated and non-vaccinated patients. The blastic transformation activity of lymphocytes increased or decreased between these two points of time in about 40% of the patients exhibiting a normal or strong reactivity, in both the vaccinated and non-vaccinated groups (6/16 and 7/17, respectively), but no tendency could be discovered in the direction of the changes. In some patients an enhancement of the initially weak reactivity was observed in

both the vaccinated and non-vaccinated groups, is evidence against an immune depression.

Lymphocyte blastic transformation measurements 3 months postoperatively, after convalescence, indicated the favourable situation of the vaccinated cases. Nevertheless, the data during this period may have been influenced by uncontrollable effect such as tumour recurrence, intercurrent diseases, etc., and therefore the results should be evaluated with care. Even so, it may be stated that our results point to the possibility of prolonged immune stimulation by means of vaccination.

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DETECTION OF MAIN CORE PROTEINS OF SIMIAN C-TYPE VIRUSES AND HUMAN RETROVIRUS HTLV AND ANTIBODIES TO THEM IN PATIENTS WITH LYMPHOID MALIGNANCIES

F. D. Tóth, L. Váczi, B. Szabó, Jolán Kiss, A. Réthy, A. Kiss, B. Telek, Ilona Kovács, Cs. Kiss and K. Rák

Institute of Microbiology, Second Department of Medicine, and Department of Paediatrics, University Medical School, Debrecen

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Peripheral leukocytes or lymph node cells and blood plasma samples from patients with lymphoid malignancies were investigated for immunological markers of BaEV, GaLV and HTLV. Antigens and antibodies were shown with radioimmunoassay. Antigen related to the p30 core protein of BaEV could be detected in each cell type of leukaemias and lymphomas. Antigen related to the GaLV p30 was found mainly in B- and O-cell forms, while that related to the p24 protein of HTLV could be detected only in two T-cell malignancies. Antibodies reactive with these antigens showed a similar distribution.

The critical question concerning retroviruses is whether they are involved in human carcionogenesis and, if so, to what extent. A recent development of continuously growing cell lines from patients with lymphoid malignancies has led to the isolation of C-type retroviruses. Most of them are very closely related to C-type viruses of primates [1]. The human T-cell leukaemia/lymphoma virus (HTLV) was first isolated from a US patient with cutaneous T-cell lymphoma [2]. The isolation of HTLV from patients in different parts of the world supports recent serological-epidemiological studies [3–5] indicating that this virus is more wide-spread than previously believed.

Our previous studies [6] with indirect membrane immunofluorescence technique showed the presence of antibodies to simian C-type viruses and HTLV in blood plasma samples from patients with malignant lymphoproliferative diseases. In the present work, we report the results of a radioimmunoassay study on the presence of primate oncovirus-specific antigens and antibodies in Hungarian patients with different lymphoid malignancies.

Ferenc D. Tóth, Lajos Váczi, Béla Szabó, Jolán Kiss, Antal Réthy
Institute of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

Attila Kiss, Béla Telek, Kálmán Rák
Second Department of Medicine, University Medical School
H-4012 Debrecen, P.O.B. 20, Hungary

Ilona Kovács, Csongor Kiss
Department of Paediatrics, University Medical School
H-4012 Debrecen, P.O.B. 32, Hungary

Materials and methods

Purification of leukocytes and lymph node cells. Erythrocytes were removed by lysis in 0.83% ammonium chloride solution. For details see D. Tóth et al. [7].

Preparation of cell extracts. One ml of phosphate buffered saline (PBS) pH 7.4 containing Ca^{++} and Mg^{++} and supplemented with ovalbumin (0.5 mg/ml), 0.5% Nonidet P-40 and 2 mM phenyl-methyl-sulfonyl fluoride (PMSF) was added to 10^8 leukocytes of lymph node cells. The cells were disintegrated by five ultrasonic treatments (1.5 A, 10 sec). The treated samples were then incubated at 37 °C for 15 min and finally centrifuged at 18 000 g for 15 min at 4 °C. The supernatants were used as competing antigens in competitive radioimmunoassay. Proteins were quantitated by the method of Lowry et al. [8].

Purification of p30 polypeptides from primate oncoviruses. The M7 strain of baboon endogenous virus (BaEV) and the gibbon ape leukaemia virus (GaLV) were provided by the Pfizer Laboratories (Maywood, N.J., USA). The BaEV and GaLV p30 proteins were purified by phosphocellulose column chromatography as described by Strand and August [9]. Viral antigens were labelled with ^{125}I using the chloramine method [10].

HTLV p24 antigen was supplied by M. Robert-Guroff and R. C. Gallo from the National Cancer Institute (Bethesda, Maryland, USA).

Immune sera. Goat immune sera to BaEV p30 and GaLV p30 were provided by R. Wilsnack (Huntingdon Research Center, Baltimore, Maryland, USA). Goat immune serum raised against HTLV p24 was sent by M. Robert-Guroff and R. C. Gallo (Bethesda, Maryland, USA). Precipitating rabbit anti-goat immune serum and goat anti-human IgG serum were from Hyland Co. (Costa Mesa, California, USA).

Competitive radioimmunoassay (RIA). The antiserum was added at dilutions which precipitated approximately 50% of the labelled antigen, and the competitive inhibition of binding of the ^{125}I-labelled antigen was measured. At first 200 μl of cell extract and 20 μl of anti-p30 or anti-p24 serum were incubated at 37 °C for 1 h. Then 10 μl of ^{125}I-labelled viral antigens were added and the mixture was incubated at 37 °C for 3 h. Thereafter 20 μl of rabbit anti-goat serum were added to the samples which were subsequently incubated for 1 h at 37 °C and then for 18 h at 4 °C. After termination of incubation, the immune complexes were sedimented (1000 g, 15 min) and washed twice in 0.5 ml of buffer at 4 h intervals at 4 °C to allow a reaggregation of small, soluble immune complexes. Radioactivity of the sediments resuspended in buffer was assessed in a gamma scintillation counter. The same buffer as used for preparation of cell extracts was employed as diluent and for washing.

Radioimmunoprecipitation assay (RIP). The experimental conditions described by Kurth et al. [11] were utilized throughout the present study. The carrier and washing buffer contained 2 mM PMSF. As it was done in RIA experiments, all solutions were centrifuged just before assay to remove possible aggregates. Ten μl of virus antigen, 70 μl of carrier buffer and 20 μl of human blood plasma sample at twofold dilutions (started from 1 : 5) were incubated for 3 h at 37 °C. Then antihuman IgG serum was added in 20-fold excess over the test plasma and the samples were incubated for 1 h at 37 °C. Incubation was terminated after 18 h at 4 °C. Centrifuging of immune complexes (1000 *g*, 15 min) was followed by two washes. Radioactivity of the sediments was assessed in a gamma scintillation counter.

Results

1. *Frequency of BaEV p30, GaLV p30 and HTLV p24.* Extracts from peripheral leukocytes of patients with lymphoid leukaemias and from lymph node cells of patients with lymphomas were tested by competitive RIA (Table I). The antigen related to the p30 core polypeptide of BaEV could be detected in each cell type of acute lymphoid leukaemia (ALL), chronic lymphoid leukaemia (CLL), non-Hodgkin lymphoma (NHL) and in Hodgkin lymphoma (HL). The presence of GaLV p30-like antigen was connected mainly with O- and B-cell types of ALL, CLL and NHL. Out of 21 T-cell malignancies, only two samples were positive for the antigen related to HTLV p24. Peripheral leukocytes of 87 healthy persons and biopsized lymph nodes of 14

Table I

Frequency of BaEV p30, GaLV p30 and HTLV p24 antigens in malignant lymphoproliferative diseases

Diagnosis	Cell type	Antigen		
		BaEV p30	GaLV p30	HTLV p24
ALL	T	2/12*	0/12	1/12
	O	3/15	6/15	0/15
CLL	B	2/8	3/8	0/8
NHL	T	3/9	0/9	1/9
	B	4/10	4/10	0/10
HL	—	2/8	3/8	0/8

* Positive samples/number of samples tested

Abbreviations: ALL = acute lymphoid leukaemia; CLL = chronic lymphoid leukaemia; NHL = non-Hodgkin lymphoma; HL = Hodgkin lymphoma

patients with reactive hyperplasia were used as control materials. Antigen related to BaEV p30 could be detected in three cases of reactive lymph node hyperplasia. No control samples contained GaLV-, or HTLV-specific antigens.

2. *Quantitative expression of main core antigens in cell samples.* Table II shows the degree of antigen expression in samples positive for GaLV p30 and HTLV p24 polypeptides, and the clinical data of the corresponding patients. The antigen content of samples is expressed in ng of competitor per mg of total protein. The amount of antigen competing with GaLV p30 is higher in ALL than in other lymphoid malignancies. In ALL the level of GaLV p30-specific competitor seems to be parallel with the ratio of blastic elements. It is notable that the HTLV p24-specific antigen could be found in two T-cell malignancies of mature cell type. These two patients were adults. Peripheral leukocyte samples from their close family members proved to be consequently negative for HTLV p24.

3. *Frequency of antibodies reacting with BaEV p30, GaLV p30 and HTLV p24.* Detection of antibodies by RIP was carried out in blood plasma samples of patients listed in Table I, except the T-cell ALL cases. In the T-cell ALL group more samples were tested for antibodies than for competing antigens (Table III). The distribution of antibodies of different specificities was similar to that of virus-specific antigens. Antibodies reacting with BaEV p30 antigen could be found in O-, B- and T-cell types of ALL, CLL, NHL and in HL. Anti-GaLV p30 antibodies were detected in plasma samples of patients with O- and B-cell malignancies and in one case of T-cell ALL. Anti-HTLV p24 antibodies were found in two patients (one with T-cell ALL and one with T-cell lymphoma). Control plasma samples were taken from the

Table II

Quantitative expression of GaLV p30 and HTLV p24 antigens in peripheral leukocytes and lymph node cells

Patient		Diagnosis	Cell type	Leukocytes		ng antigen/mg protein	
No.	age (years)			G/l	% blast	GaLV p30	HTLV p24
1	7	ALL	O	2.8	10	5.8	—
2	0.5			5.6	34	7.4	—
3	9			4.3	15	4.2	—
4	11			26.4	96	8.9	—
5	14			4.5	30	6.7	—
6	16			100	89	11.2	—
7	29		T	4.5	0	—	14.3
8	76	CLL	B	46	1	3.2	—
9	61			39	3	2.4	—
10	78			52	15	1.9	—
11	7	NHL	B		0	2.8	—
12	10				0	1.7	—
13	55				14	3.4	—
14	37				54	2.9	—
15	46		T		0	—	24.2
16	4	HL	—			3.6	—
17	12					7.2	—
18	47					5.9	—

Abbreviations: see Table I

Table III

Frequency of antibodies reacting with BaEV p30, GaLV p30 and HTLV p24 antigens in malignant lymphoproliferative diseases

Diagnosis	Cell type	Antibodies reacting with		
		BaEV p30	GaLV p30	HTLV p24
ALL	T	6/20*	1/20	1/20
	O	4/15	5/15	0/15
CLL	B	0/8	2/8	0/8
NHL	T	0/9	0/9	1/9
	B	1/10	2/10	0/10
HL	—	1/8	0/8	0/8

* Positive plasmas/number of plasmas tested
Abbreviations: see Table I

Table IV

Titres of antibodies reacting with GaLV p30 and HTLV p24 in plasma samples of patients with malignant lymphoproliferative diseases

Patient		Diagnosis	Cell type	Leukocytes		ng of precipitated antigen*	
No.	age (years)			G/l	% blast	GaLV p30	HTLV p24
1	7	ALL	O	2.8	10	5.2	—
2	9			5.6	7.4	3.8	—
3	11			26.4	96	4.6	—
4	14			4.5	30	7.2	—
5	16			100	89	6.1	—
6	34		T	4.7	4	8.4	—
7	29			4.5	0	—	24.2
8	76	CLL	B	46	1	3.8	—
9	61			39	3	2.9	—
10	50	NHL	B		2.8	4.6	—
11	7				3.4	3.7	—
12	46		T		0	—	17.7

* Nanograms of viral antigens precipitated by 10 μl of plasma diluted 1 : 10
Abbreviations: see Table I

same persons, whose peripheral leukocytes or lymph node cells served as control in competitive RIA studies. None of them had antibodies reacting with the major core polypeptide of BaEV, GaLV or HTLV.

4. *Titres of antibodies reacting with the main core polypeptides.* Table IV shows the amount of antibodies reacting with BaEV p30, GaLV p30 and HTLV p24. The antibody titres are estimated for 10 μl of plasma diluted 1 : 10. The highest values were observed in two samples containing HTLV p24-specific antibodies. These plasma samples were from those patients, whose tumour cells contained HTLV p24-specific antigen. Their close family members had no HTLV-specific antibodies. In all cases, the presence of virus-specific antibodies was connected with the progressive phase of lymphoid malignancies. It should be noted that patient No. 6 was a young mother, whose baby developed O-cell ALL in the age of 6 weeks. The baby had GaLV p30-related antigen (Table II, No. 2). The other family studies gave negative results.

Discussion

In this report we have shown that human lymphoid tumour cells contain antigens related to the main core polypeptides of BaEV, GaLV and HTLV. The BaEV p30-like antigen could be detected not only in lymphoid tumours, but in normal lymphoid cells, too. The presence of antigens related to structural components of simian oncoviruses in human cells seems to be supported by the detection of different families of human DNA sequences related to retroviruses of other mammals [12–14]. One of these DNA segments has been shown to be related to the proviruses of AKR murine leukaemia virus and BaEV [12]. Another segment of the human genome [13] has DNA sequence homology to the gag and pol genes of Moloney murine leukaemia virus [13]. Thus, the appearance of BaEV- and GaLV-related antigens in human cells could be the consequence of derepression of the human endogenous gag sequences. Moreover, the possibility of transmission of an infective agent related to GaLV cannot be excluded in the case of the ALL baby (Table II, No. 2) born by a leukaemic mother (Table IV, No. 6).

Expression of HTLV antigens and antibodies specifically reactive with structural proteins of HTLV have been found in white patients with T-cell malignancies [17, 18] in different parts of the world. Recently, a new human retrovirus, called HTLV-II, was identified in [17] and isolated from [18] cultured cells of a patient with hairy cell leukaemia; this isolate is related to, but quite distinct from, all other HTLV isolates. Other viruses belonging to the HTLV family have been isolated from patients with acquired immunodeficiency syndrome [19]. The origin of antigen related to HTLV p24 in our two patients with mature T-cell malignancies is yet not clear, because their close family members were consequently negative for HTLV p24 and antibodies to them. An analogous observation has been reported on white patients [20]. Injection of cell-free materials into baboons, originally negative for HTLV, induced appearance of HTLV-specific immunologic markers and development of lymphomatous diseases in them. Final clarification of this question needs the comparison of the p24-like antigen detected by us with the main core polypeptide of different HTLV serotypes.

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A NEW *SALMONELLA* SEROTYPE *SALMONELLA ARIZONAE* (28 : z : z_{10}) WITH UREASE-POSITIVE CHARACTER

(A NOTE)

M. KÁDÁR, MÁRIA M. ÁDÁM and L. LE MINOR

National Institute of Hygiene, Budapest, Hungary and WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France

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A new *Salmonella* subspecies III serotype: *Salmonella arizonae* 28 : z : z_{10} urease-positive variant, isolated from a sewage treatment finishing pond is described.

Salmonellae normally do not split urea. The urease test is therefore used for the rapid differentiation of *Salmonella* from *Proteus vulgaris* and *Proteus mirabilis* which are all lactose-negative and H_2S-positive on triple sugar iron agar. According to our knowledge there is a publication on a urease-positive *Salmonella cubana* strain [1] isolated from urine. In this note we describe the circumstances of the isolation of a urease-producing *Salmonella arizonae* which at the same time is a new serotype.

In the course of a routine environmental monitoring programme the new *Salmonella* serotype was isolated from a finishing pond water sample, near to the town Keszthely on 26th September, 1983. There are two parallel series of two shallow lagoons which serve for the finishing treatment of the disinfected effluent of the town's sewage treatment plant and are alternately loaded at intervals of 3 to 4 weeks.

While only strains belonging to *Salmonella* subspecies I have ever been isolated from the secondary treated sewage flowing into the finishing pond, different serotypes of *Salmonella* subspecies III have often been detected from the effluent water. It was supposed that *S. arizonae* had reached the water with the excreta of the fauna living in the abundant vegetation of the ponds.

MIHÁLY KÁDÁR, MÁRIA M. ÁDÁM
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

LÉON LE MINOR
WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur
28, Rue du Docteur Roux, 75724 Paris Cedex 15, France

Materials and methods

Five hundred ml of the water samples were membrane filtered and incubated overnight in Preuss [2, 3] enrichment broth at 37 °C. Isolation was performed on salmonella selective media [4, 5]. Biochemical and serological reactions were described by Kauffmann [4] and Le Minor [5].

Results

According to biochemical features (see Table I), the isolate belonged to *Salmonella* subspecies IIIb which corresponds to *S. arizonae* subgenus III, possessing two H phases [6, 7]: it is galacturonate-, glutamyltransferase- and glucuronidase-positive. On the other hand, the strain is lactose-, ONPG- and malonate-positive, d-tartrate- and KCN-negative. It differs in the following characters: dulcitol-positive, gelatinase-negative, H_2S-negative, gas from glucose negative and principally in splitting urea in one day in the urea-indole broth [8] derived from the medium of Ferguson and Hook [9] and similarly in Christensen's medium [10]. We could not observe segregation of urease-negative colonies: all of them possessed and maintained this feature. No concomitant DNA band could be observed by gel electrophoresis. Therefore we can not advance an argument in favour of the plasmidic localization of the genes which code the urease, as it was shown in *Escherichia coli*, *Providencia stuartii* and *Streptococcus faecium* [11, 12, 13].

The isolate belonged to serogroup M, its detailed antigenic structure was $28_1, 28_3$, it differed only by some minor antigens from the antigens of *S. dakar*.

Table I

Biochemical properties of S. arizonae 28 : z : z_{10}

Adonitol	—	D-Xylose	+
L-Arabinose	+	ONPG	+
Cellobiose	—	Methyl red 37 °C	+
Dulcitol	+	Voges–Proskauer 37 °C	—
D-Fructose	+	Urea (Ferguson)	+
D-Galactose	+	Indole	—
D-Glucose (gas)	+ (—)	H_2S	+
Glycerol (gas)	$+^{5-6}$	Gelatin (film)	—
m-Inositol	—	Ammonium citrate	—
Lactose	+	d-, l-, i-Tartrate	$—^{7}$
D-Maltose	+	Galacturonate	+
D-Mannitol	+	γ-Glutamyltransferase	+
D-Mannose	+	β-Glucuronidase	+
Melibiose	$+^{2}$	Malonate	+
Raffinose	—	Mucate	—
L-Rhamnose	+	Lysine decarboxylase	+
Salicin	—	Ornithine decarboxylase	+
D-Sorbitol	+	Arginine dihydrolase	$—^{7}$
Sorbose	—	Tryptophan deaminase	—
Sucrose	—	Tetrathionate reductase	+
D-Trehalose	+	KCN	—

The isolate possessed the H antigens z and z_{10}. The cultures in the corresponding phase absorbed serum Hz (*S. poona* phase 1) and absorbed serum Hz_{10} (*S. glostrup* phase 1), but not completely. In this way the strain analyzed proved to be a new serotype of *S. arizonae*.

Isolation of the same serotype failed in further samplings. The isolate of the new serotype has been included in the Hungarian Collection of Medical Bacteria, Budapest, under accession number 40047.

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TOXINS AS VIRULENCE FACTORS OF BACTERIAL ENTERIC PATHOGENS

(A REVIEW)

I. KÉTYI

Institute of Microbiology, University Medical School, Pécs

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Introduction

It is a paradox that while some two decades ago, beside the endotoxin, the so called neurotoxin of "Shiga" was accepted as the only toxic virulence factor of bacterial enteric pathogens, its real role in the pathomechanism is dubious still today.

IVÁN KÉTYI
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

The story of the toxic nature of Asiatic cholera reflects the change of our views in this field. Soon after the discovery of the causative agent, Robert Koch pointed out that cholera is a toxin-mediated disease. However, for various reasons, this prediction was confirmed considerably later. After the turn of century, laboratories of the industrialized world took no interest in the pathomechanism of cholera which had cessed to be of epidemiological importance. Perhaps also some fear existed to the risk of manipulating with agents unknown in their own country but capable of causing so dangerous epidemics in the past. There were technical problems, too: the classical exotoxins, e.g. tetanus or diphtheria can be easily tested in laboratory animals by giving minimum amounts parenterally. Thus, a generally accepted assumption based on experimental results was advanced: exotoxins are highly toxic materials if given to laboratory animals by parenteral or especially by intravenous route. The cholera toxin does not fit into this scheme (about 10 μg of purified cholera toxin corresponds to the LD_{50} value, whereas the same amount of tetanus toxin is equal to 4×10^5 LD_{50}) and only the pandemic appearance of the El Tor biotype, the multiplied efforts of reinvestigations and the renewed application of the isolated gut model by De and Chatterje [1] led to the discovery of cholera and related toxins [2, 3].

1. Enterotoxins

There is no generally accepted definition of enterotoxins: in a narrow sense these are protein exotoxins which in natural circumstances are effective on the bowel epithelial cells causing fluid loss by the accumulation of cAMP or cGMP — without cellular damages. This definition exclude some related toxins such as Shiga and clostridial enterotoxins and the not well defined cyto- or verotoxins. It is true, that at least in some cases their relatedness suggest a definition in a broader sense.

1.1. *The enterotoxin of Vibrio cholerae*

The causative agent of Asiatic cholera — including the classical type of *V. cholerae* and its El Tor biotype — is a non-invasive pathogen. There is a possibility that some additive factors (motility, mucinase) play a role in the pathogenesis, but according to our present knowledge there is only one virulence factor of overhelming importance, the exotoxin.

The enterotoxin of *V. cholerae* or Cholera Toxin (CT) or choleragen, purified first by Finkelstein [4] is a protein exotoxin with a molecular weight of 84 000 and consists of two, so called protomers: the "A", light subunit (29 000 d) and the "B", heavy subunit (55 000 d) associated by non covalent bonds

[5]. The A subunit is synthesized as a single polypeptid chain, but in protease producing strains it was isolated in a nicked form: polypeptide A_1 (23 000 d) and A_2 (6000 d) linked by a disulphide bond and noncovalent interactions [6]. The B subunit consists of five identical polypeptides (11 600 d) noncovalently associated. The A_1 chain is attached to the B subunit through the A_2.

The enzymatically active part of the toxin is the A_1 polypeptide which invades the sensitive cells and activates the adenyl-cyclase enzyme by a NAD-dependent ATP-ribosylation of the GTP binding component [5, 7]. The B subunit is the receptor-binding component of the toxin. This B protomer is atoxic (the A protomer is only very slightly toxic) and seems to be identical with the natural atoxic product choleragenoid [8, 9]. The choleragen treated with formaldehyde or glutardehyde gives perhaps only a very similar product [10].

Studying the pharmacological effect of cholera toxin it has been very early observed that it acts primarily by augmenting a secretory process, without impairing the absorption. The enhanced secretion is independent from e.g. arterial pressure, depending only on the dysfunction of epithelial cells. Experiments performed in perfusion chambers show a chloride secretion with concomitant loss of other ions and water across the membrane without inhibiting the glucose-enhanced sodium absorption. Essentially, a normal *succus entericus* is secreted [2].

Already van Heyningen et al. [11] have suggested that a ganglioside may be the receptor for CT. Later it has been proved by several methods that the monosialoganglioside G_{M1} is the exact receptor structure [12, 13]: Ceramide — glucose — galactose — *N*-acetylgalactosamine — galactose: *N*-acetyl-neuraminic acid (sialic acid).

The binding between the polypeptide of B protomer and 5–6 G_{M1} molecule occurs through their oligosaccharide chains [14]. G_{M1} being a rather ubiquitous component of the animal plasma membrane, CT may be bound to other tissues than bowel [15]. Therefore, the accumulation of cAMP and in some cases measurable changes may be observed in other tissues or cells, too. Nevertheless, the classical models used the natural target, the bowel. These include the ligated small bowel loops of rabbit [1], dog [16, 17] or rat jejunum [18]. Suckling rabbits [19], mice [20] and hamsters [21] are used widely as well as adult dogs [22] or the so called sealed adult mice [23]. The change in permeability caused by toxin is extensively employed in the permeability (or blueing) test (PF) on rabbit skin [24] or frog skin [25], as well as in the foot pad test in mice or rats [26]. Some cells are sensitive enough for the assay of CT, e.g. earlier the isolated fat cells of the rat [27], and subsequently the cell lines of Y1 [28], CHO [29], or Vero [30] were used.

For the study of a human disease in animal model the suckling rabbit test [19], the fluid loss and the ion transport detected by the chronic dog test

[17], and the so called Thiry–Vella loop were mostly used. For a rapid assay generally the cell culture tests are applied [28, 29]; using the CHO (Chinese Hamster Ovary cells) line the reading is somewhat easier, because the effect of CT is expressed by cell elongation and not by rounding as it occurs in the Yl adrenal cells. Because the cell cultures used in virology or in toxin test (e.g. diphtheria) react mostly with cytotoxic change, the effect exerted by CT is called cytotonic effect. The differentiation of cytotoxic and cytotonic effects of enterotoxins (in a broader sense) was used by Keusch and Donta [31] for classification, namely cytotonic enterotoxins and cytotoxic enterotoxins (e.g. "Shiga-toxin" or *Clostridium perfrigens* enterotoxin). The absence of the cytotoxic effect of CT, as well as the fact proved in several animal models and also in man that neither cholera infection, nor CT cause histological lesions, are very important. The lesion is purely functional, therefore the water and ion resorption of the gut is not impaired. Hence the principle and practice of the so important oral rehydration therapy [32].

The production of CT by *V. cholerae* strains is specified and regulated chromosomally. The *tox* gene was found in histidine *(his)* linkage [33]. The coding of protomer A and B is regulated coordinatedly by the *htx* and *ltx* loci [34], which are the allelic forms of the same regulator gene resulting hypertoxinogenic or hypotoxinogenic mutants. There are data [35] showing differences among the organization of the toxin genes of different strains of *V. cholerae*. The regulator mutants *htx* have practical significance in the yield during the purification of toxin derived from the high level of the produced CT. More important is the separated coding sequences of protomer A and B. An A^-B^+ strain was isolated by Honda and Finkelstein [36] through nitrosoguanidine mutagenesis of *V. cholerae* (strain Texas Star-SR), which is a promising vaccine candidate [37].

The production of separated A and B subunits occurs cytoplasmatically on free polysomes [38]. The transport of protomers through the inner membrane reaches the periplasmatic space, where the binding of CT happens. The release of CT seems to be very effective.

The cholera toxin, being a high molecular weight protein, is immunogenic. Experiments performed on the neutralizing mechanism of antitoxins show that this is primarily directed against the B protomer [39]. There are antibodies against the A protomer, however, the anti-B antibodies are not only overwhelming in a quantitative sense, but their relative neutralizing capacity is also much higher. This fact corresponds to the concept of Finkelstein [3] about the structure of the holotoxin, namely, the B protomer seems to cover the A polypeptide. Thus the protective mechanism of antitoxins is the inhibition of toxin to bind to the G_{M1} ganglioside receptors.

The importance of the local formation of antibodies was pointed out very early by Burrows et al. [40]. Somewhat later Pierce and Gowans were [41]

able to demonstrate by immunofluorescence the appearance of specific antibody-containing plasma cells in the lamina propria of experimental animals. Among the immunoglobulin classes the importance of sIgA was already demonstrated by Kaur et al. [42]. Because of the efficiency of IgG antibodies it was supposed that the long-lasting relative anti-cholera resistance among adults in endemic areas [43] is based on IgG antibodies [39].

The above-outlined experiences concerning cholera immunity (including antitoxic and antibacterial immunity) serve as an important theoretical basis for developing vaccines. Two basic features must be considered: (i) the antitoxic response is directed against the B subunit, therefore this protomer is necessary and sufficient for antitoxic immunity (e.g. Texas Star-SR strain); (ii) the local formation of antibodies is important, therefore most investigators favour the oral route of vaccination [44–46]. After all, it must be emphasized that at present we cannot determine which role and significance the antitoxic and the antibacterial factors play in immunity against cholera.

1.2. *The enterotoxin of non-Ol vibrios*

Non-Ol (NAG) vibrios occasionally cause sporadic cases or small outbreaks of cholera-like watery diarrhoea, frequently in cholera epidemic regions [47], but sometimes in cholera-free areas, too [48]. In the culture supernatant of such strains an enterotoxin was demonstrated with a close antigenic and biological resemblance to the cholera toxin [49, 50]. The purified non-Ol toxin may be classified into two types on the basis of molecular composition [51, 52]: one is seemingly identical with the cholera toxin, the other shows minor molecular differences (smaller B subunits, etc.). The latter observation is supported by genetic evidences. Kaper et al. [53] reported that the DNA sequence encoding enterotoxin in some non-Ol strains is not identical with the allelic region of Ol *V. cholerae*. Nevertheless, there are data about such molecular heterogeneity between the *tox* genes of Ol *V. cholerae* reference strain 569B and of an El Tor strain, too [54].

1.3. *The enterotoxin in Vibrio parahaemolyticus*

The marine, halophilic *V. parahaemolyticus*, an agent frequently associated with food poisoning, was mostly studied in Japan [55, 56]. The illness is usually mild, but sometimes severe and fatal watery diarrhoea occurs and in such cases, like in cholera, histological damages in the bowel are absent, even in fatal cases. The enteropathogenicity of *V. parahaemolyticus* seems to be associated with two toxic substances: (i) a heat-stable, so called K-(Kanagawa-)-haemolysin and (ii) a supposed enterotoxin-like substance.

The existence of the enterotoxin was supposed on the basis that the culture supernatant or the purified material of the vibrio gave positive blueing and ligated rabbit ileal loop tests and mice responded with diarrhoea to feeding with the bacteria [57]. The results became more convincing when a cytotonic effect on CHO cells was described [58].

The characteristic pathological and histopathological signs observed after the oral administration of *V. parahaemolyticus* culture supernatant and changes in man have been attributed to the combined effect of enterotoxin and K-haemolysin.

1.4. *The heat-labile enterotoxin of Escherichia coli*

Enterotoxin producing strains of *E. coli* were first isolated by veterinary microbiologists from pigs and newborn calves suffering from diarrhoeal disease [59]. The culture supernatant of these strains or the living organism caused bowel dilatation and fluid accumulation in the ligated loops of the host animal [60]. Similarly to cholera, no histological damage was observed, and the crude toxic substance was ineffective by intravenous administration [61]. Later some contradictory data on the enterotoxin were clarified by recognizing the existence of a high molecular weight, heat- and acid-labile toxin (LT) and of a low molecular weight, heat- and acid-stable toxin (ST), which are frequently produced simultaneously [2].

The significance in human diseases of enterotoxins of *E. coli* was demonstrated in Calcutta by showing loop-test positive strains from patients suffering of an acute, cholera-like disease [62].

The heat-labile enterotoxin of *E. coli* resembles choleragen in different respects. Its molecular weight is about 91 000 d [63], being composed of an A protomer with a molecular weight between 25 000–29 000 d [64] and of a B-protomer of approximately 59 000 d [64, 65]. The A protomer is synthesized as a single polypeptide chain, occurring also in a nicked form with an enzymatically active A_1-like polypeptide (21 000 d) linked by a disulphide bond to the A_2 chain. The B protomer consists of five noncovalently linked identical (11 800 each) peptide subunits [65].

LT exerts an effect quite analogous to that of CT: it increases the intracellular level of cAMP due the A_1-polypeptide activating the NAD-dependent ribosylation of the adenylate cyclase [66, 67].

On the basis of the above-described molecular and biological analogues between LT and CT it is understandable that the biological assay tests applied for choleragen are equaly useful for LT, too.

Beside the outlined molecular and biological analogy between CT and LT, the A and B protomers share common antigenic determinants [68–70].

The amino acid sequence of the B-region and the aminoterminal region of the A_1-polypeptides show an homology of 80% [71, 72].

The binding of LT to the G_{M1} ganglioside receptor has been proven [73], but data on the binding properties between CT and LT differ both qualitatively and quantitatively. First of all, there are reports, e.g. of Pierce [74] and of Holmgren [75] showing that the B-region of CT preincubated with the ligated rabbit ileal loop prevented the binding of CT, but not of LT. In reciprocal experiments [73] the B protomer of LT could block the fluid accumulation induced both by LT and CT. Holmgren therefore concluded that beside G_{M1} an unidentified glycoprotein may serve as the predominant receptor for LT [73].

It seems that there is some evolutional divergence among LTs: Takeda and Murphy [76] have described an LT with a minor biological modification (cytotonic for CHO, but not for Yl cells) the production of which is a consequence of lysogenic conversion. A similar LT (LT') was described by us [77] without any connection to lysogeny. Green et al. [78] reported on a new, chromosomally coded LT, neutralized neither by anti-LT nor by anti-CT sera. There seems to be an antigenic heterogeneity also among typical LTs: Gyles showed only a partial neutralization between two LTs of porcine origin [79]. Finkelstein [80] found no cross reaction between purified LTs of his (human strain 339_t5) and of Dorner's (porcine strain P263) in the Ouchterlony test. Similar conclusions were drawn by other investigators [81, 82].

These sometimes conflicting results were interpreted by Clemens and Finkelstein [83] so that the separately synthesized A polypeptide is cell bound and during natural release or preparation may couple to irrelevant proteins. On the other hand, apart from the above-mentioned heterogeneity caused by an artefact there is an antigenic difference between LT of human (LT_H, LT-H) and porcine (LT_P, LT-P) origin [64, 84, 85], which is residing in the B-subunits [86].

The LT — as compared to CT — seems to be more of cell bound nature [87]; the release of the toxin may be induced by polymyxin B [88], mitomycin C [89], or by lincomycin [90]. According to Gemski et al. [91] the mitomycin C induction does not affect the LT release directly, but causes a release by lysis through the induction of a temperate phage lytic cycle. Most probably the varying effectivity of release leads to a broad range of difference in the amount of the produced toxin [92]. A recent publication [93] has shown that the release of LT is controlled by a chromosomal locus.

In contrast to the chromosomally specified choleragen, the enterotoxins of *E. coli* are coded by plasmids. The existence of these so called "Ent" plasmids was demonstrated in enterotoxigenic strains isolated from animals [94, 95], as well as from human patients [96, 97]. Some of these Ent plasmids are coding only ST [98], only LT [99], others both ST and LT [98]. The rate is:

30–40% for LT^+ST^+, 40–50% for LT^-ST^+ and 20–30% for LT^+ST^- [100]. These rates depend (i) on the host animal, e.g. calf strains are predominantly ST plasmid carriers [101], or (ii) on the presence of the adhesion factor: the pig specific adhesin K88 is frequently associated with LT^+ST^+ plasmid, while 987P and K99 only with ST production. The human adhesion factor CFA/I associates frequently with LT—ST, but CFA/II only with ST [102]. This association (iii) is connected with the O-serogroup of the enterotoxigenic strain [102]. The association between Ent plasmid, adhesion factor and O-serogroup can be interpreted by the so called "clonal spreading" phenomenon: strains with given antigenic structure pick up coding sequences (plasmid or chromosomal) for adhesins and Ent plasmid (and eventually also for other factors beneficial for the survival of this bacterial clone), and such "well fitted" clones spread all over the world.

First So et al. [103] cloned the coding sequence of LT from the relatively large plasmid P307. P307 is 50×10^6 d in mass and the coding sequence was demonstrated to be 1.2×10^6 d. Subsequently Dallas et al. [104] determined the presence of two cistrons: *elt*A and *elt*B coding the A protomer (LT—A) and the B protomer (LT—B) of LT, respectively. Their order (promoter–*elt*A–*elt*B) shows a similar structure to the CT gene. Dallas [105] compared the *elt* genes of human *(elt_H)* and of porcine *(elt_P)* origin. Beside the overall structural identity, he found the *elt*B_P cistron somewhat larger than the *elt*B_H in agreement with the finding of the somewhat larger molecular weight of LT—B_P. The *elt*A cistrons were proven to be identical. It is interesting that the flanking sequences of *elt*H and *elt*P genes are quite divergent. This may provide a further evidence that the LT gene was carried originally by a transposon. Yamamoto and Yokota [106] described an LT gene which having a transposon structure was flanked by repeated sequences. The transposon theory could explain not only the already mentioned irregular LTs or the also mentioned rare findings, namely that the LT coding sequence was carried chromosomally [78] or by a temperate phage [76], but the general characters of Ent plasmids, too. As already mentioned, the Ent plasmids, which are relatively large (at least 50×10^6 d), frequently incorporate the ST gene (proven to be of transposon nature) and are frequently associated with genes coding adhesins, too (e.g. CFA/I+ST [107, 108], CFA/I+LT+ST [108], CFA/II+LT+ST [109]). There are also associated or recombinant plasmids which carry Ent genes and resistance determinants of an R plasmid [110].

Recognizing the plasmid specified nature of LT it is understandable that the first assumption [111] denied any correlation between enterotoxigenicity and antigenic (O-group) structure. Soon after the first serological investigations it has become clear that this assumption is incorrect, and there is a very definite association between serogroups and sometimes even serotypes and the nosological unit of enterotoxigenic *E. coli* (ETEC) [112–115]. The most impor-

Table I

The most important serogroups of ETEC

Host	Serogroups of *E. coli*
Man	O6, O8, O15, O25, O63, O78
Pig	O8, O20, O45, O64, O101, O138, O141 O147, O149, O157
Calf and lamb	O8, O9, O20, O101

tant serogroups are summarized in Table I according to the data of Gaastra and De Graaf [102].

The antigenic nature of LT and its cross reactivity with CT predict an antibody response after illness caused by LT producing ETEC strains [116]. In experimental challenge studies of Levine et al. [100] serum IgG antibody response with LT neutralizing capacity was detected in 77% of ill volunteers; sIgA antibodies were also demonstrated in the intestinal fluid [100].

Immunization must be directed towards stimulation of intestinal mucosal immunity [100]. Such experimental vaccines are in progress, necessarily taking into account the importance of immunity also against ST, as well as its antibacterial (anti-adhesine) effectivity [100].

1.5. *The heat-stable enterotoxin of Escherichia coli*

The heat-stable enterotoxin (ST), already mentioned in part 1.4 of this review, is a low molecular-weight, acid- and heat-stable (100 °C, 15–30 min) toxin. The ST has two distinct types: the methanol soluble ST_A (class 2, or STI), which is active in infant mice (useful assay test) and in neonate piglets, but not in weaned pigs [117–119]. The other, methanol insoluble ST (ST_B, or class 1, or STII) is active in weaned pigs, but not in suckling mice. A simultaneous production of ST_A and ST_B may occur [118].

The overwhelming majority of studies deals with ST_A, whereas our knowledge of ST_B is rather poor (see later).

It should be mentioned that ST_A is a rather heterologous group, e.g. Kapitany et al. [120] purified two ST_A's differing in heat resistance, and also in amino acid content. After all, it seems that the differences in STs are not connected with the animal host, from where the *E. coli* strains originated [121].

The molecular weight of ST_A is approximately 2000 d [122–124]. It is not only acid-, and heat-stable, but also resistant against proteases, nucleases, lipases, phospholipase C and amylase [125, 126].

