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NON-TOXIN VIRULENCE FACTORS OF BACTERIAL ENTERIC PATHOGENS (A REVIEW)

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Introduction

Since the solution of some problems of the enteric pathogenicity of bacteria a new "golden era" seems to arrive in the research of this particular field of bacteriology. Sometimes by means of new approaches such as our growing knowledge of plasmids, sometimes by rediscovery of old methods e.g. the Dean test for cholera toxin, newly discovered factors of virulence, or a new interpretation of known factors are the results of this research. Our points of view have, however, also changed: some of the factors are not determining or not essential components of the virulence, but having an additive role one or more of these factors gained a marked influence of the pathogenicity. Generally speaking, it must be emphasized that many enteric pathogens are or may be important agents in extraintestinal infections, too. With the development of hygienic, nutritional and socio-economic aspects there is a declin-

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ing significance, especially *quo ad vitam*, of enteric disturbances and the extra-intestinal infections gain more and more significance. According to data of the Hungarian National Institute of Hygiene summarized by Lányi [1], about 6000 strains of *Escherichia coli* are isolated annually in Hungary from diarrhoeal diseases without fatal consequences, but 60 000 strains originate from extra-intestinal infections which often have severe direct and frequently late sequelae e.g. pyelonephritis.

1. The adhesins

1.1. *Mannose sensitive, common fimbriae*

In 1955 when Duguid et al. [2] published the discovery of filamentous appendages called "fimbriae" in *E. coli*, adhesiveness a new aspect of the pathogenic process has been recognized. Later the working group of Duguid [3–6] described numerous important features of fimbriae such as their haemagglutinating capacity and epithelial adherence, their distribution among *Enterobacteriaceae*, their different types, the inhibitory effect of D-mannose, antisera, etc. (Table I).

The direct haemagglutinating activity as well as the epithelial adhesiveness suggested a pathomechanism in which the fimbriae may have an important function. It is paradoxical that in spite of much effort the results are inconclusive and mainly negative. Today it is impossible to exclude the mannose sensitive haemagglutinating group (MSHA) of fimbriae in enteric or

Table I

Types of common fimbriae according to DUGUID [7]

Fimbria classification	Host genera carrying fimbriae	Morphological data			Haemagglutination properties
		width (nm)	length (μm)	No./cell	
1	*Escherichia, Klebsiella, Enterobacter, Shigella, Salmonella*, etc.	7–10	0.2–2.0	50–400	MS
2	A few *Salmonella* serotypes	identical with type 1			non-haemagglutinating
3	*Klebsiella, Serratia,*				
3	*Proteus*	5	0.2–2.0	400–700	MR on tannic acid pretreated cells
4	*Proteus*	4	2–6	?	MR
5	*Pseudomonas echinoides*	5	2–6	10–20 polar	MS
6	*Klebsiella ozaenae*	10	10	10–20	non-haemagglutinating

MS = D-mannose sensitive, MR = mannose resistant haemagglutination capacity. The haemagglutinating activity has mostly a wide spectrum for the red blood cells of different animals

other adhesiveness, but if such activity exists, it seems to be rather weak and of limited significance. On the other hand, the characteristic in vitro surface pellicle formation of broth cultures of fimbriated strains with their high O_2 consumption and the consequent rapid growth may suggest an environmental advantage [8]. According to Duguid et al. [9] this effect can be demonstrated in experimental oral salmonella infection of mice.

There is a similar problem of adhesivenes or its lack in the case of extra-intestinal, especially of urinary tract infections. Under experimental conditions common fimbriae of *Klebsiella pneumoniae* seem to be the mediators of colonization foci on the bladder epithelium of rats [10]. Concerning human uroepithelial adhesiveness Schaefter et al. [11] observed a high bacterium/epithelial cell ratio (5000 : 1) optimal for *E. coli*, considering it a sign of low receptor density for common fimbriae. Microbiologists often take into account only the epithelial surface, without its rich glycocalyx and mucous cover. In this respect, Ørskov et al. [12] demonstrated that the common (type 1) fimbriae of *E. coli* have adhesiveness not to the epithelial surface, but to mucus. The substance carrying receptors in the uromucoid is the Tamm–Horsfall glycoprotein rich in D-mannose. According to the Ørskovs' opinion the common fimbriae are not virulence factors, but even the "mucus trapping" is an important part of the host defence.

A negative opinion emerged from the statistical point of view, too, as type 1 fimbriae are present in 50–70% of *E. coli* strains, being more common than are the pathogenic strains [13].

Some misinterpretation may have arisen from the data of haemagglutination activity, or from in vitro experiments carried out with epithelial cells of different origin. In respect of species specificity the haemagglutination activity does not reflect the supposed epithelial adhesiveness in a given animal [14], or the adhesiveness e.g. to buccal epithelial cells does not necessarily mean adhesiveness to e.g. uroepithelial cells [15].

There is general agreement to designate all mannose sensitive haemaglutinins (MSHA) as common fimbriae, though some exceptions in the MS character (see Table I) are known.

1.2. *Mannose resistant adhesins*

Determining the antigenic structure of a pig pathogenic *E. coli*, a new K antigen: K88a,b was described in 1964 [16]. Furher reports then showed the transmissibility and plasmid coded nature of the antigen determinant as well as its pure protein nature and fimbrial morphology [17–19]. The important pathogenic function in piglets of the K88 fimbriae serving as adhesins in the upper part of the small bowel was shown by Smith and Linggood [20] and Jones and Rutter [21]. In contrast to the type 1 fimbriae, the K88 factor

is associated with some O serogroups of *E. coli* (O8, O45, O138, O141, O147 O149 and O157) mostly together with the production of enterotoxins [22] (Table II). K88$^+$ cells are haemagglutinating guinea-pig and chicken red blood cells in a mannose resistant manner [23]. In contrast to common fimbriae the K88$^+$ character is stable on solid agar media, but is not expressed under 18 °C. In their original paper Ørskov et al. [16] distinguished two antigenic variants: K88a,b and K88a,c and later a third one, K88a,d was also described [24]. It seems that for these antigenic determinants specific receptors are present in the host. In 1975 Sellwood et al. [25] have already proved that the K88 receptors are on the brush border of epithelial cells of the small bowel and they found pigs without such receptors which were resistant against K88$^+$ enterotoxic *E.coli* (ETEC) strains. Today five pig phenotypes are known [26] showing full resistance or sensitivity against the antigenic variants of K88.

In the similar disease of calves and lambs another adhesin, named K99, was found [27, 28]. Later Moon et al. [29] discovered that K99 can be an adhesive factor for pig pathogenic ETEC strains, too. In the same year, 1977, a third pig specific adhesin 987P was demonstrated [30]. The latest finding (1982) is a second calf specific adhesive factor F41 [31], but its epidemiological significance has not yet been elucidated. The adhesins are not only specific for the host organism, but like K88 they are closely associated with some *E. coli* serogroups: the K99 with O8, O9, O20 and O101 [28, 32]; the 987P with O9, O20 and O141 [33, 34]; the F41 with O9 and O101 [31]. In the case of K99 of piglet origin an association exists with the O-groups O64 and O101 [29, 35] (Table II).

Adhesin K99 agglutinates horse and sheep erythrocytes in a mannose resistant way, while the F41 agglutinates in addition human and guinea pig red blood cells [28, 31, 35]. The 987P is a non-haemagglutinating adhesin [36].

Table II

Important characteristics of adhesins carried by enterotoxic E. coli

Adhesin	Origin	Association with serogroups of *E. coli*	Erythrocyte haemagglutinated	Association with		
				ST	LT	ST+LT
K88	pig	08, 045, 0138, 0141, 0147, 0149, 0157	guinea-pig, chicken	+	+	+
K99	calf, lamb	08, 09, 020, 0101	horse, sheep	+	—	—
	pig	064, 0101				
987P	pig	09, 020, 0141	none	+	—	—
F41	calf	09, 0101	human, horse, sheep, guinea-pig	+	—	—
CFA/I	human	015, 025, 063, 078	human, bovine, chicken	+	+	+
CFA/II	human	06, 08	bovine, chicken	—	—	+

The association of K99, 987P and F41 with enterotoxin production differs from K88 in that they are only heat-stable toxin (ST) producers [31, 32, 34, 37]. See Table II.

In enterotoxic enteropathies of domestic animals there is a marked age specificity. Moon and Whipp [39], as well as Walker and Nagy [40] showed the gradual decrease in ETEC susceptibility and in the number of colonized bacteria in piglets from birth to the age of 5 weeks. Similarly, 3-day-old calves or lambs are resistant to ETEC infection. There are data [33] that bacterial growth in the pig intestine selectively enforces the development of fimbriae. In model experiments working with $K88^+$ and $K99^+$ *E. coli* strains [41] we demonstrated in suckling mice an enrichment of the fimbriated state at the age of 3 to 10 days, while after the 14th day a rapid loss of fimbriation was observed. On the 14th day of life mice start eating solid food and a change occurs in the bowel flora, as bacteria belonging to the *Bacteroides* genus appear. This may be a coincidence; the mechanism of environmental (intestinal) influence on fimbriation is unknown.

Specific adhesins carried by human pathogenic ETEC strains were also found. In 1975 Evans et al. [42] described such a "Colonization Factor". After the discovery of a second adhesin the former was designated as CFA/I (Colonization Factor Antigen I) and the new one as CFA/II. These adhesins are also host specific factors carried by a limited number of O-serogroups. CFA/I occurs predominantly in O78 strains, less frequently in O15, O25 and O63 strains. The serogroups O6 and O8 are CFA/II carriers. CFA/I is haemaglutinating human, bovine and chicken, the CFA/II bovine and chicken red blood cells not inhibited by mannose. They are relatively stable on solid agar media, not expressed under 18 °C, like K88 and the other MRHA's (Table II).

The genetics of the MRHA host specific adhesins uniformly show that this phenotype is coded by plasmids except factor 987P. The determinant of K88 is carried by an associate type plasmid [17] of about 90 Md which is conjugative, or by a nonconjugative one of about 51 Md. In the nonconjugative plasmid there are also frequently *raf* (raffinose utilization) genes [44] and these genes are flanked by direct repeats of IS1, forming a transposon [45]. The K88a,b determinant itself, coding at least five polypeptides, was located by the gene cloning technique in a 3.4 Md DNA frgament [46]. The K99 adhesin is coded by a conjugative plasmid [47], its size being about 52 Md [48]. The fragment carrying the K99 determinant was 4.5 Md as determined by gene cloning [49]. The reference strain of CFA/I (H10407) contains a nonconjugative plasmid of 60 Md coding ST (heat-stable enterotoxin) as well as CFA/I [42]. Analogous data were obtained concerning the other CFA/I^+ ST^+ ETEC strains. Similarly, an association exists between enterotoxin production and CFA/II expression. They are also coded by a singular nonconjugative plasmid of about 60 Md, but in this case the strains are ST + LT producers [50].

In some cases there is a remarkable lability in the phenotypic expression of the adhesins. In case of K99 this is a well-studied phenomenon, which is similar to an on-off switch mechanism [51]. The same may be observed with 987P [33] which is coded by chromosomal genes. Moreover, the type 1 common fimbriae show a character like phase variation [52]. A phenotypic repression of K99 caused by glucose [53] is of catabolic type [54], and also L-alanine acts effectively, repressing K99 and F41 [55]. In some pathological processes this variation between fimbriated and non-fimbriated state may provide an environmental advantage (see later).

1.2.1. *Mannose resistant adhesins for the human uroepithelium*

The inconclusive, even negative experience concerning common fimbriae, as well as the success with MR adhesins in enterotoxic enteropathies were strong motivating factors in the search of MR adhesins responsible for uroepithelial attachment. Such MRHA strains were frequently found among extraintestinal *E. coli* isolates [56], having the characteristic narrow spectrum of haemagglutination capacity [57, 58]. These strains of *E. coli* were analysed for fimbrial antigens by Ørskov et al. [59]; they observed that beside a non functioning fimbria type, called pseudotype 1, MR adhesin designated F7 was present. The F7 shows two fimbrial structures ($F7_1$ and $F7_2$) and its binding capacity to buccal and urinary epithelial cells has been shown. Another, antigenically distinct fimbrial antigen, designated F8 and carried by urinary *E. coli* strains, was found by Svanborg-Edén in Sweden (see Ørskov et al. [13]). One of these strains was isolated in our laboratory by Kuch et al. [60]. This type of MR fimbria (F8), provisorically designated "119" occurred in our *E. coli* isolates from urinary tract infections in 40% in contrast to the faecal frequency of this HA pattern of 2.1% found by Evans et al. [61]. The phenotypic expression of 119 (F8) is inhibited under 18 °C. Its K88-like fimbrial structure was revealed by electronmicroscopy. There is no proof of its plasmid or chromosomal coding origin. The adhesive capacity of 119 (F8) to isolated urinary epithelial cells, and also to the patient's epithelial cells excreting 119 $(F8)^+$ bacteria was also demonstrated [62]. In a suckling mouse model 119 (F8) proved to be an important virulence factor [63] demonstrated also on the basis of immunological protectivity [64].

Certainly F7 and F8 are not the only uroepithelium specific adhesins of *E. coli*. Clegg [65] has recently demonstrated an antigenic heterogeneity among the MR adhesins of the studied uropathogenic *E. coli* strains.

The F7 and F8 similarly to other host specific adhesins are carried by *E. coli* strains belonging to a limited number of serogroups. Ørskov et al. [13] could observe F7 only in O6 : K2 strains, and F8 only in O18a, c and O75 strains. In agreement with Clegg further adhesins may exist: adhering strains

were also found in the O1, O2, O4, O16 and O78 serogroups without F7 or F8 fimbriae. Recently Czirók et al. [66] have studied CFA⁻MR adhesive strains of *E. coli* of faecal origin. The strict correlation of F7 and F8 with specific serogroups has been confirmed, even F8 was carried by serotype O18a,c: K5: H–, but never by O18a,c : K12 : H5 or O18a,c : K5 : H7. Of course, such findings could be explained simply by the clonal spread of an F8$^+$ strain. The quoted authors stressed the diagnostically misleading role of simultaneously carried pseudotype 1 fimbriae.

Little is known about the receptors of the uroepithelium for MR adhesins. However, it appears that a α-D-Galp-(1—4)-β-D-Galp structure constitutes the active receptor element missing from the $\bar{p}$ phenotype of P blood group antigens. The serological heterogeneity of fimbriae does not mean that they differ in receptor specificity, too [67]. Some data show the inherited nature and individual variation of ligand-receptor interactions. Women with recurrent urinary tract infections have a higher incidence and longer duration of vaginal colonization by *E. coli* [68] and their vaginal epithelial cells bind higher numbers of bacteria than those of healthy donors [69]. Similar observations were made concerning the periurethral cells of children [70]. Svanborg-Edén and Jodal [71] also found a higher binding capacity of the excreted uropathogenic *E. coli* in patients with recurrent infections, than did the cells of donors with no such history.

Unfortunately there are few and in some respect incomplete data about the urinary adhesion of *Proteae*. Among urinary *P. mirabilis* isolates MRHA character was found in 12% by Green and Thomas [58]. Silverblatt [72], and Silverblatt and Ofek [73] described a correlation between the fimbriated state, in vitro measured MR epithelial adhesion, and experimental rat infectivity.

1.2.2. *Haemagglutination pattern (haemagglutination typing)*

The data presented indicate or even suggest the existance of unknown adhesins. To prove the occurrence of a new adhesin is not always easy. Methodologically there is a possibility (i) to test the binding activity on isolated or cultured epithelial cells, or on brush border preparations. This approach is not convenient for large scale screenings. (ii) A further possibility is the serological determination. In this respect the simple slide agglutination is frequently misleading owing to the presence of other fimbriae, so preference should be given to immunoelectron microscopy or crossed immunoelectrophoresis. (iii) A simple possibility is offered by haemagglutination performed with and without D-mannose. It is useful if the eventual occurrence of non-haemagglutinating adhesins (e.g. 987P) and the misleading results caused by other fimbriae carried simultaneously are taken into consideration. Evans et al. [74] elaborated

Table III

Haemagglutination typing scheme of E. coli according to Evans et al. [74]

HA type	Haemagglutination with erythrocytes of				
	human	bovine	chicken	monkey[1]	guinea-pig
I-A	R[2]	R	R	—	—
I-B	R	R	R	S[3]	S
II-A	—	R	R	—	—
II-B	—	R	R	S	S
II-C	S	R	R	S	S
III-A	—	—	S	S	S
IV-A	S	—	S	S	S
IV-B	—	—	—	—	—
V-A	R	—	—	—	—
V-B	—	R	—	—	—
V-C	—	—	R	—	—
V-D	—	—	—	R	—
V-E	—	—	—	—	R
V-F	R	—	S	S	S
VI-A	R	—	R	R	—
VI-B	R	—	R	R	S
VI-C	R	—	—	R	—
VI-D	R	—	S	R	S
VI-E	R	—	R	R	R
VI-F	R	—	S	R	R
VII-A	R	R	—	—	—
VII-B	R	R	—	S	S
VII-C	R	R	S	S	S

[1] African green monkey
[2] mannose resistant haemagglutination
[3] mannose sensitive haemagglutination
— no haemagglutination

a HA typing scheme using human, bovine, chicken, monkey and guinea-pig erythrocytes with and without D-mannose (Table III).

2. Chemotaxis

In the pathomechanism of enterotoxic enteropathies caused by *E. coli* at least two essential and cooperative factors are taking part: adhesion and toxin production. The analogous disease caused by *Vibrio cholerae* shows the

well proved significance of toxin, but the adhesiveness of the vibrio is still discussed. The presence of fimbriae was demonstrated on the vibrio cells [75]. Fimbriae are heterogeneous like those of *E. coli*. The haemagglutination caused by some of them was inhibited by D-mannose, others by L-fucose [76]. Fimbrial haemagglutination resistant against both mannose and fucose was observed also by Finkelstein et al. [77] and Guentzel et al. [78].

In animal models, intestinal colonization of *V. cholerae* was observed for a short period both in the ligated rabbit ileal loop [79] and in orally infected infant mice [80]. Nelson et al. [81] demonstrated by scanning electron microscopy an early (one hour after infection) but transient (7–9 h) colonization in the ligated rabbit loop model and the infant rabbit bowel, but they questioned the role of fimbriae.

Guentzel and Berry [82] and Yancey et al. [83] found that motility was an important factor of virulence as about a 100-fold reduction of virulence was observed in the case of non-motile mutants.

According to Freter and O'Brien [84, 85] and Freter et al. [86, 87] staining or electronmicroscopic procedures generally fail to demonstrate the mucous gel, because it is easily washed away. The gel represents a barrier, but this barrier can be penetrated even if inefficiently by inert particles or non-motile bacteria. If motile bacteria, as in the case of wild-type vibrios, are guided by chemotactic gradients, they may easily penetrate into the deep layers of the intervillous spaces. This association is to some extent reversible and the bacteria as Freter et al. [87] have said, must keep running fast just to stay in place against the natural flow of mucus.

According to Freter, *E. coli* passively penetrates across the mucous barrier and a limited number arrives at the brush border. By the aid of its strong adhesins it can colonize this surface. On the other hand, vibrios actively penetrate across the mucous barrier and this penetration is continuous. So high numbers reach the epithelial surface and maintain there a loose or no contact with the epithelial surface.

3. Mucinase

The importance of mucus on epithelial surfaces was stressed by Ørskov et al. [12] in connection with the common fimbriae, as well as by Freter [84–87] in respect of the pathomechanism of *V. cholerae*. Therefore, attention was turned upon the enzymatic factors acting against the mucus.

In 1948 Burnet and Stone [88] described the mucinase production of *V. cholerae*. In the generally used guinea-pig animal model Freter [89] demonstrated that the bowel fluid of infected animals also contains mucinase so its role as a virulence factor had become accepted [88–92]. Recently, however,

Schneider and Parker [93] purified the mucinase of *V. cholerae* and characterized it as an alkaline protease degrading both proteins and glycoproteins. Its role in the pathomechanism remains unknown, only its helper role in active penetration and in the nutrition of vibrios in the mucus has been supposed. Mucinase or mucinolytic enzymes are produced by *E. coli* and by a few serotypes (2a, var. X, and var. Y) of *Shigella flexneri* [94, 95], too. There are no reliable data about their eventual function.

Beside mucinase *V. cholerae* strains produce neuraminidase [96, 97] which removes the terminal sialic acid residues from glycoproteins, and in addition an ill-defined group of proteases. The regulation of mucinase, neuraminidase, proteases, and perhaps the release of toxin seems to be interrelated [98], therefore the avirulance of protease deficient mutants and their eventuel role in pathogenesis needs further investigations.

4. Virulence factors of invasivity

4.1. *Penetration*

The term penetration is used in a specific sense for the unknown process in which bacteria are moving through the epithelial barrier of mucous surfaces. In some cases they are invading the epithelial cells themselves and multiply in them (shigellae) while others passing the epithelium are taken up by the macrophages of the submucosal tissues (salmonellae).

The most investigated pathogens in this respect are the shigellae. The term penetration was used originally by the Walter Reed Army Team [99] studying this first step of pathomechanism on HeLa cells. Isolating different classes of mutants or producing recombinants in virulence the penetration ability proved to be a separate virulence factor [100]. The mechanism of penetration is not clear. It seems that the O antigen has no role in it, because R mutants with intact penetration capacity were successfully isolated [101]. The eventual role of some kind of cytotoxin could neither be proved nor ruled out. According to Ogawa et al. [102] epithelial cell degeneration occurs simultaneously with the endocytosis of shigellae and so it was supposed that some cytotoxic material was produced. This assumption was then supported by in vitro studies performed with cultured macrophages and shigellae of virulent and avirulent character. Phagocytosis took place in both cases, but the ingested virulent bacteria killed the host cell [103]. Other conclusions were drawn by Bondarenko [104] who using different *E. coli* × Shigella hybrids stated that penetration, cytotoxicity and intracellular multiplication were separate factors and there is no proof that a cytotoxin was needed for penetration. In other words, it is not clear whether penetration was an active process or a passive one. The microcinematographic studies of Ogawa et al. [105] of

shigella penetration showed that the first step is a strong membrane attachment observable only with virulent bacteria. This attachment is followed by a rapid movement of the membrane and a pinocytosis of virulent bacteria was observed in HeLa and Henle 407 cells. Hale et al. [106] studied a virulent strain of *S. flexneri* on Henle 407 cultured intestinal epithelial cells. From the histological features of penetration and its inhibition by phagocytosis inhibitors they concluded that penetration was an endocytotic process similar to phagocytosis, but induced only by virulent shigellae. Electron micrograph showed bacteria in the membrane-limiting vacuoles resembling phagosomes.

Testing of penetration ability of isolated cells (HeLa, HEp2, Henle 407) is difficult if the fimbriated bacteria adhere heavily to the cells [107]. In such cases the use of 10-day-old chick embryos is advisable. The selective loss of penetration capacity is evident from the fact that the bacteria are fully virulent on intravenous inoculation and avirulent when inoculated into the allantoic cavity [108]. Furthermore the Serény test [109] generally used for testing the virulence of shigella strains shows a more complex virulence ability: penetration, intraepithelial multiplication and destruction.

The fact that avirulent mutants are easily isolated from wild-type shigella is noteworthy. This opportunity is offered partly by an unknown correlation between virulence and colonial morphology [110, 111] and by the relative lability of virulence. It is well-known that the lability of some bacterial character frequently means that the coding gene(s) are carried by a plasmid. Such a hypothesis was formed in 1971 by Stenzel [112] but the first attempt to prove such suppositions was done by Kopecko et al. [113] in 1979. Change of translucent (T) virulent colonies into opaque (O) avirulent ones was found at a frequency of 10^{-4}–10^{-5} without reversion but no plasmid loss could be observed. Later it was reported [114] that a plasmid of 120–140 Md size is responsible for the penetrating ability of *S. flexneri*.

It is of special interest that the so-called enteroinvasive *E. coli* (EIEC), the causative agent of a both clinically and histologically dysentery-like disease [115] is harbouring the same plasmid [116].

In the case of *S. sonnei* the only difference in respect of virulence is the rapid loss of O-specific side chain (phase I) and strains in phase II are avirulent. Recent observations of Kopecko et al. [117] and Sansonetti et al. [118] have show a non-conjugative plasmid of 120 Md size responsible for the I phase antigenicity as well as for virulence.

In the case of salmonella penetration there is a slight difference compared to shigellae. According to the electron microscopic studies of Takeuchi [119] carried out with a virulent strain of *Salmonella typhi-murium*, a degeneration of microvilli occurred if the bacteria were at a distance of about 35 nm. Degeneration was observed only in the close vicinity of bacteria. The bacteria are invading not only through the brush border, but also through the junc-

tions between epithelial cells. Around the bacteria invading the brush border a cavity can be seen which is frequently open toward the intestinal lumen. After this opening is closed, the integrity of the brush border is restituted and the salmonellae are phagocyted by the submucosal macrophages. In contrast to shigellae no salmonella multiplication occurs in epithelial cells.

4.2. *Tissue invasivity and serum resistance*

4.2.1. *The O antigens*

It is a well known fact that R mutants [120] or even SR mutants of Gram-negative bacteria are avirulent or show a reduced virulence [121, 122]. The reduction of virulence of R mutants, e.g. of *S. typhi-murium* in mice may be million-fold. On the other hand, R strains are capable of colonization and penetration [123]. The defect in the O-side chain generally means a defective resistance against host factors. R mutants in the intestinal canal are increasingly sensitive against fatty acids [124] and in the tissues they sometimes do not multiply at all due to B cell and macrophage activity [125, 126]. These and numerous other data clearly indicate the primary importance in pathogenicity of the O-specific side chain, affording protection against the host defence mechanisms.

It is also well-known that not only the existence of O-antigen, but its quality, its chemical composition has also an influence on the virulence. The best examples are given by the *Salmonella* genus, for which there is a suitable, naturally sensitive mouse model (i), the numerous serotypes differ from each other only in slight changes of the O-antigen (ii), and a possibility exists to exchange O-antigens by transduction or conjugation (iii).

Mäkelä et al. [127] determined the virulence of the parent *S. typhi-murium* (O antigen: 1, 4, 12) and its isogenic transductants by intraperitoneal inoculation of mice. The exchange of O antigen 1, 4, 12 for 1, 9, 12 resulted in a reduction of virulence and the transductants carrying O antigen 6,7 were nearly avirulent. Between O antigen factors O4 and O9 there is a very slight difference: the terminal sugar is abequose in the former, while another dideoxyhexose, tyvelose, in the latter. In a reciprocal experiment [128] the 9, 12 O antigen changed to 1, 4, 12 in a *S. enteritidis* strain and a significant increase in its virulence was observed. Comparing such isogenic clones carrying the O antigens 1, 4, 12 or 6, 7 the reduced virulence of the latter was correlated with the increased speed of blood clearance [129]. Because no bactericidal effect was found, the reduced virulence was interpreted as the result of an enhanced phagocytosis.

In *S. flexneri* the original, unmodified O-specific side chain is represented only by the so called var. Y and all the serotypes are the results of lysogenic

conversion modifying the side chain mostly by alphaglycosyl groups [130]. Studying a parent var. Y strain and some of its lysogenic convertants in the chick embryo model [131], the lysogenic derivatives were shown to have an increased virulence in their tissue growing capacity.

In respect of *E. coli*, according to Medearis et al. [132] the gradual loss in the sugar units of lipopolysaccharide correlates with the gradual loss in virulence.

The interpretation of the observed effect on virulence by subtile changes in the O-specific side chain may only be speculative as little is known of its eventual effect on the composition or structure of the outer membrane. Still, the loss or two major proteins (34K and 36K) out of the four major outer membrane proteins of *S. typhi-murium* did not affect the mouse virulence [133]. On the other hand, there are observations where the change in the side chain was followed by an increased antibiotic sensitivity, which in itself may suggest a change of the outer membrane [134].

4.2.2. *The K antigens*

Our data in this respect are naturally limited to *E. coli*. It is generally accepted that the K antigens of Gram-negatives are less significant in virulence as compared to Gram-positive microbes, because of the existing O antigen. In contrast, in some cases at least the capsular variants of *E. coli* are phagocyted less easily than their non-capsulated isogenic pairs [135, 136].

In extraintestinal infections certain K antigens seem to have some special role. This group of K antigens is genetically determined by a locus in *serA* linkage and not by the common *his*-linked locus. Furthermore, they belong without exception to the common chemical type of the acidic polysaccharide (PS) K-antigens. Its most frequent and most important types are K1, K2, K3, K5, K12 and K13 [136]. Among urinary strains the most frequent K-types are, according to Evans et al. [137], the K1 (29%), furthermore K2, K12 and K13. According to Minshew et al. [138] the K1, K2, K3, K5, K12 and K13 are additive virulence factors in urinary tract infections. It is noteworthy that a considerable part of urinary strains isolated mainly from infections consists of R mutants, but they are $K1^+$ [138, 140].

The antigen K1 has a specific significance in neonatal meningitis: about 85% of strains isolated from such patients are $K1^+$ [141]. The chemical structure of K1, which is an alpha-2,9-linked N-acetyl neuraminic acid polymer, is structurally identical with the capsular B-type structure of *Neisseria meningitidis* [142]. Another frequent *E. coli* agent of neonatal meningitis carries the K92 antigen, being in antigenic relationship with the C-polysaccharide of *N. meningitidis* [143]. A third K-type with similar pathogenicity carries antigen K100 which is cross-reactive with the capsular PS of *Haemophilus influen-*

zae type b [144]. These antigenic relationships are certainly not coincidences in respect of the clinical symptoms caused, but their background is not clear. Also, in cases of *E. coli* sepsis occurring predominantly in newborns [139, 140] but also in adults [145] carrying a high mortality, K1$^+$ *E. coli* strains showing an increased antiphagocytic resistance [145] are found quite frequently.

Some kind of specific affinity of the above mentioned K-types was demonstrated in an animal model, too. In orally infected infant rats K1$^+$ strains showed a 70% colonization rate and a few days later about 60% of the animals had bacteriaemia and 15% also meningitis [146]. Bortolussi et al. [147] stressed the age specificity of K1$^+$ strains in infant rats. By the gene cloning technique the K1 operon was inserted in a 35 kb long fragment through a vector miniplasmid into the K12 strain of *E. coli* [148]. This perfectly avirulent host strain showed an increased serum resistance, in agreement with the observation of Van Dijk et al. [149] that the K antigens of *E. coli* usually inhibit the alternative pathway of complement activation. The experiments of Pitt [150] concerning the age specificity of K1 showed a defective bactericidal effect of infant sera. This is not in contradiction with the data of Stevens et al. [151] namely that not only the quality of K1, but its quantity is also important in the opsoninophagocytic resistance. These authors also showed that the K1 antigen is a complex one and a neuraminidase sensitive second minor antigen has no role in resistance. The correlation of the quantity of antigen K1 with the virulence in urinary tract infections expressed in opsonino–phagocytic resistance was also stated by Björksten and Kaiser [152] and by Howard and Glynn [153].

It seems from these data that some acidic PS K antigens are playing a more or less specific role in the pathogenicity of *E. coli*, mainly in extraintestinal infections. Further work is needed to elucidate the mechanism(s) of virulence mediated by these K antigens.

4.2.3. *Outer membrane proteins*

The normal serum is generally bactericidal also for the S variants of Gram-negative bacteria. The mechanism of this bactericidal effect involves actions of antibodies, the activation of classical or alternative pathway of complement and other proteins, e. g. lysozyme. Some strains, however, are resistant against this system and this so-called serum resistance is an important factor of bacterial invasiveness. In the previous paragraphs the role of O and K antigens has been summarized, but it is clear that these polysaccharide constituents cannot always explain the tissue or serum resistance of their host bacteria. Recently there has been a considerable progress in the understanding of structure and composition of the outer membrane of bacteria. The ratio

and importance of its protein components are in itself suggesting their role in the environmental resistance.

In 1976 Reynard and Beck [154] reported that the plasmids R1 and R100 caused an increased serum resistance in *E. coli* K12. Taylor and Hughes [155] could not confirm this concerning K12. Their data show the essential importance of an intact O-side chain, demonstrating a significant increase in the serum resistance of an S strain after transmission of R1, NR1, and other R and Col plasmids. The level of serum resistance was directly influenced also by the copy number of plasmid NR1, suggesting a direct effect caused by the plasmid. Moll et al. [156] obtained an enhanced serum resistance by R plasmid R6–5 and they demonstrated by the gene cloning technique that one of the two surface exclusion proteins, the TraT-protein is responsible for the observed insensitivity. In R5–6$^+$ bacteria the TraT-protein would be one of the major structural proteins of the outer membrane, with about of 21 000 copies per cell. The authors suggested that not the TraT-protein in itself, but its interaction with other surface components leads to the observed effect. This makes it understandable that e.g. the plasmid R100 in some strains offers protection against serum while in others it fails to do so [155]. There are other outer membrane proteins also conferring serum resistance: Binns et al. [157] localizing the serum resistance determinant of ColV, I–K94 plasmid found it outside of the *tra* region (the *iss* gene).

The mechanisms by which the above structural changes of the outer membrane cause an insensitivity to serum may be different, but according to present knowledge the most probable one is an inhibition of the activation or the binding of complement [158]. Binns et al. [159] have compared the *tra*T and *iss* genes in their phenotypic effects by the use of gene cloning. Both genes were found to be similar in their effects both qualitatively and quantitatively. On the base of complement consumption tests they concluded that not the formation, but the action of the terminal complex (C6–C9) was inhibited. In an earlier paper of Wright and Levine [160] it was already suggested that the terminal complex of complement is formed at the junctions between the inner and outer membrane. The effect may be very specific, because point mutations in the *tra*T gene causing an overproduction of a TraT-like protein fail to protect bacteria from complement [156].

The serum resistance acquired by an occasional plasmid pick up may also have an important epidemiological importance. E.g. *S. wien* isolated in France prior to 1970 had been antibiotic sensitive and caused only mild sporadic cases of salmonellosis. Since 1970 the same serotype (clone) acquired multiple antibiotic resistance and caused severe infections [161].

On the other hand, not all the plasmids and not in all of the strains increased the serum resistance [155]. Moreover, urinary *E. coli* strains were shown [162] to carry multiple plasmid species, with an average number of 4.7.

The number of plasmids carried by a strain showed an inverse correlation with virulence. There are data [163] showing that the increase of the genetic material may prolong generation time and slow down growth, causing thereby a decreased virulence.

4.2.4. *Iron chelators*

The requirements for bacterial multiplication on mucous membranes, in animal tissues or even in serum are low molecular weight nutrients, mineral salts and an appropriate pH. Enteric bacteria have simple nutritional requirements and most mutational defects in biosynthetic capacity do not influence multiplication only because the exogenous sources of such materials are available. Of course, sometimes the mutational events lead to avirulence due to the lack of exogenous supply, or to some pleiotropic effects which lead to other defects or damages. In the past decade studies concerning the solubilization and transport of iron were followed by growing interest.

Iron is essential for most organisms including bacteria by serving electron transport reactions. In animal hosts the iron is not available for bacteria because it is bound to haemosiderin, myoglobin, haemoglobin, etc., and in minute amounts to the iron-binding proteins transferrin and lactoferrin. These binding proteins have a high association constant to ferric ion, therefore the amount of free iron in equilibrium with these proteins is only about 10^{-18} M. This level is far from the requirement of bacterial growth. Therefore the bacterial multiplication in serum and tissues needs iron chelating agents with similar association constants to those of transferrin and lactoferrin [164].

E. coli growing in iron-deficient media secretes 2,3-dihydroxybenzol-L-serine (DHBS) derivatives and among them the main product is enterochelin, the cyclic trimer of DHBS. Enterochelin is capable of removing iron from its transferrin complex [165]. Enterochelin produced by *S. typhi-murium* has been shown to be a virulence factor [166]. In the presence of antibodies (enterochelin being antigenic) the synthesis or excretion of enterochelin is inhibited and this is the case when *E. coli* strains producing enterochelin were found to be unable to grow in sera [167].

A second chelator system, the siderophor is not produced by *E. coli*, but the ferrichrome complex is taken up by them [168]. It forms with iron a ferritrihydroxamate compound with a high stability constant of 10^{29} [169]. A third high affinity iron transport system is that of ferric citrate [170].

For the uptake of ferric iron complexes receptor proteins in the outer membrane are required. These are specific for ferric enterochelin [171], for ferrichrome [172], as well as for ferric citrate [171] and for further transport all of them share a common requirement for a further protein, which is the product of the *ton*B gene [171].

In 1974 Smith [173] observed that invasive strains of *E. coli* isolated from sepsis or meningitis are predominantly harbouring colV plasmid. This was supported in further studies [174] which also showed a prolonged survival of colV$^+$ strains in the alimentary tract. This virulence factor bound to the presence of colV plasmid was independent from colicin V production or immunity [175]. Furthermore, a separate gene of plasmid colV was observed to be connected with virulence by coding a new iron chelator system [176, 177], with an extracellular iron chelating compound and a non-diffusible product taking part in the transport process across the membrane. This siderophore is a hydroxamate compound [178] identical with the aerobactin produced by *Aerobacter aerogenes* [179]. Aerobactin may be the sole siderophore product of certain *Salmonella* strains [179] and *S. flexneri* types [180].

It is of special interest how aerobactin production ensure an advantage for invasive *E. coli* strains which synthese other siderophores. According to Warner et al. [179] the most important chelator, enterochelin, is chemically and enzymologically less stable and less soluble than aerobactin and its iron complexes are pH dependent and its synthesis and iron release require much metabolic energy.

4.2.5. *Haemolysins*

In the previous paragraph it was stated that the iron supply for bacteria depends on the siderophores in animal tissues. An exception to this is the lysis of red blood cells which provides large amounts of iron for bacteria that can assimilate heme compounds [181, 182]. This in itself may support the idea that haemolysin production may be considered one of the virulence factors.

As early as 1921 Dudgeon et al. [183] suggested that haemolytic activity might be important in the virulence of *E. coli* for the urinary tract. It is known that *E. coli* strains isolated from extraintestinal infections are more haemolytic than the faecal strains. Minshew et al. [56] found this ratio to be 49% in extraintestinal isolates. The faecal ratio was found to be between 8% and 18% [184, 185]. Haemolytic *E. coli* strains were isolated from urine in 42% and from blood in 29% by Evans et al. [137].

Four different types of *E. coli* haemolysin have been distinguished [186]. Among them the most important ones are the alpha- and beta-haemolysins. The beta-type is cell bound [185] and seems to play no role in virulence [186]. Alpha-haemolysin is a cell-free, diffusible and filterable protein produced during the log-phase growth of *E. coli* perhaps by modification of a precursor molecule present in meat or blood of the medium [183, 188–190]. Alpha-haemolysin is the only type considered to be a virulence factor.

There are numerous reports about the general toxicity, or lethal effect of haemolysin in mice [185], or in chick embryos [56, 187], its necrotic effect

on rabbit skin [191], and the production of a lethal, haemorrhagic oedema in mice inoculated by nasal instillation [187, 192]. After intravenous inoculation of haemolytic *E. coli* strains, lysosomal disruption and focal necrosis was observed in the rat kidney [193]. In other animal models of urinary tract infection the haemolysin proved to be a virulence factor [63, 194, 195]. There are reports also about the in vitro cytotoxic effect of haemolysin in cell cultures of chick embryo fibroblast cells [196], or human amniotic AV-3 cells [63].

The above data originate from experiments performed with haemolytic strains or crude haemolysin. With partially purified haemolysin Rennie and Arbuthnott [190] failed to demonstrate mouse or rabbit toxicity. On the other hand, Cavalieri and Snyder's [197] 19-fold purified product showed in nanogram amounts a cytotoxic effect running parallel with the high haemolytic activity.

The difficulties in purification cause problems in the molecular characterization of haemolysin. According to the above data [190, 197] the measured molecular weight is about 300 000 d, but while some found it in pure protein composition [190], others demonstrated a considerable carbohydrate content [197] which may have been an endotoxin contamination. There is some uncertainity even in molecular weight determination: the above high molecular weight may be the consequence of aggregation, and according to Williams [198] monomeric haemolysin has a molecular weight of 120 000 d.

Further problems concern the production and release of haemolysin. According to Springer and Goebel [199] two transport processes are involved in the secretion of haemolysin. In the first energy-dependent process the haemolysin is released through the inner membrane and accumulates in a small pool in the periplasmatic space. In the second, energy-independent, but temperature-dependent process the haemolysin is released through the outer membrane.

The genetic background of alpha-haemolysin production is also a matter of discussion. In an early paper of Smith and Halls [200] the plasmid coded nature of the alpha-haemolysin of *E. coli* was already reported and the plasmid was designated as Hly. This finding was supported by the studies of Goebel and Schrempf [201] and Goebel et al. [202]. In a later work of this team [203] the investigated Hly plasmid was found to be an associate type with a molecular size of 41 Md and the DNA segment directing the haemolysin synthesis and secretion of 3.2 Md size. This segment was supposed to contain at least 3 cistrons, two of which are coding the synthesis and the third the secretion.

Some other data are seemingly contradictory to the above findings. First of all, that Hly plasmids belong to different compatibility groups [204, 205], furthermore most of the haemolytic strains of *E. coli* of human origin were found to produce chromosomally coded haemolysin [206]. These contradictions could be explained by the finding that the plasmid-mediated haemolysins have generally homologous gene sequences irrespective of the compati-

bility group of the plasmids [205]. Moreover, both plasmid and chromosomal coded haemolysin genes were shown to share homologous DNA sequences [207]. These data may perhaps suggest the transposon nature of the gene(s) of alpha-haemolysin integrated in some cases into plasmids and in other cases into the bacterial genome.

The serogroup distribution of the haemolytic ability of *E. coli* seems like the other virulence factors to be rather limited. The most frequent haemolytic serogroups among pig pathogens are O8, O138, O139 and O141 [185], and in humans O4, O6 and O18a, c [192], less frequently O20, O75, O115 and O147 [208].

Among other genera of enteric bacteria the high incidence of haemolytic *Proteus morganii* strains isolated from urine is noteworthy. Preliminary studies [209] showed a higher mouse and chick embryo virulence of haemolytic strains and a decrease in their virulence after the loss of haemolytic ability following actinomycin-D treatment. The latter observation may suggest a plasmid coded nature. Moreover, on the basis of cross-neutralization experiments, the alpha-haemolysin of *E. coli* and *P. morganii* seem to be identical [210].

We have no data about the significance of the frequent incidence of haemolysin production among *Serratia marcescans* strains [204] or of the *V. cholerae* biotype *el-tor* [211]. The vibriolysin of the latter was found to be cytotoxic, cardiotoxic and lethal in experimental animals. On the other hand, the lethal vibriolysin of *V. parahaemolyticus* [212] proved to be closely related to the human pathogenicity of the strains [213].

5. Relationships of virulence factors. Conclusions

Most of the virulence factors discussed in the previous paragraphs are not the only elements of pathogenicity. Sometimes they are providing an essential, sometimes an accessory additive factor of the pathogenic process.

Considering the role of adhesins, in general they seem to be important factors of virulence. Their loss may allow the microbes to be swept away by fluid or other movements and the infective process will not develop at all. In other cases the adhesiveness may be of secondary significance: e.g. in the case of *V. cholerae* some data suggest that even without colonization the active penetration of mucus by chemotactic movements of the vibrios provides enough toxin for causing clinical symptoms. Similarly, with pathogens penetrating across the epithelium like salmonellae and shigellae, their adhesion might be of secondary importance.

On the other hand, even in cases where the adhesion is an important first step of the infectious process, the advantage of adhesins turns to a disadvantage for the pathogen reaching the submucosal tissues. Bacteria with

appendages of hydrophobic nature such as fimbriae, are easily phagocyted both by macrophages and granulocytes [214, 215]. This was clearly demonstrated by Silverblatt and Ofek [73] in experimental *Proteus mirabilis* infection of rats. They found that well-fimbriated bacteria showed a higher infectivity rate and were predominantly isolated from the renal pelvis. On the other hand, the majority of bacteria which reached the renal cortex were non-fimbriated. The controversial functioning of fimbriae in the different steps of infection is balanced in different ways. One of them may be the labile expression of fimbriae permitting changes between fimbriated and non-fimbriated states. Its genotypic (plasmid coded) and phenotypic features have already been discussed. Another possibility may be provided by altering or masking the fimbrial surface hydrophobicity by another surface component, e.g. by K antigens.

The latter possibility was demonstrated with *E. coli* strains isolated from urinary tract infections: a high frequency of adhesiveness was associated with a similarly high frequency of some acidic polysaccharide K antigens [56, 137].

The importance of some other virulance factors may depend on the particular pathogenic microorganism and on the particular site of the infection. It is easy to understand that a factor like haemolysin should have no or little role in the pathogenesis of cholera, because *V. cholerae* biotype el-tor is not invasive but produces a potent toxin. This is also true for the enterotoxic enteropathies caused by *E. coli*. In these conditions, Smith and Linggood [216] observed no role of haemolysin production in contrast to its importance in parenteral infections. In addition, haemolysin may be a significant virulence factor of *E. coli* when it is invading submucosal tissues or causing bacteriaemia. The focal necrotic lesions caused by it [193] or securing the iron supply of the agent [181, 182] are sufficiently important in themselves. Futhermore, the haemolysin may by its leukocidal action be a counterbalancing factor of the phagocytosis promoting effect of fimbriae [187]. It is therefore not surprising that there is a high coincidence rate between adhesiveness and the haemolytic character in the urinary isolates of *E. coli* [56, 58] suggesting perhaps a synergistic effect. Similar relations were observed in extraintestinal isolates of *E. coli* in respect of ColV (aerobactin), haemolysin and/or adhesiveness [56].

These data suggest that the aforementioned combinations of different virulence factors have evolved under the effect of natural selection. According to present knowledge the most easy way to build up such a pathogen is done by picking up plasmids, and it has been mentioned in this review that the virulence factors listed are predominantly plasmid coded. Furthermore, sometimes more than one coding sequence of virulence factors is carried by an associated, composite plasmid element. It is therefore only natural that most reports describe some virulence factor (mostly enterotoxin) determinant carried by an R plasmid [217–221], and there are data on heat-stable enterotoxin

(ST) determinants which together with some adhesin coding sequences are carried by one plasmid: like ST + K88 [222], ST and K99 [223], ST + CFA/I [224], and ST + CFA/II [50]. The frequent presence of ST in these combinations is explained by its transposon nature [225] permitting easy integration into different genomes.

It seems that the continuous evolution of enteric pathogens is due even today to natural selection. The most suitable clones that pick up different plasmids or are further amplified by transposons, are spreading and may arise new problems in bacteriological diagnostic work, epidemiology and clinical therapy.

REFERENCES

1. Lányi, B.: Nat Congr Hungarian Association of Microbiology Debrecen, Hungary 1982.
2. Duguid, J. P., Smith, I. W., Dempster, G., Edmunds, P. N.: J Pathol Bacteriol **70,** 335 (1955).
3. Duguid, J. P., Gillies, R. R.: J Pathol Bacteriol **74,** 397 (1957).
4. Duguid, J. P.: J Gen Microbiol **21,** 271 (1959).
5. Gillies, R. R., Duguid, J. P.: J Hyg (Lond.) **56,** 303 (1959).
6. Duguid, J. P., Gillies, R. R.: J Pathol Bacteriol **75,** 519 (1958).
7. Duguid, J. P., Clegg, S., Wilson, M. I.: J Med Microbiol **12,** 213 (1979).
8. Old, D. C., Corneil, I., Gibson, L. F., Thomson, A. D., Duguid, J. P.: J Gen Microbiol **51,** 1 (1968).
9. Duguid, J. P., Darekar, M. R., Wheater, D. F. W.: J Med Microbiol **9,** 459 (1976).
10. Fader, R. C., Davis, C. P.: Infect Immun **30,** 554 (1980).
11. Schaefter, A. J., Amundsen, S. K., Schmidt, L. N.: Infect Immun **24,** 753 (1979).
12. Ørskov, I., Ferencz, A., Ørskov, F.: Lancet **1,** 887 (1980).
13. Ørskov, I., Ørskov, F., Birch-Andersen, A., Klemm, P., Svanborg-Edén, C.: Bacterial Vaccines. In Robbins, I. B., (eds): Seminars in Infectious Disease. Vol. 4. Thieme-Stratton Inc., New York 1982. pp 97–104.
14. Moon, H. W., Isaascon, R. E., Pohlenz, J.: Am J Clin Nutr **32,** 119 (1979).
15. Svanborg-Edén, C., Ericksson, B., Hanson, L. A.: Infect Immun **18,** 767 (1977).
16. Ørskov, I., Ørskov, F., Sojka, W. J., Wittig, W.: Acta Pathol Microbiol Scand **62,** 439 (1964).
17. Ørskov, I., Ørskov, F.: J Bacteriol **91,** 69 (1966).
18. Stirm, S., Ørskov, F., Ørskov, I., Mansa, B.: J Bacteriol **93,** 731 (1967).
19. Stirm, S., Ørskov, F., Ørskov, I., Birch-Andersen, A.: J Bacteriol **93,** 740 (1967).
20. Smith, H. W., Linggood, M. A.: J Med Microbiol **4,** 467 (1971).
21. Jones, G. W., Rutter, J. M.: Infect Immun **6,** 918 (1972).
22. Sojka, W. J.: Vet Bull **41,** 509 (1971).
23. Parry, S. H., Porter, P.: Immunology **34,** 41 (1978).
24. Guinée, P. A. M., Jansen, W. H.: Infect Immun **23,** 700 (1979).
25. Sellwood, R., Gibbons, R. A., Jones, G. W., Rutter, J. M.: J Med Microbiol **8,** 405 (1975).
26. Bijlsma, I. G. W., Nijs, de, A., Frik, J. F.: Antonie van Leeuwenhoek **47,** 467 (1981).
27. Smith, H. W., Linggood, M. A.: J Med Microbiol **5,** 243 (1972).
28. Ørskov, I., Ørskov, F., Smith, H. W., Sojka, W. J.: Acta Pathol Microbiol Scand [B] **83,** 31 (1975).
29. Moon, H. W., Nagy, B., Isaacson, R. E., Ørskov, I.: Infect Immun **15,** 614 (1977).
30. Isaacson, R. E., Nagy, B., Moon, H. W.: J Infect Dis **135,** 531 (1977).
31. de Graaf, F. K., Roorda, I.: Infect Immun **36,** 751 (1982).
32. Guinée, P. A. M., Jansen, W. H.: Zentralbl Bakteriol [A] **243,** 245 (1979).
33. Nagy, B., Moon, H. W., Isaacson, R. E.: Infect Immun **16,** 344 (1977).
34. Moon, H. W., Kohler, E. M., Schneider, R. A., Whipp, S. C.: Infect Immun **27,** 222 (1980).
35. Burrows, M. R., Sellwood, R., Gibbons, R. A.: J Gen Microbiol **96,** 269 (1976).
36. Isaacson, R. E., Richter, P.: J Bacteriol **146,** 784 (1981).
37. Smith, H. W., Gyles, C. L.: J Med Microbiol **3,** 387 (1970).

38. Gaastra, W., de Graaf, F. K.: Microbiol Rev **46,** 129 (1982).
39. Moon, H. W., Whipp, S. C.: J Infect Dis **122,** 220 (1970).
40. Walker, P. D., Nagy, L. K.: In Berkeley, R. C. W., Melling, J., Linch, J. M., Rutter, P. R., Vincent, B. (eds): Microbial Adhesion to Surfaces. Ellis Horwood Ltd., Chichester, England 1980.
41. Kétyi, I., Kuch, B., Verténvi, A.: Acta Microbiol Acad Sci Hung **25,** 77 (1978).
42. Evans, D. G., Silver, R. P., Evans, D. J. Jr., Chase, D. G., Gorbach, S. L.: Infect Immun **12,** 656 (1975).
43. Evans, D. G., Evans, D. J. Jr.: Infect Immun **21,** 638 (1978).
44. Shipley, P. L., Gyles, C. L., Falkow, S.: Infect Immun **20,** 559 (1978).
45. Schmitt, R., Mattes, R., Schmid, K., Altenbuchner, J.: In Timmis, K. N., Pühler, A. (eds): Plasmids of Medical, Environmental and Commercial Importance. Elsevier North Holland, Amsterdam 1979.
46. Mooi, F. R., de Graaf, F. K., van Embden, J. D. A.: Nucleic Acid Res **6,** 849 (1979).
47. Smith, H. W., Linggood, M. A.: J Med Microbiol **5,** 243 (1972).
48. So, M., Boyer, H. W., Betlach, M., Falkow, S.: J Bacteriol **128,** 463 (1976).
49. Van Embden, J. D. A., de Graaf, F. K., Schouls, L. M., Teppema, J. S.: Infect Immun **29,** 1125 (1980).
50. Peneranda, M. E., Mann, M. B., Evans, D. G., Evans, D. J. Jr.: FEMS Microbiol Letter **8,** 251 (1980).
51. Morris, J. A., Thorns, C., Scott, A. C., Sojka, W. J., Wells, G. A.: Infect Immun **36,** 1146 (1982).
52. Brinton, C. C. Jr.: Transact N. Y. Acad Sci **27,** 1003 (1965).
53. Guinée, P. A. M., Veltkamp. J., Jansen, W. H.: Infect Immun **15,** 676 (1977).
54. Isaacson, R. E.: Infect Immun **28,** 190 (1980).
55. de Graaf, F. K., Klaasen-Boor, P., van Hees, J. E.: Infect Immun **30,** 125 (1980).
56. Minshew, B. H., Jorgensen, J., Counts, G. W., Falkow, S.: Infect Immun **20,** 50 (1978).
57. Ljungh, A., Faris, A., Wadström, T.: J Clin Microbiol **10,** 477 (1979).
58. Green, C. P., Thomas, V. L.: Infect Immun **31,** 309 (1981).
59. Ørskov, I., Ørskov, F., Birch-Andersen, A.: Infect Immun **27,** 657 (1980).
60. Kuch, B., Pál, T., Emődy, L.: Acta Microbiol Acad Sci Hung **27,** 317 (1979).
61. Evans, D. J. Jr., Evans, D. G., Du Pont, H. L.: Infect Immun **23,** 336 (1979).
62. Kuch, B., Pál, T., Emődy, L.: Lancet **2,** 107 (1982).
63. Kétyi, I.: Acta Microbiol Acad Sci Hung **28,** 393 (1981).
64. Kétyi, I.: Acta Microbiol Acad Sci Hung **29,** 123 (1982).
65. Clegg, S.: Infect Immun **35,** 745 (1982).
66. Czirók, É., Ørskov, I., Ørskov, F.: Infect Immun **37,** 519 (1982).
67. Källenius, G., Svenson, S. B., Möllby, R., Korhonen, T., Winberg, J., Cedergren, B., Helin, I., Hultberg, H.: Scand J Infect Dis Suppl **33,** 52 (1982).
68. Stamey, T. A., Sexton, C. C.: J Urol **113,** 214 (1975).
69. Fowler, J. E., Stamey, T. A.: J Urol **117,** 472 (1977).
70. Källenius, G., Winberg, J.: Lancet **2,** 540 (1978).
71. Svanborg-Edén, C., Jodal, U.: Infect Immun **26,** 837 (1979).
72. Silverblatt, F. J.: J Exp Med **140,** 1696 (1974).
73. Silverblatt, F. J., Ofek, I.: J Infect Dis **138,** 664 (1978).
74. Evans, D. J. Jr., Evans, D. G., Young, L. S., Pitt, J.: J Clin Microbiol **12,** 235 (1980).
75. Tweedy, J. M., Park, R. W. A., Hodgkiss, W.- J Gen Microbiol **51,** 235 (1968).
76. Jones, G. W., Freter, R.: Infect Immun **14,** 240 (1976).
77. Finkelstein, R. A., Arita, A., Clements, J. D., Nelson, E. T.: Proc 13th Joint Cholera Research Conference, Atlanta, Georgia 1977. p. 137.
78. Guentzel, M. N., Wong, G. G., Gay, T. V.: Proc 13th Joint Cholera Research Conference, Atlanta, Georgia 1977. p. 77.
79. Srivastava, R., Sinha, V. B., Srivastava, B. S.: J Med Microbiol **13,** 1 (1980).
80. Baselski, V. S., Parker, C. D.: Infect Immun **21,** 518 (1978).
81. Nelson, E. T., Clements, J. D., Finkelstein, R. A.: Infect Immun **14,** 527 (1976).
82. Guentzel, M. N., Berry, L. J.: Infect Immun **11,** 890 (1975).
83. Yancey, R. J., Willis, D. L., Berry, L. J.: Infect Immun **22,** 387 (1978).
84. Freter, R., O'Brien, P. C. M.: Infect Immun **34,** 215 (1981).
85. Freter, R., O'Brien, P. C. M.: Infect Immun **34,** 222 (1981).
86. Freter, R., O'Brien, P. C. M., Macsai, M. S.: Infect Immun **34,** 234 (1981).
87. Freter, R., Allweiss, B., O'Brien, P. C. M., Halstead, S. A., Macsai, M. S.: Infect Immun **34,** 241 (1981).
88. Burnet, F. M., Stone, J. D.: Aust J Exp Biol Med Sci **26,** 71 (1948).

89. Freter, R.: Bact Proc 56 (1954).
90. Singer, E., Wei, S. H., Hoa, S. H.: J Immunol **59,** 341 (1948).
91. Lam, G. T., Mandle, R. J., Goodner, K.: J Infect Dis **97,** 268 (1955).
92. Lankford, C. E.: Ann NY Acad Sci **88,** 1203 (1960).
93. Schneider, D. R., Parker, C. D.: J Infect Dis **145,** 474 (1982).
94. Formal, S. B., Lowenthal, J. P., Galindo, E.: J Bacteriol **75,** 467 (1958).
95. Formal, S. B., Lowenthal, J. P.: Proc Soc Exp Biol Med **25,** 227 (1956).
96. Burnet, F. M., Stone, J. D.: Aust J Exp Biol Med Sci **25,** 227 (1947).
97. Gottschalk, A.: Biochem Biophys Acta (Amst.) **23,** 645 (1957).
98. Schneider, D. R., Parker, C. D.: J Infect Dis **138,** 143 (1978).
99. LaBrec, E. H., Schneider, H., Magnani, T. J., Formal, S. B.: J Bacteriol **88,** 1503 (1964).
100. Falkow, S., Schneider, H., Baron, L. S., Formal, S. B.: J Bacteriol **86,** 1251 (1963).
101. Okamura, N., Nakaya, R.: Infect Immun **17,** 4 (1977).
102. Ogawa, H., Honja, S., Takasaka, M., Fujiwara, T., Imaizumi, K.: Jap J Med Sci Biol **19,** 23 (1966).
103. Yee, R. B., Buffenmyer, C. L.: Infect Immun **1,** 459 (1970).
104. Bondarenko, V. M.: Vestn Akad Med Nauk (SSSR) **11,** 23 (1973).
105. Ogawa, H., Nakamura, A., Nakaya, R.: Jap J Med Sci Biol **21,** 259 (1968).
106. Hale, T. L., Morris, R. E., Bonventre, P. F.: Infect Immun **24,** 887 (1979).
107. DuPont, H. L., Formal, S. B., Hornick, R. B.: N Engl J Med **285,** 1 (1971).
108. Rauss, K., Kétyi, I., Angyal, T.: Acta Microbiol Acad Sci Hung **14,** 131 (1967).
109. Serény, B.: Acta Microbiol Acad Sci Hung **4,** 367 (1957).
110. Cooper, M. L., Keller, H. M., Walters, W. E.: J Immunol **78,** 160 (1957).
111. Kerekes, L.: Acta Microbiol Acad Sci Hung **9,** 123 (1962).
112. Stenzel, W.: Zentralbl Bakteriol [A] **216,** 67 (1971).
113. Kopecko, D. J., Holcombe, J., Formal, S. B.: Infect Immun **24,** 580 (1979).
114. Kopecko, D. J., Sansonetti, P. J., Baron, L. S., Formal, S. B.: In Levy, S. B., Clowes, R. C., Koenig, E. L. (eds): Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids. Plenum Press, New York 1981. p. 111.
115. Edwards, P. R., Ewing, W. H.: Identification of Enterobacteriaceae. 3rd ed. Burgers Publishing Co., Minneapolis 1972. p. 113.
116. Sansonetti, P. J., d'Hauteville, H., Formal, S. B., Toucas, M.: Ann Microbiol (Paris) **132**A, 351 (1982).
117. Kopecko, D. J., Washington, O., Formal, S. B.: Infect Immun **29,** 207 (1980).
118. Sansonetti, P. J., Kopecko, D. J., Formal, S. B.: Infect Immun **34,** 75 (1981).
119. Takeuchi, A.: In Schlessinger, D. (ed.): Microbiology. American Society of Microbiology, Washington 1975. p. 134.
120. Roantree, R. J.: In Kadis, S., Weinbaum, G., Ajl, S. J.: (eds): Microbial Toxins. Academic Press, New York 1971.
121. Nakano, M., Saito, K.: Nature **222,** 1085 (1969).
122. Valtonen, V.: J Gen Microbiol **57,** 28 (1969).
123. Tannock, G. W., Blumenshine, R. V. H., Savage, D. C.: Infect Immun **11,** 365 (1975).
124. Wardlaw, A. C.: Proc Soc Gen Microbiol **1,** 50 (1974).
125. Morris, J. A., Wray, C., Sojka, W. J.: Br J Exp Pathol **57,** 354 (1976).
126. O'Brien, A. D., Scher, I.: Abstr Ann Meet ASM E 105 72 (1979).
127. Mäkelä, P. H., Valtonen, V. V., Valtonen, M.: J Infect Dis **128,** Suppl S81 (1973).
128. Valtonen, M. V., Plosila, M., Valtonen, V. V., Mäkelä, P. H.: Infect Immun **12,** 828 (1975).
129. Valtonen, M. V.: Infect Immun **18,** 574 (1977).
130. Simmons, D. A. R.: Bact Rev **35,** 117 (1971).
131. Kétyi, I.: Infect Immun **9,** 931 (1974).
132. Medearis, D. N. Jr., Camitta, B. M., Heath, E. C.: J Exp Med **128,** 399 (1968).
133. Valtonen, M. V., Nurminen, M., Johansson, V., Mäkelä, P. H.: Infect Immun **18,** 454 (1977).
134. Ferro-Luzzi Ames, G., Spudich, E. N., Nikaido, H.: J Bacteriol **117,** 406 (1974).
135. Sjöstedt, S.: Acta Pathol Microbiol Scand **63,** 1 (1946).
136. Ørskov, F.: J Infect Dis **137,** 630 (1978).
137. Evans, D. J. Jr., Evans, D. G., Höhne, C., Noble, M. A., Haldane, E. V., Lior, H., Young, L. S.: J Clin Microbiol **13,** 171 (1981).
138. Minshew, B. H., Jorgensen, J., Svanstrum, M., Grootes-Reuvecamp, G. A., Falkow, S.: J Infect Dis **137,** 648 (1978).
139. Cheasty, T. R., Gross, J., Rowe, B.: J Clin Pathol **30,** 945 (1977).
140. McCabe, W. R., Kaijser, B., Olling, S., Uwaydah, M., Hanson, L. A.: J Infect Dis **138,** 33 (1978).

141. Robbins, J. B., McCracken, G. H., Gotschlich, E. C., Ørskov, F., Ørskov, I., Hansen, L. A.: N Engl J Med **290,** 1216 (1974).
142. Kasper, D. L., Winkelhake, J. W., Zollinger, W. D., Brandt, B. L., Artenstein, M. S.: J Immunol **110,** 262 (1973).
143. Glode, M. P., Robbins, J. B., Teh-Yung Liu, Gotschlich, E. C., Ørskov, I., Ørskov, F.: J Infect Dis **135,** 94 (1977).
144. Bradshaw, M. W., Schneerson, R., Parke, J. C., Robbins, J. B.: Lancet **1,** 1095 (1971).
145. Weinstein, R., Young, L. S.: J Clin Microbiol **8,** 748 (1978).
146. Glode, M. P., Sutton, A., Moxon, E. R., Robbins, J. B.: Infect Immun **16,** 75 (1977).
147. Bortolussi, R., Ferrieri, P., Wannamaker, L. W.: Infect Immun **22,** 480 (1978).
148. Silver, R. P., Finn, C. V., Vann, W. F., Aaronson, W., Schneerson, R., Kretschmer, P. J., Garon, C. F.: Nature **289,** 696 (1981).
149. Van Dijk, W. C., Verbrough, H. A., Van der Tol, M. E., Peters, R., Verhoef, J.: Infect Immun **25,** 603 (1979).
150. Pitt, J.: Infect Immun **22,** 219 (1978).
151. Stevens, P., Chung, L. C., Young, L. S.: Infect Immun **29,** 1055 (1980).
152. Björksten, B., Kaijser, B.: Infect Immun **22,** 308 (1978).
153. Howard, C. J., Glynn, A. A.: Immunology **20,** 767 (1971).
154. Reynard, A. M., Beck, M. E.: Infect Immun **14,** 848 (1976).
155. Taylor, P. W., Hughes, C.: Infect Immun **22,** 10 (1978).
156. Moll, A., Manning, P. A., Timmis, K. N.: Infect Immun **28,** 359 (1980).
157. Binns, M. M., Davies, D. L., Hardy, K. G.: Nature **279,** 778 (1979).
158. Ogata, R. T., Levine, R. P.: J Immunol **125,** 1494 (1980).
159. Binns, M. M., Mayden, J., Levine, R. P.: Infect Immun **35,** 654 (1982).
160. Wright, S. D., Levine, R. P.: J Immunol **127,** 1152 (1981).
161. LeMinor, S.: Med Malad Infect **2,** 441 (1972).
162. Waalwijk, C., van der Bosch, J. F., MacLaren, D. M., de Graaff, J.: FEMS Microbiol Letter **14,** 171 (1982).
163. Anderson, J. D., Eftekhar, F., Aird, M. Y., Hammonds, J.: J Clin Microbiol **10,** 766 (1979).
164. Bullon, J. J., Rogers, H. J., Griffiths, E.: Curr Top Microbiol Immunol **80,** 1 (1978).
165. Rogers, H. J., Synge, C., Kimber, B., Bayley, P. M.: Biochim Biophys Acta (Amst.) **497,** 548 (1977).
166. Rogers, H. J.: Infect Immun **7,** 445 (1973).
167. Yancey, R. J., Breeding, S. A. L., Lankford, C. E.: Infect Immun **24,** 174 (1979).
168. Hartmann, A., Braun, V.: J Bact **143,** 246 (1980).
169. Raymond, K. W., Carrano, C. J.: Acc Chem Res **12,** 183 (1979).
170. Frost, G. E., Rosenverg, H.: Biochim Biophys Acta (Amst.) **330,** 90 (1973).
171. Hancock, R. E. W., Hantke, K., Braun, V.: J Bacteriol **127,** 1370 (1976).
172. Wayne, R., Neilands, J. B.: J Bacteriol **121,** 497 (1975).
173. Smith, H. W.: J Gen Microbiol **83,** 95 (1974).
174. Smith, H. W., Huggins, M. B.: J Gen Microbiol **92,** 335 (1976).
175. Quackenbush, R. L., Falkow, S.: Infect Immun **24,** 562 (1979).
176. Williams, P. H.: Infect Immun **26,** 925 (1979).
177. Williams, P. H., Warner, P. J.: Infect Immun **29,** 411 (1980).
178. Stuart, S. J., Greenwood, K. T., Luke, R. K. J.: J Bacteriol **143,** 35 (1980).
179. Warner, P. J., Williams, P. H., Bindereif, A., Neilands, J. B.: Infect Immun **33,** 540 (1981).
180. Payne, S. M.: J Bacteriol **143,** 1420 (1980).
181. Kaye, D., Hook, E. W.: J Immunol **91,** 65 (1963).
182. Linggood, M. A., Ingram, P. L.: J Med Microbiol **15,** 23 (1982).
183. Dudgeon, L. S., Wordley, E., Bawtree, F.: J Hyg **20,** 137 (1921).
184. Cooke, E. M.: J Pathol Bacteriol **95,** 101 (1968).
185. Smith, H. W.: J Pathol Bacteriol **85,** 197 (1963).
186. Jorgensen, S. E., Short, E. C., Kurtz, H. J., Mussen, H. K., Wu, G. K.: J Med Microbiol **9,** 173 (1976).
187. Emődy, L., Pál, T., Safonova, N. V., Kuch, B., Golutova, N. K.: Acta Microbiol Acad Sci Hung **27,** 333 (1980).
188. Lovell, R., Rees, T. A.: Nature **188,** 755 (1960).
189. Snyder, I. S., Koch, N. A.: J Bacteriol **91,** 763 (1966).
190. Rennie, R. P., Arbuthnott, J. P.: J Med Microbiol **7,** 179 (1974).
191. Cooke, E. M., Ewins, S. P.: J Med Microbiol **8,** 107 (1975).
192. Kétyi, I., Emődy, L., Kontrohr, T., Verténvi, A., Pácsa, S., Avdeeva, T. A., Safonova, N. V., Golutova, N. K.: Acta Microbiol Acad Sci Hung **25,** 307 (1978).
193. Fry, T. L., Fried, F. A., Goven, B. A.: Invest Urol **13,** 47 (1975).

194. Van den Bosch, J. F., Postma, P., de Graaff, J., MacLaren, D. M.: J Med Microbiol **14,** 321 (1981).
195. Van den Bosch, J. F., Emődy, L., Kétyi, I.: FEMS Microbiol Letter **13,** 427 (1982).
196. Chaturvedi, U. C., Mather, A., Khan, A. M., Mehrotra, R. M. L.: J Med Microbiol **2,** 211 (1969).
197. Cavalieri, S. J., Snyder, I. S.: J Med Microbiol **15,** 11 (1982).
198. Williams, P. H.: FEMS Microbiol Letter **5,** 21 (1979).
199. Springer, W., Goebel, W.: J Bacteriol **144,** 53 (1980).
200. Smith, H. W., Halls, S.: J Gen Microbiol **47,** 153 (1967).
201. Goebel, W., Schrempf, H.: J Bacteriol **106,** 311 (1971).
202. Goevel, W., Royer-Pokora, B., Lindenmaier, W., Bojard, H.: J Bacteriol **118,** 964 (1974).
203. Noegel, A., Rdest, U., Springer, W., Goebel, W.: Molec Gen Genet **175,** 343 (1979).
204. LeMinor, S., LeCoueffic, É.: Ann Microbiol (Paris) **126**B, 313 (1975).
205. De La Cruz, F., Müller, D., Ortiz, J. M., Goebel, W.: J Bacteriol **143,** 825 (1980).
206. Hull, R., Hull, S., Minshew, B., Falkow, S.: Curr Chemother Infect Dis. Proc 11th ICC and 19th ICAAC 1283 (1980).
207. Goebel, W., Noegel, A., Müller, D., Rdest, U., Hughes, C.: Abstr Congr Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids, Santo Domingo, Dominique 1981.
208. Czirók, É.: Acta Microbiol Acad Sci Hung **27,** 71 (1980).
209. Emődy, L., Vörös, S., Pál, T.: FEMS Microbiol Letter **13,** 329 (1982).
210. Emődy, L.: Unpublished observations.
211. Honda, T., Finkelstein, R. A.: Infect Immun **26,** 1020 (1979).
212. Takeda, Y., Takeda, T., Honda, T., Miwatani, T.: Biken's J **21,** 1 (1978).
213. Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K., Yamai, S.: J Bacteriol **100,** 1147 (1969).
214. Bar-Shavit, Z., Goldman, R., Ofek, I., Sharon, N., Mirelman, D.: Infect Immun **29,** 417 (1980).
215. Mangan, D. F., Snyder, I. S.: Infect Immun **26,** 520 (1979).
216. Smith, H. W., Linggood, M. A.: J Med Microbiol **4,** 467 (1971).
217. Gyles, C. L., Palchaudhuri, S., Maas, W. K.: Science **198,** 198 (1977).
218. Gyles, C. L., Falkow, S., Rollins, R.: Am J Vet Res **39,** 1438 (1978).
219. Echeverria, P., Murphy, J. R.: J Infect Dis **142,** 273 (1980).
220. Stieglitz, H., Fonseca, R., Olarte, J., Kupersztoch-Portnoy, Y. M.: Infect Immun **30,** 617 (1980).
221. Mazaitis, A. J., Maas, R., Maas, W. K.: J Bacteriol **145,** 97 (1981).
222. Franklin, A., Söderlind, O., Mölby, R.: Med Microbiol Immunol **170,** 63 (1981).
223. Isaacson, R. E., Moon, H. W.: Amer J Vet Res **39,** 1750 (1979).
224. Smith, H. R., Cravioto, A., Willshav, G. A., McConnel, M. M., Scotland, S. M., Gross, R. J., Rowe, B.: FEMS Microbiol Letter **6,** 255 (1979).
225. So, M., Heffron, F., McCarthy, B. J.: Zentralbl Veterinaermed [B] **18,** 489 (1979).

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EFFECT OF A NEW FUNGICIDE (PNDP) ON THE MINOR NUCLEOTIDE CONTENT OF *FUSARIUM OXYSPORUM*

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The minor nucleotide constituents of rRNA from the plant pathogenic fungus *Fusarium oxysporum* were investigated. When the fungus was grown for 8 days in aerated liquid culture the following minor nucleotides were identified from 18S and 25S rRNA: m^6A, m^2A, m^2_6A, m^1G, m^2G, m^2_2G, m^3C, Cm, m^5C, m^3U, Um, cm^5U, rT and ψ. PNDP ($\pm$ threo-1-phenyl-2--nitro-1,3 diacetoxy-propane), a new fungicide of unknown biochemical mode of action, caused a great decrease in the minor nucleotide content of the fungus. Only seven minor nucleotides were present in the rRNA of the fungus grown in PNDP-containing medium. A hypermodified nucleotide, mcm^5S^2U, was found in treated but not in untreated fusarium. On comparing the effect of PNDP with that of known protein synthesis inhibitors such as cycloheximide and chloramphenicol, it was concluded that PNDP acted similarly to cycloheximide, a cytoplasmic protein synthesis inhibitor. It is suggested that the mode of action of PNDP may be the inhibition of the synthesis of RNA modifying enzymes of *F. oxysporum*.

Although a number of rRNA primary sequences have been determined, little information is available on the modified nucleotides which are formed during the post-transcriptional maturation of rRNAs. No such information is available on the rRNA of *Fusarium oxysporum*, either.

The present paper describes the minor nucleotide constituents of the 18S and 25S rRNA of *F. oxysporum* and shows the changes induced in these constituents by PNDP, a new fungicide which, probably by its effect on the synthesis of cytoplasmic modifying enzymes, causes a great decrease in minor nucleotides.

Materials and methods

Isolation and purification of RNA. Total RNA was extracted from 8-day-old aerated liquid cultures of *F. oxysporum* grown in modified Czapek medium [1] at 25 $\pm$ 2 °C in diffuse light. PNDP treatment was carried out by adding 8 μl of 1000 mg/l DMSO stock solution to 1 l sterilized liquid medium, to a final concentration of 0.08 mg/l. This concentration is the EC_{50} value of PNDP (the 50% inhibitory concentration) for *F. oxysporum*. The culture medium was inoculated with 1 ml of 10^8/ml conidial suspension obtained from a 4-week-old culture of the fungus grown on potato–dextrose agar. Mycelia of the fungus were filtered in vacuo and

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frozen with liquid nitrogen, then homogenized and extracted by the phenol method as described by Kirby [2].

Crude RNA fractions were separated by Sepharose 4B column chromatography, giving pure 18S and 25S fractions. This method has been used for the separation of proteins, polysaccharides and glycoproteides [3], for tRNA fractions [4] and for obtaining DNA-free RNA preparations [5], and mRNA preparations [6] but this was the first occasion when Sepharose 4B has been used for the fractionation of rRNA extracts.

The separation was done as follows. 5000 A_{260} units of crude RNA extract dissolved in 20 ml distilled water were loaded on a 3.5 × 40.0 cm Sepharose 4B column previously equilibrated with 0.7 M $(NH_4)_2SO_4$ solution. Elution was carried out with a salt gradient of 0.7 M $(NH_4)_2SO_4$ to distilled water. The $(NH_4)_2SO_4$ was dissolved in buffer A, which consisted of 0.01 M sodium acetate, 0.01 M $MgCl_2$, and 0.001 M Na_2EDTA, pH 4.5.

The column purified rRNA from low molecular weight RNAs (tRNAs and hydrolysates) and separated 18S and 25S RNA as well. The homogeneity of the purified fractions was checked by gel-electrophoresis as described by Loening [7, 8].

Hydrolysis and fractionation of RNA by anion exchange chromatography. Acid hydrolysis of the RNA was carried out by using 1 N HCl at 100 °C for 1 h. The anion exchanger Dowex 1 × 8 was used in acetate form, prepared as described by Cohn [9]. Then 1000 A_{260} units of the hydrolysate were loaded on the column, which was equilibrated with ammonium acetate buffer pH 9. A stepwise elution was carried out by using 0.3 M ammonium acetate pH 9, 0.5 M ammonium acetate pH 5.4 and 0.5 M ammonium acetate pH 1, in that order. The pH was adjusted with concentrated HCl. The column size was 0.8 × 50 cm, and the flow rate was 24 ml/h.

The fractions were pooled, desalted on a Sephadex G-10 column and further analysed by thin-layer chromatography.

Thin-layer chromatography. Ten A_{260} units of nucleotides emerging as individual peaks from the Dowex 1 × 8 columns were applied to MN-300 cellulose thin-layer plates. The chromatogram was developed in two dimensions according to Björk and Stevensson [10], and the constituents were identified on the basis of their UV spectrum [11].

Results

Minor nucleotides in the 18S RNA of F. oxysporum. The material in fraction 1 eluted from the Dowex 1 × 8 column (Fig. 1) contained m^3C and m^3U, while fraction 2 consisted of m^1G, m_2^2G, m^6A, m^2A, rT, G and A together with an unidentified minor component (Fig. 2). The 3rd peak contained m^3U, the 4th m_6^2A, the 5th m^1A, the 6th Um and U. Fraction 7 consisted of Cm and m^5Cm in addition to Ap. The 8th peak contained Ap, Gp and three methylated derivatives, viz. m^2Gp, m_2^2Gp and m^1Gp. Fraction 9 did not contain any

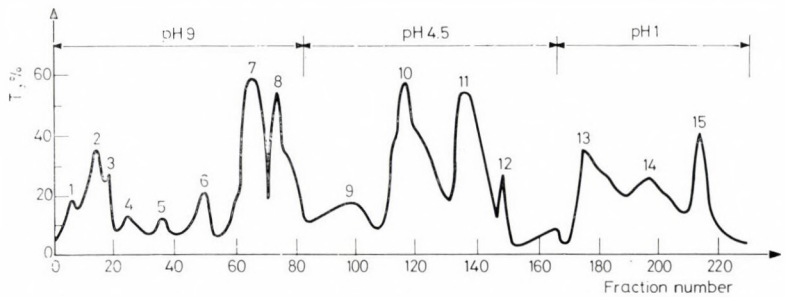

Fig. 1. Chromatography on Dowex 1 × 8 column of an acid hydrolysate of 18S rRNA extracted from *F. oxysporum*

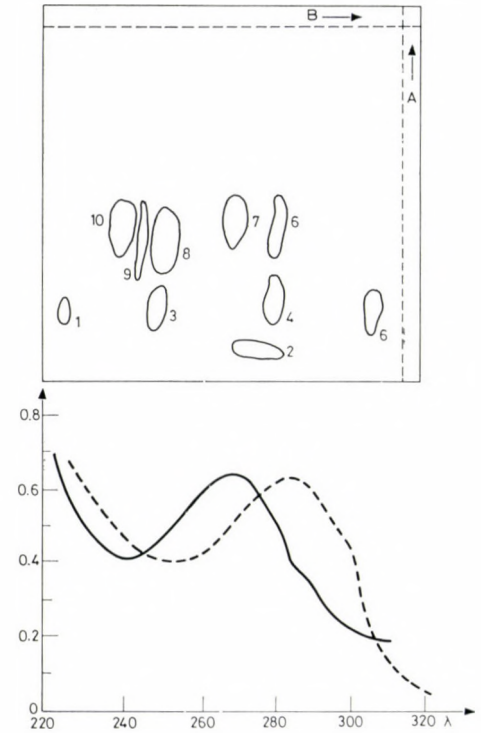

Fig. 2. Spectra of the unidentified minor component (n. 1) of fraction 2 of Dowex 1 × 8 column from 18S rRNA of *F. oxysporum.* ——— at pH 1; ····· at pH 13

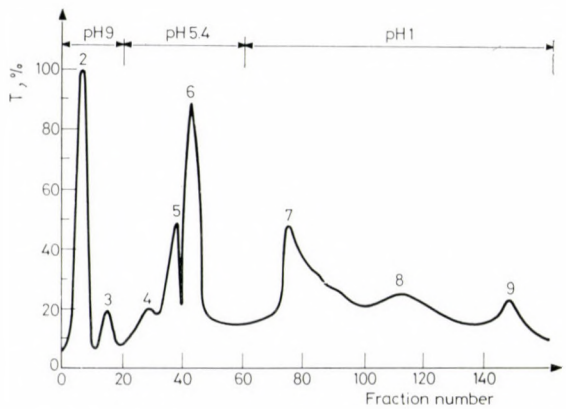

Fig. 3. Chromatography on Dowex 1 × 8 column of an acid hydrolysate of 25S rRNA extracted from *F. oxysporum*

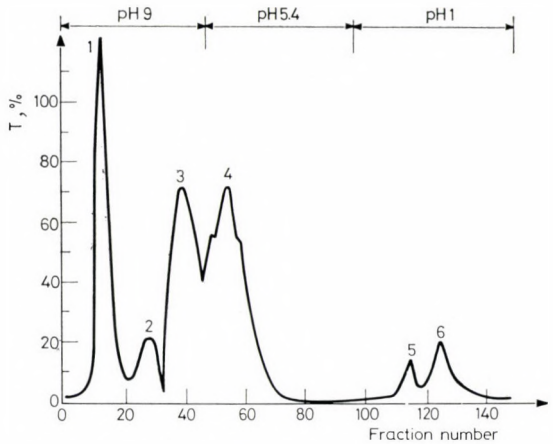

Fig. 4. Chromatography on Dowex 1 × 8 column of an acid hydrolysate of 18S + 25S rRNA extracted from *F. oxysporum* treated with PNDP

modified nucleotide. The 10th fraction contained Ap, Up and rTp and the 11th Up and ψp. The last three fractions, eluted at pH 1, consisted of pAp and pCp.

Minor nucletotides in the 25S RNA of F. oxysporum. Since the G and A derivatives are slightly soluble at alkaline pH and partly precipitate at pH 9, the first elution buffer used with the Dowex 1 × 8 column. This precipitate was fraction 1 and it contained A, C and m^1G. The 2nd fraction eluted from the column (Fig. 3) consisted of m^3U, m^1A and m_2^2G. The constituents of the 3rd peak could not be identified. Peak 4 contained m^6A and peak 5 Ump, rTp and cm^5U. In fraction 6, m^2Ap was identified besides Gp and Ap. The acidic fractions 7, 8 and 9 contained pAp, pUp and m_2^2Gp, respectively.

Minor nucleotides in the 18S + 25S RNA of F. oxysporum treated with PNDP. The chromatographic profile of the treated fusarium RNA (Fig. 4) contained fewer peaks than that of untreated RNA. The alkaline precipitate contained m^1G, m_2^2G, G and A, the first eluted peak C, Cm, A and U. m_2^2A, U and m^3U were identified in peak 2, and A, Gp and m^2G in peak 3. Besides Gp and m^2A, fraction 4 contained a hypermodified thio-derivative, mcm^5S^2U, which was not identified from untreated fusarium. The 5th peak contained Gp, Ap and m^2G, and the 6th pAp and pUp.

Table I lists the minor nucleotides identified from the 18S and 25S rRNA of untreated *F. oxysporum* and from the 18S + 25S rRNA of PNDP-treated fungus. Table II shows the differences observed in minor nucleotide content when comparing untreated and PNDP-treated fungus rRNA.

Table I

The minor nucleotides of 18S and 25S rRNA of untreated and 18 + 25S rRNA from PNDP-treated F. oxysporum

Minor component	Untreated 18S rRNA	Untreated 25S rRNA	PNDP-treated 18 + 25S rRNA
Adenine derivatives			
m^6A	+	+	−
m^1A	+	+	−
m^2A	+	+	+
m_6^2A	+	−	+
Guanine derivatives			
m_2^2G	+	+	+
m^1G	+	+	+
m^2G	+	−	+
Cytosine derivatives			
m^3C	+	−	−
Cm	+	−	+
m^5C	+	−	−
Uridine derivatives			
m^3U	+	+	+
Um	+	+	+
cm^5U	+	+	−
ψ	+	−	−
rT	+	+	−
mcm^5S^2U	−	−	+

Table II

Minor nucleotide components of untreated and PNDP-treated rRNA of F. oxysporum

Minor nucleotides detected in both untreated and PNDP treated rRNA	Minor nucleotides detected in untreated rRNA only	Minor nucleotides detected in PNDP treated rRNA only
m_6^2A	m^6A	mcm^5S^2U
m_2^2A	m^1A	
m^2G	m^3C	
m^1G	m^5C	
m^2G	cm^5U	
Cm	ψ	
m^3U	rT	
Um		

Discussion

In the present work the minor nucleotide composition of rRNA from *F. oxysporum*, a plant pathogenic fungus, and the effect of PNDP, a new fungicide, was studied.

Fifteen minor nucleotides could be identified in 18S rRNA. These were, m^6A, m^1A, m^2A, m^2_6A, m^1G, m^2G, m^2_2G, m^3C, Cm, m^5C, m^3U, Um, cm^5U, rT and ψ. Several of them (m^2_6A, m^2G, m^3C, Cm, m^5C and ψ) could not be demonstrated in the 25S rRNA of untreated *F. oxysporum*. As it is known, 25S rRNA contains less minor components than 18S. The presence of m^2_6A in 18S rRNA was characteristic. An interesting finding was the presence of rT in fungus RNA. This minor component has been identified only in the 16S + + 23S RNA of prokaryotic *Escherichia coli* [12–14] and in the 18S + 25S RNA of wheat [15]. This was the first case when it has been found in a fungus species.

Treatment with PNDP caused a great decrease in the minor nucleotide content of *F. oxysporum* RNA. The 18S + 25S RNA isolated from fungus grown on PNDP-containing medium contained only 7 minor nucleotides. An interesting hypermodified component, mcm^5S^2U, was identified from the treated fungus RNA but was not present in the untreated fungus.

The inhibitory effect of PNDP on the formation of minor nucleotides is similar to that of cycloheximide, a cytoplasmic protein synthesis inhibitor [15]. Cycloheximide is known to inhibit the synthesis of RNA modifying enzymes which are proteins found in small amounts; they are therefore suitable markers of the inhibition of cytoplasmic protein synthesis.

It is suggested that the decrease of modified nucleotides of PNDP-treated *F. oxysporum* is caused by the inhibition of de novo synthesis of RNA modifying enzymes and so the biochemical mode of action of PNDP is based on the inhibition of cytoplasmic protein synthesis.

REFERENCES

1. Surico, G., Graniti, A.: Phytopath Med **16,** 30 (1977).
2. Kirby, K. S.: Isolation of nucleic acids with phenolic solvents. In Grossman, L., Moldave, K. (eds): Methods in Enzymology. Vol 12. Pp 87–99. Academic Press, New York 1968.
3. Rimerman, R. A., Hatfield, G. W.: Science **182,** 1268 (1973).
4. Holmes, W. M., Hurd, R. E., Reid, E. R., Rimerman, R. H., Hatfield, G. W.: Proc Natl Acad Sci USA **72,** 1068 (1975).
5. Cornelis, P.: Plant Sci Letters **11,** 3 (1978).
6. Zeichner, M., Stern, R.: Biochemistry **16,** 1378 (1977).
7. Loening, U. E.: Biochem J **102,** 251 (1967).
8. Loening, U. E.: J Mol Biol **38,** 355 (1968).
9. Cohn, W. E.: Methods of isolation and characterisation of mono- and polynucleotides by ion exchange chromatography. In Colowick, S. P., Kaplan, N. O. (eds): Methods in Enzymology. Vol 3, pp 724–743. Academic Press, New York 1957.

10. Björk, G. R., Stevensson, I.: Biochim Biophys Acta **138,** 430 (1967).
11. Venkstern, T. V., Bayew, A. A.: Spectra of nucleic acid compounds. (IFI) Plenum Press, New York 1968.
12. Fellner, P., Sanger, F.: Nature **219,** 236 (1968).
13. Fellner, P., Ehresman, C., Ebel, J. P.: Nature **225,** 26 (1970).
14. Branlant, C., Widada, J. S., Krol, A., Fellner, P., Ebel, J. P.: Biochimie **57,** 175 (1975).
15. Rácz, I., Király, I., Lásztity, D.: Planta **142,** 263 (1978).

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DIAGNOSTIC EXPERIENCE IN TWO CASES OF LEGIONELLOSIS

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Sera of 30 patients with atypical pneumonia were tested for antibodies against the representative strains of *Legionella pneumophila* 1–6 serogroups and 4 species of legionella-like organisms. Investigations were carried out with indirect immunofluorescence assay. The sera of 2 patients gave a titre of 2048 and 1024 with serogroup 5 and 4, respectively. The high anti-legionella IgM titres were indicative of recent infections. Legionellae were not detected in transtracheal aspirate and in autopsy material.

The epidemiological data about the ubiquitous character of the recently discovered Gram-negative rods, legionellae, and the incidence of epidemics and sporadic cases caused by these organisms in the past five years (1–4) led us to deal with the diagnostic procedure of the disease. Since 1981 sera of 30 patients with atypical pneumonia have been tested with indirect immunofluorescence assay (IFA) [5] for antibodies against the representative strains of *Legionella pneumophila* 1–6 serogroups and 4 species of legionella-like organisms (LLOs): *Legionella bozemanii*, *Legionella dumoffii*, *Legionella gormanii*, *Legionella micdadei*. The present study reports on two positive cases of legionellosis with special emphasis on the laboratory diagnosis of the disease.

Report of two cases

Case 1. B. L. a 58-year-old female Swiss citizen, with a history of bronchial asthma since childhood, recurrent pneumonias, and transient cardiac failure was admitted two days after her arrival to Hungary with fever, sickness, diarrhoea in a distressed state.

Case 2. L. F. a 44-year-old male patient was admitted with diarrhoea since 6 days, fever, severe dyspnoea and prostration with a history of schizophrenia, alcoholism, and a rapid loss of weight of about 25 kg.

Clinical features, treatment, laboratory findings. In both cases auscultation findings of a right side pneumonia were confirmed by X-rays. Immediate tetracycline and erythromycin

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therapy combined with diuretics and strophantoside in Case 1 was followed by subsequent regression of the clinical, laboratory and X-ray status, so the patient was discharged on the 13th day.

In Case 2 after unsuccessful treatment with gentamicin and tetracycline and later penicillin and methicillin, erythromycin introduced on the 6th day failed to reverse the progressive deterioration. The most striking feature was unconsciousness without any laboratory signs of cerebral hypoxia. The patient died on the 16th day of illness.

Characteristic laboratory findings in Case 2 on the 2nd day were, ESR: 95 mm/h; RBC: 2.9×10^{12}; WBC: 16×10^{9}; band cells: 0.45; neutrophils: 0.45; lymphocytes: 0.10. On the 6th day practically the same results were obtained with Se bi: 17.1 μmol/l; SGOT: 23 IU/l; SGPT: 30 IU/l; BUN: 13.4 mmol/l; se creatinine: 122 μmol/l.

Epidemiology. Both patients acquired infection in the summer. Case 2 in the hottest days of August, when the incidence of legionellosis is the highest. In Case 1 the air-conditioning system on the plane between Zurich and Vienna and in the bus on the way to Budapest was notable beside the fact that two more members of the tourist group had a respiratory infection with fever during the trip.

Case 2 worked as a warden in a changing room of trade port workers. The room was directly connected with a steamy bath-room with small tipping windows as the only ventilation.

Diagnosis. In both cases, throat swabs, sputum, stool, urine and blood samples were taken daily. In Case 2, transtracheal aspirate was obtained on the 6th day. Lung tissue obtained post mortem was ground with sterile PBS prior to cultivation.

All the specimens were cultivated using routine bacteriological methods, the transtracheal aspirate and lung tissue also by methods developed for isolation of *L. pneumophila:* in FG bouillon, on FG and on CYE agar [6], as well as on the same media supplemented with the selective agents like vancomycin (Lilly) 4 μg/ml, polymyxin B (Pfizer) 50 IU/ml, trimethoprim (EGYT) 2.5 mg/ml. All media were incubated in candle jar at 35 °C at least for 10 days.

Serology. Serum samples in Case 1 and Case 2 taken on the 3rd and 13th day of the disease, respectively, were examined for legionella antibodies by IFA [5], and for *Mycoplasma pneumoniae* antibodies by metabolism inhibition test, for complement fixing antibodies to adeno, RS, influenza A, B, *Herpes simplex* viruses. The serum sample of Case 2 was also tested for *Chlamydia psittaci* antibody.

Conjugates for direct immunofluorescence assay (DFA), antigens of different serogroups, antihuman immunoglobulin conjugated with FITC (containing antihuman IgA, IgM, IgG) for IFA were received from the Center for Disease Control (CDC), Atlanta, USA, antihuman IgM and antihuman IgG conjugates were Hyland products. Antigen from *L. pneumophila* 1 (Philadelphia) was prepared in our laboratory by boiling the bacterial suspension in water bath for 15 min [5].

Investigations with IFA were carried out by a Zeiss (Jena) Fluoval microscope with HBO 200 high density mercury lamp, starting with a 32-fold basic dilution.

Necropsy in Case 2 was carried out 2 h after death. To demonstrate legionellae in lung and liver, formalin-fixed, paraffin-embedded sections as well as smears of scrapings of the formalin-fixed lung tissue were stained with toluidine blue, Gimenez, and by modified Dieterle silver impregnation [5]. *L. pneumophila* reference strain 4 (received from CDC) cultivated on CYE agar was mixed with homogenisate and with scrapings of formalin-fixed lung tissue to yield a positive control staining. Scrapings and sections of lung tissue were also examined by DFA.

For transmission electron microscopy (TEM) 1 mm^3 cubes of the most inflammatory areas of the formalin-fixed lung tissue were obtained. Sections were washed in 0.1 M PBS and postfixed in 2% osmium tetroxide. Dehydration and embedding were carried out by routine methods. Sections were cut on a Reichert OMU 2 ultramicrotome. Semithin sections were stained with toluidine blue, ultrathin ones were cut with diamond knife, stained with uranyl acetate and lead citrate, and examined with JEM 6 C transmission electron microscope.

Epidemiological survey. In Case 1 no environmental survey could be undertaken. In Case 2, environmental samples were taken from showerheads, taps, plugholes in the bath-room where the patient was a warden. All specimens were cultivated in FG bouillon, on FG agar and CYE agar supplemented with selective agents (see above). To gain some information about the local risk of infection, sera of 12 persons using the same bathroom were tested for 6 serogroups of *L. pneumophila.*

Results

Cultural examination. Throat, sputum and stool samples contained no pathogenic bacteria. Urine, blood and lung tissue cultured on routine and special media, were sterile. Transtracheal aspirate yielded only *Haemophilus influenzae.* In lung and liver sections no bacteria could be detected by any of the aforementioned stainings.

Results of the serological tests are given in Table I.

Gross pathological features. Confluent fibrinous bronchopneumonia with concomitant pleuritis in each lobe of the right lung as well as marked purulent--fibrinous inflammation in the dilated bronchi were observed.

Histological features. A mass of cells with foamy cytoplasm and a great number of erythrocytes occupied the alveoli. Macrophages with elongated chromatin rich nuclei were observed. Nearly all bronchioli were filled with abundant mass of neutrophils, the lung epithelium was remarkably damaged.

In lung and liver sections no bacteria were detected, nor was a specific fluorescence observed by any of the DFA conjugates. In smears of scrapings of the formalin-fixed lung tissue slightly curved corynebacterium-like rods were seen when stained with toluidine blue, the same forms in red colour were detected when stained according to Gimenez. These bacteria failed, however, to give specific fluorescence by DFA procedure following decolorization of the smears.

By TEM round or oval structures 0.35–0.7 μm in diameter were detected in the cytoplasm of the intraalveolar neutrophils and macrophages. The number of organisms per cell varied between 2–12 (Figs 1, 2). The double envelope enclosing these structures and the electron-dense layer between the envelopes were consistently demonstrated.

Table I

IFA antibody titres in sera of patients

Antibodies	Antigens (*L. pneumophila* serogroups and LLOs)				
	1, 2, 3, 6 LLOs*	4	5	1, 2, 3, 5, 6 LLOs**	4
Ig	< 32			< 32	
IgM		256	1024		1024
IgG		64	2048		128

* *M. pneumoniae* metabolism inhibition test, titre: 512

** *M. pneumoniae* growth inhibition and C. *psittaci* complement fixing antibody: negative

RS, influenza A, B, *Herpes simplex*, adenovirus complement *fixing antibodies:* negative in both patients

LLOs: *L. bozemanii, L. dumoffii, L. gormanii, L. micdadei*

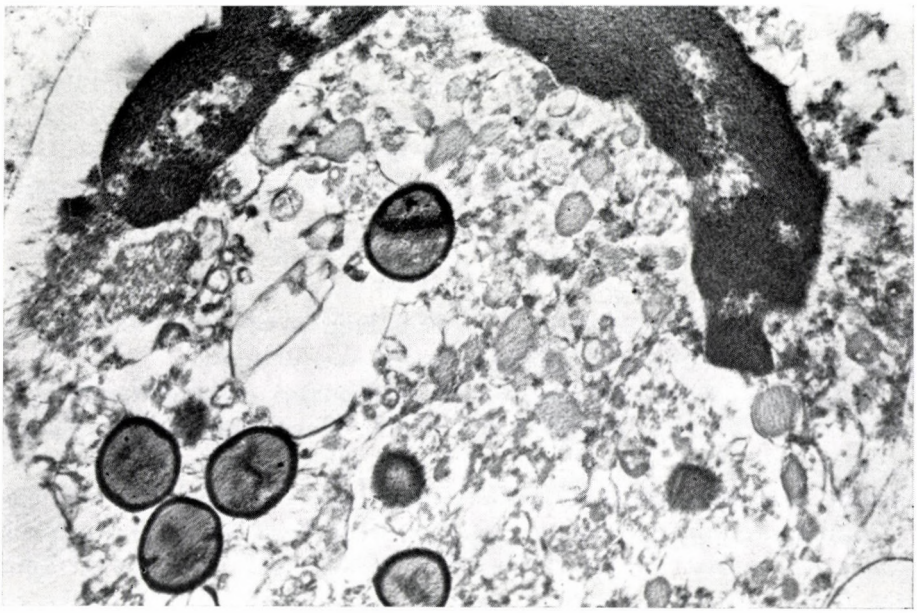

Fig. 1. Electron micrograph of bacteria in a macrophage in formalin-fixed lung tissue stained by uranyl acetate and lead citrate. × 16 700

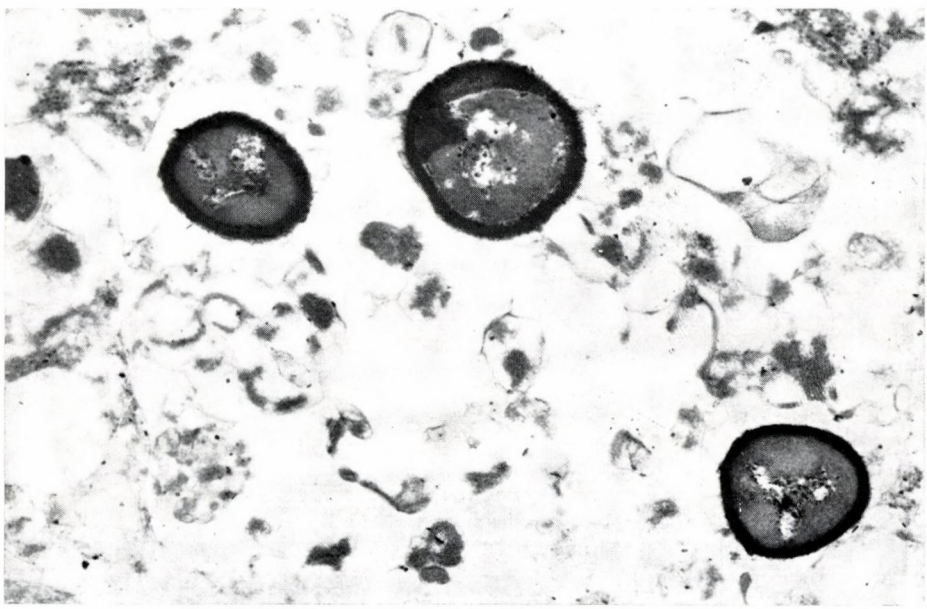

Fig. 2. Vacuoles in bacteria characteristic of legionellae. × 66 800

Epidemiological survey. From the environmental samples *L. pneumophila* was not isolated on any of the special selective media. From the 12 symptomless persons examined, one person's serum showed a titre of 128 against *L. pneumophila* 2 and 6.

Discussion

Atypical pneumonia in both patients presenting with extrapulmonary symptoms like diarrhoea was characteristic of legionellosis [6]. Similarly, confusedness or a delirious state like in Case 2 is also a typical feature. Laboratory findings in Case 2 were also in accordance with those reported in the majority of legionellosis cases. Elevated transaminase values point to the toxic effect of the causative agent.

The recommended way of laboratory diagnosis is as follows.

1. For demonstration of legionella in sputum, transtracheal aspirate, bronchial washing, pleural fluid, etc., and in formalin-fixed, paraffin-embedded tissue sections the DFA method, in scrapings of formalin-fixed tissue and in imprints of fresh frozen tissue DFA and light microscopy with Gimenez or toluidine blue staining and modified Dieterle silver impregnation are the most rapid procedures. Silver impregnation is a valuable method for demonstrating legionellae in paraffin-embedded tissue sections as well. Failure to demonstrate legionella organisms in lung tissue sections by DFA might be due to the fact that most of the bacteria are intracellular, lie at different levels and are shrunken by histological processing. The majority may disintegrate under the effect of cellular defense mechanisms. No explanation is given for the presence of corynebacterium-like rods in the smears of scrapings of formalin-fixed lung tissue in Case 2.

2. The most specific diagnostic method, the isolation of legionella is rarely successful in clinical specimens. In addition to the extremely fastidious character and slow growth of legionella, the organism in the specimen may be inhibited by drugs or its growth antagonized by the associated microflora. Culturing of DFA negative clinical specimens seldom results in recovery of legionellae. Among a total of 891 specimens, there was only 1 DFA negative with positive culture while 33 were DFA positive and negative in culture [4]. In our Case 2 the lung tissue cultures were sterile and the transtracheal aspirate yielded only *H. influenzae.* The latter was considered a concomitant organism. The frequency of isolation can be increased by propagating the bacteria in the guinea-pig, but owing to the lack of security equipment we have dispensed with the procedure.

3. The common test for serodiagnosis of legionellosis is IFA, that provides the diagnosis comparatively late. Nevertheless, legionellosis has been diagnosed on the basis of the antibody response perhaps more often than any

other bacterial disease. The titre of IFA antibodies begins to increase 7 to 10 days after the onset of illness and reaches the peak in the 3rd to 4th week. Fourfold titre rises to at least 128 in specimens taken at an appropriate time from patients with compatible clinical illness are generally accepted as serologic evidence of legionellosis, but a single titre of 256 is also considered a presumptive evidence of infection at an unknown time. Though we had no opportunity to test more than one serum sample of each patient, the IgM titre of 2048 against serogroup 5 in Case 1 and of 1024 against serogroup 4 in Case 2 provided evidence of a recent infection. In the serum of Case 1, in addition to antibody to serogroup 5 a moderately elevated titre of 256 to serogroup 4 was found. This finding corresponded with data proving that *Legionella* strains belonging to different serogroups may give cross-reaction in serological tests.

In Case 1 the remarkable *M. pneumoniae* metabolism inhibition titre would suggest some concern about the diagnosis. *M. pneumoniae* antibody is frequently detected in sporadic cases of legionnaires' disease, while in mycoplasma pneumonia legionella antibodies are much less frequent [7]. A simultaneous occurrence of the two antibodies is supposed not to be due to cross-reaction, although no reliable explanation is available for the phenomenon. In Case 1 besides the typical clinical course, the elevated antilegionella IgM titre supported the diagnosis of legionellosis as opposed to mycoplasma infection.

The gross pathological appearance of legionella pneumonia varies, but it is probably more common that it causes a nodular bronchopneumonia that tends to become confluent. Abundance of macrophages, erythrocytes and cells with foamy cytoplasm in the aveoli is not a common histological feature of bacterial bronchopneumonia. The morphological characteristics of the round and oval elements within the cytoplasm of intraalveolar macrophages and neutrophils detected by TEM were consistent with those of a small Gram-negative bacterium. Elongated forms were rarely found. The electron-lucent vacuoles that are considered typical of the ultrastructure of *L. pneumophila* were, however, consistently detected. Still, legionella cannot be differentiated from other small Gram-negative bacilli by conventional electron microscopic methods, only by the immunoferritin technique [8].

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REFERENCES

1. Jørgensen, K. A., Karsager, B., Johanssen, G., Freund, L. G., Wilkinson, H. W.: Scand J Infect Dis **13,** 133 (1981).
2. Wewalka, G.: Zentralbl Bakteriol [A] **249,** 261 (1981).
3. Weekly Epidem Rec **55,** 149 (1980).
4. Blackmon, J. A., Chandler, F. W., Cherry, W. B., England, A. C., Feely, J. C., Hicklin, M. D., McKinney, R. M., Wilkinson, H. W.: Am J Path **103,** 429 (1981).
5. Jones, G. L., Hébert, G. A.: "Legionnaires' " the disease, the bacterium and methodology. U. S. Department of Health, Education and Welfare, Center for Disease Control, Atlanta 1979.
6. Jones, G. L., Hébert, G. A.: "Legionnaires' " the disease, the bacterium and methodology. U. S. Department of Health, Education and Welfare, Center for Disease Control, Atlanta 1978.
7. Grady, G. F., Gilfillan, R. F.: Ann Intern Med **90,** 607 (1979).
8. Rodgers, F. G.: J Clin Pathol **32,** 1195 (1979).

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ENTEROTOXIN PRODUCTION BY *YERSINIA ENTEROCOLITICA* IN FOOD SAMPLES

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Milk and cold meat samples were contaminated with 7 various serogroups of *Yersinia enterocolitica* strains. The infected food samples were incubated under different conditions of growth, at different temperatures and for different periods of time, then the number of colony forming units was determined and enterotoxin production was assayed by the suckling mice test. The *Y. enterocolitica* strains multiplied well under varying conditions of growth, but enterotoxin production could be detected only in the meat samples when incubated under shaking at 25 °C for 48 h. It may be assumed that preformed yersinia enterotoxin is absent from food stored under normal conditions.

Pai and Mors [1] were the first to report that several *Yersinia enterocolitica* strains produced heat stable (ST) enterotoxin. Since experiments have confirmed enterotoxin production only under 30 °C and in vitro, its pathogenetic role is questionable [1–5]. At the same time *Y. enterocolitica* strains of different serogroups have been isolated from food, especially from milk, dairy products and meat products [3, 6–8]. In Hungary *Y. enterocolitica* strains were isolated from butcher's meat [9] and in other countries several *Y. enterocolitica* strains capable of enterotoxin production have been isolated from various food products [3]. This suggested the possibility that the enterotoxin was produced in food contaminated with *Y. enterocolitica*, stored at its optimum temperature, i.e., below 30 °C, and the preformed enterotoxin was one of the factors responsible for the symptoms registered. The experiments to be reported were undertaken to find an answer to this question and to study the epidemiology of possible food infections caused by *Y. enterocolitica*.

Materials and methods

Y. enterocolitica strains. The following strains were used: three strains isolated in the National Institute of Hygiene (Nos 47071, 71210, 81371); one strain (No. 22) of serogroup O3, isolated earlier from human enteritis and maintained in stock agar; two strains, one of serogroup O5 (No. 11337) isolated from smoked sausage, and one of serogroup O7,8 (No. 14117) isolated from another sausage and maintained in the lyophilized state; and finally a strain of serogroup O9 (No. 98010) maintained in stock agar.

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Culture media. The foods selected for artificial contamination were pasteurized milk and a kind of smoked sausage. The milk was obtained under sterile conditions and distributed in 25 ml volumes into 250 ml flasks. The sausage was homogenized in distilled water and then sterilized in steam for 20 min. Syncase broth media served as controls [1]. Cultivation took place at 25 °C in a shaker thermostate (Psycroterm, New Brunswick) for 1 to 4 days and in the refrigerator for 2 and 3 weeks.

Germ counting was performed by the dilution technique using nutrient agar and counting the colony forming units.

Filtrates. The cultures were centrifuged at 17 000 g for 30 min. The supernatants were filtered through a membrane filter (No. 1121 Göttingen, pore size 0.45 μm).

The heat-stable enterotoxin (ST) was tested following the method of Dean et al. as modified by Jacks and Wu [10]. Three mice in every group received 0.1 ml filtrate orally by tube. Four hours later the animals were sacrificed and the ratio gut weight per remaining body weight was determined. The reaction was regarded as positive if the ratio was higher than 0.090.

Results

With the usual storage of food and the optimum conditions of enterotoxin production, the growth rate and the capacity for enterotoxin production were studied under various conditions i.e. at different incubation periods and different cultivation temperatures both in stationary and shake cultures. The food samples and the control broth media were infected with *Y. enterocolitica* strains that had proved capable of enterotoxin production in the preliminary experiments, in a concentration of 10^2 germs/ml. Growth of the strains was checked by germ counting (Table I). The strains showed good growth both at room temperature and in the refrigerator, and survived storage for several days or weeks. The germ count in the milk sample contaminated with the strain No. 14117 serogroup O7,8 was determined only if growth had taken place under circumstances where enterotoxin production was most probable. Strain No. 22 was used only for the infection of cold meat since the occurrence of serogroup O3 was expected mainly here. High germ counts were obtained in these cases, too. Filtrates were prepared from the samples in which the germ count and enterotoxin content had been determined (Table II). The values printed in italics designate positive response. Independently of the conditions of cultivation, no enterotoxin production could be detected in the milk samples though they had been contaminated with 3 different serotypes capable of enterotoxin production under specific circumstances in Syncase broth media. The strains of serogroups O3; O7,8 and O5 produced enterotoxin in the cold meat only after incubation at 25 °C under shaking for 2 days. Then some strains belonging to serogroup O3 and another strain of serogroup O9 were studied in cold meat (Table III). Strain No. 22 produced enterotoxin only under specific conditions. The other 3 strains, also growing well, did not produce enterotoxin under optimum conditions either, which was not the case when growth took place in Syncase broth media. It is worth noting that, as shown in Table III, entrotoxin was produced in the control media only if conditions were the same as with cold meat.

Table I

Growth of Y. enterocolitica strains in food samples

Incubation time and temperature		Strains and their serogroups			
		No. 47071 O3	No. 11337 O5	No. 14117 O7, 8	No. 22 O3
Milk					
1 day	22 °C	1.2×10^{8}*	4.2×10^{8}	·	·
1 day	25 °C**	5×10^{8}	3×10^{8}	·	·
2 days	22 °C	3.4×10^{8}	2×10^{5}	·	·
2 days	25 °C**	6×10^{9}	2×10^{8}	2.6×10^{7}	·
3 days	22 °C	·	2×10^{7}	·	·
4 days	22 °C	·	1×10^{7}	·	·
2 weeks	4 °C	8×10^{8}	1×10^{7}	·	·
3 weeks	4 °C	·	1.6×10^{4}	·	·
Sausage					
1 day	22 °C	4×10^{9}	3.7×10^{8}	1.4×10^{9}	1×10^{7}
1 day	25 °C**	·	3.6×10^{8}	·	5.6×10^{8}
2 days	22 °C	1×10^{8}	1.2×10^{9}	1.2×10^{8}	2×10^{9}
2 days	25 °C**	2×10^{9}	1.5×10^{11}	2×10^{10}	3×10^{8}
3 days	22 °C	1×10^{7}	1×10^{9}	1.1×10^{8}	1×10^{9}
4 days	22 °C	6×10^{8}	3×10^{9}	1.6×10^{10}	5×10^{9}
2 weeks	4 °C	1×10^{11}	1×10^{8}	7×10^{10}	1.3×10^{9}
3 weeks	4 °C	2×10^{9}	1×10^{9}	2×10^{9}	4×10^{9}
Syncase					
1 day	22 °C	1.5×10^{9}	1.0×10^{9}	4×10^{9}	1.7×10^{9}
1 day	25 °C **	6.2×10^{9}	5×10^{9}		4×10^{8}
2 days	22 °C	1×10^{9}	2×10^{9}	9×10^{7}	5×10^{9}
2 days	25 °C**	2.8×10^{10}	3×10^{9}	2×10^{9}	2×10^{10}
3 days	22 °C	·	1×10^{8}	1.1×10^{8}	5.2×10^{8}
4 days	22 °C	·	·	1.5×10^{8}	2×10^{9}
2 weeks	4 °C	1.7×10^{9}	2×10^{8}	7×10^{9}	1×10^{8}
3 weeks	4 °C	·	3.4×10^{8}	6×10^{9}	4×10^{9}

* Germs/ml
** Shaken
· Not examined

Discussion

We could use comparatively fresh cultures in the experiments and this was of importance since the enterotoxic activity rapidly decreases during maintenance in stock agar [11]. The extensive study of serogroup O3 strains

was justified by their high frequency in Hungary. As stressed by Szita et al. [12, 13], pigs might well represent a reservoir of this serogroup so that many meats are probably contaminated. The study of strain O9 was based on its being the second most frequent one in Hungary.

Table II

ST enterotoxin production by Y. enterocolitica strains in food samples

Incubation time and temperature		Strains and their serogroups		
		No. 47071 O3	No. 11337 O5	No. 14117 O7, 8
Milk				
1 day	22 °C	0.046*	0.055	·
1 day	22 °C**	0.051	0.071	·
2 days	22 °C	0.068	0.062	·
2 days	25 °C	0.060	0.065	0.057
3 days	22 °C	·	0.053	·
4 days	22 °C	·	0.054	·
2 weeks	4 °C	0.053	0.056	·
Sausage				
1 day	22 °C	0.057	0.074	0.059
1 day	25 °C**	·	0.063	·
2 days	22 °C	0.080	0.063	0.059
2 days	25 °C**	*0.112*	*0.095*	*0.119*
3 days	22 °C	0.073	0.080	0.081
4 days	22 °C	0.057	0.060	0.058
2 weeks	4 °C	0.062	0.057	0.062
3 weeks	4 °C	0.067	·	0.076
Syncase				
1 day	22 °C	0.042	0.057	0.045
1 day	25 °C**	0.078	0.071	·
2 days	22 °C	0.055	0.050	0.057
2 days	25 °C**	*0.116* .	*0.122*	*0.112*
3 days	22 °C	·	0.063	0.050
4 days	22 °C	·	0.072	0.062
2 weeks	4 °C	0.054	0.051	0.060
3 weeks	4 °C	·	·	0.062

* Quotient of gut weight/body weight minus gut weight in suckling mice. ST positive: values higher than 0.090 (italicized figures)

** Shaken

· Not determined

Table III

ST enterotoxin production by Y. enterocolitica strains in sausage

Incubation time and temperature		Strains and their serogroups			
		No. 22 O3	No. 71210 O3	No. 81371 O3	No. 98010 O9
Sausage					
1 day	22 °C	0.061*			
1 day	25 °C**	0.059			
2 days	22 °C	0.060			
2 days	25 °C**	*0.091*	0.068 (germ count: 1×10^8/ml)	0.071 (germ count: 1×10^8/ml)	0.068 (germ count: 2×10^8/ml)
3 days	22 °C	0.050			
4 days	22 °C	0.052			
2 weeks	4 °C	0.054			
3 weeks	4 °C	0.067			
Syncase					
1 day	22 °C	0.058			
1 day	25 °C **	0.057			
2 days	22 °C	0.068			
2 days	25 °C**	*0.166*	*0.150*	*0.100*	*0.118*
3 days	22 °C	0.062			
4 days	22 °C	0.051			
2 weeks	4 °C	0.055			
3 weeks	4 °C	0.060			

For explanation see Table II

The presence of *Y. enterocolitica* in food products is not very common, but its significance must not be neglected. Serogroups responsible for human disease have repeatedly been grown from food. Schiemann [3, 7] could isolate serogroups O5, O7,8 and O6,30 from fresh milk and O3 and O5 from pork products, but the pathogenic agent was detected also in pasteurized milk. Black et al. [14] reported an epidemic caused by serogroup O8 isolated from cocoa. In Hungary, Adamis et al. [15] described cases of enteritis attributable to food contamination as in the absence of other possible factor they isolated *Y. enterocolitica* of serogroup O3 from the faeces. Asakawa et al. [16] also considered infected food to be the factor responsible for an epidemic. Although bacteria are killed during pasteurization, the possibility exists of a subsequent contamination and heat treatment might also prove insufficient with some strains [14–18]. Our results also confirm that *Y. enterocolitica* grows well and

preserves its viability in food for a long time. All these data stress the importance of food control with respect to *Y. enterocolitica*. Enterotoxin production in milk samples contaminated by 3 different strains out of the 7 used in the experiments could not be detected. In the contaminated meat the serogroups O7,8; O5 and two O3 strains produced enterotoxin only if they had been incubated under shaking at 25 °C for two days. Thus, presence of preformed enterotoxin will mean no danger if the examined food types are stored in the customary way. That, however, does not yet settle the problem. Only few samples and raw filtrates were used by us for enterotoxin detection. We could find only one report on the subject. In this paper Francis et al. [18] observed good growth of the strains in infected milk but only a few produced enterotoxin. We were the first to study the possibility of enterotoxin production in sausages but food poisoning due to *Y. enterocolitica* enterotoxin might still not be excluded on account of its absence in spite of its special features like production below 30 °C, resistance to boiling and gastric acid having been observed. Therefore, further experiments must be conducted in this field. Of course, the pathogenicity of *Y. enterocolitica* might be connected not only with its enterotoxin production; the role of other factors should be examined as well.

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REFERENCES

1. Pai, C. H., Mors, V.: Infect Immun **19,** 908 (1978).
2. Pai, C. H., Mors, V., Seemayer, T. A.: Infect Immun **28,** 238 (1980).
3. Schiemann, D. A.: J Food Prot **43,** 360 (1980).
4. Schiemann, D. A.: Infect Immun **32,** 571 (1981).
5. Velin, D., Emődy, L., Pácsa, S., Kontrohr, T.: Acta Microbiol Acad Sci Hung **27,** 299 (1980).
6. Aldova, E., Cerná, J., Janecková, N., Pegrimkova, J.: Čs Hyg **8,** 395 (1975).
7. Schiemann, D. A., Toma, S.: Appl Environ Microbiol **35,** 54 (1978).
8. Hanna, M. O., Zink, L. D., Carpenter, Z. L., Vanderzant, C.: J Food Sci **41,** 1254 (1976).
9. Gilányi, J., Svidró, A., Szita, J.: Egészségtudomány **21,** 385 (1977).
10. Jacks, T. M., Wu, V. J.: Infect Immun **9,** 342 (1974).
11. Velin, D., Emődy, L.: Acta Microbiol Acad Sci Hung **29,** 227 (1982).
12. Szita, J., Káli, M., Rédey, B.: Acta Microbiol Acad Sci Hung **18,** 113 (1971).
13. Szita, J., Svidró, A., Kubinyi, M., Nyomárkay, I.: Acta Microbiol Acad Sci Hung **27,** 103 (1980).
14. Black, R. E., Jackson, R. J., Tsai, T., Medvesky, M., Shayegani, M., Feeley, J. C., Mac Leod, V. I. E., Wakelee, A. M.: N Engl J Med **298,** 76 (1978).
15. Adamis, É., Svidró, A., Straub, I., Szita, J.: Egészségtudomány **19,** 16 (1975).
16. Asakawa, Y., Akahane, S., Kagata, N., Noguchi, M., Sakazaki, R., Tamura, K.: J Hyg (Lond.) **71,** 715 (1973).
17. Hughes, D.: J Appl Bacteriol **46,** 125 (1979).
18. Francis, D. W., Spaulding, P. L., Lovett, J.: Appl Environ Microbiol **40,** 174 (1980).

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HUMAN ADENOVIRUS INFECTION IN PHYTOHAEMAGGLUTININ TREATED MICE

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The course of infection by human adenovirus type 12 has been studied in mice treated with phytohaemagglutinin (PHA) 1 day after infection or those infected with the virus 3 days after PHA treatment. Virus antigens were demonstrated by immunofluorescence in the cells of the thymus, spleen and lymph nodes and in some cases the supernatant of the cell homogenate was found to contain infective viruses. Virus antigens occurred sporadically in the cells of the lymphoid organs of control animals infected only with adenovirus. Neither virus antigens nor infective virus could be detected in mice that were not infected by virus and not treated with PHA as controls. The results indicate that PHA treatment increased the sensitivity of mice to human adenovirus infection.

The pathomechanism of adenovirus infections needs elucidation from several points of view. A considerable number of children and adults carry in their adenoids and other tissues the virus or its specific components. Whether in virus carriers the latent virus may become active and under what effects is one of the questions awaiting clarification [1–5]. In earlier studies some of the T-lymphocytes of virus carriers were found to contain virus antigens especially during relapses, and in febrile recurrences even infective virus was cultivated from the lymphocytes [6, 7]. After infecting the lymphocytes of healthy persons with adenovirus, virus-specific antigens were detected in the cells without the formation of infective virus. On the other hand, if the lymphocytes were stimulated by PHA prior to virus infection, the virus antigens and also the infective virus were found to replicate in them [8]. In the present experiments it was attempted to determine inPHA treated animals the pathomechanism of infection and whether or not its course was affected by the treatment. Type 12 human adenovirus has been chosen for examinations in view of its affinity to the immune system and its malignant effect in animals [9].

Materials and methods

Experimental animals. 293 CFLP mice of 35–40 g body weight purchased from LATI (Gödöllő, Hungary) were used. In individual experiments only animals of identical sex were included.

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Phytohaemagglutinin. The animals were treated with Difco PHA—P. The optimum quantity and time of effect were determined by titration. A 1% solution was prepared and 0.3 ml or in some cases 0.4 ml was given intraperitoneally.

Virus infection. Mice were infected intraperitoneally with 2500–3000 CP units of type 12 human adenovirus. The virus was propagated and titrated on HEp-2 tissue culture.

Demonstration of virus antigens. Immunofluorescence. Cytological preparations of the lymphoid organs prepared on slides were dried at room temperature and fixed in acetone. The technique of Coons and Kaplan in our modification was applied both in direct and indirect form [6, 10]. For direct examination the preparations were treated with an immune serum produced in the rabbit against type 12 adenovirus and conjugated with fluorescein isothiocyanate. For indirect examinations the preparations were treated with adenovirus type 12 immune serum and stained with fluorescein isothiocyanate-conjugated anti-rabbit globulin. Immunofluorescence examinations were performed with a Zeiss-Fluoval microscope at ×600 magnification.

Complement fixation. Virus antigens were demonstrated in the supernatant of the organs by complement fixation's microtechnique [11].

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

Recovery of virus. For virus propagation parts of the spleen, liver, thymus and lymph nodes were ground with quartz sand and the supernatant was grown on HEp-2 tissue culture.

Examination of lymphoid organs. Absolute lymphocyte count. After determination of the leukocyte count in Buerker's chamber, smears were stained with Giemsa and in knowledge of the WBC the absolute lymphocyte count was determined.

Cytological examination. Cytologic preparations made from spleen, thymus and lymph node cells were stained with Giemsa and the morphology of the cells was evaluated under the light microscope.

Lymphoid organ weight. The relative weight of the spleen and thymus as well as the spleen-thymus index of mice sacrificed at the end of the experiment were determined as follows:

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

$$\text{Lymphoid organ index} = \frac{\text{mean relative lymphoid organ weight in experimental group}}{\text{mean relative lymphoid organ weight in control group}}$$

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

Results

As indicated by several preliminary experiments, a significant effect could be expected only in groups where in the organism the effect of virus and PHA was optimum. This optimum was found at 24 h with the adenovirus and at 72 with PHA treatment. Accordingly two groups were studied, i.e. the course of the infection of PHA treatment was applied 24 h later (group V + + PHA) in 66 mice; the course of infection if the animals were infected with adenovirus 72 h after PHA treatment (group PHA + V), in 59 mice.

The animals were sacrificed and examined on the 4th day after virus infection or PHA treatment.

The following control groups were studied with both experimental groups: 47 uninfected and untreated controls (group C) and 60 PHA treated animals (group PHA). These mice were sacrificed 3 days after treatment while the

61 controls infected only with virus (group V) were sacrificed 24 h after the infection.

In group V + PHA thymus weight changed very slightly, the spleen index increased 1.5 times, the peripheral lymphocyte count increased significantly. With Giemsa staining the majority of the thymus and spleen cells displayed lymphoblast characteristics with mitotic cell formation and in some cases polynuclear cells. Virus particles were not detected in thymus, spleen and liver cells. Immunofluorescence revealed the presence of antigens in 3–5% of the thymus, spleen and lymph node cells, while virus antigens also in the supernatant of cell homogenates with complement fixation. On HEp-2 tissue culture the same supernatants yielded adenovirus, in some cases even in infective form, though only in low titre.

In group PHA + V the relative spleen and thymus weight increased slightly, the peripheral lymphocyte count decreased, the majority of thymus and spleen cells showed lymphoblast characteristics with numerous mitotic cells and polynuclear lymphoid cells. Occasionally, virus particles were seen in thymus, spleen and liver cells. Immunofluorescence revealed the presence of adenovirus antigens in 5–7% of the thymus, spleen and lymph node cells. Virus antigens were demonstrated with complement fixation in the supernatant of cell homogenates. Adenovirus was recovered in some cases even in infective form from the supernatant kept on HEp-2 tissue culture, mainly if the animal had been treated with large PHA doses. Figure 1 shows the relative spleen weight and absolute lymphocyte count. Results concerning spleen index, virus antigen and infective virus are demonstrated in Table 1.

No signs referring to illness were found in the animals infected with type 12 adenovirus. As compared to the uninfected and untreated controls, in group V, the peripheral lymphocyte count decreased after 24 h. The same virus ef-

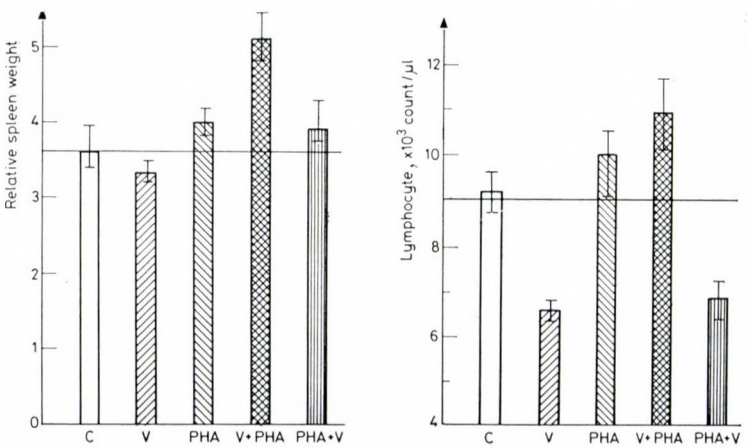

Fig. 1. Relative spleen weight and absolute lymphocyte count

Table I

Effect of adenovirus infection and PHA treatment on groups of mice

Groups	Spleen index	Virus antigen, %	Infective virus
V + PHA	1.50	3–5	+
PHA + V	1.07	5–7	++
V	0.92	0.5	—
PHA	1.10	—	—
Control	1.0	—	—

fect seemed to be responsible for the changes in group PHA + V where virus antigens were occasionally revealed while in group V where only 0.5% of the cells showed some dubious fluorescence.

In the control group treated solely with PHA, body weight and relative thymus weight increased slightly, but the relative spleen weight showed a more marked increase after 72 h. The lymphocyte count increased and blast-like mononuclear leukocytes appeared. Numerous blast cells were found in the thymus and spleen. Neither infective virus nor virus antigens were demonstrable in groups C and PHA, which had not been infected with virus.

Discussion

There is an increasing number of data to show that viruses are localized first in the lymphoid organs of animals infected with human adenovirus [12, 13]. Latent types of adenovirus may not only persist in the tonsils and adenoids of man but may also infect the circulating lymphocytes. In some cases infective virus has been isolated from the lymphocytes [7, 14, 15]. Mononuclear leukocytes obtained from cord blood were successfully infected in vitro with latent adenovirus types and the viruses persisted in the cells without causing manifest changes [16]. According to other data not only virus antigen but also significant amounts of infective virus were produced in cord blood lymphocytes if they were stimulated with PHA prior to infection with adenovirus [17]. This observation was confirmed also by our earlier experiments carried out with mononuclear leukocytes of blood donors [8]. The non-specific stimulating effect of PHA was found to increase the sensitivity of mice to human adenovirus. This was indicated by the observation of a significant amount of virus antigen in some cells of the immune organs and some of them even contained infective virus. This effect could be observed when PHA treatment had preceded by 3 days the virus infection or if it followed the virus infection by 1 day.

The fact that in animals infected with adenovirus, viral antigens were only occasionally present in the lymphoid organs, proves that the antigens were synthesized de novo. The possibility of an incidental presence of a murine adenovirus was excluded by the observation that neither virus antigens nor infective virus were found in the mice which had not been infected.

Several agents are known to stimulate the cells of the lymphoid organs both in man and animals. The stimulating effect of PHA has been studied mainly in mice [18–20]. On the basis of these experiments one may assume that an adenovirus infection would manifest itself with the production of infective viruses if the organism has already been stimulated by another agent. On the other hand, an adenovirus infection affecting an unstimulated organism would result in the production of virus antigens and perhaps in the development of a latent infection or virus carrier state [14, 21]. Though data referring to such a phenomenon are still scarce [18, 22], it is assumed that latent virus infections may be activated by different factors and then they may result in relapses or even in the development of malignancy.

REFERENCES

1. Schlesinger, R. W.: Advanc Virus Res **14,** 1 (1969).
2. Nász, I., Kulcsár, G., Dán, P., Sallay, K.: J Infect Dis **124,** 214 (1971).
3. Kulcsár, G., Vutskits, Zs., Nász, I., Dán, P., Léb, J.: Zentralbl Bakteriol **215,** 506 (1970).
4. Yunis, E. J., Hashida, Y.: Pediatrics **51,** 566 (1973).
5. Flint, S. J.: In Tooze, J. (ed.): DNA tumor viruses. Cold Spring Harbor Monogr 10B. 1980. P. 383.
6. Sallay, K., Dán, P., Geck, P., Kulcsár, G., Nász, I.: Arch Derm Forsch **241,** 15 (1971).
7. Kulcsár, G., Sallay, K., Dán, P., Nász, I., Horváth, J.: Fogorv Szle **70,** 345 (1977).
8. Schranz, V., Kulcsár, G., Dán, P., Horváth, J., Nász, I., Barinskij, I. F., Ugryumov, E. P.: Acta Microbiol Acad Sci Hung **26,** 1 (1979).
9. Trentin, J. J., Yoshiro, Y., Taylor, G.: Science **137,** 835 (1962).
10. Coons, A. B., Kaplan, M. H.: J Exp Med **91,** 1 (1950).
11. Takátsy, Gy.: Acta Microbiol Acad Sci Hung **3,** 191 (1955).
12. Horváth, J., Kulcsár, G., Dán, P., Nász, I.: Kísérl Orvostud **31,** 244 (1979).
13. Horváth, J., Kulcsár, G., Nász, I., Geck, P., Ongrádi, J.: Kísérl Orvostud **33,** 124 (1981).
14. Van der Veen, J., Lambrieux, M.: Infect Immun **7,** 604 (1973).
15. Andiman, W. A., Jacobson, R. I., Tucker, G.: N Engl J Med **297,** 100 (1977).
16. Andiman, W. A., Miller, G.: J Infect Dis **145,** 83 (1982).
17. Perrinet, M. C., Chardonnet, Y., Sohier, R.: Ann Microbiol Inst Pasteur **125**B. 575 (1974).
18. Ward, J. M., Young, D. M.: J Amer Vet Med Ass **169,** 952 (1976).
19. Gamble, C. N.: Blood **28,** 175 (1966).
20. Epstein, L. B., Smith, C. W.: J Immunol **100,** 421 (1968).
21. Nász, I., Kulcsár, G., Dán, P., Sallay, K., Vértes, L., Geck, P., Keskeny, S., Horváth, J.: Acta Microbiol Acad Sci Hung **20,** 191 (1973).
22. Ongrádi, J., Kulcsár, G., Dán, P., Nász, I., Horváth, J.: Intervirology **14,** 310 (1980).

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ENCAPSULATION AND MOUSE-VIRULENCE OF *SERRATIA MARCESCENS* STRAIN SM-1 AND ITS VARIANTS IN RELATION TO COLONIAL MORPHOLOGY

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With the addition to a soft-agar medium of rabbit anti-flagella serum inhibiting mobility, strain SM-1 of *Serratia marcescens* produced single colonies. Strain SM-1 and its variants A and B exhibited three kinds of colonial form. The parent strain showing extra large round-type growth in the medium had the highest cell volume index and mouse virulence, and a large capsule was seen on electron microscopy. An intermediate cell volume index and a remarkably lower mouse virulence were observed with variant A, which exhibited diffuse-type growth in the medium, although no definite extracellular feature was shown. The variant B, showing compact-type growth in the medium, represented the lowest cell volume index, mouse avirulence and was not encapsulated. Mouse virulence of the parent strain was assumed to be related to encapsulation which protects against phagocytosis.

Serum-soft agar and soft-agar technique have been applied for the investigation of cell surface properties of several species of non-motile bacteria such as *Staphylococcus aureus* [1], *Streptococcus pyogenes* [2], *Streptococcus pneumoniae* [3], *Klebsiella pneumoniae* [4], *Klebsiella ozaenae* [5] and *Escherichia coli* [6]. Although colonial size in the media varied depending on the species, an almost similar colonial morphology was observed in encapsulated organisms of these strains except for strains of *S. aureus* [1]. However, no paper has been concerned with the application of a motile bacterial strain in this technique. To apply the soft-agar technique to motile bacterial strains, for the examination of *Serratia marcescens*, the soft-agar medium was supplemented with anti-flagella serum.

Materials and methods

Strains. Strain SM-1 of *S. marcescens* used throughout the experiments was freshly isolated from a human patient. The strain showed large round colonies on soft-agar medium containing rabbit anti-flagella serum. Two variants, A and B, obtained by repeated subculturing, exhibited diffuse and compact colonial morphologies, respectively. Serogroup of the strain SM-1 was O5 determined by agglutination test using conventional specific antisera (Toshiba Chemical Co. Ltd., Tokyo, Japan).

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Preparation of rabbit anti-flagella serum. Strain SM-1 was cultured in trypticase soy broth (Difco) at 37 °C for 24 h and the flagella fraction was obtained by the method of Kobayashi et al. [7]. Of the fraction a volume of 0.3 ml containing 100 μg of purified flagella protein per ml, was injected into the marginal ear vein of rabbits weighing 2.5 kg (Japanese white rabbit, Nihon Clea Farm Co. Ltd., Tokyo, Japan) on three successive days weekly for 4 weeks. Ten days after the final injection, the rabbits were exsanguinated, the sera were separated and the anti-flagella antibody activity was determined by the soft-agar technique. The twofold serial dilution of the rabbit antisera which produced definite single colonies of the organism was considered one unit of the activity.

Anti-flagella serum-soft agar technique. The organisms cultured in brain heart infusion broth (BHI, Difco) at 37 °C for 24 h were diluted $1 : 10^{-6}$ with sterile saline. One tenth ml of this cell suspension and two units of rabbit anti-flagella serum were combined with 10 ml of BHI containing 0.15% (w/v) agar (Bactoagar, Difco) at final concentration. These were kept at 4 °C for 30 min, cultured at 37 °C and colonial morphology of the organisms was determined after 24 h.

Determination of mouse virulence. Strain SM-1 and the variants A and B were cultured in BHI broth, harvested by centrifugation at 7000 *g* for 30 min, resuspended in sterile saline, then the turbidity was adjusted nephelometrically to 1.0 OD at 430 nm. Viable cell number of the cell suspension was 6.43×10^8 to 7.21×10^8 per ml as estimated by the plate count method. The cell suspensions were diluted twofold with saline. Of these, 0.5 ml and 4.5 ml of 5% (w/v) mucin (Wilson type) in saline were injected intraperitoneally into a group of 5 mice each weighing approximately 15 g (DD strain, Nihon Clea Co. Ltd., Tokyo, Japan). The number of dead animals was recorded for a period of two weeks.

Viability of the strain and its variants in mouse peritoneal cavity. To determine the viability of strain SM-1 and the variants, A and B, injected into the peritoneal cavity of mice, the cell suspensions were obtained as mentioned above, and their turbidity was similarly adjusted to 0.5 OD.

The viable cell number was 1.85×10^8 to 2.20×10^8 per ml. Of the suspensions, 0.5 ml was injected intraperitoneally to each of 5 mice. One, 3, and 15 h after the injection peritoneal fluid was withdrawn and the viable cell count was determined [8] and the average cell number of 3 experiments was noted.

Cell volume index and biological properties. To determine the cell volume index of strain SM-1 and its variants, the organisms cultured in BHI were harvested by centrifugation, washed once with sterile saline and the indices were recorded [8] by using a Hopkins tube. To examine the biological properties of them, a conventional enterobacterial identification kit (ID test, EB 20, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) involving the following tests was used: fermentation to inositol, mannose, sorbose, arabinose, rhamnose and raffinose. In addition, lysine decarboxylase, arginine decarboxylase, the Voges–Proskauer reaction and utilization of malonate and arginine were examined. Deoxyribonuclease was determined by DNase medium (Difco).

Electron microscopy. The organisms cultured on BHI medium at 37 °C for 24 h were harvested, prefixed with 7% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 4 °C overnight, then fixed again with 0.1% (w/v) osmic acid at room temperature for 5 h. The fixed materials were dehydrated in ethanol and acetone, then embedded in Quetol 812 (Nisshin EM Co. Ltd., Tokyo, Japan). Ultrathin sections were prepared by an ultramicrotome (Type MT-1, Ivan Sorval Inc., Norwalk, Conn.), mounted with rabbit anti-strain SM-1 serum [9] and stained with uranyl acetate. Morphological observations were made by electronmicroscopy (Model 100B, Japan Electron Optics Co. Ltd., Tokyo, Japan).

Results

Colonial morphology of strain SM-1 and its variants in rabbit anti-flagella serum-soft agar. When rabbit anti-flagella serum was added to soft-agar medium, single colonies were observed. This technique was affected by the concentration of agar, salt and the pH of the medium. Optimum conditions were as follows: 0.15% (w/v) agar, 0.75% (w/v) salt and pH 7.0. Under these conditions, the colonies of strain SM-1 and its variants A and B exhibited large

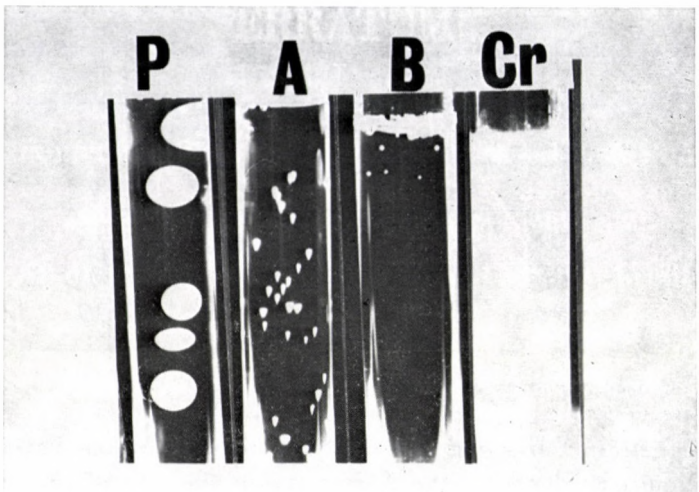

Fig. 1. Colonial morphology of strain SM-1 of *S. marcescens* (P) and variants (A) and (B) in soft-agar containing rabbit anti-flagella serum and strain SM-1 cultured in soft-agar containing normal rabbit serum (Cr)

round, diffuse and compact type growth, respectively, as shown in Fig. 1. The growth of variants A and B throughout the tube in soft-agar medium containing normal rabbit sera was excluded. In these cases, the large round colonies of strain SM-1 were converted to compact-type through diffuse-type growth after repeated subcultures. Also, variants A and B reverted to large round-type growth in mouse passages.

Biological properties. Strain SM-1 and its variants A and B showed identical biological properties. They fermented inositol, mannose, sorbose and sucrose without gas production, but did not ferment arabinose, rhamnose and raffinose. They were positive for lysine decarboxylase and negative for arginine decarboxylase. They did not utilize malonate and arginine and gave a positive Voges-Proskauer and deoxyribonuclease tests and displayed red pigmentation on BHI agar plate.

Cell volume index. Cell volume indices of strain SM-1 and variants A and B were 1.37, 1.17 and 1.00, respectively, suggesting an encapsulation in the parent strain.

Mouse virulence. Injection of 9.80×10^4 cells of strain SM-1 in mucin suspension killed 100% of the mice and of variant A 4.56×10^6 cells were required to cause a similar mortality. Of variant B, however, not even $1.02 \times \times 10^8$ cells were lethal.

Toxicity for mice. Of the parent strain and its variants A and B, 1.0 mg of heat killed vaccine killed 20 to 40% of the animals, and 2.0 mg of any strain was lethal to 80 to 100% (Table I). Such uniform results indicate that all organisms contained almost equal amounts of endotoxin.

Table I

Mouse toxicity of heat-killed vaccine prepared from parent strain SM-1 of Serratia marcescens and its variants A and B

Heat-killed organisms (mg)	SM-1	variants	
		A	B
2.0	5/5*	4/5	4/5
1.0	1/5	1/5	2/5
0.5	0/5	0/5	0/5

* No. of dead/No. of used

Fate of strain SM-1 and its variants in mouse peritoneal cavity. To elucidate the mouse virulence of the parent strain the fate of the organisms injected intraperitoneally was observed. When 2.0×10^8 of the parent strain and its variants in saline suspension were injected intraperitoneally to the mice, the parent strain gradually multiplied to 10^{10} in 15 h (Fig. 2) when it killed the animals. With variant A no multiplication was observed in 3 h, then the count gradually decreased and no animal died. (These results have not been included in Fig. 2.) With variant B, the viable cell number decreased rapidly to reach 10^4 at 15 h after the injection and all animals survived (Fig. 2).

Electron microscopy. The outermost layer of the parent strain was surrounded by high electron dense material indicating the existence of a capsular substance. No such finding was observed with the variants A and B (Fig. 3).

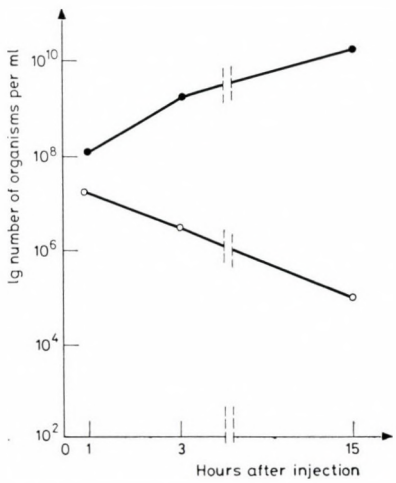

Fig. 2. Recovery of strain SM-1 of *S. marcescens* and variant B after intraperitoneal injection in mice; ●——● and ○——○ indicate strain SM-1 and variant B, respectively

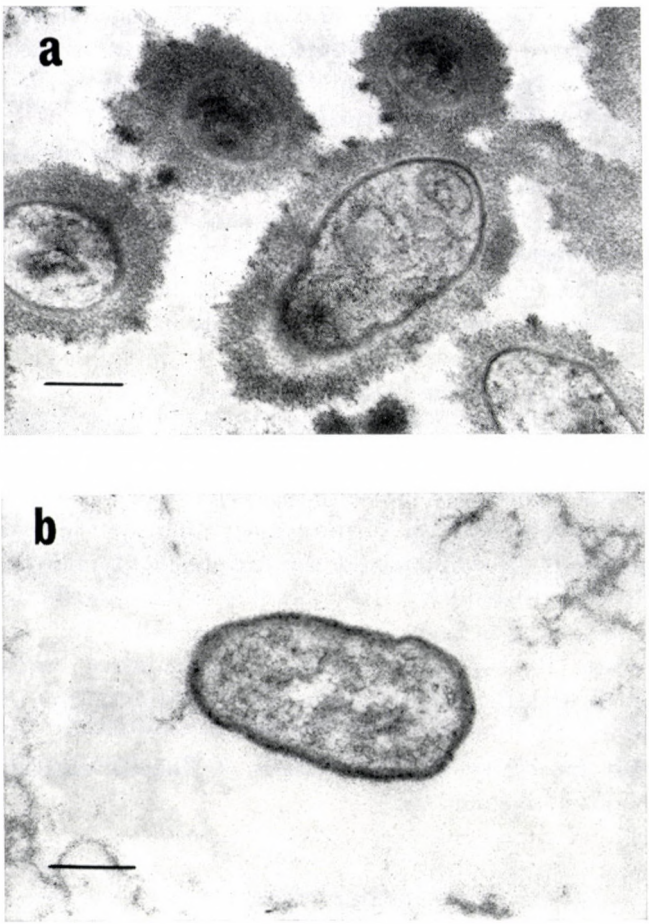

Fig. 3. Ultra-thin section of strain SM-1 (a) of S. *marcescens* and variant B (b) treated with rabbit anti-SM-1 serum

Discussion

Serum-soft agar and soft-agar techniques have been applied by several authors for the selection of non-motile encapsulated bacterial strains [1–6]. Although semi-solid agar plate containing anti-flagella serum was also used for the identification of flagella antigen of *Salmonella* sp. [10], with these techniques the colonial morphology of motile organisms has not been investigated. In our experiments distinct single colonies were produced in soft-agar containing anti-flagella serum with strain SM-1 of *S. marcescens* and its variants. The parent strain exhibiting large round-type growth proved to be mouse virulent and encapsulated, while its variants A and B, showing diffuse and compact-

type growth were less mouse virulent and avirulent, respectively, and variant B was not encapsulated while the cell surface of the intermediate type variant A was not clearly defined. Still, the biological properties of the parent strain and the variants A and B were identical.

Numerous investigators have emphasized that the surface component of microorganisms such as their capsule or slime materials played an important role in pathogenicity [11–14] and these components of the organisms are regarded as virulent factors.

In the present experiments, the encapsulated parent strain gradually increased its viable cell number in the peritoneal cavity of mice and caused their death. With the not encapsulated variant B the viable cells disappeared in the peritoneal cavity and the animals survived.

These findings are believed to reflect the importance of the surface properties and seem to suggest that the surface material such as the capsule this species of bacteria apparently protects against phagocytosis. These results coincided with findings made in Gram-negative non-motile bacterial strains in soft agar medium [4–6], emphasizing the antiphagocytic role of their capsule and its relation to pathogenicity. Interconversion by flagella antiserum of a strain of *Salmonella* sp. has been observed on semi-solid agar plate containing anti-flagella serum [10, 15]. In the present experiments no antigenic conversion was observed with the parent strain and its variants even after repeated subcultures.

Extra-high specificity and conversion of flagella antigen remain the technical problems of the method.

REFERENCES

1. Yoshida, K., Ekstedt, R. D.: J Bacteriol **96,** 902 (1968).
2. Takahashi, M., Ichiman, Y., Maruoka, Y.: St. Marianna Med J **8,** 297 (1981).
3. Takahashi, M., Ichiman, Y., Kono, E., Yoshida, K.: St. Marianna Med J **9,** 205 (1981).
4. Takahashi, M., Yoshida, K., San Clemente, C. L.: Can J Microbiol **23,** 448 (1977).
5. Takahashi, M., Iwami, S., Yoshida, K.: Microbiol Immunol **22,** 361 (1978).
6. Takahashi, M., Ichiman, Y., Yoshida, K.: Acta Microbiol Acad Sci Hung **28,** 413 (1981).
7. Kobayashi, T., Pinker, J. N., Koffler, H.: Arch Biochem Biophys **84,** 342 (1959).
8. Yoshida, K., Takeuchi, Y.: Infect Immun **2,** 523 (1970).
9. Ohtomo, T., Yoshida, K., San Clemente, C. L.: Infect Immun **31,** 798 (1981).
10. Enomoto, M., Iino, T.: J Bacteriol **86,** 473 (1963).
11. Fukumoto, T., Mitsuyama, M., Takeya, K., Nomoto, K.: J Gen Microbiol **119,** 225 (1980).
12. Morse, S. I.: Nature **186,** 102 (1960).
13. Schwarzman, S., Boring III, J. R.: Infect Immun **3,** 762 (1971).
14. Yoshida, K., Ichiman, Y., Ohtomo, T.: Jap J Microbiol **20,** 209 (1976).
15. Le Minor, L., Edwards, P. R.: Ann Inst Pasteur **99,** 469 (1960).

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SEMI-CRUDE HUMAN LEUKOCYTE INTERFERON PRODUCTION IN A SIMPLE MEDIUM

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Semi-crude human leukocyte interferon (specific activity at least 10^5 international units per mg protein) was produced by replacement of priming medium with protein poor medium before the production period. A simpler medium than Eagle's medium was suitable to obtain substantial quantities of interferon.

Human leukocyte interferon (HuIFNα) is now being produced in large quantities for clinical purposes. In recent years several purification procedures resulting in IFNα preparations of high specific activity have been described, but these methods usually require an initial purification step. Acid ethanol extraction of human leukocyte interferon according to Cantell and Hirvonen [1] has been useful for the initial purification of large amounts of interferon. Still, as reported by Erickson and Paucker [2] in our hands the procedure gave variable results. Therefore another way was tested to increase the specific activity of IFNα preparations, namely by producing it in protein poor medium.

Materials and methods

Preparation of crude interferon. Crude human interferon was produced in human buffy coat cells with Sendai virus according to the method of Cantell et al. [3–5]. Briefly, buffy coats were utilized within 24 h of collection. Red blood cells were removed by treatment with 0.83% NH_4Cl solution, and purified leukocytes were suspended to a final concentration of 10^7 cells/ml in Eagle's medium (Glasgow modification), supplemented with 25 μg/ml neomycin and 2.5 mg/ml human agamma serum. The agamma serum was produced by removing most of the globulin by $(NH_4)_2SO_4$ precipitation according to Cantell and Pyhälä [6]. In some experiments ethanol extracted human serum albumin was used (produced by Institute for Serobacterial Research and Production Human, Budapest). Volumes of 20 ml leukocyte suspensions in 100 ml flasks were shaken or 3000 ml cell suspensions in 6000 ml wide-neck round bottom flasks were agitated by a magnetic stirrer (Cantell, personal communication) in 37 °C water bath. Priming interferon was added to give a final concentration of 100 IU/ml. Interferon production was induced by the addition of 1/20 vol of Sendai virus after the 2 h priming period as described previously [7], and the incubation was continued overnight. Cells and debris were removed by centrifugation, and the supernatants were stored at −20 °C.

Interferon assay. Interferon assays were performed in a microassay system as described previously [8] using CV-1 cells and vesicular stomatitis virus [VSV] as challenge virus. All inter-

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feron units are expressed in international units (G-023-901-527 reference was obtained from the National Institutes of Health, Bethesda).

Partial purification of Sendai virus. Infected chorioallantoic fluids of developing embryos were centrifuged at 3000 *g* for 30 min, followed by a second centrifugation at 40 000 *g* for 30 min. The pellet containing approximately 10% of the initial protein content was dissolved in 1/30 vol of phosphate buffered saline (PBS) pH 7.6, supplemented with 2.5 mg/ml human agamma serum. The partially purified virus was stored at −70 °C.

Haemagglutination (HA) and infectivity assay. HA was determined by chick erythrocytes as previously described [7]. Infectivity was expressed in terms of 50% egg infection dose (EID_{50}).

Protein determination. Protein concentration was determined by the method of Lowry et al. [9] using bovine serum albmin as the standard.

Results

Interferon production in different media. Earlier we have used Eagle's medium (Glasgow modification) for interferon production. The medium was maintained at pH 7.4 by CO_2–bicarbonate buffer. When a less complex medium, such as balanced salt solution (BSS) i. e. Eagle's medium without amino acids and vitamins was used, the yield of interferon was not reduced (Table I). A medium without calcium (Jocklik modified Eagle's medium) was not suitable for optimal interferon production. The yield was also reduced when the CO_2–bicarbonate buffered Eagle's medium was replaced by phosphate buffered Hanks' solution PBS, even if Hanks' solution was supplemented with amino acids and vitamins.

Effect of protein concentration on the yield of interferon. Experiments in shaken flasks were done to determine the optimum serum concentration for interferon production. Figure 1 shows that the maximum yield of IFN was produced in BSS containing 2.5 mg/ml human agamma serum, and that albumin extracted by alcohol was not adequate for IFN production.

Table I

Interferon production by human leukocytes in different media

Medium	Eagle's medium (Glasgow modification)	BSS*	Eagle's medium	Hank's solution	Hank's solution with vitamins and amino acids**	PBS
Titre of IFN***, IU/ml	49 300	47 570	21 840	22 530	24 800	4040
Per cent	—	96.5	44.3	45.7	50.3	8.2

* Balanced salt solution (Eagle's medium without amino acids and vitamins)

** Hank's solution supplemented with vitamins and amino acids in quantities identical as in the Glasgow modified Eagle's medium

*** Data are from three different experiments. Leukocyte cultures of 20 ml/10^7 cell/ml contained 2.5 mg/ml human agamma serum

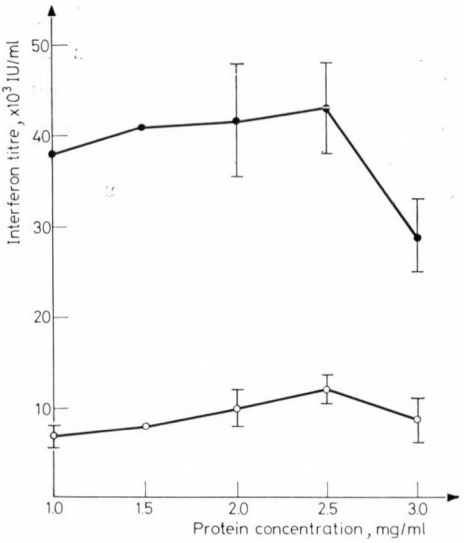

Fig. 1. Effect of protein concentration on interferon production by leukocytes. Leukocyte cultures of 20 ml contained 10^7 cell/ml in BSS supplemented with different amounts of human agamma serum ●——● or ethanol extracted human albumin ○——○

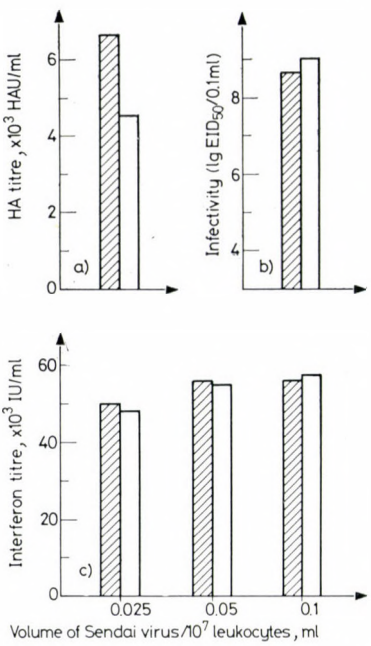

Fig. 2. Parameters of crude and partially purified Sendai virus. Haemagglutination activity (A), infectivity (B) and interferon inducing capacity (C) of crude (shaded) and partially purified (open) Sendai virus. Concentrated, purified Sendai virus material was diluted to the original volume (30-fold) in BSS before use. Values are means of results of two independent experiments. (C) Leukocyte cultures of 20 ml contained 10^7 cell/ml in BSS supplemented with 2.5 mg/ml agamma serum

Interferon induction by partially purified Sendai virus. Sendai virus partially purified by high-speed centrifugation was as active as crude virus for interferon induction (Fig. 2). After centrifugation the pellet contained 67% of the initial haemagglutination activity and all of the infectivity.