Two human [124, 127] and a porcine ST_A were analysed [123] for their amino acid sequence: they were composed of 18–19 amino acids. Six of these

were half-cystines, and basic amino acids were absent. The four amino acids at the amino-terminal end could be removed without a loss of biological activity [127].

Treatment of intestinal tissues with *E. coli* ST_A results in an increased fluid accumulation, preceded by increased cyclic GMP levels [128–130]. It is assumed that the ST_A activates the guanyl cyclase [128, 130] by an unknown mechanism.

The STs are not immunogenic, but if conjugated adequately, e.g. with bovine serum albumin (ST—BSA), antibodies may be produced. No cross reaction was found with LT or even with ST_B [131].

The ST_A receptor was successfully isolated from the brush border of rat small intestine epithelium. It seems to be a protein of about 100 000 d [132]. The binding of ST_A to this receptor protein may be directed by a disulphide bridge [132].

The assay of ST_A may be carried out on 3 days old suckling mice inoculated intragastrically [133] or orally [134]. The ligated ileal loop test is also useful. ST_A^+ strains of animal origin are optimally tested in loops of the same species [135]. A new possibility is the use of the so called "sealed adult mice" model [23]. ST conjugated with bovine immunoglobulin [136] or with bovine serum albumin [131] raises anti-ST antitoxins and antisera produced in this manner are suitable for radioimmunoassay methods [131, 136].

As already mentioned, the production of STs are specified by plasmids. The ST gene, as determined by gene cloning technique, is a transposon of about 1.7 kilobasis (kb) of magnitude flanked by inverted Insertion Sequences 1 (ISl [137]). Similar results were obtained by studies performed on two STs of porcine origin [138]. According to Latke et al. [138] the coded protein is of about 10 000 d and the end product (ST_A) was gained by a proteolytic processing mechanism. ST_A (as well as LT) genes were cloned also from a human strain [139] with analogous findings: the ST gene formed a transposon of 1.5 kb. The findings about the transposon nature of ST_A gene may explain the heterogeneity of ST plasmids, as well as easy association or recombination with other plasmids (R, Col) of this gene [140].

ST_A (and also LT) producing colonies were detected by radiolabelled ST-(LT) coding DNA sequences used as hybridization probes for homologous DNA sequences of *E. coli* colonies grown and lysed in situ on nitrocellulose filters [141].

Very little can be mentioned about ST_B because its exact role in pathogenicity is not known: the only ST_B producing *E. coli* strains were unable to cause disease in experimentally infected piglets [142] and the assay of ST_B could be done only in jejunal loops of pigs [143]. As already pointed out, there is no antigenic relationship between ST_A and ST_B [131], and they represent genetically distinct sequences [144]. Recently, Lee et al. [145] cloned the ST_B gene from

the 60×10^6 d plasmid 711(P307). The ST_B is coded by a DNA sequence of 806 bp (basis pair) and its transposon nature has been neither proven nor excluded (there are repeated sequences, but not in the close vicinity of the ST_B gene). The nucleotide sequence of ST_B is quite different from that of ST_A; the coded ST_B protein is composed by 71 amino acids [145].

As referred to part 4.1. of this review the concept of an anti-enterotoxic vaccine, beside LT, must consider also the ST_A [100]. Since STs are not antigenic, an effective experimental vaccine may be produced with synthetic ST_A cross-linked to the B-subunit of LT. This gives a satisfactory protection in rabbits [146] and rats [147].

1.6. *Enterotoxins of salmonellae*

Several authors demonstrated that salmonellae are able to provoke fluid accumulation in the ligated ileal loops of the rabbit [148, 149]. The enterotoxic nature of the above phenomenon was described by Koupal and Deibel [150], who showed the presence of a heat-stable factor in cultures and in their supernatants active in the suckling mouse test. An enterotoxin of heat-labile nature was partially purified from a *S. typhi-murium* strain by Sandefur and Peterson [151]. Its molecular weight, approximately determined on Sephadex G-100 column, was about 90 000 d; it was active in the blueing (PF) test and caused elongation in CHO culture. Both effects were successfully neutralized by cholera antitoxin [151], or by anti-cholera B subunit serum [152]. The salmonella enterotoxins gave, of course, a positive loop test, too [153]. The release of the LT-like enterotoxin seems to be rather poor, but may be induced by e.g. mitomycin C [154]. A crude lysate of a *S. enteritidis* strain isolated by us was strongly positive in the suckling mouse test (ST-effect), positive in the rabbit loop test, in the blueing reaction, in mouse foot oedema test, and caused elongation of the CHO cells. This latter was effectively neutralized by *E. coli* anti-LT serum [155].

Little is known of the receptor of the salmonella LT-toxin; a mixture of gangliosides, which are inhibitory to choleragen, inhibited also the activity of LT-like toxin on CHO cells [154].

The production of enterotoxins among salmonellae seems to be widespread, but some strains are very weak producers. Studying nearly 400 isolates Kühn et al. [156] found that in their collection the strongest producers were *S. typhi-murium* strains, almost 70% of them being positive by the PF test. *S. agona* (30%) and *S. dublin* (20%) were next in order.

The pathological role of the enterotoxins in gastroenteritis caused by salmonellae is still in doubt. The production of enterotoxins may play role in the loss of fluid and electrolytes. This assumption was supported by experiments [157] where a protective effect against living *S. typhi-murium* was demonstrat-

ed in loop test by immunizing the rabbits with procholeragenoid (heat inactivated, purified CT), as well as by a passive immunization with cholera antitoxin.

1.7. *The enterotoxin of Shigella flexneri*

An enterotoxin with unique features was described by us [158, 159] in 1978. The enterotoxin was produced by a strain of *S. flexneri* 3a. The partially purified enterotoxin was strongly active in suckling mice and in the early (4-hour) ligated rabbit loop test, furthermore it was acid- and heat-stable (100 °C, 30 min). It failed to give the LT-specific biological reactions (Yl, CHO cells, PF test, mouse foot oedema test). In contrast to these ST-like characters its estimated molecular weight (Sephadex G-100 column and Amicon ultrafiltration) was in the order of 100 000 d and it proved to be immunogenic. In neutralization experiments performed in suckling mice model, not only the homologous anti-shigella ST serum, but also antitoxins against *E. coli* LT and choleragen had neutralizing capacity.

Among others, a strain of *S. flexneri* type 2a described as a producer of "Shiga-like" toxin [160], was demonstrated to produce also an antigenic ST of the above high molecular weight [161].

The role of this (or other) enterotoxin(s) in the pathomechanism of shigellosis remains unclear. Similarly to that in salmonellosis, it may be a diarrhoeal factor, since in the case of bacillary dysentery an early phase of watery diarrhoea followed by the colitis is characteristic. Kinsey et al. [162] carried out experiments on monkeys with oral and intracoecal administration of *S. flexneri*. In the latter case, in contrast to oral feeding, only colitis developed without the prodromal watery diarrhoea.

1.8. *Enterotoxins in other enteric bacteria*

There are numerous reports on the occurrence of enterotoxin, chiefly ST production, by other different genera of *Enterobacteriaceae*. Klipstein and Engert [163] characterized and purified an ST produced by *Klebsiella pneumoniae* and *Enterobacter cloacae* [164]. They concluded that in the so called tropical sprue such enterotoxinogenic strains may have some role [165]. Boulanger et al. [166] showed two different ST-like enterotoxins of *Aeromonas hydrophila* differing in antigenic structure, as well as in pharmacological mechanism from each other and from the ST of *E. coli*. A large scale investigation was carried out by Jánossy and Tarján [167] including 170 strains of *Aeromonas*. Testing in suckling mice ST occurred most frequently (79%) among *A. hydrophila* strains. ST producers were also found in *A. punctata* subsp. *caviae* and subsp. *punctata*, and *A. salmonicida* subsp. *achromogenes*.

There are plenty of informations about the ST-like enterotoxin of *Yersinia enterocolitica* both of human and of animal origin. Pai et al. [168] found 65% of strains producing ST, with very high frequency among human isolates. The most frequent ST producers belonged to serogroups O3, O8, O5, 26, O6, 30 and O9. There was a correlation between ST production and rhamnose fermentation: rha$^+$ strains produced ST only in 10%, while rha$^-$ strains in 90%.

The mechanism of the action of *Yersinia* ST seems to be analogous to the *E. coli* ST, since activation of guanyl cyclase [169] and accumulation of cGMP have been observed [170]. In *Y. enterocolitica* only a methanol soluble substance exerts ST activity [171, 172].

The role of this ST in the enteritis caused by *Y. enterocolitica* is questionable. First of all, ST is produced only under 30 °C of incubation temperature [173]. In a model experiment carried out with various food samples inoculated artificially with ST$^+$ *Yersinia*, the presence of ST in the samples was detectable only after two days of incubation and shaking at 25 °C. Accordingly, food poisoning due to a preformed ST may practically be excluded [174].

In general, it can be concluded that the enterotoxin, especially ST genes may be widespread among enteric bacteria, sometimes in modified forms. The spreading of the genes may be facilitated by their transposon nature. Wadström et al. [175] reported that Ent$^+$ strains were isolated from 37% of Ethiopian infants suffering from acute enteritis. Among the isolates *E. coli* occurred in 38% only, the others were *Klebsiella* (15%), *Enterobacter* (12%), *Citrobacter* (11%), *Aeromonas* (11%), *Proteus* (7%), *Serratia* (2%), and *Pseudomonas* (1%). The stability of toxin production and its eventual role in the pathomechanism remains still questionable.

2. The entero-neurotoxin of Shigella dysenteriae 1

The long history of the toxin produced by *S. dysenteriae* 1 (Shiga) is an extraordinary conflicting area of toxin research. The production of an exotoxin by "Shiga" was discovered already in the year of 1903 by Conradi [176] who described a special neurological effect on rabbits after introduction of the crude toxin intravenously. Kraus and Dörr [177] described the late appearance of exotoxin during cultivation and its capacity to evoke an antitoxic response. The effect of a partially purified toxin on rabbits was described by Flexner and Sweet [178]: a flaccid paralysis appears first in the front legs and then slowly on the hing legs of the rabbit. Softening of the grey matter of the spinal cord and small haemorrhages in the brain were also observed.

It should be noted that the sensitivity of different animal species is extraordinary divergent. E.g. the lethal dose for the mouse is 700 times greater than for the rabbit and guinea pigs are practically resistant [179].

The "Shiga-toxin" was first purified by chemical methods in 1937 by Boivin and Mesrobeanu [180], but a pure toxin was produced only by van Heyningen et al. [181]. Using this pure toxin Bridgewater et al. [182] reinvestigated the neurological changes caused by this "neurotoxin" in rabbits. The final conclusion of this study has been that the term "neurotoxin" is a misname, since the "Shiga-toxin" attacks primary the endothelium of the blood vessels and the neurological lesions are the results of a secondary effect. Accordingly, in their opinion this toxin is a "vascular toxin" [182].

The next "turn" of the history of "Shiga-toxin" was connected with the observation that the heat-labile exotoxin of *S. dysenteriae* 1 causes fluid accumulation in ligated rabbit ileal segments [183]. This seems to be a curious effect produced by a vascular toxin, since the toxin has no vascular permeability effect on rabbit skin (the blueing test is negative). Somewhat later Keusch et al. [184] reported that the fluid secretion was not connected with an enhanced level of cAMP. The fact that the purified "Shiga-toxin" (the same Sephadex fractions) caused fluid accumulation and also neurological and cytotoxic lesions, led Keusch et al. [185] to conclude that the "neuro-" (vascular) and enterotoxins were identical. In view of the fluid accumulating and cytotoxic effect of this toxin, Keusch and Donta [31] classified enterotoxins into two groups: cytotonic enterotoxins (e.g. CT) and cytotoxic enterotoxins (e.g. "Shiga-toxin"). Besides the above biological features, the recently discovered structure of the "Shiga-toxin" fits also well into its classification as an enterotoxin. The toxin is a protein with a molecular weight of approximately 68 000 d and consists of two components: A (30 000 d) and B containing 6–7 subunits of the latter (each of about 5000 d). The A chain can be converted into two polypeptides [186]: A_1, the active material (27 500 d) is linked by noncovalent bindings and with a disulphide bridge to the A_2 polypeptide (3000 d).

In contrast to the (classical) enterotoxins, the effect of the "Shiga-toxin" is not an activation of adenosine cyclase, but an inhibition of protein synthesis [187], on the level of the peptide chain elongation. The ADP ribosylation of EF-2 is not demonstrable, the inactivation of the 60S subunit of ribosomas occurs with an unknown mechanism [188].

It is only assumed that the B component is responsible for the receptor binding of the holotoxin. In fact, B subunits do not bind to sensitive HeLa cells [186]. It is supposed that this binding needs some conformational changes, e.g. an association forming a B oligomer [186].

Beside these biological activities, a very characteristic feature of the toxin is the cytotoxic effect. The number of sensitive cell lines is limited: e.g. most of the HeLa cell lines are sensitive, but e.g. Yl is resistant, not being able to absorb the toxin perhaps because of the lack of receptors [189]. An other useful cell culture is the Vero line [190]. It has been mentioned [190] that some resistant cells do absorb the toxin, that is, the defect in sensitivity is not due

to the lack of receptors, but to the missing internalization of the cell bound toxin [190]. The most important fact is that the cytotoxic effect manifests also on the epithelium of the bowel of sensitive animals [191].

The toxin receptor is a glycoprotein bearing an oligosaccharide with terminal beta-1,4-linked *N*-acetyl-D-glucosamine [189].

The release of the "Shiga-toxin" is rather poor (about 1 mg/g dry weight [181]). In a very low yield it can be obtained by allowing the organism to autolyse [191]. The method of van Heyningen [191] using KOH extraction at pH 11 of heat-killed bacteria is generally used. Release of the toxin may also be obtained by phage lysis [192].

The genetic background of the toxin production is not elucidated.

The pathological role of "Shiga-toxin" in human dysentery is an open question still today. Neurological symptoms in human dysentery are very rare: only two cases were observed [179] among 3800 patients suffering from Shiga dysentery. In experiments carried out in monkeys, Branham et al. [193] failed to provoke clinical dysentery even with high doses of toxigenic *S. dysenteriae* 1. After all, it is well known that the essential part of shigella infections is the epithelial penetration followed by the intracellular multiplication [194, 195]. According to Keusch et al. [196] the "Shiga-toxin" has an essential role — like choleragen — in the diarrhoeal symptoms of dysentery. The experiments of Gemski et al. [197] gave negative answer to this point. Using a wild-type, toxinogenic *S. dysenteriae* 1 strain and its mutants without penetration ability, or without toxinogenicity they did not found any difference in the symptoms caused by the wild-type or by the atoxigenic mutant. The mutant producing toxin, but having no penetration ability was, however, unable to cause dysentery. According to these authors [197], penetration and intracellular multiplication are themselves — in absence of the exotoxin, too — sufficient to cause the loss of fluid and electrolytes.

After all, we cannot exclude the role of some cytotonic or cytotoxic enterotoxins in the prodromal watery diarrhoea in bacillary dysentery.

It was already mentioned in this chapter that soon after its discovery, the ability of "Shiga-toxin" to induce immuno response (neutralizing antibodies) was demonstrated [177]. The toxin can be assayed by using these antitoxins either in neutralization mouse test (L+ dose) or in flocculation test [181, 198].

The dubious role of "Shiga-toxin" in the clinical symptoms of human "Shiga dysentery" makes the protective role of antitoxins also doubtful. According to van Heyningen [191] the antitoxin has no therapeutic role and value in "Shiga dysentery". The toxin is readily transformed to toxoid e.g. by formaldehyde [199]. Immunizing monkeys with a such formalin-toxoid McIver et al. [200] observed very severe clinical dysentery after a challenge with a virulent strain, though the antitoxins were present in a high level.

2.1. *The occurrence of "Shiga-", or "Shiga-like" toxins in other enteric bacteria*

In a sensitive HeLa system Keusch et al. [201] investigated patients sera after an infection caused by *S. flexneri*, *S. sonnei* and *S. dysenteriae* 2. Most of them contained neutralizing antibodies against "Shiga-toxin". The authors concluded that "Shiga-like" toxins are produced also by other shigellae. The cytotoxic effect of a strain of *S. flexneri* 2a analysed by O'Brien et al. [160] was cross-neutralized by "Shiga-antitoxin". As mentioned in part 1.7. of this review, we reinvestigated this strain [161] and found that the cross-neutralization by "Shiga-antitoxin" was only partial. Furthermore, the semipurified toxin also carried the feature of the unique shigella *(S. flexneri)* ST, proven by successful neutralization with an anti-ST serum of *S. flexneri*.

In a study on the enterotoxin production of salmonellae [155], one of our *S. enteritidis* strains showed LT and ST activity, as well as a cytotoxic feature. Active Sephadex fractions were neutralized by an anti-LT serum (of *E. coli*) and partially also by an "anti-Shiga" serum. In a reciprocal experiment, antitoxic salmonella serum did not neutralize the "Shiga-toxin", indicating a unilateral antigenic relationship. Two other *Salmonella* strains: *S. kapemba* and *S. thompson* showed no LT and ST activity but exerted cytotoxicity. Surprisingly, this cytotoxic effect could be neutralized not only by "Shiga-antitoxin", but by anti-LT serum, too.

O'Brien et al. [202] tested LT^-, ST^-, and Serény-$test^-$ *E. coli* strains isolated from infants suffering from diarrhoeal disease. Among the 13 isolates 11 produced cytotoxin in different quantities. These toxins were enterotoxic in rabbits, lethal for mice and inhibited protein synthesis in HeLa cells. The latter effect was fully neutralized by "Shiga-antitoxin". One of the purified toxins [203] derived from a classical enteropathogenic *E. coli* (EPEC) strain. Beside the "Shiga-analogous" biological activities the purified toxin showed an identical relative heat-resistance (65 °C, 30 min) and identical isoelectric point (7.03 ± 0.02). Analogous to the "Shiga-toxin" it consisted of an A and several copies of B subunit. There was apparently some difference in the molecular weight: the "Shiga-like" toxin of *E. coli* was 48 000 d, while the control "Shiga-toxin" preparation (strain 60R) was 58 000 d.

These findings support the view of O'Brien and LaVeck [203] that a whole "family" of "Shiga-like" toxins exist. Furthermore, it may be assumed that this family has relationships with enterotoxins, as well as with different cytotoxins.

3. Cyto- (Vero-) toxins

In the year 1977 Konowalchuk et al. [204] described a cytotoxin produced by several strains of *E. coli*. This cytotoxin was effective on Vero cells, but not on Yl or CHO. Furthermore, it had no antigenic relationship to LT of *E. coli*,

was heat-labile and its molecular weight was estimated between 10 000 and 30 000 d. After purification [205], the predominant, active molecule was 28 000 d. Giugliano et al. [206] tested different cell lines for sensitivity. The so called Vero-toxin (VT) was effective — beside the Vero cells — also on adult rhesus kidney cells (LLC—MK_2) and on human embryonic HFS cells.

There are assumptions that in the pathomechanism of EPEC some kind of cytotoxins may have some role. According to the data of O'Brien et al. [202, 203] already cited (2.1.) these are "Shiga-like" toxins. According to a generally accepted opinion, today we cannot exclude that the above or other kinds of cytotoxins are involved in the pathomechanism [207]. The occurrence of the production of VT among EPEC strains was investigated by Scotland et al. [208]. Only about 10% of the isolated EPEC strains showed VT production and the presence of VT was associated with some serogroups: namely, all the VT^+ strains belonged to the O26 and 0128 serogroups. According to Caprioli et al. [209], the toxic factor of EPEC, its molecular weight being approximately 70 000—80 000 d, is not identical with VT.

A comparison of VTs of human and porcine *E. coli* strains was reported by Blanco et al. [210]. They could demonstrate some differences in biological activities.

Cytotoxicity among *Aeromonas* strains was also observed [211, 212].

The first report on some heat-labile cytotoxins produced by salmonellae was published in the paper of Mesrobeanu et al. [213]. Otherwise, experiments studying the HeLa (or Henle 407) invasivity of salmonellae showed both the destruction of cells [214] and even the inhibition of protein synthesis [215].

Sonic extracts of a strain of *S. enteritidis* exerted cytotoxic effect, and inhibited protein synthesis in Vero- and isolated intestinal epithelial cells, indicating that cellular damages in salmonella infections are associated with this cytotoxin [216, 217].

Considering data on entero- or "Shiga-like" toxins of shigellae, and findings on cytotoxins of *Escherichia* and *Salmonella* strains, one can easily accept the assumption of Polotsky [218] that all invasive enteric pathogens necessarily produce some kind of cytotoxin. Reports on the cytotoxic alpha-haemolysin of *E. coli* or *Proteeae* have been discussed elsewhere [219].

4. The edema disease principle (EDP) of Escherichia coli

The oedema disease of weaning pigs is caused by *E. coli* belonging to serogroups O138, O139 and O141. The animals show gastric, colonic, palpebral and subcutaneous oedema, and later they may develop paralytic symptoms [135]. The small intestinal content of the diseased pigs or extracts of the above-mentioned *E. coli* cultures, if administered intravenously, elicit a characteristic

oedema [220]. Oral application of these strains produces also a characteristic disease [221].

The supposed toxic factor — the Edema Disease Principle (EDP) — was partially purified by Clugston and Nielsen [222]. They estimated its molecular weight between 50 000 and 100 000 d. According to Kurtz et al. [223] the primary effect of EDP is a generalized vasculitis; others [224] compared it to the "Shiga-toxin" and therefore regarded EDP as an *E. coli* neurotoxin. Buxton and Thomlinson [225] considered EDP as an antigen which gives rise to a hypersensitive reaction, rather than as a toxin.

Since EDP is a heat-labile protein, precipitable by ammonium sulphate, stable in sodium deoxycholate and insoluble in weak acids [222], its exotoxin nature, as well as its being a vascular toxin, has been generally accepted [226]. The observed neurological symptoms may be the consequence of vascular damages and also of hypertension [226]. For a correct answer, experiments with a pure EDP are needed.

5. (Entero-) toxins produced by Clostridia

Clostridia are not regarded to belong to enteric bacteria in a strict sense, but because they are frequently found in the bowel, cause enteric disorders by cytotoxins or enterotoxin-like substances, it is reasonable to give here a short summary.

5.1. *Food poisoning caused by Clostridium perfringens type A*

It was discovered already in 1945 [227] that type A strains of *C. perfringens* can cause food poisoning characterized by diarrhoea and abdominal cramps. An animal model was elaborated by Hausschild et al. [228], who infected lambs orally or intra-intestinally. Ligated intestinal segments of lambs also showed fluid accumulation [229]. The rabbit loop test positivity and its strong correlation with pathogenicity in monkeys and humans was proven by Duncan and Strong [230] using cell free filtrates of *C. perfringens*.

The so called perfringens enterotoxin is produced only during sporulation [231]. When ingested, vegetative cells sporulate in the alkaline intestinal environment [232] and about 10^8 viable cells are sufficient to produce a toxin level to cause symptoms. As the spores survive initial cooking, foods contaminated prior to heating may be vehicles of clostridial food-borne illness.

According to Frieben and Duncan [233] the enterotoxin is a structural component of the spore coat. Its molecular weight is estimated as 35 000 d [234]. Antitoxin response in patients with acute food poisoning was demonstrated [235].

5.2. *Enteritis necroticans caused by Clostridium perfringens type C*

There is an illness with high mortality known as enteritis necroticans caused by type C of *C. perfringens* [236]. It occurred after the second World War in Germany ("Darmbrand" [237]), but it was studied also in Papua New Guinea as the so called "pig-bel" observed in association with "pig-feasting" [238]. Enteritis necroticans is caused by an enterotoxin produced by type C strains of *C. perfringens*. It is biologically and immunologically similar to that of type A strains [239]. The severe lesions are assumed to be caused by the beta-toxin, which is normally inactivated by proteases (primarily by trypsin) of the bowel [236]. The occurrence of "pig-bel" is associated with the feeding habits of Papuas: their daily diet is sweet potato which leads to a hypoproteinaemia and to a pronounced inhibition of trypsin synthesis. The infection happens on the occasional "pig-feasting" due to the consumption of infected meat [240].

It seems that the pathogenicity of *C. perfringens* type C is not restricted to humans. The presence of this agent and its beta-toxin was shown also in pigs suffering from necrotic enteritis [241].

5.3. *Toxins of Clostridium difficile*

During the last years it was clearly elucidated that the so called pseudomembraneous colitis is caused by *C. difficile* [242]. The Syrian hamster, used as a model, responds with ileocaecitis [243]. Toxinogenic *C. difficile* was also found in healthy infants in a high (90%) frequency [244, 245], but less than in 4% of healthy adults [243]. It is noteworthy that the pseudomembraneous enterocolitis seems to be in association with antibiotic therapy: the disease was observed most frequently after administration of ampicillin [246], clindamycin [247], lincomycin [248], and cephalosporins [249]. The nature of this relation is unclear [250].

The toxin production of *C. difficile* was already detected in 1937 [251], i.e. the culture supernatants of strains contained a heat-labile substance and caused oedema, respiratory arrest and death if injected into guinea pigs subcutaneously. It has been found later that two toxins are produced [252]: toxin A is cytotoxic, produces lethal haemorrhagic caecitis in hamsters, causes haemorrhagic fluid accumulation in rabbit ileal loops and in infant mice, gives positive skin permeability reaction [253]. This A toxin, called enterotoxin, was purified by Taylor et al. [254]. Toxin B is a very potent cytotoxin exerting cytopathic effect on hamster fibroblast cells and in human amnion cell cultures, but it causes no fluid accumulation. Minimal doses of toxin B, or "cytotoxin" are lethal for mice [255, 256]. The cytotoxin and not the enterotoxin seems to stimulate the guanylate cyclase activity and inhibits the adenylate cyclase [257].

The A and B toxins are antigenically distinct [258] on the basis of cross-neutralization experiments. Toxin B shows an antigenic relationship with of one of *C. sordellii* [259].

Vaccinating hamsters with formalin toxoid prepared from both toxins showed that both of them were involved in the pathomechanism of ileocaecitis of this model animal and most probably also in human pseudomembraneous colitis [252].

Beside the cultivation of *C. difficile* (which is inconclusive in case of infants) the tissue culture assay is a valuable test in the diagnosis. Enzyme immunoassay seems to be an easy, specific and very sensitive method [260].

6. Conclusions and remarks

It was not the purpose of this review to discuss the problem of the endotoxin of Gram-negative bacteria and its role in the pathomechanism of bacterial enteric diseases. This does not mean the underestimation of the significance of endotoxin, which is still far from being a fully elucidated, closed field of research. This problem cannot be discussed in sufficient detail in a chapter of a review like this, but should be the subject of an entire separate and voluminous article.

According to our present knowledge the endotoxin does not exert its effect in the bowel, unless it is liberated in various tissues and body fluids. The liberation requires the disruption of bacteria. The multiple effect resulting in clinical symptoms depends in a large extent on the quantity, the dynamics and the localization of the release of endotoxin.

Similarly, the present review does not deal with some virulence factors, e.g. haemolysins, which may also exert toxic effects (see the preceeding review of the author [219]).

Summarizing the outlines of data concerning enterotoxins, first of all their widespread occurrence among enteric pathogens and opportunistic pathogens should be emphasized. This does not mean that the role of enterotoxins in the pathomechanism is equally important: sometimes they play an essential and sometimes an additive part and we cannot prove or exclude the possibility that during evolution this character has had an influence on the pathogenicity of the organism.

The transposon nature of the ST_A gene, which has been undoubtedly proven, seems to be true at least in evolutional sense also for LT. This is not only an answer to the observed spreading, but also a significant example of the special way of evolution in prokaryotes, acquiring already "tested" genes in a short way by vectors (plasmids, phages).

It is not easy to produce clear cut categories for enterotoxins, "Shiga" and "Shiga-like" toxins or cytotoxins. Some kinds of overlapping features can

be observed and hypothetically they seem to form a common family of gene products, diverged earlier or later during the evolution.

In the cases where the above toxins are playing a primary role in the pathomechanism, e.g. in *V. cholerae* or ETEC, the role and significance of toxins seems to be better elucidated. A promising field of research is provided by diseases, e.g. salmonellosis and shigellosis where the toxin may or may not have an additive role.

Without a better understanding of clostridial toxins and their genes, it is as yet questionable whether these toxins, which are biologically similar to those of Gram-negatives, have originated from a common ancestor gene or are results of a parallel evolution.

Generally speaking, the new field opened by the discovery of toxins produced by enteric pathogens may also influence the future of therapy by developing pharmacons with inhibitory ability, as well as elaborating specific prevention, at least in some diseases.

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LIPID CONTENT AND ESR DETERMINATION OF PLASMA MEMBRANE ORDER PARAMETER IN *CANDIDA ALBICANS* STEROL MUTANTS

M. Pesti, L. Horváth, L. Vígh and T. Farkas

Department of Microbiology, József Attila University, Szeged, and Institute of Biophysics and Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged

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Sterol intermediates of ergosterol biosynthesis in seven sterol mutants of *Candida albicans* were determined by gas-liquid chromatography. Only one of them could synthetize ergosterol, while in the others sterol biosynthesis was blocked beyond zymosterol. Alterations in sterol composition were correlated with a slight increase in saturation and a decrease in the chain length of fatty acids, and increases in phosphatidylinositol and phosphatidic acid, and decreases in phosphatidylcholine and phosphatidylserine contents. During exponential growth, as measured on their protoplasts using the intercalated fatty acid spin probe, 5-doxylstearic acid, these single mutants exhibited higher plasma membrane order parameters than their ergosterol-producing parental strain, designated *33 erg*$^+$, as follows: *erg-12* > *erg-16* > *erg-37* > > *erg-2* > *erg-20* > *erg-40* > *erg-41* > *33 erg*$^+$. The mutants displayed significantly higher phase-transition temperatures, measured in a reconstituted lipid–water dispersion, than their parental strain.

The plasma membrane is a multicomponent system and plays an important role in a number of cellular processes. One of its constituents in yeasts is ergosterol, the end-product of sterol biosynthesis. Some aspects of its importance and function were discussed previously [1].

Study of the sterol mutants of the unicellular, petite-negative, human pathogenic eukaryotic microbe, the yeast *Candida albicans*, is a possibility to learn more about the role of ergosterol and its precursors in the plasma membrane processes [2]. It was shown in these experiments that, compared to the ergosterol-producing parental strain, an ergosterol-less mutant of *C. albicans* displayed no alteration of its plasma membrane ultrastructure as revealed by freeze etch electron microscopy [3], but it had an increased activity [4] of membrane-bound chitin synthetase (EC 2.4.1.16) and an increased spontaneous ion leakage [5].

We have especially been interested to learn the background of the observed phenomena, what kind of intermediates of ergosterol are accumulated in these mutants, and how the alterations influence the composition of other lipids. The ESR method is one of the best to obtain direct information about

Miklós Pesti
Research Institute, Seed Production and Trading Co.
H-6601 Szentes, P.O.B. 41, Hungary

László Horváth, László Vigh, Tibor Farkas
Biological Research Center, Hungarian Academy of Sciences
H-6701 Szeged, P.O.B. 521, Hungary

the plasma membrane fluidity alterations of sterol mutants. Thus in this paper we also report ESR order parameter data on the plasma membrane of the ergosterol-producing strain of *C. albicans* and its sterol mutants, as a knowledge of the complex alterations in the lipid composition of sterol mutants [5] does not permit a direct prognosis of the probable change in plasma membrane fluidity.

Materials and methods

Strains. Sterol mutants of *C. albicans erg-2*, *erg-16* and *erg-37* originated from the adenine-requiring ergosterol-producing strain *33 erg*$^+$, while mutants of *erg-12*, *erg-20*, *erg-40* and *erg-41* were obtained from the threonine- and methionine-requiring ergosterol competent strain *35 erg*$^+$ [5]. Three of these have been deposited in the American Type Culture Collection, Rockville: ATCC 44829 *(33 erg*$^+$*)*, ATCC 44830 *(erg-16)* and ATCC 44831 *(erg-2)*.

Culture conditions and protoplast formation. Media, maintenance of strains and culture conditions for lipid analysis and protoplast formation have been described earlier [4].

Lipid analysis. Extraction of lipids and determination of fatty acids by gas-liquid chromatography (GLC) was carried out as described earlier [6]. Quantitative determination of the individual phospholipids such as phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) was achieved by two-dimensional thin-layer chromatographic separation [7].

For GLC analysis of the non-saponifiable sterol extracts, the cells were treated with n-heptane [5]. Extracts were evaporated and acetylated with acetic anhydride–pyridine (5 : 1) or silanized with Sylon BT 2—3—3029 (Supelco Inc.) when necessary. Extracts were dissolved in acetone or benzene and analysed in a JEOL JGC-20K (FP) gas chromatograph. Quantitation was performed using a Packard Integrator (Model 603) connected to the GLC. The carrier gas was argon with 60 ml/min rate for all columns: (i) 10% SE-30 on a Gas Chrom Q (80–100 mesh) 5-foot glass column at 265 °C; (ii) 3% OV-17 on a Diatomile CLQ (100–120 mesh) 3-foot glass column at 271 °C; (iii) 10% on a Super Coport 1-2140 (80–100 mesh) 5-foot glass column at 300 °C. Relative retention times (RTTs) of sterols were calculated relative to cholestanol acetate (OV-17), cholestane (SE-30) and cholesterol (SP-2100).

Reproducibility in RTT was OV-17 $\pm 1\%$, SE-30 $\pm 2\%$, SP-2100 $\pm 1.5\%$. The RTTs of *Candida* sterols were compared with those of authentic components from yeast mutants obtained from Dr. A. M. Pierce (Simon Fraser University, Canada) and Dr. D. N. Kirk (University of London, U. K.) and with the gas chromatography data of Bard et al. [8].

Preparation of samples for spin labelling experiments. The protoplast suspension (10^9 cells/ml) was diluted five-fold in 1 M mannitol prior to the addition of spin probe. A 4 μl aliquot of a 5 mg/ml ethanol stock solution of 5-doxylstearic acid was then added to 1 ml cell suspension and the mixture was gently shaken to facilitate spin probe incorporation.

Finally, to remove ethanol the protoplast suspension was sedimented at low speed (1000 *g*, 5 min) and resuspended in 100 μl final volume. Ethanol-mediated spin probe incorporation has the advantage that it can be controlled very conveniently and quantitatively. Under the above conditions, magnetically resoluted spectra were obtained, i.e. no isotropic triplet due to unincorporated spin probes was observed [9].

Electron spin resonance measurements. Electron spin resonance (ESR) spectra were recorded with a JES-PE-1X JEOL (Japan) spectrometer. The protoplast suspension was added to the micro flat cell (Scanlon, USA) and thermostated at 22 °C. The order parameter was calculated from the splitting of the inner ($2A_{\min}$) and outer ($2A_{\max} = 2A_{||}$) extremes,

$$S = \frac{A_{||} - A}{26.3\text{G}}$$

where A was determined from the inner splitting ($2A_{\min}$) by Gaffney's method as described in [10]. The phase transition of total lipid extracts was measured in lipid–water dispersion. Total lipids were hydrated with 0.1 M NaCl + 5 mM $CaCl_2$ (pH 7).

Results

Lipid composition. The representative sterol mutants examined were chosen from 23 mutants of different origin on the basis of the ultraviolet absorption spectra and thin-layer chromatographic patterns of their sterol extracts [5]. The representatives originated from two ergosterol-producing strains having different auxotrophic markers [5], but they gave exactly the same GLC spectrum and so only *33 erg*$^+$ was studied further. RTT data on the applied sterol standards are listed in Table I. The RTTs of sterol components on OV-17, SE-30 and PS-2100 are given in Table II, while the relative amounts of identified sterols of *C. albicans* strains and their presumed enzyme defects are to be seen in Table III. All mutants with one exception contained zymosterol in significant amounts. Considering the main pathway in ergosterol synthesis and the data accumulated up to this time [1, 2, 8, 11] the tabulated data mean, (i) the *erg-12* strain accumulated cholesta-7,24-dien-3β-ol and ergosterol, which may be a consequence of a leaky mutation of 5,6-dehydrogenase; (ii) the *erg-2* and *erg-40* mutants accumulated Δ^8-sterols, indicating a block of $\Delta^8 - \Delta^7$ isomerase; (iii) the *erg-20* and *erg-37* mutants contained one of the last products of ergosterol biosynthesis, ergosta-7-en-3β-ol, suggesting the inactivation of 5,6-dehydrogenase or 22,23-dehydrogenase or both; (iv) the *erg-16* and *erg-41* mutants accumulated ergosta-7,22-dien-3β-ol, which presumably resulted from an enzymatic block of 5,6-dehydrogenase. For mutants deficient in the same enzyme, different amounts of accumulated intermediates of the same type could be detected.

Table I

Nomenclature of applied sterol standards and their relative retention times in separations on chromatographic columns of OV-17, SE-30 and PS-2100

Nomenclature			Relative retention times		
chemical name	trivial name	common name	OV-17	SE-30	PS-2100
		squalene	0.88		0.52
		lanosterol			1.52
Cholesta-8,24,-dien-3β-ol	$\Delta_C^{8,24}$	zymosterol	3.90	2.05	1.13
Ergosta-5,7,22-trien-3β-ol	$\Delta_E^{5,7,22}$	ergosterol	4.20	2.15	1.16
Ergosta-5,7-dien-3β-ol	$\Delta_E^{5,7}$		4.38		
Ergosta-7,24/28/-dien-3β-ol	$\Delta_E^{7,24(28)}$	episterol	4.59		
Ergosta-8,24/28/-dien-3β-ol	$\Delta_E^{8,24(28)}$	fecosterol	4.90		
Ergosta-7-en-3β-ol	Δ_E^{7}		5.04		
Cholesta-7,24-dien-3β-ol	$\Delta_C^{7,24}$				1.51
Ergosta-7,22-dien-3β-ol	$\Delta_E^{7,22}$				1.63

Table II

Relative retention times of sterols of Candida albicans strains in separations on various chromatographic columns

Column	Strain	Relative retention times					
OV-17	33 erg+	1.62	3.22	4.20	5.05		
	erg-2	3.90	4.90				
	erg-12	1.63	4.19				
	erg-16	0.77	1.82	3.91	4.39	4.59	5.04
	erg-20	0.76	3.90	4.59	5.05		
	erg-37	3.89	4.59	4.91			
	erg-40	3.89	4.92				
	erg-41	0.88	3.91	4.60	5.04		
SE-30	33 erg+	1.28	1.55	2.15	2.85		
	erg-2	2.06	2.54				
	erg-12	1.29	2.15				
	erg-16	2.05	2.33				
	erg-20	2.05	2.33				
	erg-37	2.05	2.33	2.53			
	erg-40	2.05	2.52				
	erg-41	2.04	2.33	2.86			
PS-2100	33 erg+	0.76	1.16	1.34	1.51		
	erg-2	1.12	1.18				
	erg-12	0.36	0.76	1.16	1.51		
	erg-16	1.16	1.19	2.09			
	erg-20	1.12	1.18				
	erg-37	1.13	1.29				
	erg-40	1.13	1.53				
	erg-41	0.51	1.12	1.29	2.08		

The fatty acid compositions of the total lipid extracts of the *C. albicans* strains are given in Table IV. The general trend was a slight decrease in the sterol mutants as concerns both the average fatty acid chain length and the relative proportion of unsaturated acids. Despite some variations, the levels of hexadecatrienoic (16 : 3), stearic (18 : 0) and gamma-linolenic (18 : 3) acids increased at the expense of oleic (18 : 1) acid. These changes were reflected in the major phospholipids. PC, PE and PI accounted for more than 80% of the total phospholipids in *33 erg*$^+$ (Table V). The tendency was that in the sterol mutants PC and PS decreased, while PI and PA increased significantly compared with *33 erg*$^+$.