Production of semi-crude human interferon. As Fig. 1 shows, production of IFN in substantial quantities requires a high serum concentration, but this serum demand is essential only during the priming period [10]. Since the adsorption of Sendai virus to the leukocytes is complete within 5 min as measured by HA (data not shown) and since interferon is not produced for 3 h after infection, it was supposed that between these points of time the priming medium of high protein content could be removed and replaced by the protein poor production medium. Figure 3 shows that the optimum time of exchange of priming medium with prewarmed production medium containing 0.25 mg/ml protein was 10 min after induction. Using a higher protein concentration (1 mg/ml) the yield of interferon was not increased, but production medium without protein led to only minimal interferon production. If the priming medium was changed before induction by Sendai virus the interferon inducing capacity of leukocytes was completely retained (Fig. 3, column B). Table II shows the properties of IFN preparations produced by medium exchange before and after induction, compared to interferon produced without medium

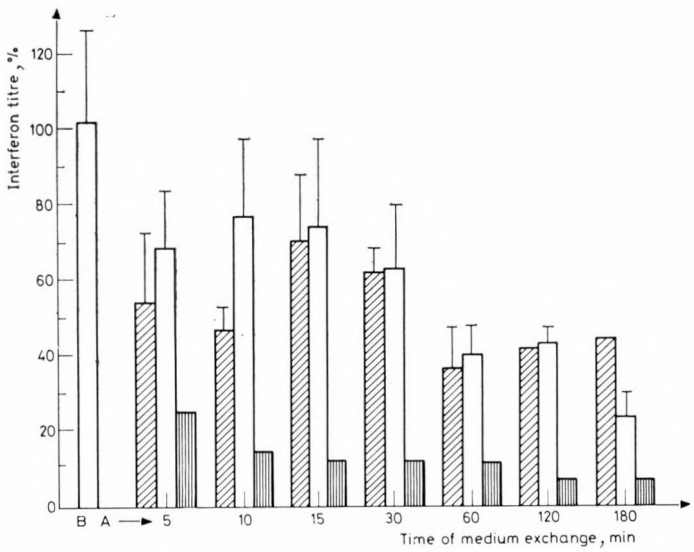

Fig. 3. Time of medium exchange and interferon yield. Priming medium was exchanged by pelleting (800 g, 10 min, room temperature) and resuspension of cells immediately before Sendai virus infection (column B) or 5–180 min after Sendai virus infection (columns A). Exchanging media contained 1.0 (shaded) 0.25 (open) and 0.0 (horizontally striped) mg/ml human agamma serum. Cultures of 20 ml contained 10^7 leukocytes/ml. All yields were expressed in per cent of IFN titre of the unchanged control (average titre 73 970 IU/ml). The priming medium and the production media were BSS

Table II

Properties of crude and semi-crude interferon produced by medium exchange

	Crude IFN (no medium exchange)	Semi-crude IFN*	
		medium exchange before induction	medium exchange 10 min after induction
Titre of IFN, IU/ml	73 970	75 250	56 880
Protein content, mg/ml	2.75	0.36	0.29
Specific activity, IU/mg protein	2.69×10^4	2.09×10^5	1.96×10^5

* Medium with 2.5 mg/ml agamma serum was replaced by medium containing 0.25 mg/ml agamma serum. Preparations were the same as used Fig. 3. Data obtained in four different experiments

Table III

Properties of crude and semi-crude interferon produced in large volume

	Crude IFN (no medium exchange)	Semi-crude IFN*	
		medium exchange before induction	medium exchange after induction
Titre of IFN, IU/ml	56 310	48 470	10 960
Specific activity, IU/mg protein	2.02×10^4	1.56×10^5	3.7×10^4

* Medium with 2.5 mg/ml agamma serum was exchanged for medium containing 0.25 mg/ml agamma serum before induction by partially purified Sendi virus or 10 min, after induction by crude Sendai virus. Data obtained in five independent experiments

exchange. The specific activity of the preparations was increased about 7-fold and exceeded the value of 10^5 IU/mg protein, so they may be called semi-crude interferon preparations.

Semi-crude interferon production in large volume. The versatility of medium exchange to increase the specific activity of IFN preparations was tested using large volumes. In five independent experiments leukocyte cultures of 3000 ml were incubated in 6000 ml round bottom flasks agitated by a magnetic stirrer. Each experiment included IFN preparations produced by medium exchange before, and 10 min after, virus infection, as well as crude IFN preparations produced without medium exchange (Table III). Substitution for the priming medium of medium containing 0.25 mg/ml agamma serum after Sendai virus infection resulted in interferon samples with lower titres than those obtained in the small volume experiments (Table II). If the medium exchange was carried out before Sendai virus infection, titres close to the control were

obtained. The specific activity of these IFN preparations exceeded the value of 10^5 IU/mg protein. To reduce the egg protein content in the interferon samples, partially purified Sendai virus was used in these experiments.

Discussion

Purification methods for interferon resulting in high specific activity generally require an initial purification step. Since the choice of these procedures is limited and recovery is variable, we have chosen a different approach to improve the specific activity of our IFNα preparations. The aim of this work was to test the use of protein poor medium for interferon production.

The results presented clearly proved that the IFN inducing capacity of leukocytes incubated in protein rich medium during the priming period is retained in protein poor medium (Fig. 3). In small volumes the reduction of protein concentration by medium exchange before or 10 min after Sendai virus infection led to an improvement in specific activity of the interferon to more than 10^5 IU/mg protein (Table II). In large volumes only a medium exchange before virus inoculation resulted in such an increase in specific activity (Table III). If the medium exchange was carried out after virus infection, low titres and specific activities were obtained. The cause of the different results obtained with small and large volumes, when the medium exchange was carried out after virus infection, is not clear. It should be noted that the time required for medium exchange in large volume experiments was longer than with small volumes, and the induction process being in its early phase was probably disturbed during the medium exchange period.

We have routinely used medium exchange before virus infection for the production of semi-crude IFN in large quantities. Since the IFN produced by crude Sendai virus contains impurities originating from allantoic fluid, we have used partially purified virus for induction.

In the other part of this work we have compared different media in respect to their versatility for IFNα production. The simple and autoclavable balanced salt solution was equivalent to the expensive Eagle's medium for IFN production (Table I). Calcium was essential for optimal interferon yields; on the other hand, an adequate buffer system was more important than the presence of amino acids and vitamins (Table I). Over a one year period in our laboratory only the balanced salt solution was used for production of crude leukocyte interferon in large volumes and the average titre was 63 200 IU/ml.

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REFERENCES

1. Cantell, K., Hirvonen, S.: J Gen Virol **39,** 541 (1978).
2. Erickson, J. S., Paucker, K.: Anal Biochem **98,** 214 (1979).
3. Strander, H., Cantell, K.: Ann Med Exp Biol Fenn **44,** 265 (1966).
4. Strander, H., Cantell, K.: Ann Med Exp Biol Fenn **45,** 20 (1967).
5. Cantell, K., Hirvonen, S., Mogensen, K., Pylälä, L.: In Waymouth, D. (ed.): In vitro. Tissue Culture Association, Rockville 1974. p. 35.
6. Cantell, K., Pylälä, L.: J Gen Virol **20,** 97 (1973).
7. Béládi, I., Tóth, M., Mécs, I., Tóth, S., Taródi, B., Koltai, M.: In Kono, R., Vilcek, I. (eds): Proc. International Conference on Clinical Potentials of Interferon in Viral Diseases and Malignant Tumors. Japan Medical Research Foundation, Tokyo 1982. p. 31.
8. Mécs, I., Béládi, I.: In Ikič, D. (ed.): Proc. Symposium on Preparation, Standardisation and Clinical Use of Interferon. Yugoslav Academy of Sciences and Arts, Zagreb 1977. p. 23.
9. Lowry, O. H., Rosebrough, N. I., Farr, A. L., Randall, R. J.: J Biol Chem **193,** 265 (1951).
10. Goore, M. Y., Dickson, J. H., Lipkin, S., DiCuollo, C. J.: Proc Soc Exp Biol Med **142,** 46 (1973).

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LINKING COMPONENTS BETWEEN CAPSOMERS IN THE ADENOVIRUS CAPSID

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The interhexonal space within the adenovirus capsid is bridged by interhexonal connective elements. These linking components have been detected also between pentons and the five peripentonal hexons surrounding them. According to the results obtained by Markham's rotational integration technique the connective elements between the capsomers are interlacing the whole virion. It could be confirmed in several cases that the linking elements consisted of two approximately parallel elements. This means that each hexon is linked by six times two parallel linking components with its six nearest neighbours in the virus capsid.

The adenovirus virion is a regular icosahedron measuring 70–90 nm in diameter [1, 2]. The capsid is built up of 252 capsomers of which 12 are situated on the vertices of the icosahedron forming with their projections the pentons. The other 240 capsomers are hexons, which constitute the triangular faces and edges of the virus capsid. Hexon capsomers show a hexagonal array in the virus capsid and have six neighbours whereas the pentons are surrounded by five neighbouring hexon capsomers.

In earlier examination of the crystallization of highly purified soluble hexons, tightly packed two-dimensional crystalline arrays were detected besides the three-dimensional tetrahedral hexon crystals [3]. In these two-dimensional crystalline arrays the hexons show a hexagonal packing, similarly to the structure of the virus capsid [4]. High-resolution electron micrographs revealed that the space between the neighbouring hexons was bridged by fine connective elements in the two-dimensional hexon crystalline arrays [5, 6].

The following report gives information on the interhexonal linking components in the adenovirus capsid as well as on the existence of connective elements between the peripentonal hexons and pentons.

Materials and methods

Preparation of virions. Adenovirus type 1 propagated on permanent HEp-2 cell culture was used for the study of virions. Infected cells were subjected to repeated freezing and thawing as well as to ultrasonic disintegration and the cell debris was eliminated by centrifugation.

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From the supernatant the virions were purified by ultracentrifugation of double CsCl cushion and by repeated ultracentrifugation on CsCl density gradient. Adenoviruses isolated on primary rabbit kidney cell culture from the intestinal tract and different organs of 6–8-week old rabbits suffering from nonbacterial diarrhoea were also studied [7].

Electron microscopy. For electron microscopic examinations the virions were adsorbed to carbon-formvar-coated grid and negatively stained with 1% uranyl acetate of 2% phosphotungstic acid. The preparations were examined in a JEM 100B electron microscope at 60 kV with a basic magnification of 25 000—50 000. The negatives were enlarged to a magnification of approximately 300 000 for study. The disturbing background granulation was eliminated by the technique of Yabe et al. [8]; printing was done at a slightly underfocussed position. For serial examinations selected virions with capsids containing well visible connective elements between certain capsomers were rephotographed and the negatives were used in the next examination. For examination of the radial symmetry of the connective elements and for enhancing images the rotational integration technique of Markham et al. [9] was used.

Results

Analysis of high-resolution micrographs revealed that the distances separating the hexons in the adenovirus capsid were bridged by fine linking elements (Fig. 1/a). Owing to these connective structures forming bridges at some sites between capsomers, the slits may appear as holes in the capsid. On the micrograph of a selected hexon processed by Markham's rotational technique with a rotational angle of 60°, the connective elements were regularly visible in the virus capsid. The connections between the given hexon and its six neighbours as well as between the hexons surrounding the central hexon were clearly seen (Fig. 1/b). If a hexon situated at the edge of the capsid and surrounded by a large symmetric area was subjected to 60° rotation, a regular network of connective elements became visible according to the hexagonal packing of the hexon capsomers. This network is distinct between the members

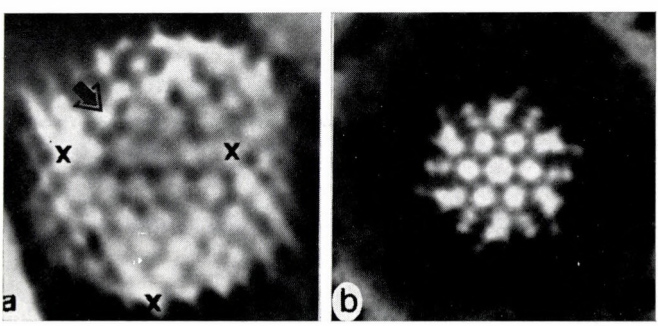

Fig. 1. (a) Electron micrograph of adenovirus type 1. The virion is viewed from the 2-fold symmetry axis (edge). Pentons on the vertices of a triangular face are marked with X. Hexagonal array of the hexon capsomers and connective elements linking the neighbouring hexons are visible at some sites in the capsid. Hexon serving as centre of Markham's rotational integration is shown by arrow; (b) Image processed by rotational integration of the hexon marked on the electron micrograph of the virion. Angle of rotation, 60°. The central hexon is connected with its six neighbours by clearly visible connective elements. The original profile of the central hexon and other parts of the virion are blurred owing to the disturbed symmetry

of the first and second hexon rows surrounding ring-like the central hexon (Fig. 2/a, b). The distance i.e. the length of the connective elements between the capsomers, is about one third of the diameter of the hexon. Considering the fact that the hexon studied (Fig. 2/a, b) is situated on one of the edges, the micrograph proves the presence of the linking components between hexons on the edges and near the edges. If a triangular face of the virion is subjected to 120° rotation (three-fold symmetry axes) the micrograph shows clearly the regular network of connective elements among the six hexons situated at the face of the virus capsid (Fig. 3).

On the rotational integration picture of the penton which has five neighbours according to the five-fold symmetry, processed by an angle of 72° (Fig. 4/a, b), the connective elements between the penton and peripentonal

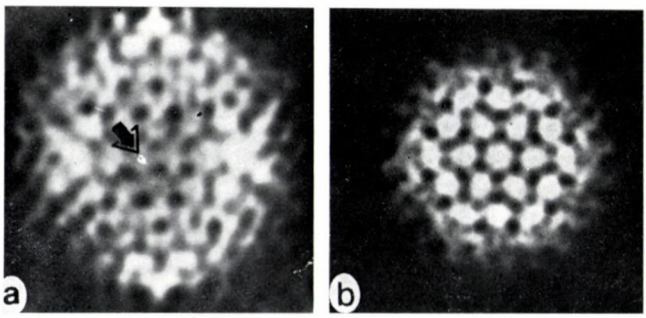

Fig. 2. (a) Hexon capsomer at edge serving as centre of Markham's rotational integration is shown by arrow on the electron micrograph of an adenovirus isolated from the rabbit; (b) Picture viewed by Markham's rotational integration of the hexon marked on the electron micrograph of the virion. Angle of rotation, 60°. Regular network of linking structure is seen in the hexagonal array of hexon capsomers. The network is well visible between the members of the two hexon rows surrounding ring-like the central hexon. Further parts of the virion are blurred owing to disturbed symmetry

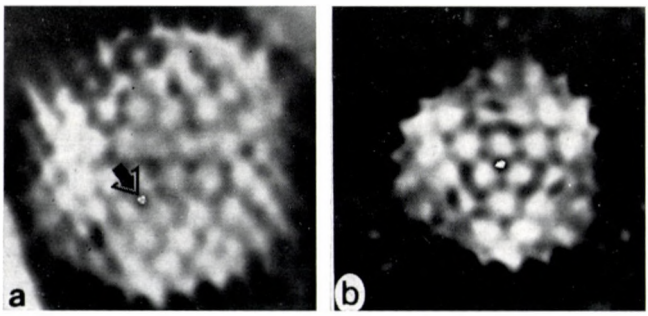

Fig. 3. (a) On the electron micrograph of adenovirus type 1 the centre of a triangular face of the virion, i.e. the centre of the space among three hexons in the middle of the face, serves as centre of Markham's rotational integration; (b) Rotational integration picture of the given area of the virion. Angle of rotation, 120 °C. Regular network of connective elements between hexons of a triangular face is well discernible

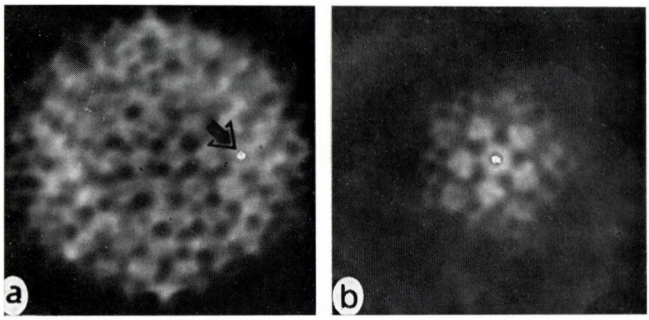

Fig. 4. (a) Penton serving as centre of Markham's rotational integration is shown by arrow on the electron micrograph of an adenovirus isolated from the rabbit; (b) Rotational integration picture of the penton and peripentonal hexons. Angle of rotation, 72°. The five peripentonal hexons surrounding the penton and the linking components connecting them to the penton are well visible. Other parts of the virion are blurred

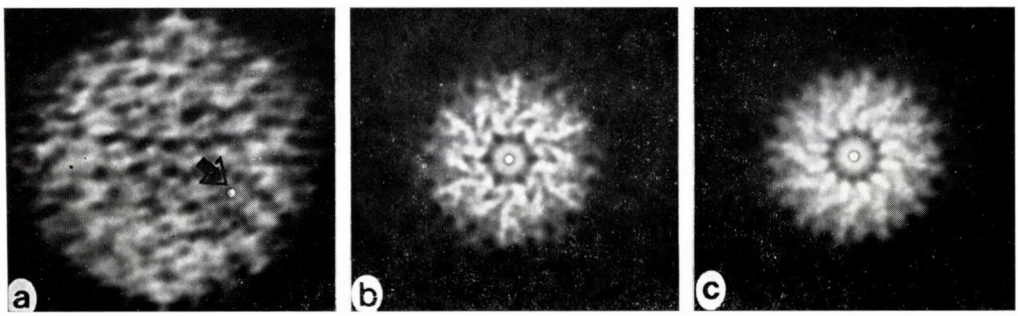

Fig. 5. (a) Hexon serving as centre of Markham's rotational integration is shown by arrow on the electron micrograph of a rabbit adenovirus; (b) Rotational integration picture of the hexon marked on the electron micrograph of the virion. Angle of rotation, 60°. The central hexon is linked to the six neighbouring hexons by six pairs of approximately parallel connective elements; (c) Rotational integration picture of the hexon marked on the virion. Angle of rotation, 120°. Twelve connective elements are discernible between the central hexon and its six neighbouring hexons. The profile of the central hexon and of the neighbouring hexons and other parts of the virion are blurred owing to disturbed symmetry

hexons are detectable and the five-fold symmetry of the vertex capsomer is well discernible.

Direct analysis of the electron micrographs revealed virions which indicated that hexons are linked to their neighbours by not one but two connective elements. This has been confirmed by the micrographs prepared at a 60° angle of rotation of the hexon marked on the virion of Fig. 5/a. This showed that the central hexon is connected to its six nearest neighbouring hexons by six pairs of approximately parallel connections i.e. by 12 elements (Fig. 5/b). As there are 12 periodically repeating elements, the study of the same hexon at a rotational angle of 30° displayed even more clearly the existence of 12 linking components between the given hexon and its six nearest neighbours (Fig. 5/c).

Discussion

Adenovirus hexons have hexagonal basal and middle part whereas their end facing the surface of the virion capsid is approximately triangular in shape [5, 10–12]. The hexagonal lower parts ensure a construction according to a closed hexagonal packing for the capsomers in the virus capsid. The part of the hexon facing the virion surface is narrower than the others, the capsomers appear to be separated by a distance. Slits have been demonstrated between the capsomers of other viruses, too [13]. In the case of adenoviruses it appears that owing to the close packing of the lower hexagonal parts these slits are very narrow and only slightly penetrable towards the inner part of the virion [14]. As the hexons become narrower towards the virion surface the slits become wider. Owing to the connective elements bridging the slits, they appear as holes in the virus capsid (Figs 1/a, 2/a, 4/a).

These connective elements form a network between the hexons all over the virion and are detectable also between the penton and its surrounding hexons. With Markham's rotational integration technique the linking structures were found to be double fibres similar to the interhexonal elements seen in two-dimensional crystalline arrays [6]. If the connective elements show the form of one thick fibre the phenomenon may be due either to the adhesion of two thin fibres along the longitudinal axis or to the uneven distribution of the negative stain. Thus, the hexon capsomers in the virus capsid are most likely also linked by six pairs of approximately parallel connective elements to their six nearest neighbours similarly as in the structure of the two-dimensional crystalline array. In experiments with 12 exposures at a 30° rotational angle the image improves owing to the periodically repeated elements and this fact confirms not only the existence of the 12 connective elements but also the approximately symmetric location of the 12 connective elements between a given hexon and its six neighbours.

REFERENCES

1. Wigand, R., Bartha, A., Dreizin, R. S., Esche, H., Ginsberg, H. S., Green, M., Hierholzer. J. C., Kalter, S. S., McFerran, J. B., Petterson, U., Russel, W. C., Wadell, G.: Intervirology **18,** 169 (1982).
2. Matthews, R. E. F.: Intervirology **17,** 59 (1982).
3. Nász, I., Ádám, É.: Orvostudomány **28,** 383 (1977).
4. Ádám, É., Nász, I.: Intervirology **13,** 1 (1980).
5. Ádám, É., Nász, I.: Intervirology **15,** 37 (1981).
6. Ádám, É., Nász, I.: Virology **114,** 265 (1981).
7. Bodon, L., Prohászka, L., Ádám, É., Nász, I.: Acta Vet Acad Sci Hung **27,** 73 (1979).
8. Yabe, Y., Sadakana, H., Isono, H.: Virology **96,** 547 (1979).
9. Markham, R., Frey, S., Hills, G. J.: Virology **20,** 88 (1963).
10. Nermut, M. V., Perkins, W. J.: Micron **10,** 247 (1979).
11. Burnett, R. M., Grütter, M. G., Markovic, Z., White, J. L.: J Supramol Struct Suppl **3,** 92 (1979).

12. Burnett, R. M., Grütter, M. G., Markovic, Z., White, J. L.: In Balaban, M., Sussman, J. L., Traub, W., Yonath, A. (eds): Structural aspects of recognition and assembly in biological macromolecules. Balaban International Science Services, Rehovot and Philadelphia 1981.
13. Howatson, A. F.: J Mol Biol **13,** 959 (1965).
14. Nermut, M. V.: Arch Virol **64,** 175 (1980).

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COMPARISON OF PASSIVE HAEMOGGLUTINATION AND RADIAL IMMUNODIFFUSION FOR C-REACTIVE PROTEIN MEASUREMENT

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(Received February 8, 1983)

Passive haemagglutination technique for C-reactive protein measurement using a commercially available C-polysaccharide and single radial immunodiffusion showed a close correlation. The haemagglutination method is very convenient to both routine clinical laboratories and experimental research.

C-reactive protein (CRP) was the first acute phase reactant discovered in human serum, increasing rapidly in amount during bacterial infection, in inflammation and tissue injury. CRP determinations are of significance mainly to estimate the activity of rheumatic disease, for the evaluation of therapy, and the diagnosis of myocardial infarction [1–4].

Determination of CRP is based on its specific reaction with antiserum or on the specific Ca^{++}-dependent binding with C-polysaccharide. The widely used methods are precipitation, radial immunodiffusion and latex agglutination. A passive haemagglutination technique using pneumococcal C-polysaccharide-coated sheep red blood cells was also developed [5]. Since each of these assays have advantages, we have compared these methods of measurement.

Materials and methods. Fifty four serum samples from 23 heterogeneous surgical cases were tested before operation and on the first and fifth day after surgery.

Single radial immunodiffusion (RID) was performed in 1% agar gel containing 0.5% anti-human CRP rabbit serum (Behringwerke). The sample volume was 8 μl, and 0.05 M sodium barbital/barbituric acid buffer pH 8.6 was used. After loading, the plates were left at room temperature for 48 h, washed with saline, dried and stained with Coomassie Brilliant Blue. The standard human serum was kindly provided by Dr. M. B. Pepys (Royal Postgraduate Medical School, London). CRP content of the standard serum was 104 μg/ml. For evaluation linear standard curves were made from each plate

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plotting the squares of the diameters of the precipitate rings as a function of the concentration. Each serum was filled in three wells and the average of the precipitate rings being in the range of the standard curve was considered.

Passive haemagglutination (HA) was carried out with sensitized sheep red blood cells (SRBC). Freeze-dried C-polysaccharide (Institute Human for Serobacteriological Production and Research, Hungary) was dissolved in

Table I

CRP Concentrations determined by radial immunodiffusion (RID) and passive haemagglutination (HA)

Patients	CRP concentration					
	before surgery		after surgery			
			first day		fifth day	
	RID μg/ml	HA titre	RID μg/ml	HA titre	RID μg/ml	HA titre
1	2	96	120	768	108	512
2	2	24	ND	ND	61	256
3	4	48	49	768	10	128
4	5	64	45	512	12	128
5	3	48	43	512	12	96
6	2	64	27	192	32	256
7	ND	ND	ND	ND	25	128
8	21	128	68	512	18	128
9	ND	ND	106	1024	80	512
10	5	64	21	256	50	512
11	ND	ND	10	64	6	64
12	ND	ND	54	384	ND	ND
13	12	128	57	512	64	384
14	ND	ND	43	384	30	128
15	2	48	78	512	94	512
16	2	16	52	512	40	384
17	5	16	62	1024	42	512
18	6	64	66	512	25	384
19	3	64	82	1024	33	256
20	35	256	ND	ND	98	768
21	ND	ND	14	128	ND	ND
22	ND	ND	17	192	ND	ND
23*	—	—	112	1024	—	—

ND: not done
* Reference serum

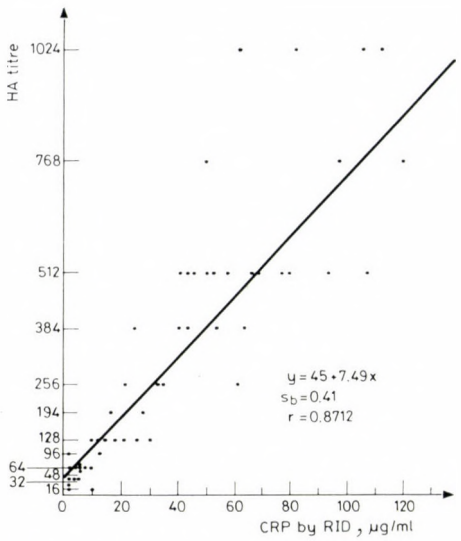

Fig. 1. Results of haemagglutination (HA) and radial immunodiffusion (RID) tests. s_b = standard deviation of the slope; r = correlation coefficient

saline (10 mg in 2 ml) and 100 µl washed packed sheep red blood cells (SRBC) were added. The suspension was incubated at 37 °C for 2 h. After three washings a 1% SBRC suspension in saline was prepared.

The sera to be tested were heat-inactivated at 56 °C for 30 min, and diluted serially in U bottom microtitre plates in two rows, beginning with 1 : 2 and 1 : 3 dilutions. All dilutions were made in saline in 50 µl volume. Fifty µl C-polysaccharide-coated 1% SRBC was added to each well. The plates were incubated at 37 °C for one hour. Sera agglutinating above 100-fold dilution (titres of or above 1 : 128) were considered pathological. Analysis of regression was used for statistical evaluation.

Results and discussion. The 13 sera containing less then 10 µg/ml CRP, the upper limit of the normal range [6] according to the RID, agglutinated in less than 96-fold dilution, 100-fold being the highest dilution at which normal sera were agglutinated [5]. Two sera with 10 µg/ml CRP gave titres of 1 : 64 and 1 : 128, while one serum with 12 µg/ml CRP did not reach the titre of 1 : 128. All the remaining samples showed elevated CRP levels as determined by both methods. There was a linear correlation between the values (regression coefficient, $r = 0.8712$, $p < 0.01$) (Fig. 1). The CRP titre in the standard human serum was between 1 : 512 and 1 : 768 (104 µg/ml). The reproducibility of the measurement was within one dilution step.

The CRP level of 54 samples measured by both techniques is shown in Table I. With both methods, the low blood CRP level before surgery, the high

concentration on the first day and the decrease by the fifth day after surgery are clearly shown.

Results obtained by HA correlated well with the RID values, therefore HA can be used as an alternative method of serum CRP assay. For more exact measurements, radioelectro-immunoassay [7], fluorescent immunoassay [7, 8], nephelometric assay [9], enzyme immunoassay [10] and radio-immunoassay [11] have been introduced.

In clinical practice, however, the exact knowledge of low levels has no significance. Besides, the HA test is a simple procedure which gives results in 4–5 h in contrast to the days required by RID. HA can be used for CRP determination from animal sera as well, without any species specific anti-CRP serum.

REFERENCES

1. Pereira Da Silva, J. A., Elkon, K. B., Hughes, G. R. V., Dick, R. F., Pepys, M. B.: Arthr and Rheum **23,** 770 (1980).
2. Becker, G. J., Waldburger, M., Hughes, G. R. V., Pepys, M. B.: Ann Rheum Dis **39,** 50 (1980)
3. Russell Jones, R.: J Roy Soc Med **73,** 143 (1980).
4. Kushner, I., Broder, M. R., Karp, D.: J Clin Invest **61,** 235 (1978).
5. Gál, K., Miltényi, M.: Schweiz Med Wochenschr **88,** 310 (1958).
6. Pepys, M. B.: Lancet **1,** 653 (1981).
7. Kindmark, C. O.: Scand J Clin Invest **29,** 407 (1972).
8. Anne, L., Eimstad, W., Bellett, N., Fisher, C.: Clin Chem **27,** 1075 (1981).
9. Van Lente, F., Marchand, A., Galen, R. S.: Clin Chem **27,** 1075 (1981).
10. Gushaw, J. B., Briscoe, R., Eimstad, W. M., Chang, C., Geenwood, H. M.: Clin Chem **27,** 1075 (1981).
11. Claus, D. R., Osmand, A. P., Rewurz, H.: J Lab Clin Med **87,** 120 (1976).

ERRATUM

DNA-METHYLATING ACTIVITY OF MYCOBACTERIA

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

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DECREASED PERMEABILITY OF GLYCEROL IN AN ERGOSTEROL-LESS MUTANT OF *CANDIDA ALBICANS*

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(Received September 10, 1982)

In a comparative study, an ergosterol-less nystatin-resistant mutant of *Candida albicans* and its ergosterol-producing nystatin-sensitive parental strain were investigated. The sterol mutant showed a more significantly decreased growth yield, respiration and glycerol-uptake activity than the parental strain as the consequence of its altered plasma membrane lipid composition.

As the end-product of sterol biosynthesis in yeasts, ergosterol plays an important role in the maintenance of the structure and function of the plasma membrane [1]. Sterol mutants of *Candida albicans*, most of them ergosterol-less [2], have been isolated and showed the following main characteristics in comparison to their parental, ergosterol-producing strains. (i) They exhibited altered phospholipid compositions [3]. (ii) The absence of ergosterol did not result in alteration of the ultrastructure of the plasma membrane as revealed by freeze-etch electron microscopy [4]. (iii) The specific activity of membrane-bound chitin synthetase (EC 2.4.1.16) of two ergosterol-less mutants was significantly increased [5]. (iv) They had increased plasma membrane order parameters and higher phase transition temperatures as measured by intercalated fatty acid spin probe with 5-doxylstearic acid [6]. (v) One of them showed a two-times higher spontaneous ion leakage in distilled water, as measure by conductometrically [2].

These data demonstrating basic alterations in the plasma membrane functions of the sterol mutants, have prompted us to study their solute uptake processes. Glycerol, which penetrates into yeast cells by simple physical diffusion [7], was the first carbon source examined.

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Material and methods

Strains and culture conditions. A detailed description and the characteristics of sterol mutants of *C. albicans* following nitrosoguanidine treatment were published earlier [2]. In these experiments an ergosterol-producing adenine-requiring strain, designated 33 erg$^+$ (ATCC 44 829), and its ergosterol-less progeny, designated erg-2 (ATCC 44 830), were used. Growth and harvesting conditions to obtain a mass of log-phase cells have been reported previously [5]. Assimilation of gylcerol on minimal medium (MM) is described elsewhere [2]. The growth of cultures shaken at 200 rpm in liquid MM media containing various glycerol concentrations was followed photometrically at 660 nm.

Measurement of respiration. The oxygen-uptake of glycerol-grown yeasts following one-hour starvation in Sörensen phosphate buffer pH 6.8 was measured in Warburg vessels containing glucose-free liquid MM supplemented with glycerol of different concentrations [7].

Uptake of glycerol. Glycerol-uptake activity was determined by adding 0.925 KBq of (2-^{3}H) glycerol (7.4 MBq/μmol) (25 μl) to 1 ml of starved cultures as described by Gancedo et al. [7], using Packard–Tricarb 2650 liquid scientillator. Glycerol was purchased from BDH Co. Ltd., Poole, England, radioactive (2-^{3}H) glycerol from Radiochemicals, Amersham, England.

Results and discussion

Glycerol can be utilized as a source of carbon and energy in yeasts [7] In preliminary experiments it was found that the ergosterol-producing 33 erg$^+$ strain of *C. albicans* could grow on the surface of a medium containing 1.1 mM of glycerol, while its ergosterol-less *erg*-2 and other mutants did not multiply at all on this medium [2]. Figure 1 shows the growth curves of these two strains in the presence of different concentrations of glycerol: these support the former observation that a concentration of glycerol about ten times higher is required to obtain the same yield for the *erg*-2 mutant as for the 33 erg$^+$ strain. No significant difference was, however, found between the two strains in the assimilation and growth yield of glucose at different concentrations (0.56 mM–56 mM).

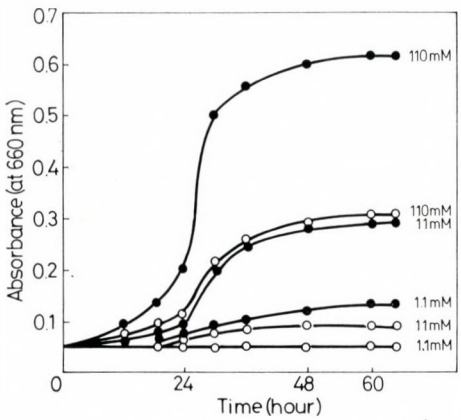

Fig. 1. Effect of different concentrations of glycerol on growth of *C. albicans* ergosterol-producing 33 erg$^+$ strain (●——●) and its ergosterol-less erg-2 mutant (○——○)

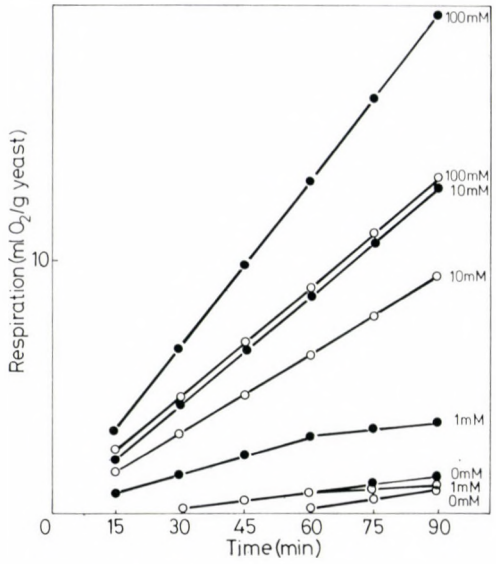

Fig. 2. Respiration of glycerol by *C. albicans* 33 erg$^+$ strain (●——●) and its erg-2 mutant (○——○). Oxygen uptake was measured in Warburg vessels at 30 °C. The initial concentrations of glycerol are indicated in mM

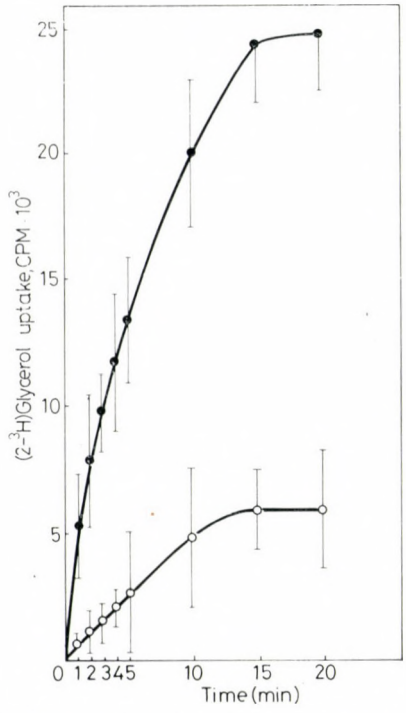

Fig. 3. Glycerol uptake of *C. albicans* 33 erg$^+$ strain (●——●) and its erg-2 mutant (○——○). Samples were withdrawn at the time indicated

In contrast to the result of Gancedo et al. [7] concerning the respiratory activity of *Candida utilis*, the *C. albicans 33 erg*$^+$ strain cannot use glycerol at very low concentration (1 mM) at the same initial rate as at higher concentration (100 mM, Fig. 2). At the same time, the erg-2 mutant required a higher glycerol concentration to produce the same respiratory activity as that of the 33 erg$^+$ strain. These results suggested that the entrance efficiency of glycerol in the ergosterol-less erg-2 mutant at low concentration is so small that it does not suffice for multiplication of the cells, and at higher concentration it is enough only for a decreased growth yield.

The uptake of glycerol was measured in both strains, as shown in Fig 3. The amount of glycerol taken up by the 33 erg$^+$ strain was about five times that of the erg-2 mutant, expounding the former results. These observations are in good agreement with the idea of Gancedo et al. [7] who explained the difference between the glycerol uptakes of *C. utilis* and *Saccharomyces cerevisiae* by essential differences in the plasma membrane between these two yeast species. Similar findings were previously reported for the permeation of glycerol and other non-electrolytes into liposomes prepared from a variety of synthetic and natural phospholipids [8] which showed a decreased glycerol permeation when the membrane lipids were converted to the gel state.

Our results may be interpreted as follows. In the erg-2 mutant, the absence of ergosterol and thus the accumulation of zymosterol and fecosterol [3] and significant alterations in the phospoholipid content [5] resulted in a decreased uptake of glycerol at each examined concentration at 30 °C. An about ten times higher concentration of glycerol in the solute was required to obtain the same growth rate, assimilation and respiration activity as for the parental 33 erg$^+$ strain. The phenomenon seems to be a consequence of the differences in plasma membrane composition and rigidity of the two strains.

Acknowledgement. We thank Miss Gizella Altordai for technical assistance.

REFERENCES

1. Parks, L. W.: CRC Crit Rev Microbiol **6,** 301 (1978).
2. Pesti, M., Paku, S., Novák, E. K.: Acta Microbiol Acad Sci Hung **29,** 55 (1982).
3. Novák, E. K., Paku, S., Zala, J., Horváth, I., Vigh, L., Pesti, M.: Proc 21st Hungarian Meeting of Biochemist, Veszprém 1981. p. 221.
4. Pesti, M., Novák, E. K., Ferenczy, L., Svoboda, A.: Sabouraudia **19,** 17 (1981).
5. Pesti, M., Campbell, J. McA., Peberdy, J. F.: Curr Microbiol **5,** 187 (1981).
6. Horváth, L. I., Pesti, M.: Proc 21st Hungarian Meeting of Biochemists, Veszprém 1981. p. 249.
7. Gancedo, C., Gancedo, J. M., Sols, A.: Eur J Biochem **5,** 165 (1968).
8. Demel, R. A., Bruckdorfer, K. R., Van Deenen, L. L. M.: Biochem Biophys Acta **225,** 321 (1972).

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ISOLATION OF *CHLAMYDIA TRACHOMATIS* FROM URETHRAL SCRAPINGS OF MEN

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Two strains of *Chlamydia* were isolated in McCoy cell cultures and hens' yolk sacs from urethral scrapings of men suffering from "nonspecific" urethritis. Their identification as *Chlamydia trachomatis* was based on cytoplasmic inclusions staining with iodine and on indirect immunofluorescence with anti-LGV serum. Both tests were performed in McCoy cells.

In the last two decades, many authors have shown that *Chlamydia trachomatis* is the commonest aetiological agent of non-gonococcal urethritis; the organism was detected in 35–50% of the patients tested [1]. A team of American researchers examined 1600 cases including men with "nonspecific" urethritis and women with cervicitis. *Neisseria gonorrhoeae*, *Candida*, *Herpesvirus*, *Trichomonas* and *Chlamydia* strains were isolated and among these *Chlamydia* had the highest incidence. It has therefore been assumed that *Chlamydia* infection is the most frequent venereal disease of man [2].

Isolation of *C. trachomatis* from human urethra has not been reported from Slovakia so far. Kazár's [3] report suggesting that nonspecific urethritis may be a manifestation of chlamydial infection did not arouse interest in this country. Detection of chlamydia-like microorganisms in urethral scrapings has suggested that *Chlamydia* may contribute to the pathogenesis of nonspecific urethritis in Slovakia as well [4].

Reports from abroad on the importance of chlamydial infection of the human urogenital organs and the frequent observation of "nonspecific" urethritis in men have prompted us to devote more attention to *Chlamydia* as a possible pathogenic agent.

Materials and methods

Patients. Thirteen men aged 22 to 34 years were examined for "nonspecific" urethritis. In 6 patients oozing of a little mucous discharge from the urethra was observed before urination in the morning. The patients denied having veneral disease in the preceding six months and stated that they had not undergone wide-spectrum antibiotic therapy. Their urethral discharges

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were tested for *N. gonorrhoeae*, *Trichomonas* and *Mycoplasma* before the isolation of *Chlamydia* was attempted.

Sampling. Smears were prepared on glass slides from the mucous discharge from the urethral orifice before urination in the morning. The smears were dried, fixed in Bunsen flame, stained according to Giménez or Giemsa and examined under the microscope. At the same time, samples were taken from the urethra with nasopharyngeal cotton-wool swabs. The swab was introduced into the urethra to a depth of 3–4 cm, it was rotated carefully to avoid mucosal bleeding. After preparing impression slides, the swabs were immersed in growth medium for *Mycoplasma* and shaken. Discharge for isolation of *Chlamydia* was taken from the urethra with a blunt curette, without anaesthesia. Bleeding was avoided. The mucosal scrapings were stored in collecting medium.

Media and cell cultures. The growth medium for McCoy cells consisted of Eagle's minimum essential medium (MEM), 88%; inactivated fetal calf serum, 10%; glutamine, 1%; 100-fold concentrated vitamins for tissue culture, 1%. Ten μg gentamicin, 100μg vancomycin and 4 μg amphotericin were added per 1 ml medium. The same medium was used for suspending urethral discharge, except that it did not contain glutamine and vitamins.

Preparation of scrapings for isolation of Chlamydia. The urethral scrapings to be inoculated into the yolk sac were suspended in 2 ml PBS of pH 7.1, containing 0.4 mol/litre sucrose [5], and 50 μg vancomycin and 100 IU of streptomycin per ml. The scrapings to be inoculated into McCoy cell culture were suspended in 4 ml of the collecting medium.

Both suspensions were kept at room temperature for 10 h, then at —20 °C overnight. On the next day, the suspensions were inoculated into the yolk sac of 7-day-old embryos and cell cultures, respectively. The McCoy cells were cultivated for 3 or 4 days at 37 °C in 50 ml flasks, each containing 6×10^5 cells in 8 ml growth medium.

Isolation of Chlamydia. One ml cell suspension containing about 1×10^5 cells was measured into flat-bottom test-tubes and a coverslip was placed on the bottom of each tube. The cultures thus prepared were incubated at 37 °C for 48 h. Then the growth medium was exchanged for the collecting medium that contained the dispersed urethral scrapings. After centrifugation at 27 °C for one hour at 2700 g, the cell cultures were incubated at 35 °C for 48 or 72 h.

Suspensions of urethral scrapings were injected into the yolk sac of 6 seven-day-old chick embryos, each 0.3 ml per yolk sac. The eggs were candled twice a day. The embryos that died by the 72nd h following inoculation were discarded. From the yolk sacs of the embryos that died later, smears were prepared on slides and stained according to Giménez [6]. The smears were examined for elementary bodies at × 500 magnification.

To demonstrate chlamydial inclusions, smears prepared from cell cultures 36 to 72 h after inoculation were stained according to Giménez or treated with iodine according to Rice [7].

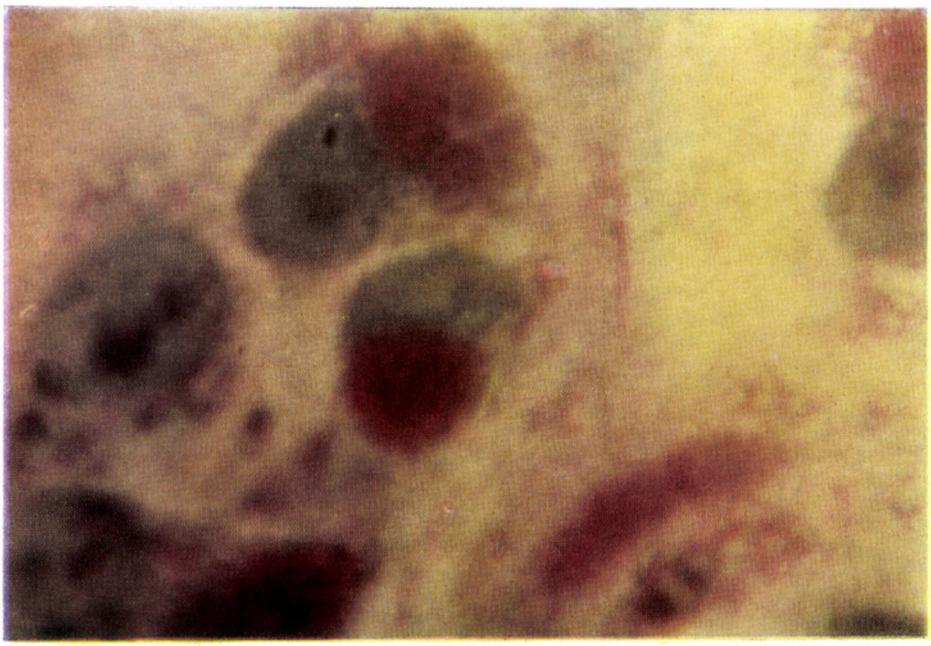
Indirect immunofluorescence with anti-LGV rabbit serum was also used for detection of inclusions.

Results

The urethral samples obtained from six patients proved to be negative for *N. gonorrhoeae*, *Mycoplasma*, and *Trichomonas* microscopically; those from two further patients were unsuitable for isolation of *Chlamydia* because of the great density of Gram-positive cocci and corynebacteria.

In four stained urethral samples, red dust-like micro-organisms were seen in the cytoplasm of epithelial cells. The limit of their visibility was at the magnification of ×500. In the smears obtained from two of the four patients, 20 to 30 polymorphonuclear leucocytes were counted in several fields of vision.

From the samples obtained from the four patients whose microscopic preparations were positive for elementary bodies, two strains of *Chlamydia* were isolated in both McCoy cells and yolk sacs. The strains were designated V. B. and Cz_1. Of the two samples in which polymorphonuclear leucocytes were present, only one yielded *Chlamydia*.



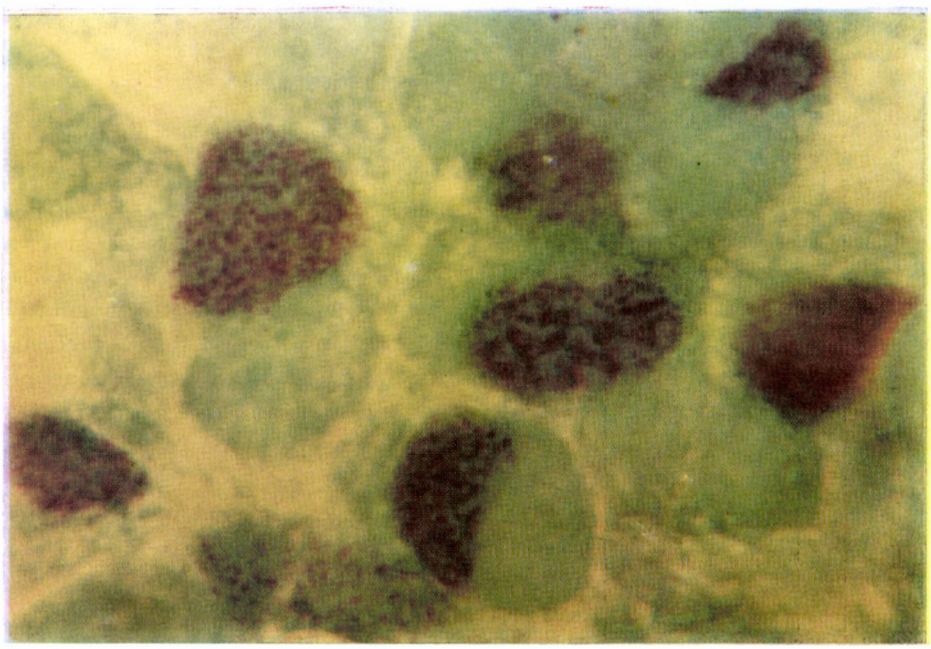


Fig. 1. Chlamydia inclusions in McCoy cells. Giménez stain

Fig. 2. Chlamydia elementary bodies in inclusions. Giménez stain

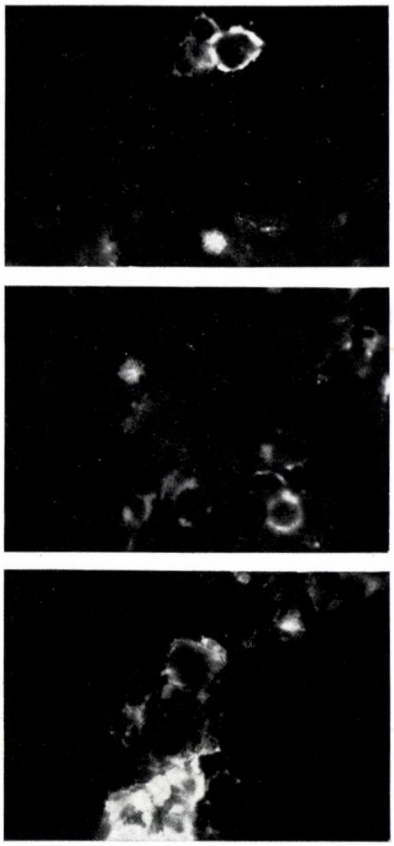


Fig. 3. Chlamydia inclusions in the cytoplasm of McCoy cells. Indirect immunofluorescence

Chlamydial inclusions were demonstrated microscopically in certain cultured cells stained according to Giménez or Giemsa, 35 h after inoculation. In the following 36 h period, the inclusions grew in number, to 4–7 inclusions in some of the cells (Fig. 1). In the smears stained according to Giménez, the inclusions, which varied in size and shape, appeared red, in striking contrast with the pale blue cytoplasm. Numerous elementary bodies were clearly visible in certain inclusions (Fig. 2).

The glycogen matrix of the iodine-treated chlamydial inclusion bodies stained dark (brown) in contrast to the pale yellow cytoplasm.

Indirect IF appeared positive in the inclusions (Fig. 3).

Both strains were isolated in yolk sacs, too. When dispersed positive yolk sacs diluted to 10^{-3} were inoculated into cell cultures, on the average 700 inclusions were counted in each coverslip culture after 4 days incubation.

The isolates have been classified into the species *C. trachomatis* on the basis of the positive iodine and IF reactions.

Discussion

In the last few years, chlamydial infection of the urogenital organs have been studied by many authors. Although veneral diseases seem to be commoner in industrially well-developed countries than elsewhere [1], no special attention has been paid to the occurrence and consequences of chlamydiosis.

In our first attempts to isolate *Chlamydia*, we selected patients with clinically obvious nonspecific urethritis (mucous urethral discharge) and patients with great masses of bacteria in their urethral discharge were excluded. In selecting patients for isolation of *Chlamydia*, we attribute a considerable importance to the observation reported by Munday et al. [8], viz., that the isolation was more successful from urethral samples containing polymorphonuclear leucocytes.

Of the cell cultures used for isolation of *Chlamydia*, McCoy cells appear to be the most suitable; they are 3 to 4 times as sensitive as the cells of the yolk sac [9].

In accordance with other investigators [1, 10], we have found that isolation of *C. psittaci* from placentas of aborting ewes [11] or cows [12] is much more laborious and time-consuming in yolk sacs than in cell cultures. For isolation from the male urethra, we used two substrates for comparative purposes. We wished to utilize the observation of Blackman et al. [13], who, using cells stored in the frozen state, attained better reproducibility with suspensions prepared from yolk sacs than with those prepared from cell cultures.

Among the methods used for staining inclusions, the iodine reaction seems to be suitable for the identification of *C. trachomatis* because only the inclusions produced by this species stain with iodine. A positive iodine reaction and positive IF with anti-LGV serum allow to differentiate *C. trachomatis* from *C. psittaci*.

In conclusion, we have isolated two strains of *C. trachomatis* in the yolk sacs of 7 day chick embryos and in McCoy cell cultures, from cases of "nonspecific" urethritis. We have proved the occurrence of this infection in Slovakia and call attention to it.

Acknowledgements. We are indebted to Drs A. Bartha and L. Varju (Research Institute for Veterinary Science of the Hungarian Academy of Sciences Budapest) for the McCoy cell line and for kind assistance in maintaining the line. We are grateful to Professor J. Schachter (WHO International Reference Centre for Trachoma and other Chlamydial Infections, University of California Medical Center, San Francisco, USA) for the rabbit anti-LGV serum and thank Mr. V. Fejfar for excellent technical assistance.

REFERENCES

1. Schachter, J., Caldwell, M.: Ann Rev Microbiol **34,** 285 (1980).
2. Schachter, J., Hanna, L., Hill, E. C., Massad, S., Shepard, C. W., Conte, J., Ir. Cohen, S. N., Meyer, K. F.: J Am Med Ass **12,** 1252 (1975).

3. Kazár, J.: Lek Obzor **11,** 617 (1976).
4. Hlavatý, P., Kazár, J., Farkas, J.: Bratisl Lek Listy **67,** 9 (1977).
5. Weiss, E., Dressler, H. R.: (1962). cit. Storz, J.: Chlamydia and Chlamydia-induced Diseases. Springfield, III. USA, Thomas Publ. 1971. p. 228.
6. Giménez, D. F.: Stain Technology **39,** 135 (1964).
7. Rice, C. E.: Am J Ophthal **19,** 1 (1936).
8. Munday, P. E., Johnson, A. P., Thomas, B. J., Taylor-Robinson, D.: J Clin Pathol **33,** 177 (1980).
9. Gordon, F. B., Harper, I. A., Quan, A. L., Treharne, J. S., Dwyer, R. S. C., Garland, I. A.: J Infect Dis **120,** 451 (1969).
10. Storz, J.: Chlamydia and Chlamydia-induced Diseases. Springfield, III. USA, Thomas Publ. 1971. Pp 79–81.
11. Sádecký, E.: JHEMI **22,** 127 (1978).
12. Sádecký, E.: JHEMI **25,** 51 (1981).
13. Blackman, H. J., Yoneda, C., Chandler, R. D., Schachter, J.: Am Soc for Microbiology **12,** 673 (1977).

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IMMUNOSEROLOGY OF *PSEUDOMONAS AERUGINOSA* INFECTIONS IN MAN

I. FOUR TYPES OF INTERACTION BETWEEN HOST AND BACTERIUM

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Continuous survey of clinical symptoms, bacteriological findings and anti-LPS antibodies in 39 acute and 9 chronic patients at a respiratory department revealed four interaction-types between *Pseudomonas aeruginosa* and host: I, clinical complications with a serological response; II, the same without serological answer; III, rise of specific antibodies without clinical symptoms; and IV, no clinical or serological reaction despite the presence of *P. aeruginosa*. Exogenous factors like massiveness or mode of infection (e.g. instrumental) determined mainly the type of interaction in the absence of immune-antibodies. *P. aeruginosa* colonization longer than a few days turned generally into manifest or subclinical infection. The lack of antibody production in severe infection was likely a consequence of an immune-paralysis, elicited by a massive infection. Antibody production was lower in subclinical than in manifest infection, yet IgG-type antibodies increased not only in the latter, but always in the former, too.

The toxic effect of lipopolysaccharides (LPS) and exotoxin A are the two main lethal factors in *Pseudomonas aeruginosa* infection [1–3]. Haemagglutination, precipitation, opsonization and the serogroup-specific protective effect are based on the presence of anti-LPS antibodies (Ab) [4, 5]. Thus anti-LPS Ab-s may play a decisive role in the development and outcome of *P. aeruginosa* infections. Pseudomonas colonization and infection in man, however, do not elicit uniform reactions, especially not in Ab production. To characterize the various types of these reactions, clinical symptoms and anti-LPS titres were consecutively followed up in patients with *P. aeruginosa* infection in a respiratory intensive care unit.

Patients and methods

Acute cases. In the acute respiratory intensive care unit of László Hospital for Infectious Diseases in Budapest, 39 patients (17 males and 22 females) were examined during their whole hospital stay. Their age varied from 14 to 76 (average, 47.6) years. The underlying diseases were tetanus in 19, meningo-myelitis in 7, polyradiculitis in 6, myasthenia gravis in 5 patients, subarachnoidal haemorrhage and cerebral embolism in one case each. The illness was considered mild (transient clinical symptoms without respiratory insufficiency) in 4, moderate (expressed, slowly improving clinical symptoms and transitory respiratory insufficiency) in 7, severe (life-threatening situation, with several days of artificial respiration) in 20 cases and the underlying illness was the primary cause of death in 8 patients. Tracheotomy or intubation followed by artificial respiration lasting for a minimum of 24 hours was performed in 34 patients.

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Chronic cases. The 9 chronic patients (average age, 35.5 years) studied during two and a half months had respiratory insufficiency which made tracheotomy and artificial respiration necessary. The underlying diseases were myasthenia gravis in 3, amyotrophic lateral sclerosis in 2 patients, sequelae of encephalitis, apoplexy, anoxaemic cerebral laesion and recovery after tetanus in one case each. The illnesses were in a stationary phase except the slowly recovering tetanus.

Bacteriological surveillance. Samples were taken from all the patients every 3rd or 4th day, and during improvement from the acute cases every week. The axillary and inguinal area of the skin, nose, throat or trachea, ear, urine, feaces, pus and in septic patients the blood as well as the environment (instruments, taps, sinks, furnitures, etc.) were sampled and cultured by the usual methods. *P. aeruginosa* strains originating from the patients and environment were serogrouped on the basis of Lányi's revised antigenic scheme [6]. Cases were grouped according to the duration of the presence of *P. aeruginosa*. Single isolations were included in group "A". Colonizations in the range from four to ten days (two or more positive cultures) fromed group "B", and those over ten days, group "C".

Serological response. Blood samples were taken from the acute patients every 2nd–4th day during the acute phase of illness, and every 6th or 7th day during convalescence. Chronic patients were sampled every 3rd week. All sera from a given patient were stored at —20 °C and examined in an experiment using two double dilution series. Passive haemagglutination was performed with sheep erythrocytes sensitized by purified *P. aeruginosa* LPS [7]. Antibodies determined for 9–13 different serogroups occurring in the patient or environment, were regarded as the total antibody (TAb) titre. When sufficient amounts of sera were available from 26 acute and 8 chronic patients they were titrated also after 0.2 mol/litre 2-mercaptoethanol treatment to determine the level of IgG-type Ab-s. Electrophoretic serum fractions [8], the main IgG classes [9], titres of 9 natural Ab-s [10] and phytohaemagglutinin induced blast-transformation rate of lymphocytes [11] were determined in paralllel in the same blood samples.

Infections. General and local clinical symptoms accompanied by a positive bacteriological finding were considered a bacteriological complication. Severity grades of complications were categorized as follows: (1) mild: temperature below 38 °C, good general condition without intensive local symptoms, vast tissue destruction or considerable pus production; (2) moderate: expressed local clinical symptoms, temperature exceeding 38 °C; (3) severe: septic state, i.e. intermittent pyrexia, heavy prostration, rapidly developing anaemia, erythrocyte sedimentation rate about 100 mm/hour, with either negative or positive blood cultures—as only a minority of haemocultures yielded *P. aeruginosa* in consequence of vigorous, intravenous administration of antibiotics from the early onset of complications; (4) lethal: fatal sepsis.

When in a patient two different infectious complications were due to the same *P. aeruginosa* serogroup, only the more severe one was taken into consideration. Infectious complications caused simultaneously or successively by two distinct serogroups were considered separate infections. Two different but equally serious complications in the same patient each by itself contributing to death were categorized as two lethal infections. Out of nearly 200 patients only those were included whose categorization was unambiguous.

Accordingly, the serological answer in 66 pseudomonas infections of acute patients was evaluated (7 bronchitis, 29 pneumonia, 10 urinary tract infections, 8 purulent rhinitis by nasogastric tube, 7 thrombophlebitis, 2 enteritis, 1 infected bedsore, 1 injection-abscess and 1 peritonitis). Distribution by severity of the infection was: mild 12, moderate 21, severs 13 and fatal 20. In 9 other thrombophlebitis infections (2 mild, 3 moderate and 4 severe), when *P. aeruginosa* was cultured only from the skin at the site of the venous cannula, a significant rise in both TAb and IgG specific Ab-s confirmed the pseudomonas infection. These 9 infections were not evaluated together with the former 66 ones, when the duration of the presence of *P. aeruginosa* had to be considered.

The underlying disease of the acute patients culminated generally near admission: *P. aeruginosa* colonized them on the 1st–4th day of hospital stay; then the infections initiated, and culminated after 10–40 days of hospital care, when the underlying disease already improved, or when the patient completely recovered from it. For a few days 6 patients received corticosteroids in a low dose. Other immunosuppressive drugs were not administered. None of the patients had critical leukopenia ($\leq$ 1G/1; $\leq$ 1000/mm^3).

In the 9 chronic patients during the $2^1/_2$ months observation period the serological response to 4 mild bronchitis and to 2 mild tube-rhinitis could be evaluated.

Different *P. aeruginosa* serogroups colonized furthermore at 17 times the acute and at 26 times the chronic patients without any clinical symptoms. The serological reaction against these serogroups was also followed up.

In the acute patients on 32 and in the chronic cases on 15 occasions, another facultatively pathogenic bacterium (*Proteus*, *Klebsiella*, *Escherichia coli*, *Acinetobacter calcoaceticus*, *Sta-*

phylococcus aureus) accompanied the *P. aeruginosa* at the site of infection or colonization; in the remaining cases *P. aeruginosa* grew in pure culture. Further 17 infections in the acute and 5 in the chronic patients were caused by other facultatively pathogenic bacteria. In 15 additional infections (mainly thrombophlebitis) the causative agent remained unidentified. However, the pseudomonas infections dominated in all acute cases and bacteriological complications of other origin were only mild and transient.

Statistical analysis. Sera not reacting at 1 : 10 dilution were regarded as having a 1 : 5 titre to maintain them in calculating the geometrical mean of the different titre-groups.

The variation coefficient in per cent was calculated by the following formula applicable for geometrical dilution series:

$$\text{variation coefficient in per cent} = 100 \text{ num lg} \sqrt{\frac{N \Sigma (\lg x_i)^2 - (\Sigma \lg x_i)^2}{(N-1)(\Sigma \lg x_i)^2} N}$$

The difference (D) between two average values was expressed in dilution steps, using the equation $2n = a/b$, where a and b are the two averages.

In comparing two groups of titre-values, Wilcoxon's or Mann–Whitney's method was employed, as required by mathematical conditions. To compare two groups of data not belonging to geometrical series, the χ^2-test with Yates's correction was used. In the tables the correlation coefficient of two related groups of TAb and IgG titres with its degree of probability is also given.

Results

Acute patients

Types of interaction. Four types of interaction were distinguished between the patients and *P. aeruginosa.*

(I) Local and general clinical symptoms accompanied by a rise of homologous specific anti-LPS Ab-s (when *P. aeruginosa* grew in pure culture, TAb = four-fold, in mixed infections TAb + IgG each = four-fold, or when IgG was not determined, Tab alone, = 16-fold): manifest pseudomonas infection (complication or illness) with immune-reaction.

(II) Clinical symptoms without serological response (maximum one dilution step rise in TAb or IgG titre, as in all mixed infections IgG was measured): manifest infection with no specific humoral immune-reaction.

(III) Specific immune-answer (see interaction-type I) without clinical symptoms: subclinical (larvate, latent) infection.

(IV) Presence of *P. aeruginosa* without clinical and serological reaction (see interaction-type II): colonization.

Out of the 75 manifest *P. aeruginosa* infections evaluated 59 plus the 9 thrombophlebitis cases belonged to interaction-type I, and 7 to type II. Interaction-types III and IV were represented on 5 and 12 occasions, respectively. From the 39 acute patients 18 showed only type I, 4 only type II interaction. Four patients were only colonized (type IV). Two or more interaction-types were observed in parallel in the remaining 13 patients, as follows: I + II in 1, I + III in 4, I + IV in 6, I + II + IV in 1 and II + III in 1 patient.

Interaction-types and serogroups. All pseudomonas serogroups of the Lányi–Bergan scheme, except O1 and O15, occurred in the patients. Interaction

was due to O6 on 26, O11 on 22, O3 on 8, O2 on 8, O10 on 7, O4 on 7, R-strains (formerly Lányi O8 and O9) on 7, O9 on 5 occasions, and O7, O9, O12 on one occasion each. Accordingly, strains of serogroups O6 and O11 were present in

Table I

Interaction-type, sort and severity or site of infection
Acute patients

Sort of infection in I + II	Interaction-type										Site of interaction in III + IV
	I				II				III	IV	
	Mild	Moderate	Severe	Lethal	Mild	Moderate	Severe	Lethal			
Tracheobronchitis	2	4	1	—	—	—	—	—	—	7	Trachea
Pneumonia	1	7	5	10	—	1	1	4	—	—	
Urinary tract	1	3	3	3	—	—	—	—	—	—	
Thrombophlebitis**	3	5	7	2	—	—	—	—	—	—	
Others***	6	4	—	1	1	—	—	—	5	5	Skin, nose, ear
Total	13	23	16	16	1	1	1	4	5	12	

* I = Clinical symptoms with antibody production; II = clinical symptoms without antibody production; III = symptomless rise in antibody titre; IV = colonization: no clinical symptoms and no antibody production
** and injection abscess
*** Purulent rhinitis by nasogastric tube, enteritis, bedsore, peritonitis

Table II

*Interaction-type, severity of infection and duration of harbouring P. aeruginosa**
Acute patients

Severity of infection	Interaction-type**												I–IV, total			
	I			II			III			IV						
	a***	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a–c
No manifest infection	—	—	—	—	—	—	2	2	1	11	1	—	13	3	1	17
Mild	3	4	4	1	—	—	—	—	—	—	—	—	4	4	4	12
Moderate	1	12	7	—	—	1	—	—	—	—	—	—	1	12	8	21
Severe	—	2	10	—	1	—	—	—	—	—	—	—	—	3	10	13
Lethal	—	3	13	1	2	1	—	—	—	—	—	—	1	5	14	20
Total	4	21	34	2	3	2	2	2	1	11	1	—	19	27	37	83

* Without 9 thrombophlebitis, when duration of harbouring *P. aeruginosa* could not be stated
** See Table I
*** a = one positive culture; b = 4–10 days long harbouring; c $\geq$ 11 days long harbouring

half of the interactions. From the 40 *P. aeruginosa* strains found on instruments (catheters, cannulas, tubes etc.) 36 belonged to these two serogroups ($p < < 0.001$). These nosocomial serogroups did not, however, predominate significantly in the severe infections: 17 out of 38 mild and moderate complications and 23 out of 37 septic cases were caused by these two serogroups. The distribution of different serogroups in manifest (interaction-types I and II) and subclinical (type III) infections or in colonizations (type IV) also showed the same pattern (data not shown).

Interaction-type, kind of disease, severity of complication and site of colonization. It was characteristic that in the presence of *P. aeruginosa* some complication developed in 3/4th of the cases (types I and II; Table I).

Interaction-type II cases all but one (a mild enteritis) were severe pneumonias with fatal outcome in four patients. The underlying disease was tetanus in 3, meningoencephalomyelitis in 2 and myasthenia gravis in 1 patient; *P. aeruginosa* responsible for the complications belonged to six different serogroups. Death due to pneumonia occurred after 6–25 days of hospital care.

Interaction-type and duration of harbouring P. aeruginosa. Presence of *P. aeruginosa* for a short time (a single positive sample) was associated mostly with interaction-type IV; on the other hand, a colonization longer than three

Table III

Interaction-type and reciprocal homologous specific antibody titres in acute patients

TAb	Interaction-type							
	I				II			
	S_1	S_2	D	p	S_1	S_2	D	p
N	68	68			7	7		
$\bar{x}$	40.8	532.7	3.71	<0.001	24.4	32.8	0.43	NS
Vc	176.1	157.4			160.9	198.2		
p/II	=0.12	<0.001			—	—		
p/III	NS	NS			=0.06	<0.01		
p/IV	NS	<0.001			NS	NS		
IgG	S_1	S_2	D	p	S_1	S_2	D	p
N	58	58			3	3		
x	≤5	57.3	≥3.52	—	≤5	≤5	?	—
Vc	?	180.8			?	?		
p/III	?	NS			?	?		
D/TAb	≥3.03	3.22			≥2.29	2.71		
r		0.6005						
p/r		<0.001						

(Table III continued)

TAb	III				IV			
	S_1	S_2	D	p	S_1	S_2	D	p
N	5	5			12	12		
$\bar{x}$	69.6	367.6	2.40	=0.02	47.6	75.5	0.67	NS
Vc	163.3	125.4			250.4	214.3		
p/II	—	—			—	—		
p/III	—	—			—	—		
p/IV	NS	<0.05			—	—		
IgG	S_1	S_2	D	p	S_1	S_2	D	p
N	4	4			9	9		
$\bar{x}$	≤5	33.6	≥2.75		≤5	≤5	?	—
Vc	?	125.5			?	?		
p/III	—	—			?	?		
D/TAb	≥3.80	3.45			≥3.25	≥3.92		
r		0.6623						
p/r		NS						

Interaction-types: see Table I; TAb = total antibody (~ IgM); S_1 = first sample taken at admission; S_2 = the sample yielding highest antibody titre; D = difference of two mean titres expressed in dilution step; p = probability of difference between S_1 and S_2; N = number of samples; $\bar{x}$ = average of titres; Vc = variation coefficient in per cent; p/II, III, IV = level of difference as compared to group II, III, IV; D/TAb = = difference of TAb and IgG mean titres expressed in dilution step; r = correlation coefficient of related IgG and TAb titres; p/r = probability level of correlation; NS = not significant

days evoked in all cases except one an immune-reaction and/or clinical symptoms (interaction-types I, II or III; Table II). Long harbouring was found mainly in interaction-type I, parallel with the ongrowing severity of infections.

Interaction-type and Ab titre. Changes in the homologous specific Ab titres against all cultured serogroups were characterized by comparing the titre of the first sample (S_1) taken on admission (natural Ab) with the peak titre (S_2; immune-Ab) found in the patient (Table III).

TAb titre. In clinical complications (interaction-types I and II) the average in S_1 of Ab-s was lower than in latent infection (type III) or colonization (type IV). During the immunological reaction TAb titres augmented in type I to a 13 times, in type II only to a 5 times higher level.

IgG level. From the 74 S_1 only six yielded specific IgG Ab-s in the starting (1 : 10) dilution step. The increase of IgG Ab-s in types I and III was of the same magnitude as in the TAb level. In types I and III a strong correlation existed between TAb and IgG Ab titres.

Chronic patients

Serogroups. Except serogroup O15, all the others were found in the 9 bedridden patients, who harboured them during the whole observation period. Serogroup O6 and O11 strains formed only one fourth of all the cultured *P. aeruginosa.*

Interaction-type, severity of infection, site and duration of harbouring P. aeruginosa. In the chronic patients interaction-type III was not observed. All pseudomonas complications were mild. In contrast with the acute patients, *P. aeruginosa* caused illness only in a minority of the interactions, whereas in the great majority only colonization (type IV) occurred.

A given serogroup was present longer in the chronic patients: in half of the interactions harbouring lasted longer than 10 days (group "C").

Interaction-type and Ab titre. (i) *TAb level.* In type I the average titres of the S_1 corresponded to that of acute patients (Table IV). The rise during complications in the chronic patients was lower according to the mild character of their infections. In type IV, S_1 titres against all serogroups regardless of colonization were two times higher than in the acute patients.

(ii) *IgG titres.* Out of the 30 S_1 of the chronic patients, 25 contained IgG type Ab-s at least at a 1 : 10 dilution. In three of the remaining 5 cases, compli-

Table IV

Interaction-type and reciprocal homologous specific antibody titres in chronic patients

TAb	Interaction-type											
	I				II				IV			
	S_1	S_2	D	p	S_1	S_2	D	p	S_1	S_2	D	p
N	4	4			2	2			26	26		
x	47.6	226.3	2.25	=0.08	113.1	160.0	0.50	—	99.0	116.2	0.23	NS
Vc	186.0	146.2			127.0	0.0			145.9	144.8		
p/IV	=0.18	=0.20			—	—			—	—		
Ig G	S_1	S_2	D	p	S_1	S_2	D	p	S_1	S_2	D	p
N	4	4			2	2			24	24		
x	10.0	47.6	2.25	<0.05	14.1	28.3	1.0	—	31.8	50.9	0.68	NS
Vc	227.7	148.5			559.0	275.4			185.6	122.6		
p/IV	<0.05	NS			—	—			—	—		
D/TAb	2.25	2.25			3.0	2.50			1.64	1.19		
r	0.8704	0.7305			—	—			0.6135	0.5312		
p/r	NS	NS			—	—			<0.01	<0.01		

For abbreviations, see Table III

cations due to the homologous *P. aeruginosa* developed. The average Ab titre in S_1 in interaction-type I was only one third of the S_1 Ab level in type IV (Table IV). The Ab rise in type I corresponded to that of the TAb titres. In S_2 of the chronic patients the ratio IgG/TAb was 2–10 times higher than in the acute patients. There was a close correlation between the IgG and TAb titres in interaction-types I and IV in the chronic patients, too.

Discussion

In the majority of the cases, the underlying disease of our patients was non-fatal [12]. The immunological answer seemed to have been influenced considerably neither in these patients nor in those, who died of subarachnoidal bleeding, cerebral embolism, tetanic seizure or cerebral oedema.

Passive haemagglutination applied to determine the anti-LPS TAb level of the patients shows first of all IgM Ab-s [13–15]. As this method measured in our patients IgG titres 3–4 dilution steps lower than TAb, the latter represented practically IgM Ab-s. Therefore, no direct comparisons of IgG and TAb titres, but only changes in their level or their ratio were considered.

Several observations revealed the existence of a symptomless pseudomonas carrier state [16–18], which may [3, 19, 20] or may not [19, 20] be accompanied by a specific immune reaction. Even clinical symptoms are not always connected with a rise in specific Ab-s [21]. However, attempts have not been made to separate these distinct reaction-types, although their neglect may lead to false results and conclusions.

The parallel occurrence of two or three different interaction-types was observed in one-third of the patients. Thus, not the individual inclination of the immune-system, but exogenous factors like the virulence of *P. aeruginosa* or the massiveness of the infection may determine the interaction-type in man.

There was no proof of an undoubtedly higher virulence of the nosocomial strains. The higher percentage rate of the nosocomial serogroups in septic cases was the consequence of their high incidence on instruments employed frequently on these patients. The data presented support rather the opinion that the *P. aeruginosa* serogroups do not differ in pathogenicity [22, 23].

Acute patients exhibited mainly interaction-type I, in accordance with other observations [3, 16–18]. It may be supposed, however, that among extrahospital cases the ratio of interaction-types III and IV is higher [24].

In the two chronic patients with interaction-type II symptoms of viral infection (rhinitis, sore throat and bronchitis) accompanied the presence of *P. aeruginosa* in their trachea: thus, in all probability, only a pseudomonas colonization occurred.

Out of the 7 infections of interaction-type II in the acute patients one was a mild enteritis; it may be supposed that an infection of other aetiology

occurred which was accompanied only by pseudomonas colonization (interaction-type IV). On admission, the other 6 patients exhibited a normal Ab titre (natural Ab-s) against 9–12 different *P. aeruginosa* serogroups including those which caused infection later. Moreover, in 3 out of these patients an increase in titre against other pseudomonas serogroups responsible for type I or III infections was observed. The cause of the lack of a specific serological answer in these 6 infections may be assumed from the course of disease. The underlying diseases as well as the infective serogroups differed widely in these patients. All the 6, however, suffered from pneumonia, which developed soon after tracheotomy during artificial respiration and was characterized by fast and vast tissue destruction. It may thus supposed that these patients were exposed to an unusually massive infection which, as an extreme antigenic assault, may result in the depression of the serological response [25–27].

Both in acute and chronic cases pseudomonas illness developed mainly when the patient had a lower TAb (and a low or no IgG) level against the colonizing strain in the S_1 in contrast to that of types III and IV.

Analyzing the occurrence of interaction-types III and IV, corresponding to literary data [28, 29] we found a correlation in the acute cases between the colonization of *P. aeruginosa* and the general condition of the patient. During a mild underlying disease the pseudomonads failed to colonize; in moderate underlying disease only a transient colonization occurred (a single positive culture); whereas latent infection was observed only in severe or lethal underlying disease (data not shown).

When *P. aeruginosa* was present longer than 3 days in the acute patients, colonization usually turned into a subclinical or manifest infection. Accordingly, *P. aeruginosa* penetrated the mucocutaneous barrier generally in a few days.

In contrast, all chronic patients carried continually pseudomonads and lived in peaceful commensalism with them, in spite of their good general condition. The interactions belonged mainly to type IV; type III was not observed. This changed pattern of interaction-types in chronic patients as compared to acute ones may be connected with the elevated TAb titre and the presence of IgG Ab-s in 25 out of 30 chronic S_1; moreover, in type IV the average IgG level in S_1 corresponded to the mean titre found in S_2 of acute patients with interaction-type III. In S_2 of the chronic patients the ratio of IgG/TAb-s was about twice higher than in the S_2 of the acute patients. All these tally with the increased IgG fraction of the chronic patients [9], and refer to a prolonged antigen stimulus following a manifest or subclinical infection. Thus, the presence of immune-antibodies in the S_1 influences very profoundly the reaction to pseudomonas (re-)infection and the development of type of interaction in man.

The S_1 of acute patients still free of pseudomonas complications contained only natural antibodies (only TAb $\sim$ IgM) against *P. aeruginosa*. However,

6 out of 74 S_1 of the acute patients yielded IgG anti-LPS Ab-s, too. Hence, a recent *P. aeruginosa* infection is not verified by the mere presence of low titre IgG Ab-s [30], only their fourfold rise or rather the simultaneous increase of IgG and IgM Ab-s can prove it: in our patients, independently from clinical symptoms in each infection, the specific immune-reaction comprised an IgG answer, too.

The increase of Ab-s in type III was less than in type I. This seems to indicate a lower antigen-stimulus in symptomless cases.

Finally, the close correlation between TAb and IgG titres both in acute and chronic patients refers to a linkage between IgM and IgG pseudomonas anti-LPS Ab production in man.

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REFERENCES

1. Young, L. S.: J Infect Dis **142,** 626 (1980).
2. Pollack, M., Young, L. S.: J Clin Invest **63,** 276 (1979).
3. Cross, A. S., Sadoff, J. C., Iglewski, B. H., Sokol, P. A.: J Infect Dis **142,** 538 (1980)
4. Crowder, J. G., Devlin, H. B., Fisher, M., White, A.: J Lab Clin Med **83,** 853 (1974).
5. Young, L. S.: J Infect Dis **126,** 277 (1972).
6. Lányi, B., Bergan, T.: In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Vol 10, pp 93—168. Academic Press, London 1978.
7. Ádám, M. M., Kontrohr, T., Horváth, E.: Acta Microbiol Acad Sci Hung **18,** 307 (1971).
8. Petrás, Gy., Kiss, S., Juraszek, J., Merétey, K.: Acta Med Acad Sci Hung **35,** 249 (1978).
9. Petrás, Gy., Merétey, K.: Zentralbl Bakteriol [A] **245,** 96 (1979).
10. Petrás, Gy., Németh, Cs : Acta Microbiol Acad Sci Hung **26,** 265 (1979).
11. Petrás, Gy., Gallyas, A., Merétey, K.: Zentralbl Bakteriol [A] **248,** 374 (1980).
12. McCabe, W. R., Jackson, G. G.: Arch Intern Med **110,** 847 (1962).
13. Robbins, J. B., Kenny, K., Suter, E.: J Exp Med **122,** 385 (1965).
14. Benedict, A. A.: Nature **206,** 1368 (1965).
15. DelGuercio, P., Tolone, G., Andrade, F. B., Biozzi, G., Benaghi, R. A.: Immunology **16,** 361 (1969).
16. Morehead, C. D., Houck, P. W.: Am J Dis Child **124,** 564 (1972).
17. Baird, I. M., Slepack, J. M., Kauffman, C. A., Phair, J. P.: Antimicrob Agents Chemother **10,** 626 (1976).
18. Bruun, J. N., MacGarrity, G. J., Blakemore, W. S., Coriell, L. L.: J Clin Microbiol **3,** 264 (1976).
19. Klieneberger, C.: Z Immunitaetsforsch **2,** 686 (1909).
20. Saku, K.: J Jpn Assoc Infect Dis **49,** 358 (1975).
21. Crowder, J. G., White, A.: J Lab Clin Med **75,** 128 (1970).
22. Ansorg, R.: Med Microbiol Immunol **167,** 269 (1979).
23. Ullmann, U., Schmülling, R. M.: Zentralbl Bakteriol [A] **247,** 241 (1980).
24. Petrás, Gy., Bognár, Sz.: Acta Microbiol Acad Sci Hung **28,** 367 (1981).
25. Andersson, J., Sjöberg, O., Möller, G.: Transplant Rev **11,** 131 (1972).
26. Coutinho, A., Gronowicz, E., Bullock, W. W., Möller, G.: J Exp Med **139,** 74 (1974).
27. Waksman, B. H.: Clin Exp Immunol **28,** 363 (1977).
28. Johanson, W. G., Higuchi, J. H., Chaudhuri, T. R., Woods, D. E.: Ann Rev Resp Dis **121,** 55 (1980).
29. Ramphal, R., Small, P. M., Shands, J. W., Fischlschweiger, W., Small, P. A.: Infect Immun **27,** 614 (1980).
30. Shigeta, S., Yasunaga, Y., Ogata, M.: J Clin Microbiol **8,** 489 (1978).

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IMMUNOSEROLOGY OF *PSEUDOMONAS AERUGINOSA* INFECTIONS IN MAN

II. EFFECT OF NATURAL AND IMMUNE ANTI-LPS ANTIBODIES ON PSEUDOMONAS COLONIZATION AND INFECTION

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In 39 acute patients of a respiratory unit a comparatively high *Pseudomonas aeruginosa* anti-lipopolysaccharide antibody level present on admission prevented colonization by the homologous pseudomonas serogroup. At lower natural antibody titres symptomless colonization occurred, and in patients with the lowest initial titres, later *P. aeruginosa* complications developed. A low antibody level also predisposed to pseudomonas infection in 9 chronic patients. When colonization occurred at high antibody titres, the presence of *P. aeruginosa* was only transient; however, the titre had no effect on the further duration of harbouring *P. aeruginosa*. Anti-LPS antibodies may play an important role not only in the outcome of pseudomonas infection, but also in other respects of pseudomonas–man interaction.

In a previous paper [1] we have shown that in an acute respiratory ward the pseudomonas serogroups [2] O6 and O11 caused nosocomial infections, whereas in the chronic ward the patients in good general condition harboured *Pseudomonas aeruginosa* continually, and the incidence of serogroups O6 and O11 was lower.

Moderate titres of total (immune-) antibodies (TAb∼IgM) and IgG pseudomonas anti-LPS (immune-) antibodies (Ab) seemed to prevent the development of pseudomonas illness in chronic patients [1]. Differences in the anti-pseudomonas Ab titre in the first samples taken on admission (S_1; natural Ab-s) of acute patients who showed later different types of interaction with the microorganism [1] suggested that these Ab-s may also influence the development of pseudomonas invasion.

The present paper deals with the effectiveness of TAb-s and IgG Ab-s in protection against pseudomonas colonization and illness and with the influence of these Ab-s on the duration of harbouring *P. aeruginosa*.

Patients and methods

Data of the 39 acute and 9 chronic patients examined were described in detail in a previous paper [1]. The acute patients had 66 and the chronic patients had 6 clinical complications due to *P. aeruginosa* with (interaction-type I) or without (interaction-type II) a serological

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response. In each of these infections the duration of harbouring *P. aeruginosa* could be proven bacteriologically. In 9 further thrombophlebitis infections a significant rise in both TAb and IgG specific Ab-s verified the infection, but the endpoint of the presence of *P. aeruginosa* could not be determined. Subclinical infection (interaction-type III) or colonization (type IV) could be observed 17 times in the acute and 26 times in the chronic patients.

The duration of the presence of *P. aeruginosa* in interaction-types I–IV was expressed by the period of time between the first and last positive bacteriological cultures. Single isolations were classified in group "A", harbouring periods of 4 to 10 days in group "B", and group "C" consisted of cases when pseudomonas had been present for more than 10 days.

For comparison, *P. aeruginosa* anti-LPS Ab-s against 23 sero- and subgroups were determined in three groups of 50 healthy blood donors each. The methods and statistical analysis were the same as decribed formerly [1].

In evaluation the difference (D) of two titre averages was expressed in dilution steps, using the formula $2^D = a/b$, where D is the difference in dilution step, and a and b are the two mean titres.

Results

Healthy controls

As the homologous average titres in the three groups did not differ significantly, data for the 150 controls were evaluated together. TAb-s were present in the basic 1 : 10 dilution from 32.7% (R-strains: formerly Lányi's serogroup O9) to 100.0% (serogroup O11a, 11b) of the samples. Against certain sero- or subgroups (O2a, 2d, 2e; O4a, 4b,; O6a, 6d; O9a; O9a, 9b; O11a, 11b) titres of 1 : 320 were measured on 14 occasions. Mean titres for the sero- or subgroups differed widely. The lowest (1 : 6.91) was for R-strains (formerly Lányi's serogroup O9), the highest (1 : 45.54) for O11a, 11b, the D between them being 2.7.

IgG type Ab-s were measured in 50 controls. One sample yielded Ab-s in 1:80, one in 1 : 40 and four in 1 : 10 dilution. In these six positive samples the homologous TAb ($\sim$ IgM) titres were considerably higher than the average. In the majority of high TAb titres, however, homologous IgG Ab-s were undetectable even at the starting 1 : 10 dilution.

S_1 *Ab titre and the development of colonization or infection*

TAb titre

Acute cases. To highlight the protectivity of TAb-s, the data were arranged in three groups as follows: (1) titres against serogroups found in the near environment of the patients, but not colonizing them; (2) titres against serogroups causing later manifest infection (interaction-types I and II); (3) titres for serogroups colonizing the patients without giving rise to clinical symptoms (types III and IV). Titre values of the two nosocomial (O6, O11) and the other sero- or subgroups were evaluated separately in each group. Mean titres calculated in this way were compared with the matched average titres of healthy controls: the control group contained the normal average value for a serogroup so many times as it occurred in the corresponding group.

D-s for titre averages in the examined and control groups for non-nosocomial strains in a diminishing order were (Table I): environmental strains > interaction-type III plus IV strains > type I and II strains. For nosocomial *P. aeruginosa* the corresponding order was: interaction type III plus IV strains > environmental strains > interaction-type I and II strains.

Table I

Reciprocal of initial (S_1) pseudomonas anti-LPS total antibody levels to serogroups colonizing and not colonizing in patients with acute or chronic underlying diseases

Origin of *P. aeruginosa* interaction-type*	Parameters	Acute patients			Chronic patients		
		Serogroups			Serogroups		
		nosocomial**	non-nosocomial***	total	nosocomial	non-nosocomial	total
Environment — control group	N	36	58	94	10	36	46
	x	22.6	20.8	21.4	23.9	18.7	19.7
	Vc	232.6	174.2	188.7	179.2	166.7	169.3
Environment — patients	N	36	58	94	10	36	46
	$\bar{x}$	56.6	40.5	46.0	149.3	80.0	91.6
	Vc	182.3	189.4	185.2	154.8	163.6	163.5
	D	1.33^{a}	0.96^{b}	1.10	2.64	2.10	2.22
Type I and II — control group	N	40	35	75	—	—	6
	x	23.3	19.5	21.4	—	—	20.8
	Vc	183.5	164.3	171.6	—	—	169.1
Type I and II — patients	N	40	35	75	—	—	6
	$\bar{x}$	52.8	28.6	39.6	—	—	63.5
	Vc	171.2	168.4	166.8	—	—	187.9
	D	1.18^{c}	0.55^{b}	0.89	—	—	1.61
Type III and IV — control group	N	8	9	17	5	21	26
	x	23.9	16.1	19.4	22.7	19.5	20.1
	Vc	208.9	144.5	178.8	182.5	170.6	173.1
Type III and IV — patients	N	8	9	17	5	21	26
	x	103.8	27.2	51.1	139.3	91.3	99.0
	Vc	168.4	221.0	230.7	131.1	148.7	145.9
	D	2.12^{ca}	0.76	1.40	2.62	2.22	2.30

* See "Patients and methods"
** O6 and O11
*** All except nosocomials
N = number of titres; $\bar{x}$ = mean titre; Vc = variation coefficient in percent; D = difference in dilution step between examined and control groups; a, b, c = significance level of difference between two indexed values: $^{a}p = 0.09$, $^{b}p = 0.09$, $^{c}p = 0.06$; NS = not significant

Chronic cases. D-s between examined and control mean titres were the same against environmental and colonizing (type IV) strains. Average TAb titres against nosocomial and non-nosocomial strains likewise showed no difference (Table I). The D values were, however, lower when later a manifest infection (types I and II) occurred.

IgG titre

Chronic cases. In each of the S_1 samples of the 9 patients with chronic illness, Ab titres against 5–9 colonizing or environmental serogroups were determined (67 measurements). IgG type Ab was absent only 7 times in the starting (1 : 10) dilution. During the investigation period (two and half months in the chronic ward) in 3 out of these 7 occasions manifest infection, and in 2 colonization by the homologous serogroup occurred.

IgG titre-groups of chronic cases are compared in Table II with one another, as in the S_1 of control cases IgG Ab-s occurred only occasionally.

Table II

Reciprocal of initial (S_1) pseudomonas anti-LPS IgG titres to serogroups colonizing and not colonizing in patients with chronic underlying diseases

Origin of *P. aeruginosa:* interaction-type	param-eters	Serogroups		
		nosocomial	non-nosocomial	total
Environment	N	8	29	37
	$\bar{x}$	51.9	30.8	34.4^{a}
	Vc	153.5	175.5	172.2
	r	0.716	0.514	0.538
	p	<0.05	<0.01	<0.001
Type I and II	N	—	—	6
	$\bar{x}$	—	—	11.2^{ab}
	Vc	—	—	239.9
	r	—	—	0.815
	p	—	—	<0.05
Type IV	N	5	19	24
	x	40.0	29.9	31.8^{b}
	Vc	169.8	191.9	185.6
	r	0.913	0.553	0.614
	p	<0.05	<0.02	<0.01

r = correlation coefficient between corresponding IgG and total antibody (see Table I) titres; p = significance level of r; a = $p < 0.02$; b = $p < 0.05$

For other abbreviations see legend to Table I

Average Ab titres against environmental and colonizing (interaction-type IV) strains were identical. On the contrary, the average IgG Ab titre in S_1 against strains causing infection later (interaction-types I and II) were significantly lower.

A close, significant correlation between corresponding IgG and TAb titres existed in each group.

Table III

Reciprocal of initial (S_1) pseudomonas anti-LPS total antibody titres to serogroups harboured for different periods after taking blood samples by patients with acute and chronic underlying diseases

Duration of harbouring *P. aeruginosa*	Parameters	Acute patients			Chronic patients		
		Serogroups			Serogroups		
		nosocomial*	non-nosocomial**	total	nosocomial	non-nosocomial	Total
A	N	7	12	19	2	11	13
control	$\bar{x}$	24.9	18.0	20.3	23.9	20.3	20.8
group	Vc	122.7	161.8	170.6	131.6	182.7	185.3
A	N	7	12	19	2	11	13
single	$\bar{x}$	88.3	28.3	43.0	160.0	124.4	129.3
isolation	Vc	188.0	262.7	238.8	156.0	135.8	136.5
	D	1.83	0.65	1.09	2.74	2.61	2.63
B	N	13	14	27	—	—	3
control	$\bar{x}$	22.5	17.9	19.9	—	—	17.5
group	Vc	125.6	168.4	172.3	—	—	164.6
B	N	13	14	27	—	—	3
4–10 days	$\bar{x}$	52.2	26.4	36.6	—	—	63.5
	Vc	152.4	163.6	164.1	—	—	124.9
	D	1.22	0.56	0.88	—	—	1.86
C	N	22	15	37	6	10	16
control	$\bar{x}$	23.9	18.9	21.8	23.9	18.4	20.3
group	Vc	123.9	173.4	178.2	131.6	179.3	172.1
C	N	22	15	37	6	10	16
$\geq$ 11 days	$\bar{x}$	53.1	26.9	40.8	113.1	80.0	91.1
	Vc	185.1	167.3	186.0	132.6	167.4	154.1
	D	1.15	0.51	0.90	2.24	2.12	2.16

* O6 and O11

** All except nosocomials

N = number of titres; x = mean titre; Vc = variation coefficient in percents; D = difference in dilution step between examined and control group

S_1 Ab titre and period of harbouring P. aeruginosa

TAb level

Acute cases. Ab titres in the S_1 of the 9 thrombophlebitis infections, in which duration of the presence of *P. aeruginosa* could not be detected, were excluded from evaluation. Data of the patients were compared as previously to that of healthy controls. The D between S_1 of patients and controls for both nosocomial and non-nosocomial serogroups diminished in the order group A $>$ B $\sim$ C (Table III).

Chronic cases. The D value of group A surpassed that of group B or C (Table III).

IgG titres

As healthy persons had rarely IgG type Ab-s, they could not be used as controls, and as S_1 samples of acute patients yielded only exceptionally IgG type Ab-s, they could not be evaluated.

Table IV

Reciprocal of initial (S_1) pseudomonas anti-LPS IgG titres to serogroups harboured for different periods after taking blood samples by patients with chronic underlying diseases

Duration of harbouring *P. aeruginosa*	Parameters	Serogroups		
		nosocomial	non-nosocomial	total
A	N	2	10	12
Single	$\bar{x}$	40.0	49.3	47.6
isolation	Vc	184.4	154.3	154.7
	r	—	0.561	0.591
	p	—	=0.09	<0.05
B	N	—	—	3
4–10 days	$\bar{x}$	–	–	15.9
	Vc	–	–	241.6
	r	—	—	0.822
	p	—	—	NS
C	N	6	9	15
$\geq$ 11 days	$\bar{x}$	25.2	13.6	17.4
	Vc	210.6	226.0	231.6
	r	0.839	0.557	0.681
	p	<0.05	NS	<0.01

r = correlation coefficient between corresponding IgG and total antibody (see Table III) titres; p = significance of r

For other abbreviations see legend of Table III

For chronic cases, the average peak titres were higher in group A than in groups B and C (Table IV).

A close correlation was found between IgG and TAb titres in these groups, too.

Discussion

Natural Ab-s (NAb) against Gram-negative microorganisms belong mainly to the IgM class [3–7]. Sera of healthy controls and the S_1 of acute cases yielded besides IgM low titre anti-pseudomonas IgG Ab-s only in 10%. Thus, Ab-s detected in S_1 of the acute patients taken before the development of *P. aeruginosa* infection, are natural antibodies (NAb).

On the contrary, in chronic patients harbouring continuously different circulating pseudomonas serogroups, the elevated TAb titres and the presence of IgG Ab-s at least in 1 : 10 dilution [1] corresponded to immune-Ab-s.

A poor general condition increases the adhesion of *P. aeruginosa* to epithelial cells [8–10]. This could have promoted pseudomonas colonization in the acute patients. Acute cases with high NAb level in their S_1 remained, however, free from infections with homologous, non-nosocomial *P. aeruginosa* serogroups. In other cases, with lower NAb titres, colonization occurred, but no clinical symptoms appeared (interaction-types III and IV). Still less NAb-s predisposed to pseudomonas illness due to non-nosocomial serogroups (interaction-types I and II). Infections by nosocomial strains usually did not occur from the environment but were associated with instrumental interventions [1]; a higher NAb level helped to avoid pseudomonas illness, but with a low titre pseudomonas complications developed. Accordingly, colonization and invasion by *P. aeruginosa* were modified by the patient's NAb level.

In the chronic cases low TAb titres (without IgG Ab-s) also failed to prevent infection. On the other hand, higher TAb and IgG titres against later colonizing or not colonizing serogroups were usually uniform. This indicates that an elevated immune anti-LPS Ab level induced very likely by former pseudomonas infections [1] no longer influenced the colonization of *P. aeruginosa*. In such a situation, the colonization may depend rather on persisting anti-pilus Ab-s [10], blocking Ab-s against anti-LPS Ab-s [11] or non agglutinating Ab-s, which develop a few weeks or months after infection [12].

The relation between anti-LPS Ab titre and the duration of harbouring *P. aeruginosa* was more simple: in all groups (acute and chronic cases, TAb and IgG level, nosocomial and non-nosocomial strains) a high average Ab titre in S_1 resulted later in brief (group A) colonization. The initial Ab level had, however, no further influence on the presence of *P. aeruginosa*, as the D-s or average titres did not differ in groups B and C.

It is questionable whether the influence of anti-LPS Ab-s on the duration of colonization or the development of infection was the result of a direct or an indirect effect. The protective capacity of human gamma-globulin in pseudomonas infection [13–16] supports the former possibility. The bactericidal action of IgM type NAb-s on complement sensitive Gram-negative microorganisms [17, 18] is also a direct effect. Nevertheless, since the NAb level is associated with immune-reactivity [3, 19], a high Ab titre might merely indicate the greater ability of the immune-system to prevent pseudomonas colonization or infection [20].

Finally, it should be emphasized that the results presented are only statistical relations. Individually, a number of different factors (virulence of *P. aeruginosa*, massiveness of infection, reactivity of the immune-system, etc.) will determine besides anti-LPS Ab-s the type of interaction between host and bacterium.

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REFERENCES

1. Petrás, Gy., Ádám, M. M., Lányi, B., Szentmihályi, A., Konkoly Thege, M.: Acta Microbiol Hung **31,** 91 (1984).
2. Lányi, B., Bergan, T.: In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Vol 10, pp 93—168. Academic Press, London 1978.
3. Backhausz, R., Lajos, J., Merétey, K.: Ann Immunol Hung **10,** 143 (1967).
4. Michael, J. G., Rosen, F. S.: J Exp Med **118,** 619 (1963).
5. Landy, M., Weidanz, W. P.: In Landy, M., Braun, W. (eds): Bacterial endotoxins. Rutgers University Press, New Brunswick 1964. pp 275—290.
6. Young, L. S., Armstrong, D.: J Infect Dis **126,** 257 (1972).
7. Merétey, K., Elekes, E., Kocsár, L.: Ann Immunol Hung **11,** 157 (1968).
8. Johanson, W. G., Pierce, A. K., Sanford, J. P., Thomas, G. D.: Ann Intern Med **77,** 701 (1972).
9. Johanson, W. G., Higuchi, J. H., Chaudhuri, T. R., Woods, D. E.: Ann Rev Resp Dis **121,** 55 (1980).
10. Woods, D. E., Straus, D. C., Johanson, W. G., Berry, V. K., Bass, J. H.: Infect Immun **29,** 1146 (1980).
11. Guttman, R. M., Waisbren, B. A.: Clin Exp Immunol **19,** 121 (1975).
12. Moody, M. R., Kessel, R. W. I., Young, V. M., Fiset, P.: Infect Immun **21,** 905 (1978).
13. Rosenthal, S. M., Millican, R. C., Rust, J.: Proc Soc Exp Biol Med **94,** 214 (1957).
14. Fisher, M. W., Manning, M. C.: J Immunol **81,** 29 (1958).
15. Waisbren, B. A., Lepley, D.: Arch Intern Med **109,** 712 (1962).
16. Jones, R. J., Lowbury, E. J. L.: Lancet **2,** 623 (1965).
17. Michael, J. G., Whitby, J. L., Landy, M.: J Exp Med **115,** 131 (1962).
18. Roantree, R. J., Rantz, L. A.: J Clin Invest **39,** 72 (1960).
19. Joó-Szabados, T., Körmendy, I., Zimonyi, I.: Ann Immunol Hung **11,** 165 (1968).
20. Pollack, M., Young, L. S.: J Clin Invest **63,** 276 (1979).

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IMMUNOSEROLOGY OF *PSEUDOMONAS AERUGINOSA* INFECTIONS IN MAN

III. SITE OF INFECTION, DURATION OF THE PRESENCE OF *PSEUDOMONAS AERUGINOSA* AND ANTIBODY RESPONSE

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In 39 patients of a respiratory intensive care unit the intensity of the serological response to the purified LPS of the causative *Pseudomonas aeruginosa* was found to change according to the site of infection. The highest titres were found in septic cases, when the antigenic assault reached all the immune-competent cells in the body. Short presence (only one positive bacteriological culture) of *P. aeruginosa* at the site of inflammation resulted in a low or moderate rise in antibody titre. Ten days were enough for the development of a maximum total antibody (~ IgM) response, while IgG type antibodies moderately grew further when the presence of *P. aeruginosa* lasted more than 10 days. Only a 16-fold increase in total antibodies per se or a 4-fold rise in both total (~ IgM) and IgG antibodies confirmed the pseudomonas infection.

In spite of the decisive role of pseudomonas anti-LPS antibodies (Ab) in the interactions of host and microorganism [1, 2], data on factors influencing the production of these Ab-s in man are rather incomplete. As the site of infection and the duration of harbouring the microorganism vary videly in pseudomonas infections, the connection between these factors and the serological response was investigated.

Patients and methods

In the respiratory intensive care unit of the László Central Hospital for Infectious Diseases, Budapest, 39 patients with acute illness were investigated. Their underlying disease, the bacteriological surveillance, passive haemagglutination experiments to determine total antibody (TAb; ~ IgM) and IgG level, etc. have been described in detail in a previous paper [3]. The positive serological response of these patients was evaluated in 68 manifest (interaction-type I of host and bacterium [3]) and 5 subclinical (interaction-type III [3]) pseudomonas infections. The manifest infections were: 7 tracheobronchitis, 23 pneumonia, 10 urinary tract infections, 16 thrombophlebitis, 8 rhinitis by nasogastric tube, 1 enteritis, 1 bedsore, 1 injection abscess and 1 peritonitis. Mild were 13, moderate 23, severe (septic state) 16, and fatal 16 cases. A definition of the grades of severity has been given in the former paper [3]. In subclinical infections *P. aeruginosa* colonized the skin, ear, nose or trachea.

Infections were grouped besides the site of complication according to the period of presence of *P. aeruginosa* as follows: group "A" = only one positive culture; group "B" = harbouring *P. aeruginosa* for 4 to 10 days (two or more positive bacteriological samples); group "C"

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= pseudomonas harbouring for more than 10 days. In 9 thrombophlebitis infections the duration of the presence of *P. aeruginosa* could not be determined; these infections have not been evaluated.

Mathematical-statistical analysis was done as previously [3]. The difference (D) between two titre averages was expressed in dilution-steps using the formula $2^D = a/b$, where a and b are the two mean titres.

Results

Ab titres in 68 manifest (type I) and 5 subclinical (type III) pseudomonas infections

TAb level

Average Ab titres against the infection-causing strain in the first blood samples (S_1) taken on admission and that of the peak titres (S_2) in different infections are shown in Table I.

D value between S_1 and S_2 (3.71) in pseudomonas complications (interaction-type I) was significantly higher ($p < 0.02$) than in subclinical infections (interaction-type III; $D = 2.40$).

D values decreased in the following order: D thrombophlebitis > urogenital infection > pneumonia > other infections of interaction-type I > bronchitis > subclinical infections.

D in thrombophlebitis and urinary tract infections surpassed significantly that in other complications except pneumonia. There was a significant difference in D values between pneumonia and bronchitis, too.

IgG level

IgG Ab was detectable only in 4 of the S_1-s in the first dilution step (1 : 10). Therefore, average titres for S_1-s were taken uniformly as 1 : 5, and the rise in the titres was compared to this value (Table II).

IgG response in manifest (type I) infections was significantly higher ($p < 0.05$) than in subclinical (type III) infections.

D-s diminished in a similar order than in the case of TAb-s: D thrombophlebitis > urinary tract infection > pneumonia > bronchitis > subclinical infections > other infections of type I.

The average S_2-titre in thrombophlebitis surpassed (in the majority significantly) that of other complications.

A close, significant correlation existed between the corresponding TAb ($\sim$ IgM) and IgG titres, except in subclinical infections and "other" infections of type I.

Table I

Changes in anti-LPS total antibody titre during infection with P. aeruginosa according to host-bacterium interaction-type and site of infection

Interaction-type*	*P. aeruginosa* illness	Serum sample	No. of samples	Average titres	Variation coefficient, %	p/D
	Bronchitis	S_1	7	48.76	136.4	a,
		S_2	7	262.51	137.6	bb,
		D		2.43		ccc
		p		<0.01		
	Pneumonia	S_1	23	47.93	167.5	
		S_2	23	550.48	148.5	a,
		D		3.52		d
		p		<0.001		
I	Urinary tract infection	S_1	10	37.32	214.3	
		S_2	10	640.0	152.2	bb,
		D		4.10		e,
		p		<0.001		f
	Thrombophlebitis**	S_1	17	43.40	148.1	ccc,
		S_2	17	1280.0	141.0	d,
		D		4.88		ggg,
		p		<0.001		hhh
	Others	S_1	11	25.73	236.1	
		S_2	11	170.41	160.0	e,
		D		2.73		ggg
		p		<0.01		
III	Others	S_1	5	69.64	163.3	
		S_2	5	367.58	125.4	f,
		D		2.40		hhh
		p		=0.02		

* I = Clinical symptoms with antibody production; III = symptomless rise in antibody titre

** and injection abscess

S_1 = serum sample taken on admission; S_2 = serum sample yielding peak antibody titre; D = difference between mean titres in dilution step; p = significance of difference between mean titres; p/D = significance in D between groups marked by the same letter: a, d, e, f = $p < 0.05$; bb = $p < 0.01$; ccc, ggg, hhh = $p < 0.001$

Table II

Changes in anti-LPS IgG titre during infection with P. aeruginosa according to host–bacterium interaction-type and site of infection

Interaction-type	*P. aeruginosa* illness	Serum sample	No. of samples	Average titres	Variation coefficient, %	p/S_2
	Bronchitis	S_1	7	≤ 5.0	—	
		S_2	7	44.16	176.3	a
		D		≥ 3.14		
		r		0.929		
		p/r		<0.01		
	Pneumonia	S_1	19	≤ 5.0	—	
		S_2	19	51.64	166.3	b
		D		≥ 3.37		c
		r		0.546		
		p/r		<0.02		
I	Urinary	S_1	9	≤ 5.0	—	
	tract	S_2	9	68.58	206.5	d
	infection	D		≥ 3.78		
		r		0.695		
		p/r		<0.05		
	Thrombophlebitis*	S_1	15	≤ 5.0	—	a,
		S_2	15	105.56	201.5	b,
		D		≥ 4.40		eee,
		r		0.715		ff
		p/r		<0.01		
		S_1	8	≤ 5.0	—	
		S_2	8	23.78	142.5	c,
	Others	D		≥ 2.25		d,
		r		0.664		eee
		p/r		$=0.08$		
III	Others	S_1	4	≤ 5.0	—	
		S_2	4	33.64	125.5	
		D		≥ 2.75		ff
		r		0.133		
		p/r		NS		

* and injection abscess; r = correlation coefficient; p/r = significance limit of r; p/S_2 = = significance of D between S_2 mean titres

For other abbreviations see legend of Table I

Table III

Increase in anti-LPS total antibody titre to environmental and infection-causing P. aeruginosa serogroups according to the duration of harbouring P. aeruginosa

Duration of harbouring *P. aeruginosa*	Serum sample	Parameters	Nosocomial strains*	Non-nosocomial strains**	Total
		N	36	58	94
	S_1	x	56.6	40.5	46.0
		Vc	182.3	189.4	185.2
Ø		N	36	58	94
Environment		$\bar{x}$	279.7	191.5	221.3
	S_2	Vc	141.5	160.7	154.5
		D	2.31	2.24	2.27
		p	<0.001	<0.001	<0.001
		N	2	4	6
	S_1	$\bar{x}$	28.3	28.3	28.3
		Vc	—	270.0	219.2
A		N	2	4	6
One positive sample		$\bar{x}$	160.0	226.3	201.6
	S_2	Vc	156.0	166.5	157.3
		D	2.50	3.0	2.83
		p	—	NS	=0.02
		N	11	12	23
	S_1	$\bar{x}$	51.5	26.7	36.5
		Vc	145.4	169.3	162.9
B		N	11	12	23
4–10 days	S_2	$\bar{x}$	773.2	359.2	518.3
		Vc	130.3	153.7	145.2
		D	3.91	3.75	3.83
		p	<0.001	<0.001	<0.001
		N	22	13	35
	S_1	$\bar{x}$	53.1	29.1	42.5
		Vc	231.5	164.5	183.2
C		N	22	13	35
≥ 11 days		$\bar{x}$	681.6	356.0	535.5
	S_2	Vc	147.9	192.5	164.6
		D	3.68	3.62	3.66
		p	<0.001	<0.001	<0.001

* O6 and O11

** All serogroups except nosocomials

S_1 = serum sample taken on admission; S_2 = serum sample yielding peak antibody titre; N = number of antibody titres; $\bar{x}$ = mean titre; Vc = variation coefficient, percents; D = difference between mean titres; p = significance of difference between S_1–S_2 mean titres; NS = not significant

Duration of the presence of P. aeruginosa and Ab response in 59 manifest (type I) and in 5 subclinical (type III) infections

TAb level

S_1 and S_2 titres to strains cultured only from the environment of a given patient served as controls. The mean titres for these strains increased by 2.27 dilution steps (D) during the infectious process (Table III). The rise for the nosocomial (O6, O11; [3]) and non-nosocomial serogroups was equal.

The increase in the mean titre of serogroups belonging to group "A" (only one positive culture) was only by a half dilution step higher than that of environmental strains.

In group "B" and "C" (harbouring *P. aeruginosa* from 4 to 10 days, or longer) the D was 1.5 dilution steps higher than in the control group (environmental strains). The D value in group "C" corresponded to that of group "B".

Table IV

Peak anti-LPS IgG titres to infection-causing P. aeruginosa serogroups according to the duration of harbouring P. aeruginosa

Duration of harbouring *P. aeruginosa*	Parameters	Nosocomial strains*	Non-nosocomial strains**	Total
A	N	2	1	3
One positive	$\bar{x}$	—	—	20.0
sample	Vc	—	—	131.9
	N	8	9	17
B	$\bar{x}$	47.6	43.2	45.2
4–10 days	Vc	177.5	167.3	171.4
	r	0.910	0.974	0.806
	p/r	<0.01	<0.001	<0.001
	N	21	12	33
C	$\bar{x}$	91.3	42.4	69.1
≥ 11 days	Vc	166.2	270.1	185.2
	p	=0.06	NS	=0.10
	r	0.557	0.768	0.664
	p/r	<0.01	<0.01	<0.001

* O6 and O11

** All serogroups except nosocomials

r = correlation between IgG and corresponding total antibody titres; p/r = significance limit of r; p = significance of difference between mean titres of groups B and C

For other abbreviations, see legend of Table III

IgG level

Peak titres were compared statistically with each other, as the Ab titres in S_1-s could not be determined.

Presence of P. aeruginosa for more than 10 days at the site of infection increased the IgG titres further; mean titres of group "C" were significantly higher than the average values in group "B" (Table IV). The difference was mainly due to the change in titre to nosocomial serogroups.

Ratio of IgG/TAb-s was higher in the S_2 of group "C" than of group "B" (data not shown). The difference was more expressed in the nosocomial serogroups.

Both in group "B" and "C" a closed, significant correlation existed between the corresponding TAb and IgG titres.

Discussion

Despite the changed reactivity of circulating human blood lymphocytes to the LPS of the infection-causing *P. aeruginosa* serogroup [4], anti-LPS Ab-s are produced in the lymph organs [5, 6]. Animal experiments have revealed that pseudomonas anti-LPS Ab production is less when at different sites the antigenic stimulus is limited to the regional lymph nodes [7–10]. The same mechanism has been supposed to occur in our patients with localized infections (infected decubitus, rhinitis by nasogastric tube, bronchitis, etc.). As plasma cells produce Ab in equal quantities [11], in pathological processes connected mostly with sepsis (thrombophlebitis, urinary tract infection, pneumonia), the peak Ab titres were significantly higher, due probably to an antigenic assault reaching all the immune competent cells in the body.

As regards the order of decreasing serological response according to the localization of pseudomonas infections, our findings correspond to those obtained by agar-gel precipitation [12].

The association between the duration of antigenic stimulus and the intensity of serological response offered a possibility to verify the criteria of *P. aeruginosa* infection described in a preceeding paper [3]. In the case of only one positive bacteriological culture accompanied by clinical symptoms of infection, the rise in mean TAb titres to the colonizing serogroups was nearly the same as that to the serogroups found only in the environment (7-fold and 5-fold, respectively). As in certain patients to environmental serogroups even 8-fold increases in TAb titres occurred, the TAb titre ($\sim$IgM) per se verifies a pseudomonas infection only at a 16-fold rise.

On the contrary, there was a marked difference in IgG Ab titres to serogroups in the environment and causing transient infections (one positive bacte-

riological sample). In the former case, there was no detectable IgG Ab titre, while to infection-causing strains (clinical signs of inflammation at the site of pseudomonas colonization) specific IgG Ab-s appeared at least in 1 : 10 dilution in every instance, even in mild infections. Accordingly, a four-fold rise in TAb titre with the appearence of IgG Ab-s in 1 : 10 dilution (or a four-fold increase in both TAb and IgG Ab-s) is indicative of pseudomonas infection.

The moderate serological response to a brief harbouring of *P. aeruginosa* referred to a weak antigenic stimulus.

The presence of *P. aeruginosa* at the site of inflammation for ten days was sufficient for the development of a maximum Ab response in TAb-s: further exposure to the microorganism did not influence the intensity of the TAb answer.

On the other hand, the presence of *P. aeruginosa* for more than 10 days resulted in a higher average IgG peak titre, however, bordering only the mathematical significance. This tallies with the higher IgG : TAb ratio in group "C", and agrees with the elevated IgG titres in chronic, bedridden patients harbouring *P. aeruginosa* continuously [3].

The usually significant correlations between corresponding IgG and TAb ($\sim$ IgM) titres confirmed our former assumption [3, 13] of the close correlation between the production of these two Ab-s.

The results presented should be taken into consideration in human vaccination trials.

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REFERENCES

1. Crowder, J. G., Devlin, H. B., Fisher, M., White, A.: J Lab Clin Med **83,** 853 (1974).
2. Young, L. S.: J Infect Dis **126,** 277 (1972).
3. Petrás, Gy., Ádám, M. M., Lányi, B., Szentmihályi, A., Konkoly Thege, M.: Acta Microbiol Hung **31,** 91 (1984).
4. Gallyas, A., Petrás, Gy., Ádám, M. M., Merétey, K.: Zentralbl Bakteriol [A] **250,** 497 (1981).
5. Fauci, A., Pratt, K.: J Exp Med **144,** 674 (1976).
6. Greaves, M., Janossy, G., Doenhoff, M.: J Exp Med **140,** 1 (1974).
7. Beh, K. J., Lascelles, A. K.: Immunology **42,** 577 (1981).
8. English, L. S., Adams, E. P., Morris, B.: J Exp Med **144,** 586 (1976).
9. Mikkelsen, D. S.: Acta Pathol Microbiol Scand **73,** 373 (1968).
10. Reynolds, H. A.: J Infect Dis **130,** S134 (1974).
11. Nathans, D., Fahey, J. L., Potter, M.: J Exp Med **108,** 121 (1958).
12. Høiby, N.: Acta Pathol Microbiol Scand Sect C **85,** 142 (1977).
13. Petrás, Gy., Ádám, M. M., Lányi, B., Konkoly Thege, M., Szentmihályi, A.: Acta Microbiol Hung **31,** 101 (1984).

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ESCHERICHIA COLI COL V PLASMIDS AND THEIR ROLE IN PATHOGENICITY*

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Out of 1474 *Escherichia coli* strains belonging to 70 serogroups and 247 serologically not identified ones, colicin V producers were found in serogroups O78, O1, O7, O18 and also among the not identified strains. The molecular weight of Col V1 plasmid from the standard strain was 70 Mdal. The Col V plasmids carried by *E. coli* O78 strains isolated from a hospital outbreak in Hungary had a molecular weight of 78 Mdal and also 78 Mdal was the molecular weight of the Col V plasmid carried by Rivier's strain (designated 23), which had caused meningitis in Switzerland. The molecular weight of Col V plasmids of O1: K1, O21, O161 and serologically not identified *E. coli* strains isolated from sporadic cases was of 94 to 119 Mdal. In the case of three strains the increase of LD_{50} values, which means the decrease of virulence, resulted in the loss of colicin V production and the loss of Col V plasmid. It was demonstrated by introduction of a transposon, inactivating colicin V production, into a wild type *E. coli* strain that the production of colicin was not essential for the increase of virulence controlled by Col V plasmid. In the case of one strain the loss of both R and Col V plasmid resulted in a decrease of virulence. No plasmid other than R and Col V was carried by this strain. The virulence determining gene could be eliminated together with both plasmids, which means that this gene could be attached equally to Col V and R plasmid.

Smith [1] in 1974 observed that in experimental animals *Escherichia coli* strains caused a higher number of sepsis after acquisition of Col V plasmid and the pathogenicity decreased after elimination of Col V plasmid. Smith and Huggins [2] then reported on a significant correlation between Col V plasmid carrier state of *E. coli* and its septicaemic effect in chickens and calves.

Earlier we have found that *E. coli* O78 strains causing meningitis and sepsis in newborn infants were producing colicin V. The O78 strains isolated from sporadic cases of enteritis or healthy carriers (nurseries) were not colicinogenic or were characterized by other types of colicin. *E. coli* strains obtained from a hospital outbreak showed significantly lower LD_{50} values for mice than did the Col V non producers [3].

In the present examinations Col V plasmids known from the literature and isolated in Hungary were compared on the basis of transferability and mo-

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lecular weight. The intention was to apply a molecular analysis for plasmid epidemiology.

The strains were examined also for mouse pathogenicity before and after plasmid curing and then controlled by electrophoresis.

Materials and methods

Strains. A total of 1721 *E. coli* strains originating from different clinical cases and from different hospitals in the period January, 1980 to March, 1982, were examined for serological and colicinogenic properties.

The following strains were for determination of molecular weight of plasmids: wild-type *E. coli* strains isolated in Hungary R981, R789, R1528 (O78); 8/1, (O9); 465/81 (O 161); 121/82 (O21); 128/82 (O1: K1), 278/82 (O1: K1); 287/82 (O1: K1); and *E. coli* 23 (O78) received from Rivier [4]; Col V1 CA7S, received from Frédéricq [5]. Col V and R plasmid transferability was examined in 30 antibiotic resistant strains carrying Col V plasmids.

The recipient strain for R and Col plasmid transfer was *E. coli* K12 J53 rif^r.

Serological examination. Agar slant cultures grown at 37 °C for 18 h were suspended in 5 ml physiological saline, adjusted to contain 10^9 cells/ml and heated at 100 °C for $2^1/_2$ h. The suspension was added at 0.05 ml amounts to 0.5 ml diluted pooled and group sera. The isolates were considered to belong to a defined O group if agglutination occurred in one of the absorbed O sera to the titre of the homologous type strain. Determination of K1 antigen was carried out with K1 serum produced by strain *E. coli* O2 : K1 : 44 (U9/41) and with Rowe's K1 phages: ABCDE [6].

Colicin type determination. Colicin type was determined with Frédéricq's indicator an colicin producing strains [5], by the method of Lewis [7] and Alwis and Thomlinson [8].

Antibiotic sensitivity. Antibiotic sensitivity was examined with Resistest disks (Human, Budapest) to streptomycin, chloramphenicol, tetracycline, kanamycin, neomycin, ampicillin, gentamicin, nalidixic acid and sulphonamide, using Mueller–Hinton agar.

Culture medium. After conjugation the cultures were streaked on agar medium containing broth extract and antibiotics at inhibitory concentrations (rifampicin 250 μg/ml, tetracycline 20 μg/ml, ampicillin 30 μg/ml, chloramphenicol 20 μg/ml, kanamycin 20 μg/ml, streptomycin 30 μf/ml).

Mouse infection assay. Mouse pathogenicity was examined by intraperitoneal injection of CFLP mice weighing 16–18 g, according to Smith [1].

Plasmid elimination. Elimination was carried out using ethidium bromide, (Bouanchaud et al. [9]), by UV irradiation (Watanabe and Fukasawa [10]) and with mitomycin C (Hardy and Meynell [11]).

Determination of molecular weight. For preparation of plasmid DNA the rapid method of Birnboim and Doly [12] was used. Electrophoresis was done on 0.8% agarose gel, for $2^1/_2$ h at 40 mA. Preparation of large plasmids was carried out according to the method of Crosa and Falkow [13].

Standard plasmids in molecular weight determination were as follows: R27 (Inc H) 112 Mdal; RA1 (Inc A) 86 Mdal; R1 (Inc F II) 62 Mdal; RN3 (Inc N) 33 Mdal; S-a (Inc W) 23 Mdal.

Rapid method for plasmid determination. Agarose-gel electrophoresis was carried out as described by Eckhardt [14].

Tn1 transposition. Tn1 transposition into *E. coli* Col V^+ strain was carried out according to the method of Quackenbush and Falkow [15].

Results

Frequency of colicinogenicity. Out of the examined 1474 *E. coli* strains belonging into 70 serogroups and 247 serologically not identifiable strains 39.2% were colicinogenic (Table I). Colicinogenicity occurred most frequently in serogroups O78, O1, O151, O7, O75, O2, O6, O124.

Table I

Frequency of colicinogenicity in E. coli strains

Serologically	No. of strains	Colicinogenic	
		No.	%
Identified	1474	580	39.3
Non-identified	246	99	40.2
Total	1720	679	39.5

Table II

Serogroup distribution of V colicin producing E. coli strains

Serogroup	No. of strains	No. of Col+ strains	No. of Col V+ strains
O1	18	16	8
O2	27	15	3
O7	16	13	5
O9	1	1	1
O18	83	33	6
O21	1	1	1
O23	8	3	2
O78	19	17	13
O111	160	55	2
O161	1	1	1
ONt	246	99	16
Total	580	254	58

Nt = Not groupable

Determination of colicin types. Table II shows those *E. coli* serogroups which contained Col V producing strains. Out of the examined 70 serogroups Col V producing strains were found in 11 serogroups and among serologically not typable strains (ONt). Most of the Col V producing strains fell in serogroups O78, O18, O1, O7 and ONt. Out of 19 strains of serogroup O78 17 were colicinogenic and 13 of them produced colicin type V. Out of 580 strains colicinogenicity was observed in 254 and 58 of them produced colicin type V.

Comparative examinations on Col V plasmids. Col V plasmids were compared on the basis of transferability and molecular weight, determined by agarose gel electrophoresis. None of the examined 30, V type Col plasmids

were transferable into *E. coli* J53. (Crosses were carried out without mobilization.)

Table III presents the data of the plasmid carrier strains. The analysis of plasmids is demonstrated in Figs 1–3.

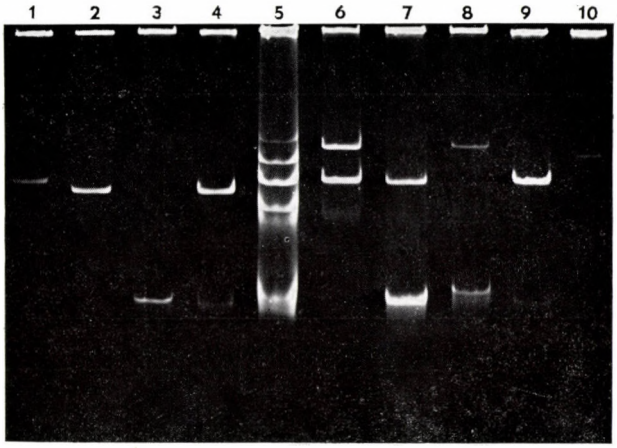

Fig. 1. Agarose gel electrophoresis of purified R and Col V plasmid DNA. Plasmids from the *E. coli* strains, lanes: 1, R789 R$^+$ Col V$^+$ (two plasmid bands); 2, R789 R$^+$ Col V$^-$ (one plasmid band); 3, plasmid free J5–3; 4, J5–3 R$^+$ (transconjugant of R789 R$^+$ Col V$^-$); 5, reference plasmids: RA1 (Inc A) 86 Mdal; RI (Inc FII) 62 Mdal; RN3 (Inc N) 33 Mdal; S-a (Inc W) 23 Mdal; 6, R981 R$^+$ Col V$^+$ (two plasmid bands); 7, R981 R$^+$ Col V$^-$ (one plasmid band); 8, R981 R$^-$ Col V$^+$ (one plasmid band); 9, J5–3 R$^+$ (transconjugant of R981 R$^+$ Col V$^-$); 10, CA7S VI$^+$

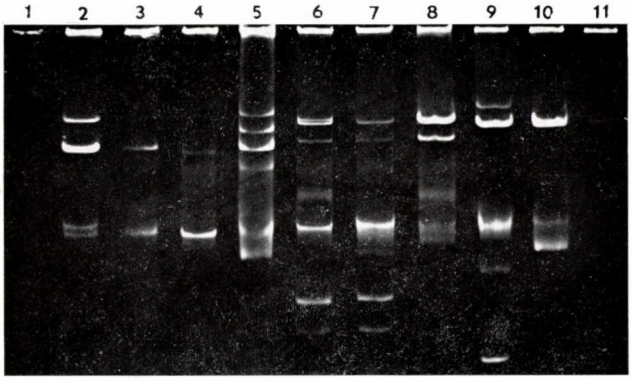

Fig. 2. Agarose gel electrophoresis of purified R and Col V plasmid DNA. Plasmids from the *E. coli* strains, lanes: 1, plasmid free J5–3; 2, RI528 R$^+$ Col V$^+$ (two plasmid bands); 3, RI528 R$^+$ Col V$^-$ (one plasmid band); 4, J5–3 R$^+$ (transconjugant of RI528 R$^+$ Col V$^-$); 5, reference plasmids, see Fig. 1; 23 R$^+$ Col V$^+$I$^+$ (5 plasmids bands, two bands close to each other correspond to Col V + I); 7, 23 R$^+$ Col I$^+$V$^-$ (four plasmid bands); 8, J5–3 R$^+$ Col I$^+$ (transconjugant of 23 R$^+$ Col I$^+$V$^-$); 9, 8/1 R$^+$ Col V$^+$ (three plasmid bands); 10, J5–3 R$^+$ (transconjugant of 8/1 R$^+$ Col V$^-$); 11, CA7S VI$^+$

Table III

Data of E. coli host strains

Designation of strain	Serogroup	Antibiotic resistance	R-plasmid	Col-plasmid
R789	078	Cm Tc Ap Su	+	V^+
R789/12/10	078	Tc Su	+	V^-
J53/789/12/10	·	Tc	+	V^-
R981	078	Cm Tc Ap Su	+	V^+
R981/8	078	Cm Tc Ap Su	+	V^-
R981/46	078	—	—	V^+
J53/981/8	·	Cm Tc Ap Su	+	V^-
R1528	078	Cm Tc Ap Su	+	V^+
R1528/3	078	Cm Tc Ap Su	+	V^-
J53/1528/3	·	Cm Tc Ap	+	—
23	078	Sm Tc Su	+	V^+ I^+
23/1	078	Sm Su	+	V^- I^+
J53/23	·	Tc	+	V^- I^{+*}
8/1	09	Sm Cm Tc Ap Km Su	+	V^+
J53/8/1	·	Ap Km	+	V^-
CA7S	·	—	—	V^+
465/81	0161	Ap	+	V^+
121/82	021	Tc Su	+	V^+
128/82	01 : K1	(Su)	+	V^+
278/82	01 : K1	(Su)	.	V^+
287/82	01 : K1	(Su)	.	V^+

·Not examined * Not colicinogenic

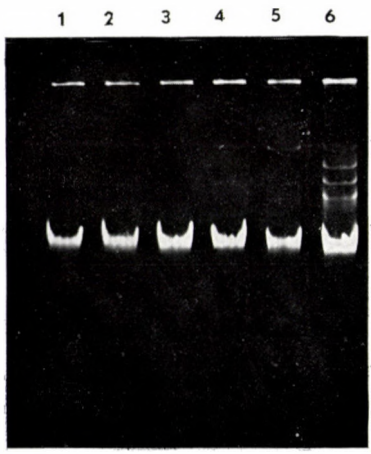

Fig. 3. Agarose gel electrophoresis of purified R and Col V plasmid DNA. Plasmids from th8 *E. coli* strains, lanes: 1, 465/81 R^+ Col V^+; 2, 121/82 R^+ Col V^+; 3, 128/82 R^+ Col V^+; 4, 278/e2 R^- Col V^+; 3, 287/82 R^- Col V^+; 6, reference plasmids: R-27 (Inc H) 112 Mdal; R1 (Inc FII) 62 Mdal; RN3 (Inc N) 33 Mdal; S-a (Inc W) 23 Mdal

Figure 1 shows the agarose gel electrophoretic picture of two wild-type *E. coli* O78 V^+R^+ strains and their Col V^- or R^- eliminants and an Col V1 plasmid carried by CA7S strain (Frédéricq).

Figure 2 shows the agarose gel electrophoresis of two Hungarian wild-type *E. coli* strains (O78 and O9), an *E. coli* 23, O78 Col V strain originating from Lausanne and from the CA7S (Frédéricq) (Col V1) strain.

Table IV

Molecular weight values of Col and R plasmids of different origin determined by electrophoresis in agarose gel

Designation of strain	Molecular weight (Md)			Epidemiological and clinical data (excretion), year
	Col V	Col I	R	
R789	78	—	33	
R789/12/10	—	—	30	Hospital outbreak Bp. 1.
J53/789/12/10	—	—	30	Infant sepsis (CSF), 1974
R981	78	—	33	
R981/8	—	—	33	Hospital outbreak Bp. 1.
R981/46	78	—	—	Infant sepsis (navel), 1974
J53/981/8	—	—	33	
R1528	78	—	33	Hospital outbreak Bp. 1.
R1528/3	—	—	33	Infant enteritis (faeces), 1975
23	78	70	44	Hospital, Lausanne (Rivier)
23/1	—	70	44	Septicaemia (blood), 1979
8/1	100	—	64	Hospital Bp. 2.
J53/8/1	—	—	64	Salmonellosis (faeces), 1979
CA7S	70	—	—	Gratia 1925
465/81	119	—	.	Hospital Bp. 3. Symptomless (urine), 1981
121/82	119	—	.	Hospital Bp. 4. Pyelonephritis (urine), 1982
128/82	94	—	.	Hospital Bp. 1. Symptomless pregnant (vagina), 1982
278/82	94	—	.	Discharged Symptomless (faeces), 1982
287/82	104	—	.	Hospital N. Symptomless premature infant (nose), 1982

· Not examined

Figure 3 shows the Col V and R-plasmids of 5 wild-type *E. coli* strains and 4 reference plasmids.

Table IV shows the molecular weights of the plasmids determined by agarose gel electrophoresis, presented in Figs 1, 2 and 3.

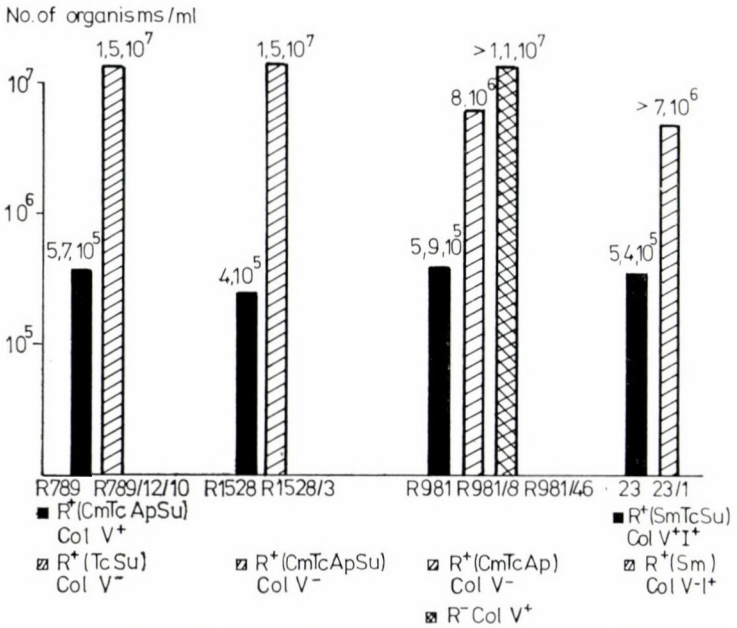

Fig. 4. LD_{50} values for *E. coli* R+ Col V+ and of their different eliminants

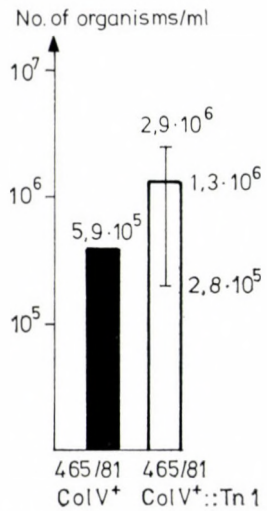

Fig. 5. LD_{50} values for *E. coli* 465/81 Col V+ and 465/81 Col V+: : Tn1 (lacking colicin V activity

Table V

Differences of LD_{50} values for the original and the treated strains

Designation of strain	Original strain		Treated strain		Significance
	Injected bacteria	No. of mice died/infected	Injected bacteria	No. of mice died/infected	
R789 $R^+Col\ V^+$	1.5×10^6	11/20	9×10^5*	0/20	$p < 0.001$
R1528 $R^+Col\ V^+$	2.8×10^6	14/20	2.1×10^6*	10/80	$p < 0{,}001$
R981 $R^+Col\ V^+$	1×10^6	11/20	4.1×10^5**	0/20	$p < 0.001$
23 $R^+Col\ V^+$	1.6×10^5	7/20	1.3×10^5*	0/20	$p < 0.001$
C465/81 Col V^+	2.2×10^6	8/20	2.0×10^6***	10/20	$p = 0.5$

* Col V^- cured strain; ** R^- cured strain; *** Col V^+: : Tnl strain

Comparative results of mouse pathogenicity and agarose gel electrophoresis experiments. The strains tested for virulence in mice were examined for plasmid bands and molecular weight. An increase of LD_{50} values and a loss of plasmid and production of V type colicin was observed in strains (R789, R1528, 23). For Rivier's 23 $R^+V^+I^+$ strain, a decrease of virulence was observed simultaneously with the of Col V plasmid loss.

In one strain (R981) the loss of the R and Col V plasmids was accompanied by an increase in the LD_{50} value (Fig. 4).

Tn1 transposition into Col V^+ wild strain and mouse infection assay. As a donor strain we used *E. coli* UB 281 (pM R5) pro^- $meth^-$ nal^r Km^r Tc^r Ap^r $Carb^r$, which was harbouring a Tn1 transposon containing thermosensitive plasmid. Tnl transposon determining carbenicillin resistance was transposed into a *E. coli* 465/81 Col V^+ strain according to the method of Quackenbush and Falkow [15]. After the insertion of transposon the strain had become resistant to carbenicillin. Col V^- colonies were selected from which the Col V plasmid could be demonstrated by agarose gel [14]. No significant difference was found in the LD_{50} values of Col V^+ colicin producer and Col V^+: : Tnl colicin non producer strains (Fig. 5 and Table V).

Discussion

Col V plasmids known from literary data are different in respect of transferability and molecular weight [16]. In the present examination the molecular weight of Col V plasmids carried by *E. coli* strains R789, R981, R1528 was 78 Mdal; these strains were isolated from meningitis and sepsis cases from a hospital outbreak in Hungary. The molecular weight of Col V plasmid carried by strain *E. coli* 23 (Lausanne) was also 78 Mdal; this strain was associated with severe meningitis in Switzerland. Col V1 plasmid of the

E. coli CA7S strain differed in molecular weight from the above plasmids; its molecular weight was 70 Mdal. The molecular weights were uniformly 33 Mdal of the R plasmids derived from the *E. coli* O78 strains isolated from one and the same outbreak in Hungary. The R plasmid of the Col V$^-$ eliminant of wild-type *E. coli* strain R789 determined Tc Su resistance only, Cm Ap resistance was absent. There was a small distance between the original plasmid band and the Tc Su controlling plasmid band; instead of 33 Mdal it was 30 Mdal. It has been suggested that Cm Ap resistance was controlled by transposons and these transposons were eliminated in the Col V$^-$ derivant.

Quackenbush and Falkow [15] reported that colicin V synthesis was inactivated by transposon insertion, while colicin V activity was not essential to Col V plasmid mediated virulence enhancement. This experiment was repeated by us using an *E. coli* Col V$^+$ strain isolated in Hungary. Our results are in agreement with the finding of Quackenbush and Falkow [15]. Williams [17] and Williams and Warner [18] reported that the enhanced virulence of invasive strains of *E. coli* carrying Col V plasmids was due to a novel plasmid-mediated iron uptake system.

We found in some cases that the loss of Col V plasmids was associated with a decrease of virulence (increase of LD_{50} value). In the case of one strain the loss of R plasmid resulted in a decrease of virulence in the presence of Col V plasmid. No other plasmid than R and Col V were carried by the examined strains.

On the basis of these experiments it is suggested that the virulence mediating gene may be associated either with Col V or R plasmids.

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REFERENCES

1. Smith, H. W.: J Gen Microbiol **83,** 95 (1974).
2. Smith, H. W., Huggins, M. B.: J Gen Microbiol **92,** 335 (1976).
3. Milch, H., Czirók, E., Madár, J., Semjén, G.: Acta Microbiol Acad Sci Hung **24,** 127 (1977).
4. Rivier, D.: Bull Inst Pasteur **77,** 375 (1979).
5. Frédéricq, P.: Rev Belge Path Méd Exp **19,** 1 (1948).
6. Gross, R. J., Cheasty, T., Rowe, B.: J Clin Microbiol **6,** 548 (1977).
7. Lewis, M. J.: Lancet **1,** 1389 (1968).
8. De Alwis, M. C. L., Thomlinson, J. R.: J Gen Microbiol **74,** 45 (1973).
9. Bouanchaud, D. H., Scavizzi, M. R., Chabbert, Y. A.: J Gen Microbiol **54,** 417 (1969).
10. Watanbe, T., Fukasawa, T.: J Bacteriol **81,** 679 (1961).
11. Hardy, K. G., Meynell, G. G.: J Gen Microbiol **73,** 547 (1972).
12. Birnboim, H. C., Doly, J.: Nucleic Acids Res **7,** 1513 (1979).
13. Crosa, J. H., Falkow, S.: In Gerhardt, Ph. (ed.): Manual of Methods for General Bacteriology. American Society for Microbiology, Washington D. C. 1981. pp 266–283.
14. Eckhardt, Th.: Plasmid **1,** 584 (1978).
15. Quackenbush, R. L., Falkow, S.: Infect Immun **24,** 562 (1979).
16. Lane, H. E. D.: Plasmid **5,** 100 (1981).
17. Williams, P. H.: Infect Immun **26,** 925 (1979).
18. Williams, P. H., Warner, P. J.: Infect Immun **29,** 411 (1980).

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EFFECT OF INTERFERON ON ANTIBODY--DEPENDENT CELLULAR CYTOTOXICITY (ADCC) IN CHICKENS

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The influence of chicken leukocyte interferon (IFN) on the antibody-dependent cellular cytotoxicity (ADCC) of peripheral blood mononuclear cells of chicken has been investigated in vivo and in vitro. The cytotoxic effect was studied on sheep red blood cells (SRBC) coated with chicken anti-SRBC serum. Cytotoxicity was estimated using a ^{51}Cr release assay system. A single intravenous injection of crude IFN led to an enhanced ADCC 6 to 12 h after the IFN injection, whereas by 24 h the activity had decreased. The ability of IFN to augment the cytotoxicity was not lost after phagocytic cell depletion. In vitro, the ADCC was boosted within 1–18 h by IFN, in a dose-dependent manner. We assume that the augmenting agent in vitro is IFN itself, as purified IFN had a similar activity, but mock IFN and trypsin-treated IFN showed no enhancing effect.

In connection with the various effects of interferon (IFN) on immune responses, much attention has been focussed on the ability of IFN to augment the cytotoxic reactivity of natural killer (NK) cells and also to increase the antibody-dependent cellular cytotoxicity (ADCC). The ADCC reaction measures the cytotoxicity of unsensitized effector cells against IgG-coated target cells different in origin. While the effects of IFN on mouse and human NK cells [1, 2] and the ADCC [3, 4] have been well studied, no data are available about these effects on chicken leukocyte IFN.

We have recently shown [5] that human adenovirus enhances the ADCC in chickens, and we assumed that IFN induced by the virus was responsible for the effect. Earlier studies from this laboratory [6] proved that adenoviruses induce IFN in chickens, and it thus seemed of interest to examine the effect of chicken IFN on the ADCC. In this paper we describe the results of experiments dealing with the effects of chicken IFN on the ADCC in vivo and in vitro in a xenogeneic test system, using antibody-coated sheep red blood cells (SRBC) as target cells. We present evidence that chicken leukocyte IFN enhances the ADCC both in vitro and in vivo.

Materials and methods

Chickens. Six-week-old birds of the White Leghorn strain (Hyline Poultry Farm, Johnston, Iowa) were used for experiments in vivo. To study the in vitro effect of IFN, effector cells were obtained from 8–12-week old birds.

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Effector cells. Peripheral blood mononuclear cells were isolated from heparinized chicken blood, obtained by heart puncture. Mononuclear cells were separated from the interphase of fresh blood after discontinuous density gradient centrifugation on Ficoll-Uromiro gradient by the method of Böyum [7]. The cells were subsequently washed three times in PBS, and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). In some experiments phagocytic cells were removed as follows: Ficoll-Uromiro-separated lymphocytes (5×10^7/ml) were incubated at 37 °C for 30 min in the presence of 10 mg/ml iron powder (Gaf. New York). Phagocytic cells were removed with a magnet, and the remaining cells were washed twice, and then diluted in RPMI 1640 medium containing 10% FCS. In these preparations 99% of the cells were classified as lymphocytes when assayed after May–Grünwald–Giemsa staining. Cell viability was monitored by exclusion of 0.2% trypan blue, and the cell number was adjusted to the appropriate dilution.

Preincubation of effector cells with IFN-α. In RPMI 1640 medium supplemented with 10% FCS, mononuclear cells (5×10^6/ml) were incubated with 100–200 IU/ml crude IFN or with 6–3200 IU/ml purified IFN (specific activity 6×10^5 IU/mg protein) for 1–18 h. Control cells were treated with an equivalent dilution of mock chicken leukocyte IFN. In some experiments trypsinized IFN having no antiviral activity was used as control.

In the experiments to examine the species-specificity of chicken leukocyte IFN, human effector cells were also treated with chicken IFN-α.

Inoculation of birds with chicken IFN-α. Two ml crude IFN (1000 IU/ml) was injected intravenously into 6-week-old chickens. Each experimental group consisted of 6 or more chickens. Two ml mock IFN was used as control in the same way.

Target cells. Blood from sheep was collected in Alsever solution and stored at 4 °C until used. Immediately prior to use, the SRBC were washed three times in PBS. Washed SRBC (10^8 cells) were labelled with 200 μCi sodium (^{51}Cr) chromate (LNK, Poland) at 37 °C for 60 min, then chicken anti-SRBC antiserum was added in a final dilution of 1 : 400, and incubation was continued for a further 30 min. Labelled erythrocytes were washed three times in RPMI 1640 medium and adjusted to a concentration of 5×10^5–1×10^6/ml in RPMI 1640 medium containing 10% FCS.

Anti-SRBC chicken serum. A 6-month-old bird was immunized at 2-week intervals with 0.1 ml/100 g of a 30% SRBC suspension. Four days after the second inoculation the chicken was exsanguinated. The serum was heat-inactivated at 56 °C for 30 min and the agglutinin titre was determined.

ADCC assay. All tests were performed in a total volume of 200 μl per U-shaped well in microtitre plastic plates. The effector-to-target cell ratios were 20:1, 10:1, 5:1 and 2.5: 1. All tests were performed in triplicate and plates were centrifuged at 300 g for 3 min before incubation at 37 °C for 18 h in 5% CO_2-air atmosphere. Tests were terminated by removal of supernatants, the radioactivity of which was determined with a gamma-counter. Spontaneous isotope release was determined from control wells containing target cells only. The percentage cytotoxicity was calculated from the formula:

$$\text{percent cytotoxicity} = \frac{\text{cpm in supernatant} - \text{cpm in spontaneous release}}{\text{total incorporated activity}} \times 100$$

Spontaneous release was calculated as

$$\text{percent Sp} = \frac{\text{cpm from targets in medium}}{\text{total incorporated activity}} \times 100$$

The spontaneous release ranged between 2 and 6%. Sensitizing antisera did not cause an increase in ^{51}Cr release in the absence of effectors. The cytotoxicity at a ratio of 20 : 1 was used for comparison on the basis of the dose-response curve.

IFN production, purification and assay. Chicken leukocyte IFN (IFN-α) was prepared as described previously [8] and purified by a slight modification of Fantes' method [9]. The assay of the antiviral activity of IFN was performed in chick embryo fibroblast cells inoculated with Sindbis virus as challenge [10]. Interferon titres were expressed in comparison to a reference standard IFN preparation from chick embryo fibroblast (MRC Research Standard A, 62/4, Mill Hill, London) in IU/ml. Mock IFN was prepared and purified similarly, without virus inducer.

Trypsin treatment of IFN. Chicken leukocyte IFN was incubated with crystalline trypsin (Worthington, 1 mg/ml) at 37 °C for 1 h, and 2 mg/ml trypsin inhibitor (Soybean, Serva) was then added.

Results

Considerable ADCC activity was found in the peripheral blood mononuclear cells of 6-week-old chickens, as well as in 3-month-old birds. The cytotoxicity level against SRBC coated with chicken anti-SRBC antibody varied around a mean of 45% in the control birds.

Augmentation of ADCC in vitro by chicken IFN-α. Table I shows the results of 5 representative experiments. In a concentration of 100–200 IU/ml, the crude IFN significantly increased the ADCC activity. Representative experiments are presented since variations were encountered in the basal level of cytotoxicity of the birds, but repeated experiments always yielded similar results. Within any single experiment, standard deviations were less than 2% and are therefore not included in the data shown.

During the studies there was some concern as to whether the IFN in the crude preparation was itself responsible for the augmentation of the ADCC.

Table I

Effect of chicken IFN-α on ADCC in vitro

Experiment	IFN IU/ml	Cytotoxicity, %
1	0	54
	200	79
2	0	64
	200	89
3	0	46
	200	80
	mock*	49
4	0	21
	200	59
	mock	19
5	0	30
	200	56
	mock	32
	trypsin-treated*	32

In these experiments mononuclear cells (5×10^6/ml) were cultured with or without IFN-α for 18 h. After the incubation period cells were washed twice in RPMI 1640 medium and the ADCC assay was then performed

* Mock and trypsin-treated IFN-α were used in a dilution equivalent to that of IFN-α

It can be seen from Fig. 1 that the effect on the ADCC activity was indeed due to IFN, since the purified preparation of chicken leukocyte IFN was as active as the crude material (Table I) even after a 1 h incubation period. Mock IFN and trypsin-treated IFN did not show any significant augmenting capacity (Table I).

As pilot experiments with crude IFN had shown that preincubation of the cells with IFN for 1 h without further washing gave a sufficient augmentation of the ADCC activity (data not reported), no further attempts were made

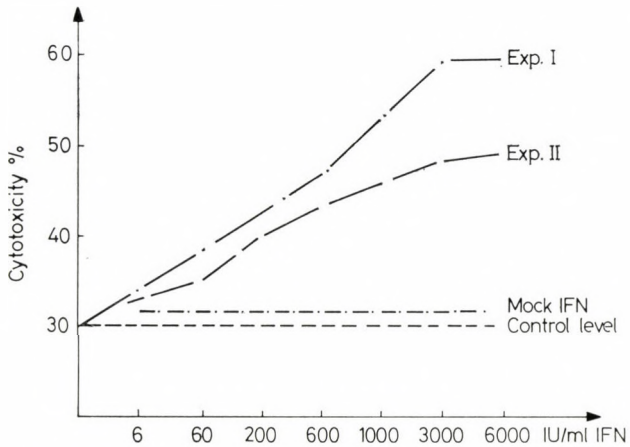

Fig. 1. Effect of purified IFN on ADCC of chicken lymphocytes. Mononuclear cells were incubated for 18 h (Experiment I) or for 1 h (Experiment II) with purified IFN or mock IFN at various concentrations. Effector-to-target ratio = 20 : 1

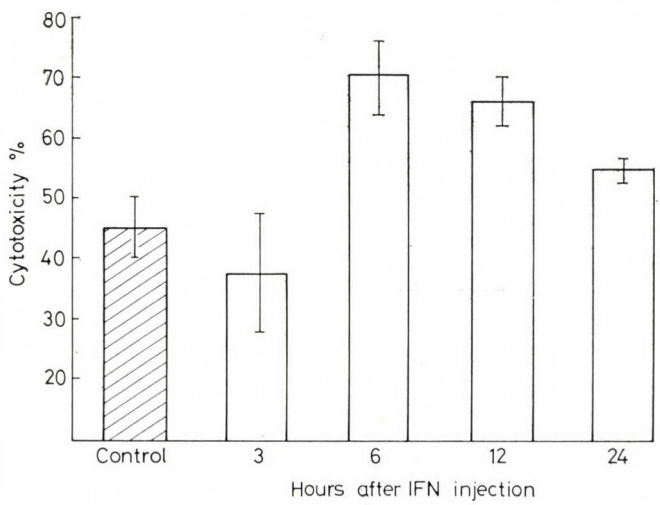

Fig. 2. In vivo augmentation of ADCC by IFN in chickens. Bars represent mean percentage cytotoxicity ±SD for groups of 6 chickens. Effector-to-target ratio = 20: 1

Table II

Influence of phagocytic cell depletion on ADCC-enhancing effect of chicken leukocyte IFN

Chickens	Cytotoxicity, %*	
	Depleted lymphocytes**	Control lymphocytes
Control No. 1	44	52
Control No. 2	42	54
IFN-treated No. 1***	56	65
IFN-treated No. 2	52	67

* E : T ratio = 20 : 1

** Phagocytic cells were removed with iron powder and a magnet, and the remaining cells were washed twice and then diluted in RPMI 1640 medium containing 10% FCS. Control lymphocytes were subjected to the same process but without iron treatment

*** Peripheral blood mononuclear cells of IFN-inoculated chickens were obtained 6 h after injection; 20 ml crude IFN (1000 IU/ml) was injected i.v. into 10-week-old chickens

to increase the preincubation period with purified IFN. The enhancement of the ADCC by purified IFN-α seemed to be dose-dependent (Fig. 1).

Chicken leukocyte IFN failed to augment the human ADCC reaction against human red blood cells, nor did it enhance the human NK reaction against K-562 cells (data not given).

ADCC in chickens following IFN injection. The ADCC was studied 3, 6, 12 and 24 h after crude IFN inoculation. An elevated cytotoxicity (75%) was seen at 6 h after IFN administration and the cytotoxicity was still above the basal level after 12 h, but by 24 h it had decreased (Fig. 2).

Enhancement of ADCC in absence of phagocytic cells. To study whether monocytes played an important role in the increase in ADCC after IFN inoculation, cell preparations from 2 control and 2 IFN-treated chickens were depleted of phagocytic cells by means of iron powder and a magnet. The lymphoid cell preparations used without depletion contained about 5% non-lymphocytic cells. After depletion, smears were subjected to May–Grünwald–Giemsa staining when only 0.5–1% of the cells proved to be monocytes. As it can be seen from Table II, the ADCC of these preparations increased to a similar extent after IFN injection. It is worthwhile to mention that the basal cytotoxicity was reduced after removal of the monocytes, indicating that not only lymphocytes but also monocytes take part in the ADCC.

Discussion

The results of these studies indicate that chicken IFN, similarly to human and mouse IFN, augments the ADCC reaction both in vitro and in vivo.

There is a difference in the kinetics of the IFN-induced enhancement of the ADCC in vitro and in vivo. One hour exposure of lymphocytes to IFN in

vitro is sufficient to cause an increase in the cytotoxicity. These data are in agreement with those of Herberman et al. [11] and de Landazuri et al. [2]. We and others have found that further washing of cells to free them from IFN after the 1 h incubation period did not alter the experimental result [12, 13]. Moreover, IFN is frequently added to the cytotoxicity assay simultaneously with the effector cells at time 0 [14, 15]. The increase in the ADCC however, could not be detected until 6 h after IFN injection (Fig. 2). Similar results were obtained by Einhorn et al. [14] with human IFN. They suggested that, in vivo, factors other than IFN act first on the cells and suppress the cytotoxic activity. In this context it is of interest that they observed a decreased cytotoxicity 6 h after IFN injection of their patients. Most of these patients developed fever. As Fig. 2 shows, 3 h after administration of chicken leukocyte IFN there was a high deviation in the mean value of the ADCC, because some chickens had a decreased ADCC activity. Similarly, Flexman and Shellam [16] reported that after inoculation of rats with IFN there was a transient decrease in the cytotoxicity level after 3 h, and augmentation was observed only after 6 h.

IFN boosted the ADCC activity in vivo and in vitro in all experiments, but the absolute cytotoxicity level varied. Herberman et al. [3] have observed that human individuals display differences in responsiveness to IFN treatment. This might be analogous to the situation in mice, in which the sensitivity to IFN was found to vary from strain to strain, and could be influenced by previous in vivo exposure to activating stimuli [17]. In our experiments the level of cytotoxicity decreased after removal of phagocytic cells, but the extent of augmentation of the ADCC remained the same. However, the augmentation in these experiments (Table II) was less marked than with the use of 6-week-old birds, probably due to discrepancies in weight and age of the birds and the amount of IFN used. We used Ficoll-Uromiro-separated chicken peripheral blood mononuclear cells as effector cells, containing less than 5% monocytes, and the number of the latter fell to 1% after carbonyl iron treatment. Though the level of cytotoxicity decreased after depletion, the augmentation was as marked as it had been before depletion. Hence, we presume that the effector cells responsible for the elevated ADCC after IFN treatment are not the monocytes; it is probably rather K cells that play and important role in the reaction.

Recently, Chi and Thorbecke described ADCC to allogeneic red blood cells in chicken, using spleen cells as effector cells [18]. In contrast to our results, they found monocytes to be the main effector cells in their system. The apparent contradiction might be due to the different methods of isolation and depletion of monocytes. Lymphocytes and monocytes reflect different requirements for antigen density on the target, for distribution of the antibody on the target and for dilution of antisera, as has recently been reviewed [19]. Keaney et al. [20] reported that monocytes appear to be the sole effector cells

when the target cells are sensitized in suboptimum concentration with the antisera, and therefore the ADCC activity of the monocytes may be masked by that of the lymphocytes when target cells are optimally sensitized with antibodies. In our experiments we used antisera at a final dilution of 1 : 400, and we therefore suggest that the effector population comprised mainly lymphocytes.

Our data proved that chicken IFN, similarly to mammalian IFN, has an immunomodulating effect on the ADCC in birds. In the present experiments augmentation of the ADCC was demonstrable in chickens 6 h after IFN administration. The kinetics of augmentation correlated well with our previous data which revealed an ADCC augmentation after adenovirus inoculation [5]. The increase in the ADCC reaction 14 h after virus infection may have been due to the IFN synthetized 6–8 h after inoculation [6]. Our present observation therefore provides further indirect evidence that adenovirus augments the ADCC via an IFN-dependent mechanism. The direct demonstration of the role of IFN in stimulating the ADCC after virus inoculation will be possible if antibodies to chicken leukocyte IFN become available.