Fluidity measurement. The order parameter (S) results for the examined strains in the exponential growth phase, measured on protoplasts (Table VI). Lower values of S represent simply less ordered membranes, and vice versa. The parental *33 erg*$^+$ strain had the most fluid membrane. The decreasing order of the parameter was *erg-12* > *erg-16* > *erg-37* > *erg-2* > *erg-20* > *erg-40* > > *erg-41* > *33 erg*$^+$.

Table III

Relative amounts of identified sterols (in per cent) in C. albicans strains, and their proposed enzymatic block

Strain	Squalene	Lanosterol	Zymosterol	Ergosterol	Episterol	Fecosterol	Ergosta-7-en-3β-ol	Ergosta-7,22-dien-3β-ol	Cholesta-7,24-dien-3β-ol	Ergosta-5,7-dien-3β-ol	Unknown	Enzyme defect
33 erg$^+$				71.5			14.3	tr	3.0		11.2	none
erg-2		1.7	42.7			55.6						$\Delta^8 - \Delta^7$ isomerase
erg-12				64.9					29.8		5.3	leaky
erg-16			24.7		36.2		13.8	25.3		tr		5,6-dehydrogenase
erg-20			42.6		49.3		6.1				2.0	22,23-dehydrogenase
erg-37			60.8		32.0		7.2					22,23-dehydrogenase
erg-40		2.1	44.1			53.8						$\Delta^8 - \Delta^7$ isomerase
erg-41	2.3		33.1		32.1		14.0	18.5				5,6-dehydrogenase

tr = trace (0.1–1.5%)

Table IV

Fatty acid compositions (per cent by wt) of total lipid extracts of C. albicans 33 erg+ strain and its sterol mutants

Strain	Percentage of total								Unsat./ Sat.
	16 : 0	16 : 1	16 : 2	16 : 3	18 : 0	18 : 1	18 : 2	18 : 3	
33 erg+	19.1	10.5	0.9	2.2	4.3	35.0	25.7	2.3	3.24
erg-2	19.1	9.4	1.7	3.7	6.0	35.7	20.2	4.2	2.98
erg-12	16.1	12.6	1.2	6.3	8.1	26.5	24.2	5.0	3.13
erg-16	18.0	10.8	1.3	3.7	4.1	40.9	19.3	1.9	3.52
erg-20	20.6	13.8	0.8	4.5	7.7	25.5	21.2	5.9	2.53
erg-37	19.0	11.2	2.1	6.2	7.6	22.6	27.8	3.5	2.76
erg-40	20.1	9.9	1.0	2.8	8.6	17.4	32.8	7.4	2.50
erg-41	15.3	12.7	1.6	5.9	6.1	25.7	25.3	7.4	3.67

Table V

Phospholipid compositions of lipids extracted from the ergosterol-producing parental Candida albicans strain 33 erg+ and its sterol mutants

Strain	Percentage of total				
	PC	PE	PI	PS	PA
33 erg+	53.9 ± 5.9	16.1 ± 2.8	10.6 ± 3.9	13.2 ± 2.5	6.2 ± 2.0
erg-2	33.2 ± 5.0	20.7 ± 2.9	25.3 ± 4.0	5.7 ± 0.9	15.3 ± 5.9
erg-12	34.9 ± 1.3	22.1 ± 3.2	16.1 ± 0.6	8.8 ± 2.5	18.1 ± 2.7
erg-16	48.0 ± 5.7	14.1 ± 1.3	23.3 ± 2.7	4.3 ± 0.9	10.3 ± 2.9
erg-20	36.7 ± 4.3	19.1 ± 3.7	14.3 ± 5.9	10.3 ± 6.0	19.6 ± 5.4
erg-37	44.1 ± 2.5	14.4 ± 1.8	21.7 ± 0.5	10.2 ± 3.6	9.6 ± 5.6
erg-40	40.4 ± 6.1	31.5 ± 2.5	11.9 ± 0.9	6.4 ± 1.2	9.8 ± 2.7
erg-41	40.4 ± 4.9	14.6 ± 4.9	17.2 ± 6.8	11.7 ± 6.1	16.1 ± 7.5

± Standard deviation

Table VI

Membrane order parameters (S) of the ergosterol-producing C. albicans strain 33 erg+ and its sterol mutants

Strain	S	
33 erg+	0.611 ± 0.003	(4)
erg-2	0.640 ± 0.005	(5)
erg-12	0.677 ± 0.008	(4)
erg-16	0.671 ± 0.010	(3)
erg-20	0.635 ± 0.013	(4)
erg-37	0.666 ± 0.013	(5)
erg-40	0.627 ± 0.005	(5)
erg-41	0.613 ± 0.003	(5)

Data are means ±S.D. Numbers of experiments in parentheses. Error in measurement: ±0.003

The temperature-dependence of the order parameter of 5-doxylstearic acid-labelled yeast lipid dispersions is seen in Fig. 1. The phase-transition temperatures of the plasma membrane lipids are not higher than 15 °C, and all these order parameter values therefore indicate fluid-phase properties. The

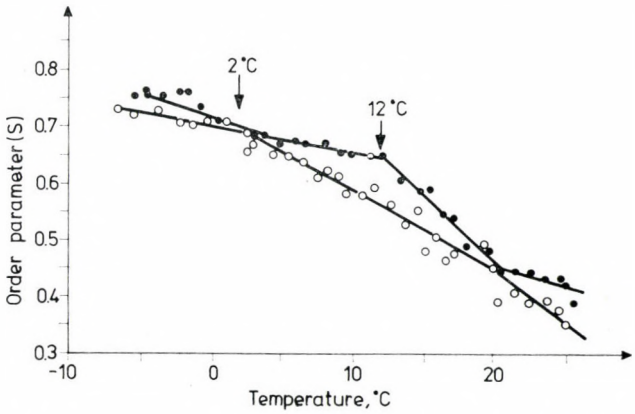

Fig. 1. Phase-transition temperature of *C. albicans 33 erg*$^+$ (○——○) and *erg-40* (●——●) strains measured on reconstituted lipid extract

order parameter vs. temperature plot exhibited a break at the phase-transition temperature (T_M) [12]. Another break could be seen at 20 °C for both the ergosterol-less and the ergosterol-producing strains pointing to a further phase transition. Hexagonal structures which may be produced by phospholipids and sterols in the plasma membrane could contribute to the existence of this phenomenon. For the four representative strains tested, the following lower, and probably characteristic T_M values were obtained: *33 erg*$^+$ 2 °C; *erg-2* 10 °C; *erg-16* 8 °C; and *erg-40* 12 °C.

Discussion

One of the most important function of the large amount of sterols in biological membranes is to rigidify their structure in the liquid crystalline phase and to maintain their permeability. The microviscosity index of cholesterol in the human erythrocyte phospholipid mixture is 12.1, while the values for the individual phospholipids range only from 0.5 to 3.4 at 37 °C [13]; thus the mixing ratio basically determines the fluidity of membranes. In wild-type strains of yeasts, ergosterol is the main sterol; the sterols accumulated in the cells can attain 0.15% of the dry weight. In sterol mutants containing different ergosterol intermediates this value can be as high as 0.72% [14].

Qualitative and quantitative alterations in sterol composition may result in at least two effects. It has been suggested that the quantity of sterols in cells itself cannot be responsible for the functional changes of the plasma membrane [15]. At the same time, very little information has emerged about the structural function and importance of various ergosterol intermediates.

It is a fact that, in the examined sterol mutants of *C. albicans*, zymosterol and precursors following zymosterol in the ergosterol biosynthesis accumulated in significant amount instead of ergosterol, with one exception *(erg-12)*. These alterations seemed to induce an adaptation in phospholipid composition, resulting in decreased amounts of PC and PS (described as fluidizing lipids by Shinitzky and Henkart [13]) and in increased amounts of PI, PE and PA (considered to be rigidifying lipids).

The consequence of these complex alterations in the lipid composition of the mutant cell must be reflected at the plasma membrane level, and could be evaluated by measurement of the plasma membrane order parameter. All sterol mutants exhibited a higher plasma membrane rigidity than that of their parental strain, similarly as in *Saccharomyces cerevisiae* [15]. Surprisingly, *erg-12*, an ergosterol-producing mutant, had the highest order parameter, certainly as a consequence of $\Delta^{7,24}$ sterol accumulation and the elevated levels of PI, PE and PA phospholipids. A direct correlation between the type of accumulated sterols and the alterations in other lipid components could not be demonstrated in these experiments. However, a comparison of the plasma membrane order parameter values with the phase-transition points strongly suggested that a lower fluid-phase order indicated a lower phase-transition point (Fig. 1).

The higher plasma membrane order parameter values, the altered enzymatic activity of membrane-bound chitin synthetase (EC 2.4.1.16) [4], the increased spontaneous ion leakage (measured conductometrically in distilled water) and the significantly reduced cell permeability to glycerol [5] of the sterol mutants compared to those for the parental strain showed that a decrease in, or the lack of ergosterol, in the mutant cell brings about a complex alteration in the amounts of other lipid components. In spite of this compensatory mechanism, the living mutant cells cannot regain their original plasma membrane function and low rigidity.

To feel the complexity of the situation one may consider that if the block of ergosterol biosynthesis would result in the missing of any sterol in the plasma membrane, it would lead to significant fluidity increase. As in reality a relevant decrease i.e. higher rigidity was detected, it refers to the fact that one or more intermediates or precursors of ergosterol biosynthesis were incorporated into the membrane endowing it with a higher level of packing. Model results seem to support this idea as ergosterol precursors incorporated into artificial membranes really increased the order parameter [10]. The case seems to

be more complicated with the mutant *erg-12* as it has the highest ergosterol content in contrast to its two-fold nystatin resistance and the highest order parameter. Thus the question arises about the cytological localization of the ergosterol and about the nature, amount, and ratio of its substituent(s) in the plasma membrane.

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CHARACTERIZATION OF R-PLASMIDS CODING FOR AMPICILLIN RESISTANCE IN *SALMONELLA TYPHI-MURIUM*

S. NIKOLNIKOV

National Institute of Hygiene, Budapest

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Eight *Salmonella typhi-murium* strains coding resistance to ampicillin were chosen from 38 strains isolated in different counties of Hungary in 1981, and their plasmids were characterized by agarose gel electrophoresis. Incompatibility groups and molecular weights of transferable R-plasmids coding resistance to ampicillin were determined and restriction enzyme analyses were done. The studies showed that among R-plasmids coding for ampicillin resistance in *S. typhi-murium* strains IncIα group plasmids with a molecular weight of 66 Mdal were dominant in Hungary. According to their cleavage patterns, the examined plasmids formed two groups. Both types contained some fragments identical in size, and they are supposed to be connected evolutionarily.

Timoney [1] reported that twelve percent of the *Salmonella typhi-murium* strains of animal origin examined in New York State during the period 1973–1976 were sensitive to ampicillin, tetracycline, kanamycin, neomycin, chloramphenicol and streptomycin. Resistance to ampicillin and chloramphenicol was the most frequent. Cherubin et al. [2] showed that the proportion of ampicillin-resistant *S. typhi-murium* strains increased significantly in 1971–1978.

Investigating *S. typhi-murium* strains isolated in New Zealand in 1980, Anderson [3] showed that the resistance determinant to ampicillin was mediated by extrachromosomal genetic elements, the plasmids. The plasmids carrying ampicillin resistance genes have been characterized by molecular weight.

According to Milch et al. [4] the proportion of ampicillin-resistant *S. typhi-murium* strains was found to be constantly low in Hungary, not higher than 2% of the examined strains (in 1979 — 18 strains, 0.9%; in 1980 — 33 strains, 1.2%; in 1981 — 38 strains, 1.6%).

In the present study eight isolates were chosen from 38 ampicillin-resistant *S. typhi-murium* strains isolated during 1981 and their plasmids were characterized for incompatibility, phage restriction, fertility inhibition and molecular weight. The purified plasmid DNAs were digested by restriction enzymes and restriction endonuclease cleavage patterns were compared.

SERGEY NIKOLNIKOV
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Materials and methods

Strains. Selected *S. typhi-murium* strains were isolated from patients by Public Health Station laboratories in Hungary in 1981, and were sent to the Department of Phage Research of the National Institute of Hygiene for determination of phage sensitivity.

Determination of the biochemical and phage types. Phage typing of *S. typhi-murium* was performed according to the international method of Felix–Callow [5], the examined *S. typhi-murium* strains were phage typed by Anderson's phage series [6]. Biochemical types were determined on the basis of reactions in rhamnose and inositol [7].

Antibiotic sensitivity was examined with Resistest disks (Institute for Serobacteriological Production and Research Human, Budapest) to tetracycline, kanamycin, chloramphenicol, streptomycin, neomycin, ampicillin, gentamicin, carbenicillin, azlocillin and doxycycline.

Transfer of plasmids from wild strains to recipient *Escherichia coli* J5—3 [8] was carried out by conjugation [9]. For the selection of transconjugants ampicillin was used.

Media, determination of fi character and phage restriction were described previously [4].

Incompatibility test. The incompatibility of plasmids was examined according to Grant et al. [10] with the modification described by Bird and Pittard [11]. Datta's and Chabbert's reference plasmids were used to examine the incompatibility of plasmids [12].

Isolation of plasmid DNA. Large scale plasmid DNA preparations by the polyethylene glycol precipitation method of Humphreys et al. [13] were followed by ethidium bromide–CsCl density gradient centrifugation. The small scale method was used to prepare DNA as described by Birnboim and Doly [14].

Restriction enzyme digestion. Restriction enzymes *EcoRI*, *Bgl*II and *Xba*I were obtained from the Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged. Restriction enzyme digestions of plasmid DNAs were performed in buffers as recommended by New England Biolabs [15].

Agarose gel electrophoresis and estimation of molecular weight. Agarose gels were prepared as described by Maniatis et al. [16]. Standard plasmids [12] in molecular weight determination were as follows: S-a (IncW) 23 Mdal, RN3 (IncN) 33 Mdal, Rl (IncFII) 62 Mdal, RAl (IncA) 86 Mdal.

Results

Results of phage- and biotyping. The strains were isolated in different parts of the country (Table I). Biotypes 2 and 3 were represented by four strains each. Biotype 2 and biotype 3 strains were either untypable or fell according to the Felix–Callow scheme into phage type 2b. With type phages of Anderson, two strains belonged to phage type U135, and four strains to U133. One strain was non-typable, and a further one could not be determined.

Antibiotic resistance and transferable determinants of antibiotic resistance. All the strains chosen contained markers of antibiotic resistance against ampicillin, carbenicillin and streptomycin. In addition, three strains had markers of kanamycin resistance, one strain markers of azlocillin resistance, while the strain designated 477 had resistance markers against chloramphenicol, tetracycline and doxycycline.

In most cases only the markers of ampicillin and carbenicillin could be transferred by conjugation (Table I). In three strains the transfer of kanamycin resistance marker was also possible. In the case of strain 477, markers of tetracycline resistance appeared in the transconjugant. Transfer was carried out without mobilization.

Characterization of plasmids. In each transconjugant we identified a plasmid. Their molecular weight was 66 Mdal by gel elecrophoresis. The plasmids proved to be incompatible with strains R64 (IncIα), R144 (IncIα), R66a (IncIω) and were classified into incompatibility group Iα. All the eight plas-

Table I

Characterization of S. typhi-murium strains carrying R-plasmids

Designation of strains	Place of origin (county or district of Budapest)	Biotype	Phage type according to		Resistance pattern*	Resistance pattern transferred to *E. coli* K12
			Felix–Callow	Anderson		
44	Békés	3	nt**	U135	Sm, Ap, Cb, Km	Ap, Cb, Km
63	Heves	2	nt	U133	Sm, Ap, Cb	Ap, Cb
395	Pest	3	nt	U133	Sm, Ap, Cb	Ap, Cb
477	Budapest XIII	3	nt	nt	Sm, Ap, Cb, Km, Cm, Tc, Do	Ap, Cb, Tc, Do, Km
659	Budapest IX	2	nt	U133	Sm, Ap, Cb, Km	Ap, Cb, Km
1384	Veszprém	3	nt	U135	Sm, Ap, Cb, Azl	Ap, Cb, Azl
1527	Vas	2	2b	nd***	Sm, Ap, Cb	Ap, Cb
1586	Veszprém	2	2b	U133	Sm, Ap, Cb	Ap, Cb

* Abbreviations: Ap = ampicillin, Cb = carbenicillin, Tc = tetracycline, Do = doxycycline, Azl = azlocillin, Km = kanamycin, Sm = streptomycin, Cm = chloramphenicol

** Non-typable

*** Not determined

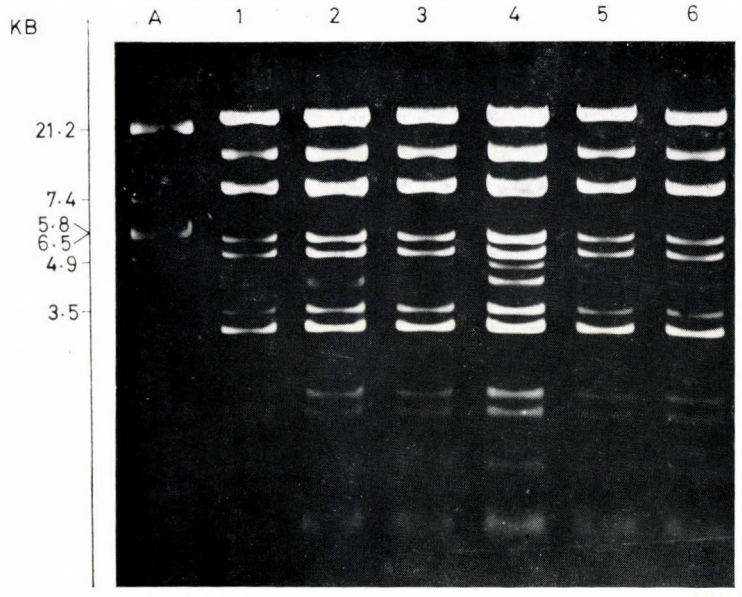

Fig. 1. Agarose gel electrophoresis of *Eco*RI fragments of R-plasmid DNA from *S. typhi-murium* belonging to incompatibility group Iα. A = λ DNA digested by *Eco*RI (size marker in KB). Plasmid DNA digestions: 1 = 1384, 2 = 395, 3 = 1586, 4 = 1527, 5 = 44, 6 = 63

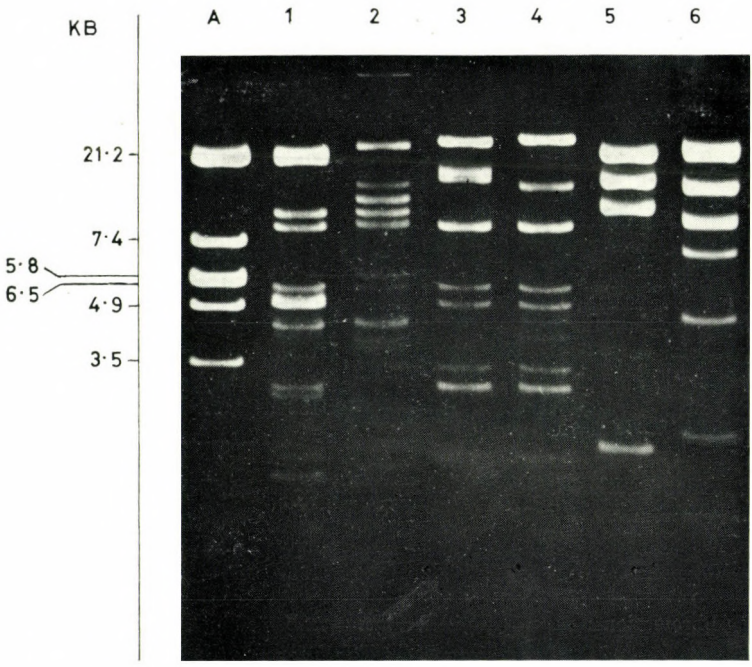

Fig. 2. Digestion of 477 and 659 designated plasmid DNAs in IncIα group by *Bgl*II, *Eco*RI and *Xba*I. A = λDNA digested by *Eco*RI (size marker in KB). 1 and 2 = 477 and 659 plasmid DNAs digested by *Bgl*II; 3 and 4 = 477 and 659 plasmid DNAs digested by *Eco*RI; 5 and 6 = 477 and 659 plasmid DNAs digested by *Xba*I

mids were fi^-. The plasmid designated 477 had an inhibitory effect for phages T4, F4, F5, 23, 24, Ø, while other plasmids had no such effect.

In the case of seven plasmids, fully identical plasmid patterns were found after *Eco*RI digestion (Fig. 1.: 1–6 lanes, Fig. 2.: 4 lanes). The pattern of the plasmid designated 477 (Fig. 2.: lanes 1, 3, 5) did not prove identical with other plasmids in incompatibility group Iα; a close relationship could, however, be seen on the basis of the fragments similarly mobile to those of plasmid DNA on gel patterns after digestion by restriction endonucleases *Eco*RI and *Bgl*II.

Discussion

The examined strains of *S. typhi-murium* which were non-typable according to the scheme of Felix-Callow, or belonged to phage type 2b, were classified along with the phage type of Anderson to phage types U133 and U135. According to Threlfall et al. [17, 18] in England *S. typhi-murium* strains belonging to incompatibility group Iα and carrying plasmids with resistance determinants of ampicillin, kanamycin and streptomycin belonged to phage types U193 and U204, the most frequent ones in Europe.

R-plasmids of *S. typhi-murium* containing resistance determinants of kanamycin and azlocillin beside those of ampicillin and carbenicillin, and which, in the plasmid designated 477, also contained resistance determinants of tetracycline and doxycycline, belonged to incompatibility group Iα, and were isolated with a frequency increasing year by year as compared with other incompatibility groups [19–21].

Examining plasmids of incompatibility group Iα, Bird and Pittard [22] found them to be incompatible also with an Iγ plasmid, and so they evaluated them as non-typical I plasmids. The plasmids examined by us have shown incompatibility also with a plasmid of the IncIω group, so they belong to non-typical Iα plasmids.

All the eight plasmids were fi^- and had a molecular weight of 66 Mdal. The molecular weights of the plasmids belonging to the I plasmid complex known from the literature were similar in value [12].

In the case of seven plasmids, completely identical patterns were found after *Eco*RI digestion, which indicates their common origin. DNA restriction patterns of the plasmid designated 477 were different from those of other IncIα plasmids, but they contained some fragments of identical size.

Chabbert et al. [23] had plasmid DNAs of different Iα incompatibility groups digested by *Eco*RI restriction enzyme and characterized them by agarose gel electrophoresis. The cleavage pattern of DNA shown by their plasmids was similar to those in our examinations after *Eco*RI digestion, still there was no full identity to be established (different number of fragments).

The analysis of plasmids shows that those of the examined Iα incompatibility groups are of common origin, and it is to be assumed that these plasmids are highly frequent in *S. typhi-murium* strains in Hungary.

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PHAGE TYPES AND EPIDEMIOLOGICAL SIGNIFICANCE OF *SALMONELLA ENTERITIDIS* STRAINS IN HUNGARY BETWEEN 1976 AND 1983

VERA G. LÁSZLÓ, ERZSÉBET SZ. CSÓRIÁN and JUDIT PÁSZTI

National Institute of Hygiene, Budapest, and Pest County Public Health Station, Budapest

(Received May 24, 1984

In Hungary, 14 819 human *Salmonella enteritidis* strains were isolated between 1976 and 1983. Phage type was determined of 10 132 human strains originating from 6852 foci, and of 711 strains isolated from animals and water in this period. The human strains were typable in 99.4% and they belonged into 21 phage types. Five phage types (1, 4, 7, 16 and 17) were more frequent than 1%. Phage type 7 predominated among the strains isolated between 1976 and 1980, including 65.6%–89.3% of the strains. There was a change in the prevalence of phage types from 1980–1981, as phage type 7 was ousted by phage type 1. The date of the change in the predominance of phage types coincided with the considerable increase of *S. enteritidis* isolates; the number of isolates was nearly fivefold in 1980 of that in 1976. Phage type 7 frequent in the first period proved to be homogeneous; the strains could not be subdivided either by the temperate phages carried by them or by other phages. The incidence of phage types 1 and 7 was nearly the same among the strains derived from animals, food, water and hygienic control examinations, and there was no temporal difference in the frequency of the two phage types as it was observed among the human strains. The human strains originated in 49.5% from outbreaks and in 50.5% from sporadic cases in the country. Of the strains examined for phage type during the eight-year period, 41.9% were isolated from 23 field epidemics, 84 community outbreaks and 757 family infections. Analysing the regional spread of *S. enteritidis*, the increase in the number of isolates was the highest in counties Tolna, Bács-Kiskun, Somogy and Győr-Sopron. The predominance of phage type 1 was observed in counties Békés, Borsod, Csongrád, Győr-Sopron, Hajdu-Bihar, Pest and Tolna. It was obvious in the case of county Tolna that the source of infection was contaminated egg and baby chicken. Phage type 7 predominated in counties Komárom, Vas and Veszprém. Phage type 4 circulated in counties Csongrád and Pest, phage type 17 in county Fejér and phage type 2 in county Hajdú-Bihar.

Because of the increasing number of infections caused by *Salmonella enteritidis* it was desirable to subdivide this serotype by phages. The first scheme of *S. enteritidis* phage typing was proposed by Lilleengen in 1950 [1]. Using four phage preparations, 16 strains were tested and 8 different lytic reactions were differentiated. Anderson [2] worked out a system which was applied only by him. In Poland, Macierewicz et al. [3] elaborated a phage-typing system, which was modified on several occasions. In the first version by means of 7 phage preparations 11 lytic reactions were distinguished by testing 605 strains. In the second version 25 phage types were determined by the addition of five phages. Though the method had epidemiological advantages, it has given some

VERA G. LÁSZLÓ, ERZSÉBET SZ. CSÓRIÁN
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

JUDIT PÁSZTI
Pest County Public Health Station
Váradi u. 37–41, H-1032 Budapest, Hungary

unrepeatable typing reactions and therefore a third version was worked out, which consisted of 9 phages (six phages from the former set and three new ones) and allowed the distinction of 20 phage types [4]. As the lytic reactions of preparations I and I/6585 were homogeneous, the latter was withdrawn from the set [5]. Gershman published a phage typing method to subdivide *S. enteritidis* strains [6]. The examined 183 strains were subdivided into 17 phage types using 8 phages isolated from sewage. It was supposed that the number of phage types will increase after the examination of further strains. The characteristic patterns of the phage types observed were reproducible and the cultures isolated from a common outbreak belonged to identical types. Some of the phages affected other, serologically different salmonellae. On the basis of this fact a single phage typing set, consisting of 50 phages was developed to differentiate 58 serovars belonging to serogroups A, B, C1, C2, D, E1, E2, E3 and E4 [7]. The method was modified and by the help of 27 phages [8] 1245 cultures belonging to serogroups A, B, C1, D1, D2, E1, E2, E3, G1, K, N were typed.

The present paper gives an account of the phage typing results carried out between 1976 and 1983.

Materials and methods

Bacterial strains. Phage type was determined of a total of 10 843 *S. enteritidis* strains (10 132 human and 711 strains of animal and water origin) isolated in Hungary between 1976 and 1983, and 31 cultures isolated in the German Democratic Republic.

Propagating strains and type phages. The modified method of Lalko and Macierewicz was used [4]. The type phages and propagating strains are listed in Table I. Phages 1, 2, 3, 4 and 7 (according to the Hungarian designation) were isolated from *Salmonella paratyphi-B* strains, phages 5, 6 and 8 from *S. enteritidis* strains. These were adapted to the propagating strains listed in Table I. The propagating strain of phage 1 was a *Salmonella typhi-murium* strain, the other seven propagating strains were *S. enteritidis* strains.

Table I

S. enteritidis type-phages and propagating strains

Type phages		Propagating strains
Hungarian	Polish	
1	IV/8956	8956
2	III/10/7530	9396
3	III	885
4	I	6585
5	VI	11/K
6	VII	2106
7	IV	99/M
8	V	64/M

Propagation of phages was carried out by the "cutting out" method, starting from one single plaque. To obtain bacteria-free phage preparation G5 glass filter was used. Phages were used in RTD (routine test dilution) determined by titration of the phage on the propagating strain and on the phage type standard strains.

Determination of phage types. The 2 h broth culture of the strain to be typed was poured over an agar plate, the excess being drawn off. After the culture had dried, type phages in routine test dilution (RTD) were spotted on the plate, incubated at 37 °C overnight and the lytic pattern was read and phage type was given according to the phage-typing scheme.

Phage typing scheme is given in Table II, which shows the lytic patterns and both the Hungarian and Polish phage types because there are differences between them. The set which we received consisted of 17 standard strains, therefore the Hungarian scheme omitted phage types 9, 11 and 19 (Polish phage type designation) and in 9 cases the phage types differed. Five new phage types were added (1a, 2a, 3a, 4a, 10d). Table II shows the designation of standard strains, too.

Induction of lysogenic strains by mitomycin C. Mitomycin C (10 μg/ml) was added in 0.1 ml quantity to 1.9 ml of the 2 h broth culture of the lysogenic strain (final concentration, 0.5 μg/ml) and after 30 min it was centrifuged (3000 rpm, 20 min); to the sediment 2 ml prewarmed broth was added and incubated at 37 °C for 3 h. After centrifugation the supernatant was dropped on the surface of an agar plate which was poured over with the broth culture of the indicator strain and incubated overnight.

Media. Oxoid nutrient broth No. 2 and agar were used.

Table II

Phage typing scheme for S. enteritidis

Phage type		Type-phages								Phage type standard strains
Hungarian	Polish	1	2	3	4	5	6	7	8	
1	1	SCL	OL	OL	SCL	SCL	SCL	SCL	SCL	7115
2	2	SCL	SCL	OL	—	SCL	SCL	SCL	SCL	9396
3	3	SCL	—	—	SCL	SCL	SCL	SCL	SCL	9381
4	4	—	SCL	CL	SCL	SCL	SCL	SCL	SCL	7068
5	5	—	SCL	SCL	—	SCL	SCL	SCL	SCL	7761
6	6	—	—	CL	SCL	SCL	SCL	SCL	SCL	885
7	7	—	—	—	SCL	SCL	SCL	CL	CL	253/81
8	8	—	±	±	+++	+	SCL	+	+++	64M
9	10	—	—	±	—	SCL	SCL	SCL	SCL	4296
10	12	SCL	—	—	—	+++	—	SCL	SCL	4106
11	13	—	++	SCL	—	±	SCL	+	SCL	10562
12	14	—	+++	++	—	—	—	—	SCL	9510
13	15	—	+++	++	—	±	—	±	—	7530
14	16	—	—	—	—	SCL	—	++	—	11/K
15	17	—	—	±	—	—	SCL	±	± (++)	2106
16	18	—	—	—	—	—	—	SCL	+++	2480
17	20	—	—	—	—	—	—	±	SCL	1122
1a	—	SCL	OL	SCL	SCL	SCL	SCL	SCL	—	214/83
2a	—	SCL	SCL	SCL	—	SCL	SCL	SCL	—	1254/81
3a	—	SCL	—	—	SCL	SCL	SCL	SCL	—	1368/83
4a	—	—	SCL	SCL	SCL	SCL	SCL	SCL	—	484/83
10d	—	SCL	—	—	SCL	—	—	SCL	SCL	514/83

Results

Phage type distribution of human strains. Phage type was determined of a total of 10132 *S. enteritidis* strains isolated from 9826 persons and from 6852 foci, in the years 1976 to 1983. The strains were isolated from patients and asymptomatic excretors. Table III shows the distribution of phage types in percentages. The strains and foci were typable in 99.4% and 99.2%, respectively, and 21 phage types were encountered. Non-characteristic types were found among the strains and foci in 0.2% and 0.3%, respectively. In the period of examination five phage types, i.e. 1, 4, 7, 16 and 17 occurred more frequently than 1%. In certain years there was an increase in the incidence of some of the phage types, e.g. phage type 1a in 1977, 1982 and 1983, phage type 2 in 1981,

Table III

Phage type distribution of human
(Hungary, 1976–1983)

Phage tyoe	1976		1977		1978		1979	
	S	F	S	F	S	F	S	F
1	5.1	6.3	5.5	7.0	13.8	14.9	18.6	18.8
1a			1.0	1.4				
2								
2a								
3							0.3	0.4
3a								
4	7.1	1.9	1.8	2.0	7.1	2.2	13.1	2.0
4a	1.0	1.2						
5							0.1	0.2
6			0.3	0.3	0.2	0.3		
7	81.8	84.4	89.3	86.7	75.3	77.7	65.6	75.6
8							0.1	0.2
9								
10					0.2	0.3	0.3	0.4
11								
12								
13								
14	1.0	1.2	0.3	0.3	0.2	0.3		
15								
16	0.5	0.6	0.3	0.3	0.4	0.5		
17	1.5	1.9	0.3	0.3	1.2	1.6	1.2	1.5
Not characteristic			0.5	0.7	0.6	0.8	0.1	0.2
Not typable	2.0	2.5	0.7	1.0	1.0	1.4	0.6	0.7
Total number of strains	198	160	384	299	481	363	733	537

S = strain
F = focus

phage types 4a and 14 in 1976. In 1977 the strains and foci belonged to phage type 7 in 81.8% and 89.3%, respectively. In 1980–1981 there was a change in the predominance of phage types, the incidence of phage type 7 strains and foci decreased to 56.9% and 31.3%, respectively. The decreasing tendency continued and in 1983 phage type 7 strains were found only in 23.8%. The frequency of phage type 1 was 5.1% in 1976, it increased to 33.8% and 46.2%, respectively, in the period of the change in phage-types in 1980 and 1981, and it was as high as 66.5% in 1983. The incidence of phage type 17 was higher than 5% in 1980; it reached a maximum of 13.2% in 1981 and decreased in the next years.

Phage type 4 occurred among the foci in 1.9%–2.2%, among the strains in 1.8%–13.1%, between 1976 and 1979. The difference between the values was due to the outbreaks caused by strains of phage type 4.

S. enteritidis strains and foci, in percentage

1980		1981		1982		1983		Total	
S	F	S	F	S	F	S	F	S	F
33.8	33.6	46.2	41.9	63.1	59.2	66.5	58.1	47.0	41.8
0.9	0.7	0.2	0.2	1.1	1.7	0.9	1.5	0.6	0.8
0.1	0.1	2.5	2.7	0.6	0.8	0.2	0.5	0.5	0.8
0.2	0.2	0.1	0.1					0.1	0.1
0.5	0.7	0.5	0.6	0.3	0.4	0.2	0.3	0.3	0.4
						0.2	0.4	0.1	0.1
0.7	0.9	0.4	0.6	0.9	1.4	1.7	2.5	2.2	1.5
						0.1	0.1	0.1	0.1
				0.1	0.1			0.1	0.1
		0.3	0.3	0.2	0.2	0.2	0.3	0.1	0.2
56.9	55.0	31.3	33.7	26.7	26.6	23.8	27.7	40.6	43.9
		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
						0.1	0.1	0.1	0.1
				0.1	0.1	0.2	0.1	0.1	0.1
0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.3	0.1	0.1
						0.1	0.1	0.1	0.1
						0.1	0.1	0.1	0.1
								0.1	0.1
				0.1	0.2	0.1	0.1	0.1	0.1
0.9	1.3	4.0	4.4	0.5	0.8	0.4	0.5	1.1	1.4
5.1	6.2	13.2	14.0	5.3	7.1	4.1	6.0	5.6	6.9
0.3	0.5	0.4	0.4	0.1	0.2	0.2	0.4	0.2	0.3
0.4	0.6	0.7	0.9	0.7	1.0	0.6	0.8	0.6	0.8
1774	1228	1768	1388	2158	1347	2636	1530	10 132	6852

The frequency of foci of phage type 16 was 1.3% and 4.4% in 1980–1981, and was less than 1% in the other years. Figure 1 shows the percentage distribution of the frequent phage types according to strains and foci, and the change in predominance of phage types; phage type 7 was ousted by phage type 1.

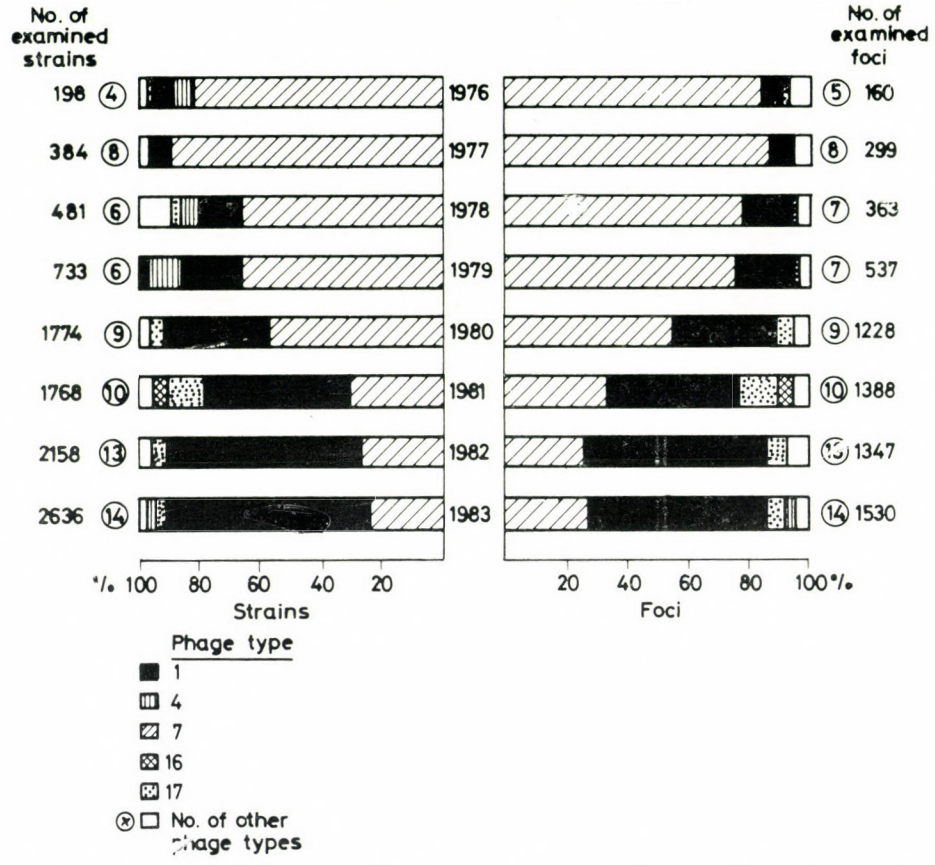

Fig. 1. Distribution of frequent phage tipes of human *S. enteritidis* strains and foci (1976—1983)

Examination of the lysogenic state of strains of phage type 7. Because of the high incidence of phage type 7 among the human strains examined in 1976 and 1977 (81.8% and 89.3%, respectively), attempts were made to subdivide this phage type. The lysogenic state and the lytic spectra of the carried phages were examined by testing 60 strains of phage type 7 isolated from different areas of the country (from counties Bács-Kiskun, Komárom, Nógrád, Pest, Szolnok, Vas, Veszprém and from Budapest), in 1976 and 1977. As inducer, mitomycin C and as indicator strains the phage type strandard strains and wild type strains of phage types 1, 4, 6, 7 and 17 were used. The lytic spectra

of the temperate phages liberated from the strains of phage type 7 were identical, only the strains of phage type 1 were lysed by them.