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REFERENCES

1. Djeu, J. Y., Heinbaugh, J. A., Holden, H. T., Herberman, R. B.: J Immunol **122,** 175 (1979).
2. de Landazuri, M. O., Lopez-Botet, M., Timonen, T., Ortaldo, J. R., Herberman, R.: J Immunol **127,** 1380 (1981).
3. Herberman, R. B., Ortaldo, J. R., Bonnard, G. D.: Nature **277,** 221 (1979).
4. Ortaldo, J. R., Pestka, S., Slease, R. B., Rubinstein, M., Herberman, R. B.: Scand J Immunol **12,** 365 (1980).
5. Mándi, Y., Bakay, M., Béládi, I.: Cell Immunol **69,** 395 (1982).
6. Pusztai, R., Bakay, M., Mucsi, I., Béládi, I.: Acta Virol **13,** 71 (1969).
7. Böyum, A.: Scand J Clin Lab Invest **21,** (Suppl 97) 77, (1979).
8. Mucsi, I., Pusztai, R., Béládi, I., Bakay, M.: Acta Virol **14,** 453 (1970).
9. Taródi, B., Pusztai, R., Berencsi, K.: Acta Virol **19,** 393 (1975).
10. Pusztai, R., Béládi, I., Bakay, M., Mucsi, I.: J Gen Virol **4,** 169 (1969).
11. Herberman, R. B., Ortaldo, J. R., Mantovani, A., Hobbs, D. S., Kung, H., Pestka, S.: Cell Immunol **67,** 160 (1982).
12. Hokland, P., Berg, K.: J Immunol **127,** 1585 (1981).
13. Ullberg, M., Merrill, J., Jondal, M.: Scand J Immunol **14,** 285 (1981).
14. Einhorn, S., Blomgren, H., Strander, H.: Int J Cancer **26,** 419 (1980).
15. Saksela, E., Timonen, T., Cantell, K.: Scand J Immunol **10,** 257 (1979).
16. Flexman, J. P., Shellam, G. R.: Immunology **44,** 311 (1981).
17. Herberman, R. B., Holden, H. T., Bonnard, G. D., Kay, H. D., Tagliabue, A., Djeu, J. Y., Mantovani, A., Riccardi, C.: In Terry, W. D., Yamamura, Y. (eds): Immunobiology and Immunotherapy of Cancer. Elsevier North Holland Inc., Amsterdam 1979. pp. 87–102.
18. Chi, D. S., Thorbecke, G. J.: Cell Immunol **64,** 258 (1981).
19. Buglio, A. F., Levy, P. C., Shaw, G. M.: In Terry, W. D., Yamamura, Y. (eds): Immunobiology and Immunotherapy of Cancer. Elsevier North Holland Inc., Amsterdam 1979. pp. 137—145.
20. Keaney, M., McPhail, S., Joduin, C., Richter, M.: Immunology **40,** 205 (1980).

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SOME CHARACTERISTICS OF LYSOGENS OF *MYCOBACTERIUM SMEGMATIS*

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By the use of radioactive labelling it was observed that mycobacteriophage butyricum (By) enter *Mycobacterium smegmatis* strain Rabinowitz (M. sm. R.) cells, lysogenise them but do not form plaques. Mycobacteriophage Rabinowitz (V72) and phage By proved to be heteroimmune; that is, they superinfect lysogens of one another productively. Spontaneous lysis frequencies of two different mycobacterium strains lysogenised by phage By [M. sm. R. (By); M. sm. b. (By)] and that of the double lysogenic M. sm. R. (V72, By) strain were compared. The spontaneous lysis frequency of the two monolysogenic strains was found to be similar (10^{-4}–10^{-2}) indicating that transition into the vegetative replication cycle of By phages occurs also in M. sm. R. cells. Spontaneous lysis frequency of the double lysogenic strain was found to be influenced by the medium used.

It was reported previously that the plating efficiency of mycobacteriophage butyricum (By) was less than 10^{-8} on cells of *Mycobacterium smegmatis* Rabinowitz (M. sm. R.), and 5×10^{-1} on its lysogenic variant (M. sm. R. (V72)) compared to the value obtained on the original host *Mycobacterium smegmatis* butyricum (M. sm. b.). This difference in plating efficiency was shown to arise not in consequence of an altered phage adsorption to the M. sm. R. (V72) strain, as the same degree of adsorption could be detected to both Rabinowitz strains corresponding to that of the original host [1].

Phages By not only form plaques on M. sm. R. (V72) cells, but also lysogenise them and that results in a double lysogenic mycobacterial strain [1].

This paper describes experiments designed to obtain information referring to (i) By phage penetration into M. sm. R. bacteria; (ii) lysogenization of M. sm. R. and M. sm. b. cells by phage By, and (iii) spontaneous phage production of different mycobacterial strains carrying prophage By.

Materials and methods

Bacterial and bacteriophage strains. The bacteria and mycobacteriophage used were *M. smegmatis* strain Rabinowitz (M. sm. R.) and its phage Rabinowitz (designated V72), and *M. smegmatis* strain butyricum (M. sm. b.) and its phage butyricum (designated By), and the double lysogenic *M. smegmatis* strain Rabinowitz (M. sm. R. (V72, By)) of our own culture

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collection. Streptomycin resistant strains of M. sm. b. and M. sm. R. were isolated in our laboratory. Preparation, concentration and purification of phages were described elsewhere [2].

Media and buffer. Bouillon and YRP media were used in the experiments [1]. Trishydroxymethyl-aminomethane buffer (0.05 M, pH 7.4) containing 0.004 M $CaCl_2$ was used to store phages and to make dilutions.

Assay of phage titres. Phage techniques followed those described by Adams [3]. Plates and soft agar containing 1.5 and 0.6% agar were prepared from YRP or bouillon media.

Isolation of lysogenic M. sm. R. and M. sm. b. strains. Cells grown overnight were centrifuged, resuspended in fresh medium to reach an absorbancy of 0.2–0.4 at 660 nm. After two hours incubation at 37 °C the cells were pelleted and resuspended in Tris buffer to obtain again an absorbancy of 0.2–0.4 at 660 nm. Bacteria were infected with phage By at a multiplicity of 1–5. Adsorption was allowed to proceed for 30 min, then the degree of phage adsorption and the number of cells surviving By phage infection were determined. Colonies formed by cells surviving By phage infection were investigated for lysogeny in the following way: each colony was transferred to (i) solid YRP medium, (ii) solid YRP medium covered with M. sm. b. lawn. The surviving M. sm. R. colonies were also transferred to solid medium covered with M. sm. R. lawn. In these conditions lysogenic colonies form plaques on a corresponding bacterial lawn in consequence of spontaneous lysis occurring in lysogenic cells. Colonies showing signs of spontaneous lysis were transferred to YRP medium containing 0.1% Tween 80, grown up, and plated. Developed colonies were transferred again to solid medium and to solid medium covered with bacterial lawn. This procedure was repeated several times, and at last stable lysogenic strains were isolated both from M. sm. R. and M. sm. b. strains.

Determination of spontaneous lysic frequency. Lysogenic strains were grown overnight at 37 °C in YRP or bouillon medium containing 0.1% Tween 80. Cells were centrifuged and washed twice with the medium to remove free phages, then resuspended in the medium, diluted, and plated. Some of the plates were overlayered with soft agar containing 50 μg/ml streptomycin and the streptomycin resistant indicator bacteria. Plates were incubated overnight at 37 °C. Spontaneous lysis frequency was calculated:

$$\frac{\text{Number of plaques on resistant bacteria}}{\text{Number of colonies on corresponding plate}}$$

Infection of M. sm. R. strain with ^{3}H-labelled phage By. Phage By DNA was labelled with ^{3}H-uracil according to the method described by Somogyi and Földes [4].

For the infection bacteria grown overnight were centrifuged, resuspended in fresh medium to reach an absorbancy of 0.4 at 660 nm and infected with ^{3}H-labelled By phage at a multiplicity of 1–5. At intervals titres of free phages were determined. To measure DNA uptake by the cells, samples of the infected culture were centrifuged, washed twice in ice-cold 0.05 M Tris buffer pH 7.4 containing 0.001 M EDTA, and heated in 5% trichloroacetic acid at 80 °C for 60 min. The samples were centrifuged again and radioactivity of the supernatant was determined in a Packard 3330 scintillation counter using Bray scintillation cocktail.

Results

Infection of M. sm. R. cells with ^{3}H-labelled phage By. M. sm. R. bacteria were infected with ^{3}H-labelled phage By at a multiplicity of 1–5 to investigate penetration of phage DNA into the cells. From time to time samples were taken to determine (i) phage adsorption by measuring the titre of free phages; (ii) DNA uptake of the cells by measuring their radioactivity as described in Materials and methods.

Figure 1 shows that a considerable radioactivity could be detected in the cells reaching its maximum 180 min after infection. The time course of the radioactivity curve correlated with the decrease in titre of the free phages, indicating that phages By not only adsorb to M. sm. R. cells, but their DNA penetrates into them.

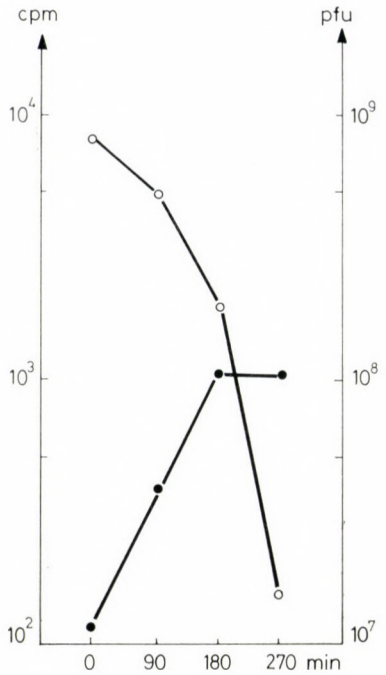

Fig. 1. Infection of M. sm. R. cells with ^{3}H-labelled phage By. ○——○ titres of free phages (pfu); ●——● radioactivity measured in cells (cpm)

Lysogenization of M. sm. b. and M. sm. R. strains with phage By. The contradiction that DNA of By phages penetrates into M. sm. R. cells but does not form plaques suggested the possibility of lysogenization of cells by phage By. To check this, M. sm. R. and M. sm. b. cells were infected with phage By and the colonies developed from cells surviving phage infection were investigated for lysogeny as described in Materials and methods. Out of the M. sm. R. colonies developed after phage infection, not a single one was found to show lysis of M. sm. R. lawn in consequence of the spontaneous phage production; on the contrary, some of the colonies lysed M. sm. b. lawn. Some of the surviving colonies of M. sm. b. strain also proved to be lysogenic. The lysogenic strains obtained were designated as M. sm. R. (By) and M. sm. b. (By).

In further experiments the phage-susceptibility of both M. sm. R. and M. sm. b. strains lysogenized by phage By was studied by superinfecting them with phage By and phage V72, respectively. Plating efficiencies were determined so that the number of plaques obtained on the original (non-lysogenic) host was taken as the unit. Adsorption of the phages to the lysogenic strains was also determined. Data are shown in Table I. Both phages could adsorb to both lysogenic strains; By phages did not give plaques on either lysogenic strain and phage V72 gave plaques only on M. sm. R. (By) strain.

Table I

Superinfection of M. sm. R. (By) and M. sm. b. (By) with V72 and By mycobacteriophages, respectively

Bacterical strains	Adsorption of phages		Plating efficiency	
	By	V72	By	V72
M. sm. b. (By)	93%	84%	10^{-8}	10^{-8}
M. sm. R. (By)	89%	73%	10^{-7}	1,5

Table II

Spontaneous lysis frequency of the monolysogenic mycobacterium strains

Experiment	M. sm. b. (By)	M. sm. R. (By)
1	6.2×10^{-2}	1.1×10^{-2}
2	1.7×10^{-3}	6.8×10^{-3}
3	1.2×10^{-2}	5.8×10^{-2}
4	4.2×10^{-3}	4.0×10^{-3}

Table III

Spontaneous phage production of M. sm. R. (V72, By) strain in YRP and bouillon media

Experiment	Relative number of cells producing phage			
	By	V72	By	V72
	in YRP medium		in bouillon medium	
1	2.5×10^{-4}	1.6×10^{-3}	2.5×10^{-3}	1.5×10^{-4}
2	3.5×10^{-4}	4.7×10^{-2}	1.6×10^{-2}	1.9×10^{-4}
3	1.2×10^{-3}	2.1×10^{-2}	9.8×10^{-2}	1.1×10^{-3}

Spontaneous phage production of lysogenic mycobacterium strains. Spontaneous phage production of M. sm. b. (By), M. sm. R. (By), and of the double lysogenic M. sm. R. (V72, By) strain [1] was investigated by the streptomycin overlyer technique. The soft agar contained 50 μg/ml streptomycin to kill lysogenic cells. At this concentration the antibiotic had no effect on the plating efficiency either of phage By or of phage V72. Table II shows that the spontaneous lysis frequencies of the two monolysogenic mycobacterium strains were similar and varied between 10^{-4}–10^{-2}. Results were independent from the media used in the experiments.

In the case of the double lysogenic mycobacterium strain it was found that spontaneous lysis frequencies referring to the two phages differed by at

least one order of magnitude. It is of interest that a reversed ratio of By or V72 phage producer cells has been observed using YRP or bouillon medium (Table III).

Discussion

Only a small proportion of mycobacteria adsorb phages in a phage-infected culture, while the sensitive cells adsorb hundreds of phages without showing the phenomenon of "lysis from without" [5, 6]. Mycobacterium–mycobacteriophage systems cannot therefore be investigated by the "mixed infection" or "multiplicity activation" methods [7–9]. We could overcome these difficulties by the use of lysogenic strains, especially by studying the spontaneous lysis of the latter.

Earlier we have shown that By phages adsorb to, but do not give plaques on M. sm. R. cells [1]. By the use of ^{3}H-labelled By phage we could observe in the present work that DNA of By phages even penetrate into M. sm. R. cells. Considering this observation together with our previous finding, namely that By phages can lysogenise the lysogenic M. sm. R. strain, resulting in a double lysogenic strain (M. sm. R. (V72, By)), we hypothesized that By phages are able to lysogenise also the original M. sm. R. strain. The hypothesis could be proved by isolation of an M. sm. R. (By) strain as described in Results.

The fact that By phage forms plaques on M. sm. R. cells lysogenised by phage V72 [1], and the present finding that V72 forms plaques on M. sm. R. cells lysogenised by phage By (Table I), mean that By and V72 phages are heteroimmune; that means that they superinfect lysogens of one another productively.

Spontaneous lysis frequency proved to be similar in our two strains lysogenised by phage By (Table II). Transition of By prophages into vegetative replication cycle in M. sm. R. cells seems to be in contradiction to the observation that By phages do not form plaques on these cells. This contradiction needs further studies; it may, however, be hypothesized that not only some vegetative replication is necessary for lysogenization [10, 11] but an established lysogeny may also facilitate the initiation of the vegetative replication cycle.

The interesting results referring to the spontaneous lysis frequency of the double lysogenic strain need also further studies.

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REFERENCES

1. Háber, K. J., Vajda, B. P., Földes, I.: Acta Microbiol Acad Sci Hung **28,** 407 (1981).
3. Vajda, B. P., Háber, K. J.: Am Rev Resp Dis **114,** 245 (1976).
3. Adams, M. H.: Bacteriophages. Interscience Publishers, New York 1959.

4. Somogyi, P. A., Földes, I.: Ann Microbiol (Paris) A **134,** 19 (1983).
5. Somogyi, P. A.: Thesis, Hungarian Academy of Sciences, Budapest 1980.
6. Kölbel, H.: In Juhász, S. E., Plummer, G. (eds): Host-virus relationships in Mycobacterium, Nocardia and Actinomyces. Charles C. Thomas, Springfield, Ill. 1970. pp 29—38.
7. Uetake, H., Toyama, S., Hagiwara, S.: Virology **22,** 202 (1964).
8. Heip, J., Rolfe, B., Schell, J.: Virology **59,** 356 (1974).
9. Potts, T. V., Christensen, J. R.: J Virol **14,** 1319 (1974).
10. Stent, G. S., Fuerst, C. R.: Virology **2,** 737 (1956).
11. Levine, M.: Virology **3,** 22 (1957).

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SERIAL DETERMINATION OF COMPLEMENT AND SPECIFIC ANTIBODY TITRES IN *PSEUDOMONAS AERUGINOSA* INFECTION

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Serial examination of the complement system and specific antibody titre of 12 surviving patients and 8 lethal cases suffering from *Pseudomonas aeruginosa* infection showed that except the onset of infection, until the development of septic shock, the level of the complement components corresponded to or exceeded the average normal value. In reversible septic shock the complement titre decreased significantly and in irreversible shock the values were even lower. Activation of the complement system occurred on 10 occasions via the classical and on 42 occasions via the alternative pathway. The number of activations grew parallel with the severity of the infection. Activation through the classical way was generally more intensive. During the whole infectious process not the individual characteristics of the *P. aeruginosa* present unbroken, but the pathological events and the specific antibody level determined the mode (alternative or classical pathway) of complement activation. The specific antibody level of the surviving patients significantly surpassed the titres of the lethal cases until the development of shock. Not an insufficiency of the complement system but the relative lack of specific antibodies was mainly responsible for the fatal outcome of *P. aeruginosa* infections.

The great number of papers on *Pseudomonas aeruginosa* are almost impossible to survey. Our knowledge on the development and changes of the humoral immunity in pseudomonas infection is fragmentary. The present report deals with the level of the different complement (C) components and with the specific antibody (Ab) titres at the time of C activation during the whole infectious process.

Patients and methods

Patients. Ten male and ten female patients of a respiratory ward were examined. Their average age was 47.6 years. Eighteen had undergone tracheotomy and were given artificial respiration for more than 24 h. The underlying diseases were tetanus in 9, polyradiculitis in 5, meningoencephalomyelitis in 4 cases, myasthenia gravis and subarachnoideal haemorrhage in one patient each. The underlying diseases were mild (transient clinical symptoms without respiratory disturbance) in 2, moderate (expressed clinical symptoms with respiratory insufficiency of short duration) in 1, severe (life-threatening situation) in 14, and lethal in 3 cases.

Bacteriological samples were taken in the acute phase of infection twice, and later once weekly from the patients' skin (axillary and inguinal region), nose, throat or trachea, ear, faeces,

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urine and pus. Facultatively pathogenic bacteria, other than *P. aeruginosa*, as *Klebsiella*, *Proteus*, *Escherichia coli*, *Staphylococcus aureus*, etc. colonized the patients only for a few days if at all, without causing any local symptom.

The infective *P. aeruginosa* strains belonged to serogroups O2a,2d,2e, O3, O4a,4b, O6a,6b, O9a, O10a,10c, O11a,11b of the Lányi–Bergan scheme [1] or contained R antigens (Lányi's former groups O8 and O9). With three exceptions the same serogroup could be detected during each infection.

P. aeruginosa infections. In the 20 patients a total of 39 *P. aeruginosa* infections such as tracheobronchitis, pneumonia, urinary tract infection, thrombophlebitis, enteritis, purulent rhinitis in consequence of nasogastric tube, injection absess, infected decubitus were registered. Beside a positive bacteriological result local and general clinical symptoms pointed to a complication due to pseudomonas in every instance.

Only the minority of blood cultures yielded pseudomonas in consequence of vigorous, intravenous administration of antibiotics from the early onset of complications. The severity of infections was determined mainly on the basis of local and general clinical signs; transient bacteriaemia was observed also in nearly symptomless cases.

Three patients had mild (complication without intensive local signs, extensive tissue destruction and pus production; temperature below 38 °C and good general condition), 4 moderate (expressed local symptoms, temperature exceeding 38 °C), 5 severe (septic state: intermittent fever, heavy prostration, erythrocyte sedimentation rate about 100 mm/hour, rapidly developing anaemia, in spite of a negative haemoculture, too) and 8 lethal (lethal sepsis) *P. aeruginosa* infections.

Criterion of septic shock was a septic condition with a blood pressure below 70 mm Hg for more than 60 min, accompanied by a marked decrease in urinary output. Four reversible septic shocks developed in the 5 severe cases, whereas 9 reversible and 8 irreversible shocks in the 8 lethal ones.

The pseudomonas infections started generally between the 3rd and 10th day of hospital care and culminated with the improvement of, or sometimes after complete recovery from, the underlying disease.

Serum samples. Blood samples were obtained on admission and during the whole hospital stay in the acute phase every 3rd ot 4th day, in convalescence every 6th or 7th day. All serum specimens from a given patient were stored at —20 °C and tested simultaneously.

Each blood sample was categorized after the stage of infection, as follows. (1) Free of complications; (2) onset of infection; (3) ongoing infection; (4) culmination of infection, or septic state in severe and lethal infections; (5) 1–2 h before septic shock; (6) septic shock; (7) improvement; (8) convalescence; (9) irreversible septic shock on the day of death.

Specific antibody titre. Total antibody (TAb) titre was measured by the passive haemagglutination technique of Neter et al. [2], carried out by adaptation to Takátsy's microtitrator [3, 4]. O-antigens of the infective *P. aeruginosa* serogroups purified by ultracentrifugation [3] were used in passive haemagglutination. At evaluation it was computed with the average of those Ab titres which belonged to a certain stage of the infection. Thus, from one stage only one Ab titre per patient was taken into consideration.

Serum samples were subjected to 2-mercaptoethanol treatment to determine IgG type Ab-s. Freshly prepared 0.2 mol/l 2-mercaptoethanol was added in an equal volume to five-fold diluted serum. Ab titrations were performed in two double-fold dilution series of the samples, starting from the one-tenth step. When calculating the average titres, a serum sample yielding no Ab at the starting level was considered to contain it in 1 : 5 dilution. Since the average of Ab titres represents a geometrical mean, the variation coefficient expressed in percent was calculated by the formula:

$$\text{variation coefficient } \% = 100 \text{ num lg} \sqrt{\frac{N \Sigma (\lg x_i)^2 - (\Sigma \lg x_i)^2}{(N-1)(\Sigma \lg x_i)^2} N}$$

Complement determination. In the serum samples, C4, C3, C3–9 and total complement (TC) were measured using the method of Nelson et al. [5] for C4, Mancini's radial immunodiffusion technique as modified by Kohler and Müller-Eberhard [6] for C3 with rabbit anti-human C3 serum prepared as outlined by Mardiney and Müller-Eberhard [7], the method of Townes and Stewart [8] for C3–9, and the standard method for determination of TC activity [9]. Details were given in an earlier paper [10]. The normal value for C titre was determined from the sera of 75 healthy persons [10].

Statistical analysis. Statistical analysis was done by Student's *t*-test for evaluation of C values, and by the Mann–Whitney method in the case of Ab titres. For comparing the number

Table I

Significance limits of the rate of complement titre decrease during physiological fluctuations*

Significance limit, %	T CH_{50}/ml	C4 CH_{83}/ml	C3 mg/100 ml	C3–9 CH_{50}/ml
5	4.41	9 577	14.19	5.88
1	6.34	13 622	20.99	8.29
0.1	9.32	19 717	32.45	11.85

* decrease/day

of C activations via the alternate and the classical pathways, the χ^2-test was used. The rapidity of C-activation was calculated on the basis of 25 blood samples of 5 healthy persons taken serially at 2–5 day intervals. The standard error of the mean of daily decreases in TC, C3, C4 and C3–9 found during the physiological fluctuation and multiplied with the value for the 5, 1 and 0.1% probability levels of the *t*-table corresponding to the degree of freedom gave the limit of normal rapidity of titre decrease (Table I).

Complement activation. A daily titre decrease between two sera exceeding the limit of significance was considered a sign of C activation. A significant decrease could be observed at both high and low C levels, thus an activation of the C system does not yet mean a low C value.

In the case of a significant decrease in the C4 titre as compared with the former serum sample activation beginning through the classical (C1) pathway was supposed. A significant fall in the C3 or the late components without a decrease in the C4 fraction was considered a sign of activation via the alternate (A1) pathway. A decrease solely in the TC level was not taken into consi leration.

Results

Complement component levels. C values of the 12 surviving patients suffering from mild, moderate or severe infections and of the 8 patients with lethal outcome are presented by columns in Figs 1 and 2. TC, C4, C3 and C3–9 titres of patients still free of infection and of healthy controls did not differ significantly. In the fatal cases the level of all C components was lower than in the surviving patients at the onset of infection. The greatest fall, one of 35% was in the C3 fraction. Henceforth, until the development of shock in the TC, C3 and C3–9 levels there was hardly any difference between the two groups. In sepsis, the average values of all components exceeded that of the healthy controls. One or two hours before septic shock, the titres of TC, C3 and C3–9 began to diminish. In septic shock all the four components showed a marked decrease, indicating an intensive activation of the C system. On the day of fatal shock the decreases were even greater. In the survivors, the highest TC, C3, C4 and C3–9 levels were measured during improvement.

Mode of activation of the complement system. At the onset of infection, 1–2 h before shock, and during improvement and convalescence activation could be observed only through the Al pathway. In sepsis, too, the Al mechanism predominated. In septic shock, activation via the Cl pathway was significantly

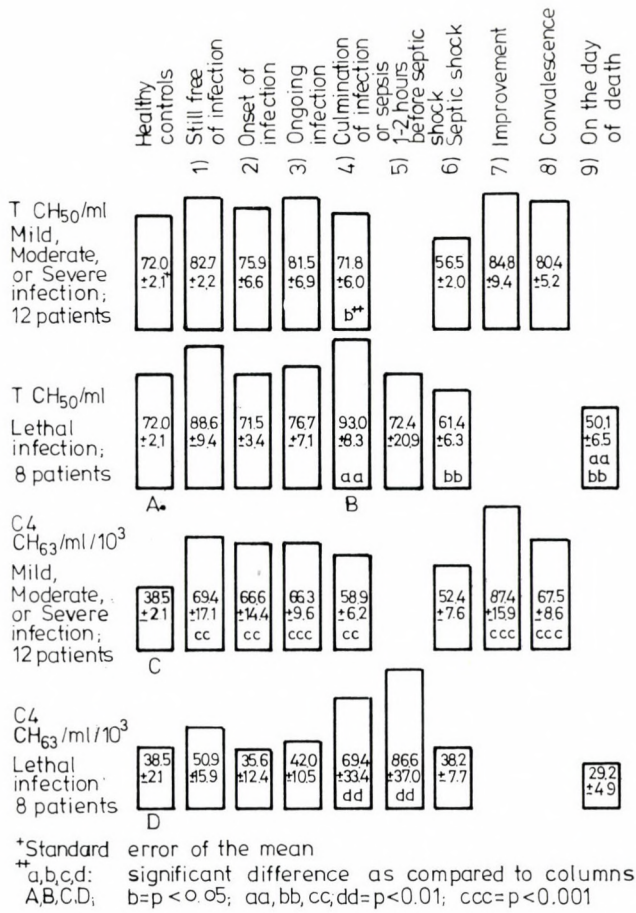

Fig. 1. Level of total complement and C4 in 20 patients before and during *P. aeruginosa* infection

more frequent than in other stages of the infection. From the 7 observed activations in lethal shock in 4 the C system has been activated by the Cl mechanism. Neither did in the remaining 4 cases including the 3 Al pathway activations, the C4 titres exceed the value of 40 000 CH_{63}/ml. The growing number of activations ran parallel with the increasing severity of infections (Table II). No activation of the C system occurred in patients still free of infection (data not shown).

Intensity of complement activation. The decrease of the level of the different C components was more expressed (lower average values) in the case of activation through the Cl than through the Al pathway (Table III). Assuming a constant production, the rate of activation, i.e. the daily decrease is probably more characteristic of the intensity of activation. Comparing

the daily decreases in titre according to activation by the Al or Cl mechanisms, activation was more rapid in the latter case in every C component (Table III). This difference was observed from the onset of infection till the development of shock and also during septic shock (data not shown), and was significant in the values of fraction C3.

Specific antibody levels during complement activation. The Ab titre of a serum sample yielding the lower C value, i.e. that obtained after activation was evaluated. From the onset of infection until the development of shock, when activation of the C system occurred by Al mechanism, the average TAb was only half of that observed with the Cl pathway. In septic shock, however, in the case of both pathways the average TAb titre was very low. The TAb level of surviving patients was always higher independently of the mode of C activation (Table IV).

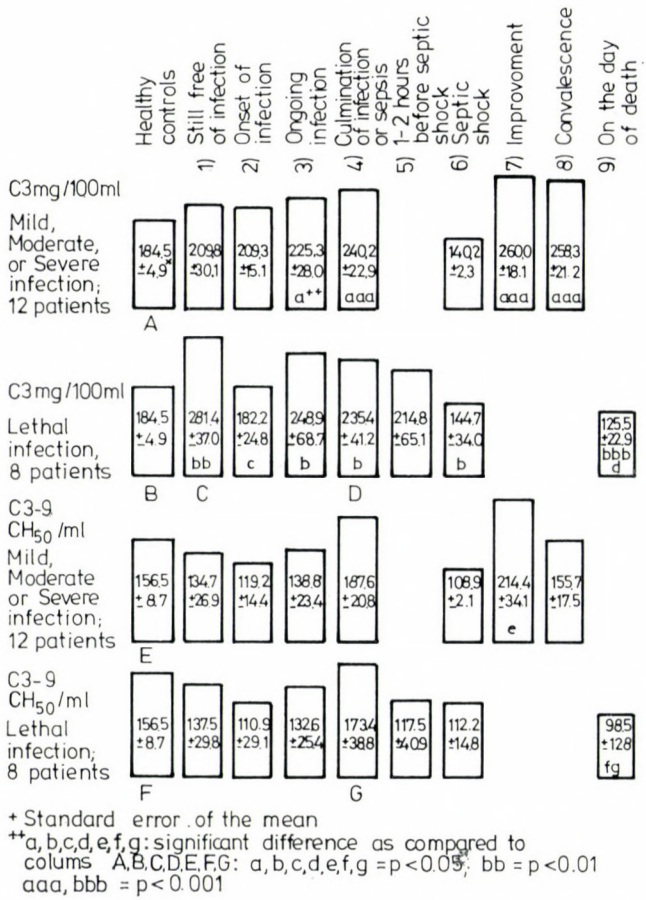

Fig. 2. Level of C3 and C3–9 in 20 patients before and during *P. aeruginosa* infection

Table II

Complement activation via classical and alternative pathways according to the severity and stages of P. aeruginosa infections

Stage*	Severity												Total (20 patients) No. of		
	Mild (3) No. of			Moderate (4) No. of			Severe (5) No. of			Lethal (8) No. of					
	serum samples	complement activations		serum samples	complement activations		serum samples	complement activations		serum samples	complement activations		serum samples	complement activations	
		Cl	Al		Cl	Al		Cl	Al		Cl	Al		Cl	Al
2	3	—	—	6	—	—	4	—	1	5	—	3	18	—	4
3	1	—	1	7	1	3	8	2	1	5	—	1	21	3	6
4	3	—	—	5	—	1	9	—	4	26	2	9	43	2	14
5	—	—	—	—	—	—	—	—	—	4	—	4	4	—	4
6	—	—	—	—	—	—	3	1	1	10	—	6	13	1	7
7	1	—	—	4	—	—	9	—	3	—	—	—	14	—	3
8	3	—	—	4	—	1	5	—	—	—	—	—	12	—	1
9	—	—	—	—	—	—	—	—	—	8	4	3	8	4	3
2–5	7	—	1	18	1	4	21	2	6	40	2	16	86	5	28
6 + 9	—	—	—	—	—	—	3	1	1	18	4	9	21	5	10
p**	—	—	—	—	—	—	—	—	—	—	—	—	—	<0.05	NS
2–9	11	—	1	26	1	5	38	3	10	58	6	26	133	10	42

* See Materials and methods — Serum samples

** Sum of stages 2–5 compared to stages 6 + 9; e.g.: 5 Cl pathway activations in 86 samples compared to 5 activations in 21 samples

Cl = classical pathway; Al = alternative pathway; NS = not significant

Table III

Average complement and mean rate of decrease in activation via the alternative or classical pathway

Parameters studied	Mode of activation	No. of activations	T (CH_{50}/ml)	C4 (CH_{63}/ml)	C3 (mg/100ml)	C3—9 (CH_{50}/ml)
Average of fractions	Al	42	71.87* ± 3.61	71 238 ± 7 889	189.46 ±12.28	129.77 ± 6.37
	Cl	10	53.89 ± 9.39	36 120 ± 8 348	129.22 ±23.09	102.56 ±10.96
	p		< 0.05	< 0.05	< 0.05	= 0.06
Daily decreases	Al	42	— 3.93 ± 0.87	+ 2 828** ± 508	—14.64 ± 2.56	—12.02 ± 2.10
	Cl	10	— 5.86 ± 2.68	—18 281 ± 3 322	—28.54 ± 7.03	—13.82 ± 4.77
	p		NS	< 0.001	< 0.05	NS

* Mean ± standard error of the mean
** Positive: increase
Al = alternative pathway; Cl = classical pathway; NS = not significant

A similar pattern was observed in the specific IgG titres with the exception that no significant difference was found even before septic shock between Al or Cl pathway activation.

Discussion

Phagocytosis and intracellular "kill" are the most important factors against *P. aeruginosa* infection [11–13]. For the perfect functioning of this system specific Ab-s and C are needed [11, 14–16].

According to recent investigations the LPS of different Gram-negative microorganisms activates in different ways and at different degrees the C system [17–20]. Moreover, differences could be observed even among strains belonging to the same species [21]. This might explain that in Gram-negative bacteriaemia, activation of the C system could occasionally be observed through either the Al or Cl pathway [22].

Different *Pseudomonas* strains did not activate the C system identically in vitro either [23]. In opsonization experiments *Pseudomonas* was found to activate by turns mainly the Al [16] or the Cl [11, 24] pathways, while most authors observed a more or less parallel activation of the other pathway, too [11, 23–25]. In our material, there was no difference among the *P. aeruginosa* O-serogroups in activating the C system by the Al or Cl mechanism.

The disadvantage of our methods is that they indicate only indirectly the activation of the C system, by the decrease of the level of different components. Thus, when the increased consumption was counterbalanced by an

increased production, no activation could be observed. At the onset of infection and in shock, however, the decrease in the C level may be ascribed not only to the consumption but also to the increased permeability of the capillaries, when part of the serum proteins is filtered out from the circulation into the interstitial space in proportion to their diffusion coefficient [26, 27]. In our lethal cases the decrease of the IgG level (molecular weight: 160 000) was 29% at the onset of infection and 35% in septic shock, whereas that of C4 (molecular weight: 206 000) 30% and 51%, that of C3 (molecular weight: 180 000) 35% and 43%, respectively, which refers to that in our patients the C components were not only filtered out from the intravascular space but had become activated. As C3 and C4 differ only slightly in molecular weight, the increased capillary permeability could not influence the observed rate of activation occurring through the Al and Cl pathways.

It is doubtful whether the direct measurement of the C3 cleavage products would have increased the number of activations. Owing to their quick turnover, a transient increase of the C3b and C3d concentration might have remained unobserved since blood sampling was performed only at 2–4 day intervals. The greater transcapillary escape rate of the cleavage products due to their molecular weight being lower than that of C3 would probably also contribute to their being left unobserved.

The present patient material was more homogeneous than that in other examinations [10, 22, 28, 29], as all the 20 patients suffered exclusively from *P. aeruginosa* infection, and none of them had C deficiency before infection (Figs 1, 2).

Similarly to other Gram-negative infections [22, 28, 29], until the development of shock the C level was either normal or elevated both in the survivors and the lethal cases except at the onset of infection, whereas in both reversible and irreversible septic shock the C titres were significantly decreased. We could confirm the observations that activation of the C system may precede the development of shock caused by Gram-negative bacteria [10, 28].

According to our data, C3, the late components and TC decreased at a higher rate i.e. more rapidly during activation of Cl than of the Al pathway (Table III). This may explain the higher efficiency of cell-lysis and opsonization occurring by activation of the Cl pathway [30–32] and might indicate that, in agreement with data in the literature [11, 23–25], not only the Cl pathway but to some extent also the Al pathway became activated and the conversion through both of them was additioned in the late components.

In our patients the ratio of Cl and Al pathway activations varied with the consecutive stages of the infection, in spite of the presence of the same *P. aeruginosa* serogroup. Many authors share the opinion that the mode of C activation is influenced by the Ab titre, i.e. in the case of a low level it occurs via the Al pathway and a high Ab concentration promotes the Cl mechanism

[20, 33–37]. Activation of the Al pathway in our cases occurred at the onset of infection on three occasions in lethal cases and once in a severe infection when the Ab titres were still low ($\bar{x} = 1/56.6$). At the beginning of lethal infections the granulocyte count decreased by 18% and the thrombocyte count by 26% (unpublished data) and the response to phytohaemagglutinin of the lymphocytes decreased significantly [38]. At the same time changes in the concentration of certain protein fractions and immunoglobulins indicated an increased permeability of the capillaries [26, 27]. The temperature of the patients rose in these days and slight circulatory disturbances could be observed. All these referred to the effect of endotoxin. One may thus assume that in our patients at the onset of infection and probably also later the activation of the Al pathway at a low Ab concentration was a consequence of the presence of LPS [37].

Table IV

Specific antibody titres at complement activation by classical and alternative pathways in Pseudomonas aeruginosa infections. Reciprocals of geometrical means

Stage of infection	Classical pathway								
	Surviving cases*			Lethal cases			Total		
	CA	TAb	IgG	CA	TAb	IgG	CA	TAb	IgG
2–5**	3	806.4 (144)	31.8 (261)	2	320.0 —	28.3 (140)	5	557.1 (139)	30.3 (203)
6 + 9	1	640.0 —	80.0 —	4	47.6 (250)	8.4 (205)	5	80.0 (253)	13.2 (282)
p§	—	—	—		—	—		NS	NS
2–9†	4	761.1 (135)	40.0 (220)	6	89.8[a] (221)	12.6 (215)	10	211.1 (205)	20.0 (239)

Stage of infection	Alternative pathway								
	Surviving cases*			Lethal cases			Total		
	CA	TAb	IgG	CA	TAb	IgG	CA	TAb	IgG
2–5**	11	726.0 (168)	59.9 (139)	17	125.3[c] (173)	12.3[c] (209)	28	249.8 (190)	22.9 (277)
6 + 9	1	640.0 —	40.0 —	9	74.1 (250)	10.8 (203)	10	91.9 (252)	12.3 (209)
p§		—	—		NS	NS		<0.05	NS
2–9†	16	612.9 (161)	48.4 (226)	26	104.4[c] (198)	11.7[b] (205)	42	204.9 (200)	20.1 (260)

* Mild, moderate and severe infection
** See Materials and methods — Serum samples
§ Significance between groups 2–5 and 6+9
† Including the data of stages of improvement and convalescence
CA = No. of complement activations; TAb = total antibody titre, variation coefficient in percent in brackets; NS = not significant
[a, b, c] Significant difference as compared to survivors: [a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$

During the development of infection until the onset of septic shock the TAb and IgG levels of the survivors surpassed two to six times those observed in the lethal cases. When in this period activation had taken place according to the Cl mechanism, the specific TAb level was more than twice higher than in the case of Al pathway activation (Table IV). Thus the high TAb concentration had in fact contributed to the activation of the Cl pathway. This was due to the fact that the PS part facing the cell surface of the LPS of *P. aeruginosa* [39] invading the circulation in sepsis, forms an immune-complex with the Ab and activates the Cl pathway [37]. Similarly as cell-lysis and opsonization, phagocytosis, kill and elimination of the bacteria from the circulation occurs more rapidly and effectively in this case [23, 30–32], so activation of the Cl pathway before the onset of septic shock at high Ab level may indicate a better prognosis.

In irreversible septic shock, activation of the Cl pathway occurred at very low TAb and IgG Ab levels. It is supposed that in irreversible septic shock the granulocytes and RES cells cannot totally eliminate from the circulation the high number of damaged microorganisms and their degradation products. This results in endotoxaemia when the lipid-A released from the decomposed bacteria and attached to Cl_q is activating the Cl pathway directly even in the absence of Ab-s [16, 19, 36, 40]. Thus, our data show that in human *P. aeruginosa* infection not the individual characteristics of the infective strain [23] but the pathological events and the specific Ab level determine the changing mode as well as the intensity of C activation during the whole process. In our cases it was not an insufficiency of the C system that was mainly responsible for the lethal outcome. The relative lack of circulating Ab-s may play the decisive role in it, inducing favourably the activation of the less efficient Al pathway.

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REFERENCES

1. Lányi, B., Bergan, T.: In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Vol 10, pp 93–168. Academic Press, London 1978.
2. Neter, E., Bertram, L. F., Zak, D. A., Murdock, M. R., Arbesman, C. E.: J Exp Med **96,** 1 (1952).
3. Ádám, M. M., Kontrohr, T., Horváth, E.: Acta Microbiol Acad Sci Hung **18,** 307 (1971).
4. Takátsy, G.: Acta Microbiol Acad Sci Hung **3,** 191 (1955).
5. Nelson, R. A., Jensen, J., Gigli, I., Tamura, N.: Immunochemistry **3,** 111 (1966).
6. Kohler, P. F., Müller-Eberhard, H. J.: J Immunol **99,** 1211 (1967).
7. Mardiney, M. R., Müller-Eberhard, H. J.: J Immunol **94,** 877 (1965).
8. Townes, A. S., Stewart, U. R.: Bull Johns Hopkins Hosp **117,** 349 (1965).
9. Mayer, M. M.: In Kabat, E. A., Mayer, M. M. (eds): Experimental Immunochemistry. 2nd ed. Charles C Thomas, Springfield, Ill. 1961. Pp 133–240.
10. Füst, G., Petrás, Gy., Ujhelyi, E.: Clin Immunol Immunopathol **5,** 293 (1976).
11. Young, L. S., Armstrong, D.: J Infect Dis **126,** 257 (1972).

12. Bjornson, A. B., Michael, J. G.: J Infect Dis **128S**, S182 (1973).
13. Crowder, J. G., Devlin, H. B., Fisher, M., White, A.: J Lab Clin Med **83,** 853 (1974).
14. Johnston, R. B., Klemperer, M. R., Alper, C. A., Rosen, F. S.: J Exp Med **129,** 1275 (1969).
15. Bjornson, A. B., Michael, J. G.: Infect Immun **5,** 775 (1972).
16. Bjornson, A. B., Michael, J. G.: J Infect Dis **130S**, S119 (1974).
17. Loos, M., Bitter-Suerman, D., Dierich, M.: J Immunol **112,** 935 (1974).
18. Morrison, D. C., Ulevitch, R. J., Henson, P. M., Cochrane, C. G.: J Reticuloend Soc **18S,** A23 (1975).
19. Ulevitch, R. J., Cochrane, C. G., Henson, P. M., Morrison, D. C., Doe, W. F.: J Exp Med **142,** 1570 (1976).
20. Morrison, D. C., Kline, F.: J Immunol **118,** 362 (1977).
21. Leist-Welsh, P., Bjornson, A. B.: Infect Immun **26,** 99 (1979).
22. Robin, M., Intrator, L., André, C., Lagrue, G., Rapin, M.: Nouv Presse Méd **3,** 883 (1974).
23. Peterson, P. K., Kim, Y., Schmeling, D., Lindeman, M., Verhoef, J., Quie, P. G.: J Lab Clin Med **92,** 883 (1978).
24. Forsgren, A., Quie, P. G.: Infect Immun **10,** 402 (1974).
25. Inada, K.: Jpn J Exp Med **50,** 13 (1980).
26. Petrás, Gy., Kiss, S., Juraszek, J., Merétey, K.: Acta Microbiol Acad Sci Hung **25,** 249 (1978).
27. Petrás, Gy., Merétey, K.: Zentralbl Bakteriol [A] **245,** 96 (1979).
28. Fearon, D. T., Ruddy, S., Schur, P. H., McCabe, W. R.: N Engl J Med **292,** 937 (1975).
29. McCabe, W. R.: N Engl J Med **288,** 21 (1973).
30. May, J. E., Green, I., Frank, M. M.: J Immunol **109,** 595 (1972).
31. Mergenhagen, S. E., Snyderman, R., Philips, J. K.: J Infect Dis **128S,** S86 (1973).
32. DesPrez, R. M., Bryan, C. S., Hawiger, J., Colley, D. G.: Infect Immun **11,** 1235 (1975).
33. Klemperer, M. R., Austen, K. F., Rosen, F. S.: J Immunol **98,** 72 (1967).
34. Bokisch, V. A., Top, F. H., Russel, P. K., Dixon, F. J., Müller-Eberhard, H. J.: N Engl J Med **289,** 996 (1973).
35. Mayer, M. M.: Sci Am **299,** 54 (1973).
36. Young, L. S.: J Infect Dis **130S,** S111 (1974).
37. Cooper, N. R., Morrison, D. C.: J Immunol **120,** 1862 (1978).
38. Petrás, Gy., Gallyas, A., Merétey, K.: Zentralbl Bakteriol [A] **248,** 374 (1980).
39. Meadow, P. M.: In Clarke, P. H., Richmond, M. H. (eds): Genetics and biochemistry of Pseudomonas. Wiley and Sons, New York 1975. pp 67–98.
40. Galanos, C., Rietschel, E. T., Lüderitz, O., Westphal, O.: Eur J Biochem **19,** 143 (1971).

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INTEREFERENCE BETWEEN HUMAN HEPATITIS A VIRUS AND AN ATTENUATED APATHOGENIC AVIAN VIRUS*

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The effect of an attenuated apathogenic avian bursa virus on the course of human hepatitis A viral infection was studied in marmoset monkeys. The monkeys were infected with human hepatitis A virus, then superinfected with avian bursa virus one and three weeks after initial inoculation with human hepatitis A virus. The superinfected monkeys did not show the characteristic serum glutamic pyruvic transaminase (SGPT) elevation. Also their liver biopsies showed no pathologic changes. The virus control animals exhibited six times higher SGPT enzyme elevation than the superinfected groups, and hepatitis was detected by histopathology. This experiment, as known to us, is the first in which a definite interference was documented using a nonpathogenic virus against a highly pathogenic and clinically significant human virus. This should be considered a successful experiment demonstrating that the use of an apathogen virus for the cure of a virus-induced disease is a realistic possibility.

Although intensely studied for the last sixty years, viral hepatitis remains a major public health problem. Viral hepatitis is caused by several different viruses. Hepatitis A virus (HAV) accounts for 60–80% of the reported cases, and hepatitis B virus (HBV) is responsible for 10–30% [1]. In addition, based on the lack of HAV and HBV antibodies, the existence of at least a third type of viral hepatitis (non-A, non-B) and possible others were suggested [2]. Hepatitis A is transmitted primarily by the fecaloral route and occurs endemically and epidemically. The transmission takes place by contaminated water, food and drink [3]. Hepatitis B is transmitted by direct inoculation and by close personal contact, and is usually endemic. Hepatitis non-A, non-B is currently recognized primarily in post-transfusion patients [3].

HAV infection remains a major public health problem. There is no vaccine or special treatment available. Marmoset monkeys are a suitable experimental model for studying HAV [4]. The virological, serological and

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pathological changes that characterize HAV infections and type A hepatitis in human patients can be reproduced experimentally in these animals [5]. However, the signs and symptoms are less remarkable in monkeys than in humans. The aim of this study was to determine whether the infection of marmosets by HAV could be altered by administering an avian bursa vaccine [6] postinfection.

Materials and methods

Human hepatitis A virus. Faecal specimens were obtained during the acute phase of hepatitis A (MS-1 prototype) from an infected chimpanzee. Inocula for intramuscular inoculations consisted of a 5% fecal suspension (w/v) diluted in phosphate-buffered saline (pH 7.2), clarified (300 g for 30 min.), filtered (0.45 nm filter prewashed with Hank's balanced salt solution containing 1% albumin). The inoculum was diluted 1 : 500 and 0.5 ml inoculated per animal. This dose of virus induces hepatitis in inoculated animals usually within five to six weeks.

Avian bursa vaccine. Marmoset monkeys were inoculated with the Bursa Disease Vaccine (Sterwin Laboratories, Inc. Millsboro, Delaware; Bursa-vac No. G-603). This vaccine is used for the prevention of avian bursa disease. Each marmoset monkey was inoculated with 50 units orally and 50 units intranasally on days described below.

Animals. In an exploratory study before the start of the main experiment, the effect of avian bursa virus was studied in four adult, wild- caught and/or colony-born and reared red-bellied marmoset monkeys *(Saguinus labiatus labiatus)*. Four monkeys served as uninoculated controls.

In the main experiment, the monkeys were divided into five experimental groups of four animal each. In each animal the baseline enzyme values were determined prior to inoculation. Baseline data of three consecutive weeks included serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). Percutaneous liver biopsies were performed on the third week. Each group was treated in this following manner: Group 1, inoculated with saline; Group 2, inoculated with HAV; Group 3, inoculated with HAV and treated with bursa vaccine one week post-inoculation; Group 4, inoculated with HAV and treated with bursa vaccine three weeks post-inoculation; and Group 5, inoculated with HAV and treated with bursa vaccine five weeks post-inoculation. The treatments with bursa vaccina were repeated four consecutive days in each vaccinated animal.

During the experiment, each animal was bled weekly and percutaneous liver biopsies were performed bi-weekly, except for the fifth and sixth weeks. SGOT/SGPT levels were determined for each serum sample. Serum (approximately 0.2 ml) was stored at -20 °C for future evaluations. Tissue sections of the liver biopsies were histopathologically evaluated for evidence of hepatitis. All animals were sacrified at 10 weeks, and necropsies were performed. All major tissues were saved in 10% buffered formalin. In this report, we emphasized the two major parameters of viral hepatitis A, the presence or absence of elevated enzyme level (SGPT) and the presence or absence of histological evidence of hepatitis.

Results

In the exploratory experiment, there was no evidence that the avian bursa virus affected the animals. The SGPT and liver biopsies remained in normal range.

In the main experiment, the baseline data remained within a normal range. The data during the viral exposures are demonstrated in Figs 1, 2 and 3 and are described below.

Group 1. Transaminase levels remained normal and there was no histologic evidence of hepatitis throughout the experiment.

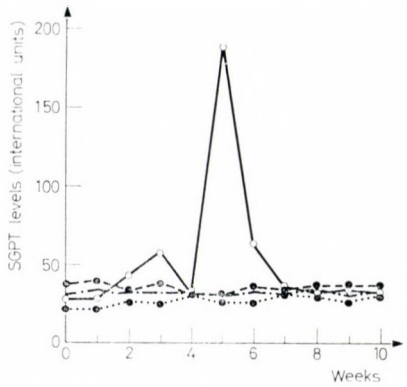

Fig. 1. Interference between human hepatitis A virus and an attenuated apathogenic avian bursa virus. ●——● Group 1, saline control ○——○ Group 2, HAV inoculated, ●····● Group 3, HAV + bursa virus 1 week post inoculation, ·—·—· Group 4, HAV + bursa virus 3 weeks post inoculation

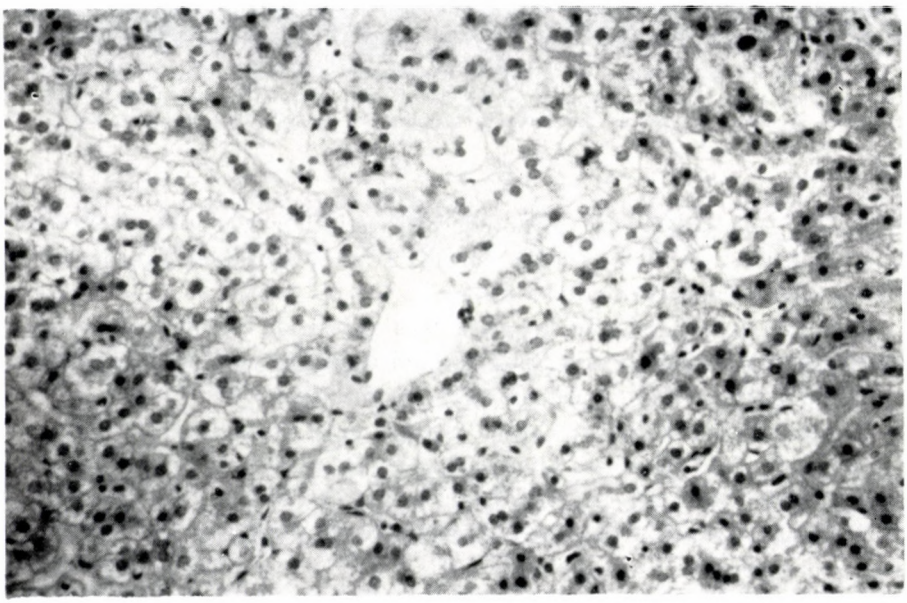

Fig. 2. Liver biopsy from marmoset monkeys inoculated with HAV. Biopsy was done five weeks after inoculation with HAV. There is focal leukocytic infiltration, hepatocellular necrosis and cellular degeneration. Haematoxylin-eosin stain, ×150

Group 2. SGPT levels remained normal until the fifth week post-inoculation. On week five, the average SGPT level was six times the baseline value and there was histological evidence of hepatitis (Figs 1 and 2). On week six, average SGPT levels were twice the baseline, value, and there was still evidence

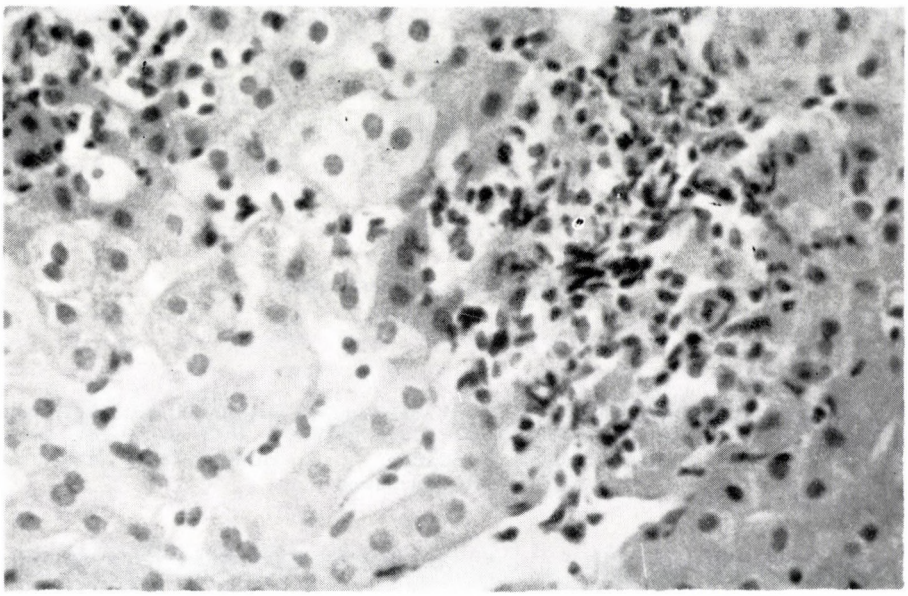

Fig. 3. Liver biopsy from marmoset monkeys inoculated with HAV followed by inoculation with avian bursa virus one week later. Biopsy was done five weeks after inoculation with HAV. The normal architecture of the liver can be recognized. Haematoxylin-eosin stain. ×100

of hepatitis microscopically, although inflammation was not as severe as seen on week five. At seven weeks, the SGPT levels and liver biopsies became and remained normal through 10 weeks.

Group 3. No evidence of hepatitis was observed; SGPT levels remained normal and liver histology remained normal through week 10 (Figs 1 and 3).

Group 4. The results were the same as for Group 3.

Group 5. SGPT levels remained normal until week five at which time there was a fivefold increase. Contamitantly, there was histological evidence for hepatitis. On week six, the SGPT levels returned to twice the baseline, and there was still evidence of hepatitis microscopically similar to that observed in Group 2 at week six. At seven weeks, the SGPT levels remained normal and liver biopsy morphology returned to normal, and it remained normal through 10 weeks.

All animals were sacrificed on week ten and autopsies were performed. No gross pathology was observed in any of the animals. Tissues from liver, lung, kidney, spleen and brain were processed and slides were made for microscopic evaluations. No liver pathology was observed and all other tissues appeared normal.

In addition to the SGPT enzymes, the SGOT enzymes were also determined. The levels of the SGOT enzymes did not show significant changes in

different groups during the course of this experiment. It should be noted that the SGPT, and not the SGOT enzymes are the most sensitive indicator of hepatitis in marmoset monkeys [5].

Discussion

One of us (LKC) made an observation that concurrent viral infections can significantly influence the outcome of viral and hypothetical viral diseases in humans [7, 8]. By systematic data collection, the most remarkable inhibitions were seen in the human herpes [7] and hepatitis viral infections. Under carefully controlled and monitored conditions the apathogenic bursa virus interfered with both the human hepatitis A and B viral infections. The interference was recognized in spite of the fact that HAV is an RNA and HBV is a DNA virus, and they differ from each other in a lot of characteristics. The bursa virus is a double-stranded RNA virus. This peculiar RNA structure of the bursa virus may possibly esplain why both hepatitis viruses are affected.

For evaluation of these observations, suitable animal models were sought to study the interference under experimental conditions. It was found that the effect of an oncogenic animal virus can be favourably influenced by human influenza virus [7]. In a preliminary experiment, it was also found that the avian encephalitis virus markedly (70%) reduced the mortality of mice preinoculated with a strain of rabies virus. The other six viruses in the same experiment — avian Newcastle, duck plague vaccine, avian infectious bronchitis, bursa virus, canine hepatitis and canine distemper — had little or no effect on mortality [9]. From this rabies experiment previously mentioned, it can be concluded than when viral interference is used, the key point is to find the suitable interfering virus which is apathogenic to the host and which is capable of eliminating the harmful effect of the pathogenic virus. In another experiment, interference was also found between Rous' sarcoma, Marek's disease and avian bursa viruses [9]. The mechanisms of interference need a great deal of further exploration. Among others, there are possibilities that the apathogenic virus interferes by (i) inhibition of the attachment of the pathogenic virus on the cellular receptor, (ii) intercellular inhibition of viral reproduction, assembly and maturation n cells by the challenging virus, (iii) interferon production, (iv) stimulation of immuno-response induced by the superinfecting apathogenic virus.

In our most recent in vivo experiment [10], we succeeded enhancing the virus interference phenomenon by adding to the interfering viruses an antihistaminic compound (chlorpromazine), [11].

The fifth group of four animals was superinfected five weeks after HAV infection. This group did not show differences in SGPT and liver histopathology from the positive control group. Very likely, the reason is that in marmoset

monkeys the detectable symptoms are already present at five weeks, similar to the illustration in Figs 1 and 2. In humans, in which the course of the diseas is more severe and lasts longer than in monkeys, the beneficial effects of the interfering virus could last probably much longer. Even when clinical signs are already present, the superinfecting suitable virus is capable of interrupting the pathological process in any phase of the disease. Experiments have been initiated using animal models for the study of the effects of apathogenic viruses on human hepatitis B and non-A, non-B viral infections.

REFERENCES

1. Byran, J. A., Gregg, M. B.: Am J Med Sci **270**, 271 (1975).
2. Conrad, M. E.: Am J Hematol **1**, 356 (1976).
3. Deinhardt, F.: Am J Clin Pathol **65**, 890 (1976).
4. Deinhardt, F., Holmes, A. W., Capps, R. B., Popper, H.: J Exp Med **125**, 673 (1967).
5. Deinhardt, F.: Adv. Virus Res., **20**, 113 (1976).
6. Hitchner, S. B.: In Hofstad, M. S. (ed): Diseases of Poultry, 7th ed. (1978).
7. Csatary, L. K., Romvary, J. J., Toth, B., Tauber, L. N.: J Med **13**, 1 (1982).
8. Csatary, L. K., Lancet **2**, 825 (1971).
9. Csatary, L. K., Romvary, J. J.: Under publication (1984).
10. Csatary, L. K., Romvary, J. J.: Under publication (1984).
11. Csatary, L. K., Lancet **2**, 338 (1972).

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YEAST AS PROTEIN SOURCE FOR HUMAN NUTRITION (A REVIEW)

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(Received March 7, 1983)

Introduction

The increasing pressure of population and predicted world food shortage have created a demand for new protein sources for human nutrition. This has mainly been seen in underdeveloped countries where increasing malnutrition is an important problem requiring an urgent solution.

Intensive research has been carried out to find and develop cheap sources of protein such as yeast, fungi, bacteria and algae. Biomass containing at least 50% of protein can be produced by each of these groups of microorganisms and the term Single Cell Protein (SCP) has therefore been applied to the products. Nevertheless, due to their high content of protein and their rapid growth on a variety of substrates, microorganisms are potentially a significant source of protein for nutritional use [1]. The main incentive for the widespread research on their utilization as food is provided by authoritative prognoses concerning world food supplies and population increase [2].

Since 1967, conferences and symposia on Single Cell Protein have pointed out some problems concerning many toxicological, psychosocial, physiological and nutritional factors associated with the use of SCP. Furthermore, several authors [2–8] have summarized most of the knowledge of different types of SCP as a source of food for human and animal nutrition. Many economic, processing, nutritional, safety, regulatory and functional criteria must be fulfilled before SCP will be generally available and acceptable.

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SCP for human food has been successfully accepted when grown on various sources of substrate such as molasses, starch, hydrocarbons, waste by-products, ethanol and methanol [2, 9–15]. But the use of SCP directly as human food may be hampered by several serious shortcomings [16]. Most of SCP sources contain pharmacoactive or toxic substances.

Food yeast is the only example of SCP which is currently produced for use as human food [17]. Nowadays the most popular food yeasts are coming from *Saccharomyces* and *Candida* genera and their potential as a source of protein for human consumption will be analysed below.

Yeast technological processes

Keeping in mind the above considerations, much of the research and development on SCP has been concentrated on the production of biomass. For potential human use, however, biomass from yeast should be regarded as a protein-rich raw material requiring further processing before it would become valuable as a high protein food [4]. It has been demonstrated that protein concentrates or isolates prepared from SCP have a better nutritional quality than the original intact cell [1, 2] because of their nucleic acid content, the presence of undesirable physiologically active components, the deleterious effects of cell wall material on protein bioavailability and the lack of requisite and discrete functional properties of intact cells [4].

Many methods have been developed to improve the overall nutritional properties and efficiency of yeast protein [1, 2, 18–21].

Most of the technological processes generally begin by rupturing the cell. This is followed by protein extraction by one or a combination of various methods. Cell rupture and protein extraction are made by means of endogenous or exogenous enzymes, mechanical devices, chemical solvents or using physico-chemical properties [22–29]. For yeast cell rupture Asenjo and Dunnill [30], have recently described an economic and elegant method in which they isolated a complex of yeast-lytic enzyme on a pilot scale from *Cytophaga* species and immobilized this enzyme complex on soluble polymeric carbohydrates from which they obtain further rupture of yeast cells. Mosqueira et al. [31] have developed a mechanical-centrifugal study of bakers' yeast which showed a good correlation between laboratory and industrial scale of protein functional properties. Other methods have also been discussed in the literature [4, 32–36].

Nevertheless, all these methods are time consuming and fail to produce a yeast protein concentrate or isolate with certain established safety and superior nutritional quality. In spite of this, PAG guideline No. 12 [37] is a general guide for producers on processes for products from SCP for human

consumption. The end product must be submitted to intensive evaluations in order to prove with certainty its safety and good nutritional characteristics for man [38, 39].

Another procedure is frequently needed for nucleic acid reduction to levels required for human consumption. A process of producing isolated yeast protein low in nucleic acid by adding exogenous nucleases to protein separated from ruptured yeast cells, has been reported [26] and, Limblom [32] and Hedenskog and Ebbinghaus [40] showed a decrease in nucleic acid by combining ionic strength, heat treatment and acid precipitation with alkaline protein extraction. Sinskey and Tannebaum [41] reviewed the possible advantages

Table I

Nucleic acid content, per cent, in some single cell protein sources

Candida lipolytica	9.0—11.5
Saccharomyces cerevisiae	6.0— 8.0
Saccharomyces carlsbergensis	6.0— 7.5
Candida tropicalis	8.5— 9.7
Candida utilis	7.5—10.5
Pseudomonas sp.	17.0—18.5
Spirulina	3.3— 4.6

and disadvantages of the methods for nucleic acid removal from SCP. From all of these papers it is clear that the known processes for nucleic acid reduction have not solved the problem of obtaining a suitable final product.

Conventional procedures of the reduction of nucleic acid and extraction of yeast protein can produce extensive proteolysis by endogenous proteases reducing the total yield of protein. Nucleic acids constitute one of the main factors limiting the use of yeast as a food source for human nutrition. The content of nucleic acids in various microorganisms is shown in Table I.

Uric acid is the final metabolic product formed in man from the purine moiety of nucleic acids. In man the level of uric acid in plasma and urine is affected by two main factors: the purine and protein contents.

Uric acid is insoluble at physiological pH. When the blood uric acid level is high, crystals may form in the joints, causing gout or gouty arthritis [16], because man lacks the enzyme uricase for oxidizing uric acid to the substance allantoin which is eliminated in urine. Thus the nucleic acid in yeast cells must be reduced by any process since the daily intake of nucleic acid from yeast should not exceed 2 g on dry weight basis [42]. Many procedures for nucleic acid reduction have been assayed [20, 25, 26, 43–45].

A well-known problem is that some SCP products from technological processes provoke alterations of the proximal part of the renal tubuli, being the nephrotic factor induced by treatment with 0.1 N sodium hydroxide

during manufacturing. This fact has been discovered to be due to lysinoalanine, a toxic derivative of lysine [46–48].

During yeast treatment denaturation of protein, racemization of amino acids, beta-elimination and cross-linking of certain amino acids and formation of potentially antinutritive compounds, can be a serious problem because functional proterties are significantly destroyed and utilization of yeast protein is limited in food application [4, 49, 50]. Functional properties of yeast proteins must be evaluated if one wants to know how these new proteins behave in specific food systems and if they can be used as an additive in diet, to replace or simulate conventional proteins in different foods and their capacity for production of new proteinaceous foods [4]. Characteristic properties of solubility, flavour and texture together with the foaming and emulsifying capacity of yeast proteins have extensively been studied [21, 34, 51, 52] although we should need more information about the functional properties of yeast if one would wish to introduce it in the food system.

The yeast cell wall is another factor significantly affecting the nutritional value of yeast. An important decrease in yeast digestibility occurs because the cell wall does not allow the action of gastrointestinal hydrolases.

The molecular structure of the yeast cell wall has been postulated by Kidby and Davies [53], who described a net formed by a complex of the polysaccharides glucans and mannans with a disulfide bridge containing protein as a linking point. This structure is very difficult to break. It can be hydrolysed by specific enzymes present in the intestinal tract of some snails.

Apart from decreasing the digestibility and, of course, the nutritional value, the cell wall may cause adverse reactions such as allergic effects, toxicological reactions, gastrointestinal disturbances, etc., which may also impair the biological value of yeast. Ernst et al. [54] showed in a series of metabolism studies where rats were fed a diet containing 10% protein provided by whole yeast or destroyed yeast cell wall that intact yeast was significantly lower in biological value than the destroyed yeast cell wall. At the same time Ocio and Viñaras [55] concluded that whole brewer's yeast impaired body weight gain in rats due to difficulty in digestive hydrolysis of the yeast cell wall.

Thus, a process to disrupt or modify the cell wall structure is necessary for most of the yeast strains. In this sense, Kinsella and Shetty [4] have developed a method employing succinylation to render a yeast protein with improved functional properties and a low nucleic acid level with reducing degradation of proteins by endogenous proteases during incubation. In another paper [1] they have discussed the effect of soluble thiol reagents on the extractability of protein from yeast cells. The thiol reagents activated significantly the activity of glycosidases and endogenous proteolytic enzymes. They showed that reduction of disulfide bridges of the protein in the cell wall by thiol reagents, may facilitate the release of the bound inactive glycosidases in the

active form and, at the same time, these reagents may cause destruction of specific polypeptide inhibitors of the endogenous proteases.

Thus, some improved method for yeast protein concentrates or isolates with many applications for industrial food must be developed in the near future.

Toxicological aspects

Because of technological processes, the yeast protein may result in an undesirable or harmful product since it is exposed to treatments which may produce toxicological effects. The toxicological aspects of the use of solvents in food technology are summarized in the 14th report of the Joint FAO/WHO Expert Committee on Food Additives [56] as follows: (a) treatment with solvents may affect the nutritive value of foodstuffs, (b) residues of solvents may have toxic effects, (c) impurities in solvents and additives to solvents may remain in the extracted food and have toxic effects, and (d) a solvent may react with the constituents of a foodstuff to form toxic products.

Many authors have shown the effects of the most frequently used treatments for SCP production as protein modification or food conventional impairment up to a certain toxic level [2, 57–59]. For example, toxicological consequences of the Maillard reaction on food products have extensively been reviewed in animal experiments [60]. Alkali-treated proteins and lysinoalanine products were found to cause a renal alteration characterized by nuclear and cytoplasmic enlargements, with alterations in DNA synthesis, mitosis and nucleoproteins [46, 61]. The nephrotic factor was found to be heat stable [7].

Toxicological tests employ more or less similar schemes [62] which are summarized in Table II. In spite of this, several national and international organizations have elaborated guidelines and protocols for the various types of food additives and contaminants [38, 39, 63–73]. These guidelines and protocols serve as a general recommendation rather than as a series of mandatory procedures for testing novel sources of proteins.

Table II

Toxicological test for single cell protein
De Groot [73]

Test	Time
Sub-acute feeding test	2 weeks
Sub-chronic study	3 months
Chronic study	2 years
Multigeneration study	3 generations
Teratogenicity study	20 days
Mutagenicity study	15—20 days

Some yeast samples were subjected to subchronic toxicological studies in rats by BP Proteins Ltd [74], other studies were performed in pigs, chickens, dogs, rabbits and cats. It was concluded that alkane-grown yeasts do not have toxicological or deleterious effects and similar results were obtained with the same yeasts in one and two-year feeding studies in rats [75–78].

Furthermore, these results have only permitted yeast grown on hydrocarbons to be used for feed because the principal factors which impede its generalized use in human food are safety and tolerance, although other crucial factors are also involved. For instance they do not exceed the level of amino acids required for normal tissue function, because various metabolic mechanisms do not permit such an excess. Intake of large amounts of amino acids may produce toxic reactions in which the plasma concentration of the administered amino acid rises to very high levels [79]. In Table III the factors involved are summarized; for tolerance the data in Table IV must be taken into account.

Table III

Safety factors involved in evaluation of yeast protein
FAO/WHO PAG guideline [38]

Nutritional adeyuacy of test diet
Identity of test animals
Natural toxicants
Microbiological toxins
Extraction residues
Multilevel feeding of test protein
Comparability of thest and basal diets
Highest feasible feeding level of test protein

Table IV

Tolerance of yeast protein
Yudkin [106]

Social factors	Psycho-physiological factors
Religious and social habits	Inheritance
Social class	Allergicity
Instruction in subject of nutrition and hygiene	Acceptability
Publicity	Palatability

Mauron [80] in 1975 wrote: "neither yeast, bacteria nor fungi will make their entry into food during the next years for safety reasons and because there is no need and no commercial demand for them as long as the huge plant protein reservoir is not better used for food purposes". I believe safety

reasons, at least in yeast, may be obtained and the last years have shown that there is a need of another new source of protein than plant protein and mankind has in this moment the possibility to find them.

Nutritional aspects

Nutritional value of food protein must be predicted in order to establish its quality. Quoting PAG guideline No. 6 [38] "Ideally, the nutritional evaluation of protein foods should be made in relation to their potential role in the diet of the population for whom they are intended. Whereas this would preclude assigning a single numerical rating to a given protein which under all conditions of use would avoid the fallacy of assuming that such a rating, based on a single level as the sole source of nitrogen to the test animal, provides a true measure of its nutritional value as a supplement to the diet or to specific food for man". Determination of protein quality is conducted with a variety of biological, chemical or microbiological methods. Table V shows the methods used to determine the nutritional quality of a food protein. Most of these methods have, however, some advantages with respect each other and none of them may by itself prove the final nutritional quality of a protein. To set out the nutritional quality of a protein, a battery of these methods must be performed, preferably on different animal species although the rat is more widely used than others. Nevertheless, there is much controversy about the adequacy of the various procedures [81] because many authors believe that there are only two parameters to evaluate the nutritive value, growth and nitrogen balance.

In yeast many nutritional evaluation have been performed in animals, and have shown that yeast has an acceptable nutritional value as a source of protein for feeding purposes [2, 55, 82–84]. Nutritional value for some strains of yeast is shown in Table VI.

Table V

Classical methods of nutritional quality measurement

Chemical test	Reference	Biological test	Reference
Chemical Score (CS)	[107]	Protein Efficiency Ratio (PER)	[115]
Essential Amino Acid Index (EAA)	[108]	Nitrogen Protein Utilization (NPU)	[116]
Available Amino Acid	109, 110, 111]	Liver Protein Utilization (LPU)	[117]
		Biological Value (BV)	[118, 119]
Digestibility in vitro	[112, 113, 114]	Net Protein Ratio (NPR)	[120]

Table VI

Nutritional value of different yeast strains

Species	BV	D	NPU	PER
Saccharomyces cerevisiae	58.8	80.7	47.5	1.99
Saccharomyces carlsbergensis	66.5	84.7	55.8	2.24
Strawberry yeast	65.6	76.3	50.1	2.30
Candida utilis	42.8	83.3	35.7	1.76

On the other hand, there are numerous methods for nutritional evaluation. Many authors have tried to develop new single, economical, rapid and potential methods for testing food while it is manufactured and to assess the nutritional quality of the end product. Harper [85] wrote that "There are two main reasons for developing methods for protein evaluation. The first is to provide a procedure for ranking protein according to its efficiency of utilization under a set of standard conditions. The second is to predict the efficiency of utilization of protein as a source of nitrogen and amino acids for meeting the amino acid requirement of man and animals. These are not different ways of saying the same thing, as is often thought, but are distinctly different objectives". In this sense I want to call attention to the work of Pion [86] who studied the relationship between the blood and muscle levels of lysine, threonine, isoleucine and methionine and the levels of these amino acids in the original diet; he found that growth and intake were affected only when the amino acid levels were lower than the requirements supposedly due to the nutritional quality of the diet and he measured the efficiency of utilization of protein as a source of nitrogen and amino acids.

Omstedt et al. [87] utilized the measurement of protein synthesis in vitro, as a rapid biological evaluation of protein quality to evaluate the effect of processing on the nutritional value of *Saccharomyces cerevisiae*. They demonstrated that incorporation of radioactive amino acids into protein by muscle ribosomes is proportional to the quality of the test protein source after feeding rats for 6 days. With this method they found a good comparison with the nitrogen efficiency ratio (NER). Because of environmental and seasonal factors there are, however, variations in the level of protein synthesis between experiments. They have attempted to avoid this by including standard diets as reference in each experiment. But that did not solve the problem because there are variations between the values obtained in different experiments with the reference diets casein and wheat gluten.

Eggum [88] estimated the blood urea level after 5 days feeding of rats as an indicator of protein quality. He found that the blood urea level decreased with increasing quality of the protein in the diet. But the experimental condi-

tions must be standardized, especially in relation to the protein content of the diet and to the time after feeding at which blood samples are taken. The nutritional consequences of the Maillard reaction have also been analysed. Availability of some essential amino acid is strongly reduced in rats by the formation of the Amadori compound derivatives [89–93]. These compounds are formed by reaction of sugar carbonyl groups with the amino group of the amino acids in presence of an alkali medium or by storage of dehydrated and semi-moist foods [94]. The Maillard reaction has also been studied from the point of view of physical consequences on food. Browning is one of the major problems leading to a darkening of protein colour, insolubility, and the subsequent nutritional loss and a bitter flavour [94]. For these reasons processed foods must be evaluated for biological quality and physico-functional properties, and also for toxicological effects.

Thus, from analysis in animals, extrapolation of nutritional data to human beings is more acceptable than are the toxicological ones, especially if they were performed on animals having nutritional aspects similar to those of humans. As PAG guideline No. 7 [39] recommends, final evaluation must be realized on humans. The nutritional test recommended by guideline No. 7 for human evaluation are: Biological Value, Digestibility and Net Protein Utilization; they are to be performed in the same manner as in animal evaluation. Unfortunately, limited information has been published concerning humans fed yeast.

Chemical and biological evaluation of the nutritive value of biomass and protein concentrate from *Candida utilis* have been done by Kuzela et al. [95, 96]. They have measured in weanling rats the digestibility, biological value, NPU, and PER, and have suggested the potentialities of the protein concentrate of *C. utilis* and other similar preparations in meeting the protein needs of domestic animals.

Wolf et al. [97] evaluated the biomass of *Torula* and a *Saccharomyces* extract by the measurement of EAA, PER, and NPU for establishing some recommendations about the criteria that must be followed for acceptability of these yeasts for human food.

The nutritional effect of modified proteins by chemical treatment were also analysed [98, 99]. It was found that modified essential amino acids become partially unavailable when these proteins are given to rats and compared with rats fed unmodified proteins. An excess of amino acids in the diet may also produce adverse nutritional consequences. Antagonisms and amino acid imbalances arise from feeding one amino acid in excess inducing a poor utilization of the diet [79].

Human fed yeast test

Since the first years of this century, experimental tests were done to explore yeast consumption as a food for humans [100]. During World War I and II many thousand tons of yeast were consumed in Germany and Japan by the army, prisoners of war and the civilian population [3]. Nevertheless, all these trials showed contradictory results and we have little information about the factors responsible for the unfavourable reactions to the feeding of several types of SCP to human subjects.

It has been reported that humans fed molasses yeast of not more than 15 g daily showed unacceptable gastrointestinal reactions [101, 102]. It is believed that the agent employed to avoid foam and microbial contamination was responsible for the reaction. Various trials were performed in the Massachusetts Institute of Technolog to investigate the effect of feeding different amounts of yeast to human beings [3, 103]. They fed batches of *Torula* yeast grown on sulphite and glucose media in several different experiments on human volunteers. No gastrointestinal disturbances were observed in 72 subjects fed 45 to 135 g *Torula* yeast daily for 60 days, while 12 to 23 individuals consuming the sulphite-grown yeast in quantities of 90 or 135 g daily, respectively, developed a painless and apparently self-limiting desquamation of the skin of the palms and soles. This disappeared spontaneously before or soon after the yeast feeding had been discontinued. An antigen prepared from the yeast did not evoke a dermal reaction in any subject, so the response was not due to an allergic process. It was assumed that some impurities in the culture medium were the cause of the effect.

Another trial of this group was the evaluation of two new single cell products, one a bacterium and another a yeast grown on ethanol as substrate. Two groups of 50 subjects were fed 12 and 20 g, respectively, per day. There was no indication of any problem in an extensive program of preclinical testing in different animal species. Over 80% of the experimental subjects consumed the material daily during a 6-month tolerance trial without demonstrable consequence. However, nearly 20% developed sudden nausea, vomiting, diarrhoea and prostration after consuming the material uneventfully for a few days or a few weeks.

In the same study, tests were performed of serum total protein, albumin, alkaline phosphatase, glutamate oxalacetic transaminase, glutamate pyruvic transaminase, glucose and uric acid; and urinary sugar, acetone, 17-hydroxysteroids, 17-ketosteroids and microscopic examination of the sediment; all tests gave normal values.

Later Abrahamsson et al. [104] performed an experiment with yeast grown on ethanol at the same intake level but only for 6 weeks. Only one of their 20 subjects developed nausea and vomiting during the sixth week.

After an interval of two weeks, without consuming the yeast or experiencing symptoms, the same subjects were again fed the material and both nausea and vomiting returned.

In some primitive communities large quantities of yeast are consumed as a current food, but it is not known whether they suffered gastrointestinal and dermal reactions. Still, according to the Thirteenth Expert Committee FAO/WHO [105], traditional use of a material does not constitute evidence of its safety.

In other studies of human feeding of certain SCP sources have shown other pathophysiologic reactions such as pain and oedema of the great toe [16]. These symptoms may be due to the high nucleic acid content of the cells used in these trials.

Conclusion

Extensive information on yeast as a source for human nutrition is still lacking in spite of the great interest in the field. In any case, we have to take into account that yeast for human consumption must meet the following requirements: (a) specific chemical and functional properties, (b) a prescribed level of safety and (c) good nutritional characteristics. The last two points must be evaluated in animals and humans. Should these points be reached successfully, then we shall be able to recommend the introduction of yeast protein into the human food system.

REFERENCES

1. Shetty, K. J., Kinsella, J. E.: Biotech Bioeng **20,** 755 (1978).
2. Kihlberg, R.: Ann Rev Microbiol **26,** 427 (1972).
3. Scrimshaw, N. S.: In Tannenbaum and Wang (eds): Single Cell Protein II. MIT Press, Cambridge, Mass. 1975, p. 24.
4. Kinsella, J. E., Shetty, K.J.: In M. Friedman (ed.): Nutritional Improvement of Food and Feed Proteins. Plenum Press, New York 1978, p. 38.
5. Tannenbaum, S.: Single Cell Protein. Mateles and Tannenbaum (eds) MIT Press, Cambridge, Mass. 1968, p. 343.
6. Pokrovsky, A.: In Tannenbaum and Wang (eds): Single Cell Protein II. MIT Press, Cambridge, Mass. 1975, p. 475.
7. Oser, B. L.: In Tannenbaum and Wang (eds): Single Cell Protein II. MIT Press, Cambridge, Mass. 1975, p. 484.
8. Shacklady, C.A.: In Tannenbaum and Wang (eds): Single Cell Proteins II. MIT Press, Cambridge, Mass. 1975, p. 489.
9. Cooney, C.L., Levine, D.W., Snedecor, B.: Food Technol **29,** 32 (1975).
10. Litchfield, J.H.: Food Technol **31,** 175 (1977).
11. Rose, A., Morrison, J.S.: The Yeast. Vols 1–3. Academic Press, New York 1971.
12. Shacklady, C.: Outlook Agric **6,** 102 (1970).
13. Tannenbaum, S.: In Whitakers and Tannenbaum (eds): Food Proteins. Avis Publ. Co, Westport, Conn. 1977, p. 315.
14. Gounelle de Pontanel, H.: In Guenelle de Pontanel, H. (ed.): Les levures cultivées sur alcanes. Symposium d'Aix en Provence. Paris 1972.

15. Humphrey, A.E.: In Tannenbaum and Wang (eds): Single Cell Protein II. MIT Press, Cambridge, Mass. 1975, p. 1.
16. Calloway, D.H.: Single Cell Protein. Davis, P. (ed.) Academic Press, New York 1974, p. 129.
17. Worgan, J.T.: In Porter and Rolls (eds): Protein in Human Nutrition. Academic Press, New York 1973, p. 47.
18. Johnson, J.C.: Yeast for food and other purposes. Noyes Data Corp, Park Ridge, New York 1977.
19. Maul, S.B., Sinskey, A.J., Tannenbaum, S.R.: Nature **228,** 181 (1970).
20. Robbins, E.A., Sucher, R.W., Schuldt, Jr., Sidoti, E.H., Seeley, D.R., Newell J.A.: U.S. Patent No. 3,887,431 (1975).
21. Linbolm, M.: Biotech Bioeng **16,** 1495 (1974).
22. Morgen, L., Lindblom, M.: Biotech Bioeng **16,** 261 (1974).
23. Phaff, H.J.: In Feeney and Whitaker (eds): Food Proteins. Am Chem Soc. Washington, D.C. 1977, p. 244.
24. Machek, F., Fencl, Z., Beran, K., Behalová, B., Sillinger, V., Kejmar, J.: Biotech Bioeng Symp No. 4, 977 (1974).
25. Imada, A., Sinskey, A.J., Tannenbaum, R.: Biotech Bioeng **14,** 103 (1972).
26. Engler, C.R.: Biotech Bioeng **23,** 765 (1981).
27. Newell, J.A., Robbins, A.E., Seeley, R.D.: U.S. Patent No. 3,886,555 (1975).
28. Engler, C.R.: Biotech Bioeng **23,** 765 (1981).
29. Edebo, L., Magnusson, K.E.: Pure Appl Chem **36,** 3 (1973).
30. Asenjo, J.A., Dunnill, P.: Biotech Bioeng **23,** 1045 (1981).
31. Mosqueira, F.G., Higgins, J.J., Dunnill, P.: Biotech Bioeng **23,** 335 (1981).
32. Linblom, M.A.: Lebensmitt Wiss Micro-Technol **7,** 295 (1974).
33. Vananuvat, P., Kinsella, J.E.: Biotech Bioeng **20,** 1329 (1978).
34. Vananuvat, P., Kinsella, J.E.: J Agric Food Chem **23,** 613 (1975).
35. Lee, C.H., Tsang, S.K., Ukrakube, R., Rha, C.K.: Biotech Bioeng **21,** 1 (1979).
36. Hedenskog, G., Mogren, H.: Biotech Bioeng **15,** 129 (1973).
37. FAO/WHO PAG guideline No. 12. On the production of single cell protein for human consumption. Geneva 1970.
38. FAO/WHO PAG guideline No. 6. For preclinical testing of novel sources of protein. Geneva 1970.
39. FAO/WHO PAG guideline No. 7. For human testing of supplementary food mixture. Geneva 1970.
40. Hedenskog, G., Ebbinghaus, L.: Biotech Bioeng **14,** 447 (1972).
41. Sinskey, A.J., Tannenbaum, S.R.: In Tannenbaum and Wang (eds): Single Cell Protein II. MIT Press, Cambridge, Mass. 1975, p. 158.
42. Edozien, J.C., Udo, U.U., Young, V.R., Scrimshaw, N.S.: Nature **228,** 180 (1970).
43. Gierhart, D.L., Potter, N.N.: J Food Sci **43,** 1705 (1978).
44. Lindblom, M.: Biotech Bioeng **19,** 199 (1977).
45. Lindblom, M., Mogren, H.: Biotech Bioeng **16,** 1123 (1974).
46. Woodard, J.C., Short, D.D.: J Nutr **103,** 569 (1973).
47. Sternberg, M., Kim, C.Y., Schwende, F.J.: Science **190,** 992 (1975).
48. De Groot, A.P., Shump, P., Feren, V.J., Van Beek, L.: J Nutr **106,** 1527 (1976).
49. Friedman, M.: In Friedman, M. (ed.): Protein Crosslinking: Nutritional and Medical Consequences. Plenum Press, New York 1977.
50. Chefter, J.C.: In Whitaker and Tannenbaum (eds): Food Proteins. Avis Publishing Co, Wesport, Conn. 1977, p. 404.
51. Kinsella, J.E.: Handbook of Food and Nutrition. CRC Press, Cleveland, Ohio 1978.
52. Kinsella, J.E., Shetty, K.J.: Proc ACS Symp. Poor-El, A. (ed.) ACS Publications, Am Chem Soc, Washington, D.C. 1978.
53. Kidby, D.K., Davies, R.: J Gen Microbiol **61,** 327 (1970).
54. Ernst, L.K., Ignat, E.V., Rogozhin, A.D., Sukhanov, B.P.: Gol skokhozyaistvennykh Nauk No. 11, 23 (1974).
55. Ocio, E., Vinaras, R.: Rev Butr Animal **12,** 75 (1974).
56. FAO/WHO. Fourteenth Report of Expert Committee on Food Additives. Technical Report Series No. 462. Geneva 1971, p. 9.
57. Hirs, C.M., Timmasheff, S.: Methods in Enzymology. Vol. 25. Academic Press, New York 1972.
58. Cohen, L.A.: Ann Rev Biochem **37,** 695 (1968).
59. Ferando, R.: Collection FAO. Alimentation et Nutrition No. 2. FAO, Rome 1970.
60. Mauron, J.: Proc IV Int Congress Food and Science Technology.

61. Gould, D.H., McGreger, J.T.: In Friedman, M. (ed.): Protein Crosslinking: Nutritional and Medical Consequences. Plenum Press, New York 1977, p. 29.
62. Dalderup, L.M.: Council of Europe. Medical Fellowship No. 7018, 1970.
63. FAO/OMS. 17e Rapport du Comité Mixte des Additifs Alimentaires. Série de Rapports Techniques No. 539. Genève 1973.
64. OMS. Série des Rapports Techniques No. 426. Genève 1969.
65. OMS. Série des Rapports Techniques No. 482. Genève 1971.
66. WHO. Technical Report Series No. 546. Geneva 1974.
67. WHO. Technical Report Series No. 384. Geneva 1967.
68. Association of Food and Drug Officials of U.S. Appraisal of the safety of chemicals in foods, drugs and cosmetics. Texas State Department of Health, Dallas, Tex. 1959.
69. FDA Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. Toxicol Appl Pharmacol **20,** 419 (1971).
70. FDA Advisory Committee on Protocols for Safety Evaluation: Panel on Reproduction. Toxicol Appl. Pharmacol **16,** 264 (1970).
71. Environmental Protection Agency. Federal Register 37 No. 183 19383 (1972).
72. Taylor, J.C., Lucas, E.W., Gable, D.A., Graber, G.: Single Cell Protein. Davis, P. (ed.) Academic Press, New York 1974, p. 179.
73. De Groot, A.P.: Single Cell Protein. Davis, P. (ed.) Academic Press, New York 1974, p. 75.
74. Shacklady, M.C.A.: Levures Cultivées sur Alcanes. Gounelle de Pontanel, H. (ed.) Symposium d'Aix en Provence. Paris 1972, p. 89.
75. De Groot, A.P., Til, H.P., Feron, V.J.: Fd Cosmet Toxicol **8,** 267 (1970).
76. De Groot, A.P., Til, H.P., Feron, V.J.: Fd Cosmet Toxicol **8,** 499 (1970).
77. De Groot, A.P., Til, H.P., Feron, V.J.: Fd Cosmet Toxicol **9,** 787 (1971).
78. Engel, C.: Levures cultivées sur alcanes. Gounelle de Pontanelle, H. Symposium d'Aix en Provence. Paris 1972.
79. Mumro, H.N.: Friedman, M. (ed.): Nutritional improvement of food and feed protein. Plenum Press, New York 1970, p. 29.
80. Mauron, J.: Bibl Nutr Diet No. 21, 147 (1975).
81. Mauron, J.: In Porters and Rolls (eds): Protein in Human Nutrition. Academic Press, New York 1973, p. 1.
82. Pronczuk, A., Roskowski, W., Bartnik, J.: Technol Rolno-Spozyw **8,** 269 (1973).
83. Skorkowska-Zieleniewska, J., Pronczuk, A., Roszkowski, W., Bartnik, J.: Technol Rolno-Spozyw **8,** 281 (1973).
84. Yanez, E., Ballester, D., Gattas, V.: Arch Latinoam Nutr **24,** 263 (1974).
85. Harper, A.E.: In Porters and Rolls (eds): Protein in Human Nutrition. Academic Press, New York 1973, p. 349.
86. Pion, R.: In Porters and Rolls (eds): Protein in Human Nutrition. Academic Press, New York 1973, p. 329.
87. Omstedt, P., von der Decken, A., Hedenskog, G., Mogren, H.: In Tannenbaum and Wang (eds): Single Cell Protein II. MIT Press, Cambridge, Mass 1975, p. 553.
88. Eggum, B.O.: In Porters and Rolls (eds): Protein in Human Nutrition. Academic Press, New York 1973. p. 329.
89. Sgabieri, V.S., Tanaka, M., Chichester, C.O.: J Nutr **103,** 657 (1973).
90. Tanaka, M., Lee, T.C., Chichester, C.O.: J Nutr **105,** 989 (1975).
91. Finot, P.A., Bujard, E., Motto, F., Mauron, J.: In Friedman, M. (ed.): Protein Crosslinking: Nutritional and Medical Consequences. Plenum Press, New York 1977, p. 343.
92. Barbiroly, G., Mazzaracchio, P., Cuisa, W.: Protein Crosslinking: In Friedman, M. (ed.): Nutritional and Medical Consequences. Plenum Press, New York 1977, p. 449.
93. Erbersdobler, H.F.: In Friedman, M. (ed.): Protein Crosslinking: Nutritional and Medical Consequences. Plenum Press, New York 1977, p. 367.
94. Labuza, P.T.: In Friedman, M. (ed.): Protein Corsslinking: Nutritional and Medical Consequences. Plenum Press, New York 1977, p. 379.
95. Kuzela, L., Masek, J., Zalabak, V., Kejmar, J.: Bibl Nutr Dieta **23,** 169 (1976).
96. Kuzela, L., Ruzickva, J., Masek, J., Kejmar, J., Frankova, S.: Baroda J Nutr **5,** 25 (1978).
97. Wolf, A., Hrivnák, D., Maresova, P., Svabova, M.: Cs Hyg **21,** 285 (1976).
98. Bjarnason, J., Carpenter, K.J.: Brit J Nutr **24,** 313 (1970).
99. Bjarnason, J., Carpenter, K.J.: Brit J Nutr **23,** 859 (1969).
100. Bunker, H.J.: Biochemistry of Industrial Microorganisms. Rainbow, C., Rose, A.H. (eds). Academic Press, New York 1963.
101. Goyco, J.A., Asenjo, C.F.: Puerto Rico J Publ Hlth **23,** 471 (1947).

102. Goyco, J.A., Asenjo, C.F.: J Nutr **38,** 517 (1949).
103. Scrimshaw, N.S.: In Gounelle de Pontanel, H. (ed.): Levures cultivées sur alcanes. Symposium d'Aix en Provence. Paris 1972, p. 215.
104. Abrahamsson, L., Hambraeus, L., Hofrander, Y., Vahlquist, B.: Nutr Metab **13,** 186 (1971).
105. FAO/WHO. Thirteenth Report. Expert Committee on Food Additives. WHO Technical Report Series No. 445. Geneva 1970.
106. Yudkin, J.: In Gounelle de Pontanel, H. (ed.): Levures cultivées sur alcanes. Symposium d'Aix en Provence. Paris 1972, p. 171.
107. Mitchell, H.H., Bolck, R.J.: J Biol Chem **163,** 599 (1946).
108. Oser, B.L.: In Albanese, A.A. (ed.): Newer Methods of Nutritional Biochemistry. Vol. 1. Academic Press, New York 1959.
109. Lakin, A.L.: In Posters and Rolls (eds): Protein in Human Nutrition. Academic Press, New York 1973, p. 175.
110. Carpenter, K.J.: Biochem J **77,** 604 (1960).
111. Finley, J.W., Friedman, M.: Cereal Chem **50,** 101 (1973).
112. Seffner, G.A., Eckfeldt, G.A., Spector, H.: J Nutr **60,** 105 (1956).
113. Saunders, R.M., Conner, M.A., Booth A.N., Bickoff, E.M., Kohler, G.O.: J Nutr **103,** 530 (1973).
114. Akeson, W.R., Stahmann, M.A.: J Nutr **83,** 257 (1964).
115. Osborne, T.B., Mendel, L.B., Ferry, E.L.: J Biol Chem **37,** 223 (1969).
116. Bender, A.E., Miller, D.S.: Biochem J **53,** 7 (1953).
117. Mokady, S., Viola, S., Zimmermann, G.: Brit J Nutr **23,** 491 (1969).
118. Thomas, K.: Arch Anat Physiol 219 (1909).
119. Mitchell, H.H.: J Biol Chem **58,** 873 (1924).
120. Bender, A.E., Doell, B.H.: Brit J Nutr **11,** 138 (1957).

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POSSIBLE ROLE OF LINCOMYCIN-THERAPY IN THE GENETIC ALTERATION OF A STAPHYLOCOCCUS EPIDEMIC POPULATION

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In a staphylococcosis epidemic occurring in a child community, the proportion of inducible lincomycin resistance has risen significantly in the bacterium population. This conveyed the possibility that lincomycin or its derivatives may induce a lincomycin resistance in *Staphylococcus aureus* as it is already known in streptococci. Examination of human and animal samples obtained during lincomycin treatment showed that lincomycin had no role in the induction of resistance; the agent can effectively be applied against pathogens of the above-mentioned phenotype. Immunological examination of serum samples provided opportunity for a more exact localization of the protein-linkage of lincomycin.

As it is known, the resistance against antibiotics belonging to the macrolide-lincosamide-streptogramin B group (MLS antibiotics) can be inducible or constitutive in type. In inducible resistance the inducing agent, through altering the conformation of mRNA, causes a reversible dimethylation of the adenins of 23S rRNA; the modified ribosomas have a low affinity to MLS antibiotics, therefore they do not inhibit translation [1–4]. Most often, the inducing agent is some subinhibitor-concentrated macrolide antibiotic [1, 3], but in the case of streptococci it can also be lincomycin (Lm) [2]. Constitutive resistance is the outcome of a mutation taking place on the R-determinant, the adenins of the ribosoma are present in dimethylated form, even without the presence of an inductor [1].

Such type of MLS-resistance based on ribosoma-modification is common among microorganisms [4, 5]. In addition, the R-determinants of *Staphylococcus aureus* and different streptococcus species show a phylogenetic relation on the basis of DNA-hybridization; relying on experiments in vitro, a genetic substance, exchange through transposon, "gene-epidemy" [4, 5], may be presumed. It seemed therefore interesting to examine whether Lm had a role in inducing MLS-resistance also in staphylococci.

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The idea of the possible resistance-inducing effect of Lm and its derivatives emerged during a staphylococcal epidemic in the second half of 1976, in three nurseries being in close contact through the exchange of babies [6, 7]. The *S. aureus* strains isolated proved to be Lm-sensitive and some of them contained such R-determinant that specifies inducible MLS-resistance (ind$^+$ character) [8]. Though as a result of parenteral Lm-therapy introduced on the basis of laboratory results the morbidity rate diminished somewhat, the epidemic persisted and pathogenic strains were continuously isolated from both the patients and the environmental samples till the end of 1981. Between 1977 and 1979 the proportion of ind$^+$ strains in the *S. aureus* population of the nurseries grew from the original 2.4% to 48.9% [6]. It was therefore studied whether Lm had a role in the change of the genetic composition of the population.

Materials and methods

Bacterial strains. S. aureus strains used in the induction examination are shown in Table I.

Antibiotic sensitivity tests. The semiquantitative antibiotic sensitivity of bacteria was tested on DST agar (Oxoid, London) by disc diffusion method [9] with "Resistest" discs (Institute for Serobacteriological Production and Research Human, Budapest). Determination of MICs was carried out by the agar dilution method [9].

Analysis of in vitro induction. Induction of Lm-resistance was carried out on solid culture medium by the modified method of Malke et al. [2]. Bacteria were grown simultaneously on plates containing subinhibitory concentrations of antibiotics (erythromycin 0.3 μg/ml; Lm: 0.01 or 0.1 μg/ml) at 37 °C for 24 h, then they were inoculated onto plates containing 4 μg/ml Lm, and further incubated at 37 °C.

Experimental Lm-treatment. Wistar R rats and New Zealand rabbits were treated intramuscularly with 20 mg/kg body weight Lincocin (Upjohn, Puurs) every 12 h for 2 days. Blood was obtained from the rats by bleeding and from the rabbits by heart-puncture. Human tests were performed on 4 healthy male persons aged 25–30 years. In two cases the dosage was 0.5 g Lincomycin hydrochloride (V/O Medexport, USSR) orally 3 times daily for 3 days, and in two cases 600 mg Lincocin® was given in intravenous infusion. Time of blood-drawing was chosen so that the Lm-level in the serum should fall under the MIC. Thus the presumed inductor substance can still be present, but the Lm in the sample cannot delay the growth of bacteria.

Antimicrobial assay was carried out with radial diffusion [10]. The multiplesensitive *S. aureus* LK9001 was used as indicator strain (see Table I). The plates containing 25 ml DST agar were seeded by flooding with 3 ml of the 3 h broth culture of the indicator strain so as to give a confluent growth. Wells 1 cm in diameter were made in the agar and into these were measured 0.1 ml volumes of the calibrating Lm-dilutions and samples. After one half hour of diffusion at room temperature the samples were incubated at 37 °C for 16 h. Lm-concentration in the sample can be read off the diagram obtained on basis of the size of the inhibition zones.

Analysis of the resistance-inducing effect of samples. Culture medium was prepared at a ratio of 1:1 from serum obtained after each Lm-treatment or from rat-liver and -kidney homogenate in 8% DST agar prepared with Sörensen buffer pH 8.5. The control culture media contained unprocessed serum or physiological NaCl solution instead of serum. From the 3 h broth culture the bacteria were inoculated onto plates then after a 90 min phenotypical expression at 37 °C, Lm discs were put on the lines of inoculation and incubated overnight at 37 °C.

Physico-chemical analysis of the sera. Immune electrophoresis was performed according to Scheidegger [11]. "DISC"-polyacrilamide gel electrophoresis (PAGE) was carried out as described by Ornstein [12] and Davis [13].

Table I

Characterisation of the s. aureus strains used in induction experiments

Strain	Origin	Resistance			Lm-MIC μg/ml
		Pc	Em	Lm	
PM1151	A	R	R	R_i	1.0
PM1007	A	R	R	R_i	1.0
PM1486	A	R	R	R_i	1.0
PM1661	A	R	R	R_i	1.0
PM1020	A	R	R	R_i	0.75
PM1954	A	R	R	R_i	0.75
PM246	A	R	R	S	0.5
LK9001	B	S	S	S	0.1

Pc = penicillin; Em = erythromycin; Lm = lincomycin; R = resistant; R_i = resistance induced by Em; S = sensitive

Origin:

A = strain originating from nursery epidemic (isolated and identified at the Department of Bacteriology, National Institute of Hygiene, Budapest);
B = strains originating from patients of László Hospital (isolated and identified in our laboratory)

Results

Inductional experiments. To decide whether Lm was responsible for induction of resistance of *S. aureus* strains causing the epidemic, bacteria were grown on agar plates containing Em and Lm at subinhibitory concentration and inoculated onto plates containing Lm at a concentration higher than MIC. According to the test only erythromycin (Em) could induce the Lm resistance of these strains in vitro.

Next human and animal sera were studied for induction. When examining Lm sensitivity on media made with sera, the size of the inhibition zone was the same as the one observed on the control agar containing no serum. Similarly, no induction has been observed on media prepared from rat liver and kidney.

Physico-chemical analysis of the sera. The arc of IgG became diffuse in the immunoelectrophoretic pattern of the serum of a healthy male treated with Lm. This finding indicated a decrease of the IgG level. This dropped at 4 h after the first administration and the decrease could still be detected 12 h after the end of treatment (Fig. 1).

According to PAGE the motility of part of the gamma globulins was accelerated by Lm treatment. Both IgM and IgG showed detachement of new fractions; their amount seemed to be stable during therapy but diminished after finishing the treatment (Fig. 2). Same patterns were shown by the electrophoresis of animal sera.

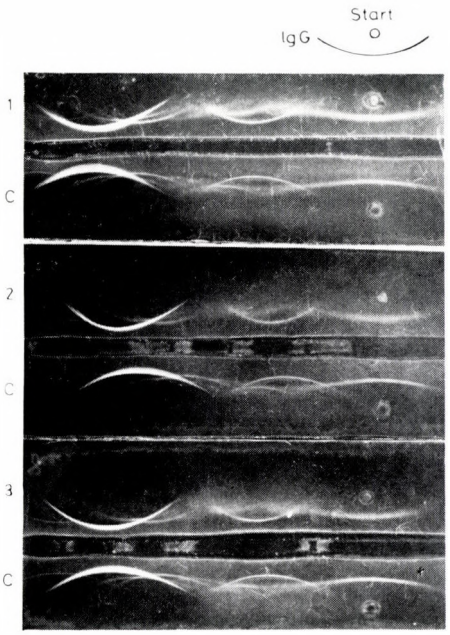

Fig. 1. Immune electrophoresis of serum of a healthy male treated with lincomycin (rate of dilution 1: 8, staining by Ponceau S). Samples: C = control; 1 = 4 h after first administration; 2 = during continuous treatment; 3 = 12 h after end of treatment

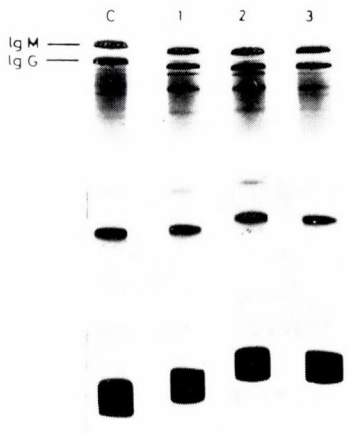

Fig. 2. Effect of lincomycin treatment on human serum proteins by comparison of PAGEs (amido black-10 B staining). Samples, see Fig. 1

Discussion

In the organism Lm is metabolized to a slight extent and is excreted in a biologically active form [14]. Therefore, some metabolite which would induce resistance cannot be found in urine, as in the case of Em [15], and had to be looked for in other materials.

In the organism Lm is present not only in unbound form but also in a microbiologically inactive, protein-bound form in serum [16, 17], liver and the kidneys [18].

Starting from the supposition that if induction exists in the organism the inducing agent might be this protein-Lm-complex, we have examined the inducing effect of the mentioned samples by using physico-chemical methods for following the linkage of Lm to proteins.

According to immunological tests, as a result of Lm treatment, the IgG level decreases and a detachment of new fractions from IgM and IgG can be observed. To our knowledge, Lm is bound to the glycoprotein fraction of the globulin component [17]. During this reversible linkage, a charge-alteration occurs: the mobility of proteins toward the anode is growing. According to our results these changes have ensued in the immune-globulins.

According to our microbiological experiments under the influence of Lm no such substance develops that could induce Lm-resistance in the *S. aureus* strains isolated from the epidemic. This means that Lm can be efficacious against pathogens of similar phenotype.

As a final conclusion it may be assumed that (i) Lm has no resistance-inducing effect on *S. aureus* either in vitro or in vivo; (ii) the selective pressure on the ind$^+$ strains must have been caused by some other factor; the coincidence of Lm-therapy and the change of resistance can only be attributed to chance; (iii) according to physico-chemical evaluation of the serum-samples obtained, Lm can link to immuno-globulins from among the serum proteins.

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REFERENCES

1. Horinouchi, S., Weisblum, B.: Proc Natl Acad Sci USA **77,** 7079 (1980).
2. Malke, H., Reichard, W., Hartmann, M., Walter, F.: Antimicrob Agents Chemother **19,** 91 (1981).
3. Weisblum, B.: In Schlessinger, D. (ed.): Microbiology–1974 American Society for Microbiology, Washington, D.C. 1975, p. 199.
4. Weisblum, B., Holder, S.B., Halling, S.M.: J Bacteriol **138,** 990 (1979).
5. Ounissi, H., Courvalin, P.: Ann Microbiol (Paris) B **132,** 441 (1981).
6. Jánosi, L., Bán, É.: Egészségtudomány **24,** 272 (1980).
7. Jánosi, L., Bán, É.: Acta Microbiol Acad Sci Hung **29,** 187 (1982).
8. Jánosi, L., Barcs, I., Bán, É.: Egészségtudomány **28,** 158 (1984).

9. Matsen, J.M.: In Sonnenwirth, A.C., Jarett, L. (eds): Gradwohl's Clinical Laboratory Methods and Diagnosis. 8th edition. C.V. Mosby Co., St. Louis—Toronto—London 1980, p. 1937.
10. Sabath, L.D., Anhalt, J.P.: In Lennette, E.H., Balows, A., Hausler, W.J. Jr., Truant, J.P. (eds): Manual of Clinical Microbiology. 3rd edition. American Society for Microbiology, Washington, D.C. 1980, p. 485.
11. Scheidegger, J.J.: Int Arch Allergy **7,** 103 (1955).
12. Ornstein, L.: Ann N Y Acad Sci **121,** 321 (1964).
13. Davis, B.J.: Ann N Y Acad Sci **121,** 404 (1964).
14. Fass, R.J.: In Kagan, B.M. (ed.): Antimicrobial Therapy. 3rd edition. W.B. Saunders Company, Philadelphia—London—Toronto 1980, p. 97.
15. Majer, J.: Antimicrob Agents Chemother **19,** 628 (1981).
16. Gordon, R. C., Regamey, C., Kirby, W.M.M.: J Pharm Sci **62,** 1074 (1973).
17. Rimmer, D.M.D., Sales, J.E.L.: Antibiotics Chemother **25,** 204 (1978).
18. Gryaznova, N.S., Koroleva, V.G., Sazykin, Yu.O., Navashin, S.M.: Antibiotiki **21,** 819 (1976).

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ABSORPTION OF VIRUSES INTO ORAL LYMPHOCYTES AND DECREASED ANTIBACTERIAL ACTIVITY OF ORAL POLYMORPHONUCLEAR LEUKOCYTES

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Antibacterial activity of the oral polymorphonuclear leukocytes and in vitro virus absorption by oral lymphocytes was studied in two patient groups and in controls. A slight decrease of phagocytosis was observed in those control cases and periodontitis patients where orolymphocytes and oral epithelial cells had absorbed viruses, but in the same subjects the bactericidal effect was significantly weaker. Periodontosis patients with a defect of polymorphonuclear cells did not exhibit such a difference. As in similar subjects the antibacterial activity was more frequently weak when their orolymphocytes carried latent viruses, the results suggest that certain individuals are prone to carry latent viruses in their lymphocytes. Lymphokines released from these carrier cells and concentrated locally might damage phagocytic leukocytes, resulting in serious local inflammation.

The immunomechanism of mixed microbial infections has not been clarified [1], because of their interaction with different parts of the immune system [2]. Recently some data have indicated the immunosuppressive effect of several viruses [2, 3] which would account for the clinical observation of frequent bacterial invasion after viral infections [2]. The data suggest a close relation between certain parts of the immune apparatus [2].

The oral cavity seemed to be an appropriate model for studying the phenomenon, because all types of circulating leukocytes may reach the gingival sulcus [4, 5]. The task of these so-called oroleukocytes and orolymphocytes is to protect the periodontium against different microbes [6]. The antibacterial activity of polymorphonuclear (PMN) oroleukocytes was found to decrease in the case of periodontal inflammations [7, 8], while an inherited defect of PMN oroleukocytes leads to serious degeneration of the gingiva [9]. There are some data on the relationship of orolymphocytes and viruses [10–12], which appear to confirm their joint aetiological role. On the basis of these observations was the correlation of bactericidal capacity and latent virus

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carriership of oral cells studied in some types of patients [11, 13]. The results showed a higher incidence of virus carriage with impaired antibacterial function of PMN cells in periodontal inflammations.

One of the possible conclusions was that virus carrying orolymphocytes release lymphokines. Similar mediators developing after virus infection have already been described [3, 14, 15]. Such lymphokines concentrated in the narrow gingival sulcus might damage PMN and other cells. Since certain patients have a tendency to become latent or persistent virus carriers, their lymphocytes may be affected. As the oral cavity is potentially at risk of virus infections, among them with herpesviruses [16], virus infection of oral cells promised to offer further information concerning the problem.

Materials and methods

Patients. Specimens were obtained from the following individuals. Group I: 32 healthy subjects of 28.3 years average age, free from periodontal illness during the last 6 months. Group II: 29 patients of 41.4 years average age, suffering from periodontitis. Group III: 8 patients of 24.2 years average age, with juvenile or postjuvenile periodontosis. In this group the PMN leukocytes were impaired [9, 13].

Collection of oroleukocytes. Collection of oral cells was carried out by a modification of Klinkhammer's method [4]: each proband rinsed the mouth for 30 s ten times consecutively with 5 ml of phospate buffered salt solution (PBS), pH 7.4. The ten suspensions were collected separately in test tubes, washed in PBS and centrifuged three times. Oroleukocyte counting was done in Bürker chamber. After preliminary experiments the exfoliative epithelial cells mixed to the oroleukocytes were ignored. Proportion of viable oroleukocytes was established by the trypan blue exclusion test [17].

Determination of phagocytic and bactericidal capacity of PMN oroleukocytes. 20 000 oroleukocytes/ml from each suspension were mixed with living *Staphylococcus aureus* grown in broth, in 1:10 cell: bacteria ratio. Bacteria were counted after mixing them to a standard red blood cell suspension. The oral cell-bacterium mixture was incubated in Parker 199 minimal essential medium (MEM 199) containing 3% rabbit serum at 37 °C for 120 min. After incubation the mixtures were washed and shaken well in PBS, then centrifuged three times to remove bacteria that adhered to the cell surface. From the last pellet smears were prepared, stained with Giemsa, and the phagocyted bacteria were counted. The bacteria phagocyted by these cells previously in vivo were counted in control samples and their number was deduced from the corresponding total bacterium number.

To determine bactericidal capacity, 1.2% Difco agar plates were used [13, 18]. At the beginning of incubation 2000 cells with bacteria in 0.1 ml volume were mixed to the agar (A), an equal quantity was mixed after incubation (B), and the same amount after bursting the cells in distilled water for 15 min (C). Colonies of discharged live bacteria were counted. Results are given as killed bacteria in per cent of total phagocyted ones, $100 \times (A-C) : (A-B)$.

In vitro absorption of viruses by orolymphocytes. From each specimen 20 000 oral cells were resuspended in 0.9 ml of MEM 199, then 1000 $TCID_{50}$ of Herpes simplex virus type 1 (HSV-1) or Adenovirus human type 1 (Ad-1) in 0.1 ml were added to the samples to determine the ability of cells to absorb viruses. Mixtures were incubated at 37 °C for 120 min, and after a wash smears were prepared for direct immunofluorescent investigation [19]. Oral cells of patients were also examined without adding viruses and only those persons were evaluated whose cells did not carry virus antigens in vivo.

To regain infectious viruses from the cells, cells from the third pellet were frozen and thawed 3 times, centrifuged, then the supernatant was placed on the confluent monolayer of HEp-2 and VERO cell cultures [19]. The characteristic cytopathic effect (CPE) was detected visually and subsequently confirmed by direct immunofluorescent technique. Uninfected cells of the probands were also examined by this method in order to exclude from the study those subjects who carried adeno- or herpesviruses in their cells.

Electron microscopy of virus infected samples. Some oral cell samples indicating absorption of HSV-1 or Ad-1 were examined by electron microscopy. An aliquot of 20 000 cells of the infected samples was washed as mentioned above, the pellet was fixed by glutaraldehyde and osmium tetroxide, then embedded in Epon 812. Ultrathin sections were cut, stained with uranyl acetate and lead citrate. The position of particles absorbed in vitro was determined by a JEOL 100B electron microscope.

Results

Each determination was done in triplicate from the last three samples; the results given reflect mean values.

Characteristics of emigrated oroleukocytes. The outflow rate of PMN oroleukocytes and their viability was different in the three patient groups (Fig. 1). In inflammatory and degenerative conditions more cells were found but their viability was considerably decreased. There were only minor differences between subgroups of virus-free subjects and those with absorbed viruses in their orolymphocytes (data not shown). Emigration of orolymphocytes showed a similar pattern, and viability in any group and subgroup varied between 99.4 and 100% (Fig. 2). Counting of monocytes was not done because of their small number. Ratio of exfoliated epithelial cells to all oroleukocytes was roughly 40:60.

Virus absorption by orolymphocytes and epithelial cells. The result of virus absorption indicated by immunofluorescent assay is shown in Table I. There was a discrepancy in the number of persons (n) given in the first column and of those whose cells took up viruses in vitro; this was due to the absorption of both viruses into both types of cell. This was considerable in Group II.

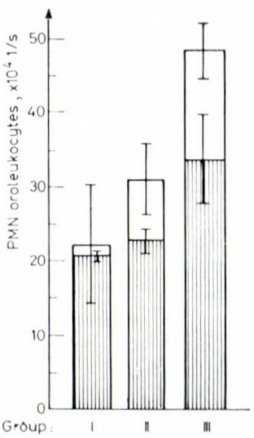

Fig. 1. PMN leukocyte emigration into the gingival space. Shaded part of columns: viable proportion of PMN cells

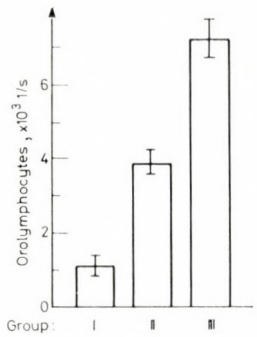

Fig. 2. Lymphocyte emigration into the gingival space

Absorption was verified by electron microscopy; the diffuse distribution of intact virions demonstrated their in vitro origin (Fig. 3).

Phagocytosis and bactericidal capacity of PMN oroleukocytes. Results are shown in Table I with details of groups and subgroups concerning virus absorption. Competence of division of groups into two subgroups proved to be significant ($p < 0.05$, $\chi^2 = 7.294$ at 2 DF), concerning virus absorption

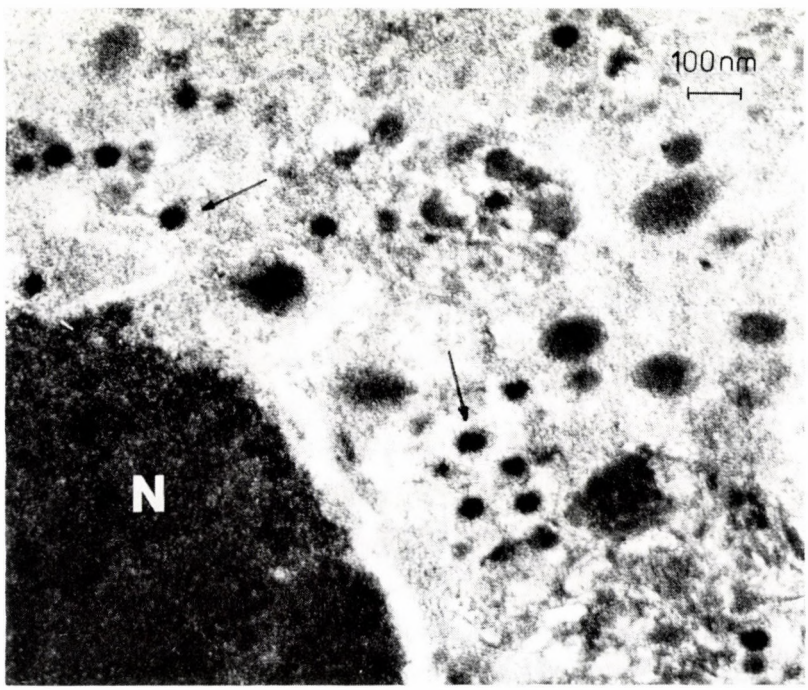

Fig. 3. Electron micrograph of part of an orolymphocyte obtained from a periodontitis patient. N = nucleus. Arrows show absorbed Ad-1 particles

and virus-free condition in the whole population. The difference in phagocytosis of all the virus-positive and virus-free subjects was not significant ($p = 0.0538$, $\chi^2 = 9.324$ at 4 DF) although it was decreased in all groups in virus-positive subjects. Bactericidal capacity was significantly impaired in those persons whose other oral cells took up viruses in vitro, independently from their group ($p < 0.001$, $\chi^2 = 19.686$ at 4 DF).

Table I

Virus absorption and antibacterial function

Group and subgroups concerning virus absorption		Absorbed viruses (in No. of persons)						Phagocytosis, No. of bacteria	Bactericidal capacity %
		Lmpho-cyte	HSV-1 Epi-thelium	Culti-vation	Lympho-cyte	Ad-1 Epi-thelium	Culti-vation	by PMN oroleukocytes	
I. Total n=32	100%							6.68±3.10	79.6±12.1
No n=24	75%							6.94±3.28	83.4± 9.2
Yes n= 8	25%	2	2	2	4	3	3	5.90±2.48	68.4±13.2
II. Total n=29	100%							5.60±2.12	50.8±17.2
No n=12	41.4%							6.56±2.48	64.5± 8.7
Yes n=17	58.6%	5	5	4	10	11	6	4.92±1.56	41.2±14.9
III. Total n= 8	100%							2.15±1.44	38.9±11.2
No n= 4	50%							2.70±1.64	40.1± 8.6
Yes n= 4	50%	—	2	2	3	—	3	1.60±1.16	37.9±14.7

A similar comparison between the subgroups of the same group indicated that phagocytosis became less effective in subjects with simultaneously absorbed virus, mainly in Group I. Probabilities in the order of groups were, $p < 0.1$ ($\chi^2 = 7.924$ at 4 DF), $p < 0.3$ ($\chi^2 = 5.164$ at 4 DF) and $p < 0.2$ ($\chi^2 = 2000$ at 1 DF), respectively, none of them reached the limit of significance. Tail probabilities of one way analysis of variance were in the order of groups 0.4192, 0.0382 and 0.3157, indicating that the decrease of phagocytosis was important only in Group II.

The bactericidal capacity showed different results. The impairment of killing intracellular bacteria in subjects whose oral cells showed affinity to viruses was found strongly significant in Groups I and II ($p < 0.001$ and $\chi^2 = 13.206$ at 3 DF in the first group, $\chi^2 = 11.675$ at 3 DF in the second group). Tail probability of one way analysis of variance reckoned on basis of impairment, confirmed the previous data, the values being 0.0012 and 0.0000. Subgroups of Group III did not differ, their bactericidal capacity was weak. Distribution of the patients was not significant ($p < 0.4$ and $\chi^2 = 2.333$ at 2 DF), and the decrease in the amount of killed bacteria was not significant either, even a homology could be suspected, the probability between subgroups having been 0.811.

Discussion

In our previous experiments [11, 13] statistical comparison of the bactericidal capacity of PMN oroleukocytes with virus carrying oral cells showed a closer relationship between the two phenomena, namely virus carrier subjects had impaired bactericidal functions, especially those with periodontitis. It is known that virus carrier lymphocytes may enter the oral cavity via the gingival space [10, 13, 16], interacting with epithelial cells of the oral mucosa and resulting in a similar virus carrier state [10]. Moreover, the narrow gingival space is suitable for the accumulation of such carrier cells which are continuously mixed to PMN leukocytes having the strict function to taking up and killing microbes. Recent studies have drawn attention to that viruses may stimulate various lymphoid and non-lymphoid cells to produce a wide variety of immune regulatory lymphokines [3, 14, 15] as gene products, and that they have a toxic influence on other cell types [14]. This effect manifests itself because of the dilution of mediators less in the blood stream than in certain tissues, among them the gingiva, where intensive immunoreactions may take place [20].

Kinetic analyses [11] and the present results suggest that normal PMN cells emerge in both healthy and periodontitic subjects; the concept of local damage was postulated by earlier investigators [5, 7], too.

Such factors that must be similar to LIF and MIF [3, 15] are responsible for the slow mobility and decreased viability as well as for the impaired bactericidal capacity of PMN leukocytes [2]. The effect of mediators must act inside PMN cells, as biochemical studies suggest [1, 21]. As the PMN cells of periodontosis patients are a priori defective [9] the mediators can alter their functions only in a slight degree.

As PMN cells were not responsible for the severe symptoms, lymphocytes came into the limelight. More types of both herpesvirus [10, 11, 16, 24] and adenovirus [22, 23] groups have already shown strong evidence to establish

latent or persistent infection in lymphoid cells. This would mean the susceptibility or rather a defect of part of lymphocytes. Instead of being sensitized by viruses in the usual manner after infection, a latent state persists due to uptake of the virus [1]. This does not affect the viability of lymphocytes, as shown by the present results. Of course, this may occur not only with adeno- and herpesviruses, as cultivation of uninfected cells of similar patients has indicated (unpublished data).

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REFERENCES

1. Gresser, J., Lang, J.D.: Progr Med Virol **8,** 62 (1966).
2. Mogensen, S.C.: Proc FEMS symposium on baterial and viral inhibition and modulation of host defences. Pisa, 1982. Abstracts, p. 14.
3. Friedman, H., Specter, S.: Proc FEMS symposium on bacterial and viral inhibition and modulation of host defences. Pisa, 1982. Abstracts, p. 18.
4. Klinkhammer, J.M., Zimmerman, S.: J Dent Res **48,** 709 (1969).
5. Skapski, H., Lehner, T.: J Periodont Res **11,** 19 (1976).
6. Goldman, H.M., Cohen, D.W.: Periodontal therapy. C.V. Mosby, Co, St. Louis 1980, p. 87.
7. Wilton, J.M.A., Renggli, H.H., Lehner, T.: Immunology **32,** 955 (1955).
8. Wilton, J.M.A., Renggli, H.H., Lehner, T.: Clin Exp Immunol **27,** 152 (1977).
9. Cianciola, L.J., Genco, R.J., Patters, M.R., McKenna, J., Oss, C.J. van: Nature **265,** 445 (1977).
10. Sallay, K., Kulcsár, G., Dán, P., Nász, I., Geck, P.: Dtsch Zahnärztl Z **30,** 570 (1975).
11. Ongrádi, J., Sallay, K., Kulcsár, G., Dán, P., Horváth, J., Nász, I.: Fogorv Szle **73,** 305 (1980).
12. Veen, J. van der, Lambriex, M.: Infect Immun **7,** 604 (1973).
13. Ongrádi, J., Sallay, K., Kulcsár, G., Dán, P., Horváth, J., Nász, I.: Fogorv Szle **72,** 215 (1980).
14. Wheelock, E.F., Toy, S.T.: Advanc Immunol **16,** 123 (1973).
15. Pham, N.D., Béládi, I., Rosztóczy, I., Tóth, M.: J Interferon Res **1,** 23 (1980).
16. Zakay-Rones, Z., Ehrlich, J., Hochman, H., Levy, R.: J Periodont **44,** 779 (1973).
17. Nowotny, A.: Basic exercises in immunochemistry. Springer-Verlag, Berlin—Heidelberg—New York 1979.
18. Hellum, K.B., Solberg, C.O.: Acta Path Microbiol Scand Sect C **85,** 1 (1977).
19. Ongrádi, J., Kulcsár, G., Dán, P., Nász, I., Horváth, J.: Intervirology **14,** 310 (1980).
20. Denman, A.M., Pelton, B.K., Appleford, D., Kinsley, M.: Transplant Rev **31,** 79 (1976).
21. Degré, M., Rollag, H.: Proc FEMS symposium on bacterial and viral inhibition and modulation of host defences. Pisa, 1982. Abstracts, p. 37.
22. Andiman, W.A., Jacobson, R.I., Tucker, G.: New Engl J Med **297,** 100 (1977).
23. Faucon, N., Desgranges, C.: Infect Immun **29,** 1180 (1980).
24. Docherty, J.J., Chopan, M.: Bact Rev **38,** 337 (1974).

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VIRULENCE FACTORS OF *ESCHERICHIA COLI*

I. MANNOSE RESISTANT HAEMAGGLUTINATING CAPACITY IS ASSOCIATED WITH SEROGROUP BUT NOT WITH SITE OF INFECTION

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Different serogroups of *Escherichia coli* strains originating from faeces (patients with enteritis 244, healthy individuals 225), urine (pyelonephritis 111, cystitis 130, asymptomatic bacteriuria 59) and other extraintestinal sources (blood 30, cerebrospinal fluid 15, wound 13, autopsy material 9, umbilicus 8, vagina 20, throat 13 and nose swabs 5) were examined for mannose resistant haemagglutination of human A erythrocytes (MRHA hum.). The most frequent serogroups were O1, O2, O4, O6, O7, O18 and O75 uniformly among faecal (26.5%), urinary (34%) and other extraintestinal (57.9%) strains. Haemagglutinating activity was significantly more frequent in these serogroups than in others ($p < 0.001$). There was no significant association between MRHA positivity and origin of the serogroups. It has been concluded that MRHA hum. property of an *E. coli* strain depends on its serogroup rather than its origin. As these serogroups are the most frequent in the intestinal flora, it is assumed that their physiological function is to colonize the bowel and their pathological significance is to provide a source for extraintestinal infection.

There are many traits to indicate the pathogenicity of *Escherichia coli* strains isolated from different conditions. In the last five years the importance of adherence to different surfaces has been in the foreground. Recognition of fimbrial structures [1], adhesive factors of enterotoxigenic *E. coli* (K88, K99, CFAI, CFAII [2–6]), and elaboration of haemagglutination (HA) patterns [7, 8] led to the discovery of a new group of *E. coli*. This group is characterized by agglutination of human A erythrocytes in a mannose resistant manner (MRHA hum. [9–11]. Analysis of different properties of MRHA hum. positive strains [9, 11–13] led to a better understanding of the pathogenic role in urinary tract infections (UTI), to their serological characterization [14, 15] and recognition of the chemical nature of their receptors [16, 17]. Occurrence of MRHA hum. positive strains in Hungary was described by Kuch et al. [18] and Czirók et al. [19].

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The aim of this study was to examine whether MRHA hum. positivity of *E. coli* has any other importance besides causing UTI.

Materials and methods

Bacterial strains were isolated from faecal samples, urine and other extraintestinal sources between January, 1979, and January, 1983. All strains were stored on Dorset egg slopes at ambient temperature as well as in lyophiles until subcultured for testing.

Faecal samples were collected from sporadic cases of enteritis (244) or healthy individuals (225), with the exception of 71 out of 99 faecal strains of patients under one year of age. These were isolated from children with enteritis in three wards of a hospital.

Urinary samples mostly originated from patients of the nephrological and urological clinics and wards of one hospital (210 strains), the rest from another hospital (29) and from different cases (61 strains). The 210 hospital strains represented 200 urine samples of 148 patients, the remaining 90 strains were isolated from urine of 81 patients.

Diagnostic criteria for classification of urinary strains were as follows. *Pyelonephritis* (111 strains): significant bacteriuria ($\geq 10^5$ bacteria/ml), pyrexia (38.0 °C), increased erythrocyte sedimentation rate (>20 mm/h), leukocytosis, pyuria, nausea, malaise, back or abdominal pain. *Cystitis* (130 strains): significant bacteriuria, accompanied by burning pain, frequent urination and pyuria. Wide bladder neck was frequently in the background of recurrent infection.

Asymptomatic bacteriuria (59 strains): significant bacteriuria without pyuria and clinical symptoms.

Other extraintestinal infections. Blood isolates (30) were collected from sepsis or urosepsis. Strains isolated from cerebrospinal fluid (15) were from cases of meningitis. The 13 wound swabs represented different purulent processes including abscesses. Nine strains were isolated from different necropsy materials of patients who died with sepsis. The samples were collected from different parts of the country.

Umbilical (8), vaginal (20), throat (13) and nose (5) swabs originated from healthy mothers and their babies staying in the gynaecological and perinatal wards of a hospital.

Serological examination of O antigens. The procedures were as described by Ørskov and Ørskov [20] except that in the case of heterologous reactions with different O antigens, antibodies were removed by absorbing the sera with all the reacting heterologous antigens.

Haemagglutination. Human group A erythrocytes were washed twice in phosphate buffered saline (PBS) and suspended to 3%. The HA activity and its inhibition by D-mannose were examined on a glass slide by mixing one drop of red blood cell suspension with or without one drop of D-mannose (2%) and one loopful of bacteria, grown on CFA medium [21] at 37 °C for 18 h.

Statistical analysis was carried out in 2×2 contingency tables and examined for significance by the χ^2 test [22].

Results

The incidence of MRHA hum. positive strains in faecal samples from patients and healthy persons of different age groups is presented in Table I. Faecal strains exerted haemagglutinating activity in 18.6% (24.6% for enteritis strains and 12% for healthy persons' strains). The difference in MRHA hum. positivity between patients and healthy persons was significant ($p<0.001$), but this was associated with the differences found in the age groups under one year ($p < 0.01$). There was no significant difference between enteritic and healthy persons of other age groups. The only considerable difference existed between enteritic patients under one year of age and enteritic patients

above 14 years of age ($p < 0.01$). A calculation was made to find out whether the high incidence of haemagglutinating strains among patients under one year of age was due to the fact that 71 of 99 enteritis strains strains were isolated in the same hospital. Thirty out of 71 "hospital" and 6 out of 28 "sporadic" strains were MRHA hum. positive, indicating no significant difference ($p > 0.1$).

Table I

Incidence of MRHA hum. positive E. coli *strains in faecal samples of patients and healthy persons*

Source	Patients with enteritis, age groups (year)					
	<1	1–2	3–5	6–14	>14	Total
No. of strains	99	28	27	22	68	244
No. of MRHA hum. positive strains	37	5	3	3	12	60
Source	**Healthy persons, age groups (year)**					
	<1	1–2	3–5	6–14	>14	Total
No. of strains	69	26	38	26	66	225
No. of MRHA hum. positive strains	11	0	3	1	12	27

The distribution of *E. coli* serogroups and haemagglutinating capacity is demonstrated in Table II. A total of 87 serogroups was isolated, but 26.5% of the strains belonged to not more than 8 serogroups. Haemagglutinating strains belonged in 78.2% to these most frequently occurring serogroups (O1, O2, O4, O6, O7, O15, O18ac and O75). Haemagglutinating activity was significantly more frequent in these serogroups than in others ($p < 0.001$).

From urine, 300 strains were isolated (Table III). MRHA hum. positivity occurred in 25.3% (27% for pyelonephritis, 27% for cystitis, 18.6% for asymptomatic bacteriuria (ABU) strains). There was no significant difference in the frequency of MRHA hum. between pyelonephritis and cystitis strains ($p > 0.99$). On the other hand there was considerable difference found between groups of pyelonephritis and ABU strains ($p < 0.02$) and between cystitis and ABU strains ($p < 0.02$).

To test the relationship between haemagglutinating activity and O antigens of the same urinary *E. coli* strains the *E. coli* isolates were serogrouped (Table III). A total of 51 serogroups was determined; 102 strains belonged to the most prevalent 8 serogroups (O1, O2, O4, O6, O7, O8, O18ac, O75), 52 of them proved to be MRHA hum. positive. Incidence of HA positivity in these serogroups was significantly higher than in other serogroups ($p < 0.001$).

Table II

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

Serogroup	Patients with enteritis	Healthy persons	Total
O1	3/9[1]	4/7	7/16
O2	1/5	2/7	3/12
O4	10/13	1/3	11/16
O6	9/13	5/6	14/19
O7	2/3	1/7	3/10
O15	3/6	5/9	8/15
O18ac	16/17	4/6	20/23
O75	1/7	1/7	2/14
Other[2]	7/78	1/90	8/168
Spontaneous agglutination	0/6	1/6	1/12
NT[3]	8/87	2/77	10/164
Total	60/244	27/225	87/469

[1] No. of strains haemagglutinating/No. of strains examined

[2] O3, O5, O8, O9, O10, O11, O12, O13, O15,56, O16, O17, O17,77, O18ab, O19,133, O20, O21, O22, O23, O25, O26, O29, 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, O106, O107, O110, O111, O112ab, O112ac, O113, O115, O121, O123, O124, O127, O128, O131, O136, O141, O142, O146, O147, O148, O149, O151, O152, O154, O156, O159, O164

[3] Not groupable

Table III

Serogroup distribution of MRHA hum. positive E. coli strains isolated from urinary tract infections

Serogroup	Pyelonephritis	Cystitis	ABU[1]	Total
O1	3/6[2]	5/6	0/0	8/12
O2	2/6	8/12	0/6	10/24
O4	1/1	1/3	4/5	6/9
O6	9/9	9/15	1/2	19/26
O7	0/2	1/2	0/5	1/9
O8	0/3	1/3	0/1	1/7
O18ac	2/5	1/1	2/3	5/9
O75	1/4	1/1	0/1	2/6
Other[3]	6/30	0/35	1/11	7/76
Spontaneous agglutination	0/7	3/6	2/3	5/16
NT[4]	6/38	5/46	1/22	12/106
Total	30/111	35/130	11/59	76/300

[1] Asymptomatic bacteriuria

[2] No. of strains haemagglutinating/No. of strains examined

[3] O3, O5, O7,16, O9, O10, O11, O12, O15, O17, O17,77, O20, O21, O22, O23, O25, O29, O29,134, O30, O36, O42, O45, O51, O56, O57, O61, O71, O78, O83, O85, O95, O96, O99, O101, O106, O107, O109, O112ab, O131, O141, O142, O149, O152, O159

[4] Not groupable

Table IV

E. coli serogroups in urine samples of patients with urinary tract infection

Patient and age (year)	Date	Serogroup	Diagnosis
H.B.	03.10. 1982	O8	
9	04.28.	NT	Cystitis
	02.28. 1983	Sp.	
N.B.	03.10. 1982	NT	
10	06.16.	NT	Chronic pyelonephritis
	02.28. 1983	NT	
S.A.	03.10. 1982	NT	
12	03.20.	NT	Chronic pyelonephritis
	04.16.	NT	
S.L.	04.28. 1982	NT	
2	06.16.	NT	Cystitis
	10.22.	NT	
T.I.	03.10. 1982	O5	
5	03.20.	O5	Cystitis
	10.22.	NT	
V.V.	03.10. 1982	O1	
4	03.20.	O1	Cystitis
	02.28. 1983	O8, NT	
K.Sz.	11.12. 1981	O30	
13	03.10. 1982	O1: K1	Cystitis
	03.20.	O1: K1	
	10.22.	O61	
K.E.	03.10. 1982	NT	
10	10.22.	O2	Cystitis
	10.31.	O2	
	02.28. 1983	NT	
S.K.	11.12. 1981	O21	
10	03.10. 1982	O21	Chronic pyelonephritis
	06.16.	O42	
	02.28. 1983	O107	
Á.E.	03.10. 1982	O6	
10	06.16.	Sp.	Chronic pyelonephritis
	10.22.	O101	
	02.28. 1983	O6	
S.D.	03.10. 1982	O1,0131	
9	04.28.	NT	Cystitis
	06.16.	NT	
	02.28. 1983	O18ac	Chronic pyelonephritis
V.K.	11.12. 1981	Sp.	
9	03.10. 1982	O152	Relapsing cystitis
	04.28.	O3	
	10.22.	O2	Wide bladder neck
	02.28. 1983	Sp.	

NT = Not groupable
Sp. = Spontaneous agglutination

In a paediatric nephrological ward and outpatient clinic 210 strains were collected from 148 patients. Patients with recurrent infections were subjected to further analysis. Only 12 out of the 148 had more than two samples (Table IV). In two patients (S.K., Á.E.) out of 5 with pyelonephritis the same O serogroup was isolated at different acute periods.

MRHA hum.positive isolates occurred most frequently (51.2%) in different non-urinary extraintestinal samples (Table V), with especially high incidence in blood, necropsy material, wound swabs, sputum and nose swabs of healthy newborn infants.

Twentyone serogroups were isolated from other extraintestinal materials. Six serogroups (O1, O2, O4, O6, O18ac, O78) were the most frequent, comprising 69 strains with 48 being MRHA hum. positive. The difference in incidence of HA activity in these 6 serogroups and in the remaining ones was highly significant ($p < 0.001$).

Table V

Serogroup distribution of MRHA hum. positive E. coli strains isolated from extraintestinal sources

Serogroup	Blood	CSF[1]	Autopsy material	Wound	Umbilicus	Vagina	Throat	Nose	Sputum	Ear	Total
O1	1/1[2]	—	—	0/1	1/1	4/4	—	1/1	—	1/1	8/9
O2	1/1	—	—	—	1/4	2/4	1/2	1/2	—	—	6/13
O4	—	2/2	2/2	1/1	1/1	—	—	—	—	—	6/6
O6	6/6	0/1	3/4	5/5	—	—	2/2	—	—	—	16/18
O18ac	3/4	0/1	1/2	—	0/1	0/3	2/4	1/1	2/2	0/1	9/19
O78	2/2	1/2	—	—	—	—	—	—	—	—	3/4
Other[3]	3/7	1/6	0/8	0/3	0/1	2/5	0/4	1/1	—	0/2	7/30
Sp. aggl.[4]	1/2	1/2	—	—	—	—	—	—	—	—	2/4
NT[5]	2/7	1/1	—	1/3	—	0/4	0/1	—	—	—	4/16
Total	19/30	6/15	6/9	7/13	3/8	8/20	5/13	4/5	2/2	1/4	61/119

[1] Cerebrospinal fluid
[2] No. of strains haemagglutinating/No. of strains examined
[3] O3, O5, O7, O12, O15, O19, O22, O23, O33,56, O75, O83, O106, O107, O110, O114
[4] Spontaneous agglutination
[5] Not groupable

On the basis of electron microscopical examinations 200 out of 224 haemagglutinating strains harboured fimbrial structures.

Discussion

According to literary data, adhesion of *E. coli* to epithelial cells of the urinary tract is correlated to mannose resistant agglutination of human A erythrocytes [9, 23]. This adhesiveness is closely associated with virulence [24].

Concerning the origin of strains, it has been observed that faecal strains caused haemagglutination less often than isolates from UTI [25]. According to these and other data there is a relationship between pyelonephritic origin and haemagglutinating capacity of strains.

As a contradiction to these data and to our expectations, however, our survey revealed no significant difference in MRHA hum. positivity between strains isolated from pyelonephritis and those isolated from cystitis. From recurrent infections of certain patients the same serogroup was isolated infrequently. MRHA hum. positivity in pyelonephritis and negativity in cystitis of the prevalent serogroups as reported by others [30] could not be observed consequently in our strains. As to whether strains isolated in the course of cystitis can be differentiated as potential causative agents of a subsequent pyelonephritis in the same patient, is a question to be solved, although a considerable difference was found between groups of pyelonephritis and ABU strains and between cystitis and ABU strains.

It is well known from the work of Evans et al. [8] that the large majority (80.2%) of the strains belonging to O1, O2, O4, O6, O7, O18 serogroups derived from UTI, show MRHA hum. positivity. This was the case in 69.5% in our material. In the material of Evans et al. [8] the stool isolates of the same serogroups were positive for MRHA hum. in 59.2%. This frequency was 54.8% for our strains. Svanborg Eden et al. [26] described that strains belonging to the eight O groups (O1, O2, O4, O6, O7, O16, O18, O75) found in 80% of patients with acute pyelonephritis, showed attachment in a higher proportion than those of the remaining O groups. Van den Bosch et al. [27] confirmed that UTI strains positive for MRHA hum. belonged to groups O1, O2, O4, O6, O8, O18ac, O23, O25, O57, O75, O128.

In our material faecal, urinary and extraintestinal strains belonged the most frequently to serogroups O1, O2, O4, O6, O7, O18, O75. These serogroups were at the same time the most frequent haemagglutinating ones (Tables II, III, V).

Briefly, the high incidence of these serogroups in blood, faecal samples, urine and their frequent MRHA hum. activity is well-known. The observation, however, that MRHA hum. positivity is not related to the source of strains, and that MRHA hum. positivity depends on their serogroup rather than their origin, has been first revealed by this study. This finding was corroborated by the fact that there was no significant difference in the incidence of MRHA hum. positivity of O1, O2, O4, O6, O18, O75 serogroups either between UTI and faecal strains ($p > 0.99$), between faecal and extraintestinal isolates ($p > 0.2$), or between UTI and extraintestinal cultures ($p > 0.1$). All these results have led to the conclusion that MRHA hum. property of an *E. coli* strain is associated with certain serogroups but not with its origin. It may be assumed that types of fimbria responsible for MRHA hum. are associated with certain O

antigens. This assumption was also supported by earlier studies [15, 28] according to which the association between O,K,H and F antigens reflects the existence of a common ancestor. Determination of K and H antigens may provide more evidence for this theory.

E. coli isolates exerting MRHA hum. activity have a peculiar F antigen [14] or a P fimbria [16] and represent a special group of adhesins. They have to be differentiated from fimbria type 1 and from adhesive factors of enterotoxigenic strains (K88, K99, 987, CFAI, CFAII) by their function.

In earlier studies [29, 30] it has been assumed that P fimbriae bind efficiently not only to the urinary but also to the intestinal epithelium. This has also been proved for some of our strains in another study [19]. Since serogroups with P fimbria occur very frequently as residents in the faecal flora it may be concluded that the physiological function of MRHA hum. positive strains is to colonize the bowel. Outside the gut, in view of their affinity to other epithelial surfaces, they have ample opportunity to cause various extraintestinal infections.

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REFERENCES

1. Duguid, J.P., Smith I.W., Dempster, G., Edmunds, P.N.: J Path Bact **70,** 335 (1955).
2. Bertschinger, H.V., Moon, H.W., Whipp, S.C.: Infect Immun **5,** 595 (1972).
3. Ørskov, I., Ørskov, F., Sojka, W.J., Wittig, W.: Acta Path Microbiol Scand **62,** 439 (1964).
4. Nagy, B., Moon, H.W., Isaacson, R.E.: Infect Immun **13,** 1214 (1976).
5. Evans, D.G., Silver, R.P., Evans, D.J. Jr., Chase, D.G., Gorbach S.L.: Infect Immun **12,** 656 (1975).
6. Evans, D.G., Evans, D.J. Jr.: Infect Immun **21,** 638 (1978).
7. Evans, D.J. Jr., Evans, D.G., DuPont, H.L.: Infect Immun **23,** 336 (1979).
8. Evans, D.J. Jr., Evans, D.G., Young, L.S., Pitt, J.: J Clin Microbiol **12,** 235 (1980).
9. Källenius, G., Möllby, R.: FEMS Microbiol Lett **5,** 295 (1979).
10. Wevers, P., Picken, P., Schmidt, G., Jann, B., Jann, K., Golecki, J.R., Kist, M.: Infect Immun **29,** 685 (1980).
11. Svanborg Eden, C., Hansson, H.A.: Infect Immun **21,** 229 (1978).
12. Vosti, K.L.: Infect Immun **25,** 507 (1979).
13. Ljungh, A., Faris, A., Wadström, T.: J Clin Microbiol **10,** 477 (1979).
14. Ørskov, I., Ørskov, F., Birch Andersen, A.: Infect Immun **27,** 657 (1980).
15. Ørskov, I., Ørskov, F., Birch Andersen, A., Kanamori, M., Svanborg Eden, C.: Scand J Infect Dis S **33,** 18 (1982).
16. Källenius, G., Möllby, R., Svenson, S.B., Winberg, J., Lundblad, A., Svenson, S., Cedergren, B.: FEMS Microbiol Lett **7,** 297 (1980).
17. Väisänen, V., Elo, J., Tallgren, L.G., Siitonen, A., Mäkelä, P.H., Svanborg Eden, C., Källenius, G., Svenson, S.B., Hultberg, H., Korhonen, T.: Lancet **2,** 1366 (1981).
18. Kuch, B., Pál, T., Emődy, L.: Acta Microbiol Acad Sci Hung **27,** 317 (1980).
19. Czirók, É., Csik, M., Börzsönyi, M.: Acta Microbiol Acad Sci Hung **28,** 119 (1981).
20. Ørskov, I., Ørskov, F.: Med Microbiol Immunol **163,** 99 (1977).
21. Evans, D.G., Evans, D.J. Jr., Tjoa, W.S.: Infect Immun **18,** 330 (1977).
22. Horváth, I.: Kvantitativ mikrobiológiai eljárások. Akadémiai Kiadó, Budapest 1974.
23. Korhonen, T.: FEMS Microbiol Lett **6,** 421 (1979).

24. Svanborg Eden, C., Hanson, L.Å., Jodal, U., Lindberg, U., Åkerlund, A. S.: Lancet **2,** 490 (1976).
25. Varian, S.A., Cooke, E.M.: J Med Microbiol **13,** 111 (1980).
26. Svanborg Eden, C., Eriksson, B., Hanson, L.Å., Jodal, U., Kaijser, B., Lidin, Jonson, G., Lindber, U., Olling, S.: J Pediatr **93,** 398 (1978).
27. van den Bosch, J.F., Postma, P., Koopman, P.A.R., de Graaf, J., MacLaren, D.M., van Brenk, D.G., Guinée, P.A.M.: J Hyg (Camb) **88,** 567 (1982).
28. Czirók, É., Ørskov, I., Ørskov, F.: Infect Immun **37,** 519 (1982).
29. Hartley, C.L., Neumann, C.S., Richmond, M.H.: Infect Immun **23,** 128 (1979).
30. Källenius, G., Möllby, R., Svenson, S.B., Helin, I., Hultberg, H., Cedergren, B., Winberg, J.: Lancet **2**, 1369 (1981).

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SYNERGISTIC INTERACTION OF BAKER'S YEAST AND IRON IN THE ENHANCEMENT OF BACTERIAL VIRULENCE

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The virulence-enhancing interaction of baker's yeast and different iron preparations (ferric ammonium citrate and iron dextran) was tested in mice challenged with *Salmonella typhi* and *Vibrio cholerae* (Inaba and Ogawa) strains. The virulence-enhancing effect of the yeast + iron combination increased significantly as compared to that of either yeast or iron alone. Toxicity assays of the single and combined baker's yeast and iron preparations by the mouse weight gain test have shown that the combinations are considerably more toxic than either single agent, probably owing to the presence of yeast. Examination of the single and combined preparations for influence on body temperature of mice has revealed a general hypothermic action, which was strongest in the combinations, owing again to the yeast. Theoretical considerations on the underlying mechanism of the virulence-enhancing effect have supported the hypothesis that the effect might be associated with the strong hypothermic action produced by baker's yeast and baker's yeast+iron combinations, in as much as hypothermia increases the production of siderophores which ensure the acquisition of iron indispensable for bacterial growth.

Iron is known to enhance the virulence of many microorganisms (for reviews see [1, 2]). We [3–5] and Ford and Hayhoe [6] have shown this effect on *Vibrio cholerae*, Kreeftenberg et al. [7] demonstrated a strong pathogenicity promoting effect of baker's yeast on *Salmonella typhi* Ty2, and we too, could confirms this finding [8].

In this paper we report on investigations into the interaction of iron and baker's yeast, viz. on their effect on the virulence of *S. typhi* and *V. cholerae* strains.

Materials and methods

Bacteria. Salmonella typhi Ty2; *Vibrio cholerae* Inaba 569 B and Ogawa 41.

Mice. CFLP outbred mice were used at an equal sex ratio.

Virulence-enhancing (VE) agents. Mucin (M). Hog gastric mucin, type 1701-w (Wilson Laboratories, Chicago, USA) was used in 5% w/v suspension according to the recommendations

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of the World Health Organization [9]. *Baker's yeast (BY).* Inactive dried baker's yeast ("Engevita", Gist-Brocades N.V., Delf, The Netherlands) was suspended in isotonic saline at 5% w/v concentration, heat-sterilized on 2 consecutive days in water bath at 100 °C for 30 min, and adjusted to pH 7.2 with 1 N NaOH before use. *Ferric ammonium citrate (FAC).* (Ammonium ferric citrate brown, Code No. 27163. BDH, Poole, England.) The solutions were prepared in distilled water and sterilized by boiling. *Iron dextran (ID).* (Imferon, Pharmacia AS, Hellerød, Denmark) without preservative; it was sterilized by autoclaving.

Virulence tests. Four or 5 groups of mice allocated at random, each consisting of 12 animals (22–24 g) were challenged intraperitoneally (i.p.) with tenfold serial dilutions of bacterial suspensions prepared from 5–6 h agar cultures in phosphate-gelatin-saline buffer pH 7.2 [9]. The VE agents were added to the suspensions immediately before challenge. Animals were observed 72 h.

Determination of germ count. The total bacterial count was assessed visually by comparison with the 4th and 5th International Reference Preparations for Opacity. The viable count was determined by counting colony-forming units in cultures obtained by the pour-plate technique.

Estimation of the toxicity of VE agents by the mouse weight gain test. Groups of mice allocated at random, each consisting of 12 animals (18–20 g) were allowed free access to food and water for not less than 2 h before inoculation. The group weights were determined immediately before treatment with the VE agent, and after it on each of 7 consecutive days, 2 h after feeding. The VE agents were applied ip in the same doses as in the virulence tests, in 0.5 ml volume.

Determination of temperature changes induced by VE agents. Groups of mice allocated at random, each consisting of 10 animals (18–20 g), received the materials i.p. Rectal temperature was determined before treatment, and 1, 2, 3, 4, 5, 6, 24, 27 and 48 h after it with an electric universal thermometer, type RM6 (ELLAB A/S, Copenhagen, Denmark), using a type A—RM4 thermocouple probe. The VE agents were applied in the same doses as in the virulence tests, in 0.5 ml volume. The mice were kept at 19–20 °C room temperature during the test period.

Statistical analysis of results. The LD_{50} values and the standard error (s.e.) (at 95% confidence limit) of the LD_{50} values were determined with the method of Reed and Muench [10]. The significance between the LD_{50} values was estimated by Fisher's F test [11]. The significance between the body weight-decreasing ability of the different materials was assessed by Student's one sample t test, and the significance between the temperature-decreasing activity of the various compounds one hour after inoculation was determined by Student's two sample t test.

Results

Virulence-enhancing effect of different agents. THE VE effect of the examined materials on the test bacteria are shown in Table I. Table II presents the significance between the VE effects of different agents.

Salmonella typhi. Comparison of the LD_{50} values gave the following sequence of VE efficiency: (1) 1 FAC + BY; (2) 2 FAC + BY; (3) M.; (4) 10 ID + BY; (5) 20 ID + BY; (6) BY; (7) 2 FAC; (8) 20 ID; (9) 1 FAC; (10) 10 ID. The VE effect of M and BY agreed with data from the literature. FAC and ID proved to be considerably less efficient than M and BY, but still had a certain VE effect, because it is well-known that the LD_{50} of *S. typhi* Ty2 without VE agents amounts to about $1–4 \times 10^6$ bacteria. It is, however, fully unequivocal that the VE effect of the FAC + BY and ID + BY combinations exceeded significantly that of BY, FAC or ID applied separately.

Vibrio cholerae Inaba. The sequence of VE activity was; (1) 20 ID + BY; (2) 1 FAC + BY; (3) 2 FAC + BY; (4) 10 ID + BY; (5) M.; (6) 2 FAC; (7) 20 ID; (8) 10 ID; (9) 1 FAC; (10) BY. Apart from some minor differences,

Table I

Virulence-enhancing effect of different agents

VE agents	*S. typhi* Ty2			*V. cholerae* Inaba 569 B			*V. cholerae* Ogawa NIH 41		
	No. of tests	LD_{50}	s.e.	No. of tests	LD_{50}	s.e.	No. of tests	LD_{50}	s.e.
5% M	4	8.32	1.81	2	37.71	4.72	2	49.05	32.30
5% BY	7	236.25	113.60	3	3.11×10^5	2.04×10^5	3	1.49×10^6	8.81×10^5
1 FAC (1 mg) 5	5	1.08×10^5	3.92×10^4	3	3.41×10^3	1.74×10^3	3	2.56×10^4	2.19×10^4
2 FAC (2 mg) 3	3	1.25×10^4	8.38×10^3	3	79.02	54.85	3	2.35×10^3	2.23×10^3
1 FAC + BY	4	1.11	0.26	2	1.65	0.94	2	2.26	0.88
2 FAC + BY	2	1.73	1.36	1	2.01	—	1	1.52	—
10 ID (10 mg)	4	1.28×10^5	7.58×10^4	3	976.05	953.15	2	448.65	405.15
20 ID (20 mg)	1	5.95×10^4	—	2	159.26	157.33	2	46.05	7.47
10 ID + BY	6	13.15	6.77	2	2.65	0.55	2	6.45	1.73
20 ID + BY	2	23.53	15.21	1	1.53	—	1	5.03	—

– Not determined; a single test was performed

Abbrevations. M = mucin; BY = baker's yeast; FAC = ferric ammonium citrate; 1 FAC = 1 mg ferric ammonium citrate; 2 FAC = = 2 mg ferric ammonium citrate; ID = iron dextran; 10 ID = 10 mg iron dextran; 20 ID = 20 mg iron dextran

the most important statement was identical with that for *S. typhi*, viz. the FAC + BY and ID + BY combinations were significantly more active than BY, FAC or ID alone.

Vibrio cholerae Ogawa. The sequence of the VE effect proved to be essentially similar to that found with the other two strains: (1) 2 FAC + BY; (2) 1 FAC + BY; (3) 20 ID + BY; (4) 10 ID + BY; (5) 20 ID; (6) M.; (7) 10 ID; (8) 2 FAC; (9) 1 FAC; (10) BY. The FAC + BY and ID + BY combinations were again significantly more efficient than BY, FAC or ID by themselves.

Repeated examinations with the three test organisms have unequivocally affirmed that combinations of iron and yeast enhance the virulence of microorganisms in a significantly greater degree than either iron or yeast applied separately.