Sensitivity to temperate phages of phage type 7 strains. The strains examined for lysogenic state were tested for sensitivity to temperate phages liberated from phage type standard strains by mitomycin C induction. Strong lysis (+++ or SCL) was displayed by the temperate phages carried by strains of phage types 2, 6, 11, 14 and 17, and weak lysis by the phages carried by strains of phage types 5, 9, 13, 15 and 16. The lytic patterns of every strain tested were identical. It was not possible to subdivide the strains of phage type 7 either by the temperate phages carried by them or by the temperate phages derived from strains belonging to other phage types.

Phage type distribution of strains of animal and water origin. Phage type was determined of a total of 711 strains isolated from animals, food products, food samples from food borne outbreaks, hygienic control examinations and from water, between 1976 and 1983. Table IV shows the phage type distribution of the strains. The strains derived from beef belonged to phage type 7 in 63% and to phage type 1 in 26%. Among the poultry strains, phage type 1 was the most frequent, the occurrence of phage type 7 was high and phage types

Table IV

Phage type distribution of S. enteritidis strains of non-human origin
(Hungary, 1976–1983)

Phage type	Total No. of strains	Beef cattle	Poultry	Egg	Other animals	Food sample	Hygienic control examination	Water
1	313	14	103	36	10	81	61	8
1a	13		2		10		1	
2	4					4		
3	7		2				3	2
3a	2							2
4	28			2		13	10	3
6	1							1
7	281	34	92	44	4	36	51	20
8	1					1		
10	6		3				3	
13	1		1					
14	1							1
16	13	3	6				4	
17	30	3	18	1		3	5	
Not characteristic	8		3		1			4
Not typable	2				1			1
Total	711	54	230	83	26	138	138	42

17 and 16 were also present. The incidence of types among the strains isolated from eggs was inversed, phage type 7 being more frequent than phage type 1. Column "other animals" involved the 10 strains of phage type 1a isolated from wild-ducks, the other strains were isolated from pheasants, dogs, cats, horses and guinea pigs. Among the strains obtained from food samples, phage type 1 was the most frequent, followed by phage types 7 and 4. The strains derived from hygienic control examinations belonged mainly to phage types 1, 7 and 4. Half of the strains originating from well water, bath water, surface water and sewage, belonged to phage type 7 and 6 other phage types were found; the phage pattern of 4 strains was not characteristic, and one strain was not typable.

Phage typing of 31 strains isolated in the GDR in 1981 and 1982. Table V shows the phage type distribution of the strains. The strains were derived

Table V

Phage type distribution of S. enteritidis strains isolated in the German Democratic Republic

Origin of strains	Phage type			Total
	4	6	7	
Human	11		12	23
Broiler chicken	3			3
Water		3	2	5

Table VI

Distribution of S. enteritidis isolations according to origin (1976–1983)*

Year	Epidemics				Sporadic cases				Total		
	Total No.	Patients	Excretors	P/E	Total No.	Patients	Excretors	P/E	No. of isolations	E/S	P/E
1976	240	146	94	1.5 : 1	361	257	104	2.5 : 1	601	0.6 : 1	2.0 : 1
1977	292	176	116	1.5 : 1	407	305	102	2.9 : 1	699	0.7 : 1	2.2 : 1
1978	226	138	88	1.6 : 1	548	394	154	2.6 : 1	774	0.4 : 1	2.2 : 1
1979	601	435	166	2.6 : 1	703	484	219	2.2 : 1	1304	0.8 : 1	2.4 : 1
1980	1651	775	876	0.8 : 1	1228	887	341	2.6 : 1	2879	1.3 : 1	1.4 : 1
1981	1032	721	311	2.3 : 1	1208	948	260	3.6 : 1	2240	0.8 : 1	2.9 : 1
1982	1462	884	578	1.5 : 1	1366	1012	354	2.9 : 1	2828	1.1 : 1	2.0 : 1
1983	1824	1260	564	2.2 : 1	1670	1251	419	3.0 : 1	3494	1.1 : 1	2.6 : 1
Total	7328	4535	2793	1.6 : 1	7491	5538	1953	2.8 : 1	14 819	0.9 : 1	2.1 : 1

* On the basis of reports of the Public Health Stations
E/S = No. of epidemic cases per one sporadic case
P/E = No. of patients per one excretor

from 3 outbreaks, sporadic human cases, broiler chicken and water. In two outbreaks, strains of phage type 4 were found; in one of them the infection was spread by chicken. The third outbreak was caused by strains of phage type 7. Phage type 4 was demonstrated among the strains isolated from broiler chicken, phage types 6 and 7 from surface water.

Analysis of epidemics caused by S. enteritidis. Out of 14 819 *S. enteritidis* strains isolated in Hungary in 1976–1983. 49.5% originated from epidemic outbreaks and 50.5% from sporadic cases. From 1980 onward, the ratio of isolations from epidemics increased significantly (Table VI). The ratio of isolations from patients and asymptomatic excretors was on the average 2.1 : 1 in 1981 and almost 3 : 1 in 1983. There was a significant difference as to the patient/excretor ratio between the epidemic and sporadic cases.

During the period of examination phage type was determined of 4232 strains originating from 23 field epidemics, 84 community outbreaks and 757 family outbreaks. Table VII presents the geographic distribution of phage types of the examined strains isolated from field epidemics. The majority was caused by strains of phage type 1. The outbreak in Tolna county was of great importance; it lasted for five months, 32 localities were involved, 453 patients and 224 asymptomatic excretors were diagnosed. The pathogenic agent was spread through contaminated eggs and baby chicken delivered from the poultry breeder in Dunaszentgyörgy. The spread of infection showed in time and space a

Table VII

Geographic distribution of S. enteritidis phage types derived from field epidemics (1976–1983)

Location (county or Budapest)	Total No. of		Phage types					
			1		4		7	
	epidemics	strains	No. of epidemics	No. of strains	No. of epidemics	No. of strains	No. of epidemics	No. of strains
Baranya	1	7	1	7				
Bács-Kiskún	1	10	1	10				
Borsod-A.-Z.	1	22					1	22
Csongrád	2	110	1	25	1	85		
Győr-Sopron	6	151	4	61			2	90
Hajdú-Bihar	1	24	1	24				
Komárom	1	19					1	19
Nógrád	1	52	1	52				
Szabolcs-Sz.	1	19					1	19
Tolna	2	436	2	436				
Vas	4	43	2	26			2	17
Veszprém	1	64					1	64
Budapest	1	110					1	110
Total	23	1067	13	641	1	85	9	341

parallelism with the regional delivery of baby chicken. *S. enteritidis* of phage type 1 was isolated uniformly from the stools of patients and from the equipment of the brooder.

In county Csongrád widespread outbreaks were caused by strains of phage type 4. The infection was conveyed by contaminated ice-creams. The strains isolated from ice-cream samples, from the confectioner and from the patients were uniformly of phage type 4.

The outbreak which took place in Budapest in 1980 affected 26 children communities [9], 206 patients and 474 asymptomatic excretors. The infection was spread by spiced butter-cream and spiced sheep cheese with butter made by the same caterer. The pathogenic agent was *S. enteritidis* of phage type 7.

It was reported simultaneously from six areas of the country in 1983 that members of different tourist groups were taken ill with enteric symptoms. The similar aetiology proved by phage typing has drawn attention to a connection betweea the cases. Every tourist group passed through the town Győr

Table VIII

Geographic distribution of S. enteritidis phage types derived from community outbreaks (1976–1983)

Location (county or Budapest)	Total No. of		Phage type 1		4		7		17		Other
			No. of		No. of		No. of		No. of		
	out-breaks	strains	out-breaks	strains	out-breaks	strains	out-breaks	strains	out-breaks	strains	No. of strains
Baranya	2	23	1	8			1	15			
Bács-Kiskún	1	3					1	3			
Békés	2	7	1	4			1	3			
Borsod-A.-Z.	2	40	1	15			1	25			
Csongrád	6	44	5	35			1	9			
Fejér	5	29	2	11					3	17	1
Győr-Sopron	12	257	8	92			4	165			
Hajdú-Bihar	1	27	1	27							
Heves	3	27	1	5			2	22			
Komárom	3	43	1	16			2	25			2
Nógrád	4	104	1	53			3	46			5
Pest	6	201	4	166	2	35					
Szabolcs-Sz.	3	26	2	17			1	9			
Szolnok	1	12	1	12							
Tolna	5	64	2	39			3	25			
Vas	5	57					5	57			
Veszprém	1	5					1	5			
Zala	1	19	1	19							
Budapest	21	223	11	140			9	76	1	7	
Total	84	1211	43	659	2	35	35	485	4	24	8

and consumed food with mayonnaise or tartare sauce at the same restaurant. The strains isolated from the patients belonged to phage type 7. The infectious agent reached the restaurant with eggs, because strains of phage type 7 were isolated also from eggs stored in the kitchen.

Table VIII shows the phage distribution of strains isolated from community outbreaks; of these, 43 were caused by strains of phage type 1, 35 by phage type 7. In two outbreaks in county Pest strains of phage type 4, in county Fejér three outbreaks and in Budapest in one outbreak strains of phage type 17 were the pathogenic agent. In children communities 37 and in weddings 12 outbreaks occurred. Out of 12 outbreaks in 10 the food was contaminated with strains of phage type 1 and in 2 cases the phage type was 7.

The number of cases was high in the kindergarten-outbreaks in Győr in 1980, which were caused by strains of phage type 7 and in a wedding in county Pest, where the strains belonged to phage type 1. The phage type distribution

Table IX

Geographic distribution of S. enteritidis phage types derived from family outbreaks (1976–1983)

Location (county or Budapest)	Total No. of		Phage type									
			1		4		7		17		Other	
			No. of		No. of		No. of		No. of		No. of	
	out-breaks	strains	out-breaks	strains	out-breaks	strains	out-breaks	strains	out-breaks	strains	out-breaks	strains
Baranya	7	15	3	7			4	8				
Bács-Kiskún	5	13	1	4			2	5	2	4		
Békés	16	42	14	37			2	4				1
Borsod-A.-Z.	46	121	29	65	1	2	11	44	1	2	4	8
Csongrád	28	67	23	54			4	11	1	2		
Fejér	40	104	13	37			13	29	14	38		
Győr-Sopron	116	307	105	282			8	19	2	4	1	2
Hajdú-Bihar	45	111	21	54	4	9	13	33			7	15
Heves	49	124	26	67			17	43	5	11	1	3
Komárom	6	13	1	2	1	2	4	9				
Nógrád	18	38	10	21			5	11			3	6
Pest	42	123	24	67	2	5	10	30	2	9	4	12
Somogy	13	32	4	12			9	20				
Szabolcs-Sz.	26	72	13	31			12	39			1	2
Szolnok	26	66	17	38	1	2	5	16	3	10		
Tolna	77	185	62	151			14	30	1	3		1
Vas	42	112	10	22			30	86	2	4		
Veszprém	32	100	6	19			21	69	3	7	2	5
Zala	18	49	9	28			8	19			1	2
Budapest	105	260	56	137	1	2	42	105	5	12	1	4
Total	757	1954	447	1135	10	22	234	630	41	106	25	61

Table X

Percentage distribution of phage types of S. enteritidis (1976–1983)

Location (county or Budapest)	Total No. of strains		Phage type 1		Phage type 4	
	O	S	O	S	O	S
Baranya	45	33	64.7	35.3	—	100.0
Bács-Kiskún	26	68	63.6	36.4	—	—
Békés	49	111	35.7	64.3	—	100.0
Borsod-A.-Z.	185	301	34.8	65.2	9.1	90.9
Csongrád	221	211	48.7	51.3	94.4	5.6
Fejér	134	332	42.5	57.5	—	100.0
Győr-Sopron	717	595	50.8	49.2	—	100.0
Hajdú-Bihar	159	314	38.1	61.9	52.9	47.1
Heves	151	209	43.1	56.9	—	100.0
Komárom	76	191	51.4	48.6	50.0	50.0
Nógrád	196	157	72.4	27.6	—	100.0
Pest	326	345	61.2	38.8	90.9	9.1
Somogy	32	88	35.3	64.7	—	100.0
Szabolcs-Sz.	117	243	35.8	64.2	—	100.0
Szolnok	78	154	40.7	59.3	50.0	50.0
Tolna	685	266	86.8	13.2	—	100.0
Vas	212	316	41.0	59.0	—	100.0
Veszprém	174	438	14.5	85.5	—	100.0
Zala	70	171	48.5	51.5	—	—
Budapest	596	1342	35.5	64.5	8.3	91.7
Total			51.1	48.9	60.7	39.3
Number of strains	4249	5883	2432	2334	142	92

O = strains isolated from outbreaks

of the strains derived from family outbreaks are summarized in Table IX. A total of 1954 strains isolated from 757 families were phage-typed. The majority originating from family outbreaks were isolated in county Győr-Sopron and in Budapest, and also in county Tolna. Phage type 1 was predominant in family outbreaks in county Győr; phage types 1, 7 and 17 were frequent in Budapest, and the widespread field epidemic which took place in county Tolna in 1983, was followed by the spread of phage type 1.

Table X presents the phage type distribution of the strains isolated from epidemics and sporadic cases. The examined 10 132 *S. enteritidis* strains were derived in 41.9% from epidemics and in 58.1% from sporadic cases. In Budapest the strains were isolated from single cases in 70% and phage type 7 was the predominant one. In county Győr-Sopron the strains were derived in 54.7% from outbreaks and the prevalence of phage type 1 was demonstrated. In county

strains derived from outbreaks and sporadic cases

7		16		17		Other	
O	S	O	S	O	S	O	S
57.5	42.5	—	—	—	—	—	100.0
17.0	83.0	—	100.0	22.2	77.8	—	100.0
25.9	74.1	—	100.0	—	—	8.3	91.7
48.7	51.3	50.0	50.0	16.7	83.3	25.8	74.2
22.5	77.5	—	—	22.2	77.8	—	100.0
21.8	78.2	10.0	90.0	26.6	73.4	100.0	—
70.6	29.4	13.3	86.7	20.0	80.0	7.1	92.9
28.4	71.6	—	100.0	—	100.0	22.1	77.9
42.2	57.8	—	100.0	52.4	47.6	23.1	76.9
25.1	74.9	33.3	66.7	—	100.0	33.3	66.7
36.5	63.5	66.7	33.3	—	100.0	84.6	15.4
17.3	82.7	33.3	66.7	25.0	75.0	38.7	61.3
25.0	75.0	—	—	—	100.0	—	100.0
34.5	65.5	—	100.0	—	100.0	28.6	71.4
20.5	79.5	—	100.0	55.6	44.4	—	100.0
27.5	72.5	—	100.0	30.0	70.0	5.5	94.5
42.4	57.6	—	100.0	30.8	69.2	—	100.0
32.7	67.3	31.3	68.7	20.6	79.4	100.0	—
16.4	83.6	33.3	66.7	—	100.0	28.6	71.4
31.4	68.6	15.8	84.2	14.1	85.9	7.4	92.6
35.4	64.6	17.7	82.3	22.9	77.1	20.5	79.5
1456	2658	20	93	130	439	69	267

S = strains isolated from sporadic cases

Tolna the strains had originated from epidemics in 72.0% and here phage type 1 predominated. In county Pest, phage types 7, 16, 17 and other types were predominant among the sporadic cases, in outbreaks phage types 1 and 4 were the frequent types. In Veszprém and Vas the majority of the strains of phage types 1, 4, 7, 16, and 17 were isolated from sporadic cases.

The incidence of *S. enteritidis* strains varied significantly in the geographic areas of the country. The yearly average ratio of isolation for 100 000 inhabitants was examined parallel with the predominance of phage types 1 and 7 (Table XI). Because phage typing of *S. enteritidis* strains started in 1976, the data were summarized from 1976 onwards. In the period of the predominance of phage type 7, between 1976 and 1980, the average of strains was 11.7 for 100 000 inhabitants and in the period of the prevalence of phage type 1, between 1981 and 1983, this value had doubled. During the two periods, the

frequency of isolates increased in every area, except in county Csongrád, where a moderate decrease appeared. The most striking was the increase in counties Bács-Kiskun and Tolna, where an eight-fold increase was observed, in county Somogy a seven-fold, in county Győr-Sopron a six-fold increase was demonstrated in the yearly average of isolations. In contrast, in Budapest, the yearly average of isolations remained unchanged and the increase was moderate in counties Békés and Komárom.

Regional distribution of phage types showed the predominance of phage type 1 in the counties Békés, Borsod, Csongrád, Győr-Sopron, Hajdu, Pest, Szolnok and Tolna. The prevalence of phage type 7 was observed in counties Komárom, Somogy, Vas and Veszprém. Phage type 4 was frequent in counties Csongrád and Pest, phage type 17 accumulated besides Budapest also in county Fejér. Phage type 16 was found besides Budapest in counties Veszprém, Győr-Sopron, Szabolcs-Szatmár and Fejér, mainly in sporadic cases. As to other phage types, phage type 2 appeared almost only in county Hajdú-Bihar.

Table XI

*Geographic distribution of S. enteritidis isolations**

Location (county or Budapest)	No. of isolations					5 year average for 100 000 inhabitants	No. of isolations			3 year average for 100 000 inhabitants
	1976	1977	1978	1979	1980		1981	1982	1983	
Baranya	18	40	20	22	65	7.6	107	58	192	27.4
Bács-Kiskún	7	3	5	15	40	2.5	61	29	195	16.8
Békés	64	50	88	60	59	14.8	34	111	67	16.4
Borsod-A.-Z.	45	9	54	35	74	5.4	116	77	191	15.9
Csongrád	53	47	129	273	219	31.4	59	169	87	23.1
Fejér	48	13	16	56	54	8.9	139	138	98	29.6
Győr-Sopron	12	12	40	38	222	15.1	365	476	270	86.1
Hajdú-Bihar	11	11	13	64	113	7.7	102	130	109	20.6
Heves	3	—	4	54	94	8.9	55	52	85	18.4
Komárom	43	27	110	247	64	30.7	73	147	131	36.2
Nógrád	6	52	7	18	113	16.5	128	33	52	29.7
Pest	20	31	20	24	150	5.1	100	211	260	19.4
Somogy	4	1	6	13	40	3.6	24	99	107	21.4
Szabolcs-Sz.	12	32	2	13	133	6.6	110	145	156	23.3
Szolnok	35	16	15	52	120	10.7	95	171	123	29.2
Tolna	14	24	27	56	73	14.8	60	44	828	115.9
Vas	34	24	44	53	170	23.0	209	165	93	54.6
Veszprém	44	24	47	51	82	12.0	102	180	78	30.9
Zala	12	10	8	14	117	8.1	31	80	35	15.4
Budapest	116	273	119	146	887	14.8	270	313	337	14.9
Total	601	699	774	1304	2879	11.7	2240	2828	3494	26.7

* Data of the National Salmonella Centre

Discussion

The occurrence and epidemiological significance of salmonellae increased throughout the world and also in Hungary. The National Salmonella Centre collecting the data within the scope of the International Salmonella Surveillance Programme gave an account on the salmonella isolations in the years 1972–1976 [10]. The yearly incidence of the human *Salmonella* and *S. enteritidis* isolates in Hungary between 1974 and 1983 is shown in Fig. 2, on the basis of data of the National Salmonella Centre. (In the number of salmonellae, *S. typhi*, *S. paratyphi-A*, *S. paratyphi-B*, *S. paratyphi-C* are not included and one isolation means one isolation per person for a given serotype.) During the period of examination, salmonellae were isolated from a total of 94 494 persons and *S. enteritidis* from 15 977 persons. The incidence of this serotype increased significantly from 1980 onwards, the number of isolates was more than five-times higher in 1983 than it was in 1974. Figure 2 shows the incidence of animal strains, too. (The data were obtained from the Central Laboratory of Veterinary Hygiene.) The number of salmonella isolates of animal origin was 31 765; it varied between 2798 and 3647 yearly and from 1978 showed an increasing tendency. The number of *S. enteritidis* isolates was 662.

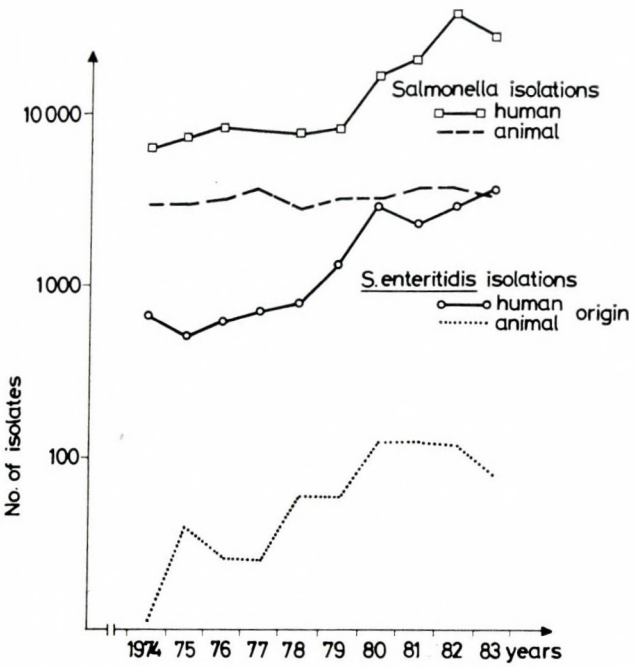

Fig. 2. Salmonella and *S. enteritidis* isolations of human and animal origin (1974—1983)

Table XII shows the order of frequency for the 10 commonest human salmonella serotypes. *S. typhi-murium* was the most frequent in each year, except in the years 1980, 1982 and 1983, when it fell to the 2nd and 3rd rank, respectively, while the 1st rank was occupied by *S. enteritidis* which earlier was the 2nd to 4th frequent serotype. The rank of *S. panama* varied between 2nd and 4th for 9 years, *S. saint-paul* ranked among the 5 commonest serotypes for 6 years, *S. infantis* belonged to the 5 most frequent serotypes since 1977. There were serotypes which were frequent only in single years, causing widespread field outbreaks (*S. give* in 1981, *S. stanley* in 1974, *S. abony* in 1978, *S. agona* in 1975, *S. derby* in 1975, 1976 and 1977, and *S. hadar* in 1980).

The common *Salmonella* serotypes among the strains of animal origin are shown in Table XIII. *S. typhi-murium* was the most frequent among the isolates of animal origin, too, it ranked in the first two places but the frequency of other serotypes differed from the human ones. *S. cholerae-suis*, *S. derby*

Table XII

The ten most frequently reported human Salmonella serotypes in Hungary (1974–1983)*

Rank	Serotype	Year/rank									
		1974	1975	1976	1977	1978	1979	1980	1981	1982	1983
1	*S. typhi-murium*	1	1	1	1	1	1	2	1	3	3
2	*S. enteritidis*	2	4	2	3	3	2	1	2	1	1
3	*S. panama*	3	6	3	2	4	3	4	4	4	4
4	*S. saint-paul*	4	5	4	7	7	5	7	5	5	6
5	*S. stanley*	5	7								
6	*S. anatum*	6			8	8		8	10	10	
7	*S. derby*	7	2	5	4	6	6	9	8	8	7
8	*S. thompson*	8	10	6			7	6		7	
9	*S. infantis*	9	8	9	5	2	4	3	3	2	2
10	*S. abony*	10			10	5					
11	*S. agona*		3	7							10
12	*S. manhattan*		9			10					9
13	*S. london*			8			9		9		
14	*S. brandenburg*			10	6						
15	*S. give*				9				6		
16	*S. bareilly*					9					
17	*S. bovis-morbificans*						8	10	7		5
18	*S. hadar*						10	5			
19	*S. oranienburg*									6	
20	*S. heidelberg*									9	
21	*S. gold-coast*										8
Total No. of salmonella isolations		6152	7250	8165	7913	7580	7995	11190	11845	13704	12700

* Data of the National Salmonella Centre

and *S. anatum* were the most common till 1980, and from 1978 onwards, *S. infantis*. The rank of *S. enteritidis* was 29th to 13th between 1974 and 1979, and was among the 10 most common from 1980 onward. The rank of *S. panama* was 11th, 12th and 11th in 1975, 1976 and 1979, respectively, and in the other years it was among the 10 most frequent serotypes.

According to the WHO reports, *S. enteritidis* has a great significance worldwide in human salmonellosis. The yearly data of the countries participating in the International Salmonella Surveillance Programme showed that the epidemiological importance of this serotype was the most significant in Europe. In recent years this serotype was the most frequent in Roumania with 20–30% of the yearly isolations. Since 1980 it became the most frequent

Table XIII

The 10 most frequently reported Salmonella serotypes of animal origin in Hungary (1974–1983)*

Rank	Serotype	Year/rank									
		1974	1975	1976	1977	1978	1979	1980	1981	1982	1983
1	*S. cholerae-suis*	1	3	3	4	4	5	5			
2	*S. typhi-murium*	2	1	1	2	2	2	1	2	2	2
3	*S. anatum*	3	4	4	3	3	3	7	4	3	4
4	*S. derby*	4	2	2	1	1	1	3	3	4	3
5	*S. senftenberg*	5									
6	*S. agona*	6	6								10
7	*S. potsdam*	7									
8	*S. panama*	8			7	9		4	5	10	5
9	*S. infantis*	9	7	7	8	5	4	2	1	1	1
10	*S. stanley*	10									
11	*S. london*		5				7		9		
12	*S. manhattan*		8			10					
13	*S. bareilly*		9								
14	*S. thompson*		10	5	6	7	6		7	5	7
15	*S. give*			6	5	6	8	10		7	6
16	*S. saint-paul*			8				9	6	6	
17	*S. brandenburg*			9	9	8	10	6		8	
18	*S. livingstone*			10							
19	*S. kapemba*				10						
20	*S. hadar*						9				
21	*S. enteritidis*							8	8	9	
22	*S. gold-coast*								10		
23	*S. heidelberg*										8
24	*S. bovis-morbificans*										9
Total No. of salmonella isolations		2826	2871	3102	3571	2798	3057	3122	3647	3592	3179

* Data of the Central Laboratory of Veterinary Hygiene

one in Bulgaria and in Jugoslavia, with nearly one third of the total isolates yearly. It occupied the second rank in Norway, Spain and Sweden in recent years. It was among the 3 commonest serotypes varying yearly in Austria, Denmark, Finland, and Poland [11]. The number of salmonellosis was 29 334–41 166 in GFR in 1977–1979 [12]. *S. typhi-murium* was the most frequent, *S. panama* was the second, *S. enteritidis* the third and *S. infantis* the fourth in frequency. *S. enteritidis* was the second serotype in the USA, according to a survey carried out in 1975 [13], and among the ten most common serotypes in Canada in 1975–1976 [14].

The increasing number of infections caused by *S. enteritidis* made it necessary to introduce the differentiation of this serotype by phages. The method elaborated in Poland was based on numerous epidemiological data and proved to be applicable. The method reported by Gershman [6] was used for the differentiation of 183 strains but their epidemiological analysis has not been published. Therefore, after certain modifications the Polish method was introduced in Hungary. Determination of the phage types of 10 874 human and animal strains showed that the method was suitable for epidemiological purposes. Phage types proved to be stable in the course of repeated examinations and examination of outbreaks and several strains from one and the same persons. The results of phage typing revealed the connections between the cases of outbreaks, proved or precluded the possibility of the suspected source in development of the epidemics [15].

Analysing the results of phage typing, it has been found that phage type 7 predominated among the human strains isolated between 1976 and 1980. On the basis of the same lytic spectra of the temperate phages carried by strains of phage type 7 and the same phage sensitivity of these strains to temperate phages, it was suggested that the strains of phage type 7, spread in the country, were homogeneous. Phage type 7 was ousted by phage type 1 from 1981 onwards and the number of isolates increased significantly. The time when the change in the prevalence of phage types occurred coincided with the significant increase of *S. enteritidis* isolates (Figs 1 and 2). Analysing the distribution of *S. typhi-murium* strains, a similar change in the predominance of phage types was observed [16].

The frequency of phage types 1 and 7 was nearly the same among the strains derived from animals, foodstuffs and water, while no such a temporal difference was observed between the frequency of the two phage types as between human strains.

Comparing our results with Lalko's [5] in Poland between 1970 and 1975, examining 1847 strains isolated from 1567 persons from 673 foci, it was found that in the two countries different phage types spread except one phage type. In Poland 19 phage types were found, the most frequent were phage types 8 (31.8%), 5 (21.8%), 7 (18.0%) and 12 (7.5%). (Phage type 12* is type 10 ac-

cording to our scheme.) Among the 164 strains of animal origin the most frequent were phage type 12* (50.0%), 8 (23.8%) and 5 (15.9%). Out of these only type 7 was frequent in Hungary. Phage type 1, which was frequent in Hungary, occurred only in one human case but in no animal strain in Poland. The frequency of phage type 4 was 30% among the Polish human strains and one animal strain belonged to this type. Phage type 17 (according to the Hungarian designation) which was frequent among human strains in Hungary, occurred in Poland in 2.7%, while phage type 16 was represented only by one human and five animal strains.

According to the Polish examinations, the 30 human strains derived from one focus, in GDR belonged to phage type 7. In our examinations the strains isolated in the GDR proved to be of phage types 7, 4 and 6. According to Lalko's examinations the 259 animal strains from Bulgaria belonged to phage type 7 (70.7%) and 4 (17.8%). These results showed that different strains were circulating in Poland and Hungary, in the GDR and Bulgaria.

Suchowiak and Halat [17] reported a food poisoning caused by contaminated milk in the Wroclaw district, and Granicki et al. [18] reported an outbreak associated with *S. enteritidis* in Katowice Province where contaminated tartar-beefsteak was the source of infection. Lalko [5] described two outbreaks caused by *S. enteritidis*. The first was discovered in 1972 in the Szczecin district and the infectious source was ice cream, and the phage type of the infecting strain, 12*. The second outbreak occurred in 1975 in the Lodz district. Here tartar-beefsteak conveyed the infection with phage type 8. The strains isolated from food, as well as from sick people and from the contact asymptomatic cases represented the same phage type. It was observed that the phage types 12*, 6, 7 and 4 were isolated mainly from adults and older children, while the types 5 and 2 from infants staying in hospitals and children's homes. Examining the spread of phage types in Hungary, no connection was found between age distribution and phage types.

Antibiotic resistance of *S. enteritidis* strains and the characterization of the carried R-plasmids will be the subject of a further study.

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DIFFERENTIAL TRANSLATION OF VIROGENIC AND ONCOGENIC SEQUENCES IN MALIGNANT LYMPHOPROLIFERATIVE DISEASES AND TRANSFECTION OF CODING DNAs INTO NIH 3T3 CELLS

F. D. Tóth, L. Váczi, B. Szabó, Jolán Kiss, K. Rák, A. Kiss, Ilona Kovács, Cs. Kiss and K. Pecze

Institute of Microbiology, Second Department of Medicine, and Department of Paediatrics, University Medical School, Debrecen

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The expression of oncoviral p30 polypeptides and onc gene-specific proteins has been examined in different human lymphoid malignancies. The distribution of antigen(s) related to the p30 of BaEV lacked any specificity. Antigen(s) related to the main core polypeptide of GaLV could be detected mainly in B- and O-cell malignancies. The *myc*-encoded protein was translated at higher levels in malignant than in normal lymphoid cells. An active *src* gene was identified in three acute lymphoid leukaemias and in one non-Hodgkin lymphoma of T-cell origin. Human DNAs coding oncoviral antigens or onc gene-specific proteins could be transfected into NIH 3T3 cells. These data suggest that the synergistic effect of the *myc* and *src* genes would operate in malignant transformation of some progenitors of T-cell lineage.

The transforming genes of retroviruses are derived from normal cellular genes that are conserved among vertebrates [1, 2]. The function of these cellular gene products is not clearly defined. There is, however, evidence that virus-induced transformation is correlated with enhanced levels of expression of these genes [3, 5]. Recent data suggest that two or more oncogenes must cooperate to transform cells [6, 7]. The development of continuously growing cell lines from patients with lymphoid malignancies has led to isolation of C type retroviruses. Most of them are very closely related to C type primate viruses [8, 9]. By using the nucleic acid hybridization assay, expression of RNA sequences homologous to DNA fragments containing different onc genes has been detected in diverse human lymphoid malignancies [10–13]. The DNA-mediated gene transfer techniques provided an approach for the detection of virogenic and oncogenic DNA sequences. DNAs from a number of human tumours were found to induce malignant transformation of NIH 3T3 cells, a

Ferenc D. Tóth, Lajos Váczi, Béla Szabó, Jolán Kiss
Institute of Microbiology, University Medical School
4012 Debrecen, P.O.B. 17, Hungary

Kálmán Rák, Attila Kiss, Károly Pecze
Second Department of Medicine, University Medical School
4012 Debrecen, P.O.B. 32, Hungary

Ilona Kovács, Csongor Kiss
Department of Paediatrics, University Medical School
4012 Debrecen, P.O.B. 20, Hungary

continuous mouse line that is contact-inhibited and highly susceptible to DNA transfection [14].

The present report deals with the detection of oncoviral p30 polypeptides and translational products of the *myc* and *src* oncogenes in human lymphoid malignancies. Another aim was to determine whether any viral or cellular gene products, detected in human cells, could be induced in NIH 3T3 cells by DNA transfection.

Materials and methods

Purification of leukocytes and lymph node cells. Heparinized blood samples from patients were centrifuged. Lymph node biopsy specimens were taken apart in RPMI 1640 media, minced, and the fragments were passed through a stainless-steel wire mesh. Erythrocytes were removed by lysis in 0.83% ammonium chloride solution [15]. Cell viability, determined by trypan blue dye exclusion, was greater than 80%.

NIH 3T3 cells were kindly supplied by Dr. C. Altaner (Cancer Research Institute of the Slovak Academy of Sciences, Bratislava, Czechoslovakia), and maintained in Dulbecco's modified MEM containing 5% fetal calf serum (Gibco Bio-Cultures Ltd., Paisley, Scotland).

Immune sera. Goat immune sera to the p30 core polypeptide of baboon endogenous virus (BaEV) and gibbon ape leukaemia virus (GaLV) were kindly provided by Dr. R. Wilsnack (Huntingdon Research Center, Baltimore, MD, USA). Rabbit antiserum to a synthetic peptide that corresponds to the *v-myc*-encoded protein lacking *gag* sequences [16] was kindly sent by Dr. K. Bister (Max-Plank-Institut für Molekulare Genetic, West Berlin). The rabbit immune serum raised against the $pp60^{src}$ was the gift of Dr. R. L. Erikson (Department of Pathology, School of Medicine of the University of Colorado, Health Sciences Center, Denver, CO, USA). Rabbit anti-goat IgG and goat anti-rabbit IgG sera conjugated with FITC were bought from Hyland Co., (Costa Mesa, CA, USA).

Indirect cytoplasmic immunofluorescence assay was performed by spotting 10^5 cells washed in PBS on slides. After air-drying the samples were fixed in acetone. The appropriate immune serum diluted 1 : 40 was applied to cells and incubated at 37 °C for 30 min in a humified atmosphere. Then the slides were washed three times in PBS. The appropriate FITC-labelled Hyland antiserum was diluted 1 : 20 and applied to the fixed cells at 37 °C for 30 min. The slides were washed again three times before examination under the fluorescence microscope. As controls, normal rabbit or goat sera were used in the first step.

Preparation of high molecular weight DNA from human cells. DNA was extracted according to the procedure of Gross-Bellard et al. [17], with minor modifications. Cells were suspended in TNE buffer pH 8.3 containing 150 mM NaCl, 2 mM EDTA, 10 mM Tris–HCl, 1% Na Dod SO_4, 1% mercaptoethanol and 1 mg per ml of protease (Sigma Chemical Co., St. Louis, MO, USA), and incubated overnight at 27 °C. Lysates were extracted twice with buffer-equilibrated phenol and once with chloroform. DNA was precipitated in 2 vol of absolute ethanol. The pellet was washed in 70% ethanol, dried, and resuspended with Hepes buffer pH 7.05 which contained 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, and 20 mM *N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulphonic acid (Hepes). For different preparations, the ratio OD_{260} to OD_{280} varied between 1.85 and 2.00.

Transfection assay. Transfections were performed by the calcium phosphate precipitation technique [18], as modified by us. The DNA was precipitated by the addition of 2M $CaCl_2$ to a final concentration of 125 mM and left at room temperature for 30 min before being added to the cultures. The DNA was sheared 10 times with a 22-gauge needle attached to a 1 ml tuberculin syringe. Portions of the DNA precipitate were then added to 25 cm^2/40 ml plastic tissue culture flask (Nunc, Roskilde, Denmark) cultures of NIH 3T3 cells so that the monolayer would be about 70–80% confluent at the time of addition of DNA; 40 μg of DNA were utilized to transfect 10^6 cells. The samples were incubated at 37 °C for 30 min and then Dulbecco's modified MEM with 5% fetal calf serum was added to them. After 5 h incubation at 37 °C, the cell cultures were washed once with growth medium, and 5 ml Dulbecco's modified MEM containing 3% fetal calf serum was added to the cultures. Focus formation was scored at 18–21 days.

Results

Detection of oncoviral p30 polypeptide-, v-myc- and v-src-specific proteins in different human lymphoid cell samples. Thirty four samples were assayed for the presence of proteins identical or related to oncoviral proteins or translational products of *v-myc* and *v-src* genes. Immunofluorescence studies of acetone-fixed cell samples indicated that antigens related to BaEV or GaLV p30 core polypeptides were located in the cytoplasm (Fig. 1). The *myc*-encoded proteins were observed in the nucleus (Fig. 2), while the *src*-encoded $pp^{60^{src}}$ stain was most intensive in the peripheral parts of the cells (Fig. 3). Eighteen of 34 samples were positive for BaEV p30-related antigen(s). The distribution of this antigen among the different groups of patients lacked any specificity (Tables I and II). GaLV p30-like antigen(s) could be detected in 10 samples; they originated mostly from patients with B- or O-cell malignancies. Results of experiments with the anti-myc immune serum showed that *myc* was expressed in almost every examined cell type but the *v-myc* encoded protein was translated at higher levels in malignant than in normal lymphoid cells (Tables I and II). *Src* was expressed at high levels in 4 cases. Tumour sources in which an active *src* gene was identified included acute lymphoid leukaemias and a non-Hodgkin lymphoma of T lymphoblast type.