Table II

Virulence-enhancing effect of different agents (P values)

VE agents	*S. typhi* Ty2	*V. cholerae* Inaba 569 B	*V. cholerae* Ogawa NIH 41
By – 1 FAC	<0.01	<0.01	<0.01
BY – 2 FAC	<0.01	<0.001	<0.01
BY – 10 ID	<0.001	<0.001	<0.001
BY – 20 ID	–	<0.001	<0.001
1 FAC – 2 FAC	<0.01	<0.001	<0.01
1 FAC – 10 ID	no significant difference	<0.001	<0.001
1 FAC – 20 ID	–	<0.001	<0.001
2 FAC – 10 ID	<0.001	<0.01	<0.001
2 FAC – 20 ID	–	<0.01	<0.001
BY – 1 FAC + BY	<0.001	<0.001	<0.001
BY – 2 FAC + BY	<0.01	–	–
BY – 10 ID + BY	<0.001	<0.001	<0.001
BY – 20 ID + BY	<0.1	–	–
1 FAC – 1 FAC + BY	<0.001	<0.001	<0.001
2 FAC – 2 FAC + BY	<0.001	–	–
10 ID – 10 ID + BY	<0.001	<0.001	<0.001
20 ID – 20 ID + BY	<0.001	–	–

– Not determined; a single test was performed

Estimation of the toxicity of VE agents by the mouse weight gain test. The average results of repeated tests are presented in Table III. Table IV shows the significance between the average weight changes (for days 1–4) in animal groups inoculated with different VE agents. The mean weight gain rates assessed in the groups treated with the different VE agents by comparison to the mean initial weights gave the following decreasing sequence of effect: (1) 2 FAC + BY; (2) 1 FAC + BY; (3) BY; (4) 10 ID + BY; (5) 20 ID + BY;

Table III

Arithmetical average weight changes in animal groups inoculated with different virulence-enhancing agents

VE agent	Average weight changes (g) on days				
	1	2	3	4	7
Nil	+1.125	+1.708	+2.541	+3.333	+4.250
Isotonic saline	+0.375	+1.250	+2.000	+2.542	+3.375
5% M	*−1.000*	+0.125	+1.334	+1.917	+3.709
5% BY	*−2.292*	*−1.167*	+0.167	+0.917	+3.367
1 FAC	*−0.676*	+0.278	+0.963	+1.752	+3.699
2 FAC	*−1.417*	+0.132	+1.437	+1.875	+3.812
10 ID	+0.333	+1.500	+2.000	+2.708	+3.791
20 ID	+0.458	+1.500	+2.042	+2.583	+4.042
1 FAC + BY	*−2.667*	*−1.458*	*−0.167*	+0.833	+3.250
2 FAC + BY	*−2.958*	*−2.375*	*−1.208*	0.000	+2.834
10 ID + BY	*−2.042*	*−0.185*	+0.597	+1.858	+4.293
20 ID + BY	*−1.875*	*−0.208*	+1.000	+1.834	+4.125

(6) 2 FAC; (7) M; (8) 1 FAC; (9) 10 ID; (10) 20 ID. The data show that (i) the agents having the strongest VE effect (2 FAC + BY, 1 FAC + BY, 20 ID + BY, 10 ID + BY) were the most toxic; (ii) the increase of the toxicity was caused by BY.

Temperature changes induced by VE agents. In Table V arithmetic mean values of several tests are presented. Table VI shows the significance between the average temperature changes in the first hour after inoculation in the groups inoculated with different VE agents. Relying upon their temperature-decreasing ability the following sequence of the VE agents could be established: (1) 1 FAC + BY; (2) 2 FAC + BY; (3) BY; (4) 10 ID + BY; (5) 20 ID + BY;

Table IV

Average weight changes on days 1–4 in animal groups inoculated with different virulence-enhancing agents (P values)

VE agent	Significance	VE agent	Significance
BY – 1 FAC	<0.02	BY – 1 FAC + BY	<0.02
BY – 2 FAC	<0.001	BY – 2 FAC + BY	<0.001
BY – 10 ID	<0.001	BY – 10 ID + BY	<0.05
BY – 20 ID	<0.001	BY – 20 ID + BY	<0.001
1 FAC – 2 FAC	<0.02	1 FAC – 1 FAC + BY	<0.02
1 FAC – 10 ID	<0.001	2 FAC – 2 FAC + BY	<0.01
1 FAC – 20 ID	<0.001	10 ID – 10 ID + BY	<0.02
2 FAC – 10 ID	<0.05	20 ID – 20 ID + BY	<0.05
2 FAC – 20 ID	<0.02		

Table V

Temperature changes in animal groups inoculated with different virulence-enhancing agents

VE agent	Average temperature changes (°C) at hours									
	1	2	3	4	5	6	24	27	30	48
Nil.	*−0.98*	*−0.93*	*−0.86*	*−0.86*	*−0.70*	*−0.76*	+0.03	*−0.61*	*−0.91*	*−0.41*
	0.108*	0.098	0.059	0.059	0.070	0.043	0.073	0.100	0.163	0.132
Isotonic saline	*−0.58*	*−0.61*	*−0.54*	*−0.62*	*−0.66*	*−0.61*	*−0.06*	*−0.63*	*−0.50*	*−0.34*
	0.245	0.207	0.169	0.178	0.202	0.274	0.198	0.326	0.300	0.220
5% M	*−3.45*	*−2.65*	*−2.06*	*−1.82*	*−1.15*	*−0.72*	*−0.35*	*−1.01*	*−0.81*	*−0.12*
	0.479	0.431	0.399	0.484	0.342	0.324	0.473	0.499	0.388	0.432
1 FAC	*−2.04*	*−1.13*	*−0.84*	*−1.02*	*−0.81*	*−0.91*	*−0.12*	*−0.76*	*−0.51*	*−0.05*
	0.537	0.374	0.204	0.325	0.342	0.301	0.498	0.300	0.322	0.273
2 FAC	*−2.74*	*−2.49*	*−2.02*	*−2.01*	*−1.70*	*−1.64*	*−0.95*	*−0.31*	*−0.44*	+0.01
	0.762	0.832	0.666	0.503	0.447	0.503	0.997	0.526	0.532	0.571
10 ID	*−0.65*	*−0.43*	*−0.65*	*−0.67*	*−0.77*	*−0.71*	*−0.41*	*−0.59*	*−0.50*	*−0.33*
	0.368	0.305	0.246	0.198	0.247	0.190	0.365	0.289	0.278	0.332
20 ID	*−0.75*	*−0.70*	*−0.57*	*−0.77*	*−0.77*	*−0.65*	*−0.42*	*−0.70*	*−0.55*	*−0.18*
	0.384	0.367	0.389	0.282	0.266	0.294	0.373	0.271	0.304	0.364
BY	*−4.63*	*−3.92*	*−3.23*	*−3.92*	*−3.45*	*−2.93*	+0.11	*−0.32*	*−0.01*	*−0.20*
	0.938	0.976	1.033	1.017	1.147	1.154	0.449	0.426	0.429	0.637
1 FAC + BY	*−5.99*	*−4.71*	*−3.33*	*−3.64*	*−3.55*	*−3.57*	*−2.19*	*−1.47*	*−1.00*	—
	1.034	0.748	0.483	0.602	0.703	0.686	1.876	1.673	1.135	—
2 FAC + BY	*−5.54*	*−4.05*	*−3.13*	*−3.12*	*−3.19*	*−3.12*	*−0.93*	*−1.30*	*−0.90*	—
	1.221	1.015	0.570	0.595	0.752	0.886	1.474	1.696	1.146	—
10 ID + BY	*−3.27*	*−2.44*	*−1.92*	*−1.34*	*−0.96*	*−0.69*	+0.18	*−0.10*	*−0.25*	+0.02
	0.679	0.443	0.500	0.398	0.470	0.346	0.463	0.389	0.642	0.378
20 ID + BY	*−3.93*	*−2.87*	*−2.70*	*−2.22*	*−1.65*	*−1.33*	0	+0.15	*−0.13*	+0.13
	0.565	0.424	0.430	0.348	0.352	0.405	0454	0.248	0.451	0.516

* s.e. (standard error)

(6) 2 FAC; (7) M; (8) 1 FAC; (9) 10 ID; (10) 20 ID. This permits the following conclusions: (i) untreated and saline-treated mice also showed a certain decrease in body temperature, presumably in response to the temperature measurement; (ii) those VE agents which proved to be most toxic in the weight gain test also accounted for the greatest temperature drop; (iii) the agents showing the highest VE effect gave rise to the most distinct decrease in temperature; (iv) for the decrease of the temperature, BY was chiefly responsible.

On the basis of the results the rank correlation coefficient (R_{RANK}) between the influence of the VE agents on the organism of the animals (weight- and temperature-decreasing effect) and their VE effect on the tested bacterium strains was determined by Kendale's method [12] (see Table VII).

Table VI

Average temperature changes in the first hour after inoculation in animal groups inoculated with different virulence-enhancing agents (P values)

VE agent	Significance	VE agent	Significance
BY – 1 FAC	>0.05	BY – 1 FAC + BY	<0.05
BY – 2 FAC	>0.05	BY – 2 FAC + BY	>0.3
BY – 10 ID	<0.001	BY – 10 ID + BY	>0.6
BY – 20 ID	<0.001	BY – 20 ID + BY	>0.8
1 FAC – 2 FAC	<0.1	1 FAC – 1 FAC + BY	<0.001
1 FAC – 10 ID	<0.02	2 FAC – 2 FAC + BY	<0.05
1 FAC – 20 ID	<0.1	10 ID – 10 ID + BY	<0.001
2 FAC – 10 ID	<0.01	20 ID – 20 ID + BY	<0.001
2 FAC – 20 ID	<0.05		

Table VII

Rank correlation coefficient (R_{RANK}) between the weight- and temperature-decreasing ability of different VE agents and their virulence-enhancing effect

Compared investigations	R_{RANK}	P_{RANK}
Weight-decreasing ability – temperature-decreasing ability	0.9151	<0.001
Weight-decreasing ability – VE effect on *S. typhi* Ty2	0.7940	<0.01
Temperature-decreasing ability – VE effect on *S. typhi* Ty2	0.8787	<0.001
Weight-decreasing ability – VE effect on *V. cholerae Inaba*	0.5757	<0.1
Temperature-decreasing ability – VE effect on *V. cholerae* Inaba	0.5515	<0.1
Weight-decreasing ability – VE effect on *V. cholerae* Ogawa	0.4666	>0.1
Temperature-decreasing ability – VE effect on *V. cholerae* Ogawa	0.4909	>0.1

Discussion

The FAC + BY and ID + BY combinations accounted not only for the highest VE, but also for the strongest toxic effect and greatest temperature drop. Analysis of the results indicated the responsibility of BY for the

marked, increased toxic and hypothermic action of these agents. The question arose by which mechanism(s) was this effect produced, because the striking synergistic VE activity of FAC + BY and ID + BY cannot be explained by a simple additive effect.

Hypothetically the non-specific toxicity of the VE agents could account for their strong VE effect. This would presuppose that at least part of the deaths had been due to strong non-specific toxic action. The supposed correlation between the non-specific and VE effect cannot, however, be reconciled with the experimental fact that treatment with VE agents without bacteria (control groups) caused only 1–2 deaths.

Another factor to be considered is the strong hypothermic action of BY, FAC + BY and ID + BY. The interrelationship between infection and body temperature has been studied extensively in part of the cases with iron considered to the third interacting factor (1, 2, 13–21). One of the characteristics of fever and inflammation is a rapid decrease in the concentration of iron in serum [22]. This hypoferraemia is accomplished by the endogenous mediator released from polymorphonuclear leukocytes, which appears to be identical with the leukocytic pyrogen [22]. The decrease in the concentration of serum iron helps to make the extracellular environment less favourable for bacterial growth and there is evidence that a combination of fever and low iron concentration inhibits the growth of some bacteria. When rabbits were infected with *Pasteurella multocida*, the concentration of iron in their plasma decreased and their temperature rose [15]. To determine whether the rise in body temperature and the fall in plasma iron may be a coordinated host defense response, *P. multocida* were grown in vitro at various temperatures and iron concentrations. At afebrile temperatures the bacteria grew equally well at low or high concentrations of iron. When, however, the temperature was raised to febrile, the growth of bacteria was inhibited by a low and not a high iron concentration [15]. Similarly, infection of the lizard *Dipsosaurus dorsalis* with *Aeromonas hydrophila* led to a decrease in the serum iron concentration [19, 20, 22]. The importance of low environmental iron concentration is reinforced by the observation that injection of infected lizards with ferric ammonium citrate greatly increased their mortality. These data support the hypothesis that one of the mechanisms behind the adaptive (or beneficial) role of fever is the reduced ability of pathogenic bacteria to grow well at elevated temperatures in an iron-poor medium. Fever-induced limitation of iron for microbial growth and toxin synthesis favour survival of the host. The interaction of host and iron metabolism are apparently of benefit to the host invaded by bacteria.

It is well-known that to acquire essential iron, bacteria produce iron-chelating components or siderophores, which are extremely efficient in removing ferric iron from the environment and making it available for growth of the microorganisms [23, 24]. These compounds have been shown to enhance

the virulence of *Escherichia coli* [25, 26], *Salmonella* [27] and *Pseudomonas aeruginosa* [28] in experimental animals. Evidence has been accumulating on the temperature sensitivity of siderophore production [13]: at high temperatures, many bacteria such as *E. coli*, *Salmonella* and *Pseudomonas* sp. produce few siderophores if any [29–32], and at elevated temperatures, these bacteria are capable to grow only in culture media containing supplemental iron.

In addition to favouring bacterial growth by enhancing siderophore production, hypothermia may have other adverse effects, too [18]. It rendered mice more susceptible to the lethal effects of Gram-negative bacterial endotoxins and to challenge with *S. typhi-murium* and *Staphylococcus aureus*. There are indications that hypothermia gives rise to leukopenia, and also depresses phagocytosis under certain conditions [18].

In the light of the above observations there is reason to postulate that the strong VE effect of FAC + BY and ID + BY correlates with the substantial hypothermia caused by these agents, and the subsequent increased siderophore synthesis. An increased siderophore production remains, however, to be proven for verification of this hypothesis.

The cause of the hypothermic effect of BY is unknown. Prashker and Wardlaw [33], Wardlaw et al. [34] demonstrated that *E. coli* endotoxin gave rise to marked hypothermia in mice. Perhaps the carbohydrate complex contained in the yeast cell wall is responsible for the hypothermic effect.

Mucin was used in the present study only as a reference preparation, and was therefore not studied for its mechanism of action. It deserves mention that Powell et al. [35] observed a considerable increase in the pathogenicity promoting effect of mucin on *S. typhi* after the addition of iron, and we observed a similar effect in *E. coli* and *Shigella* strains [36].

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REFERENCES

1. Weinberg, E.D.: Microbiol Rev **42,** 45 (1978).
2. Bullen, J.J.: Rev Infect Dis **3,** 1127 (1981).
3. Joó, I., Csizér, Z.: Symposium on Field Trials of Vaccines. Yugoslav Academy of Sciences and Arts, Zagreb 1973, p. 59.
4. Joó, I.: Progr Drug Res **19,** 546 (1975).
5. Joó, I., Zsidai, J.: J Biol Standard **5,** 341 (1977).
6. Ford, A., Hayhoe, J.P.V.: J Biol Standard **4,** 353 (1976).
7. Kreeftenberg, J.G., van Ramshorst, J.D., Loggen, H.G.: J Biol Standard **1,** 73 (1973).
8. Joó, I., Zsidai, J.: J Biol Standard **10,** 17 (1982).
9. World Health Organization: Unpublished WHO Working Document. WHO/BD/Cholera/72.3.

10. Reed, L.J., Muench, H.: Amer J Hyg **27,** 493 (1938).
11. Fisher, R.A., Yates, F.: Statistical Tables for Biological, Agricultural and Medical Research. 6th ed. Oliver and Boyd Ltd., Edinburgh 1963.
12. Kendale, M.G.: Rank Correlation Methods. Hafner Publ. Co., New York 1952.
13. Mackowiak, P.A.: Rev Infect Dis **3,** 508 (1981).
14. Grieger, T.A., Kluger, M.J.: J Physiol **279,** 187 (1978).
15. Kluger, M.J., Rothenburg, B.A.: Science **203,** 374 (1979).
16. Weinberg, E.D.: In Hoekstra, W.G., Suttie, J.W., Ganther, H.E., Mertz, W. (eds): Trace Element Metabolism in Animals. University Park Press, Baltimore 1974, p. 41.
17. Weinberg, E.D.: In Thermoregulatory Mechanisms and their Therapeutic Implications. 4th International Symposium on the Pharmacology of Thermoregulation, Oxford 1979. S. Karger, Basel 1980.
18. Roberts, N.J. Jr.: Microbial Rev **43,** 241 (1979).
19. Bernheim, H.A., Kluger, M.J.: Am J Physiol **231,** 198 (1976).
20. Bernheim, H.A., Bodel, P.D., Askanase, P.W., Atkins, E.: Br J Exp Path **59,** 76 (1978).
21. Kluger, M.J.: Pediatrics **66,** 720 (1980).
22. Merriman, C.R., Pulliam, L.A., Kampfschmidt, R.F.: Proc Soc Exp Biol Med **154,** 224 (1977).
23. Payne, S.M., Finkelstein, R.A.: J Clin Invest **61,** 1428 (1978).
24. Lankford, C.E.: CRC Crit Rev Microbiol **2,** 273 (1973).
25. Langman, L., Young, I.G., Frost, G.E., Rosenberg, H., Gibson, F.: J Bacteriol **112,** 1442 (1972).
26. Griffiths, E., Humphreys, J.: Infect Immun **28,** 286 (1980).
27. Yancey, R.J., Breeding, S.A.L., Lankford, C.E.: Infect Immun **24,** 174 (1979).
28. Cox, C.D., Graham, R.: J Bacteriol **137,** 357 (1979).
29. Rogers, H.J.: Infect Immun **7,** 445 (1973).
30. Garibaldi, J.A.: J Bacteriol **105,** 1036 (1971).
31. Garibaldi, J.A.: J Bacteriol **110,** 262 (1972).
32. Kochan, I.: Adv Chem Ser **162,** 55 (1977).
33. Prashker, D., Wardlaw, A.C.: Br J Exp Path **52,** 36 (1971).
34. Wardlaw, A.C., Boorman, L., Reid, R.: Br J Exp Path **52,** 198 (1971).
35. Powell, J.C. Jr., DeSett, C.R., Lowenthal, J.P., Berman, S.: J Biol Stand **8,** 79 (1980).
36. Joó, I.: Unpublished observations.

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EFFECT OF pH ON THE ACTIVITY OF CEFTIZOXIME

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The in vitro antibacterial activity of ceftizoxime (Cefizox™) is influenced by the ambient pH of the medium. This pH-dependent activity was observed when either the assay discs or the assay medium were buffered. For the conventional disc agar-diffusion assay, using *Bacillus subtilis* ATCC 6633, the medium buffered to pH 6 had definite advantage for measuring potency and activity. In serial dilution assay, the acidic (pH 6) medium gives better MIC values with *Staphylococcus aureus* strains. In the case of *Escherichia coli* strains, the pH of the medium (6, 7.3 and 8) appears to be of less significance.

Many years ago we found that the activity of a newly synthesized SK&F oral, phenylglycine-type cephalosporin was greatly influenced by the pH of the medium. Slight acidity (pH 6) increased the activity. Ceftizoxime, like this compound, contains only a H-atom at the 3-position of the dihydrothiazine ring. It was reasoned that this feature may convey similar pH-sensitivity to the activity of ceftizoxime. In this communication, the results obtained in in vitro experiments with ceftizoxime [1], the new broad-spectrum, beta-lactamase-stable third generation aminothiazolyl-methoxyimino cephalosporin, at various pH values are presented.

Materials and methods

Sodium ceftizoxime which is water soluble was supplied by Fujisawa SmithKline Corporation. Its chemical structure is shown in Fig. 1. Two types of assays were performed, using either the disc agar-diffusion or the two-fold serial broth dilution methods. In the first case, non-buffered agar was seeded with an appropriate dilution of spores of *Bacillus subtilis* ATCC 6633. The susceptibility discs were buffered to various pH values with McIlvaine's cirtic acid phosphate buffer and stored dry. Immediately before the assay they were saturated with the solution of ceftizoxime to contain 1 μg antibiotic per disc, and were placed on the surface of the seeded agar [2]. After overnight incubation at 37 °C, the inhibition zones were measured and recorded. Triplicate experiments were carried out with freshly prepared solutions using 4 discs for each pH value.

For the tube dilution assay, the peptone-glucose-buffered (PGB) medium [3] was used buffered with McIlvaine's citric acid-phosphate buffer [4] to pH 6, 7.3 and 8. The tubes contained 3 ml of the medium and two-fold concentrations of ceftizoxime (in the case of *S. aureus* strains 50 to 0.8 μg/ml, and in the case *E. coli* strains 3.2 to 0.05 μg/ml). Tubes were

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inoculated with 0.05 ml of the 10-times dilutions of overnight grown cultures in the same (PGB) liquid medium. After overnight incubation at 37 °C, the turbidity for growth was observed visually. The assay was repeated several times.

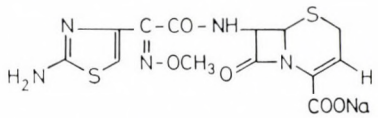

Fig. 1. Chemical structure of ceftizoxime

Results and discussion

The results of a typical disc agar-diffusion assay are presented in Table I. It can be seen that the diameters of the inhibition zones are strongly pH-dependent, as it was observed with the earlier SK&F phenylglycine-type compound which similarly to ceftizoxime contains only a H-atom at the 3-position of the cephem molecule. On this basis, pH 6 is recommended for assay of ceftizoxime when *B. subtilis* ATCC 6633 is used as the indicator organism, in particular for quality control studies.

The pH-dependent activity of ceftizoxime was also observed when the serial dilution assay was used. The MIC values for two representative *S. aureus* strains are presented in Table II and those for two *E. coli* strains in Table III. The data of the tables show that ceftizoxime is highly and almost equally active against both the beta-lactamase-producing and non-producing *S. aureus* and *E. coli* strains in liquid medium. Against the *S. aureus* strains, the MIC's are the greatest in the medium buffered to pH 6 and they diminish at neutrality and further on at pH 8. In the case of *E. coli* strains, the influence of pH on the MIC values seems to be less significant, but they still are better at slightly

Table I

Diameters of the inhibition zones in mm with the pH-ed discs saturated with ceftizoxime solution

pH of the disc	Diameter of inhibition zones in mm*
5	19
6	18
7	14
8	13
9	11
10	12
Non-buffered	12

* Assay organism: *B. subtilis* ATCC 6633

Table II

pH-Dependent activity of ceftizoxime against two S. aureus *strains in liquid medium (PGB)*

pH	Growth at μg/ml ceftizoxime															
	S. aureus 671*								*S. aureus* 674							
	C	50	25	12	6	3	1.5	0.8	C	50	25	12	6	3	1.5	0.8
6.0	++++	−	−	−	−	−	−	+	++++	−	−	−	−	−	−	±
7.3	++++	−	−	−	−	−	++	++++	++++	−	−	−	−	−	−	++++
8.0	++++	−	−	−	−	++	++	++++	++++	−	−	−	−	−	+++	++++

* Beta-lactamase producing strain
++++ Full growth
− No growth
C = Control (no drug added)

Table III

Activity of ceftizoxime against two E. coli *strains in liquid medium (PGB) buffered to three different pH values*

pH	Growth at μg/ml ceftizoxime															
	E. coli 12140								*E. coli* 211*							
	C	3.2	1.6	0.8	0.4	0.2	0.1	0.05	C	3.2	1.6	0.8	0.4	0.2	0.1	0.05
6.0	++++	—	—	—	—	—	—	++	++++	—	—	—	—	—	—	++++
7.3	++++	—	—	—	—	—	—	++++	++++	—	—	—	—	—	—	++++
8.0	++++	—	—	—	—	—	—	++++	++++	—	—	—	—	—	++++	++++

* Strong beta-lactamase producer
++++ Full growth
— No growth
C = Control (no drug added)

acidic pH. These experiments show that buffering the medium influences the MIC values of ceftizoxime, similarly as it influences its stability in aqueous solutions [5]. It is recommended that the medium should be buffered to pH 6 for potency and activity determinations, like for stability studies [5]. In weakly acidic environment ceftizoxime is least ionized and the existing molecular species has a better penetration into the bacterial cell than the ionic forms [6]. Similar phenomenon with the pH effect was recently observed with ketoconazole [7]. Ceftizoxime has been approved for clinical use in Japan [8] and in the USA [9] and will be followed in other parts of the world. Therefore it is essential that its biological determination and quality control should be performed at optimal assay conditions. In many places of the mammalian body, such as prostate, vagina, skin and inflammatory tissues, the pH is acidic. It explains also the clinical finding that ceftizoxime is highly effective in staphylococcus infections inspite of its MIC's (2–4 μg/ml) obtained in non-buffered media.

REFERENCES

1. Kamimura, T., Matsumoto, Y., Okada, N., Mine, Y., Nishida, M., Goto, S., Kawahara, S.: Antimicrob Agents Chemother **16,** 540 (1979).
2. Uri, J.V., Actor, P., Weisbach, J.A.: Experientia **35,** 1034 (1979).
3. Uri, J.V., Actor, P.: J Antibiot **33,** 501 (1980).
4. McIlvaine, T.C.: J Biol Chem **49,** 183 (1921).
5. Uri, J.V.: Experientia **39,** 1085 (1983).
6. Joklik, W.K., Willet, H.P., Amos, D.B.: Linsser Microbiology. 17th ed. Appleton-Century-Crofts, New York 1980, p. 281.
7. Minagawa, J., Kitaura, K., Nakamizo, N.: Antimicrob Agents Chemother **23,** 105 (1983).
8. Shimada, J., Shimizu, K.: J Antimicrob Chemother **10,** Suppl C, 311 (1982).
9. Parks, D., Layne, P., Uri, J.V., Ziv, D., Bass, S.: J Antimicrob Chemother **10,** Suppl C, 327 (1982).

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PATHOGENESIS AND IMMUNOPROPHYLAXIS OF BOVINE ROTAVIRUS INFECTION*

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Introduction

Rotaviruses, presently a genus within the family *Reoviridae* being recognized on behalf of their specific morphology were first detected in calves by Mebus et al. [1] in 1969. In the meantime, rotaviruses have been described in around 10 mammalian species [2]. Molecular biologists are involved with delineation of common versus species-specific properties of the virions. This paper will not deal with such aspects. Rather it will present published results of other authors as well as own results on biological and pathogenetical parameters of rotavirus infections. They show remarkable parallels between rotavirus infections of different species as well as between rotavirus infections and other viral infections of the intestinal tract which cause superficial enteritis in their respective hosts.

For the time being it appears that rotavirus infections have the profoundest clinical impact in the human and the bovine species [2]. In both species their immunoprophylactical control has been considered. As our group in Vienna has worked with calves, the present report will largely concentrate on bovine rotavirus infection. With regard to the physicians present in this auditorium some cross-links to human rotavirus infection will be put forward.

We and others have greatly benefited from the fundamental studies, which Haelterman [3], Hooper and Haelterman [4] have performed on transmissible gastroenteritis (TGE). This coronavirus infection of piglets, determined almost 40 years ago, thanks to its great economic impact and to the relative ease of experimentation with its multiparturient host, was investigated since the sixties for its pathogenetical and immunoprophylactical para-

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meters [5] as hardly another viral enteropathogen was except for human poliomyelitis.

The two most important observations of Haelterman's group were: (i) TGE virus does not cause systemic infection; in fact, it not even infects the entire intestinal wall, but merely the epithelial cells at the tips of its villi, leading to denudation of villi. (ii) Immunoprophylactical experiments made along different approaches terminated in a single effective way: institution of lactogenic immunity.

Let us dwell for a moment on these important parameters, which will form the keystones for understanding our own rotavirus results and merit due consideration of everyone engaged in specific prophylaxis of probably all rota-, corona-, calici-, astro- and additional viruses.

"Denudation of villi" stands as synonymous for the older term "superficial enteritis" and merits preference to the latter one, as histopathology does not disclose -itis, inflammation, in rapidly fatal cases. In fact death ensues loss of water and electrolytes, followed by dehydration and collapse of circulation. Similarly as TGE in piglets, bovine rotavirus denudes the villi of the small intestine in the gut [6].

"Lactogenic immunity" represents a specific form of passive immunity, mediated by maternal antibodies which exclusively neutralize virus that is freely accessible in the lumen of the gut.

Maternal antibody present in the newborn's serum (aquired in utero already by human babies, whereas in the first 24 h after birth by the calf, as we all know), is not in a position to substantially alter TGE infection of the piglet, nor rotavirus infection in the calf nor of the human baby

A third fundamental finding has only been documented in the eighties: the immunological gut–mammary link, by which B-lymphocytes sensitized in the animal's gut home in for the mammary gland, which gets colonized by them.

The well-documented synopsis published in 1982 by Butler [7] spares me the effort to go into details. Let me just mention, in flash-like form, some important parameters of this mechanism.

(i) The gut–mammary link works in so different systems as viral TGE of swine [3] and human *Vibrio cholerae* infection [8].

(ii) Certain immunoglobulin classes with remarkable resistance against proteolytic digestion account for the protective action of colostrum and milk in the recipient. Whereas in man this is acknowledged as dimeric IgA [8], in piglets dimeric IgA and pentameric IgM [9], at present there exists some controversy, whether in the calf IgG_1 [10] or IgA [11] mediate lactogenic protection. Open as this debate is presently at the scientific level, the facts merit full consideration, that bovine colostral antirotavirus immunoglobulins possess neutralizing capacity [12] and that large quantities of them pass the

calf's gut in undigested form [13]. For the sake of complete coverage of the matter let me add, that by for not all antibody involved in lactogenic immunity is produced locally in the mammary gland. Certain antibody classes are transferred pre- and postpartally and selectively from blood serum into the colostral milk [7].

(iii) A third finding essential for understanding our negative vaccination results: rotavirus antibodies present in the milk complex with the homologous virus in the gut lumen and get excreted in complexed form [14]. This process has an important consequence for the host: complexed virus apparently fails to stimulate its immune system, as de Leeuw et al. [15] and we ourselves [16] concluded from the observation that calves with maternal antibody supply very rarely develop titres inspite of rotavirus shedding.

When now presenting our findings made during two calving seasons in our University herd, one season without vaccinations, i.e. natural course of rotavirus infection, the other season with systematic vaccination, the profound influence of the parameters defined will become apparent.

Determination of rotavirus dissemination in a closed dairy herd

The closed dairy herd of Vienna Veterinary University, determined earlier as severe infected by bovine rotavirus [17], was systematically screened for rotavirus shedding and clinical diarrhoea during one calving season. ELISA, the most sensitive indicator of rotavirus-shedding [18] was used.

A total of 35 out of 48 calves excreted rotavirus. Contrary to American authors [1] virus shedding was not most pronounced during the very first days of life, but 37.5% excreters were found in the first week of life and 47.9% in their second week of life.

Let me immediately point out that invariably all mother cows of this herd possessed humoral antibodies against rotavirus, and all calves did so in their serum after resorption of colostrum. Lactogenic immunity was, accordingly, not able to suppress rotavirus takes and replication in many calves (for identical results in literature see [19]). Its beneficial effect became,however, manifest during the first week of life, where colostrum was fed daily, as compared to the more frequent viral incidence in the second week, where only milk-replacer devoid of antibody was fed. Retarding onset of infection has a beneficial effect on disease as all cattlemen know, and in fact merely 3 calves died out of 43 diarrhoeic animals.

Diarrhoea was observed frequently up to the 5th week of life, its severest watery form being limited to the first 3 weeks. The highest incidence and most frequent watery diarrhoea was recorded in the second week, where rotavirus shedding was at its peak.

As other authors (cited by Schusser et al. [19]), we could, however, not find a systematic correlation between rotavirus shedding and diarrhoea. Furthermore, rotavirus excreters with normal faeces were found not infrequently, and, especially from the 3rd to 5th week, diarrhoeic calves with negative ELISA for rotavirus were a frequent finding.

Peroral calf vaccination under controlled field conditions

As the same University cattle herd was in addition proved to be infected by bovine coronavirus, and as an attenuated rota-corona-live-virus vaccine for oral application was available on the market (kindly supplied by Dr. Ed. Bass, Norden Labs, Lincoln, Nebraska, USA), we deemed it desirable to investigate it for its potential prophylactic effect.

As we have discussed elsewhere [16], this particular vaccine has found an ambiguous appreciation by the international community of scientists. Briefly, favourable results were reported under experimental conditions, whereas failures under field conditions. De Leeuw et al. [15] ascribed its failure as most evidently caused by neutralization of vaccine virus taking place in the recipient's gut by maternal antibodies. This is very likely the case, as our ELISA testing showed that each dose of vaccine contains only little rotavirus [16]. Astonishingly enough, the manufacturer of this vaccine disregards the need to circumvent the detrimental effect of lactogenic immunity by prescribing its peroral application within the first 24 h after a calf is born.

We, however, did our best to circumvent the expected detrimental effect of lactogenic immunity. Logical to everyone familiar with calf rearing that no diet may bypass colostrum supply within the first 24 h of a calf's life, unless the herdsman takes the enormous risk of loosing many calves from *Escherichia coli* septicaemia.

We figured out two different approaches that might enable rotavirus vaccine takes (and coronavirus vaccine takes, still under analysis in the author's laboratory), by applying two different strict time regimes to 65 calves born during one calving season. (i) Thirty-one calves of group A, consecutively born, were perorally vaccinated, day or night, during their first 6 h post natum, and only 6 h later was colostrum feeding begun. The hope was, that meanwhile vaccine virus had penetrated into gut epithelial cells, where it would be protected, at least partially, against neutralization by maternal antibodies, arriving later via gut lumen. (ii) Twenty-four other calves of group D were regularly supplied with colostrum during 48 h, thereafter fed exclusively a glucose-electrolyte solution, followed by peroral vaccination the third evening. From the 4th day onwards regular colostrum feeding was resumed, colostrum which now is known to contain few maternal antibodies only [12].

Two groups each of 5 calves were left as unvaccinated controls. Subgroups of A-calves were later revaccinated, partly perorally, partly by intramuscular route. A peroral challenge was finally made in selected calves with virulent bovine rotavirus. All these experiments were carefully evaluated by ELISA for rotavirus excretion, by antibody determinations with CF in mothers' and calves' sera, and by clinically recording diarrhoea during 21 days.

An overview of results obtained after 1st day vaccinations confirmed that invariably all mother cows had CF antibodies, mostly of medium titres in their sera. So had their calves, of remarkably similar titres. Irrespective of vaccine takes or diarrhoea observed, these passively transferred titres did not change significantly within 5 weeks after birth.

Only half of the vaccinated calves excreted rotavirus, which, without a known marker available, could represent vaccinal or field virus. Clinically, diarrhoea was observed in 23/31 vaccinees within 21 days after birth, thereby approximating its occurrence in the controls as well as in the unvaccinated calves of the previous year.

The calves vaccinated on their 3rd day of life showed higher CF titres than the A-calves, partly on account of an apparent antigenic stimulation by reinfection of their mothers (reinfections being documented in literature [17]), partly by more colostrum meals fed during their first 24 h of life. This richer passive antibody supply had the following consequences: Rotavirus excretion following peroral vaccination was even rarer in these vaccinees, being limited to merely 3/15 calves tested. Clinical diarrhoea affected only 50% of this group, whereas 74% of group A.

When summarizing our results reported as yet, no beneficial effect of our specific measures to foster rotavirus takes in vaccinees could be demonstrated. Withholding colostrum by either regime had not sufficed to allow regular takes of rotavirus in their gut. Incidence of diarrhoea became reduced by better lactogenic immunity but not on account of active immunity.

Hope for beneficial economic effects had formed one aspect of our experimentation with this rota-coronavirus market vaccine. Failing to achieve this, we were interested to learn, at which level success of vaccination became annihilated. In accordance with Dutch workers [15] we had found that determination of serum antibodies allowed no conclusions regarding evaluation of vaccination success or failure. Efforts for determination of copro-IgA seemed fruitless, since our Bernese colleagues had not mastered the technical difficulties of this procedure [13]. We therefore resorted to an indirect and a direct investigation to answer the question, whether peroral vaccination had inferred immunoprotection that had remained unrevealed by the tests described above.

Our indirect approach consisted in performing revaccinations, aimed at disclosing a possible priming effect of primovaccination by soliciting a booster effect. This phenomenon had been observed by Svennerholm et al. [8] in

Pakistani women, although with *V. cholerae*, thus a bacterial system. Non-invasive as this human intestinal infection runs, it nevertheless primes the immune-system of its hosts. In consequence, parenteral vaccination results in significant antibody boosters, which become manifest in serum, milk and saliva. Evidently then, the gut-mammary link is operative for *V. choleae* immunity.

No such priming became, however, evident by revaccinating our calves with the market vaccine. We would rather have anticipated this when revaccinating perorally, as calves in the meantime had developed age resistence. Intramuscular revaccination, however, should have resulted in significant boostering, if priming had resulted from primovaccination. It did not.

We know authors, who consider titre differences of 1.7 or 2.2 geometrical dilutions as obtained in these calves as significant, as for either value there was $p < 0.01$. But anyone envolved with seroevaluation of vaccines knows that for measuring booster effects this is statistical cheating, as long as at least 4-fold increases of titres are not achieved.

Our last and direct evaluation of peroral rotavirus vaccination was performed by subjecting calves to virulent peroral rotavirus challenge. By necessity, vaccinated calves as well as unvaccinated controls were exposed in a single experiment. Thereby several of these animals had attained ages, where they already displayed age resistence. Those vaccinees that were challenged at susceptible age nevertheless proved to be unprotected by experiencing takes of virulent rotavirus, certified by positive fecal ELISA.

Discussion

Our experiments have shown, that even when detaining maternal colostrum for critical periods of time, attenuated live-virus rotavirus market vaccine was unable to mediate protection in perorally vaccinated calves. The responsible pathogenetical and immunological parameters accounting for this failure have been elucidated in the introduction. Substantiated by our own results and insights, I shall resume our present understanding of events as follows:

Bovine rotavirus pathogenesis

I. *In the calf without colostral antibody supply*

(a) Primary infection becomes established in villus epithelia, cytolytically destroys these cells and gets excreted in large amounts in faecal matter. Little virus gains access to the lamina propria mucosae, where B-lymphocytes

mature to plasmacells. Whether T-lymphocytes become involved in defence has remained undisclosed yet. Such calves have neither protection against infection nor against disease.

(b) Reinfection may occur in the same animals as several authors have described (see Schusser et al. [19]). Accordingly, the new villus epithelia covering formerly denudated areas are again susceptible to rotavirus infection. Plasmacells in the lamina propria, secreting specific antibody, however, modulate the course of this reinfection, to such extent only, that protection against infection does not result. The concomittant protection against disease is more a sequel of age resistance than of immunity.

II. *In the calf with lactogenic immunity*

(c) Maternal antibodies plus extraneous rotavirus reach the calf's gut. Much of this antibody gets resorbed to the blood stream, much of it passes the gut in undigested form. There it gets complexed with homologous virus [14]. The resulting neutralizing effect mediates protection against infection as well as against disease as long as high colostral level persist. Later, when the level of maternal antibodies has dropped, they no more mediate protection against infection, but merely against disease.

(d) Later in life, when daily supply of maternal antibodies has ceased, physiological low-level transudation of formerly resorbed serum antibody does not suffice to protect against infection nor against disease on immunological base. Still later protection against disease results on account of age resistance.

Bovine oral rotavirus vaccination

III. *In calves reared colostrum-free experimentally*

Attenuated rotavirus vaccine leads to takes in the small intestine, which mediate protection from infection as well as disease starting between 5 to 7 days postvaccination [20, 21].

IV. *In calves with normal colostrum supply*

This means almost automatically provision of lactogenic immunity, owing to the extreme frequency of seropositive cows. Such calves are passively protected against infection as well as disease during their first days of life. As virus-antibody complexes are formed and excreted, immunization fails to develop. Consequently, such calves lack protection against infection and disease shortly afterwards. There follows an unprotected phase, only terminated by development of age resistance.

General conclusions

Active immunity failed to develop after peroral vaccination with attenuated rotavirus in calves kept under field conditions. Lactogenic immunity accounts for this failure, evidently because ingested rotavirus forms complexes with maternal antibodies and becomes excreted in the faeces, whereby antigenic stimulation of the calf's immune system is prevented.

As in large and medium-sized cattle herds practically every cow possesses rotavirus antibodies and excretes these in her colostrum, peroral live-virus vaccination of calves as recommended by the manufacturer of this market vaccine, is logically subject to failure. Colostrum with its broad spectrum of protective antibodies must be given within the first 24 h of a calf's life. Our sincere efforts made to prevent rotavirus neutralization in the vaccinees' gut by temporary withholding of colostrum failed.

Our results have confirmed other authors' findings, that maternal colostrum antibodies at titres as encountered naturally do not suffice to prevent rotavirus infections of calves, but merely retard them and render them less severe [12, 15]. It would appear then, that promoting elevated maternal antibody supply over extended periods of time would have better chances than active immunization to counteract rotavirus infections. As dimeric IgA, presumably the most beneficial antibody class has merely a half-life of 24 h [22], daily supply of high-titered colostrum would seem most suitable to mediate such prophylactic effect. Such passive prophylaxis may be achieved by either adding daily first-day colostrum during the most susceptible first two weeks of life [23, 24] or by actively vaccinating pregnant cows, which prolongs their physiological excretion period of protective antibody [25, 26].

Chances for active peroral vaccination of calves with attenuated coronavirus might seem better, as this virus is less frequently encountered in herds than rotavirus. Our preliminary examination has, however, disclosed, that in our University herd every cow possesses antibodies against bovine coronavirus also. Accordingly, prophylactic prevention would appear also more successful along extended supply of maternal antibodies than by active peroral vaccination.

Similarly, it would appear that those human virus infections which remain restricted to villus epithelial cells, could be more promisingly prevented or mitigated by passive rather than active immunoprotection. Chanock et al. [27] have discussed prospects and problems regarding immunoprophylaxis of infants against human rotaviruses. Active immunization faces, of course, the same obstacles as we encountered, complicated further by the existence of several human rotavirus types [2]. On the other hand, bovine milk immunoglobulins have proved usable on industrial level to passively protect human babies against enteropathogenic *E. coli* [28]. Such an approach should also be

extendable to prophylaxis against human enteropathogenic viruses. Under the aspects of comparative pathogenesis this would appear to promise better results and certainly be less risky than oral rotavirus vaccination of human volunteers, about which tentative results have recently been published [29].

REFERENCES

1. Mebus, C.A., Underdahl, N.R., Rhodes, M.B., Twiehaus, M.J.: Res Bull **233,** 1 (1969).
2. Holmes, I.H.: Viral gastroenteritis. Progress Med Virol **25,** 1 (1979). Karger, Basel 1979.
3. Haelterman, E.O.: Transmissible gastroenteritis of swine. Proc 17th World Vet Congress, Hanover, 1963. 5(A)102.
4. Hooper, B.E., Haelterman, E.O.: J Am Vet Med Ass **149,** 1580 (1966).
5. Pensaert, M.: Viral gastroenteritis in suckling pigs. Proc VII International Symp World Ass Vet Microbiologists, Barcelona 1982, Sobrino/Barcelona, pp. 148–150.
6. Mebus, C.A., Stair, E.L., Underdahl, N.R., Twiehaus, M.J.: Vet Path **8,** 490 (1971).
7. Butler, J.E.: In Butler, J.E. (ed.): The ruminant immune system. Plenum Press, New York 1982, pp. 3–55.
8. Svennerholm, A.-M., Holmgren, J., Hanson, L.A., Lindblad, B.S., Quereski, F., Rahimtoola, R.J.: Scand J Immunol **6,** 1345 (1977).
9. Morgan, K.L., Bourne, F.J., Newby, T.J., Bradley, P.A.: In: Butler, J.E. (ed.): The ruminant immune system. Plenum Press, New York 1982, pp. 391–411.
10. Newby, T.J., Bourne, F.J.: Immunology **31,** 475 (1976).
11. Honkanen-Buzalski, T., Sandholm, M.: Comp Immun Microbiol Infect Dis **4,** 329 (1981).
12. Ellens, D.E., de Leeuw, P.W., Straver, P.J.: Ann Rech Vét **9,** 337 (1978).
13. Zbinden, R.: Klassenspezifischer Koproantikörpernachweis gegen Rotavirus beim Kalb mit ELISA. Lizentiatsarbeit, Universität Bern/Switzerland, 1981.
14. Watanabe, H., Gust, J.D., Holmes, J.H.: J Clin Microbiol **7,** 405 (1978).
15. De Leeuw, P.W., Ellens, D.J., Talmon, F.P., Zimmer, G.N., Kommerij, R.: Res Vet Sci **29,** 142 (1980).
16. Bürki, F., Schusser, G., Székely, H.: Zbl Vet Med B **30,** 237 (1983).
17. Sibalin, M., Székely, H., Bürki, F.: Wien Tierärztl Mschr **67,** 122 (1980).
18. Ellens, D.J., de Leeuw, P.W., Straver, P.J., van Balken, J.A.M.: Med Microbiol Immunol **166,** 157 (1978).
19. Schusser, G., Hinaidy, B., Bürki, F.: Microbiologica **5,** 321 (1982).
20. Mebus, C.A., White, R.G., Bass, E.P., Twiehaus, M.J.: J Am Vet Med Ass **163,** 880 (1973).
21. Woode, G.N.: Vet Rec **103,** 44 (1978).
22. Bourne, F.J., Newby, T.J., Evans, P., Morgan, K.: Ann Rech Vét **9,** 239 (1978).
23. Bridger, J.C., Woode, G.N.: Br Vet J **131,** 528 (1975).
24. Snodgrass, D.R., Stewart, J., Talor, J., Krautil, F.L., Smith M.L.: Res Vet Sci **32,** 70 (1982).
25. Bachmann, P.A.: Proc. VII. Internat. Symp. World Ass. Vet. Microbiologists, Barcelona 1982. Sobrino/Barcelona, pp. 200–202.
26. Snodgrass, D.R., Fahey, K.J., Wells, P.W., Campbell, I.,Whitelaw, A.: Infect Immun **28,** 344 (1980).
27. Chanock, R.M., Wyatt, R.G., Kapikian, A.Z.: J Am Vet Med Ass **173,** 570 (1978).
28. Hilpert, H., Gerber, H., Amster, H., Pahud, J.J., Ballabriga, A., Arcalis, L., Farriaux, F., de Peyer, E., Nussle, D.: Food and Immunology, XIII Symp Swed Nutr Found, 1975.
29. Kapikian, A.Z., Wyatt, R.G., Levine, M.M., Yolken, R.H., Van Kirk, D.H., Dolin, R., Greenberg, H.B., Chanock, R.M.: J Inf Dis **147,** 95 (1983).

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DETECTION OF RUBELLA-SPECIFIC IgM ANTIBODIES BY A COMBINED APPLICATION OF SOLID-PHASE-IMMUNOSORBENT- AND HAEMAGGLUTINATION-INHIBITION-TECHNIQUE*

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Introduction

The determination of rubella-specific IgM antibodies is a useful method in the diagnosis of acute rubella infection. The detection of IgM rubella antibodies can be carried out by a range of various methods [1]. Some procedures require an expensive technical equipment or show results falsified by interference of rheumatoid factors and IgM reacting competitively for IgM.

Now Krech and Wilhelm [2] published a test which can be performed without sophisticated technical tools and does not show interference by rheumatoid factors and competition of IgG. The principle of this test is based on a solid-phase "capture" antibody technique in combination with the haemagglutination inhibition reaction (SPIT).

The aim of our investigation was the adaptation of this method to our own laboratory conditions using pigeon erythrocytes and Dextrose–Gelatin–Veronal buffer (DGV) instead of sheep erythrocytes and HEPES buffer described by Krech and Wilhelm [2].

Methods

Modifications of the SPIT were as follows. In a first step, polystyrene microtitre plates were coated with 100 μl chainspecific anti-human IgM (Behringwerke, FGR, dilution 1 : 1600). The plates were left overnight at room temperature. Then 0.1% fetal calf serum was added to the wells and the plates were left to stand at room temperature for 1 h. By this procedure parts of well surfaces possibly not coated by anti-human IgM should be blocked. After shak-

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ing dry, 100 μl portions of patient serum diluted in DGV were added. Heteroagglutinins and unspecific rubella inhibitors were removed by treatment of the serum with pigeon erythrocytes and heparin-manganeous chloride as previously described [3]. Then the plates were left at 4 °C for 18 h and thoroughly washed thrice.

In the following step 25 μl rubella haemagglutinin (SEVAC, Czechoslovakia) containing 2 haemagglutinating units were given to the wells. After a reaction time of 4 h at 4 °C, 25 μl pigeon erythrocytes (0.25% suspension in DGV) were added. Before reading the test, the mixture was incubated about 2 h at 4 °C.

Results

A series of 30 sera taken from patients with a recent rubella infection was comparatively tested by SPIT and haemagglutination inhibition test (HIT) with the IgM fraction separated by gel filtration as previously described [3]. The results show a higher sensitivity of the SPIT giving in all investigated sera IgM-titres in a range of 1:10–1:640. The IgM fractions had only HI titres from 1:4–1:16, sometimes of 1:64.

Furthermore, the specificity and sensitivity of SPIT could be confirmed by the serological results of 116 patients with acute rubella infection diagnosed clinically and by antibody rising of HIT. In all cases rubella-specific IgM antibodies were demonstrated. Additionally, we investigated 25 sera containing rheumatoid factors and rubella HI antibodies. These sera originating from persons without known recent rubella exposure or acute infection were negative by SPIT.

In view of these exact results we applied SPIT in our routine laboratory work. Altogether, 421 pregnants with known rubella exposure or with a suspected recent infection that could not be exactly excluded by HIT were investigated by SPIT. Of them, 88 (20.7%) showed a positive SPIT.

Discussion

The modified SPIT was characterized by an adequate sensitivity and specificity.

From the theoretical standpoint false positive reactions by rheumatoid factors and competition of IgG can be excluded. However, according to our opinion, a few possibilities of false SPIT should be discussed. Firstly, μ-chainspecific anti-human IgM of insufficient specifity cal also react with human IgG. Secondly, a cross-reaction of IgG rubella antibodies could occur, if the well surfaces of microplates are not fully coated by anti-human IgM and IgG molecules are adsorbed to uncoated areas. Consequently, the results of SPIT should not only be interpreted alone but also in connection with the patients' history and serological findings with HIT.

REFERENCES

1. Report of a WHO Scientific Group. WHO Techn Rep Ser 661, 1981.
2. Krech, U., Wilhelm, J.A.: J Gen Virol **44,** 281 (1979).
3. Sandow, D.: Beiträge zur Charakterisierung des Rötelnvirus sowie zur Serodiagnostik und Epidemiologie der Röteln. Promotion B., Leipzig 1977.

NINTH CONGRESS OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY

BUDAPEST, AUGUST 23–25, 1983

ABSTRACTS OF PAPERS

Acta Microbiologica Hungarica 31 (3), pp. 229—230 (1984)

OPENING ADDRESS

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At the opening of our Congress, the importance of which is emphasized by the participation of our highly esteemed foreign guests, it is justified to take into account the most important results and future prospects of our special scientific field.

There is no doubt that the one hundred years' development of microbiology has brought decisive results in the knowledge of microorganisms pathogenic to man, animals and plants, in decreasing their number, in the prevention and curing of infectious diseases induced by them. I wish to mention here, as the most convincing example in this field, the eradication of smallpox or the control of most of the human and animal epidemic diseases that caused great pandemia in the past.

There have also been brilliant achievements as to the knowledge of the biological role, importance and understanding of the microscopic living world surrounding us and as to the utilization of part of the capabilities and energies of this micro-world to satisfy the demands and requirements of the macro-world. With the highly promising results of the biotechnological revolution I dealt with in detail in my opening lecture of our previous Congress, in the lecture given in memory of Robert Koch.

In the year that has passed since then there has been a new discovery in our field, an immense step which may have unforeseen consequences, and may alter many of our notions such as have been so far accepted as absolutely unquestionable facts. This is the discovery made by Baross and Deming of the existence of microorganisms capable of proliferation at 250 °C or higher temperatures. These microorganisms that were sampled from crustal parts of deep-sea volcanic shelves alter not only our ideas about the conditions and limits within which we have so far been able to imagine the existence of organic life, but at the same time are suitable to suggest that life is possible in such regions of our Earth where we have regarded it impossible, and under similar conditions, on the surface of other planets, too. This discovery also refers to the assumption that life may have started amidst chemical reactions accelerated at such extremely high temperatures. These microorganisms can now

be cultured and studied in enriched sea water in titanium chamber at 250 °C, at 265 atm pressure. Their generation time is about 40 min, which, on decreasing pressure and temperature is getting longer and longer and entirely stops below 100 °C. In the course of their culturing they produce methane, carbon monoxide, hydrogen, and a small amount of nitrogen oxide. The discovery and existence of these obligately thermophilic, gas-demanding and producing microorganisms have not only disproved the chemical and biochemical dogmas on proteins, nucleic acids, lipids, i.e. the most important components of living matter, but also suggest that beyond the theoretical involvements, their importance is unpredictably great as to the whole of biology and for the new trends in industrial microbiology (large-scale production of thermostable enzymes, industrial gas production, etc.). The establishments concerning the time of the origin of living matter on our Earth also need reappraisal. In the course of the Earth's evolution life must have originated in an earlier period of cooling down, and the possibility of life on other planets that have so far been excluded from this possibility due to their high temperature, is to be reconsidered.

These results raise our recent knowledge on the micro-world to an equal level with the results of the technical revolution of our times, and they will form the essential basis of the development and persistence of the plant and animal kingdom in the new millenium.

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Bacteriology and Parasitology

EXPERIMENTAL ANIMAL MODELS IN THE STUDY OF ENTERIC PATHOGENS

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Experimental animal models are mandatory in human medical microbiology, but they are essential also in veterinary microbiology, because the price of big farm animals limits the number of experiments. The experimental animal model is not a simple forced compensation: in many cases it gives better information about some steps of pathomechanism or about virulence factors, as compared to the complex picture yielded by the natural host. Mistakes can be prevented by considering the model character of experiment and by control observations in the natural host. Animal modelling of salmonellosis, shigellosis and diseases caused by different pathogenic goups of *Escherichia coli* has been successfully applied and the possibility to study the virulence factors of *Vibrio cholerae* in this manner may also be considered.

THE CLONE CONCEPTION — A CONTRIBUTION TO THE CHARACTERIZATION OF BACTERIA OF EPIDEMIOLOGICAL AND DIAGNOSTIC IMPORTANCE

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Long time epidemilogical observation indicate that within a bacterial species particular qualified clones with outstanding virulence and epidemic spread are responsible for the epidemic process of the infectious diseases in question. These clones can be described and characterized by epidemiological laboratory methods such as phage typing, serotyping, colicinotyping, biochemotyping, analysis of plasmid pattern or protein pattern etc. and therefore discriminated according to their different genetical and phenotypical traits. In summarizing these observed traits a clone type formula can be established for each epidemiologically significant strain. The clone conception in close

connection with ecological, epidemiological and clinical studies explains the development of virulent epidemic or clinically important strains, their spread and their disappearence: it gives further insight into the dynamics of alteration and changing of the infective agents themselves and of the development of drug resistance.

ANALYSIS OF PLASMIDS DERIVED FROM MULTIPLE RESISTANT *ESCHERICHIA COLI* STRAINS

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Escherichia coli strains cultured from hospitalized patients with urinary tract infections and leukaemia, treated with antibiotics or chemotherapeutics for a long period, were examined for sensitivity to 27 antibiotics. Though the therapeutical use of most of these antibiotics was not general in Hungary, multiple resistant strains were often found. In 20 out of the examined 30 strains the presence of R-plasmid was demonstrated. The difference in antibiotic resistance between the strains derived from the two clinical groups was significant. In *E. coli* strains from urinary tract infections the number of resistance determinants per one strain was 1.7, of R-plasmid controlled determinants was 0.8, whereas in case of leukaemia these numbers were 3.2 and 2.4, respectively. Transferable cephalosporin resistance was found only among the first generation cephalosporins. Out of the examined 6 cases resistance to sulfamethoxazole-trimethoprim was determined by R-plasmids in five cases. On the basis of determination of incompatibility group, phage-inhibition and molecular weight, the R-plasmids were not "hospital-endemic-plasmids".

ISOLATION OF RESTRICTION DEFICIENT MUTANTS FROM *STAPHYLOCOCCUS AUREUS* SA113

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Further $r^- m^+$ mutants were isolated from *Staphylococcus aureus* SA113 an $r^-_{S1} m^+_{S1} r^-_{S2} m^+_{S2}$ derivative of NCTC 8325 following mutagenic (NTG) treatment. For selection mutants typing-phage $\varphi 80$ adapted to a Group 2

strain PS116 (φ80.PS116) was used. Phage sensitivity of 113/34426, one of the r$^-$ mutants increased with a value of 2–5 log to both Group 2 typing-phages and φ80 adapted to Group 2 propagating strains (φ80.PS71, φ80.PS 116). The above phages gave the same e.o.p. value on both the propagator and a delysogenized derivative of 8325 after propagation on 113/34426. In transduction experiments using Group 2 donor strains from clinical sources and, strains 8325, SA113 and 113/34426 recipients, chloramphenicol, tetracycline and erythromycin plasmids could be transferred only to 113/34426 with a frequency of 10^{-5}–10^{-7} by φ80.PS116 propagated on the donor strains. From this mutant plasmids could be transduced to 8325 with the same frequency. The use of mutant 113/34426 offers the possibility to transfer R-plasmids from the clinically important Group 2 *S. aureus* strains into 8325 for determining their incompatibility relationships with recombinant plasmids of Iordanescu et al.

TN5 CARRIES A STREPTOMYCIN RESISTANCE GENE

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In *Rhizobium meliloti*, Tn5 conferred resistance not only to kanamycin but to streptomycin, as well, in *Escherichia coli*, however, only to kanamycin Using in vitro recombinant DNA techniques, it was shown that the streptomycin resistance determinant was located downstream from the kanamycin resistance gene in the unique central region of Tn5. Expression of various cloned fragments of Tn5 suggested that both kanamycin and streptomycin resistance genes were transcribed from the same promoter. *E. coli* mutants allowing the expression of streptomycin resistance from Tn5 were isolated. The differential expression of the streptomycin resistance gene provides simple selection/counterselection conditions, using only streptomycin in transfer experiments of Tn5 between *E. coli* and *R. meliloti*.

INTRODUCTION OF SYMBIOTIC GENES OF *RHIZOBIUM MELILOTI* INTO OTHER RHIZOBIA AND AGROBACTERIUM

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Strains of *Rhizobium meliloti* able for symbiotic nitrogen fixation with the host plant alfalfa, harbour megaplasmids. Recently we have shown that one of the megaplasmids of *R. meliloti* strain 41 (pRme41*b*) carries genes responsible for symbiotic nitrogen fixation, including the structural genes of enzyme nitrogenase *(nif)* and genes involved in the early steps of nodulation *(nod)*. These findings were further supported using two approaches. (1) The *mob* region of RP4 was integrated into the pRme41*b* which was then mobilizable with R68.45. In this way the pRme41*b* could be introduced into other rhizobia and *Agrobacterium tumefaciens*. The *nod* genes of *R. meliloti* could be expressed in the new hosts, transconjugants carrying the pRme41*b* induced nodules on alfalfa. (2) R-prime derivatives of R68.45 carrying symbiotic genes of *R. meliloti* were constructed in vivo. Introduction of R-primes carrying *nod* genes into *Agrobacterium* resulted in transconjugants able to nodulate alfalfa. Physical mapping of this DNA region also indicated that genes involved in early steps of nodulation and the *nif* genes are located on a relatively short (50kb) DNA region of pRme41*b*.

DNA-PROTEIN INTERACTION IN PHAGES OF *MYCOBACTERIUM PHLEI*

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Protein patterns of Phlei phages disintegrated by different methods were investigated. At least 10–12 proteins were found associated to DNA mild disintegration conditions (by removing Ca^{++} ions). These so called internal proteins were found associated to the DNA even after CsCl equillibrium centrifugation. Sedimentation constant, buoyant density and electrophoretic mobility of DNA thus obtained were measured. By the use of other disintegration methods different results have been obtained. Thus, using $NaClO_4$ to

isolate bacteriophage DNA, 5–6 proteins attached to DNA, using temperature treatment in the presence of 0.05% SDS a relatively pure DNA could be obtained (1–2 proteins), while using the phenol method no protein associated to DNA could be detected. This latter phenomenon means that phage DNA contains no covalently bound proteins.

BIOSYNTHESIS OF THE HEPTOSE REGION OF LIPOPOLYSACCHARIDES IN GRAM-NEGATIVE BACTERIA. ISOLATION OF ADENOSINE 5'-DIPHOSPHATE-L-GLYCERO-D-MANNOHEPTOSE, THE "PROPER" SUBSTRATE OF HEPTOSE TRANSFERASE, FROM *SALMONELLA MINNESOTA* R 595 AND *SHIGELLA SONNEI* R_e MUTANTS

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A few years ago we isolated ADP–D-glycero-D-mannoheptose from a *Shigella sonnei* R mutant which incorporates into its cell wall lipopolysaccharide D-glycero-D-mannoheptose as sole heptose. With similar methods, i.e. by extraction of bacteria with perchloric acid, ion exchange chromatography, we could isolate from class R_e mutant of *Salmonella typhi-murium* and from a similar heptoseless mutant of *S. sonnei* a mixture of the nucleotides of "DD" and "LD" heptose. After paper chromatography in ethanol-butanone-0,5 M morpholinium tetraborate, pH 8.6, in 0.01 M EDTA (70:20:30; Carminatti et al.) we could isolate both of the nucleotide sugars in pure form ("DD":"LD" ratio about 2:3). The structure of ADP-L-glycero-D-mannoheptose has been verified by analytical methods as g.c., p.c., spectroscopy and determination of constituents. On the basis of our results, *S. typhi-murium* R 595 and *S. sonnei* R_e mutants can be regarded as epimeraseless rather than transferaseless ones.

STRUCTURE OF THE IMMUNODOMINANT REGION OF *ESCHERICHIA COLI* R_1 CORE TYPE LIPOPOLYSACCHARIDE

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In a previous work, we have postulated that the end-group tetrasaccharide is substituted with the immunodominant of R_1 core on the terminal galactose. This finding was supported in a comparative study with *Escherichia coli* R_4 core type lipopolysaccharide having similar terminal structure except that in the side-chain, galactose was substituted for glucose. In contrary to R_4 polysaccharide, the R_1 core was not attacked by galactose oxidase, was negative with galactose-specific lectins and the serological activity was rapidly eliminated with mild alkali and acid treatment. In passive haemolysis inhibition test, the R_1 type lipopolysaccharide/antibody system was strongly inhibited by heptoseless lipopolysaccharides, ketodeoxy-D-mannooctulonic acid (KDO) and KDO-methylglycosides.

APPLICATION OF THE INDIRECT HAEMAGGLUTINATION TEST FOR SURVEYING *MYCOPLASMA HYOPNEUMONIAE*

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Sheep erythrocytes pretreated with glutaraldehyde and tannin were sensitized with *Mycoplasma hyopneumoniae* antigen. Sera collected from 2090 conventional pigs and 2283 pigs free from *M. hyopneumoniae* were examined by the indirect haemagglutination test, using erythrocytes treated in this way. The proportion of seropositive animals was 70 to 90% in conventional fattened pig herds, 30 to 40% in 3 to 5 years old sows and boards, and 16% in backyard pigs. Titres of the reactor animals ranged between 1:16 and 1:512. One and a half per cent of pig sera collected in herds undergone *M. hyopneumoniae* eradication gave doubtful or mildly positive reactions. However, *M. hyopneumoniae* infection could not be established by gross pathological and histopathological examinations and mycoplasma isolation performed after the extermination of such animals. The indirect haemagglutination test seems to be a reliable method for screening pig herds for *M. hyopneumoniae* infection.

ISOLATION OF CO_2 DEPENDENT *SALMONELLA GALLINARUM* STRAINS FROM CHICKEN WITH FOWL TYPHOID

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Among 10–20 days old chicken of meat-type flocks an acute disease occurred with moderate losses. The pathological changes resembled fowl typhoid but yielded no bacteria by aerobic cultivation. However, *Salmonella gallinarum* grew readily from the organs of all diseased birds in increased CO_2 atmosphere. The 14 strains could be adapted to aerobic growth after 4–5 subcultures. The source of infection was an infected breeder stock and the CO_2 depended *S. gallinarum* was isolated from the dead-in-shell embryos, too. The 14 strains showed identical phage pattern (phage type 1 with *S. enteritidis* phages and phage type A with *S. panama* phages).

BIOTYPES AND SEROTYPES OF *PASTEURELLA HAEMOLYTICA* ISOLATED FROM SHEEP, GOATS AND CALVES

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Biochemical and serological characteristics of 201 *Pasteurella haemolytica* strains isolated from sheep, goats and calves succumbed to pneumonia, pleuropneumonia or septicaemia were examined. For the serological examinations the indirect haemagglutination test was used. About 92.5% of the isolates (186 strains) belonged to the A and about 7.5% (15 strains) to the T biotype. Except 8 strains they could be assigned into one of the up till now established 15 serotypes. From calves A1 was the most prevailing serotype (65% of the strains). In sheep almost all serotypes were represented, but serotypes A1 (26%), A2 (12%), A7 (10%), A9 (9.5%) and A6 (9%) occurred the most frequently. About 24% of serotype A bacteria caused septicaemic disease in sheep. Apart from one strain, isolates from goats represented a single serotype A2.

COMPARATIVE STUDIES ON TWO TYPES OF LEPTOSPIRAL CULTURE MEDIA

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Two kinds of leptospiral medium Korthof's rabbit sera and Ellinghause–Johnson's BSA were used for isolation and propagation of leptospires. In the low protein containing (BSA) medium even the fastidious *Leptospira interrogans* serovar *hardjo* was able to grow. The semisolid BSA medium is most suitable for isolation of *L. interrogans* serovar *hardjo*. *L. interrogans* serovar *pomona* from renal tissues grew in Korthof's medium when inoculated undiluted, meanwhile in sensitive BSA medium these leptospires multiplied only when appropriate dilutions were seeded. As in BSA medium leptospires multiplied at least five times more intensively than in Korthof's medium, vaccines prepared from these cultures may establish a better immunity.

CHARACTERISTICS OF THE NUTRIA-TREPONEMA

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Previously we have reported on an epidemic disease similar to swine dysentery observed in a nutriastock. In the coecum and in the faeces of the ill and dead animals spirochaets identical with *Treponema hyodysenteriae* and binding *T. hyodysenteriae* conjugates were present. The microorganism was isolated from the cadavers from an other nutriastock. There was no difference between *T. hyodysenteriae* and the nutria-treponema by light- and electronmicroscopical, biochemical and serological examinations. The treponema strains isolated from nutrias colonized the large intestine of artificially infected pigs, but caused no disease. Pigs were infected with the coecal-content of nutrias from which the treponema strains were isolated. No disease developed for one month in the infected pigs. The colon of killed pigs was fed to an other group of pigs but they also remained symptomless. After two further swine-passages 3 infected pigs became seriously ill after 5–7 days post infection with characteristic symptoms of swine dysentery. We conclude that the nutria-isolate becomes pathogenic for pigs after a few passages.

DETECTION OF *TREPONEMA HYODYSENTERIAE* ANTIBODIES BY ELISA

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So far, no serological methods serving for the detection of *Treponema hyodysenteriae* antibodies and suitable for the examination of individual animals has been available. As a sensitive and reliable test enabling the examination of large numbers of individuals within a relatively short time, ELISA seems to be the method of choice. In our studies performed so far, 48–72 h broth cultures (Trypticase soy broth) of a strain isolated from a dysenteric pig and designated B 8, and those of an apathogenic strain designated HI were used as antigen. The cells were recovered from the broth cultures by centrifugation, washed in PBS and sonicated. Serum of dysenteric sows was used as positive control, and SPF piglet serum as negative control. Anti-swine IgG–HRPO marketed by Human Institute for Serobacteriological Production and Research was used as conjugate, and OPD as substrate. As compared to the micro-agglutination test, ELISA is sensitive enough and suitable for the detection of treponemal antibodies. The great advantage of ELISA lies in the fact that it eliminates the difficulties of antigen production. Once produced, the antigen can be used in high dilution for a long time.

INCIDENCE OF AN EPIDEMIC DISEASE WITH ABSCESS FORMATION IN SHEEP (MOREL DISEASE) IN HUNGARY

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In a stock of sheep imported from France a disease involving suppuration of lymph nodes and subcutaneous abscess formation was observed. In the course of a few months all individual of the stock of 318 ewes acquired the disease. Recurrence was common. Six-eight weeks after the burst and healing of the abscess a newer, occasionally a third abscess was formed. After 12–13 months the disease gradually ceased in the adult stock, but reappeared in the lambs born during this period. From all the examined materials a slowly growing, microaerophilic, fastidious Gram-positive coccus was cultured. Merino sheep infected with the isolates, similarly to the naturally occurring cases, developed subcutaneous lymph node abscesses. The isolates showed the

characteristics of "*Micrococcus abscedens-ovis*" described as the causative agent of Morel disease. Occurrence of this disease has not so far been reported in Hungary.

DETECTION OF COMPLEMENT FIXING ANTIBODIES FOR *CHLAMYDIA* IN HUNGARIAN EQUINE HERDS

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Blood samples collected from horses of different breeds and reared for different purposes were examined by complement fixation test (CFT) to detect antibodies for *Chlamydia*. A total of 317 blood samples were collected from private farms, where the owners usually have not more than two horses and 393 sera samples were collected from big (governmental) herds. Antibody titers 1:10 or higher were considered positive. The number of examined blood samples collected at random from each farm ranged from 8 to 50. Out of the sera examined (710), 13.2% were positive. By CFT the number of positive cases from different farms varied between O and 53.3%. The highest antibody titres occurred in 8 foals less than one year old, from one farm; these foals suffered from respiratory disease when they were six to twelve weeks old. Horses reared in small number and bred for private purposes were rarely positive. According to our serological results and according to the experience of other authors elsewhere one must draw attention to the role played by *Chlamydia* as a causative factor in respiratory disease complex of horses and abortion in mares.

ROLE OF ANTIBACTERIAL MECHANISMS IN THE PATHOLOGY OF SWINE DYSENTERY

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Swine dysentery (*Treponema hyodysenteriae* infection) is a common disease of pigs kept in large-scale farms. In recent years we have observed that cases of swine dysentery did not occur in farms where pigs were fed maize silage/(3-year observation including 40 000 pigs). We have shown that, due to

the antibacterial effect of volatile fatty acids (VFA), bacterial multiplication is strongly inhibited in the intestinal tract of pigs fed maize silage, and this prevents the spread of infection. The intensity of the VFA-associated antibacterial effect in the intestinal tract depends on the quality of the feed. By enhancing the intensity of the studied natural defence mechanism by adequate feeding, the prevention of swine dysentery seems to be possible without the use of drugs.

CHARACTERIZATION OF *ESCHERICHIA COLI* STRAINS ON THE BASIS OF THEIR ADSORPTION ON $Al(OH)_3$ GEL AND HAEMAGGLUTINATION

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Szöllősy showed in 1969 that cell suspension of strains belonging to the family *Enterobacteriaceae* are bound to the surface of $Al(OH)_3$ gel with different intensity. The adsorption of the bacteria can be inhibited by phosphate ions. Strains were characterized by the numerical value of the phosphate buffer molarity causing 50% equilibrium on the gel (EC_{50}). This character is independent from serological types. All strains belonging to the serological groups causing infantile enteritis are characterized by their inability to absorb on the gel. The relation between adsorbing capacity and the behaviour with Serény test as well as the haemagglutination in the presence and absence of mannose have been studied on strains originating from patients. It was found that the majority of Serény-positive strains demonstrated weak adsorption (EC_{50} 0.01), while Serény-negative strains adsorbed more firmly (EC_{50} 0.04). The haemagglutinating *Escherichia coli* strains have been grouped as follows: Agglutinating human blood cells in the presence of mannose (MRHA), only in the absence of mannose (MSHA), agglutinating guinea pig blood cells with mannose (MRTA), only without mannose (MSTA). The haemagglutinating characteristic of strains have been compared with their EC_{50} values.

MANNOSE RESISTANT HAEMAGGLUTINABILITY OF *ESCHERICHIA COLI* IS ASSOCIATED WITH SEROGROUP BUT NOT WITH SITE OF INFECTION

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Mannose resistant haemagglutination of human erythrocytes (MRHA hum.) and O antigen of *Escherichia coli* strains originating from faeces (patients with enteritis 244, healthy individuals 225), urine (pyelonephritis 111, cystitis 130, asymptomatic bacteriuria 59) and other extraintestinal sources (blood 30, cerebrospinal fluid 15, wound 13, and nose swabs 5) were examined. Serogroups O1, O2, O4, O6, O7, O18, O75 were uniformly the most frequent in faecal (26.5%), urinary (34%) and extraintestinal (57.9%) strains. Haemagglutinating activity of these serogroups was significantly more frequent than that of the others ($p < 0.001$). There was no significant association between MRHA positivity and origin of these serogroups. It has been concluded that MRHA hum. property of an *E. coli* strain rather depends on its serogroup than its origin. As serogroups with MRHA hum. activity are the most frequent serogroups of the intestinal flora, the primary function of these strains is to colonize the bowel; outside the gut they may cause extraintestinal infections.

THE EFFECT OF ANTIBIOTIC TREATMENT ON THE IN VIVO SELECTION OF RESISTANT HAEMOLYTIC CLONES OF *ESCHERICHIA COLI*

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The appearance of resistant haemolytic *Escherichia coli* clones was studied in mice treated with a synergetic combination of antibiotics (broad range penicillin derivative + aminoglycoside). The R plasmid donor and the haemolytic recipient were given orally. Starting next day 10 animals were treated intraperitoneally with 2.5 mg carbenicillin and 2.5 mg kanamycin pro die through 7 days; 10 control animals were injected with saline. The appearance of resistant transconjugants was tested both by direct plating of faecal samples onto selective blood agar plates and after selective enrichment. Out of 80 faecal samples of mice receiving antibiotic treatment resistant haemolytic transconjugants were isolated from 13 samples by direct plating and from 27 samples after enrichment. Out of 80 parallel samples of control animals a single one resulted in a positive culture, however, only after enrich-

ment. On the basis of these results we assume that even a proper antibiotic combination, while controlling the actual infection, might facilitate the appearance of resistant haemolytic clones (and clones possessing other virulence factors) in the intestinal tract. These multiresistant clones might represent a special danger as possible sources of later extraintestinal infections.

DETECTION OF COMMON ANTIGEN AMONG VIRULENT STRAINS OF ENTEROINVASIVE *ESCHERICHIA COLI*, *SHIGELLA FLEXNERI* AND *SHIGELLA SONNEI* BY ELISA

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Using a special kind of ELISA technique the existence of common antigen characteristic only of the virulent strains of enteroinvasive *Escherichia coli* (EIEC) of different serogroups was investigated. Immune serum produced against a virulent strain of serogroup 0143 and absorbed with living and heat-treated cells of the avirulent derivative of same strain was used. This serum showed with all the virulent EIEC strains a titre higher than or equal with 1: 2000, while the avirulent ones gave titres lower than 1: 200. Comparable results were obtained with virulent and avirulent strains of *Shigella flexneri* and *Shigella sonnei*. We assume the existence of a common antigen, characteristic of the virulent strains only.

PILUS SPECIFIC VACCINE FOR PREVENTION OF BOVINE ENTERIC COLIBACILLOSIS

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Most of the bovine enterotoxigenic *Escherichia coli* (ETEC) strains possess one or both of the two pilus (fimbria) antigens: K99 and F41. With the help of these antigens they adhere to the small intestinal epithelium. Calves born to cows vaccinated with the pilus extract of a $K99^+$ *E. coli* strain (produced under laboratory conditions) were protected against an experimental challenge with about one LD_{50} of an ETEC, $K99^+$, $F41^+$ strain. Based on the above results an inactivated bacterial vaccine was produced containing piliated bacteria of the *E. coli* O101:K99, F41 and was licenced under the name

"Phylacol-K99" at the end of 1982. In 1981–1982 experimental vaccinations using the above vaccine were performed of four herds in which enterotoxic colibacillosis caused by K99$^+$ ETEC had been diagnosed. Deaths due to diarrhoea under 6 days of age were investigated between calves of 738 vaccinated and 1681 control cows. Six per cent of the control calves and 0.5% of calves receiving colostrum from vaccinated cows died at this age. Enterotoxic colibacillosis (presence of K99$^+$ ETEC) was not diagnosed in the latter group, and 70% of the tested dead calves from the control group died as a result of K99$^+$ ETEC induced enterotoxic colibacillosis. In the serum and colostrum of the vaccinated cows as well as in the serum of their calves pilus-agglutinating and ELISA antibodies were detected. Titres were similar to that induced by a foreign calf colibacillosis vaccine Vicogen.

INHIBITION OF SMALL INTESTINAL COLONIZATION OF ENTEROTOXIC *ESCHERICHIA COLI* BY *STREPTOCOCCUS FAECIUM*

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An O101: K30(A), K99: NM, STa$^+$ enterotoxic *Escherichia coli* (ETEC), an O8: K50(A): NM nonenteropathogenic *E. coli* (NEEC) and a *Streptococcus faecium* strain were studied electronmicroscopically. On the surface of both *E. coli* strains pili (fimbria) were detected. *S. faecium* was nonpiliated. In the small intestine of intragastrically infected newborn mice, the ETEC strain attained significantly higher numbers than the NEEC. Also the number of NEEC was significantly higher than that of *S. faecium*. Twelve hours before ETEC inoculation, mice were given 10-fold amounts of *S. faecium*. Controls were only infected with ETEC. No difference was detected in terms of small intestinal ETEC-colonization. A similar experiment was performed on hysterectomy derived colostrum deprived piglets. *S. faecium* could not attain as high numbers as ETEC in the small intestine of piglets. However, pigs preinoculated with *S. faecium* showed significantly less diarrhoea, weight loss and small intestinal ETEC counts as compared to the controls. It seemed that *S. faecium* could not colonize the small intestine of infant mice and pigs as well as the piliated ETEC and NEEC strains could. The inhibition of ETEC colonization by streptococci in piglets could be interpreted as a result of biochemical activity of streptococci which could also be accompanied by a nonspecific early stimulation of mucosal protective activity of the porcine small intestine.

HAEMAGGLUTINATING CAPACITY IS ABSENT IN *YERSINIA ENTEROCOLITICA*

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A total of 122 strains of *Yersinia enterocolitica* of different serogroups were examined by slide haemagglutination of detect fimbriae. Most of the strains were fresh isolates from enteritis. Erythrocytes of man, pig, sheep, guinea pig, chicken and ox were used. The test was performed at room temperature and also at 4 °C with strains serially subcultured in broth at 20 °C. None of the strains formed haemagglutinins against any types of erythrocytes used. Fimbriae detectable by the used test are at least infrequent in *Y. enterocolitica* strains and therefore their role in pathogenicity could not be established.