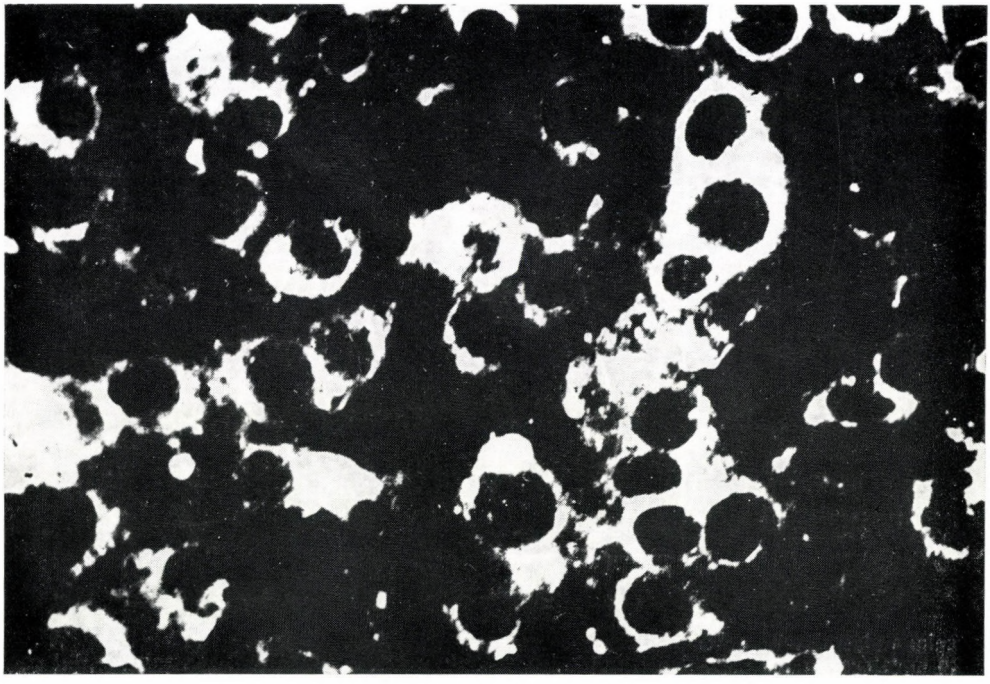

Fig. 1. Immunofluorescence staining for GaLV p30 in leukaemic cells from patient No. 11 listed in Table I

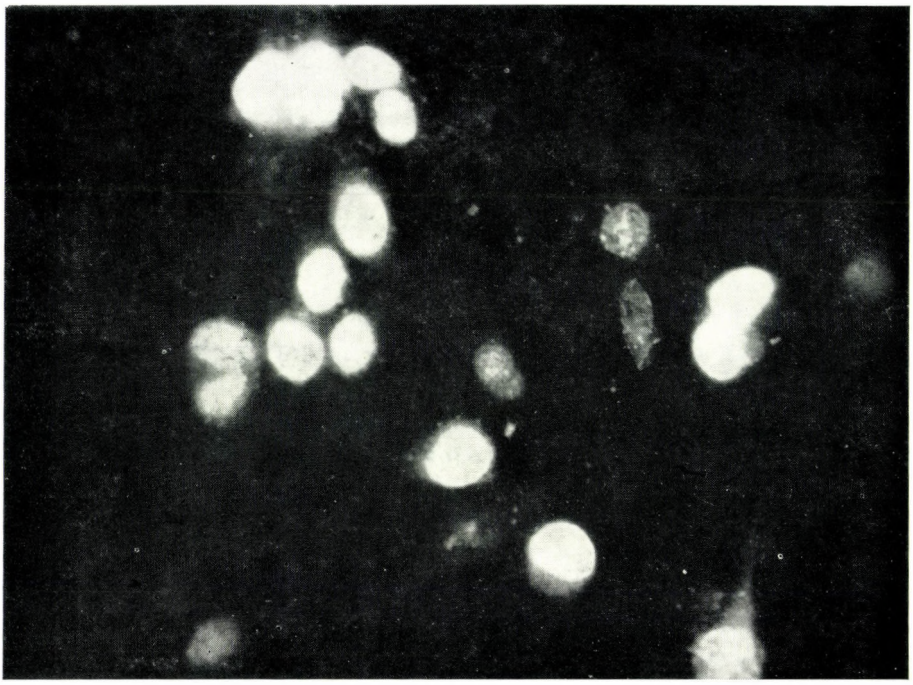

Fig. 2. Immunofluorescence by anti-p57^{myc} antibody of leukaemic cells from patient No. 6 listed in Table I

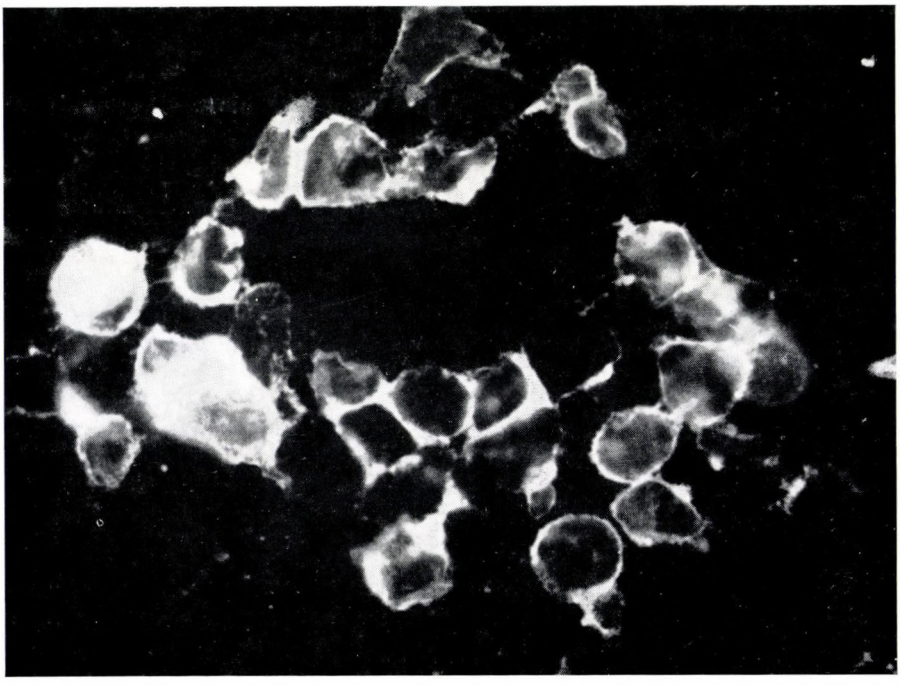

Fig. 3. Immunofluorescence micrograph of leukaemic cells (patient No. 3 in Table I) treated with anti-*pp60*^{src} antibody

Table I

Detection of p30 antigens, v-myc- and v-src-specific proteins in acute lymphoid leukaemic cell samples

No	Cell type	BaEV p30	GaLV p30	myc	src
1	mature T	+	−	+	−
2		−	−	−	−
3		−	++	++	++++
4		+	−	+++	−
5		++	−	+++	+++
6	T lymphoblast	++	−	++++	−
7		−	−	++	++++
8		++	−	+++	−
9		++	++	++	−
10		−	−	++	−
11		++	+++	+++	−
12	O	+	−	++++	−
13		++	+	+++	−
14		−	++	++	−

Table II

Detection of p30 antigens, v-myc- and v-src-specific proteins in cell samples of patients with CLL, NHL, HL, and of control persons

No	Diagnosis	Cell type	BaEV p30	GaLV p30	myc	src
1	chronic		−	+	+	−
2	lymphoid	mature B	−	−	+	−
3	leukaemia		++	−	++	−
4			−	+	++	−
5		mature T	−	−	−	−
6			−	−	+	−
7			−	−	+++	++++
8	non-Hodgkin	T lymphoblast	+	−	++++	−
9	lymphoma		+	−	+++	−
10			−	++	+++	−
11		B lymphoblast	+	−	+++	−
12			++	+++	++++	−
13			++	+	++	−
14	Hodgkin	−	−	−	+	−
15	lymphoma		−	−	++	−
16			+	−	++	−
17	reactive		+	−	−	−
18	hyper-	−	−	−	+	−
19	plasia		+	−	++	−
20			−	−	+	−

Transfection of DNA coding p30-like antigens and onc gene-specific proteins from human lymphoid cells into NIH 3T3 cells. Samples were assayed for induction of foci in monolayer cultures. The results of these experiments (Tables III and IV) showed that DNAs of lymphoid tumours induced transformation at a level significantly above the background of spontaneous transformation in control cultures exposed to high molecular DNAs of lymphoid

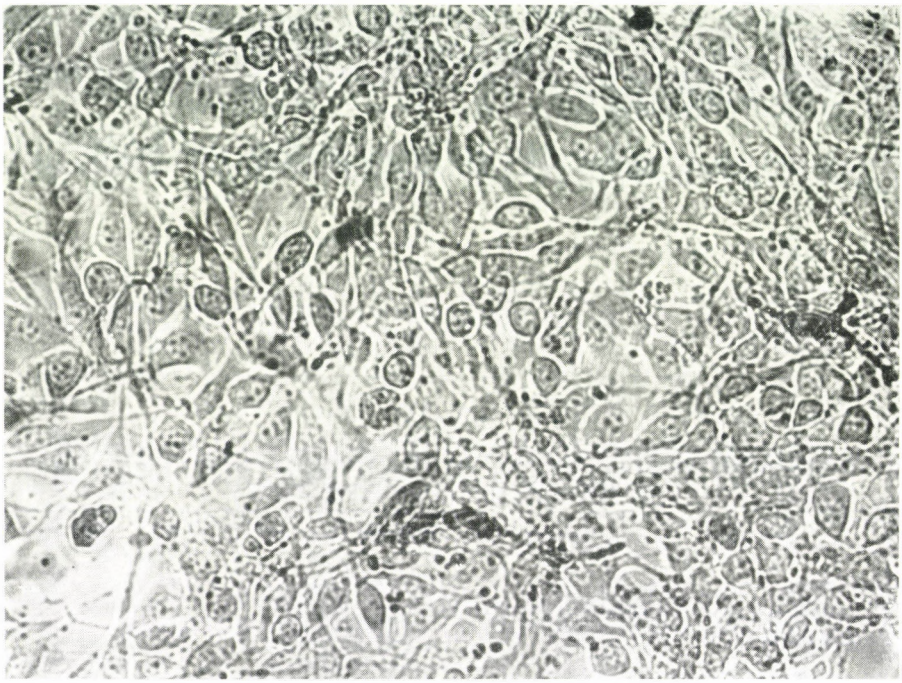

Fig. 4. Morphology of normal NIH 3T3 cells

cells from persons with reactive hyperplasia of lymph nodes. Eighteen of 30 tumour cell DNAs were scored as positive in the transfection assay. DNAs of 12 lymphoid tumours presented in Tables III and IV were negative for transformation. Foci of NIH 3T3 cells transformed by tumour cell DNAs were not similar in appearance (Figs 4 and 5).

Transformed cells were grown to mass cultures for further studies. The cells retained their transformed appearance. Detection of transforming activity was corroborated by assaying for oncoviral core polypeptides and onc gene-encoded proteins in foci derived from human DNAs. Immunofluorescence studies indicated that human DNAs coding BaEV or GaLV p30-related antigens or onc gene-specific proteins could be transfected in every investigated case (Tables III and IV).

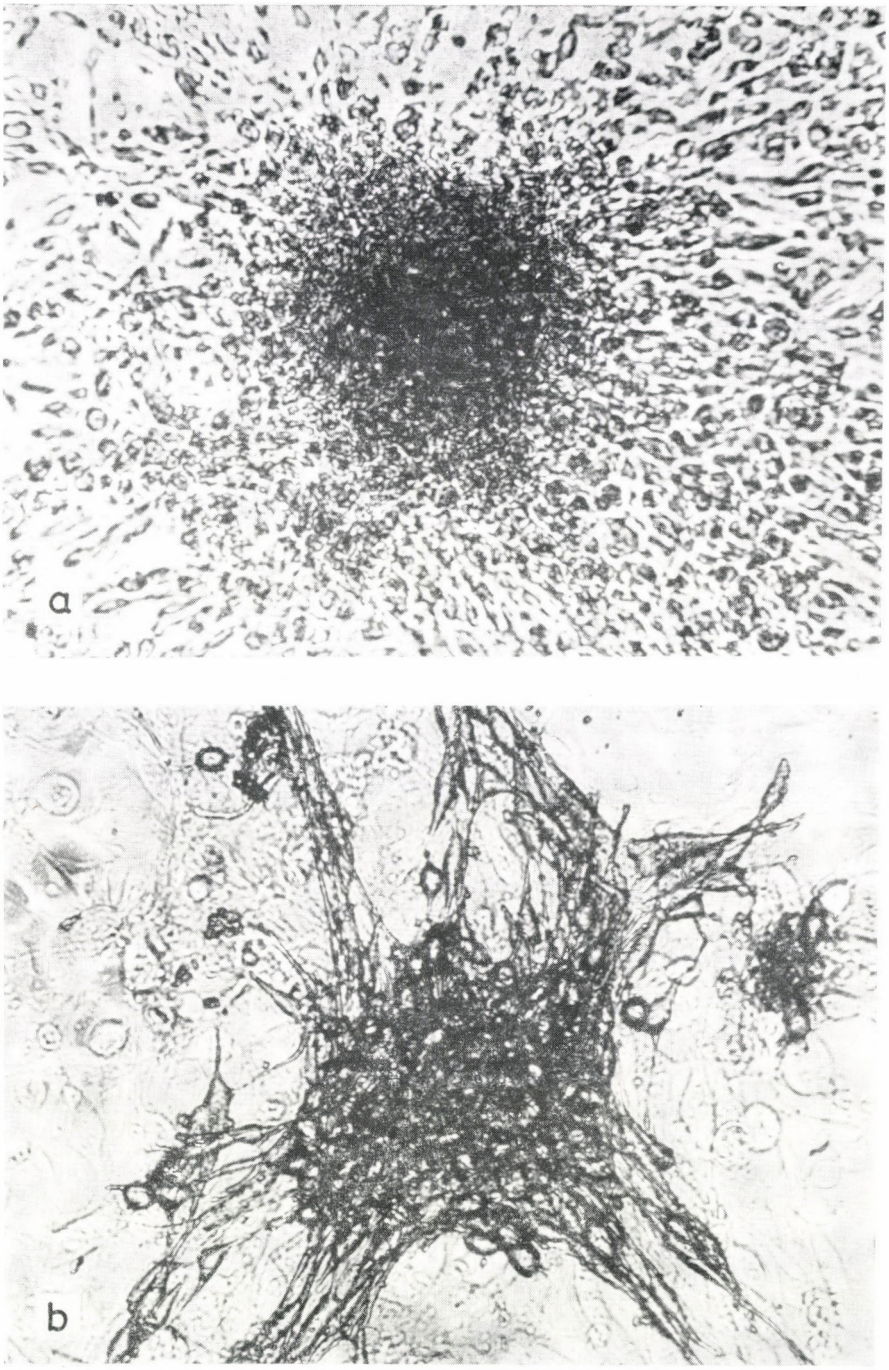

Fig. 5. (a) Representative focus of NIH 3T3 cells transformed by DNA from leukaemic lymphoblasts of patient No. 7 listed in Table III. (b) Another morphological type of transformation induced by DNA from leukaemic cells of patient 11 listed in Table III

Table III

Transfection of DNA coding p30 antigens and onc gene-specific proteins from ALL cells into NIH 3T3 cells

No	Donor DNA (cell type)	Foci per μg DNA	Translation of			
			BaEVp30	GaLVp30	myc	src
1	mature T	0.17	++	−	+	−
2		<0.005	−	−	−	−
3		0.30	−	++	++	++++
4		0.15	+	−	++++	−
5	T lymphoblast	0.17	+	−	+++	++++
6		<0.005	+++	−	++	−
7		0.24	−	−	++++	++++
8		0.09	++	−	+++	−
9		<0.005	++	++	++	−
10		0.09	−	−	+++	−
11	0	0.23	++	+++	+++	−
12		<0.005	++	−	+++	−
13		0.06	++	+	+++	−
14		0.08	−	++	+++	−

Table IV

Transfection of DNA coding p30 antigens and onc gene-specific proteins from cell samples of patients with CLL, NHL, HL, and from control persons into NIH 3T3 cells

No	Donor DNA		Foci per μg DNA	Translation of			
	Source	Cell type		BaEV p30	GaLV p30	myc	src
1	CLL	mature B	<0.005	−	+	+	−
2			<0.005	−	−	+	−
3			<0.005	++	−	+	−
4			<0.005	−	++	+	−
5	NHL	mature T	0.14	−	−	−	−
6			<0.005	−	−	+	−
7		T lympho-blast	0.18	−	−	+++	++++
8			0.17	++	−	+++	−
9			<0.005	++	−	++++	−
10		B lympho-blast	0.06	−	++	+++	−
11			<0.005	+	−	+++	−
12			0.11	++	+++	++++	−
13	HL	−	0.04	++	+	++	−
14			0.02	−	−	++	−
15			<0.005	−	−	+	−
16			0.02	++	−	++	−
17	reactive hyper-plasia	−	<0.005	+	−	−	−
18			<0.005	−	−	++	−
19			<0.005	++	−	++	−
20			<0.005	−	−	+	−

Discussion

The results indicated that primary human lymphoid tumour cells as well as normal lymphocytes express antigen(s) related to the BaEV p30 polypeptide and the *v-myc*-encoded protein. The translational product of *v-myc* was expressed at higher levels in malignant than in normal lymphoid cells. Although activation of the *myc* gene apparently contributes to the development of experimental and human lymphomas, the identified transforming gene turned out to be a new oncogene designated *Blym* [19] or *ras* [20]. An active *src* oncogene coexisted with *myc* in some T-cell malignancies and its translational product was expressed at high levels in NIH 3T3 cells, transformed by the DNA of the primary human tumour cells. From these data it has been concluded that if the *myc* gene were involved in malignant transformation, it would most likely operate in conjunction with the *src* gene in these malignant T lymphoblasts. This assumption was supported by the observation that a recombinant murine retrovirus containing the *src* gene of avian Rous sarcoma virus was shown to transform murine lymphoid progenitor cells. The transformants, committed to the T-cell lineage, expressed high levels of the $pp^{60^{SRC}}$ [21]. The only sample in which transcription of the *sis* gene had been detected was a human mature T-cell lymphoma cell line [10], but a transforming gene related to the *ras* gene family seems to be activated in some human leukaemia samples of T-cell origin [22]. This *ras* gene was demonstrated in human lymphoid tumour cells of B-cell origin, too [23]. In contrast to the wide expression of the *ras* gene, the *src* seemed to be activated only in some T-lymphoblastic human tumours. Thus, the results indicated some degree of specificity with respect to the *src* gene activated in discrete form of T cell transformation. A GaLV p30-related antigen could be found mainly in B- and O-cell malignancies. The relationship between its expression and activation of *Blym* [19] and *ras* [23] oncogenes remains to be clarified. All the samples used throughout our studies were taken from patients in the progressive stage of some lymphoid malignancy. It seems to be useful to investigate the expression of these markers in the same patients during remission. Such experiments are in progress and it is hoped that they will lead not only to a reasonable assessment of the involvement of virogens and oncogenes in lymphoid malignancies, but also to a better understanding of the role of these genes in the normal maintenance and differentiation of different lymphoid cell types.

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PRIMING OF INTERFERON PRODUCTION IN HUMAN EMBRYO FIBROBLASTS BY ALPHA, BETA AND GAMMA INTERFERONS*

I. Rosztóczy and Olga Siroki

Institute of Microbiology, University Medical School, Szeged

(Received December 13, 1984)

Pretreatment of human embryo fibroblasts with homologous alpha, beta and gamma interferons (IFNs) exerted a priming effect on both the ordinarily poly I : C-induced and the superrinduced human IFN beta production. The priming activities of HuIFN-alpha and beta were equivalent while HuIFN-gamma proved to be somewhat inferior in this respect. DEAE-dextran enhanced the efficacy of induction when it was used in combination only with poly I:C. No such effect could be observed in the superinduced cultures. A correlation was found between the amount of IFN produced by the primed or superinduced human embryo fibroblasts and the quantity of extractable polyadenylated translatable IFN mRNA in these cells.

Interferon (IFN) pretreatment of cells primes their subsequent IFN production [1]. The priming effect of IFN is commonly used to enhance the production of HuIFN-α in human buffy coat cells [2, 3]. The production of HuIFN-β in cultures of poly I : C-stimulated human diploid fibroblasts can also be primed, even when the superinduction procedure is used [4–7]. It has been demonstrated that type II IFNs also exert a priming effect [8–10]. Alpha, beta and gamma IFNs seem to have different antiviral and anticellular activities [11, 12]. In the present work, therefore, we compared their priming effects in poly I : C-stimulated human fibroblasts. With the enhancement of IFN production in mind, we also investigated the effects of DEAE-dextran and superinduction. To obtain information on the level of regulation in the process of IFN synthesis under different induction procedures, we determined the amount of IFN mRNA in stimulated human embryo fibroblasts by injecting the extracted poly A-rich RNA fraction into *Xenopus laevis* oocytes.

Materials and methods

Cell culture. The human embryo fibroblast cell line used in the present experiments originated from the lungs of a 15-week-old male human embryo. Frozen stock of low passage cells were set up and experiments were performed with cultures of 20 to 30 population doublings. Cells were cultured in Eagle's minimal essential medium (MEM) plus 10% newborn calf serum (Hungarocalf, Mezőhegyes, Hungary).

István Rosztóczy, Olga Siroki
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

Chemicals. Polyinosinic-polycytidylic acid (poly I : C) was purchased from Reanal Fine Chemicals, Budapest, Hungary; actinomycin D from Serva Feinbiochemica GmbH, West Germany; cycloheximide from Fluka, Chemische Fabrik, Buchs, Switzerland; and diethylaminoethyldextran (DEAE-dextran) from Pharmacia Fine Chemicals, Inc, Uppsala, Sweden.

Induction of interferon. For the induction of IFN, monolayer cultures were prepared in 150 cm^2 plastic tissue culture flasks (Greiner) by seeding 2×10^6 cells in 50 ml of growth medium. Confluency was usually reached following incubation at 37 °C in 5% CO_2 atmosphere for 5 to 6 days. The cultures were used 3 to 4 days after confluency. The culture medium was removed, and in experiments involving priming it was replaced by 20 ml of fresh medium containing varying concentrations of human IFNs. The specific activities of the IFN preparations used for pretreatment were as follows: 2×10^6 IU/mg protein for HuIFN-α, 1×10^6 IU/mg protein for HuIFN-β, and 1×10^5 laboratory unit/mg protein for HuIFN-γ. After incubation for 12 h the IFN was removed, and cultures were induced with 75 μg/ml poly I : C in 10 ml of serum-free medium for 1 h at 37 °C (ordinary induction). When DEAE-dextran was added to poly I : C, it was used at a concentration of 150 μg/ml. At the end of the induction period, cultures were washed three times with serum-free medium and incubated for an additional 16 h in 30 ml of growth medium supplemented with 2% newborn calf serum. When the superinduction procedure [13–15] was used, the poly I : C solution was supplemented with 60 μg/ml cycloheximide, the cultures were incubated for 220 min and then 0.75 μg/ml actinomycin D was added. After 110 min further incubation, the inducer and antimetabolite mixture was removed, and cultures were washed with serum-free medium and incubated for 12 h in the presence of 30 ml of growth medium supplemented with 2% newborn calf serum.

Interferon assay. IFN was titrated by means of a cytopathogenic effect inhibition micromethod [16] on monolayers of human embryo fibroblasts cultured in microtitre plates (Greiner), using vesicular stomatitis virus as the challenge virus. The multiplicity of infection was 0.2 plaque forming unit/cell. IFN activities were calibrated against an international reference standard of HuIFN-β (G-023 902 527) and titres were expressed in international reference units.

Interferon mRNA determination. The total cytoplasmic RNA was extracted from 75 to 100×10^6 cells, using the phenol–chloroform technique. In brief, 6 h from the beginning of induction 10 to 12 monolayer cultures were washed twice cold PBS. Cells were removed by scraping with a rubber policeman. After centrifugation, the cells were resuspended in an ice-cold buffer containing 20 mM Tris pH 7.5, 100 mM NaCl and 1 mM EDTA pH 7.5. Nonidet P 40 was added to 1%, and nuclei and cell debris were removed by centrifugation for 5 min at 2000 g. The supernatant was adjusted to 0.5% (w/v) sodium dodecyl sulphate and the total RNA was obtained by extracting 3 times with a solution containing a mixture of phenol-chloroform-isoamyl alcohol in a ratio of 50 : 50 : 1 (v/v/v) and 2 times with chloroform–isoamyl alcohol (50 : 1) at room temperature. The RNA was precipitated by the addition of 2.5 volumes of cold ethanol and 1/50 volume of 5 M NaCl. The poly A-rich RNA fraction was isolated by affinity chromatography on oligo (dT) cellulose (Type 7, PL Biochemicals), essentially as described by Aviv and Leder [17]. The poly A-rich RNA fraction was dissolved at a concentration of 1 mg/ml in sterile distilled water. The IFN mRNA contents of these preparations were assayed by translation in *Xenopus laevis* oocytes. Batches of 10 oocytes were injected with a total of 400 nl of RNA solution, and then incubated at room temperature for 30 h in 100 μl of modified Barth solution [18].

Results

The kinetics of IFN production were compared in ordinarily induced and superinduced human embryo fibroblasts. The ordinarily induced cultures were stimulated with poly I : C only; the superinduction procedure was performed as described in "Materials and methods". The aim of these experiments was to acquire information on the period of maximum IFN production, in order to determine the optimum time for IFN sampling and for RNA extraction. IFN production was faster in the superinduced cultures and the titres exceeded usually more than 50 times the IFN production observed in the ordinarily induced human embryo fibroblast (Fig. 1). Pretreatment of cells with any of

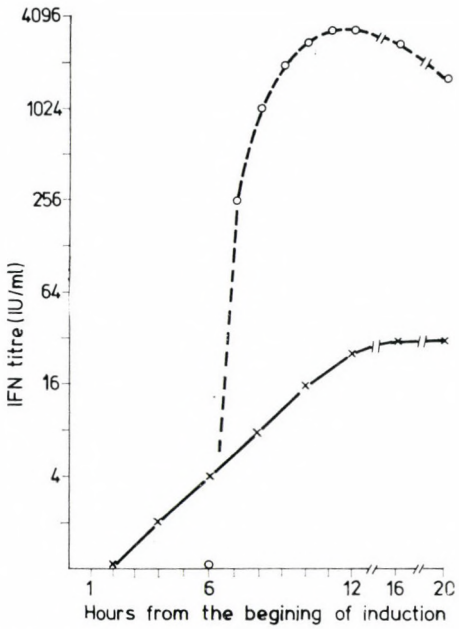

Fig. 1. Kinetics of IFN production in ordinarily induced and superinduced human embryo fibroblasts. ○———○ superinduced; ×———× ordinarily induced

the three IFN types resulted in an enhancement of IFN production. This priming effect developed in a dose-dependent manner and could be observed for both ordinarily induced and superinduced IFN production. The enhancing effect of priming was somewhat higher in the ordinarily induced cultures and the priming activity of HuIFN-γ proved to be less pronounced than those of HuIFN-α and -β. Priming with optimum doses of IFN-α and -β enhanced the ordinarily induced interferon production 8 to 10 times, while the production of superinduced cultures was increased 3 to 5 times. The corresponding values for HuIFN-γ were 4 to 5 and 2 to 3, respectively (Fig. 2).

In the presence of DEAE-dextran, poly I : C induced substantially more IFN (512 IU/ml versus 32 IU/ml). However, when the superinduction procedure was applied, DEAE-dextran was not effective and DEAE-dextran-treated cultures produced the same amount or even less IFN. Priming of cells with 100 IU/ml HuIFN-α for 12 h enhanced the IFN production in these cultures, too (Table I). IFN mRNA activity in the poly A-rich RNA fractions extracted from the ordinarily induced cells was below the sensitivity of the assay used.

Priming of cells with 100 IU/ml HuIFN-α before induction increased the IFN mRNA content to a detectable level. A more pronounced enhancement in the amount of IFN mRNA could be observed in the poly A-rich RNA fractions extracted from the superinduced cultures (Table II).

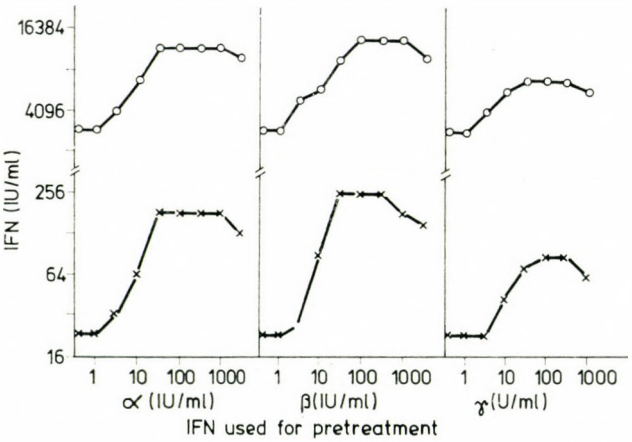

Fig. 2. Effects of HuIFN-α, -β and -γ pretreatment on IFN production of ordinarily induced and superinduced human embryo fibroblasts. ○——○ superinduced; ×——× ordinarily induced

Table I

Influence of DEAE-dextran on effectivities of ordinary induction and superinduction in primed and unprimed human embryo fibroblasts

Treatment				IFN titre
DEAE-d	Ordinary induction	Super induction	Priming	
−	−	−	−	<2
−	+	−	−	32
+	+	−	−	512
+	+	−	+	2048
−	−	+	−	3840
−	−	+	+	12 800
+	−	+	−	2560
+	−	+	+	6144

Table II

IFN mRNA content of ordinarily induced and superinduced human embryo fibroblasts

Treatment			IFN (IU/10 oocytes)
priming	induction		
	ordinary	super	
−	−	−	<2
−	+	−	<2
+	+	−	4
−	−	+	32

Discussion

Priming activity has been considered to be one of the inherent properties of IFN molecules [3, 19]. In the present work we found that HuIFN-α and -β exerted similar priming activities. The HuIFN-γ preparation used in the present study proved to be somewhat less effective than HuIFN-α and -β regarding enhancement of the production of HuIFN-β. However the optimum priming effect with HuIFN-γ was achieved with an equivalent number of antiviral units (50 to 300 IU/ml) as observed for the other two HuIFNs. The lower priming activity of HuIFN-γ in comparison with HuIFN-α and -β is at variance with the results of Tyring et al. [10], who found that partially purified MuIFN-γ was equivalent to MuIFN-α/β in its ability to enhance the production of MuIFN-α/β by L_{929} cells. Our HuIFN-γ preparation contains a substantial interleukin-2 activity (data not shown). This or other impurities can act as hyporeactivity or blocking factors [20, 21], the presence of which might influence the priming activity of HuIFN-γ, depending on their ratios in the preparation.

We do not know the reason for the lower rate of priming in the superinduced cultures, and for the ineffectiveness of DEAE-dextran when superinduction was used instead of ordinary induction. A similar tendency of changes was observed both in the IFN mRNA content of cells and in the amount of IFN produced in the present experiments. This is in agreement with the findings of other authors [22, 23], and suggests an important role for the increase in the IFN mRNA content in the mechanism by which the IFN production is enhanced in the primed and superinduced human embryo fibroblast.

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COMBINED EFFECTS OF AMANTADINE AND INTERFERON ON INFLUENZA VIRUS REPLICATION IN CHICKEN AND HUMAN EMBRYO TRACHEA ORGAN CULTURE*

KATALIN LUKÁCSI, MAGDOLNA MOLNÁR, OLGA SIROKI and I. ROSZTÓCZY

Institute of Microbiology, University Medical School, Szeged

(Received December 13, 1984)

Amantadine ($\geq$100 μg/ml) treatment of chicken or human trachea organ cultures 6 h before infection inhibited influenza virus replication. Chicken or human leukocyte IFN evoked antiviral resistance in the cells of the homologous organ cultures only at a concentration of 100 U/ml or more. Treatment of organ cultures with combinations of the two substances resulted in an additive effect on influenza virus replication in both chicken and human tissues.

The antiviral effects of amantadine and interferon (IFN) against influenza viruses are well documented both in vitro and in vivo [1–4]. Although combinations of antiviral substances can result in a greater virus inhibitory activity than that of either single agent, few studies on the combined effect of these drugs on influenza virus replication have been reported [5, 6]. Cells in organ cultures seem to preserve most of their in vivo characteristics, such as susceptibility to viruses and differentiated cellular functions [7]. The antiviral effect of IFN has been demonstrated in organ cultures, although larger doses are needed in comparison to those active in tissue cultures [8, 9].

The aim of the present study was to evaluate the effects of amantadine and IFN alone and in combination on the replication of influenza virus in organ cultures.

Materials and methods

Antiviral substances. Amantadine hydrochloride (Serva) was dissolved in distilled water at a concentration of 10 mg/ml; further dilutions were prepared in the appropriate culture media. Chicken leukocyte IFN and human IFN-α were partially purified preparations with specific activities of 6×10^4 IU and 1×10^6 IU/mg protein, respectively.

Influenza virus and titration. The A/Moscow (1019/1965) H_2N_2 strain was the same as used in a previous work [10]. Infectivity was determined by egg bit titration [11, 12].

Organ cultures were prepared according to the method of Hoorn and Tyrrell [7]. Tracheas were removed from 19–20-day-old chick embryos, and the human tissue was obtained from 16–20-week-old human embryos. Organ cultures were incubated in Eagle's MEM without calf serum.

KATALIN LUKÁCSI, MAGDOLNA MOLNÁR, OLGA SIROKI, ISTVÁN ROSZTÓCZY
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

Cell cultures. Secondary chick embryo fibroblast (CEF) and 4 to 6 passage level human embryo fibroblast (HEF) were used. Cultures were maintained in Eagle's MEM + 10% newborn calf serum.

IFN assay was carried out in flat-bottomed microtitre plates and was based on the inhibition of CPE of the vesicular stomatitis virus (VSV) [13].

Amantadine toxicity was determined using three methods. (a) Morphological changes of secondary CEF cultured in the presence of various drug concentrations were scored after 24 h by microscopic examination from + to ++++, corresponding to 25 to 100% cell damage.

(b) The ciliar activity of trachea epithelial cells in organ cultures was followed. Toxicity was estimated from the per cent of cultures which lost ciliar movement in the presence of appropriate drug concentrations.

(c) The effects of amantadine on the RNA and protein syntheses of trachea cells were assayed by determining uptake of ^{14}C leucine and ^{14}C uridine. Organ cultures were treated for 6 h with different amantadine concentrations, and then pulsed for an additional hour with 3.7×10^4 Bq/ml uridine or 3.7×10^4 Bq/ml ^{14}C leucine. Organ cultures were washed three times with saline, once with 5% TCA and once with methanol. They were dried at 37 °C for 24 h, and then solubilized in Soluene (Packard). Toxicity was expressed in terms of inhibition of incorporation of radioactive precursors in comparison with controls.

Antiviral activities of amantadine, IFN and their combinations. Trachea bits were incubated for 6 h in the presence of antiviral substances, then with 1×10^5 $EBID_{50}$/ml influenza virus for one hour. Unadsorbed virus was removed by washing the cultures three times. If not stated otherwise, the amount of virus produced was determined in samples collected 2, 14, 24, 36 and 48 h after infection.

Results

Toxicity of amantadine. In CEF cultures 200 μg/ml was the highest amantadine concentration not causing visible morphological changes. Ciliated epithelial cells proved to be somewhat more resistant, as the beating of the cilia was similar to that of the controls even after treatment with 400 μg/ml amantadine. Incorporation of ^{14}C uridine and ^{14}C leucine was inhibited at 800 μg/ml and at 400 μg/ml amantadine, respectively (Table I). The toxicity of amantadine was similar in human tissues and the presence of IFN up to 3×10^3 IU/ml in the culture media did not cause any change in the observed toxic values (data not shown).

Table I

Toxicity of amantadine in CEF and in chicken trachea organ cultures

Concentration, μg/ml	CEF cytotoxic effect	Organ cultures		
		inhibition of ciliar activity, %	inhibition (%) of ^{14}C uridine	incorporation of ^{14}C leucine
0	0	0	0	0
100	0	0	0	0
200	0	0	−10	0
400	++	0	−20	10
800	++++	10	25	46
1600	++++	70	65	83
3200	++++	100	86	95

Inhibition by single substances. On the basis of the results of toxicity tests amantadine concentrations up to 200 μg/ml were investigated. Preliminary experiments indicated that virus titres reach their maximum level in untreated cultures 12 to 16 h postinfection; we took samples for virus titration after incubation for 16 h (Fig. 1). Amantadine concentrations less than 50 μg/ml were ineffective; 100 μg/ml amantadine inhibited influenza virus replication by 90% on the average, while the effect of 200 μg/ml amantadine approached 99% inhibition.

The effects of 1 to 10 IU/ml IFN can easily be demonstrated in tissue cultures, but these concentrations of IFN did not inhibit the growth of influenza virus in organ cultures. At least 100 IU/ml or more IFN was required for a detectable virus inhibitory effect under the present experimental condi-

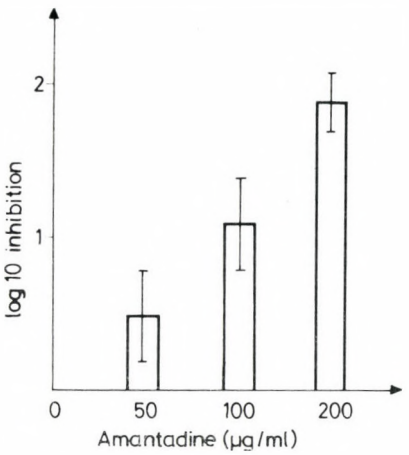

Fig. 1. Effect of amantadine on influenza virus replication

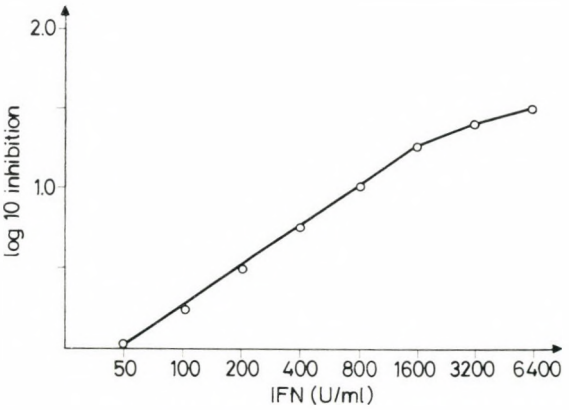

Fig. 2. Effect of chicken leukocyte interferon on replication of influenza virus in chicken embryo trachea organ cultures

tions. When higher IFN concentrations were used the dose response was proportional on a logarithmic scale (Fig. 2).

Inhibition by combinations of IFN and amantadine. Concentrations inhibiting virus replication by approximately 1 log unit were selected for the combination experiments. The single substances affected the kinetics of virus replication in different ways: amantadine caused a delay in the appearance of the peak level of virus replication. In contrast, in the presence of IFN virus titres were lower, but the kinetics of virus production remained similar to that for the controls.

In combinations, the two substances exhibited an additive effect. Since drugs were removed from the cultures at the end of virus infection period, the

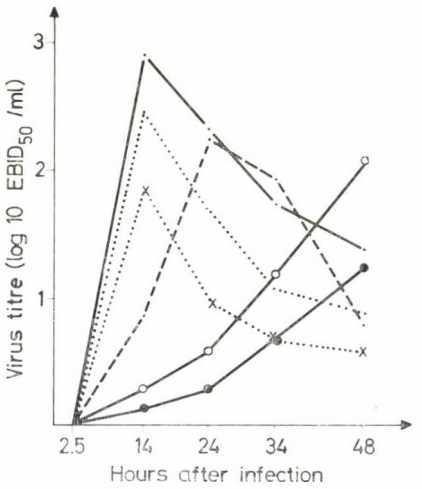

Fig. 3. Inhibition of influenza virus replication in chicken embryo trachea organ cultures by chicken leukocyte interferon and amantadine combinations. ●———● control; · · · · · IFN 1000 U/ml; × · · · · × IFN 3000 U/ml; ● · · · · ● amantadine 100 μg/μl; ○———○ amantadine 100 μg/ml + IFN 1000 U/ml; ●———● amantadine 100 μg/ml + IFN 3000 U/ml

Table II

Effects of amantadine and human IFN-α on influenza virus replication in human embryo trachea organ cultures

Treatment	log 10 inhibition of virus replication		
	2*	14	24
—	0	0	0
Amantadine 100 μg/ml	0	1.6	0.8
Hu IFN-α 1000 IU/ml	0	1.2	0.9
Amantadine 100 μg/ml + Hu IFN-α 1000 IU/ml	0	2.5	1.5

* Hours postinfection

inhibition of virus replication decreased with time and virus titres grew up to the control level or even higher at 48 h (Fig. 3). Results obtained on human embryo organ cultures with HuIFN-α and amantadine followed the same pattern of inhibition as observed in the chicken system (Table II).

Discussion

Organ cultures are considered to be superior to cell cultures: their cells do not dedifferentiate, and they therefore constitute better models for in vivo conditions. However, there are several disadvantages of organ cultures, such as the heterogeneous cell composition and limited life span. These drawbacks influenced the results of our experiments, e.g. different toxicity tests showed only approximate coincidence. This can be explained by the fact that the radioactive precursors must have been taken up not only by the ciliated cells, but also by the other structural elements of the trachea. For this reason we used amantadine in a concentration which was not toxic in any of the three tests applied. Our results correspond well with those of Burlington and Gordon [14], who found that amantadine was toxic at 2000 μg/ml after treatment for 5 h in ferret trachea organ cultures. The minimal virus inhibitory concentration of amantadine was considerably higher than that found by Oxford and Schield [15, 16] and by Grunert and Hoffmann [17] in tissue cultures. It has also been reported that IFN concentrations effective in organ cultures are higher than those which inhibit virus replication in tissue cultures [8, 9]. There are few reports on the influenza virus inhibitory effects of amantadine and IFN combinations. Our results seem to confirm the results of the original work of Lavrov et al. [5, 6], and are also in good agreement with the recently published work of Hayden et al. [18] on the effects of rimantadine and IFN combinations.

Drugs with different mechanisms of action can be additive or may exert synergistic or antagonistic effects. Lavrov et al. [5] found that a combination of amantadine and IFN was 10 times more effective than would be expected from their individual application. We consider the effect observed in our experiments to be an additive one, since the inhibition caused by the combined use of IFN and amantadine never exceeded the effects of the single agents by a factor of two. One possible explanation for this variance from the results of Lavrov et al. [5] may be the differences between organ and tissue cultures. Rimantadine has also been shown to exert an additive or synergistic effect with IFN against influenza virus in tissue cultures [16]. The data warrant the evaluation of possible combinations of amantadine and IFN for the chemoprophylaxis and chemotherapy of influenza.

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EFFECT OF AMINO ACIDS ON THE EXPRESSION OF ANTIVIRAL ACTIVITY OF DIFFERENT TYPES OF HUMAN INTERFERON

I. EFFECT OF SINGLE AMINO ACIDS*

S. TÓTH and I. MÉCS

Institute of Microbiology, University Medical School, Szeged

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It has been observed that certain amino acids may influence the antiviral activity of different types of human interferon (IFN). The effect may be enhancing or inhibitory. A qualitative survey is provided on all three types of human IFN using 23 different amino acids. A more detailed study was carried out with 10 amino acids which proved to be active towards HuIFN-α. Their influences on the synergistic antiviral effects of HuIFN-α and HuIFN-γ were also investigated. The dose-dependence curves of their effects on HuIFN-α were established.

The production of human leukocyte IFN does not require the presence of amino acids and vitamins [1–3]. They are not necessary for the priming activity of any types of human IFN and the priming effect is not influenced by them. In order to investigate whether amino acids have any influence on the properties and/or activities of the IFN system, we tested the effect of most of the naturally occurring amino acids on the antiviral activity of different types of human IFN. Preliminary results of these experiments are presented here.

Materials and methods

Human leukocyte interferon (HuIFN-α). HuIFN-α was produced in a mixed leukocyte suspension culture induced with Sendai virus (Cantell strain) by a method discussed elsewhere [1, 2, 4].

Human fibroblast interferon (HuIFN-β). HuIFN-β was induced in a human amnion (WISH) cell monolayer with 200 HAU Sendai virus/10^6 cells. Three-day-old monolayer culture (approximately 1.5×10^6 cells/dish) grown in Eagle's medium (Glasgow modification) containing 10% fetal calf serum were treated with 0.1 ml of a partially purified [2] Sendai virus suspension containing 3000 HAU/ml for 1 h. Cultures were washed two times in Hanks' balanced salt solution, and were then supplied with 3 ml fresh culture medium and kept at 37 °C in a 5% CO_2 air atmosphere. Supernatants were harvested after 20 h, and were regarded as crude HuIFN-β preparations.

Human immune interferon (HuIFN-γ). Crude HuIFN-γ preparations were made in a mixed human leukocyte culture by a routine procedure described earlier [5, 6]. Purified HuIFN-γ used in synergism experiments was kindly provided by Dr. M. Krim (Sloan Kettering Memorial Research Center, New York, USA).

Amino acids. All amino acids tested were pro analysi products of Reanal Pharmaceutical Works, Budapest, Hungary.

SÁNDOR TÓTH, IMRE MÉCS
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

Interferon assay. Antiviral assays of all three types of human IFN were carried out on the WISH human amnion cell line against vesicular stomatitis virus (VSV) in Cooke microtitre plates. Cells were incubated in Eagle's medium (Glasgow modification) containing 10% bovine serum in wells of microtitre plates for 48 h. The culture medium was then changed for a fresh one containing 5% bovine serum, and a series of twofold dilution steps of IFN samples were made. Cells were further incubated for 24 h at 37 °C in 5% CO_2 air atmosphere, then washed and challenged with 100 $TCID_{50}$ of VSV/well. Antiviral effects of IFN samples were evaluated under a light microscope after 24 h, on the basis of the cytopathic effect. Amino acids, when tested, were added to the replacement medium simultaneously with IFN samples. All titres were expressed in international units (G-023-901-527 reference standard, NIH, Bethesda). The results of IFN assays are given as percentages of corresponding controls.

Results

General findings. The effects of 23 natural amino acids were tested on the antiviral activity of HuIFN-α, β and γ. All amino acids were applied at 10 mg/ml, except L-Arg, L-Lys, and L-Try, which proved to be toxic at this concentration, and these were therefore used at 5 mg/ml concentration. Table I pre-

Table I

Effect of single amino acids on antiviral activity of different types of human interferon; a qualitative survey

Amino acid	HuIFN-α	HuIFN-β	HuIFN-γ
L-α-Alanine	Ø	Ø	Ø
L-β-Alanine	Ø	+	—
L-Arginine*	Ø	Ø	Ø
L-Asparagine	Ø	Ø	—
L-Aspartic acid	+	+	+
L-Cysteine**	—	—	—
L-Glutamine	Ø	—	—
L-Glutamic acid	—	—	—
D-Glutamic acid	Ø	+	+
L-Glycine	+	Ø	Ø
L-Histidine	—	—	—
L-Isoleucine	Ø	—	—
L-Leucine	Ø	—	—
L-Lysine*	Ø	Ø	Ø
L-Methionine	—	—	—
L-β-Phenylalanine	—	—	—
L-Oxyproline	+	—	—
L-Proline	Ø	Ø	Ø
L-Serine	+	+	+
L-Threonine	Ø	Ø	Ø
L-Tryptophan*	Ø	Ø	Ø
L-Tyrosine**	+	Ø	Ø
L-Valine	Ø	—	—

Meaning of symbols: + = enhancement; — = inhibition; Ø = no influence

* These amino acids were cytotoxic at 10 mg/ml. The results were obtained at 5 mg/ml

** Cysteine and tyrosine were applied in saturated solution (0.1 and 0.4 mg/ml, respectively)

sents a qualitative survey of the results obtained. The tested amino acids were divided into three different groups from the aspect of their activity: enhancing, inhibitory or indifferent. Each group had different members in relation to different IFN types. Some amino acids had a similar influence on all IFN types (e.g. L-Asp, L-Cys), while others had different effects on different types (e.g. L-β-Ala, L-Oxypro) or acted on only one of the three IFN types (e.g. L-α-Ala, L-Gly).

For further investigation we have chosen 10 amino acids. The basis of choice was their stimulatory or inhibitory activity towards HuIFN-α. (In the following text they will be referred to as α-active amino acids.) Table II shows

Table II

Effect of α-active amino acids on antiviral activity of different human interferon types

Amino acid	HuIFN-α (% + SD)	HuIFN-β (% + SD)	HuIFN-γ (% + SD)
L-Aspartic acid	148.4 $\pm$ 32.4	225.0 $\pm$ 18.2	157.0 $\pm$ 7.1
L-Cysteine	82.0 $\pm$ 17.5	65.0 $\pm$ 8.4	52.5 $\pm$ 0.7
L-Glutamic acid	48.8 $\pm$ 10.6	N. a.	41.0 $\pm$ 5.7
L-Glycine	212.2 $\pm$ 44.6	109.2 $\pm$ 14.4	99.3 $\pm$ 6.4
L-Histidine	12.8 $\pm$ 6.8	N. a.	N. a.
L-Methionine	31.7 $\pm$ 11.7	N. a.	N. a.
L-Oxyproline	146.2 $\pm$ 45.5	73.5 $\pm$ 17.7	N. a.
L-β-Phenylalanine	N. a.	N. a.	N. a.
L-Serine	243.0 $\pm$ 41.0	425.5 $\pm$ 89.7	480.7 $\pm$ 165.7
L-Tyrosine	149.2 $\pm$ 33.6	97.5 $\pm$ 9.2	101.2 $\pm$ 9.9

Effect of amino acids expressed as percentage of untreated corresponding IFN control titres. Data obtained in five independent experiments
N. a. = no detectable antiviral activity

Table III

Influence of five α-active amino acids on synergistic antiviral effect of HuIFN-α and HuIFN-γ

Amino acid	Percentage of nominal titre (%)	Percentage of synergistic titre (%)
Aspartic acide	399.0	126.0
β-Phenylalanine	N. a.	N. a.
Methinonine	N. a.	N. a.
Serine	859.3	270.7
Tyrosine	263.5	79.0

250 International units of HuIFN-α and 250 U (units expressed in HuIFN-α standard) of HuIFN-γ were applied simultaneously as synergistic control. The sum of the independent titres of IFN-α and -γ was regarded as nominal titre
Data are means from three independent experiments
N. a. = no detectable antiviral activity

the quantitative value of their effect on the antiviral activity of all three human IFN types.

Dose-dependence of effects of α-active amino acids. Dose-dependence experiments on HuIFN-α were carried out with all α-active amino acids, and the corresponding curves were calculated by regression analysis (second grade). It could be observed that all were saturation curves. Except for serine (Fig. 1)

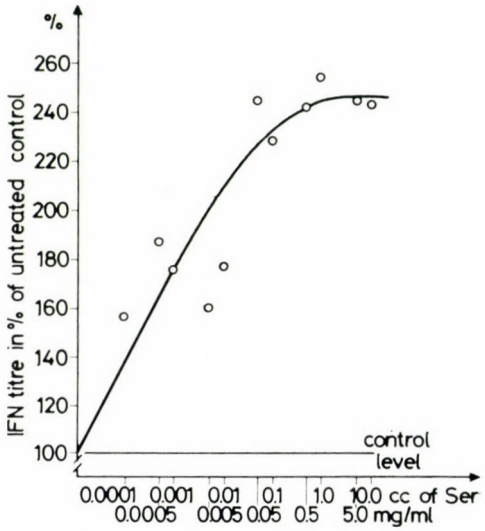

Fig. 1. Dose-dependence curve of effect of serine on antiviral activity of HuIFN-α. Data are means of results of three independent experiments

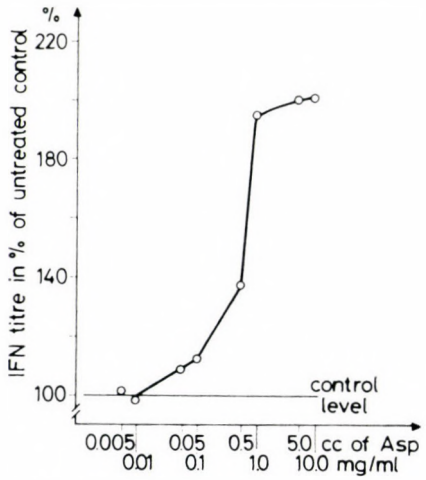

Fig. 2. Dose-dependence curve of effect of aspartic acid on antiviral activity of HuIFN-α: example of a stimulatory sigmoid saturation curve. Data are means of results of two independent experiments

which had a convex saturation curve, all curves were sigmoid in shape, regardless of the type of effect (Figs 2 and 3).

Antiviral synergism and amino acids. HuIFN-α and HuIFN-γ each stimulated the antiviral activity of the other in a synergistic way [5]. In our experiments we used HuIFN-α and HuIFN-γ in 1 : 1 ratio, and obtained 3.2–4.2 times higher titres than the nominal titre (sum of HuIFN-α and -γ applied). Testing 4 amino acids which had the same effect on both HuIFN-α and HuIFN-γ (two stimulators and two inhibitors), we found that they influenced even the synergistic (already apparent) titres in a similar direction and to a similar extent as the separate IFN types (Table III). Tyrosine, which influenced only HuIFN-α, slightly decreased the synergistic titre.

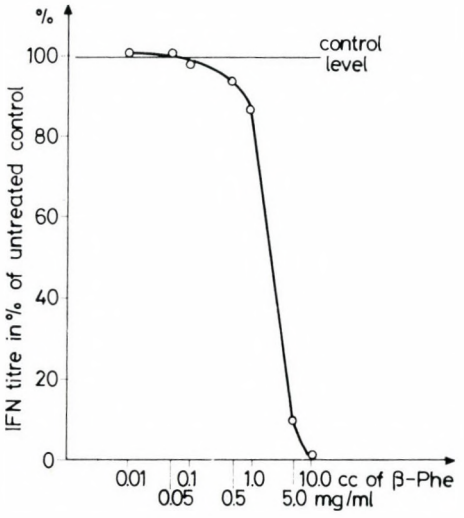

Fig. 3. Dose-dependence curve of effect of β-phenylalanine on antiviral activity of HuIFN-α: example of an inhibitory sigmoid saturation curve. Data are means of results of two independent experiments

Discussion

It is known that amino acids may exert regulatory functions on biological activities which are not related with their biochemical pathways. For example, leucine, arginine and lysine trigger insulin secretion in the presence of subliminal amounts of glycose [7]. Still, we could not find any evidence of a report on the influence of amino acids on the antiviral properties of human (or any) IFN. We have therefore examined this question. We observed (Table I) that five amino acids stimulated the antiviral activity of HuIFN-α, four that of HuIFN-β, and three of IFN-γ; while aspartic acid and serine did this with

all three types of IFN. We suggest that this general effectiveness may be of great importance in establishing their mechanisms of action. The same holds for general inhibitors such as cysteine, L-glutamic acid, histidine, methionine and β-phenylalanine. Other amino acids, acting in different ways on the different HuIFN types, might shed light on the disparities of the biological properties of human IFNs, providing a cheap and easily accessible tool for biochemical investigations. The data in Table II reveal that active amino acids may be divided further into "strong" and "weak" effector groups. When experimentation turns to the in vivo system, the "strongest" ones (serine and β-phenylalanine) should be the most promising effectors.

In our experiments we applied extremely high doses of amino acids. It can be seen from the dose-dependence curves that lower concentrations have weaker or no effect, the only exception being serine, which caused a significant enhancement even at 10^{-4} mg/ml. Under these conditions the question arises as to whether the phenomenon observed (again with the exception of serine) is some kind of artifact. Still, conclusions must not be drawn too quickly as some of our later observations provided evidence against the suggestion [8]. Meanwhile, it may be claimed that serine even in physiological concentrations was an effective stimulant of the antiviral activity of all types of HuIFNs.

Of all the observations described in the present paper, the stimulatory effect of serine on the synergism between HuIFN-α and -γ seemed to be interesting. If serine proves to be active in vivo, then the cost of treatment of viral diseases with IFN could be lowered to about one-tenth of the current one.

The mechanism of action of the antiviral effect of IFNs is still unclear. We hope that our findings will add a new aspect and a useful tool to such investigations.

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EFFECT OF AMINO ACIDS ON THE EXPRESSION OF ANTIVIRAL ACTIVITY OF DIFFERENT TYPES OF HUMAN INTERFERON

II. EFFECTS OF VARIOUS AMINO ACID PAIRS*

S. TÓTH and I. MÉCS

Institute of Microbiology, University Medical School, Szeged

(Received December 13, 1984)

Seven amino acids which influence the antiviral activity of IFN-α were tested in various pairs on human leukocyte and immune IFN and on their synergistic antiviral effect. Different kinds of interactions were observed between amino acids, ranging from extinction through competition to synergism. In some cases, different types of interactions could be observed in relation to HuIFN-α and HuIFN-γ.

It has been reported [1] that certain amino acids influence the antiviral activity of human interferons (IFNs). Some of them are even capable of affecting synergistic cooperation between HuIFN-α and HuIFN-γ. In our experiments we tested whether these effects can be modified further by applying two amino acids simultaneously. We hoped to shed light on the problem of their mode of action by analysing their interactions.

Materials and methods

All materials and methods used in this series of experiments were identical to those described in the first part of this study [1].

Results

HuIFN-α. Seven α-active (having either an enhancing or an inhibitory effect on HuIFN-α) amino acids were tested in pairs on the antiviral activity of HuIFN-α. The amino acids were added to the antiviral assay system at 5 mg/ml in fresh medium immediately prior to IFN. Table I shows the effects of the individual members of the pairs alone, their joint effect, and the probable type of their interaction. It can be seen that the most frequent result is competition, the joint effect being somewhere between the individual ones. However, some amino acids tend to predominate over their counterpart (e.g. aspartic acid seems to play an inferior role to half of its partners, while oxyproline in combination predominates over the two most powerful amino acids). The most remarkable phenomenon is the synergistic cooperation of serine with aspartic acid and with tyrosine.

SÁNDOR TÓTH, IMRE MÉCS
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

Table I

Combined effects of active amino acid pairs on antiviral activity of HuIFN-α

Combination 1 + 2	Percentage of untreated control			Probable type of interaction*
	effect of amino acid 1	effect of amino acid 2	combined effect	
Asp + Gly	141.7	119.0	119.0	Gly dominance
Asp + Met	141.7	N. a.	N. a.	Met dominance
Asp + Oxypro	141.7	109.0	154.7	addition
Asp + β-Phe	141.7	N. a.	N. a.	β-Phe dominance
Asp + Ser	141.7	274.0	468.3	synergism
Asp + Tyr	141.7	146.5	117.2	extinction
Gly + Oxypro	119.0	109.0	150.0	addition
Gly + β-Phe	119.0	N. a.	133.3	Gly dominance
Gly + Ser	119.0	274.0	168.3	competition
Gly + Tyr	119.0	146.5	124.0	competition
Met + β-Phe	N. a.	N. a.	N. a.	?
Met + Ser	N. a.	274.0	124.0	competition
Met + Tyr	N. a.	146.5	114.0	competition
Oxypro + β-Phe	109.0	N. a.	114.0	Oxypro dominance
Oxypro + Ser	109.0	274.0	130.3	Oxypro dominance or competition
Oxypro + Tyr	109.0	146.5	129.0	competition
β-Phe + Ser	N. a.	274.0	169.0	competition
β-Phe + Tyr	N. a.	146.5	93.0	competition
Ser + Tyr	274.0	146.5	584.0	synergism

Data are means of results of 3 independent experiments

* In determining the type of interaction, we considered the mathematical significance of the data obtained, as well as the competence of the assumed interaction types in comparison to other interactions of the same amino acids in other combinations

N. a. = no detectable antiviral activity observed

HuIFN-γ. Five α-active amino acids (four of them were also active towards HuIFN-γ) were tested in these experiments (Table II). Most of the data corroborate the results found with HuIFN-α. The two differences in interaction types, the predominance of aspartic acid and of β-phenylalanine over tyrosine, can be explained by the fact that tyrosine has no effect on HuIFN-γ.

Synergism of HuIFN-α and HuIFN-γ. These tests were carried out with the same five amino acids as in the HuIFN-γ experiments. Data in Table III are in very good correlation with our previous results [1] concerning the effects of single amino acids on synergism. They also confirm the suspected nature of interactions between amino acid pairs, the only exception being the competition between aspartic acid and tyrosine. It is possible that this is only an apparent competition, being in fact an extinction between a weak enhancer and a similarly weak inhibitor.

Table II

Combined effects of active amino acid pairs on antiviral activity of HuIFN-γ

Combination 1 + 2	Percentage of untreated control			Probable type of interaction*
	effect of amino acid 1	effect of amino acid 2	combined effect	
Asp + Met	225.0	N. a.	N. a.	Met dominance
Asp + β-Phe	225.0	N. a.	N. a.	β-Phe dominance
Asp + Ser	225.0	463.2	1467.3	synergism
Asp + Tyr	225.0	100.0	207.5	Asp dominance
Met + β-Phe	N. a.	N. a.	N. a.	?
Met + Ser	N. a.	463.2	164.0	competition
Met + Tyr	N. a.	100.0	28.4	competition
β-Phe + Ser	N. a.	463.2	230.5	competition
β-Phe + Tyr	N. a.	100.0	N. a.	β-Phe dominance
Ser + Tyr	463.2	100.0	897.5	synergism

See footnotes to Table I

Table III

Combined effects of active amino acid pairs on synergistic antiviral effect of HuIFN-α and HuIFN-γ

Combination 1 + 2	Percentage of untreated control			Probable type of interaction
	effect of amino acid 1	effect of amino acid 2	combined effect	
Asp + Met	121.3	N. a.	N. a.	Met dominance
Asp + β-Phe	121.3	N. a.	N. a.	β-Phe dominance
Asp + Ser	121.3	331.0	667.0	synergism
Asp + Tyr	121.3	75.7	104.3	competition
Met + β-Phe	N. a.	N. a.	N. a.	?
Met + Ser	N. a.	331.0	232.9	competition
Met + Tyr	N. a.	75.7	34.3	competition
β-Phe + Ser	N. a.	331.0	106.2	competition
β-Phe + Tyr	N. a.	75.7	N. a.	β-Phe dominance
Ser + Tyr	331.0	75.7	1315.8	synergism

See footnotes to Table I

Discussion

This study was planned to find out whether α-active amino acids have the same target point(s) in the flow of biochemical events resulting in the development of an antiviral state in IFN-treated cells, or whether they act on different ways, even though their modes of action are unknown. Table I reveals different types of interactions, which means that there is more than one mode of action

of amino acids. The picture is further complicated by the contradictory relations displayed by certain amino acids in different combinations. (For example, oxyproline predominates over β-phenylalanine, which means either a much stronger affinity for the same receptor site, or prevention of the action by blocking, while both seem to be in competition with serine and tyrosine, meaning a nearly similar affinity for the same receptor site or an identical mode of action. Further, serine and tyrosine act in a synergistic way, which excludes competition between them for a common receptor site.) To explain these contradictions, we must suppose that some of the active amino acids do not interfere with IFN receptors, and that they do not take part in those direct biochemical events which lead to antiviral state either. It is more probable that they act at a regulatory level. Considering the intricate web of interactions between them, it is possible that they influence allosteric conformations of an enzyme or some enzymes. The relations between serine and tyrosine, serine and aspartic acid, and tyrosine and aspartic acid are reminiscent of the relations of nucleotide triphosphates in the regulation of cytidine-synthetase [2]. On the other hand, the competition of these three amino acids with others may exist at a receptor level. An evidence (article in preparation) suggests that certain amino acids (e.g. cysteine) act directly on biochemical events leading to an antiviral state. We suggest, therefore, that amino acids influence the IFN-induced antiviral state at different levels and that they have different modes of action. It should be stressed that this reasoning, along with the fact that serine can be synergized by tyrosine and aspartic acid, excludes the probability of our results being artifacts, despite the high concentrations required. Last but not least, we would like to point out that the synergistic antiviral effect of HuIFN-α and -γ (Table III) can be further enhanced by serine and tyrosine applied together, by a factor of more than 13 times, which adds up to nearly 40 times the nominal titre. Such data reveal the importance of further investigations as to whether amino acids influence the IFN-induced antiviral state in vivo.

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EFFECT OF HUMAN ADENOVIRUS ON NATURAL KILLER CELL ACTIVITY IN MICE*

YVETTE MÁNDI, GY. SEPRÉNYI and ROZÁLIA PUSZTAI

Institute of Microbiology, University Medical School, Szeged

(Received December 13, 1984)

The influence of human adenovirus type 6 on the natural killer cell activity in mice has been investigated. The cytotoxic effect of splenic mononuclear cells of different mouse strains (Balb/c, CBA and BlO) against YAC-1 cells was estimated using a ^{51}Cr release system assay. The effector cells responsible for cytotoxicity were identified by Percoll density gradient fractionation as LGL cells. A single intraperitoneal injection of the virus enhanced the natural killer activity in all three strains of mice. The cytotoxicity was enhanced 24 to 48 h after the virus injection, depending on the host strains, but it then decreased and the preinjection level was reached after 72 h. A good correlation was found between the augmented cytotoxicity and interferon induction.

The natural killer (NK) system is one of the defense mechanisms which is exhibited even by nonsensitized hosts. It is thought to play a role in tumour resistance [1] and may also be effective against infections [2]. Interferon and interferon inducers are well known enhancing agents of NK cell activity in humans, animals and leukocyte cultures. Viruses such as NVN, Kunjin virus, LCM virus and murine CMV stimulate NK activity within a few days after infection in mice [3–6]. It seemed us of interest to investigate the effect of human adenovirus on NK activity in mice, and the possible mechanism responsible for the alteration in NK activity.

Materials and methods

Mice. Inbred male SPF Balb/c, and BlO mice were obtained from BRC, Szeged. The mice were 6–8 weeks old.

Virus. Human adenovirus type 6 was grown in HEp-2 cells, purified by CsCl gradient centrifugation and stored at $-70\,^{\circ}C$ in 20% glycerol. Mice were inoculated with 2.5×10^9 $TCID_{50}$ of virus diluted in physiological saline.

Preparation of effector cells. Mice were sacrificed by cervical dislocation or by bleeding. Spleen cell suspensions were prepared individually in a glass homogenizer and were passed through a stainless steel mesh. Cells were washed and resuspended in RPMI 1640 medium with gentamicin (100 μg/ml) and 10% fetal bovine serum (referred to below as culture medium).

Mononuclear cells were isolated by Ficoll-Uromiro gradient centrifugation [7] adjusted to a density of 1080. After three washings, the cells were resuspended in the culture medium and viability was tested in the Tripan blue exclusion test. The viability always exceeded 90%.

YVETTE MÁNDI, GYÖRGY SEPRÉNYI, ROZÁLIA PUSZTAI
Institute of Microbiology, University Medical School
Dóm tér 10, 6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferom

Isolation of NK cells. Discontinuous density gradients were made using isotonic Percoll solution with a slight modification of the Timonen method [8]. Briefly, 2.5 ml volumes of Percoll dilutions were layered in 15 ml tubes, starting with 70% Parcoll and shifting in 5% steps to 50%. $2–5 \times 10^7$ cells, depleted from adherent cells by incubation for 45 min in plastic dishes at 37 °C, were added at the top of the gradient, and the tubes were centrifuged at 450 *g* for 30 min at room temperature. The cells at the interface of each fraction were collected, washed twice in culture medium, counted and used in the experiments. Smears were made from each cell fraction and stained by May-Grünwald-Giemsa staining.

Cytotoxicity assay. A ^{51}Cr release assay was used, employing YAC-1 [9] cells as target cells, with labelling for 45 min at 37 °C with 20 μCi $Na(^{51}Cr)O_4$ (LNK, Poland). The effector-to-target ratios were 200 : 1 to 25 : 1. The assay incubation time was 4 h and spontaneous release was usually 12–15%. The percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{cpm in supernatant} - \text{cpm in spontaneous release}}{\text{total incorporated activity}} \times 100.$$

Interferon assay. Mice were bled and the interferon content of the serum was determined individually. Interferon was assayed by a conventional cytopathic effect inhibition assay, performed in microtiter plates [10] with adherent L929 cells as the indicator cells and vesicular stomatitis virus as the challenge virus. The titer of interferon was expressed as NIH reference (G-002-905-511. NIH, Bethesda, Maryland, USA) units (U) per ml.

Results

Considerable cytotoxicity against the YAC-1 cell line was found in the mononuclear spleen cells, of all three strains of mice. The level of cytotoxicity ranged between 20–25%, for Balb/c, CBA and BlO mice. In agreement with observations of others [11] we found that the cytotoxicity decreased in the

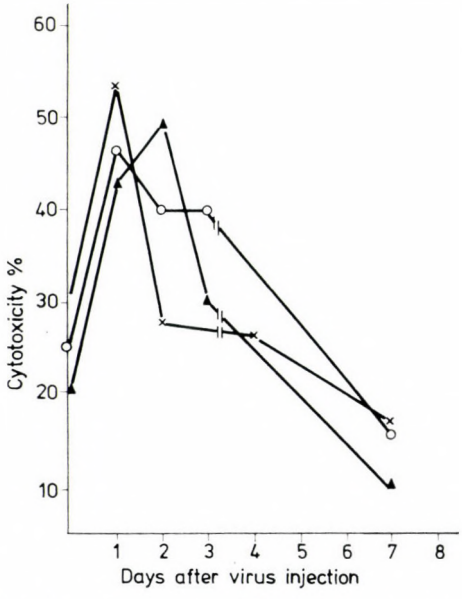

Fig. 1. Effect of human adenovirus type 6 on NK activity in mice. Mice were injected intraperitoneally with 2.5×10^9 $TCID_{50}$ of purified virus (6 mice per group). Spleen cells were tested against YAC-1 targets. Symbols represent mean percentage of cytotoxicity for groups of 6 mice. Effector-to-target ratio = 200 : 1. ▲——▲ Balb/c; ○——○ CBA; ×——× BlO

animals after 14 weeks of age, and only negligible cytotoxicity was shown at 6–8 months. Accordingly, 6–8-week-old mice were used subsequently.

Characterization of the effector cells. As mononuclear leukocytes were used without any specific purification for NK cells, in pilot experiments it was necessary to show that the cells responsible for cytotoxic activity were in fact NK cells. The method of Timonen and Saksela [8] was adapted taking into account the different physical characteristics of mouse cells [12]. Most of the LGL cells as well as the NK activity against YAC-1 cells were found in the low-density fraction after discontinuous density centrifugation at 55–60% Percoll, with densities of 1062 and 1068, respectively. A 7–10 fold increase in cytotoxicity was observed when the cells from these fractions were applied as effector cells, and the enrichment of the LGL cells was about 20–30%. The relatively low enrichment is very probably due to the low LGL percentage of the input spleen cells.

Natural cytotoxicity following adenovirus infection. Cytotoxicity was studied 1, 2, 3, 4, and 7 days after virus infection. Experimental groups of animals contained 6 mice at each time indicated. An elevated cytotoxicity was seen 24 h after adenovirus inoculation in CBA and BlO mice, with a more pronounced augmentation at 48 h in Balb/c mice. Thereafter the cytotoxicity decreased, but it was still above the basal level on the third day in the Balb/c

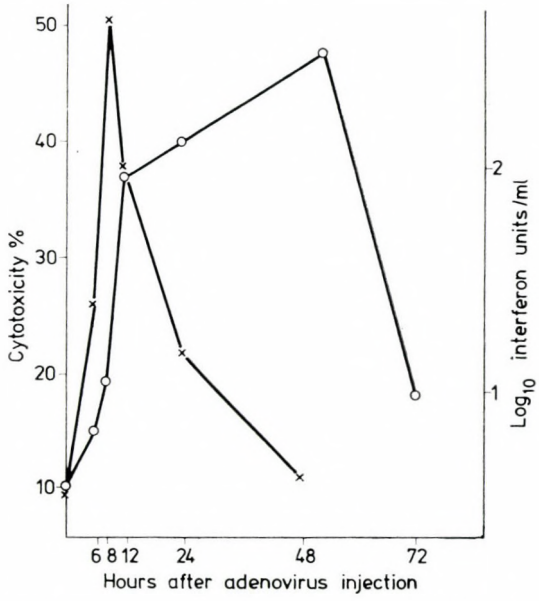

Fig. 2. Relationship between augmented cytotoxicity and serum interferon level. Balb/c mice were injected intraperitoneally with 2.5×10^9 $TCID_{50}$ of purified human adenovirus type 6. For each group (6 mice) the spleens were processed for cytotoxicity against YAC-1, and the sera were used individually for interferon assay. Symbols represent mean values for groups of 6 mice. ×——× IFN; ○——○ cytotoxicity

and CBA mice (Fig. 1). By day 7, cytotoxicity was depressed as compared to the control group.

Relationship between augmented cytotoxicity and IFN levels. After virus infection Balb/c mice (6 in each experimental group) were bled and the circulating interferon was measured in the serum of each mouse individually. The spleen cells of the same mice were processed for NK activity. An augmentation in spleen cell cytotoxicity was seen over the period 24–48 h with a return to the control levels by 72 h (Fig. 2). The serum interferon response was of shorter duration and peaked at 8 h.

Discussion

Many viruses have been shown to augment NK activity and it is widely accepted that the advent of NK cell activity is correlated with the synthesis of interferon in virus-infected animals [13]. We have recently demonstrated that human adenovirus enhances the ADCC in chickens [14] and we assumed that IFN induced by the virus was responsible for the effect [15]. In this paper we describe the induction of altered NK cytotoxicity in mice infected with human adenovirus type 6. The virus enhanced the NK activity in all three strains of mice tested, and we presume a correlation between the serum interferon and the cytotoxicity. Adenovirus type 6 has been shown to induce circulating interferon in the experiments of Berencsi et al. [16], but no correlation was found between the immunosuppressive effect of the virus and interferon induction. The immunosuppressive effect was observed by these authors when Ad_6 was inoculated 3–11 days before SRBC administration. This effect of the virus is delayed when compared with the augmentation of the natural cytotoxicity, which occurs within the first 48 h of encounter. The relatively short interval of interferon production and NK stimulation might be due to the fact that human adenovirus does not multiply in mice; however, the persistence of human adenovirus with contaminant viraemia has been described in infection of mice [17]. A rather high amount of infectious virus was therefore needed to achieve stimulation (2.5×10^9 $TCID_{50}$). Lower doses of virus had no influence on the cytotoxic activity. Theoretically, the virus infection itself could affect the effector lymphocytes, but the correlation between the kinetics of interferon production and NK augmentation lead us to suppose that interferon was responsible for the stimulation of NK activity after adenovirus infection. We are currently investigating the significance of the drop of NK activity at the 7th day of infection.

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COMPARATIVE STUDY OF ANTIPROLIFERATIVE EFFECTS OF CHLORPROMAZINE, 7,8-DIOXOCHLORPROMAZINE, AMANTADINE-N-MUSTARD, RUTIN-N-MUSTARD AND ALPHA, BETA AND GAMMA INTERFERON ON K-562 CELLS IN VITRO*

J. Molnár, B. Prágai, Klára Berencsi, Yvette Mándi and S. Földeák

Institute of Microbiology, University Medical School, and Institute of Organic Chemistry, József Attila University, Szeged

(Received December 13, 1983)

The effects of rutin-N-mustard, amantadine-N-mustard, chlorpromazine and human interferon types α, β and γ (IFN-α, -β and -γ) were studied on the DNA, RNA and protein synthesis of K-562 cells. Monocyte-mediated cytotoxicity and immune spleen cell activity were examined in the presence of the same compounds (except for IFN-β). The natural killer (NK) cell activity was tested in the presence of the two chlorpromazine compounds and the two N-mustard derivatives. Only 7,8-dioxochlorpromazine exerted an inhibitory effect on DNA synthesis. The protein synthesis of the cells was inhibited in the presence of IFN-α, -β and -γ. 7,8-Dioxochlorpromazine exerted some inhibition on both NK and immune spleen cell activity, while monocyte-mediated cytolysis was not altered. IFN-α, -β and -γ activated the cytolytic activity of monocytes and the NK activity in control experiments. Chlorpromazine, rutin-N-mustard and amantadine-N-mustard were ineffective in both tests in vitro. Rutin-N-mustard, 7,8-dioxochlorpromazine and the interferons may be assumed to have quite different antiproliferative mechanisms of actions.

Béládi et al. [1, 2] have described that some flavonoids, e.g. tannic acid, rutin, quercetin, etc., inhibit multiplication of various Herpes viruses. Later, Mucsi et al. [3] pointed out that flavonoids affect only enveloped viruses. One of the flavonoid derivatives, rutin-N-mustard, exerts a marked antitumour effect in mice [4, 5]. Some chlorpromazine derivatives which interact with various macromolecules of different cells also exerted antiviral effects in vitro [6] and antitumour effects in mice [7]. The antiviral effects of various types of interferons are well known and experiments on antitumour action, too, have been reported [8]. The three groups of compounds all display antiviral and antiproliferative effects in vivo. In the present paper, the effects of chlorpromazine, 7,8-dioxochlorpromazine, rutin-N-mustard, amantadine-N-mus-

József Molnár, Béla Prágai, Klára Berencsi, Yvette Mándi
Institute of Microbiology, University Medical School
Dóm tér, H-6720 Szeged, Hungary

Sándor Földeák
Institute of Organic Chemistry, József Attila University
H-6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

tard and human interferon of alpha, beta and gamma types (IFN-α, -β and -γ) were studied on the macromolecular synthesis (DNA, RNA and protein) of K-562 cells, the natural killer (NK) cell activity and monocyte-mediated cytotoxicity. Some preliminary experiments were also carried out to study the immunosuppressive effects of the tested compounds, by which tumour growth might have been influenced.

Materials and methods

Materials. Chlorpromazine and 7,8-dioxochlorpromazine (Fig. 1) were obtained from EGYT Pharmaceutical Works, Budapest and National Institute of Mental Health, Rockville, Md., respectively. Rutin-N-mustard and amantadine-N-mustard (Fig. 2) were synthetized by one of us (S. F.). Human IFN-α, -β and -γ were produced in the Institute of Microbiology. Tissue culture: K-562 cells grew in RPMI medium for 24 h (number of cells, 2.0×10^6/ml). Radioactive compounds, ^{3}H-thymidine, ^{3}H-uracil and ^{14}C-protein hydrolysate (Amersham) were applied from 1 μCi stock solution.

Antigen. Sheep red blood cells (SRBC) were stored in Alsever's solution at 4 °C and washed 3 times with physiological saline on the day of use. Male CELP mice 8–12 weeks old were injected intraperitoneally with 0.2 ml of a suspension containing approximately 5×10^8 erythrocytes.

Measurement of macromolecular synthesis. The effects of the compounds on DNA, RNA and protein synthesis were determined by the modified method of Rivest et al. [9]. The K-562 cells were grown in RPMI medium distributed in 1.0 ml aliquots. The compounds tested were added to the cell cultures in quantities of 0.5–50.0 μg/ml. The amount in the case of IFN-α was 1700–17 000; IFN-β from 1500–3000, and IFN-γ from 125–500 IU/ml. The cells were incu-

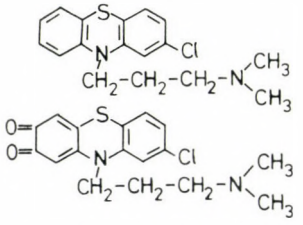

Fig. 1. Chlorpromazine (upper formula) and 7,8-dioxochlorpromazine (lower formula)

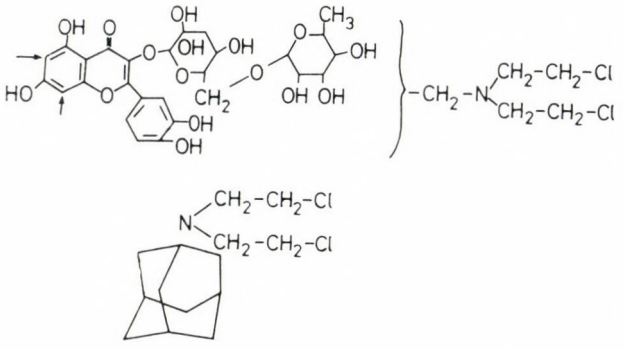

Fig. 2. Rutin-N-mustard (upper formula) and 1-bis(-2-chlorethyl)-amantadine. HCl (lower formula)

bated with the compounds for 120 min at 37 °C, and after adding 0.02 μCi thymidine, uracil or ^{14}C proteinhydrolysate to each sample, further incubated at 37 °C for 180 min. One ml of cold PBS solution was added to the samples and they were filtered through CF/C filters. The cells were washed with 5.0 ml PBS with 10 ml trichloracetic acid (10%) and finally with 1.0 ml cold ethanol (70%, v/v). The filters were dried in scintillation vials, and the radioactivity was measured in the presence of 5.0 ml scintillation fluid for 1 min using a Packard-Tri-Carb 4530 scintillation spectrometer; the results were expressed in dpm.

NK assay. Human peripheral blood mononuclear cells were isolated from healthy volunteers by Ficoll-Uromiro centrifugation [10]. The cells from the interface layers were washed three times in phosphate buffered saline (PBS) and then resuspended in RPMI 1640 medium with 10% FCS. Chlorpromazine, 7,8-dioxochlorpromazine rutin-N-mustard and amantadine-N-mustard were added to 5×10^6 lymphocytes to yield a final concentration of 0.5, 5, 10 or 50 μg/ml, respectively. Effector cells were incubated for 1 h in the presence of the above compounds and used in the cytotoxicity assay. ^{51}Cr-labelled K-562 cells were used in triplicate in microtitre plates. Effector cells and targets were diluted to obtain killer-to-target ratios of 50 : 1, 25 : 1, 12 : 1 and 6 : 1. After incubation at 37 °C for 4 h, the amount of ^{51}Cr in each supernatant was determined with a gamma counter. From the average of triplicate release values, the percentage cytotoxicity was determined via the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{supernatant cmp} - \text{spontaneous cpm}}{\text{incorporated activity cpm}} \times 100$$

Assay of effects of substances on splenic plaque-forming cells in vitro. Mice were immunized with SRBC and 4 days later the spleens were removed. A spleen cell suspension was prepared in a glass homogenizer. To 0.9 ml aliquots of two dilutions of the spleen cell suspension 0.1 ml of the tested compound was added. After incubation for 2 h in an ice-cold water bath, each cell suspension was plated in two Petri dishes and the number of HPFC was determined by the Jerne method as modified by Dreasser and Wortis [11]. The plates were incubated at 37 °C for 2 h in an air atmosphere containing 5% CO_2. High-efficiency haemolysin-producing cells, referred to as 19S HPFC, were detected following the addition of 1 ml of guinea pig complement diluted 1 : 10 and incubated for 45 min.

Isolation and culture of human monocytes. Mononuclear cells were isolated by centrifugation of citrated venous blood through Ficoll-Uromiro, and washed three times in RPMI 1640 medium. Mononuclear cells in RPMI supplemented with 5% bovine serum were added to tissue culture flasks. After incubation for 60 min at 37 °C, the flasks were washed three times to remove the nonadherent cells, and 20 ml of 0.02% EDTA were added. After incubation for 15 min at 37 °C, adherent cells were removed with a rubber policeman. Cells were washed three times in RPMI. Aliquots of 10^5 cells in 100 μl RPMI containing 5% bovine serum were added to U-bottomed micro-wells. The tested substances were added to the cell culture in 100 μl medium and cultured for 24 h.

Assay for target cell lysis. As targets, K-562 cells were used after labelling with ^{51}Cr/$NaCrO_4$ (specific activity 6–11 MBq/mg). To a 16 h culture of labelled K-562 cells monocytes were added (effector-target ratio 30 : 1), and 100 μl supernatant was then removed for radioactivity determination. The percentage of target cell-specific lysis was calculated as by the NK assay.

Results

The effects of three different types of antitumour drugs were studied on the DNA, RNA and protein synthesis of K-562 cells in vitro. 7,8-Dioxochlorpromazine exerted a marked inhibitory effect on thymidine and amino acid incorporation, but such an effect could not be observed in the chlorpromazine-treated cells.

Rutin-N-mustard and its control, amantadine-N-mustard, had no effect on the macromolecular synthesis of tissue culture cells. Surprisingly, IFN-α, -β and -γ elevated the DNA synthesis to various extents, though the RNA and protein synthesis were not altered (Table I).

Table I

Effects of chlorpromazine derivatives, N-mustard derivatives and interferons on DNA, RNA and protein syntheses of K-562 cells in vitro

Compound	Concentration (μg/ml)	Incorporation of		
		^{3}H-thymidine	^{3}H-uracil	^{14}C-amino acids (protein hydrolysate)
None (control)	0.5	363 879	7 580	39 809
	5.0			
Chlorpromazine	0.5	357 707	7 231	32 997
	5.0	347 357	7 921	40 825
7,8-Dioxo-chlorpromazine	0.5	192 644	7 361	41 121
	5.0	14 793	4 919	24 443
Amantadine N-mustard	0.5	350 745	7 342	39 344
	5.0	497 600	6 959	34 271
	50.0	513 078	5 781	28 300
Rutin-N-mustard	0.5	349 224	7 429	38 305
	5.0	439 880	8 246	43 802
	50.0	440 138	6 486	29 867
IFN-α	1 700 IU	593 914	7 506	36 738
	17 000 IU	941 331	5 560	9 977
IFN-β	1 500 IU	892 672	8 665	27 343
	3 000 IU	770 124	2 084	9 848
IFN-γ	125 IU	117 926	7 182	35 471
	500 IU	979 233	11 924	18 799

The uptake of labelled precursors was expressed in dpm

Table II shows that none of the compounds examined caused any augmentation of NK activity. 7,8-Dioxochlorpromazine even markedly decreased the cytotoxicity at a concentration of 5 μg/ml. This concentration led to up to 40% lymphocyte cell death after incubation for 4 h as assessed with the Trypan blue exclusion test. Chlorpromazine itself was ineffective at the same concentration.

Rutin-N-mustard and amantadine-N-mustard did not affect the activity of natural killer cells. Human IFN-α and -γ augmented NK activity in our previous experiments [12]. In other experiments the effects of these antitumour compounds on immune spleen cells were compared (see Tables III and IV). Surprisingly, only 7,8-dioxochlorpromazine exerted a marked inhibitory effect in the studied system. The N-mustard derivatives of rutin and amantadine had

Table II

Effects of chlorpromazine derivatives and N-mustard derivatives on NK activity in vitro

Compound and concentration (μg/ml)		Cytotoxicity % at E : T ratio	
		50 : 1	25 : 1
—	0	37	30
Chlorpromazine	0.5	38	31
	5	40	28
7,8-Dioxochlorpromazine	0.5	37	24
	5	8	4
Rutin-N-mustard	5.0	38	27
	50.0	37	27
Amantadine-N-mustard	5.0	39	29
	50.0	24	15

Table III

Effects of chlorpromazine, 7,8-dioxo-chlorpromazine, rutin-N-mustard and amantadine-N-mustard on the number of HPFC after in vitro treatment of immune spleen cells of mice

Substance and concentration (μg/ml)		Number of HPFC/spleen	Inhibition %
—	0	306 000	—
Chlorpromazine	0 5	292 800	—
	2.5	293 760	—
	5.0	376 300	—
7,8-Dioxo-chlorpromazine	0.5	254 000	17
	2.5	22 000	93
	5.0	9 600	97
Amantadine-N-mustard	0.5	314 000	—
	5.0	331 000	—
	50.0	297 000	—
Rutin-N-mustard	0.5	321 600	—
	5.0	375 000	—
	50.0	296 000	—

Table IV

Cytolytic activity of human monocytes towards K-562 cells after exposure to chlorpromazine, 7,8-dioxochlorpromazine, amantadine-N-mustard and rutin-N-mustard

Concentration of substance in culture medium (μg/ml)	Specific lysis (%) in presence of			
	CPZ	7,8-dioxo-CPZ	amantadine-N-mustard	rutin-N-mustard
—	17	17	17	17
0.5	16	16	—	8
2.5	12	11	ND	
5.0	3.8	6.7	—	11.7
50.0	ND	ND	—	9

Samples were incubated for 24 h at 37 °C

Table V

Cytotoxic activity of human monocytes towards K-562 cells after exposure to IFN-α and -β

Concentration (IU/ml)	Specific lysis (%) in presence of	
	IFN-α	IFN-β
0	17	
10	18	14
100	24	32
1 000	36	36
10 000	24	53

Samples were incubated for 24 h at 37 °C

no effect (Table III). On exposure to chlorpromazine, 7,8-dioxochlorpromazine, and rutin-N-mustard for 24 h, the monocyte-mediated cytolysis of prelabelled K-562 cells was not enhanced at any concentration tested (Table IV). As control, human IFN-α and -γ were used, which are known to enhance the cytolytic activity of monocytes. Incubation of monocytes in the presence of IFNs resulted in a significant activation of the cytotoxic potential of the monocytes (Table V).

Discussion

Many data are available on the cytotoxic effects and reactions with cellular macromolecules of N-mustard derivatives [13, 14]; however, rutin-N-mustard and amantadine-N-mustard were not investigated. The selective and particular cytotoxicity of rutin-N-mustard were reported by Molnár et al. [5], but the exact mechanism of antitumour action is still unknown. It seems that

the N-mustard substituent of this molecule plays an important role in fixing the molecule as a whole into the rutin-directed or oriented place, possibly into the cellular membrane. Rutin itself also has some antineoplastic effect in vivo [4]. Such a large molecule with a highly reactive N-mustard group cannot penetrate the cell membrane. Amantadine-N-mustard was freshly synthetized for our experiments. We had no information about the in vivo antineoplastic effect of this compound, since it had not previously been tested. It was earlier pointed out that 7,8-dioxochlorpromazine [7], rutin-N-mustard [5] and IFN [15] had antitumour or antiproliferative effects in vivo. IFN was able to inhibit the sarcoma virus-induced neoplastic transformation [8]. The NK-cell activity, which represents the natural host defence mechanism, was suppressed in tumour-bearing animals, but IFN and IFN inducers such as poly I : C or RNA were able to restimulate the NK activity [16]. One antitumour compound, 7,8-dioxochlorpromazine, displayed inhibitory action towards the macromolecular synthesis of tumour cells and NK cell activity. On the other hand, this chlorpromazine derivative exerted some immunosuppressive activity, too. We presume that these effects are due to the general toxicity of the compound on ATP-ase [17], since chlorpromazine itself had no such effects at the same concentration. However, in other experiments the chlorpromazine derivative enhanced the longevity of tumour-bearing mice [18], and showed a synergistic effect with hyperthermia in mouse solid tumours [19]. IFNs which did not exert a significant inhibitory effect on the macromolecular synthesis of K-562 cells may have had an antiproliferative effect via activation of the natural mechanism of the host defence, such as NK cells, or by enhancing the monocyte-mediated cytotoxicity, as previously shown [20]. The most interesting compound was rutin-N-mustard. This compound had no inhibitory or enhancing effects on thymidine, uracil or amino acid incorporation, NK activity or monocyte-mediated cytotoxicity. Rutin-N-mustard had no inhibitory activity on immune spleen cells and it might have an antitumour effect, very similarly as for abrin. In the latter case the in vitro pretreated tumour cells induced antitumour immunity in vivo [21]. An antitumour agent, imidazole-4-carboxamide, had a similar mechanism of action; it also increased the tumour-associated immunogenicity in vivo during treatment of leukaemic mice [22]. Such "immunization" may be based on alteration of the tumour cells, which can lead to host-versus-graft responses; however, this type of immunity differs basically from immunization against primary carcinogens such as murine osteosarcoma viruses [23], which can also reduce tumour incidence, but through an other mechanism. We conclude that 7,8-dioxochlorpromazine, rutin-N-mustard and IFNs have different antiproliferative mechanisms of action. The results suggest that it would be worthwhile to study the antitumour effects of these compounds in combination, in the hope that synergism exists between the entirely different antiproliferative effects.

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EFFECT OF ARGININE-BUTYRATE ON INTERFERON INDUCTION BY ADENOVIRUS*

B. Taródi and Rozália Pusztai

Institute of Microbiology, University Medical School, Szeged

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Treatment of human adenovirus type 12 infected chick cells with a low concentration of arginine-butyrate strongly inhibited interferon formation. At this concentration, as evaluated by the rate of protein synthesis the drug exerted no significant toxic effect on the cells. The early virusgene expression, not being affected in the butyrate-treated chick cells, it is autonomous and is not influenced by the effect of butyrate on chick cells. This is at variance with what has been observed in other semipermissive systems infected with adenovirus.

Abortive infection by human adenovirus induces interferon production in chick cells, and for a number of years we have been studying this system with the aim of understanding the events responsible for the induction of interferon. These studies have revealed that the induction does not depend on viral DNA replication [1, 2] or on the synthesis of capsid components. At the same time, the appearance of early viral antigens (P and T) in infected cells has provided evidence that viral specific transcription is one condition of induction. Some evidence implicates an early interaction between virus or virus product and chick cells [3]. The nature of this early event, however, has not yet been clarified. A possible approach to tackle this problem is the use of specific metabolic inhibitors.

Research carried out in the last few years has shown that the salts of butyric acid have a wide variety of effects on mammalian cell cultures; accordingly, this could be a valuable tool for study of the mechanism of gene activation, the regulation of cell growth, etc. (for a review, see Ref. 4). Especially since several publications discussing the possibility that butyrate, acting as a histon deacetylase inhibitor can modulate cellular gene expression, its effect on interferon gene expression has become an intriguing problem. This is obviously a complex phenomenon, which is probably controlled both by the cell type and by the type of inducer. We have therefore studied the effect of butyrate on adenovirus-induced interferon production in chick cells.

Béla Taródi, Rozália Pusztai
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

Materials and methods

Cell cultures. Primary chick embryo fibroblast cells were cultured in Eagle's medium containing 5% calf serum. HEp2 cells were grown as monolayers in glass bottles in the same medium. Monolayers of cells in 60 mm Petri dishes or 6×4 well tissue culture plates (Greiner) were used for interferon induction.

Virus. Adenovirus type 12 (Huie strain) was propagated in HEp2 cells and was purified and titrated as described previously [5]. Purified virus at a multiplicity of 25 $TCID_{50}$/cell was used for infection of chick cells.

Interferon assay. Interferon titration was carried out on secondary chick embryo fibroblast cells plated out in flat-bottomed microtitre plates (96 wells per plate) by the micromethod described previously [3], except that vesicular stomatitis virus (VSV, Indiana strain) was used as challenge virus at a concentration of 6×10^4 p.f.u./well.

Evaluation of T antigen production. Chick cells were grown as monolayers in glass coverslips, and 24 or 48 h after infection were removed from the dish, washed in phosphate-buffered saline and then fixed in acetone at 4 °C. The fixed cells were stained for T antigen and the number of T antigen-producing cells were evaluated by the indirect immunofluorescence technique described by Pusztai et al. [5].

Radioactive labelling. Chick cells were pulse-labelled with 3.7 MBq/ml ^{35}—S-methionine in methionine-free medium for 60 min at different intervals after the addition of arginine-butyrate to the cell cultures. After the labelling period, the cells were scraped off, washed and fixed with 6% trichloracetic acid. The acid-insoluble activity of the TCA-extracted cells was determined.

Arginine-butyrate. The arginine derivative of butyric acid was kindly provided by Dr. Charles Chany (Institut National de la Santé et de la Recherche Médical, Paris Cedex, France). It was dissolved in Hanks balanced salt solution.

Results

Results of preliminary experiments revealed that low concentrations of arginine-butyrate (several mM) do not exert a marked toxic effect on primary chick embryo fibroblast cells. However, some distinct changes in cell morphology could be observed. This effect of treatment is reversible and the cells resumed their normal morphology after transferring them to a butyrate-free medium. Moreover, the cells seemed to be viable after 48 h of treatment (not shown).

To determine the effective concentration of arginine-butyrate in the inhibition of interferon induction, 1–3 mM butyrate was added to the cells simultaneously with the inducer virus. Incubation was terminated 24 h or 48 h post-infection, and dose vs. response curves were constructed (Fig. 1). The 24 and 48 h curves were fairly similar, and in both cases 3 mM butyrate completely inhibited interferon formation. To ascertain whether the inhibition is due to the overall shut-off of cellular protein synthesis, the rates of protein synthesis and interferon formation were compared at intervals after concomitant infection and treatment of the cells with butyrate. The protein synthesis was measured via the incorporation of ^{35}S-methionine into trichloracetic acid-precipitable material (Fig. 2). While a dramatic reduction was observed in interferon formation, even as early as 12 h after infection, treatment of the cells with 3 mM butyrate caused only a small reduction in the rate of uptake of the radioactive precursor. We attempted to pinpoint the butyrate-sensitive step during induc-

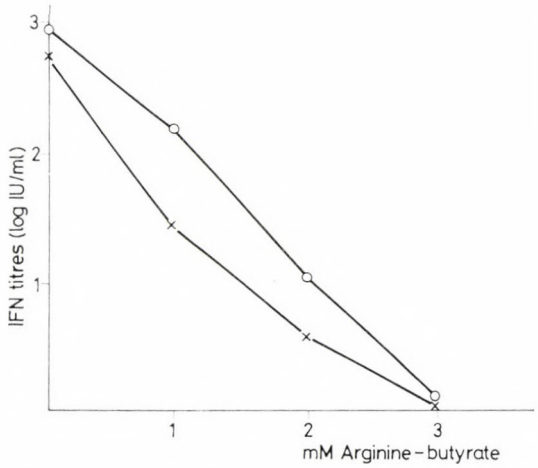

Fig. 1. Effect of arginine-butyrate on interferon induction by human adenovirus type 12 on primary chick embryo fibroblast cells. ×——× 24 h; ○——○ 48 h after induction

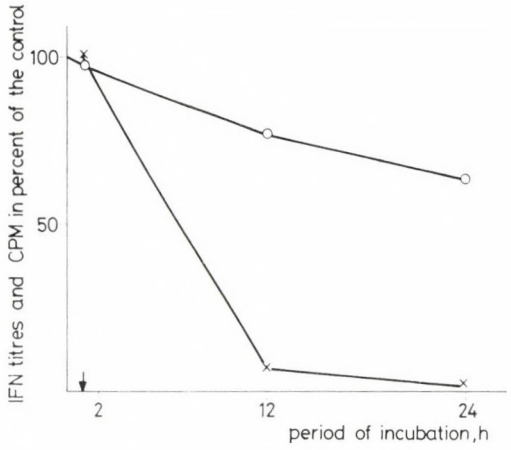

Fig. 2. Rate of interferon induction and protein synthesis in arginine-butyrate (3 mM) treated cells. ○——○ ^{35}S-methionine incorporation; ×——× IFN production

tion, and thus we applied the drug for 5, 8 and 12 h, together with the inducer (Fig. 3). Because of the slow recovery from the treatment of the cells, and the relatively slow adsorption of the inducer, the only conclusion that could be drawn is that an early event in interferon induction is involved.

It has been reported that in some interferon-producing systems, when the cells were pretreated with butyrate before virus induction, a striking enhancement of interferon yields was observed. In contrast with this, pretreatment of chick cells does not enhance the production of interferon induced by adenovirus (Fig. 4). At the same time, it seems that the effect of the drug is reversible: cells remained viable and later resumed normal interferon-producing ability.

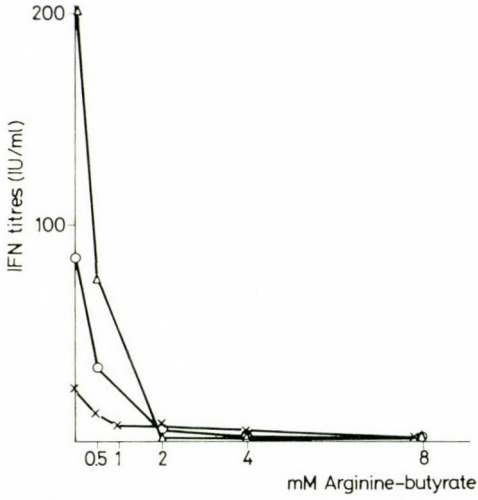

Fig. 3. Effect of arginine-butyrate treatment (4 mM) in early phase of induction. ×——× 5 h; ○——○ 8 h; △——△ 12 h postinfection

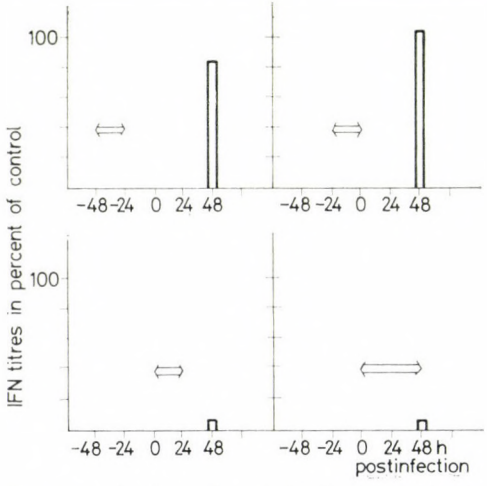

Fig. 4. Effect on interferon production of arginine-butyrate treatment applied pre- and postinfection

These results also show that the butyrate-sensitive step takes place in the first 24 hours of induction by adenovirus.

In chick cells infected with human adenoviruses, simultaneously with interferon formation, certain virus-specific events can be observed. One of the most noteworthy events is the T (tumour) antigen formation of cells infected with type 12 virus. As can be seen in Table I, butyrate strongly inhibited interferon production, but did not have any influence on T antigen formation. This is a unique observation, as in all butyrate-treated semipermissive systems tested so far, the early adenovirus gene expression was inhibited.

Table I

T (tumour) antigen formation and interferon production in human adenovirus type 12 infected and butyrate treated cells

Cell number ($\times 10^6$/well)	Arginine-butyrate concentration (mM)	IFN titre (U/ml)	T antigen positive cells (% of control)
2.5	0	566	100
2.5	2	309	88
2.5	7	<6	82
1.5	0	341	100
1.5	2	77	104
1.5	7	<6	92

Discussion

The interest in studies concerning the influence of butyrate on interferon induction by adenovirus in chick cells arose from the growing number of papers reporting on the multiple and rather curious effects of this natural fatty acid. Some data related to the modifications caused by the drug in interferon-producing systems, as well as its effects on adenovirus gene expression and replication. However, we do not know how butyrate acts when interferon formation takes place on induction by adenovirus.

The effect of butyrate on interferon production by human lymphoblastoid cells was recently examined. Cells were treated before infection with Sendai virus, and the treatment increased the subsequent yield of interferon [6, 7]. Similarly, pretreatment of the monkey cell line Gl—V_3 with the drug resulted in a markedly enhanced interferon yield [8]. We have made a different observation in our analysis of the results of arginine-butyrate treatment on interferon production induced by adenovirus in primary chick embryo fibroblast cells. The drug has a similar effect on the interferon production by chicken leukocytes (data not shown). In these experiments we used arginine-butyrate, which has a lower toxic effect on the cells than sodium butyrate. Pretreatment with 1–4 mM arginine-butyrate did not increase the interferon production in the subsequent induction. However, when the drug and the inducer were applied simultaneously, a strong inhibition was observed. Treatment with 3 mM butyrate for up to 48 h was not cytotoxic for the cells, but some slight morphological alterations were observed. Overall rates of protein synthesis, as measured by the incorporation of radioactive amino acid into TCA-precipitable material, are not significantly altered in butyrate-treated cells. It seems therefore that the drug modifies some gene expression without significantly reducing the level of protein synthesis in general. On the other hand, the effects of butyrate

are reversible: a return to the initial biochemical and morphological patterns occurs after a lag period following removal of the drug from the medium.

Sodium butyrate is an inhibitor of histon deacetylase [4, 9] and, based on the correlation between histon acetylation and transcription, the hyperacetylation caused by the inhibitory effect of butyrate may influence the cellular gene expression [10]. The drug has also been shown to affect other, non-histon chromosomal proteins, such as high-mobility group (HMG) proteins. These are located in the transcriptionally active parts of chromatin, and their modification (acetylation, phosphorylation) may play an important role in control of the gene expression [11].

In cases when enhancement of interferon production has been observed, it is tempting to speculate that the increased level of interferon may be due to an increased rate of transcription induced by the butyrate in the above-mentioned way, and that this process must be specific for interferon and possibly for some other genes. However, if the results described in this report are taken into account, it is obvious that the stimulatory effect of butyrate is controlled by the cell type too, or the induction involves a more complex regulatory mechanism.

Sodium butyrate is able to support viral DNA replication and even to activate integrated virus DNA, although it inhibits cellular DNA replication [12, 13]. Daniell [14] has reported that butyrate does not inhibit adenovirus DNA replication in permissive HeLa cells, where no detectable synthesis of cellular DNA or histon occurs. In striking contrast with this, treatment of a BHK derived cell line prevents the adenovirus DNA replication, and even the expression of early viral genes is inhibited in these semipermissive cells [15]. Adenovirus-infected chick cells are unique in this respect, too. This system is also semipermissive for adenovirus replication [16, 3]. Despite the almost complete inhibition of cellular DNA synthesis [17], in butyrate-treated cells the drug does not influence early viral gene expression, as can be seen from the number of cells producing T (tumour) antigens.

Derivatives of butyric acid produce a wide variety of effects on cells in culture; some of them are observed in almost all cells studied, while others are observed in a limited number of cells, or even vary markedly depending upon the cell type. One of the rather unique effects is that which we have observed in adenovirus-infected chick cells. Although the mechanism of interferon induction by adenoviruses is still the subject of investigation, and the mode of butyrate inhibition also remains to be determined, we assume that interferon production may result from the interaction between the trans-acting transcriptional enhancing factor encoded by the E_1 region of the adenovirus and the appropriate cellular gene(s).

Butyrate may modify this viral gene product, thereby indirectly preventing expression of the interferon gene. Further studies may clarify this

possibility. In addition, we have been provided with a system where the effect of endogenous and exogenous interferon on T (tumour) antigen formation and transformation can be studied.

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PRODUCTION OF HIGH TITRE HUMAN INTERFERON-GAMMA IN PRIMED LEUKOCYTE CULTURES*

VALÉRIA ENDRÉSZ, S. TÓTH and M. TÓTH

Institute of Microbiology, University Medical School, Szeged

(Received February 13, 1985)

Con A-stimulated leukocytes previously treated with IFN-α (1500 IU/ml for 4 h) were very suitable for the production of IFN-γ with a high titre even under large-scale conditions. The properties of the IFN produced under such conditions were identical to those of IFN-γ. The IFN-γ produced in primed cultures showed two peaks of antiviral activity corresponding to molecular weights of 51 000 and 23 000 daltons.

Human interferons (IFNs) can be divided into three groups: leukocyte (α), fibroblast (β) and immune (γ) IFNs [1]. They exert similar biological effects (antiviral and anticellular activities), though IFN-γ displays a higher antitumour activity in vivo [2] and a higher cytostatic activity in vitro [3] than IFN-α or IFN-β.

In addition to these biological effects, the IFNs possess priming activity. Cells pretreated with IFN generally yield more IFN and produce it sooner than nonprimed cells [4, 5]. In a previous publication we have compared the priming activities of human IFN-α, IFN-β and IFN-γ [6]. Pretreatment of leukocytes with any type of IFN enhanced their IFN-α and IFN-γ production, but IFN-α and IFN-β had more potent priming activities. Pretreatment of leukocytes with relatively high doses of IFN-α or IFN-β (1000–3000 IU/ml) resulted in at least a 10-fold increase in the IFN-γ production of Con A-stimulated leukocytes. Since the demand for large quantities of IFN-γ is still growing, we have tried to adapt this system to produce IFN-γ on a large scale.

Materials and methods

Interferons. Sendai virus-induced leukocyte IFN-α was purified to a specific activity (sp. act.) of 10^6 IU/mg protein, as described previously [6]. The IFN-γ was produced in Con A-stimulated leukocyte cultures and purified to a sp. act. of 10^5 U/mg protein with the same method as for IFN-α. The IFN-β preparation was a generous gift from A. Billiau (Rega Institute, Leuven, Belgium); it had a sp. act. of 10^6 IU/mg protein.

VALÉRIA ENDRÉSZ, SÁNDOR TÓTH
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

MIKLÓS TÓTH
Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences
H-6721 Szeged, P.O.B. 521, Hungary

* In commemoration of the 60th birthday of Professor Ilona Béládi as a sign of respect for her research work on interferon

Production of IFN-γ. IFN-γ production under small-scale conditions was performed as described in reference [6]. For the large-scale production of IFN-γ, leukocyte cultures of 3000 ml in wide-neck round-bottom flasks were used. Cells were maintained in suspension by agitation with a magnetic stirrer.

Quantitation of antiviral activities of IFNs. Antiviral activity was assayed on human amnion (WISH) or bovine kidney (MDBK) cells in microtitre plates (Greiner) by a cytopathic effect reduction method [6]. Titres of IFN-α and IFN-γ preparations were expressed in IU/ml and U/ml.

Measurement of antiproliferative activities of IFNs. The tests were performed in exponentially growing HEp-2 cell cultures. Cells (3×10^5) were seeded in 45 mm glass Petri dishes. One day later the cell monolayers were washed and incubated in the presence or absence of IFN. Cell growth was determined 3 days later by haemocytometer counts of viable cells using trypan blue dye exclusion. Determinations were carried out in triplicate.

pH and temperature studies. Crude IFN-α (30 000–50 000 IU/ml) and IFN-γ (1000–3000 U/ml) samples were dialysed against glycine-HCl buffer (0.1 M, pH 2) for 1–2 days. The pH was raised by further dialysis against phosphate-buffered saline (pH 7.6) for 1 day. The samples were tested for residual IFN activity. The crude IFN-α and IFN-γ samples were also tested for thermal stability at 56 °C.

Gel filtration of IFN-γ on Sephacryl S-200. Controlled Pore Glass (CPG-75) beads were used for partial purification of IFN-γ according to the method of Langford et al. [7]. The ethylene glycol and the salt from the eluted IFN fractions were removed by chromatography on Sephadex G-25 (Pharmacia). The IFN-γ was fractionated on a Sephacryl S-200 (Pharmacia) column (1.6 by 75 cm) previously equilibrated with PBS containing 5% ethylene glycol. The column was standardized with substances of known molecular weight (blue dextran, bovine serum albumin, ovalbumin and cytochrome C).

Measurement of protein content. Protein contents of IFN samples were determined by the dye binding assay with a Bio-Rad protein assay kit.

Results and discussion

IFN-α-pretreated and Con A-induced leukocytes can produce a considerable amount of IFN-γ in stationary cultures [6]. We have tried to modify this IFN production system to make it useful for large-scale production. A system currently applied to produce human IFN-α for clinical trials was adapted [8]: leukocyte cultures were primed with IFN-α and induced with Con A (see Materials and methods section). Different experiments involving slight variations in the conditions were carried out in order to determine the influence of different factors on the IFN yield (Table I).

The effect of priming was evident in the large-scale production system (Table I, A). The optimum priming period was 4 h (Table I, B). With a longer priming period, the viability of the leukocytes decreased parallel with their IFN-producing ability (data not shown). A high cell concentration (2.5×10^7/ml) was required for the efficient production of IFN-γ (Table I, C). At a higher cell concentration, the pH of the medium changed rapidly, which decreased the IFN-producing ability of the leukocytes (data not shown). In the case of IFN-γ production, similarly to the IFN-α-producing system [9], the relatively expensive Eagle's minimal essential medium could be replaced by a simple balanced salt solution (BSS) containing no amino acids or vitamins (Table I, D).

IFN-γ preparations produced under large scale conditions were tested together with crude IFN-α for stability at pH 2, and at 56 °C. IFN-γ produced

Table I

IFN-γ production on a large scale

Mode of production	Medium*	Priming with IFN-α (IU/ml)	Length of priming, h	Cell number (×10⁵/ml)	Median** titre (U/ml)
A	BSS***	0	4	2.5	380
	BSS	1500	4	2.5	2790
B	BSS	1500	1	2.5	724
	BSS	1500	2	2.5	892
	BSS	1500	3	2.5	1466
	BSS	1500	4	2.5	2200
C	BSS	1500	4	1	402
	BSS	1500	4	2.5	2625
D	Eagle's	1500	4	1	482
	BSS	1500	4	1	442

* Medium contained 2 mg/ml human gamma-globulin-free serum

** Leukocytes were stimulated with 15 μg/ml Con A, and 16 h later when the titres were maximum IFN activities of the culture supernatants were assayed. The titres shown in A, B, C and D sections are means of 1, 2, 3 and 4 experiments respectively.

*** Balanced salt solution: Eagle's medium without amino acids or vitamins

by either primed or unprimed leukocytes lost about 80–90% of its antiviral activity at pH 2 in 1 day, whereas IFN-α remained unaffected under the same conditions (data not shown). On the other hand, IFN-γ preparations lost about 70% of their antiviral activities in 30 min, at 56 °C whereas under the same conditions the IFN-α samples retained 90% of their activities (data not shown).

The cell growth-inhibitory effect of Con A-induced IFN-γ was also compared with those of IFN-α and IFN-β (Fig. 1). A significant inhibition (20%) in cell growth was achieved with 40 IU IFN-α or IFN-β, whereas 4 U IFN-γ was sufficient to obtain the same effect.

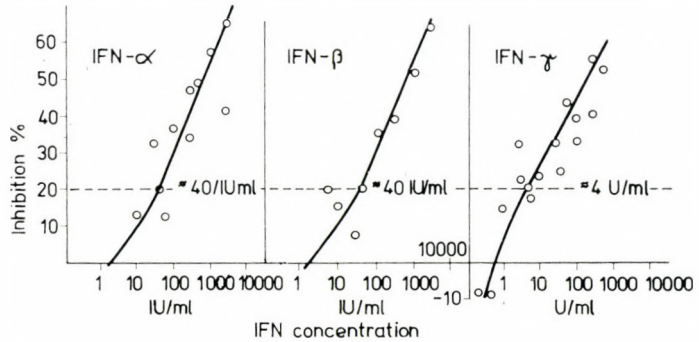

Fig. 1. Anticellular activities of IFN-α, IFN-β and IFN-γ to HEp-2 cells

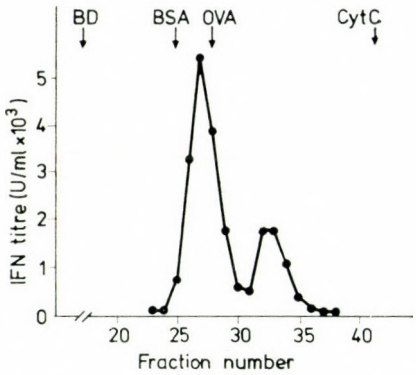

Fig. 2. Elution profile of Con A-induced IFN on Sephacryl S-200. Molecular weight markers: blue dextran (BD) 3 000 000; bovine serum albumin (BSA) 67 000; ovalbumin (OVA) 45 000; cytochrome C (CytC) 13 000

The molecular weight of Con A-induced IFN-γ was determined by Sephacryl S-200 column chromatography. With WISH cells, two peaks with antiviral activity were detected, corresponding to proteins with molecular weights of 51 000 and 23 000 daltons (Fig. 2). Neither the higher nor the lower molecular weight component of Con A-induced IFN protected MDBK cells against the challenge virus. It is probable that the two peaks correspond to the monomeric and dimeric forms of the IFN-γ molecule.

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MUTUAL SPATIAL ORIENTATION OF HEXONS IN THE ADENOVIRUS CAPSID BY ELECTRON MICROSCOPY AND MODELLING

ÉVA ÁDÁM and I. NÁSZ

Institute of Microbiology, Semmelweis University Medical School, Budapest

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On the basis of electron microscopic analysis of the virion and model experiments there are four hexon species in the adenovirus capsid which have different positional status and spatial orientation as compared to the six nearest neighbouring capsomers. The binding pattern of the polypeptide subunits according to their orientation is also different. The four hexon species are situated beside one another in the capsid and they form the capsid of 240 hexons besides the penton by repeating symmetrically 60 times, as a group of four hexons (GOF).

Electron microscopic analysis and model experiments lead to the conclusion that the mutual spatial orientation of GOF hexons can be parallel (their longitudinal axes are parallel), or the direction of their longitudinal axes is different in one or two planes, thus they form different angles with the longitudinal axes of the neighbouring hexons. In the latter cases therefore, a bend and torsion of the bindings (connective elements) can be supposed as compared to the tendency-plane of parallel bindings. Based on the different combinations of the three kinds of rotational orientation of polypeptide subunits and of the different spatial orientation of hexons, six kinds of interhexonal bindings can be found in the adenovirus capsid. The distribution and characteristics of the 690 interhexonal bindings are the following: *(1)* 240 bindings of "one-to-two" polypeptide orientation with parallel spatial orientation; *(2)* 180 bindings of "one-to-two" polypeptide with spatial orientation divergent in two planes; *(3)* 30 bindings of "one-to-one" polypeptide orientation with parallel spatial orientation; *(4)* 60 bindings of "one-to-one" polipeptide orientation with spatial orientations divergent in one plane; *(5)* 60 bindings of "one-to-one" polypeptide orientation with spatial orientation divergent in two planes; *(6)* 120 bindings of "two-to-two" polypeptide orientation with spatial orientation divergent in two planes. The dissociational sequence of the capsid suggests that the firmest bindings are the ones having "one-to-two" polypeptide orientation and parallel spatial orientation. It may be supposed that any two hexons are able to connect to each other according to all three polypeptide orientations and the connections can tolerate the bends and torsions arising from the icosahedral structure. This suggests that only one kind of hexon is existing and the adenovirus capsid is formed by a homogeneous hexon population.

Adenovirus hexon was revealed to have a pseudohexagonal lower part and a triangular top. The triangular profile of the hexon-end facing the virion surface is formed by three polypeptide subunits arranged around a longitudinal axis of three-fold symmetry. The three apices of the triangular profile are formed by one polypeptide each. The molecule has a small axial hole extending to a Y-shaped slit at the top [1, 3]. The hexagonal lower portion of the hexon ensures a closed (hexagonal) packing for the capsomers, while the triangular profile of the top, the Y-shaped slit and the three polypeptide subunits give

ÉVA ÁDÁM, ISTVÁN NÁSZ
Institute of Microbiology, Semmelweis University Medical School,
H 1445 Budapest, P.O.B. 370, Hungary

useful reference point for the establishment of the orientation of hexons and subunits in the adenovirus capsid in flat projection [3, 4].

Previous experiments have shown the mutual rotational orientation of all the polypeptide subunits of the adenovirus-building capsomers, which is characterized by the regular alternation of the "one-to-two", "one-to-one" and "two-to-two" orientation of polypeptide subunits of the neighbouring hexons. This corresponds to the 5-, 3- and 2-fold (532) rotational symmetry characteristic of the icosahedral capsid [2, 5]. It has also been shown that every hexon is joined to its six nearest neighbours by six double interhexonal connective elements [6, 7].

The present report gives information on the mutual spatial orientation of the individual capsomers to the neighbouring hexons located on different points of the virus capsid as well as the bending and the torsion of the interhexonal connections, which follows necessarily from the icosahedral structure of the capsid.

Material and methods

With direct analysis of electron micrographs and Markham's rotational integration technique it was observed in several cases that the distances between the edge and the triangular facets of the virion were wider than those among the hexons on the facets and on the edges. The distances separating the pentons and peripentonal hexons appeared similarly wider. These findings are visible in Fig. 1/a in reference [2] on the electron micrograph of the adenovirus and on the micrograph processed by rotational technique (Fig. 1/b in the same reference). The phenomenon may be explained by the deviating spatial orientation of the hexons located on different points of the virus capsid, which follows necessarily from the icosahedral symmetry of the virion. The mutual spatial orientation of hexons situated on different parts of the capsid was studied by use of a virion model. The deviation of their longitudinal axes was examined with the help of an icosahedron from which five triangular facets were absent. Steel sticks were placed according to the supposed longitudinal axes of hexons and the angles enclosed by the axes of the different hexons were measured inside or sometimes outside the icosahedron. The angles in the Figures may deviate somewhat from the values given in the text, due to a slight deformation during photographing.

Results

Group of four hexons (GOF)

The six hexons building the triangular facet and three other ones taking part in the constitution of three edges around the given triangular facet form the group of nine hexons (GON) as seen in Figs 1 and 7 [3]. Each neighbouring GON participates in the formation of the common edge.

In the icosahedral capsid, the following four species of hexon capsomers may be recognized which have different positional status to each other and different spatial orientation to the six nearest neighbouring capsomers.

(1) *Peripentonal hexons* (PPH); five hexons that surround each penton at the vertices (Hexon number 1 in Fig. 1).

(2) *GON-hexon, edge* (GON-HE); two GON-HE from two neighbouring GONs are located at the centre of each edges between the two PPHs (Hexon number 2 in Fig. 1).

(3) *GON-hexon, facet-vertex* (GON-HFV); each GON is situated on the triangular facet of the capsid. Three of the six hexons building the facet situated at the three vertices are the GON-HFVs (Hexon number 3 in Fig. 1).

(4) *GON-hexon, facet-centre* (GON-HFC); three of the six hexons building the facet situated among the three GON-HFVs are the GON-HFCs (Hexon number 4 in Fig. 1).

Out of the four hexon species, 60 can be found in each capsid. The different hexon are situated beside one another in the capsid as a group of four hexons (GOF), and they are repeated symmetrically 60 times according to the 532 symmetry axes of the icosahedron to form the virus capsid composed of 240 hexons besides the 12 penton (Figs 1 and 2). The groups of four hexons (GOFs) can be "arranged" in two ways. To a given PPH belong two neighbouring GONs. According to the first arrangement, the GON-HE, the GON-HFV and the GON-HFC belonging to the one and the same GON are joining to the PPH (Fig. 1). According to the other arrangement, the same GON-HE from one neighbouring GON is joining to the given PPH like at the first form, and from the other neighbouring GON, the GON-HFV and GON-HFC are joining (not shown). Thus the PPH and the GON-HE is the same in both forms, the difference being only whether the GON-HFV and GON-HFC are joined to them from one or the other side; i.e. viewed from the given PPH, they are on the left or on the right. The rotational orientation of these hexons and their polypeptide subunits show a 60° rotation as compared to the orientation of GON-HFV and GON-HFC in the first arrangement (Fig. 1). The spatial orientation of the four hexon species is the same with both forms; the mutual orientation of the polypeptide subunits of the hexons, however, is different both within the GOF and in joining to the neighbouring GOFs. In the first arrangement of a GOF all the five interhexonal bondings are characterized by a "one-to-two" polypeptide orientation pattern; out of the 13 interhexonal combinations with hexons of neighbouring GOFs, five had "one-to-one", four "two-to-two" and four "one-to-two" pattern. In the second arrangement, within a GOF there were one "one-to-one", two "two-to-two" and two "one-to-two" patterns. At combinations with neighbouring GOFs, three "one-to-one" and ten "one-to-two" types of polypeptide orientations were found (penton-PPH bindings were left out of consideration). Concerning the whole virus capsid, however, the number of bindings of different polypeptide orientations is the same, whether deduced from any of the two arrangement forms.

In the following, the mutual spatial orientation of the longitudinal axes of the four kinds of hexon species belonging to a GOF in relation to the neigh-

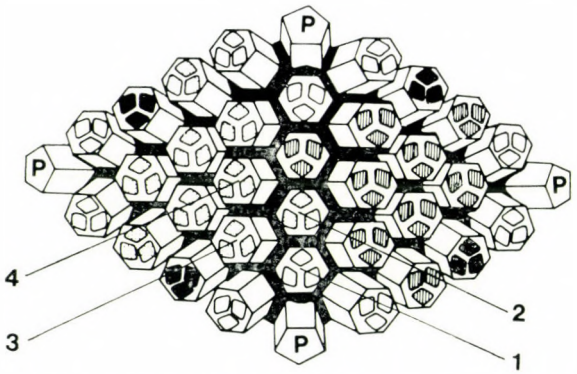

Fig. 1. Diagram of two triangular facets of adenovirus capsid showing schematically the mutual spatial orientation of capsomers having different positional status. Outlines of the three polypeptide subunits around the Y-shaped slit represent the triangular profile of a hexon top. The mutual rotational orientation of subunits are also shown. Hexons belonging to two neighbouring GONs are differently shaded. Black hexons belong to other GONs, not shown in the diagram. P = penton base; 1 = peripentonal hexon; 2 = GON-hexon, edge; 3 = GON-hexon, facet-vertex; 4 = GON-hexon, facet-centre. Hexons numbered 1—4 form the Group four hexons (GOF)

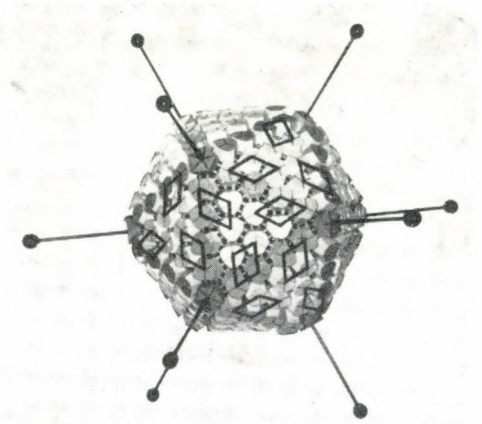

Fig. 2. Adenovirus model viewed from the 3-fold symmetry axes, showing the symmetrical repetition of GOF-hexons in the virus capsid (see Fig. 1). The model is built of hexons with hexagonal base and triangular outer-end profile. The possible mutual orientation pattern of the double interhexonal connective elements are also shown

bouring six capsomers is expounded, and also the types of polypeptide orientation is given. The first type of arrangement of the GOFs is followed in the description.

Peripentonal hexons (PPH)

The longitudinal axes of PPHs and the two GON-HE situated on the edges were supposed to be perpendicular to the plain of the edges and parallel with the 2-fold symmetry axis, so that the longitudinal axes of hexons being

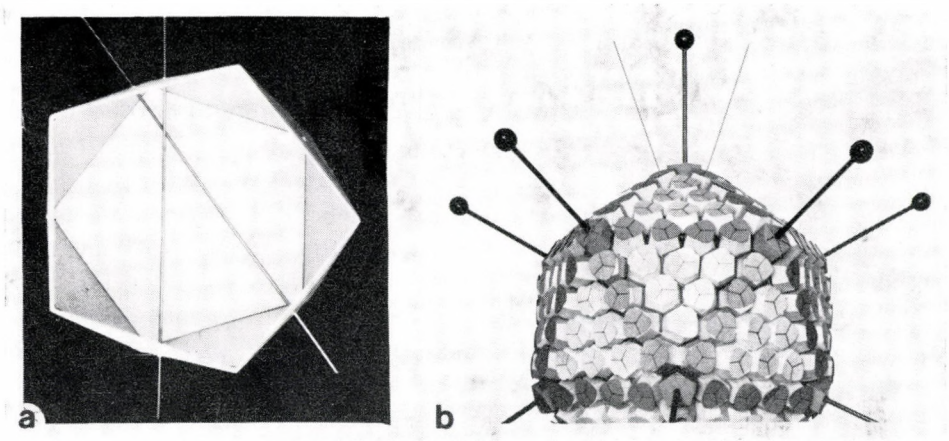

Fig. 3. Angle formed by the longitudinal axes of two neighbouring PPHs; (a) inside the icosahedron, (b) at the surface of the virion model

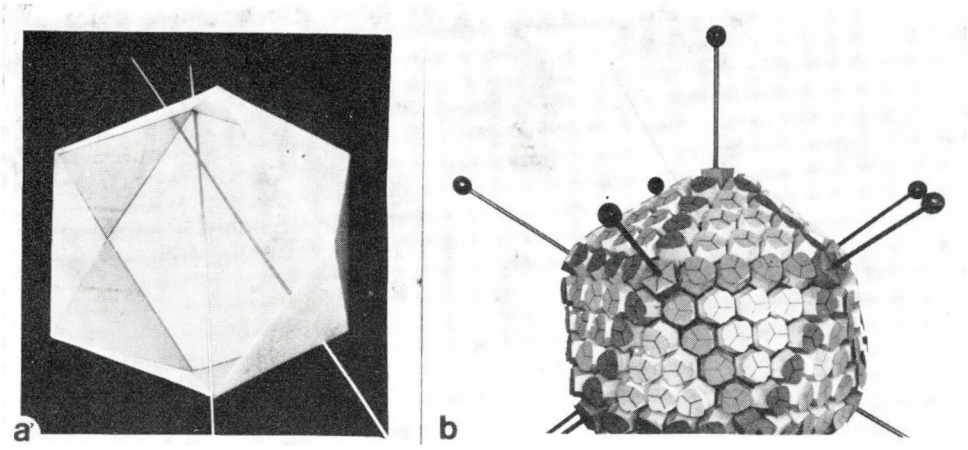

Fig. 4. Angle formed by the longitudinal axes of the penton and a PPH; (a) within the icosahedron, (b) at the surface of the virion model

in similar positions on the edges facing each other are on the same imaginary line. The imaginary lines connecting the longitudinal axes of two neighbouring PPHs and those of PPHs being in similar position on opposite edges are interesting (Fig. 3). In the case of pentons it was supposed that their longitudinal axes point radially towards the virion centre and coincide with the 5-fold symmetry axis of the icosahedron, with which the longitudinal axis of the penton on the facing vertex subsequently coincides, too (Fig. 4). The mutual spatial orientation was characterized by the angle which is formed by the longitudinal axis of the examined hexon with the longitudinal axis of the neighbouring capsomer taking part in the comparison; the angle being opened towards the surface of the virion. The angles were measured from different

definite viewpoints, if the imaginary elongated longitudinal axes are not intersecting each other but they cross at a definite distance from each other. On the basis of the experiments with the model and issuing from the structure of the icosahedron it may be supposed that a $\sim 32°$ angle is formed between the longitudinal axes of the penton and the PPHs. According to previous examinations [2], two polypeptide subunits of each PPH are oriented towards the penton and there seems to be a deviation of the connecting elements between them that corresponds to the above angle. Two neighbouring PPHs each have one polypeptide subunit that are oriented towards each other ("one-to-one" orientation); their longitudinal axes form a $\sim 36°$ angle and the bending of the connecting elements between them corresponds to that. As can be seen in Figs 1 and 2, each PPH has two neighbouring PPHs, towards which it has the same polypeptide orientation and deviation angle; besides the penton, these are the second and third neighbours. The fourth neighbour is the GON-HE, the hexon on the given edge beside the PPH, and this hexon belongs to one of the GONs forming the given edge (the GON on the left in Fig. 1). Here, the polypeptide subunits show a "one-to-two" pattern, the orientation of the longitudinal axes is parallel and the connecting elements are accordingly, not bent. The fifth and sixth neighbours of the PPH are the two GON-HFC. Out of these, one belongs to the same GON, as the fourth neighbour GON-HE, the other to the other GON adjoining. The orientation of polypeptide subunits of PPH to the fifth neighbour hexon is "two-to-one". When defining their mutual spatial orientation, we set out from taking the longitudinal axes of hexons on the triangular facets as perpendicular to the plane of the facet, parallel with each other and with the 3-fold symmetry axis of the icosahedron. They were considered also to be parallel with the longitudinal axes of hexons on the triangular facet opposite to the given one on the icosahedral virion. Out of the hexons on two opposing triangular facets none is in the same position, as the opposing facets show a 60° rotation along the 3-fold symmetry axis of the icosahedron. This rotation results in a shift as compared with each other, so that projecting the two triangular facets on each other they give a regular hexagon. Due to the hexagonal packing, the longitudinal axes of hexons situated on the facets and edges, do not intersect when fictitiously elongated, because their distance from one another corresponds to half ot the hexon diameter, but when measured from the given edge, they form an angle corresponding to the deviation between the planes of the edges and of the facets. If the longitudinal axes of hexons on the facets, however, are perpendicular to the plane of the facets and parallel with the 3-fold symmetry axis of the icosahedron (which passes between three hexons, in the centre of the two opposing facets) and do not coincide with the longitudinal axes of any hexons on the two opposing facets, then consequently, other angles being in other planes and measurable from other directions, can

also be formed between the longitudinal axes of hexons on the edges and the facets. According to this, the longitudinal axis of the PPH forms a 21° angle with the longitudinal axis of the fifth neighbouring hexon (GON-HFV) measured along the given edge (Fig. 5/a), and forms a 18° angle measured along the line connecting the vertex of the triangular facet and the centre of the opposite side (Fig. 5/b). From the direction of one of the neighbouring facets an other angle formed for other planes can be measured as ~10.5° corresponding to the two longitudinal axes of these two hexons (Fig. 5/c). According to this, at the connecting elements between the PPH and the fifth neighbouring hexon not only a bend but a proper degree of torsion should also be taken into account. The sixth neighbour of PPH is also a GON-HFV, which is a hexon belonging to the other GON connected with it (the GON to the right in Fig. 1) and being nearest to the PPH on the vertex of the other triangular facet of the virus capsid. The position of this hexon is similar to that of the fifth neighbouring hexon, so the angle formed with its longitudinal axis is also the same, as well as the bend and torsion of connecting elements. Here the orientation of the polypeptide subunits is "two-to-two".

GON-hexon, edge (GON-HE)

One of its neighbours on the edge is a PPH, to which the orientation of polypeptide subunits shows a "two-to-one" pattern, the orientation of their longitudinal axes is parallel; its second neighbour on the given edge is the other GON-HE belonging to the neighbouring GON (the GON to the right

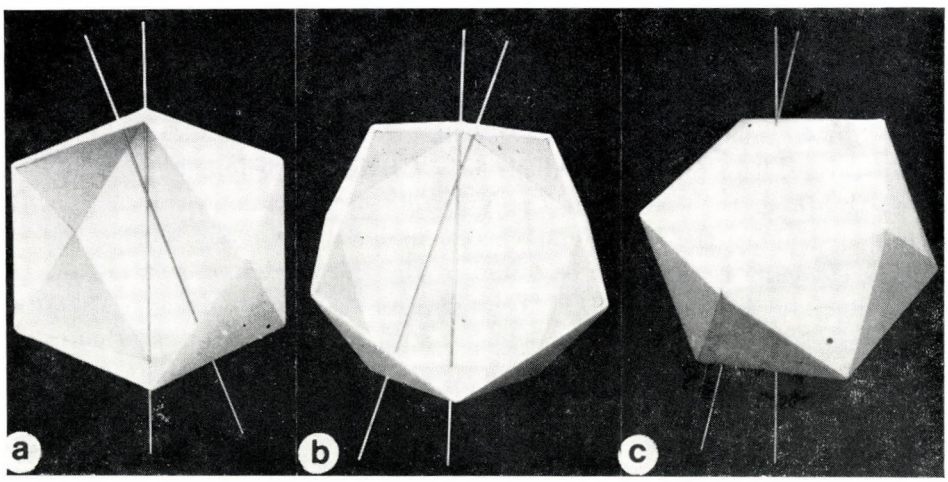

Fig. 5. Angles formed by the longitudinal axes of PPH and a GON-hexon facet-vertex; (a) seen along the edge on which the given PPH is situated, (b) seen along the line connecting the vertex and the middle of the facet, (c) viewed from the neighbouring side of the facet

on Fig. 1), their polypeptide orientation is "one-to-one", and their longitudinal axes are parallel. The third neighbour is the GON-HFV belonging to the same GON as the GON-HE (Fig. 1) and it displays a polypeptide orientation to this of a "one-to-two" pattern. Their longitudinal axes measured from the lengthwise direction of the given edge form an angle of ~21°, which correspond to the half of the ~42° bend between the plane of the two faces (Figs 6/a and b). Measured along the line connecting the vertex of the triangular facet and the centre of the opposite side, the angle is 18°. Measured from the

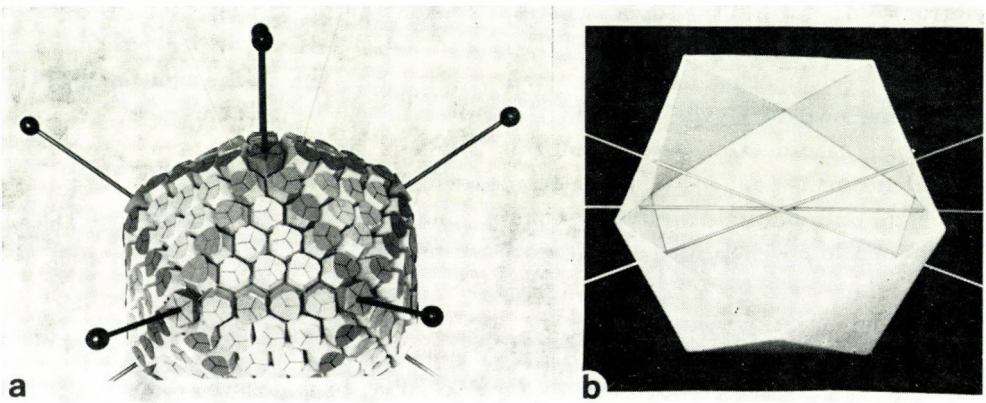

Fig. 6. Angles formed by the longitudinal axes of two GON-hexon facet-vertex; (a) at the surface of the virion model, (b) the angle corresponds to the deviation between the planes of two facets, as measured by the angle for two 3-fold symmetry axes of the icosahedron. A 2-fold symmetry axis between them demonstrates the angle formed by the longitudinal axes of hexons on the edge and on the facet seen along the edge

direction of any neighbouring triangular facet, the angle formed by the two longitudinal axes is ~10.5°. Therefore, the existence of a bend and torsion of the interhexonal elements corresponding to the above angles is a probable consideration. The fourth neighbour is a GON-HFC belonging to the same GON, to which a "two-to-one" polypeptide orientation is shown, but the angles formed by the longitudinal axes, and the bend and torsion of connecting elements is the same as found at the third neighbouring hexon. The fifth neighbour is the GON-HFV belonging to the neighbouring GON taking part in the formation of the same edge, and the polypeptide orientation to it is or a "one-to-one" pattern. The sixth neighbour is the GON-HFC belonging also to this GON. The polypeptide orientation here is "two-to-two". The latter two neighbouring hexons are in the same position on the other neighbouring triangular facet as the third and fourth neighbouring hexons, therefore the angles for the longitudinal axes and the bend and torsion of the connective elements are the same.

GON-hexon, facet-vertex (GON-HFV)

The GON-HFV shows a "one-to-two" polypeptide orientation to one of the PPH neighbours and a "two-to-two" pattern to the other one (Fig. 1). The angles formed by their longitudinal axes and the bend and torsion of connecting elements have already been expounded at the PPHs. Its other two neighbours are the two GON-HE (Fig. 4) adjoining the neighbouring PPHs on the two neighbouring edges. Out of these, with the one in the own GOF the polypeptide orientation is "two-to-one", with the other, "one-to-one". The angles between their longitudinal axes and the bend and torsion of connecting elements have already been shown at the description of the GON-HE (Figs 6[8]a and b). The fifth and sixth neighbours are two GON-HFCs, here polypeptide orientation is "one-to-two" with the one within the GOF, and "two-to-one" with the other, and their longitudinal axes parallel.

GON-hexon, facet-centre (GON-HFC)

On the neighbouring edge it has two neighbouring GON-HEs (Fig. 1). One belongs to the neighbouring GON (GON to the right on Fig. 1) showing a "two-to-two" polypeptide orientation with it). The other belongs to the own GON and GOF having a "one-to-two" orientation with it. The angles for their longitudinal axes and the bend and torsion of the connecting element are formed according to the spatial orientation of the longitudinal axes of hexons on the edge and on the facets beside the given edge. The third and fourth neighbours are the two GON-HFVs belonging to the same GON, the polypeptide orientation with the one being "one-to-two", with the other (which belongs to the same GOF) "two-to-one", and their longitudinal axes are parallel. The fifth and sixth neighbours are also the two GON-HFCs belonging to the same GON, one of them having a "one-to-two", the other a "two-to-one" orientation, the longitudinal axes being parallel with both of them.

Frequency distribution of different connections in the capsid

With the help of the triangulation number (T) [8], the number of connections (C) and the interhexonal connecting elements (IC) can be calculated on the basis of the following formulas:

$$C = 30\,T; \quad IC = 30T - 60$$

For adenovirions T = 25, the number of all connections in the virion is 750, that of the interhexonal connections without the 60 connections between the penton and PPHs is 690, as proved by the experiments (Tables I and II).

As shown previously (see Fig. 2 in reference [7]), in the case of adenoviruses these interhexonal connections are double bindings, because each hexon is connected to its six nearest neighbours by six pairs of approximately parallel elements. Electron microscopic observation suggests that double connective elements form a regular network between the hexons all over the capsid independently of the polypeptide subunit orientation pattern and are present between the penton and its surrounding hexons, too. Thus, 1500 connective elements can be considered per virion, out of which the number of interhexonal connective elements is 1380, i.e. 690 double bindings.

Table I

Summary of the different types of interhexonal bindings between neighbouring hexons in the virion

Orientation of polypeptide subunits	Spatial orientation of longitudinal axes			sum total
	parallel	forms angle in one plane(s)[1]	forms angle in two plane(s)[2]	
"one-to-two"	240 (120)[3]	—	180 (180)[3]	420
"one-to-one"	30	60	60	150
"two-to-two"	—	—	120	120
sum total	270	60	360	690

[1] Bend of interhexonal connective elements can be supposed
[2] Bend and torsion of interhexonal elements can be supposed
[3] Numbers in brackets mean the bindings within groups of four hexons out of all bindings the difference given the number of bindings between the groups of four hexons

Out of the 690 interhexonal bindings in the virion, 420 are connecting with "one-to-two" type of polypeptide orientation, and this conforms to about 61% of all the bindings. In 57% of these (240 bindings) the spatial orientation of the longitudinal axes of two compared neighbouring hexons is parallel. As this is the most frequent type of binding, it may be considered as "regular". Out of the "one-to-two" bindings, in 180 cases the spatial orientation of the longitudinal axes of two neighbouring hexons is divergent in two planes, thus here the bindings, i.e. the connective elements must necessarily be bent and twisted as compared to the tendency plane of "regular" bindings. Therefore, only 240 "regular", "one-to-two" bindings can be found in the capsid. According to the polypeptide orientation, the number of "irregular", "one-to-one" bindings per virion is 150. For 30 of these, the spatial orientation of hexons is parallel, but 60 are bent and 60 bindings are bent and twisted. Therefore, these latter ones are doubly irregular. All the 120 bindings of "two-to-two" type is the capsid are both bent and twisted, therefore they can also be considered doubly irregular.

Table II

Localization of the different interhexonal bindings in the capsid

Orientation of polypeptide subunits of neighbouring hexons	Number of interhexonal bindings		Spatial orientation of longitudinal axes of neighbouring hexons					
			parallel		forms angle in one plane[1]		in two planes[2]	
"one-to-two"	a) in one GON	15	9		—		6	
	a') in the capsid	300 (20×15)	180	(20×9)			120	(20×6)
	b) between one GON and three PPH[3]	6 (3×2)	3	(3×1)			3	(3×1)
	b') in the capsid	120 (20×6)	60	(20×3)			60	(20×3)
	altogether in capsid	420	240				180	
"one-to-one"	a) between one GON and three neighbouring GONs	9 (3×3)	3	(3×1)	—		6	(3×2)
	a') in the capsid	90 (10×9)	30	(10×3)			60	(10×6)
	b) on one vertex between five PPHs	5	—		5		—	
	b') in the capsid	60 (12×5)			60	(12×5)		
	altogether in capsid	150	30		60		60	
"two-to-two"	a) between one GON and three neighbouring GONs[4]	6 (3×2)	—		—		6	(3×2)
	a') in the capsid	60 (10×6)					60	(10×6)
	b) between one GON and three PPHs[5]	3 (3×1)	—		—		3	(3×1)
	b') in the capsid	60 (20×3)					60	(20×3)
	altogether in capsid	120					120	
Number of all interhexonal bindings		690	270		60		360	

[1] Bend of interhexonal elements can be supposed

[2] Bend and torsion of the interhexonal elements can be supposed

[3] Out of the two peripentonal hexons beside the vertices of the GON, here the hexon of anticlockwise position viewed from above (Fig. 7)

[4] Between every triangular facets there is an edge, therefore according to the 20 facets, the bindings of hexon rows on edges can occur only ten times in one virion

[5] Hexon of clockwise position (mark 3 and Fig. 7)

Fig. 7. Diagram of a triangular facet and three edges of adenovirus capsid showing tentatively the six different types of interhexonal bindings. GON-hexons are shaded and the border line of the GON is drawn. < "one-to-two" polypeptide orientation, with parallel spatial orientation; ⊘ "one-to-two" polypeptide orientation, with different spatial orientation in two planes; — "one-to-one" polypeptide orientation with parallel spatial orientation; ⊖ "one-to-one" polypeptide orientation with divergent spatial orientation in one plane; ⊖ "one-to-one" polypeptide orientation with divergent spatial orientation in two planes; ⓘⓘ "two-to-two" polypeptide orientation with divergent spatial orientation in two planes; × hexon-penton linkage

On the vasis of the rotational orientation and the spatial orientation of hexons (i.e. longitudinal axes), six types of interhexonal bindings may be supposed in the adenovirus capsid (Tables I and II, Fig. 7). *1.* "One-to-two" polypeptide orientation, with parallel spatial orientation (their number in the capsid is 240); *2.* "one-to-two" polypeptide orientation with a spatial orientation divergent in two planes (180 in the capsid); *3.* "one-to-one" polypeptide orientation with parallel spatial orientation (60); *4.* "one-to-one" polypeptide orientation with a spatial orientation divergent in one plane (60); *5.* "one-to-one" polypeptide orientation with spatial orientation divergent in two planes (60); *6.* "two-to-two" polypeptide orientation with spatial orientation divergent in two planes (120).

The arrangement of different types of bindings in the capsid

The bindings of individual hexons of different positions are the following: besides hexon-penton bindings, each PPH has two bent "one-to-one" and one bent and twisted "two-to-two" bindings. One of its two "one-to-two" bindings is bent and twisted, thus in fact each PPH has only one regular interhexonal binding. Each PPH has two GON neighbours and each GON is connected to 3×2 PPHs; to one of the PPH with two "one-to-two" polypeptide orientation pattern out of wich one is twisted and bent, to the other PPH with an also bent and twisted "two-to-two" binding (Figs 1 and 7). Between

two GONs there are only irregular bindings, two of them are bent and twisted "two-to-two" bindings and three "one-to-one" ones. Two of the latter ones are bent and twisted. Thus out of the five irregular bindings among the GONs four are doubly irregular.

Within one GON (aimed inwards) there are 15 bindings all with "one-to-two" polypeptide orientation, six of them are bent and twisted (Fig. 7). Three hexons of each GON (GON-HE) are located on an edge of the virion. These hexons have three "one-to-two" bindings, two of them are bent and twisted. Each GON has three other sort of hexons (GON-HFV), which are connected to two PPHs each. Each of these hexons has four "one-to-two" bindings, two of which are bent and twisted. Each of the three central hexons (GON-HFC) has five "one-to-two" bindings, one of them being bent and twisted. Therefore there is not one hexon in the GONs — and consequently in the whole virion — that would connect to all of its six neighbours with a regular "one-to-two" binding (Fig. 7 and Table II).

Discussion

The six kinds of interhexonal bindings determined by electron microscopy and model-experiments raise the question, whether the hexons forming the capsid of one virion are homogeneous or somewhat different according to their position in the capsid. There are signs that the hexon population may differ in exhibiting a faster or slower migrating ability, or in their isoelectric points [11]. Taking into account that there are only four kinds of hexon species (GOF) regarding their position in the capsid, it seems highly improbable that there should exist six kinds of hexon species according to the six different types of bindings. The six types of bindings arise from the combination of the three polypeptide subunit orientation patterns ("one-to-two, one-to-one and two-to-two") and from the differences of the spatial orientation of the neighbouring hexons (Tables I and II, and Figs 1 and 7). For the different spatial orientation, the corresponding bend and torsion of the interhexonal elements may be assumed. In a previous paper, analysing the structure of two-dimensional hexon crystalline arrays, we have shown that certain individual hexons within the crystal lattice were significantly displaced from their theoretically correct position [12]. Nevertheless, each hexon stays within a certain definite distance as compared to its equilibrium position determined theoretically in the lattice This proves that the interhexonal connecting elements must be flexibile and elastic to a certain extent. This, consequently, refers to the interhexonal elements in the virus capsid, too. It means that the bend and torsion arising from the icosahedral structure can be tolerated by the connective elements, even if the connections prove to be the side-chains of hexon polypeptide subunits of hexon associated protein IX [7, 13, 14].

According to electron microscopic observations on the virion, the existence of double interhexonal connective elements is generally valid in the whole virus capsid [7]. They occur not only at the most frequent "one-to-two" bindings in the capsid, but they are formed at the connections according to "one-to-one" and "two-to-two" polypeptide orientation, too. That where and which kind of polypeptide orientation is formed in the virus capsid issues necessarily from the icosahedral structure in the proper site and is the result of a 60° rotation of the given hexons along their longitudinal axes. Supposedly, every two hexons are able to connect to each other according to all three polypeptide orientation patterns. These considerations lead to the supposition that a unified, homogeneous hexon population can form a whole adenovirus capsid. It seems certain, however, that the firmness of different polypeptide orientation bindings are different, and the firmest one is the "one-to-two" binding which is proved by the dissociational sequence of the capsid. The peripentonal hexons have only two such bindings and can easily dissociate from the capsid. Within one GON, however, occur only such bindings and these can tolerate the various harmful effects the best, because the nine hexons stay together the longest. Between two GONs, however, there are no such bindings, which is in conformity with the observation that the virus capsid can relatively easily decompose into individual GONs [15, 16].

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Acta Microbiologica Hungarica 32 (4), pp. 413–414 (1985)

REVERSE CAMP PHENOMENON OF GARDNERELLA VAGINALIS

(A NOTE)

K. Csiszár

Public Health Station, Salgótarján

(Received May 2, 1984)

Gardnerella vaginalis strains inhibit the haemolysis by *Listeria monocytogenes* on human blood agar incubated in 5–10% CO_2 atmosphere or under anaerobic conditions.

Gardnerella vaginalis (former names: *Haemophilus vaginalis*, *Corynebacterium vaginale*) was described in association with "nonspecific vaginitis" by Gardner and Dukes [1]. The new genus *Gardnerella* was proposed by Greenwood and Pickett [2]. After 48 h *G. vaginalis* strains form small colonies on human blood agar incubated in an atmosphere containing 5–10% CO_2 at 37 °C. The colonies are surrounded by characteristic beta-type (gardnerella-type) haemolysis [3].

Since the first observation of Christie et al. [4], the synergetic or inhibitory effect on haemolysis by bacteria has been used as a simple, rapid method for the identification of certain species. The CAMP test worked well mostly in the identification of *Streptococcus agalactiae* and *Corynebacterium pyogenes* [4, 5]. Recently a CAMP reaction has been worked out for the presumptive identification of *Clostridium perfringens* [6]. We have observed a reverse CAMP reaction between *G. vaginalis* and *Listeria monocytogenes*.

Materials and methods

CAMP test agar. Tryptone (Oxoid L 42), 15 g; soya peptone (Oxoid L 44), 5 g; agar (Oxoid No. 1), 10 g; tap water 1000 ml; pH 7.3; sterilized at 121 °C for 30 min. To the base agar 7% fresh human blood was added and plates were poured.

Test procedure. The *G. vaginalis* control (NCTC 10287) and the strains to be tested were inoculated perpendicularly to *L. monocytogenes* (strain ATCC 19114) inoculated as a diametrical streak. The plate was incubated under anaerobic conditions in Gas Pak (Bio-Merieux), or in 5–10% CO_2 atmosphere at 37 °C for 48–72 h.

Károly Csiszár
Public Health Station
Bem út 7, H-3100 Salgótarján, Hungary

Results and discussion

The haemolysis of *L. monocytogenes* was inhibited by the *G. vaginalis* strains so that a semicircular arch of non-haemolytic area was formed at the juncture of the two kinds of organism (Fig. 1).

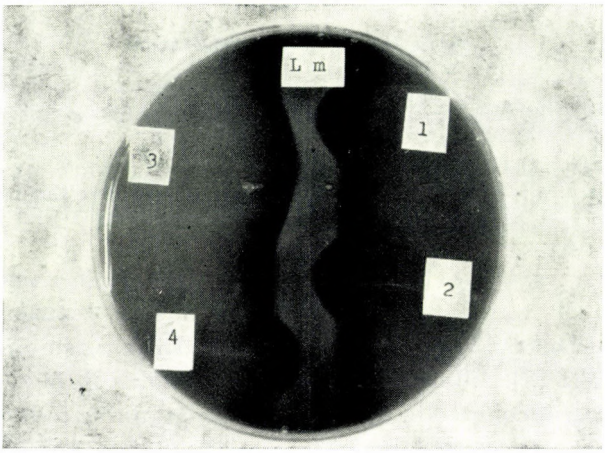

Fig. 1. G. vaginalis CAMP test on human blood agar. Lm = *L. monocytogenes;* 1 = *G. vaginalis* NCTC 10287 type strain; 2, 3, 4 = *G. vaginalis* strains isolated by the author

Besides the reference strain, reverse CAMP phenomenon was observed for 52 *G. vaginalis* strains isolated from genital and cervix mucus. Other haemolysing species isolated from vaginal mucus *(Staphylococcus aureus, Streptococcus pyogenes, S. agalactiae, C. pyogenes, Gemella haemolysans, Kingella kingae)* showed no similar phenomenon with *L. monocytogenes.*

The reverse CAMP test described here may be considered as one of the taxonomic tools suitable for identification of *G. vaginalis* strains.

Acknowledgement. I am indebted to Dr. J. SZITA, Head, Department of Bacteriology, National Institute of Hygiene, Budapest, for the identification of part of *G. vaginalis* strains.

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BOOKS RECEIVED

Barnett, J. A., Payne, R. W., Yarrow, D. Yeasts: Characteristics and Identification. Cambridge University Press, London 1983. pp. 811.

This book is the third, and latest, describing the computerized identification of yeasts. It is a new work, differing in many respects from its predecessors. The first (Barnett, J. A., Pankhurst: A new key to the yeasts, North Holland, 1974) and the second (Barnett, J. A., Payne, R. W., Yarrow, D.: A guide to identifying and classifying yeasts, Cambridge University Press, London 1979) versions were closely related to the standard manual on yeast taxonomy by Lodder, (ed.): The yeasts. A taxonomic study. North Holland, 1970. This latest book is based on new data and is completely independent of Lodder's manual.

A new and most important feature of the book is the inclusion of Chapter 7, the largest chapter of the book, containing complete descriptions of all individual yeast species. For the characterization of species, 83 physiological and 14 morphological features are used. The extraordinarily large task of checking in the majority several strains for each species for all characteristics has been carried out by D. Yarrow at the CBS Yeast Collection in Delft. As the same methods have been systematically applied, the results are fully comparable with each other.

Altogether 439 species are described, tabulated and keyed. Of these, some 130 species have been discovered since the latest edition of Lodder's book. In addition, nearly 150 species have been renamed due to nomenclatural and taxonomic changes. Despite the above-mentioned differences, there are two underlying features which are common to all three books on yeast identification. The first is the senior author, J. A. Barnett of Norwich, the driving force behind this tremendous work of classification. His coworker in the preparation of the computer programs in the last two books was R. W. Payne. The computer-made identification keys are the second common feature of the books, permitting the identification of any unknown yeast strain through the smallest possible number of tests.

There are 18 different keys for identification: some of them cover all the 439 species described, while others relate to some more specific groups e.g. yeasts of clinical importance, yeasts occurring in foods, wine or beer, or yeasts utilizing methanol or hydrocarbons. There are two alternatives for each kind of key, one applying physiological and morphological tests and another applying only the former. Though 36 different keys might well be enough to make a choice, one more key concerning yeasts commonly found in soils would still be of practical value.

Using the computer, another chapter has been compiled, showing those characters which differentiate individual species from the remainder. One single species is usually determined by 4 to 7 features. Unfortunately, there are a few species which cannot be distinguished from one another by any combination of the 97 tests, and appear to differ only in name (e.g. the pair *Saccharomyces cerevisiae* and *S. exiguus*).

Not only the preparation of the keys and tables, but also the typesetting and proofreading were performed by computer. This guaranteed a text which is almost free of printing errors (less than a dozen have been noticed) and permitted such a short printing time that all yeasts tested up to the end of 1982 could be included.

Two more unique and outstanding features of the book should be mentioned. One concerns Chapter 7, the other Chapter 10. The former describes the individual yeasts species. Each description is supplemented with a brilliant microphotograph taken by an up-to-date light-microscopic technique, the Zeiss-Nomarski differential interference contrast method, which produces very life-like pictures of the cells. Though not listed among the authors, the photographer, Mrs. Linda Barnett, well deserves to be ranked with them.

Chapter 10, the Register of Yeast Names, is also a most valuable part of the book. It contains about 3500 yeast names. The authors went back in their search to the early 19th century and checked most of the original publications. Whenever it was possible, they gave synonyms, too. Compiling this chapter together with the list of references must have been a painstaking work: indeed, the authors have succeeded in assembling the most complete collection of data ever produced on yeast species. For this reason the value of the book is increased to that of a basic reference work, that will be indispensable for future research. Moreover, the Register of Yeast Names may help bringing order into the present chaotic situation in the nomenclature of yeasts. The Register may form the basis for an approved list of yeast names, similar to that which became effective for bacteria in 1980. The book deserves praise as a whole and in its details, and the only criticism which can be made concerns its failure to be even more complete than it is. In several cases the cross-references between Chapters 7 and 10 are lacking. Also missing are the dates of descriptions supplementing the names of authors. This lack means that some uncertainty arises as to the validity of the descriptions of some species and genera. It is admitted, however, that these deficiencies come into question only from a taxonomic point of view and do not hinder success in identification.

This book provides the most up-to-date knowledge on yeast identification; it is well laid out, attractively produced, easily usable and practical. It can be thoroughly recommended for all those who participate in the routine determination of yeast identity, and it will also serve as a basic volume for anyone interested in yeast taxonomy.

T. Deák, L. Ferenczy and *E. K. Novák*

ERRATUM

Figure 1 on page 134 of the paper "Detection of Beta-Lactamase Activity with Nitrocefin of Multiple Strains of Various Microibal Genera" by J. V. URI, in Acta Microbiologica Hungarica **32** (2), 133 — 145 (1985) should be corrected:

(a) the first chemical formula's name should read "Nitrocefin";

(b) the second chemical structure is "PADAC" and the third is "Cephacetrile".

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