PHAGE TYPING OF *YERSINIA ENTEROCOLITICA* IN HUNGARY IN THE YEARS 1980–1983

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By the phage sets of Nicolle and Mollaret 1584 *Yersinia enterocolitica* strains were typed. More than 96% of the strains belonged to serogroup O3 The most frequent European phage type, VIII was found in 89.9%. Phage type IX occurred in 5%. An increase has been observed in the spreading of phage IXa. Non-classified (n.c.) strains occurred in less than 0.5%. The proportion of phage resistant strains was smaller than 1%. Results of the phage typing performed at 30 °C and 37 °C were different from those obtained at 22 °C. There is a strong lysis with phages VI, VII and VIII and a weaker one with V and X at 30 °C. At 37 °C none of the phages of the international phage set exerted lysis. A total of 827 strains were examined by the 6 Calvo phages isolated from the sewage but none of them was suitable for further classification of the strains. One of the six phages, namely ZD3 was able to evoke lysis even at 37 °C. A new phage was isolated from a strain originating from a patient in 1982. The phage named 308 was propagated on strain No. 3827. More than 1.3% of VIII strains phage type was divisible by phage 308. Because *Y. enterocolitica* is a significant agent of enteric infections and is associated with certain chronic diseases (i.e. rheumatic disorders), and the isolates are highly homogeneous (serotype 103 and 4 biotypes), it seems desirable to continue efforts to divide them by phage typing methods.

DATA TO THE PATHOGENIC PROPERTIES OF NON-O1 *VIBRIO* STRAINS ISOLATED FROM SURFACE WATER IN HUNGARY

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During 1979–1982 nine non-O1 *Vibrio* strains were isolated from surface water in Balaton region. Seven strains belonged to the following Smith's serotype: 11, 17, 19, 22 (2 trains), 106 and 342. Two *Vibrio* strains were untypable. All of them produced haemolysin and permeability increasing substance. Four out of the 6 strains studied caused necrosis in mouse footpad. Two strains had not necrotic effect but one of them killed the mice very rapidly. Enterotoxin could not be detected in culture supernate of the strains with infant mouse test. Infant mice died after infection by living culture of *Vibrio* strains. Enterotoxin production was detected also by ELISA test.

CAMPYLOBACTER ENTERITIS IN HUNGARY — EXPERIENCES OF A THREE YEAR STUDY IN PEST COUNTY

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In the years 1980–1982 culturing of *Campylobacter* was successively started in 13 laboratories of the Hungarian Public Health Service. From 5880 (1980), 31 778 (1981) and 63 837 (1982) faecal samples from patients with enteritis campylobacters were isolated in 9.2, 6.1 and 4.0%, respectively. Out of a total of 11 881 asymptomatic contact persons of different age the isolation rate was 3.1% and our of 4195 healthy people 1.7%. In Pest county the annual incidence of campylobacter enteritis was 6.8, 6.0 and 2.0% in 3403, 5942 and 6265 patients, respectively, while the incidence of salmonellosis was 3.1, 2.5 and 3.8% and that of shigellosis 0.6, 0.2 and 0.2%. Campylobacter enteritis was most frequent in October 1980 (8.6%), in February (11.4%) and May 1981 (10.2%), both in May and December 1982 (3.3%). Positive results were mostly shown among infant patients (9.7, 13.8 and 4.3%) and among small children (1–5 years, 10.1, 7.8 and 3.0%). Males were usually more frequently affected by campylobacter enteritis. Watery and slimy diarrhoea occurred in 41.6%, bloody diarrhoea in 25.9%, vomiting in 4%, abdominal pain in 25.2%, pyrexia

in 35.7%. The illness was mild in 13.4%, severe in 3.1% and lasted as an average 6.3 days. The isolates were sensitive to tetracycline in 80–91% and to ampicillin in 13–39%.

BIOCHEMICAL AND SEROLOGICAL CHARACTERISATION OF *CAMPYLOBACTER JEJUNI* STRAINS ISOLATED FROM ANIMALS AND MEN

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Growth and biochemical characteristics of 98 *Campylobacter jejuni* strains isolated from diseased broiler chicken, laying hens, sheep, dog and human beings were examined. Based on the heat stable antigens, 77 strains were serogrouped by the tube agglutination test using hyperimmune rabbit sera. All *Campylobacter* strains behaved typically in growth tests (25 °C and 42 °C), in nalidix acid and NaCl tolerance and in H_2S production, but varied widely in their glycine, bile salts, tetrazolium chloride and selenite tolerance. Using boiled antigens the strains were classified into six serogroups at least. Almost each serogroup was found also in dogs, sheep and humans.

IDENTIFICATION OF UFP ("UNCLASSIFIED FLUORESCENT PSEUDOMONAS") STRAINS BY CLINICAL BACTERIOLOGY METHODS

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One hundred and four our of 134 UFP strains isolated from surface water, swimming pool, hospital wastes and some clinical samples belonged to biotypes I and III. These strains uniformly grew at 41 °C but not at 5 °C, had polar flagella, produced fluorescent pigment, oxidase, catalase and arginine dihydrolase, utilized ammonium citrate, formed acid from glucose and were haemolytic. Different reactions were obtained (% positive) for $NO_3 \rightarrow NO_2$ (11), urease (3), ammonium acetate (98), gelatin (82), KCN (3), casein hydrolysis (97). Tween 80 hydrolysis (88), DNase (15), acetamide (3), fructose (92), galactose (44), maltose (2), mannose (14), xylose (43), 2-ketogluconate (5).

Uniformly negative characters were: $NO_3 \rightarrow N_2$, indole, H_2S, lecithinase, phenylalanine deaminase, lysine and ornithine decarboxylase, inositol, lactose, mannitol, mannose, rhamnose, salicin, sucrose, esculin and starch hydrolysis, ONPG. UFP strains of other biotypes usually failed to hydrolyse casein and gelatin and were non-haemolytic. Methods used in clinical bacteriology laboratories allow a proper differentiation of UFP and other pseudomonads.

INVESTIGATION OF THE INTESTINAL FLUID OF PATIENTS WITH CONTAMINATED SMALL BOWEL SYNDROME

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To extend the diagnostic possibilities of the "irritable small bowel" syndrome, the small bowel aspirates from 114 patients were subjected to bacteriological examination. Additionally the bacterial flora of the intestinal fluid of 53 patients with other gastrointestinal diseases and of 10 patients without gastrointestinal complaints were tested. In 85% of the patients having "irritable small bowel" syndrome, 10^6–10^7 aerobic and anaerobic bacteria per ml were found. In 48.4% the mouth flora and in 34.6% the faecal flora predominated and in 16.6% a mixed flora was isolated. The results demonstrated that the contaminated small bowel syndrome must be considered separately from the functional intestinal disease, the "irritable small bowel" syndrome.

STREPTOMYCIN RESISTANT MUTANTS OF *MYCOBACTERIUM PHLEI* AND *MYCOBACTERIUM SMEGMATIS* STRAINS EXHIBIT ALTERED PHAGE-SENSITIVITY

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Streptomycin resistant mutants of different *Mycobacterium smegmatis* strains (butyricum, friburgensis, Rabinowitz) and that of *Mycobacterium phlei* were isolated by one-step selection. These mutant strains did not show cross resistance to other aminoglycosides, like gentamicin, kanamycin or

neomycin. A streptomycin concentration-dependent decrease of the phage titres was found on titrating homologous phages on their streptomycin resistant hosts. In addition, three of eleven streptomycin resistant strains proved to have an altered phage-sensitivity to non-homologous phages.

THE ENZYME PROFILE OF *HAEMOPHILUS* SPECIES

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Haemophilus strains (27 *Haemophilus influenzae*, 9 *Haemophilus parainfluenzae* and 1 *Haemophilus haemolyticus*) were isolated from clinical samples from 25 cases of chronic bronchitis, 2 cases of meningitis and 10 swabs from oral mucosa. *H. influenzae* produced a low amount of coagulase, *H. parainfluenzae* produced lipase (some strains weakly). The organisms had no effect on fibrin or gelatin and no lecithinase, deoxyribonuclease, elastase and proteinase activity could be demonstrated. The results suggest that these enzymes play an unimportant role in developing histological damages in chronic haemophilus infections.

DETERMINATION OF SULPHONAMIDE-SENSITIVITY OF 962 STRAINS OF *ESCHERICHIA COLI*

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The sensitivity to sulphonamides of 962 strains of *Escherichia coli* cultured from animals with enteritis has been tested by diffusion and dilution methods. Comparative determinations on Mueller–Hinton (MH) medium and on a genuine medium (7GS) deficient in sulphonamide antagonists were made. (1) By diffusion, the strains were highly sensitive of 7GS-medium although they appeared resistant on MH medium. (2) The dilution method confirmed these data. The index MIC MH/7GS expresses the antisulphonamide effect of MH-medium.

CORTICOSTEROID-DEPRESSION IN THE REPRODUCTION OF EXPERIMENTAL ERYSIPELAS AND PATHOGENICITY TESTING OF *ERYSIPELOTHRIX RHUSIOPATHIAE* STRAINS IN SWINE

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According to a hypothesis of the authors the results obtained on 48 animals have confirmed that corticosteroid hormones administered to piglets can depress their processes of defence thus favouring the installation of experimental erysipelas. Piglets submitted to corticosteroid treatment before infection display increased susceptibility allowing the reproduction of septicaemic or cutaneous erysipelas. Inoculating culture dilutions intradermally the minimal contaminating dose can be titrated and thus the degree of pathogenicity of strains and their epizootic potential can be assumed. Corticosteroid-depression (CSD) offers a possibility of testing the protective value of vaccines and immune sera, as vaccinated piglets on CSD background resist to experimental infection. In comparison with other techniques, the method described possesses the advantage of constantly offering reproducible data.

MICRO METHOD FOR DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION OF ANTIBIOTICS IN CLINICAL ISOLATES OF BACTEROIDES

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The minimal inhibitory concentrations (MIC) of 8 antibiotics were determined with a micro method in 91 clinical isolates of *Bacteroides*. The micro MIC method and the disk diffusion test showed 90.1% agreement. For 50 *Bacteroides* strains, the overall agreement between the micro MIC and the classical agar dilution MIC was 89%.

SURVEY OF *MYCOPLASMA PNEUMONIAE* INFECTIONS BY GROWTH INHIBITION TEST

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Growth inhibition tests were performed in children and in adults suffering from different respiratory diseases in order to verify *Mycoplasma pneumoniae* infection. In Szabolcs-Szatmár county there was an unusually high proportion of infected persons when compared with other parts of the country. Fifty per cent of the material obtained from this county was found positive while in material obtained from the rest of Hungary this ratio was only 10%.

SODIUM-DEPENDENT UPTAKE OF TAURINE IN STAPHYLOCOCCI

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A novel uptake system for the unusual amino acid taurine was discovered in encapsulated *Staphylococcus aureus* strain M. Taurine has been shown to be a capsular polysaccharide component in this strain. Taurine uptake by cells in buffer was dependent upon Na^+, stimulated by glucose and markedly affected by temperature. Taurine uptake was a saturable process and Lineweaver–Burk plots revealed a KM of 43 μM for taurine in the presence of 10 mM NaCl. Li^+ was the only other cation that supported taurine uptake. Radioactivity taken up was not caused to efflux by high levels of unlabelled taurine, and the radioactivity did not chromatograph as taurine indicating that rapid metabolism occurs during or closely following uptake. Taurine uptake was a highly specific process that was not diminished by amino acids representing the major groups of amino acid transport systems in *S. aureus*. Sulfhydryl group reagents, electron transport inhibitors, an uncoupler and inhibitors of Na^+-linked transport processes inhibited taurine uptake. An Na^+-dependent taurine uptake system seems to be a common property of staphylococci since it was detected in most coagulase-positive and -negative staphylococcal species, encapsulated and unencapsulated, examined.

ISOLATION OF ATYPICAL *CORYNEBACTERIA*

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Two atypical *Corynebacterium* strains were isolated from two different patients; one of the strains originated from a mandibular fistula of a 60 years old man, the other one from vaginal discharge as well as throat swab of a 35 years old aborted woman. The first *Corynebacterium* belonged to F-2 group, the second one to B-3 group.

ISOLATIONS OF *CLOSTRIDIUM DIFFICILE* IN VESZPRÉM COUNTY

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For the isolation from stools of *Clostridium difficile* we examined stools of patients with diarrhoeal disease, coming from the different departments of hospitals. The cycloserine–cefoxitin–fructose–egg yolk agar described by George et al. has been used. Out of 314 faecal specimens 14 yielded *C. difficile*. *C. difficile* was implicated as aetiological agent of antimicrobial-associated diarrhoea in some of the cases. *C. difficile* was recovered from two *Campylobacter jejuni*-positive patients and from one *Salmonella enteritidis*-positive patient.

THE CHARACTERISTICS OF PHOSPHOLIPASE-C FORMATION IN *CLOSTRIDIUM PERFRINGENS* STRAINS

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The dynamism of phophlipase-C production of 60 *Clostridium perfringens* strains was investigated according to our method described before in 5 parallel samples in test tubes. The great majority of the strains (11/14) with a maximal toxin quantity above 1000 U/L reached this level in the 5th hour of the cultiva-

tion. The maximal toxin quantity of the culture supernatants decreased under 50% by the 24th hour in 21 of 28 strains when the peak of phospholipase-C activity was above 500 U/L, but when it was under 500 U/L such a decrease was shown only in 9 of 32 strains. With great probability this phenomenon is caused by factors hindering the effect of phospholipase-C. It seems that these factors were produced proportionally with the quantity of phospholipase-C. Further investigations are required to identify the nature of these factors.

DIAGNOSTIC METHODS OF *LEGIONELLA* INFECTION

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Rabbits were immunized with the antigen prepared from CDC reference strain *Legionella pneumophila* type 1. Rabbit immunoglobulins labelled with peroxidase were used to detect legionella. In 15% of 403 sera collected from healthy persons of different age antibodies could be detected in 1:16 dilution. The titre of the remaining samples was lower.

SERO- AND PHAGE-TYPES OF *LISTERIA* STRAINS

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Nasal, faecal, vaginal and milk samples were taken repeatedly at every 2nd–6th day from 50 healthy, sheep designated individually and kept together for 5 weeks maximum. Out of 814 samples 148 were positive and 152 *Listeria* strains were isolated; 75% of the strains belonged to serotype 1/2, the others to serotypes 4, 5 and 6. A few strains could not be serotyped. From a given animal, in the same time, *Listeria* strains with the same sero- and phage-type or with the same sero- but different phage-type, or rarely, with different serotype can be isolated. When the samples were taken from the same animals at different points of time, the sero- as well as phage-types were sometimes different. In the second study *L. monocytogenes* strains isolated in Borsod-Abaúj-Zemplén county from dead animals and ill patients were typed and

compared. Out of the 101 animal strains 84 belonged to serotype 1/2 and only 17 to serotype 4. In case of the human strains, 6 out of the 8 were serotype 4 and only 2 were serotype 1/2. Animal and human strains differed in phage-type.

SEPSIS OF FETUS CAUSED BY *LISTERIA MONOCYTOGENES*

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A 3100 g infant born in the 38th week of pregnancy developed sepsis and died on the second day. *Listeria monocytogenes* was cultured from the CSF and pus obtained from the eyes. During pregnancy the mother had a disease resembling grippe with a moderate pyrexia.

IDENTIFICATION AND OCCURRENCE OF COAGULASE-NEGATIVE STAPHYLOCOCCI IN CLINICAL SPECIMENS

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According to newest taxonomy, the *Staphylococcus* genus is divided into 13 species. On the basis of 24 easily performable tests an identification scheme has been elaborated for coagulase-negative species. A total of 208 strains isolated from patients was classified. In every kind of samples *S. epidermidis* occurred the most frequently (wound 25.0%; urine 29.0%; blood 54.1%; eye 53.4%; other specimens 38.9%). In wound haemolytic species (*S. epidermidis, S. haemolyticus, S. simulans, S. warneri, S. cohnii)* predominated (74.9%). The majority of strains from blood cultured was assumed to be contaminants. The incidence of oxacillin and cephalosporin resistant strains was low (13% and 8%). A considerable part of strains was resistant to other antibiotics, especially to penicillin.

IDENTIFICATION OF ANTIGENIC COMPONENTS OF *TOXOPLASMA GONDII* BY AN IMMUNOBLOTTING TECHNIQUE

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Proteins of *Toxoplasma gondii* were separated by polyacrylamide gel electrophoresis with subsequent transfer to a nitrocellulose sheet by electrophoretic blotting. Immunologically reactive polypeptides were detected by human sera with known toxoplasma antibody levels measured using enzyme immunoassay. Heavy chain specific, peroxidase-conjugated anti-human immunoglobulins were used as the indicator antibodies for the separate identification of IgG and IgM reactive polypeptides. IgG toxoplasma antibodies reacted with several antigens with molecular weights approximately from 27 kD to 67 kD while toxoplasma specific IgM seemed to detect only a few polypeptides. The toxoplasma specific IgM reactive polypeptides might have some specific role in immunopathogenesis of toxoplasmosis. Especially the molecular weight of 35 kD for the dominating IgM reactive polypeptide was observed.

ACUTE SEROUS MENINGITIS CAUSED BY *TOXOPLASMA GONDII*

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Acute serous meningitis of a 4-year-old child was observed. In the CSF the tachyzoites could be demonstrated by Giemsa staining method as well as by animal inoculation. The control of the disease could be accomplished by an adequate treatment. According to the evaluation of the smears, serological findings and literary data it was suggested that the route of infection was directly through the nasal cavity.

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Virology

COMPARATIVE ANALYSIS OF PHYSICAL AND CHEMICAL PROPERTIES AND ANTIGENIC SPECIFICITY OF HEXONS OF TWO SUBSTRAINS OF ADENOVIRUS TYPE 1

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Physical and chemical properties and the hexon antigenic structure of two substrains originating from the standard strain Ad h 1 (Ad 71) of human adenovirus type 1 were studied. Substrain Ad h 1K and substrain Ad h 1B had been passaged for a long period in cell cultures of human origin in the Kiev Institute and in the Budapest Institute, respectively. Hexon preparations were purified, stored for a long time and subjected to electrophoresis in SDS gel. The polypeptides were different in molecular weight (Ad h 1B polypeptide, 120 kD; Ad h 1K polypeptide, 105 kD). Hexons were treated with chymotrypsin and the peptides were mapped. Besides common peptides, two unique peptides were found in hexon Ad h 1K and six in hexon Ad h 1B. The results were confirmed by mapping of a mixture of chymotrypsin hydrolysates of both hexons. It seems that slight differences in the primary structure made hexon Ad h 1K more sensitive to proteases and the resulting more intense proteolysis accounted for its lower molecular weight. Immunodiffusion with monospecific sera to each of the two hexons and inclusion of Ad S-16 in the reaction gave an opportunity to identify unique antigenic determinants in each of the hexons. Results of competitive RIA also showed differences in the antigenic structure of the two hexons. The differences, which may touch the type-specific determinant, are suggestive of some intratypic variability of adenovirus hexon.

COMPARATIVE ANALYSIS OF TWO SUBSTRAINS OF ADENOVIRUS TYPE 1

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The substrains examined in this work (Ad h 1B, Ad h 1K), were the same as those characterized in the preceding paper. The synthesis of virus-specific polypeptides and the development of intranuclear inclusions were studied in infected HeLa cells. Forty hours after infection mainly major viral polypeptides II, III, IIIa, IV, V, VI and VII and nonstructural polypeptide IOOK were revealed by gel electrophoresis in lysates of cells marked with ^{14}C protein lysates. The purified hexons prepared from the two substrains were different from each other in their polypeptide spectrum and in the electrophoretic mobility of polypeptide II. By luminescent microscopy of cells fluorochromized with acriedine orange some differences were observed in the morphology of inclusions and in the degree of monolayer affection. In cell cultures infected by Ad h 1K, inclusions were seen in 80% of the cells; while late centranuclear inclusions prevailed, finely-granulated younger inclusions were also seen in part of the cells. In cells infected by substrain Ad h 1B only centranuclear inclusions were detected, in practically all monolayer cells. In cells infected by either of the substrains, ribamidil (virasol) in a concentration of 31 μg/ml prevented finely-granulated inclusions from being transformed to centranuclear, a phenomenon more distinctly seen in cells infected by Ad h 1B than in those infected by Ad h 1K.

DETECTION OF ANTIBODY-MEDIATED AGGREGATION OF ADENOVIRUS BY ETHIDIUM BROMIDE FLUORESCENCE

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Complete virions of adenovirus can be stained with ethidium bromide, and detected in agarose gels by fluorescent photography. Visible bands may be obtained when 10^8 particles (approximately 10^7 PFU) are run in one electro-

phoresis sample, which can be obtained from 10^5 to 10^7 cells of permissive tissue cultures. Purified complete virions of human adenovirus type 1 strain (Ad h 1) were reacted with serial dilutions of antisera prepared in rabbits against native or SDS-, and guanidine-HCl-treated virions of the homologous virus, against purified hexon, and fibre preparations, and heterologous (bovine, and simian) adenoviruses. Type-specific antibodies prevented the penetration of virions into the 6 mg/cm^3 agarose slab gels during electrophoresis. The antibody titres measured by this technique were found to be at least 10 times higher than those determined by immunodiffusion with soluble antigens, although only a very small proportion of antibodies react with surface epitopes.

MORPHOLOGICAL BASES OF THE INTERCAPSOMER BONDINGS IN THE ADENOVIRUS CAPSID

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The interhexonal space within the adenovirus capsid is bridged by interhexonal connective components. Linking components are detectable also between pentons and the five peripentonal hexons. According to results obtained by Markham's rotational integration technique connective elements between capsomers interlace the whole virion. It was confirmed in several cases that the linking components consisted of two approximately parallel elements. This refers to that each hexon is linked by six times two parallel linking elements with its six nearest neighbours in the capsid.

ELECTROPHORETICAL SEPARATION OF NEWCASTLE DISEASE VIRUS PROTEINS BY MEANS OF A MODIFIED COLOURED SILVER STAINING

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Polyacrylamide gel electrophoresis is a common technique in pure protein isolation. The protein contained by the excised and eluted slice samples is considered to be pure, since by Coomassie Brilliant Blue staining the samples give a one-striped pattern. Using the silver staining method, which is much

more sensitive than the conventional procedures, the once excised slice samples show a marked amount of impure proteins which can only be removed by purifying steps repeated two or three times. The modified coloured silver staining method was developed from the methods of Merril and Sammons. It requires a significantly shorter procedure time, produces a lighter yellow background, and is as sensitive as the original coloured silver staining method.

STUDY ON THE CAPSID PROTEINS OF EDS VIRUS

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EDS virus grown in duck's eggs was purified by ultracentrifugation on CsCl gradient. The capsid proteins were separated in 12% gel under reduced conditions by means of SDS-polyacryl amide electrophoresis. The protein bands of the gel were transmitted mechanically by means of pressing onto the cellulose-nitrate-acetate membrane. The membrane was stained with the immunperoxidase method, while the gel with Brilliant Blue R-250. By means of suitable molecular-weight standard, the molecular weight of protein (antigen) active with immunoperoxidase staining was determined at 17 000 dalton. After inactivation carried out with different inactivating agents (formaldehyde, ethylene imine, aziridino-ethanol, beta-propiolactone) the virus proteins were tested with the above method. Protein existing in the region of larger molecular weight showed antigen staining on the formaldehyde activity, the other inactivating agents did not elicit alteration in the protein antigen.

MOLECULAR SIZE VARIATION OF EBNA IS DETERMINED BY THE EB-VIRAL GENOME

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The size classes of the Epstein–Barr virus-(EBV)-induced nuclear antigen (EBNA) have been determined in somatic hybrids between EBV-carrying cell lines or between EBV-positive and EBV-negative lines, and in sublines of two originally EBV-negative Burkitt lymphomas, converted to EBNA positive by in vitro infection with two different EBV substrains. Partially

purified EBNA components were detected by Western blotting and subsequent visualization by antiserum/alkaline phosphatase-coupled protein A complexes. The parental Raji, P3HR-1, Namalwa and Daudi cells contained EBNA components of different molecular-weight classes. In addition, several lower molecular weight components were seen as well distinctive for each cell line. Somatic hybrids expressed the main EBNA components of both parental lines. Hybrids between EBV-carrying and EBV-genome-negative lines contained the characteristic EBNA component of the positive parent. The EBNA-positive sublines of the originally EBV-negative Ramos and BJAB lymphomas contained the main EBNA components characteristic of the virus donor strain. Thus, the EBNA components of P3HR-1 virus-converted Ramos and BJAB cells resembled each other and the P3HR-1 donor, whereas all B95–8 virus-converted lines were different. They resembled each other and the EBNA of the B95–8 cell line. It is concluded that the size variation of EBNA is determined by the viral genome.

NEW RESTRICTION ENDONUCLEASES FROM STREPTOCOCCUS ISOLATES

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Certain strains of streptococci of the viridans group cannot be transformed by foreign DNA. As a reason a thus far unknown restriction modification system may be assumed. On this theoretical basis three different restriction endonucleases were identified from standard and wild strains. The optimal purification method of the enzymes and the conditions of enzyme activity are established. Taking advantage of physical maps constructed on the DNAs of adenovirus type 1 and SV_{40} the characterization of the recognition sequence is under investigation.

PHYSICAL MAPS OF A NEW STREPTOCOCCUS RESTRICTION ENDONUCLEASE ON THE DNA OF ADENOVIRUS TYPE 1 AND SV_{40}

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One of the streptococcus restriction endonucleases having been isolated in this laboratory cuts the DNAs of adenovirus type 1 and SV_{40} to fragments ranging in number like the products of restriction enzymes of high specificity.

The location of cleavage points were, however, unknown. In the present experiments the physical maps were established by the methods of double digestions, cross digestions and the analyses of incomplete fragments.

GROUPING OF FOWL ADENOVIRUSES BASED UPON THE RESTRICTION PATTERNS OF DNA GENERATED BY BAM HI AND HIND III

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Based upon the restriction patterns of DNAs generated by restriction endonucleases Bam HI and Hind III, 17 fowl adenovirus strains representing 11 serotypes were placed into 5 groups, as follows. Group A: strains CELO and 112; Group B: strains 340 and TR-22; Group C: strains KR-5 and C-2B; Group D: strains 380, 685, SR-48, SR-49 and A-2; Group E: strains YR-36, CR-119, X-11 H-6, TR-59 and 764.

PRIMING ACTIVITY OF HUMAN INTERFERON TYPES IN COMPARISON WITH THEIR OTHER BIOLOGICAL EFFECTS

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The priming activities of different IFN types (leukocyte or α, fibroblast or β and immune or γ IFN) were compared. In α- and γ-IFN-producing systems purified α and β IFNs had a more pronounced priming effect than purified γ IFN had. The IFN production could be more efficiently influenced by IFNs in α-than in γ-IFN-producing system. Besides priming activity, other biological effects of purified α, β and γ IFN preparations were tested. Despite its low priming activity, γ IFN had a high anticellular potency, while α and-β IFNs had a high priming, but relatively low anticellular, activity. Augm enta tion of NK by α, β and γ IFNs was identical.

THE ROLE OF THE VIRAL DNA IN INTERFERON INDUCTION BY ADENOVIRUSES

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All human adenoviruses (Ad) tested so far (18 serotypes) induced interferon (IFN) production in chick cells. Ad types can be divided into two groups depending on their inducing capacities. Members of potent and nonpotent inducer groups induced IFN of high (5×10^3 IU/ml) and low (50–100 IU/ml) titre, respectively. Experiments carried out with Ad12, a representative member of potent inducers, showed that viral nucleic acid plays an important role in IFN induction. Empty capsids lacking DNA could not, complete and incomplete particles carrying the whole or part of the genome could, trigger cells to produce IFN. One fifth of the genome was sufficient for IFN induction. Hybridization experiments proved that the subgenomic DNA required for induction is located to the left end. The role of viral DNA in IFN induction was investigated in the case of Ad2 a member of the nonpotent inducers, too. In contrast to Ad12 even empty Ad2 capsids induced IFN, suggesting that the viral core is not required for IFN induction with Ad2.

CONCANAVALIN A-INDUCED INTERFERON PRODUCTION BY CHICKEN

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Interferon (IFN) was induced in leukocyte suspensions by exposure to Concanavalin A (Con A) under a variety of cell culture conditions. The induced IFN reached a maximum level 4 days after stimulation. The Con A-induced IFN is stable at pH 2, labile at 56 °C, trypsin-sensitive and species-specific. The tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) was found to potentiate IFN production in leukocyte cultures in combination with Con A.

EFFECT OF INTERFERON ON ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) IN CHICKENS

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The influence of chicken leukocyte interferon (IFN-α) on the ADCC of chicken peripheral blood mononuclear cells was investigated in vivo and in vitro. The cytotoxic effect was studied on sheep red blood cells (SRBC) coated with chicken anti-SRBC serum. Cytotoxicity was estimated in a ^{51}Cr-release assay system. A single i.v. injection of crude IFN led to an enhanced ADCC 6 to 12 h after the IFN injection, whereas the activity had decreased by 24 h. In vitro the ADCC was boosted within 1–18 h by IFN, in a dose-dependent manner. We assume that the augmenting agent in vitro is IFN itself, as purified IFN has a similar activity but both mock IFN and trypsin-treated IFN showed no enhancing effect. The extent of ADCC was the highest in the case of granulocytes as effector cells. The augmentation of ADCC by IFN-α was also most pronounced with granulocytes. It is concluded that chicken IFN, similarly to mammalian IFN, has an immunomodulating effect on the ADCC in birds.

TRANSLATION IN *X. LAEVIS* OOCYTES OF THE POLY A-CONTAINING RNA FRACTION EXTRACTED FROM SENDAI VIRUS-INDUCED L CELLS

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Total cytoplasmic RNA was isolated from Sendai virus-induced L cells at the time of their maximum interferon (IFN) production. The poly A plus RNA fraction was separated by means of oligo-dT cellulose chromatography. The molecular-weight distribution of the RNA preparations was determined by horizontal polyacrylamide gel electrophoresis. The translational activities of different RNA fractions were assayed in *X. laevis* oocytes. Microinjection of the poly A plus RNA fraction resulted in an IFN production 15–30-fold higher than that from total cytoplasmic RNA. The kinetics of IFN production by poly A plus RNA injected oocytes showed that IFN appears 4 h after injection and reaches the maximum titre after 30–40 h. The IFN production by the oocytes was found to be proportional to the amount of poly A plus RNA present in the injected preparations.

EFFECTS OF FLAVONOIDS ON VIRUS MULTIPLICATION AND ON INTRACELLULAR cAMP LEVEL

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Various flavonoids block the multiplication of herpesviruses in cell cultures. Quercetin increases the level of cAMP in Ehrlich ascites tumour cells. Several flavonoids are effective inhibitors of cAMP phosphodiesterase. Dibutyryl cAMP inhibits the multiplication of human adenovirus type 2 and herpes simplex virus in vitro. As to the mechanism of the antiviral effect of these flavonoids some connection was assumed between their antiviral effect and the increase in the cAMP level. In the present studies quercetin and quercitrin inhibited (in a concentration-dependent manner) the multiplication of human (alpha) herpesvirus 1 in HEp-2 cell culture, and the same flavonoids increased the intracellular cAMP level; rutin and hesperidin neither displayed an antiviral effect nor elevated the cAMP level.

ANTIVIRAL ACTIVITY OF TUFTSIN AND ITS ELONGATED ANALOGUES

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Tuftsin, a biologically active tetrapeptide, stimulates functions of macrophages and polymorphonuclear granulocytes and enhances the migration of normal and sensitized human cells. In the present experiments, the effect of tuftsinyltuftsin and other elongated analogues of tuftsin on the development of tumour induced by i.m. inoculation with murine sarcoma virus (MSV) was investigated and compared with the effect of tuftsin. Tumour development was followed up for 10 days. Tumour size was expressed in per cent of the increase in the volume of infected thigh and compared with the volume of intact thigh. The peptides (10 μg per injection) were injected i.p. according to four different schedules. Tuftsinyltuftsin inhibited the tumour growth independently on the schedule of administration. The best results were observed when the peptide was administered twice; 24 h before and 24 h after inoculation. Another experiment suggested that tuftsin possesses not only antitumour

but also antiviral activity. In mice infected intranasally with virus herpes hominis type 1 (HSV_1) tuftsin markedly reduced the mortality, depending on the schedule of administration. The best results (40% of protection) were observed when the peptide was administered for 6 days after infection. Similar results were obtained in mice infected with EMC virus s.c. In this case, too the rate of protection (ranging from 10% to 60%) was depending on the schedule of administration. Tuftsinyltuftsin was much more active than tuftsin.

IMMUNOLOGICAL AND HISTOCHEMICAL INVESTIGATIONS IN SHEEP IMMUNIZED WITH ATTENUATED RABIES VACCINES

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Two groups each containing 10 Merino sheep were immunized; the one with a vaccine containing fixed virus and the other with a Vnukovo-32 strain vaccine. On each of postvaccination days 4, 7, 14, 21 and 28 2 sheep of each group were bled and the route of the virus antigens in their organ samples was followed by the IF technique; further, the morphological changes in the lymphoid organs and the in vitro reactions of the lymphoid cells were investigated. Histochemical and electron microscopical investigations showed similar immunomorphological changes in both groups. However, there were differences in immuno-inductive effects, which manifested in differences in the autoradiographically-measured blastogenesis of the lymphocytes originating from lymph nodes, spleen and blood, moreover, in differences in the macrophage migration inhibition indices. Using IF antigen of the fix virus persisted longer at the inoculation site and in the lymph nodes than that of the Vnukovo-32 strain, but this latter concentrated earlier in the lymph nodes following vaccination.

IMMUNIZATION AGAINST CANINE PARVOVIRAL ENTERITIS WITH AN ATTENUATED FELINE PARVOVIRUS

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The first occurrence of parvoviral enteritis of dogs was reported only a few years ago. Since initially no vaccine prepared from homologous virus was available, the close antigenic relationship existing between the newly isolated canine parvovirus and the causative virus of feline panleucopenia—(a parvovirus) was utilized. Therefore, the live or inactivated feline panleucopenia vaccine produced abroad served for protection against the disease. Since Hungarian veterinary circles were dissatisfied with the protection provided by the vaccine containing inactivated virus, an attenuated strain of feline parvovirus was propagated in primary or secondary feline kidney cell cultures. After i.m. or s.c. administration of the propagated virus, susceptible dogs remained symptomless, and virus-neutralizing (VN) and haemagglutination-inhibiting (HI) antibodies to both the feline parvovirus and the BU/80 canine parvovirus isolated in Hungary appeared in their sera. One thousand doses of vaccine were produced from the cell-culture-propagated virus, and 900 pet dogs and 100 dogs kept in kennels were vaccinated. The disease did not occur among the immunized animals, while unvaccinated dogs kept in kennels developed severe disease, and 20 to 80% of them died. A single vaccination provided a protection lasting one year.

DEVELOPMENT OF INACTIVATED INFECTIOUS LARYNGOTRACHEITIS VIRUS VACCINE

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Infectious laryngotracheitis (ILT) occurs amongst poultry of different ages in certain areas of Hungary. To prevent the disease, there is one imported living virus vaccine available however, it caused mild disease in broiler flocks. An inactivatived oil-adjuvated vaccine has been developed. The potency of the vaccine was tested under laboratory and field conditions. SPF cockerels

of 8 weeks old were immunized and it has been established that the index of neutralization varied withing 1.6 to 1.8 as well as, all cockerels were protected against the field virus challenge. The potency of the attenuated and inactivated vaccines was compared in the practice in each of 1600 birds. The attenuated vaccine conferred complete protection in the second week, while the inactivated one assured the same protection on the 4th week only. Following the vaccination all birds of both groups were resistant against the challenge. The booster effect of the inactivated vaccine was tested 2, 3, 4, 5 and 6 weeks following the primovaccination. The best effect was found at the booster vaccination carried out on the 2nd and 3rd week, the average of the indices of neutralization ranges between 1.5 and 1.6, and between 1.9 and 2.0, respectively.

COMPARATIVE EXAMINATIONS OF DIFFERENT NEWCASTLE DISEASE VIRUS STRAINS

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For the production of inactivated vaccine against NDV, several lentogenic and mesogenic virus strains were compared. In the course of the propagation of different virus strains, crude suspensions of different infective titres were obtained, however, the immunizing power of the inactivated vaccines produced from the above substances did not agree with their infective titres. The NDV-6 strain proved to be the most appropriate for vaccine production. On the basis of literary data the heat-stability of haemagglutinin and neuraminidase antigens of virus strains used in Hungary was compared and the alteration of their infectivity at $50_{\circ}$ C was studied. From the strains under test, the LaSota strain was the most fragile, while the NDV-6 strain was the most resistant. Presumably, the stability of the surface antigens of NDV-6 strain makes the strain especially appropriate for inactivated vaccine production.

EXPERIENCES OBTAINED IN THE COURSE OF THE PRODUCTION AND OF A TRIAL OF INACTIVATED PIGEON HERPES VIRUS VACCINE

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In the early spring and late autumn, the virus attacked mainly young pigeons of 50 to 90 days old. The diseased birds were disturbed in by serious catarrhal symptoms, and many of the starving birds perished. For prevention, a water-in-oil vaccine containing inactivated pigeon-herpes virus was prepared. The virus was grown in chicken embryo fibroblast cell culture maintained in a MEM–Hanks nutritive medium containing fetal serum. Three different inactivating agents were tested. The inactivation carried out with ethylene imine proved to be the best. The adjuvant was selected from eleven different oils on the basis of a safety test. In addition to the laboratory test, a field trial was carried out in a large-scale farm. In the course of the trials, average S index calculated from the serum-neutralization tests surpassed 1.5 five weeks after the vaccination. The mortality rate for young pigeons, diminished from 30%, the average for the previous 3 years, under 3% in the issues of vaccinated parents.

A PIGEON VACCINE CONTAINING FOUR COMPONENTS

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The aim of the present studies was to develop a combined vaccine against four pigeon diseases, each prevalent in Hungary. *Salmonella typhi-murium*, *Mycoplasma gallinarum*, *Chlamydia* and pigeon herpes virus antigens were administered in 15 different compositions and the serological responses elicited were tested in SPF cockerels. In every case the oily adjuvant was applied which had proved to be the best immunostimulant. The serologic responses of carrier-pigeons to the vaccine of appropriate composition were tested on the 1st, 3rd and 12th months postvaccination. The common symptoms of the respiratory organs and the mortality of young birds stopped and the production of the carriers increased in the vaccinated flocks.

STUDIES ON INACTIVATED VACCINE AGAINST DUCK HEPATITIS

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Water-in-oil vaccines were prepared from the virus strain TN used for vaccine production against infectious duck hepatitis (IDH), and from virulent duck hepatitis virus inactivated with formaldehyde. Each of 10 day-old ducklings was immunized s.c. with the inactivated vaccine and another ten with the Anahep vaccine containing living IDH virus. The dose was 0.2 ml throughout. Three weeks after vaccination pooled blood sera of the vaccinees and those of untreated controls were examined. The S indices for the different groups were as follows: untreated control group 1.62; ducklings vaccinated with Anahep, 2.16; ducklings vaccinated with inactivated virulent virus, 2.04; ducklings vaccinated with TN virus, 2.70. With the vaccine produced from the inactivated TN virus strain field trials were preformed. The vaccine proved to be safe according to the test carried out in a breeder-flock of 300 birds vaccinated two times, each with 1.0 ml dose. Thirty day-old ducklings of the parents vaccinated in the above trial as well as 30 susceptible 6 days old ducklings were challenged s.c. with 1 ml of virulent virus of a titre $10^{4.5}$ ID_{50}/ml. Of the 30 susceptible ducklings 24, while from the issues of vaccinees one bird died of hepatitis.

THE AETIOLOGICAL ROLE OF ECHOVIRUS TYPE 6 IN MENINGITIS AND ENCEPHALITIS CASES IN 1982 IN HUNGARY

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In the period between July and November, 1982 echovirus type 6 infection was detected in 148 cases of acute CNS diseases which occurred in different parts of Hungary. The age-specific morbidity rates were the highest in children between 3 and 9 years of age but young adults were also considerably affected. The aetiology could be verified by isolation of the virus only in 10 cases since no appropriate materials were received for this purpose from the majority of the patients. Thus, the laboratory diagnoses had to be based on serological tests which were performed by the radial plaque neutralization and/or the haemagglutination inhibition techniques. A significant rise of

antibody titre was demonstrated in 53 patients including 7 yielding virus. In additional 92 patients the actual echovirus type 6 infection was verified by demonstration of specific IgM antibodies in their blood samples.

THE ROLE OF HEPATITIS NON-A NON-B (NANB) VIRUS IN CHRONIC DISEASES OF LIVER

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233 patients with a diagnosis of chronic liver disease verified by biopsy have been investigated for hepatitis B (HBV) and hepatitis Non-A Non-B virus (NANBV) infections. Micro-Hepanosticon tests were applied for diagnosis of HBV and the double immunodiffusion tests as described by Schirachi for diagnosis of NANBV. Positive results were obtained in 89% for HBV and in 11% for NANBV among the patients with chronic persistent hepatitis, in 57% for HBV and in 33% for NANBV among those with chronic active hepatitis and in 58% for HBV and in 18% for NANBV among those with cirrhosis. In some cases the NANBV diagnosis were supported also by electron-microscopic examination of blood samples. The results indicate that besides the HBV the BANBV plays a significant role in eliciting chronic disease of the liver.

STUDIES ON INFLUENZA VIRUS STRAINS ISOLATED IN THE COURSE OF OUTBREAKS AMONG PIGS IN HUNGARY

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In the spring of 1983 influenza outbreaks occurred in piggeries in Hajdú, Szabolcs and Békés counties. 4–5 months old porkers were the most severely affected. The outbreaks were characterized by sudden onset and short duration. Rhinorrhoea, catarrh, coughing, dispnoea, anorrhexia, and distress were the most common symptoms. No fatal outcome was observed. 24 influenza virus strains were isolated from the lung tissues of slaughtered animals. The strains proved antigenically related to the A/New Jersey/8/76/H1N1) strain in haemagglutination inhibition tests. A serological survey with 300 blood

samples taken from pigs in affected piggeries indicated a large scale immunological experience with the isolated virus variant. A closer analysis of structural proteins of isolated strains compared to those of some prototype strains of swine origin (A/swine/Iowa/15/30, A/New Jersey/8/76) and of a swine strain isolated in Szeged in 1981 (A/swine/Szeged/1/81/H1N1) carried out with SDS–PAGE technique revealed notable differences. The molecular weight of the HA component of isolates and of prototype strains proved to be identical but differed from that of the Szeged strain. Nevertheless, the HA component of the isolates could be split by mercapto-ethanol to HA1 and HA2 subunits only partially in contrast to the complete split in the case of prototype strains.

LARGE-SCALE ABORTION INDUCED BY INFECTIOUS RHINOTRACHEITIS (IBR/IPV) VIRUS IN A STOCK OF BEEF CATTLE

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In the Hereford stock of 200 animals bought as primiparous heifers by the stock farm Sz., 157 aborted during the 5th to 9th months of pregnancy in the first quarter of 1982. Sixty-six fetuses and three fetal membranes were brought to the institute. In more than half of the fetuses, necrotic, necrotic-inflammatory foci were observed in the liver and spleen, in a few cases even macroscopically. Ocassionally jaundice was also observed. Listeria, streptococcus, staphylococcus and fungi were demonstrated in 1, 1, 1 and 3 of the fetuses, respectively. All examinations were negative for brucella, leptospira and chlamydia. The IBR/IPV virus was found in 8 fetuses. The majority of the animals that had aborted were subjected to serological examinations and proved to be negative for antibodies to brucella, leptospires and chlamydia. Each of the 50 blood samples tested for antibodies to IBR/IPV gave positive results with considerably high titres. Jointly responsible for the large scale abortions were the high susceptibility of the stock, the high risk of infection, as well as the considerable decrease in the natural resistance of the animals.

CORONA AND CORONA-LIKE VIRUSES IN DIARRHOEAL CALVES

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Coronavirus and corona-like virus particles were detected by electron microscopy in 24.7% of the ileal and faecal samples submitted from 761 diarrhoeal calves under one month of age. Based on the morphological criteria of Woode et al. (1978), most of these (in 16% of the calves) were typical bovine Coronavirus particles. Immuno electron microscopic and immunofluorescence studies confirmed the findings of bovine coronaviruses. Most of the "corona-like" viruses morphologically resembled the "syncytia-forming" virus described by Mebus et al. (1978). Sporadically there were also virus particles that appeared morphologically as being Orthomyxovirus, Paramyxovirus virions or virions of the "Breda agent" described by Woode et al. (1982). A clear differentiation of the above particles failed in some cases. Virus particles detected in some other cases had to be classified to be unknown in calves, based on their characteristic morphology (20–28 nm long projection with 5–7 nm diameter balls on the tip). Studies on 18 nondiarrhoeal calves revealed only one calf with corona-like and none with coronavirus.

EXPERIMENTAL INFECTION OF LAYING HENS WITH EDS AVIAN ADENOVIRUS ISOLATED FROM DUCKS

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In spite of the wide occurrence of the haemagglutinating avian adenovirus in duck flocks, no data are available on its pathogenicity in laying hens infected naturally. To determine the pathogenicity of this virus, susceptible hens were infected per os with the KT/80 strain isolated from ducks. Egg production of hens decreased from the initial level of 70% to 35% in the 2nd week postinfection (PI), and the eggs showed characteristic changes. HI titres of the laying hens ranged between 1:16 and 1:512. Eggs laid after infection were collected separately at weekly intervals, and susceptible laying hens were infected with the ovalbumin per os. On the 15th day PI, hens infected with eggs collected on the 7th day PI laid pigmentless and shell-

less eggs or eggs having thin and breakable shells; their production decreased by 50% for a period of 2 weeks, then returned to normal. In the sera of these hens, HI antibodies had appeared by the 14th day PI and their titre reached the peak (1:128) 4 weeks later. Egg production of hens infected with eggs collected later remained undisturbed, and no antibodies appeared in the sera of such hens. This finding confirms our earlier experience that eggs collected from laying hen populations recovered from clinically apparent EDS lay no part in the spread of infection. On the other hand, eggs laid in the initial, acute, stage of the condition may have a role in the transmission of infection.

DETECTION OF ANTIBODIES TO HUMAN HERPES VIRUS AND CYTOMEGALOVIRUS IN A MEDITERRANEAN ISLAND POPULATION (PANAREA, EOLYAN ISLANDS)

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The present study was undertaken to determine the prevalence of CMV and HSV infections in a "semi-closed" population living in Panarea, an island of the Mediterranean sea. The survey was carried out on 130 inhabitans, 66 female and 64 male, living in the island for all the year. Antibodies to CMV and HSV were detected by complement fixation according to Komer's method. The results obtained suggest a different epidemiological situation concerning CMV and HSV compared to world and Italian data; the 29.60% positivity for CMV and the 35.61% positivity for HSV are lower than worldwide serological data. These differences could be explained by the situation of "semi-closed" population in our study; higher rates were found for women than for men. This may be an important aspect in the development of acquiring primary and secondary infections, and the relationship with fetal morbidity. In fact, when considering viral disease in pregnancy, we must remember that the true incidence is probably very much higher than we suppose, yet relatively few deleterious effects have been observed on either mother or fetus.

FOCAL DIFFERENCES IN POSTVACCINATION MEASLES IMMUNITY

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In children vaccinated against measles in the 14th month of life, the seroconversion rate was, on the average, 96.7%, and the geometric mean of the HAI titres was 99 one month after the vaccination. The mean level of HAI antibodies of the same children decreased by 15–49% by the 15th to 23rd months postvaccination (PV). The decrease was lower in localities in which a relatively high measles morbidity was observed in the PV period. In the sera of 25 out of 172 children the HAI titres were two to fourfold higher in the third half year after the vaccination than one month after the vaccination, evidently as a result of the booster effect of the circulating wild virus. In four villages where no cases of measles were registered between 1975 and 1980, the HAI titres were diminishing year by year after the vaccination and the rate of seronegative children was higher. The latter reached even to 50–80% among the children born in 1974. The rate of seronegative children vaccinated at 10–11 months of age, was more than twice higher, the geometric mean of HAI titres half of those observed among the children vaccinated over 11 months of age. In one of the villages a local measles epidemic appeared in the period of this survey; 96% of the seronegative children fell ill with measles. In two villages where measles cases were registered from time to time, the highest rate of seronegative children was about 20%. Examining the HAI antibodies of — at least — 10 children per age group and county focal differences in the rate of seronegative children were not detectable. Vaccine prepared from the Leningrad-16 strain was employed throughout.

MEASLE ANTIBODY STATUS OF HUNGARIAN POPULATION BASED ON SURVEY OF BLOOD SAMPLES COLLECTED IN SEPTEMBER, 1982

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Serum samples from 4626 subjects representing all age groups of population over 6 months of age were tested for measles antibodies by the haemagglutination inhibition technique. Rate of subjects with antibodies (≥ 2) varied between 94 and 100% in age groups above 2 years of age. Antibody status

of children living in areas where measles was epidemic in 1980–1981 did not differ significantly from that observed in other areas of Hungary. Both the geometric mean of antibody titres and the rate of subjects with antibodies proved to be relatively low in the age groups of children who had been vaccinated between 1974 and 1977 when vaccination at 10 months of age was compulsory. Before 1974 the majority and after 1977 all of the children were vaccinated after 13 months of age. Revaccination of the age groups of poor immunity seems inevitable to prevent epidemic episodes in the coming years.

ANTIBODY STATUS OF POPULATION AGAINST POLIOVIRUSES 23 YEARS AFTER INTRODUCTION OF USE OF LIVE VACCINE

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A cross-sectional polio antibody survey has been carried out with 4298 sera collected in September 1982, by the radial plaque neutralization technique. Subjects above 1 year of age proved to be positive against poliovirus type 1 in 94%, against type 2 in 95%, against type 3 in 97% and against all three types in 88%. There were only 4 triple negative subjects found who all belonged to the age group (1–2 years of age) which had not yet received the complete course of vaccination. The results proved comparable to those obtained in a similar survey in 1963. This indicates that systematic vaccination ensured the maintenance of humoral immunity status of the population on a highly satisfactory level.

ANTIBODY STATUS OF FORESTRY WORKERS VACCINATED AGAINST TICK-BORNE ENCEPHALITIS VIRUS

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Serum samples of 356 forestry workers, who had received "FSME–Immun" vaccine, have been tested for antibodies against tick-borne encephalitis virus by both haemagglutination inhibition (HI) and indirect fluorescent antibody (FA) techniques. 222 subjects received a single dose of vaccine one

month before sampling, 97 subjects received two doses about one year before sampling, whereas in 37 cases serum was taken a few years after completion of whole series of vaccination. In the sequence of the groups 59.9, 79.4 and 29.2% of persons proved to be negative by the HI technique (< 10), whereas 42.8, 40.2 and 18.9% by the FA technique (< 5). 23 subjects had a high HI antibody titre (≥ 320) suitable even for preparation of immune gamma globulin against tick-borne encephalitis.

SURVEY OF RETICULOENDOTHELIOSIS VIRUS INFECTION IN A TURKEY FLOCK

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A serological survey was done to demonstrate antibody to reticuloendotheliosis virus (REV) with indirect fluorescent antibody assay (IFA) in a selected turkey flock where tumour losses had occurred and serious egg infertility had been noticed. Virus isolation was attempted in chicken embryo fibroblast culture (CEF) from plasma samples. Serum samples collected from primary breeding turkeys at 1 day of age contained no antibody to REV (O/39), while those collected from 51 to 53 weeks old hens were positive in 15–40%. Sera from flocks with serious egg infertility were negative in one case (0/17), but were positive in two other cases (12/50; 5/10). Sixty-eight per cent of serum samples collected from broiler turkeys in the slaughterhouse contained antibody to REV. According to the IFA and electron microscopic examination, REV was isolated in CEF from 2 of 50 plasma samples collected from a 53 weeks old parent flock suffering from serious egg infertility. This is the first documentation of the presence of REV in Hungary turkey flocks.

PROPAGATION OF HEPATITIS A VIRUS (HAV) IN AGMK AND BGM CELLS

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Primary AGMK cells were infected with HAV (strain H-175). The virus production was demonstrated by measurement of increasing amounts of HAV antigens (s/HAAg) in cultures by radioimmunoassay (RIA), immunofluores-

cence (IF) and/or detection of complete virus by electron microscopy(EM). After second passage of infected cells, HAAg was detected over P/N ratio near 50. Virus harvested from this sample was used to infect tertiary AGMK cells in suspension. Monolayer cultures were developed and maintained for 10 weeks with weekly passages. The highest P/N ration (73) was detected in sample from second-passage cells, while P/N ratios 34–39 were observed up to passage 7, and later the HAAg production decreased to P/N ratio 3 in passage 10. However, still the number of physical particles was 10^6/ml, counted under EM, in this sample. The virus seemed to be strongly cell-associated, it was not released spontaneously into the medium and no cytopathic effect was observed. Using BGM cells for infection the maximum amounts of HAAg (P/N ratio 22) were detected after two weeks of incubation and this value remained relatively stable during a period of one month. In both cell cultures a typical granular cytoplasmic fluorescence was observed almost at the same time with RIA positivity. The potential value of these cell-virus systems for producing diagnostic materials is discussed.

THE TITRATION OF SPECIFIC ANTIBODIES TO MUMPS VIRUS BY MEANS OF THE SINGLE RADIAL HAEMOLYSIS (SRH) TEST

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The SRH test was applied for the demonstration of specific antibodies in the sera of patients with clinical mumps infection and in those of children vaccinated with live mumps virus vaccine. The optimum amount of virus for the sensitization of erythrocytes was found one tenth of that described by other authors. A laboratory reference preparation was prepared from mumps convalescents' sera. The reference preparation was assigned a potency of 100 units per ml, arbitrarily. In each titration session a dose-response line was established which showed the correlation between the different dilutions of the reference preparation and the diameters of the haemolytic zones produced. In this way, the antibody content of the sera to be tested could be expressed in laboratory units. The results of the titration of 403 sera showed that the SRH test was more sensitive for the demonstration of mumps-specific antibodies than the HI test was. No convincing evidence for a correlation between the results of the SRH and the HI test or between those of the SRH and the CF test was found.

COMPARATIVE TESTS FOR DETECTION OF RUBELLA-SPECIFIC IgM ANTIBODIES

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530 sera taken from pregnant women either with symptoms suggestive of rubella or with a most recent history of exposure to rubella infection were tested for specific IgM antibodies, using two different techniques as follows. (a) Separation of IgM fractions of sera by ion-exchange chromatography and testing the fractions for rubella haemagglutination inhibiting (HI) antibodies; (b) investigation of sera with haemadsorption immunosorbent technique (HIT) as described by van der Veen. Identical results were obtained by both techniques in 91% of sera. The HIT proved suitable to detect even incipient IgM antibody responses in a phase when no antibodies or very low titres coult be revealed by the HI technique. Persistence of specific IgM antibodies could be demonstrated for a longer period by the HIT than by the other method used. Thus the HIT seems especially suited for laboratory diagnosis in cases when serum samples are available only from a very early or from an extremely late stage of infection.

DIAGNOSTIC ANTIBODY TESTS FOR DETECTION OF HERPES SIMPLEX VIRUS INFECTIONS OF THE CNS: CRITERIA AND EFFICIENCY

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Based on complex laboratory examination of 18 proven herpes encephalitis cases the authors summarize their experiences especially with regard to criteria and efficiency of antibody tests carried out with FA and ELISA techniques for a relatively early aetiological diagnosis. It is concluded that 80% efficiency can be achieved if serum and CSF pairs taken at an interval of 3–4 days at the early stage of illness are examined for the presence of specific immunoglobulin classes. Demonstration of specific IgM antibodies, of significant increase in IgG antibody titre, and of a ratio ≤ 20 for serum to CSF antibody content may be the proofs for the aetiological relationship. The FA technique may be used generally but the ELISA technique is more sensitive for detection of IgM antibodies in the CSF.

DETECTION OF ANTIBODIES TO AUJESZKY'S DISEASE VIRUS BY ELISA

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Sera collected from pigs of different age groups and immunological status were examined for antibodies to Aujeszky's disease virus by the VN test and by indirect ELISA. Aujeszky's disease virus propagated in cell culture purified by ultracentrifugation in sucrose gradient and exposed to detergent was used as antigen, peroxidase-labelled anti-swine IgG as conjugate, and 1,2-phenylenediamine (OPD) as substrate. Evaluation was performed in a Titertek multichannel photometer. The so-called base level was determined on the basis of the obtained extinction values, and the 800 blood samples examined were evaluated as compared to the base level. Sera positive in the VN test were always positive also in ELISA, while the proportion of ELISA-positives among sera derived from VN-negative herds was about 10%. From titration of positive sera and evaluation of a vaccination trial it has been concluded that ELISA detects antibodies earlier and in higher serum dilutions than the VN test.

APPLICATION OF ELISA FOR THE DETECTION OF ANTIBODIES TO INFECTIOUS BRONCHITIS VIRUS

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Indirect ELISA was used for the detection of antibodies to infectious bronchitis virus in chicken. As antigen, a Beaudette-serotype virus propagated in embryonated chicken eggs and purified by ultracentrifugation was used. The positive reference serum was collected from birds vaccinated with H-120 and H-52 virus strains and subsequently inoculated with a purified virus emulsified in incomplete Freund's adjuvant, while the negative reference serum was obtained from SPF chicken of different age. The conjugate was the horseradish peroxidase-labelled IgG fraction of an anti-chicken IgGMA rabbit serum prepared according to the periodate method of Wilson and Nakane (1978), as modified by Boorsma and Streefkerk (1979). Having determined the optimum concentrations of reagents by chess-board titrations, factors influencing the specificity of the method were studied, i.e. the effect of differ-

ent sensitizing media and additives given to the conjugate- and serum-diluent (NaCl, bovine albumin, polyethylene glycol, normal rabbit serum). The specificity of the assay was verified by the inhibition test. To normalize data obtained in test performed at different times, the result of the test is expressed in antibody units/ml serum, on the basis of a positive reference serum of known antibody content. The method has been found suitable for demonstrating the quantitative changes of antiviral antibodies produced during the course of infection.

DETECTION OF ANTIBODIES TO RS VIRUS BY VIRUS-NEUTRALIZATION TEST IN FLK CELL LINE

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The diagnosis of RS (respiratory syncytial) virus infection by direct virus isolation is successful only in a minor part of the cases. Antibody titres of paired sera might have diagnostic value, however, practically a simple and reliable serological test applicable in routine diagnostic work has not been available. The complement fixation (CF) test detects only 20% of the low-titre antibodies, while the virus-neutralization (VN) test performed in the commonly-used cell cultures (such as primary or secondary calf kidney or testicle) cannot be evaluated because of the poor growth of the RS virus. The VN test was carried out according to the method of Haralambiev in FLK cell line permanently infected by bovine leucosis virus. In this cell line the cytopathic effect of RS virus appears within a few days. In cell cultures without covering layer and containing only liquid culture medium, plaques grow up to 1 mm in diameter within 4 to 5 days. Fifty per cent reduction in the number of plaques indicates presence of antibodies. The test can be done in Petri-dishes, test tubes or Takátsy's Microtitrator trays.

PRODUCTION OF BOVINE LEUKAEMIA VIRUS ANTIGEN IN LARGE QUANTITIES IN HUNGARY AND ITS COMPARISON WITH THE PITMAN–MOORE ANTIGEN

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A method for large-scale production of bovine leukaemia virus (BLV) antigen was developed for an extensive survey of BLV infection in Hungary. The antigen, consisting primarily of glycoprotein, was produced by ammonium sulphate precipitation from the supernatant of roller cultures of a permanent fetal lamb kidney cell line infected with BLV. The concentration of the antigen and of the control sera (−, +, ++, +++) of the Hungarian immunodiffusion kit was adjusted to the Pitman–Moore reagents representing the international standard. Three thousand serum samples were simultaneously examined with both antigens. The results agreed in 99%; the infection of cows was detected earlier by the Hungarian antigen in 0.9%, while by Pitman–Moore antigen in 0.1% of the cases. The first Hungarian eradication experiment conducted in an industrial-scale cattle farm, again by using the Pitman–Moore antigen collaterally with our antigen, is close to its successful end.

ENZYMATIC METHODS FOR DETECTION OF MORPHOLOGICAL ALTERATIONS PROVOKED BY VIRUS INFECTION IN EGGS

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On the effect of infectious laryngotracheitis virus (ILT) inoculated onto the chorioallantoic membrane (CAM), the activity of cytoplasmic located enzymes (LDH, MDH, GOT) augments significantly, compared to non inoculated eggs. The increased enzyme activity is due to the decay of CAM cells owing to the cytopathogenic activity of the virus. The augmentation of enzyme activity was significant, even for the 24th h following the infection of the eggs, as compared to the controls. With the progress of the time of incubation, the activity of the enzymes under test showed a sheer ascending trend. The extent of virus dilution can also be followed sensitively and it can be evaluated through determination of enzyme activity measured in the allantoic fluid.

The method is suitable to the determination of the degree of virus attenuation, as well as to the verification of the safety of inactivated viruses. It can be used for the study of every virus cytopathogenic for CAM cells.

CANCER OF UROGENITAL TRACT: OCCURRENCE OF HCMV DNA AND ITS HINDIII E FRAGMENT IN PROSTATIC CARCINOMA

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The occurrence of the entire human cytomegalovirus (HCMV) DNA and of its transforming fragment (HindIII E) was investigated in DNA samples of prostatic cancers. HCMV–DNA homologous sequences were present in 7% of normal prostate (NP), 22% of prostate hypertrophy (BHP) and 41% of adenocarcinoma of prostate (ACP). No positive in situ hybridization for HCMV–RNA was observed in section of NP tissue. At the time the transcription of HCMV–DNA was observed in 33% of BHP and 45% of ACP. The results obtained using anticomplement immunofluorescence was in close correlation with in situ hybridization results and imply some degree of HCMV association with prostatic abnormality. The results obtained using the transforming fragment (HindIII E) of HCMV–DNA in DNA–DNA reassociation kinetics was detailed.

STUDIES ON IMMUNOLOGICAL MARKERS OF SIMIAN C-TYPE VIRUSES AND HUMAN RETROVIRUS HTLV IN ADULTS AND CHILDREN WITH LYMPHOID LEUKAEMIAS AND LYMPHOMA

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Peripheral leukocytes and/or lympn node cells and blood plasmas from patients with lymphoid malignancies were investigated for immunological markers of baboon endogenous virus (BaEV), gibbon age leukaemia virus (GaLV) and human T-cell leukaemia/lymphoma virus (HTLV). For detection

of antigens and antibodies immunofluorescence, and competitive RIA or radioimmunoprecipitation were used. Antigen related to the p30 core protein of BaEV was detected in B-, O- and T-cell forms of malignancies. Antigen related to the GaLV p30 was found mainly in B- and O-cell forms, while antigen related to the p24 protein of HTLV was detected only in a few T-cell malignancies. Distribution of antibodies reactive with envelope antigens of BaEV and GaLV and of those reacting with the p24 antigen of HTLV proved to be similar. The presence of oncovirus-specific antigens and antibodies was connected with pregression of lymphoid malignancies.

STUDIES ON CYTOTOXIC EFFECT OF LYMPHOCYTES AND ANTIBODIES REACTIVE WITH ENVELOPE ANTIGENS OF ONCOVIRUSES IN DIFFERENT STAGES OF CHRONIC GRANULOCYTIC LEUKAEMIA (CGL) AND ACUTE MYELOID LEUKAEMIA (AML)

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In earlier studies lymphocytes and antibodies cytotoxic against autologous tumour cells were detected frequently in the quiescent phase of CGL, but very rarely in the blastosis of AML. Starting from these results we have compared the cytotoxic activity of lymphocyte and plasma samples from the quiescent and blastoid phase of CGL, and from the blastosis and remission of AML, respectively. Target cells were collected also from the different stages of CGL and AML. ^{51}Cr release tests showed that the lymphocytes and plasma samples from blastoid crisis of CGL had no cytotoxic activity for autologous blast cells. On the contrary, cryopreserved lymphocytes and plasmas collected from the quiescent phase of CGL proved to be cytotoxic for the autologous blast cells and their effect could be significantly blocked by oncoviral envelope antigens. A similar relationship was found between the blastosis and remission stage of AML. In the majority of cases the native and carbohydrate-free gp70 and p15 antigens of gibbon ape leukaemia virus blocked both cellular and humoral cytotoxicity. However, out of the antigens of baboon endogenous virus, only the native gp70 showed marked blocking effect.

RESTRICTION ENZYME ANALYSIS OF INTEGRATED ECOTROPIC MURINE LEUKAEMIA VIRUSES IN NORMAL AND TUMOROUS THYMUSES

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Tumours induced by chemicals develop through multiple stages where intrinsic factors may play a definite role. Endogenous murine leukaemia proviruses represent integrated parts of the mouse genome: their contribution to tumorigenesis is, however, not resolved. Earlier, we found that endogenous virus expression was independent of chemically-induced lymphomagenesis in different mouse strains. In order to follow possible changes of the integrated ecotropic proviruses, thymuses from normal and tumorous mice of different strains were analysed by Southern-blot hybridization technique, using on ecotropic-specific probe. The restriction enzyme pattern of the integrated proviruses in the normal thymuses, MNU-induced lymphomas and spontaneous tumours of four mouse strains (AKR, BDF_1, Swiss, CFLP) did not reveal any change which could be attributed to specific carcinogen action. These results present further evidence that MNU-induced lymphoma development in mice is independent of endogenous murine leukaemia virus expression.

INAPPARENT ACTIVATION OF LATENT HSV IN RABBITS

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A long-term follow-up study in 70 rabbits (inoculated into the right scarified cornea with the Kupka strain) showed establishment of latency in 86% of homolateral and 29% of contralateral Fasserian ganglia. The fragment of the right cornea yielded virus in 4.4% of animals and the right nerve root entrance area in 6%. Short episodes of virus shedding were found in 22 out of 33 rabbits by continuous swabbing of both eyes for 9 months at 3–7 day intervals (55 samples were positive out of total 4108 ones, i.e. 1.3%). Artificial stimulations were made by mechanical injury of the cornea and by chemical provocation (adrenaline, xylol keratitis) in the presence or absence of immunosuppression (CPA in the dose of 200 mg/kg body mass) in altogether 33 animals. Virus shedding from right cornea was not significantly enhanced.

However, corneal fragments yielded virus in 37% of rabbits. The recovery rate from the contralateral ganglion explants was also enhanced (63%). As evidenced by the occurrence of single neurons containing HSV antigens in the ganglia examined by serial sectioning at the time of their removal, virus activation was accompanied by inapparent transport of minute amounts of HSV to the cornea and to the brain stem. The presented results show that inapparent "round trip" of HSV need not be accompanied by any virus shedding. In addition, infection of previously unaffected contralateral ganglia may occur as well.

EFFECT OF PROGESTERONE ACTH AND PREDNISOLONE FOR THE REACTIVATION OF AUJESZKY'S DISEASE VIRUS (ADV) IN AN INFECTED FARM

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Twelve breeding gilts nine months old were chosen on the basis of VN titre in an ADV infected farm. Four groups, 3 animals in each, were formed, Group 1 and group 2 were ovariectomized two weeks before the treatment. Group 1 was treated with 400 mg prednisolone/animal/day for five days; group 2 was treated with progesterone 50 mg/animal/day for two weeks. The animals in group 3 and group 4 were not ovariectomized. Group 3 was treated with ACTH 200 IU/animal/day for five days, group 4 with prednisolone 400 mg/animal/day for five days. Nasal, pharyngeal and vaginal swab samples were collected each day for 15 days after the treatment. The swab samples were examined for ADV by inoculation of lamb kidney cell cultures. The gilts remained clinically normal and feverless throughout the experiment and no virus could be isolated.

REACTIVATION OF LATENT INFECTION OF MUTANT ADENOVIRUSES IN TISSUE CULTURE BY ENDOTOXIN

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A wild strain of human adenovirus type 5 and its temperature-sensitive (ts) 18 and 19 mutants were used to establish latent infection in HEp-2 cell cultures. For reactivation purified and radiodetoxified endotoxins obtained

from *Escherichia coli* O89 were applied in different times during latency at permissive and nonpermissive temperatures. Both wild strains and mutants could be reactivated at the permissive temperature, but only the wild strain at the nonpermissive temperature. Reactivation developed fast and on a great scale. The process is independent of the original virus concentration and of the moment of endotoxin application during the latent period, but depends on the endotoxin concentration. Possible mechanism is the clearing effect of inhibiting proteins of certain lysosomal enzymes released after damage of membranes due to endotoxin effect. These alterations in the cell are not able to substitute or complete mutant viral polypeptides. Furthermore, results suggest that lack of assembly of virus polypeptides is not the reason for latent state of adenoviruses.

LOCALIZATION OF HUMAN ADENOVIRUSES IN THE MALE GENITALS OF MICE

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According to observations of human specimen adenoviruses may be localized in male genitals. In order to investigate this phenomenon we infected CFLP mice with adenovirus types 5 and 12. Virus antigens could be detected by the immunfluorescent method from the cells of the testicles in 24% of the infected mice 1–15 days after infection, in most of the cases after 3 days. The presence of the virus antigens in the supernatant of the cell homogenates was confirmed by complement fixation test. Adenoviruses were reisolated in HEp-2 cell cultures also in infective form from the genitals of a few mice. There were neither infective viruses nor virus antigens found in uninfected mice used as controls.

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Immunology

LYMPHOCYTE SUBPOPULATIONS IN THE COLOSTRUM AND MILK OF CATTLE

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Number of lymphoid cells and within it the proportion of macrophage and lymphocyte subpopulations were examined in the colostrum and milk samples of 20 Holstein Friezien cattle using immunofluorescence, rosette cell formation and lymphocyte stimulation tests. Colostrum and milk samples were taken on the first, second, third and fifth day after parturition and on the eighth weeks of the lactation period, respectively. In the first day colostrum the number of lymphoid cells was 10^6 cells/ml in average, which decreased to 10^5 cells/ml the fifth day, and to 1–2000 cells/ml by the eighth week of the lactation. The great majority of lymphocytes T-cells represented about 40–50% and B-cells were present in about 20–30%. Among B-cells the IgA positive cells occurred in the greatest number. The lymphocytes of the colostrum like those of the blood could be transformed by mitogens, but they did not react to antigens with which the lymphocytes from the blood could be stimulated.

EFFECTS OF ADENOVIRUS ON THE HUMORAL IMMUNE RESPONSE OF MICE

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The infection of mice with human adenovirus type 6 (Ad6) was shown to elicit an immunosuppressive effect. Mice inoculated with Ad6 displayed a decreased humoral immune response to sheep red blood cells (SRBC). The suppressive effect was dependent on the sites of inoculation of the virus and SRBC. Inoculation of the virus and SRBC by the same route, i.e. intraperitoneally, resulted in a significant decrease of the humoral immune response. On the other hand, following administration of the virus and SRBC at different sites, no inhibitory effect of the virus could be detected. Some cellular requirements for the immunosuppression caused by Ad6 were examined. Pretreatment of mice with silica to depress the macrophage function prevented the immunosuppression. These results indicate that peritoneal macrophages play an important role in the immunosuppression observed.

EFFECT OF SUPERNATANT OF LYMPHOCYTES INFECTED WITH VIRUSES ON THE FUNCTION OF PERIPHERAL BLOOD LEUKOCYTES IN VITRO

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Up to the present neither the pathomechanism of the frequent secondary bacterial complications consequent upon viral infections nor their immunosuppressive effect shown also in vitro have been made clear enough yet. To investigate these phenomena, lymphocyte cultures obtained from peripheral blood were infected by several types of human adenoviruses and herpes simplex virus type 1. After a 7-day incubation period the supernatants were added to mononuclear and polymorphonuclear leukocytes obtained from blood. The supernatants of infected lymphocytes reduced the phagocytic activity on staphylococci of both types of leukocytes by 20–60% in comparison with different controls. This effect is of the same degree if adenovirus types are compared to each other, but herpesvirus had more intensive weakening. The damaging effect of supernatants was dose-dependent, and most of it was lost after longer storage. The described inhibitory effects may be related to lymphokines or some similar materials which could be produced by infected lymphocytes and by some other cells as further tests revealed.

EFFECT OF HUMAN ADENOVIRUS INFECTION ON THE LYMPHOID ORGANS OF MICE

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After having infected CFLP mice with adenovirus type 12, there were no clinical symptoms during the 15 days of observation. The virus infection reduced the number of the circulating lymphocytes already in 4 hours. The dicrease was most intensive after 24 h, then a hypercompensation followed on the 3–7 days, and after this the number of the lymphocytes returned slowly to the normal level. The weight of the thymus decreased depending on the dosage on 3rd or 4th day after infection, then approached the normal level. The weight of the spleen begin to increase after the virus infection and the maximum weight was reached on the 3rd and 4th days. It may be concluded that a moderate and temporary immunosuppression was caused by adenovirus type 12 in the lymphoid organs of mice.

HERPES SIMPLEX VIRUS INTERACTION WITH HUMAN MONOCYTES AND MACROPHAGES

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It is accepted that cells belonging to reticuloendothelial system are of primary importance for naturally occurring resistance to herpes simplex virus (HSV) infections. In previous experiments we studied the role played by rat peritoneal macrophages on HSV^{-1} infection showing the effect of immunocompromission on the "in vitro" intrinsic antiviral activity. The present study elucidates some early interactions between HSV-1 and human monocytes and macrophages, as well as the intrinsic antiviral activity using standardized models for culturing monocytes from human peripheral blood and by evaluating the "in vitro" maturation to macrophages. On both cell types we evaluated the attachment of HSV-1 by studying the kinetic of virion adsorption assaying residual (non absorbed) virus infectivity at 15, 30 and 120 min post infection. At the same time we studied the intracellular behaviour by indirect immuno fluorescence and the evaluation of single cicle growth curves on both HSV-1 infected cells. The residual infectivity of HSV-1 was higher in monocytes than in macrophages; HSV specific antigens were not observed in monocytes while viral antigens were detected in the cytoplasm and at the nuclear membrane of infected macrophages; a marked intrinsic antiviral activity of macrophages was demonstrated. These results suggest marked differences between monocytes and macrophages by HSV-1 might reflect a modification in their metabolic or differentation state.

INTESTINAL WALL ALTERATIONS OBSERVED IN CONVENTIONAL AND GERMFREE MICE TREATED WITH DETOXIFIED ENDOTOXIN AND LYMPHOTROPIC CYTOSTATIC AGENT

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Our previous experiments showed a higher resistance of germfree (Gf) mice to a lymphotropic cytostatic agent, dianhydrodulcitol (DAD) as compared with that of conventional (Cv) mice. On the basis of histological changes in the

intestinal wall it has been assumed that the cause of higher resistance is the lack of the damaging effect of endotoxin from normal intestinal flora. Therefore we have compared the effect of the endotoxin preparate on the changes of intestinal walls of Cv and Gf mice treated with DAD. Radiodetoxified endotoxin (rdLPS) was applied the day before DAD treatment. It had only a slight modifying effect on intestinal wall alterations caused by DAD, while in germfree mice alterations were decidedly aggravated by rdLPS. On the basis of these results we suppose that the different sensitivity of Cv and Gf mice to DAD is in connection with the presence or absence of normal intestinal flora.

DEMONSTRATION OF AN AUTOANTIBODY BINDING TO HUMAN MEMBRANE ATTACK COMPLEX

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Membrane attack complex (MAC) assembled from C5b through C9 complement components causes complement damage. Using immunohistological methods, in 1980 we demonstrated the presence of MAC in tissue specimens obtained by biopsy or at autopsy from patients with chronic glomerulonephritis and multiple sclerosis. On the basis of neoantigenic nature, long persistence and membrane integrated protease-resistance characteristics of MAC we postulated the development of an anti-MAC antibody response in these patients. Using indirect cyto-immunofluorescent and direct modified immunoelectrophoretic methods we have found antibodies binding to MAC in fresh sera of 6 out of 7 patients with multiple sclerosis. Using modified 3-phase immunoelectrophoresis and electrosyneresis and antibody binding to MAC has been found in sera of patients with chronic glomerulonephritis (8/8), meningitis (1/1) and normal human sera (10 persons), too. This antibody gives precipitations near to the cathode in modified 3-phase immunoelectrophoresis. This is the first report on the existence of an autoantibody binding to human membrane attack complex.

INVESTIGATION OF THE IMMUNOPATHOLOGIC ROLE OF AUTOANTIBODY BINDING TO HUMAN MEMBRANE ATTACK COMPLEX

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On the basis of the presence and characteristics of membrane attack complex (MAC) in chronic pathologic processes, the existence of an autoantibody to MAC can be expected and demonstrated by indirect immunocytologic and 2 direct immunochemical methods. Results of double-blind investigations indicated that the benignity of multiple sclerosis may be associated with high autoantibody titre to MAC. This hypothesis was modelled by examining the recovery of *Neisseria meningitidis* (strain HNCMB 122001) after treatment with fresh normal human sera. Unlike sera inactivated by heat (56 °C, 30 min), the fresh sera impair meningococci ($1–10 \times 10^6$ ml) without sensitization by specific anti-bacterial serum at 37 °C (30–60 min). The impairing effects of MAC formed on meningococci could be neutralized neither by antihuman-MAC rabbit immune sera nor by antirabbit-IgG.

PURIFICATION OF CHICKEN IgM IMMUNOGLOBULIN AND PREPARATION OF CLASS-SPECIFIC IMMUNE SERUM

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Chicken immunoglobulins are not easily purified owing to their overlapping molecular mass and electric charges. Hence class-specific anti-chicken immunoglobulin reagents are difficult to prepare and to characterize. Substantial quantities of purified IgM immunoglobulin can only be prepared by the combination of complex fractionation techniques. Serum globulin fractions salted out between 9–14% Na_2SO_4 concentrations were repeatedly fractionated by Sepharose 6B column chromatography, and the IgM-rich material eluted between 0.10–0.26 Kav values was further purified by DEAE-Sephacel ion-exchange column chromatography using a linear gradient from 0.06 to 0.4 M NaCl in 0.05 M Tris–HCl, pH 7.5 buffer for elution of the bound proteins. Distribution of proteins in the effluent fractions was detected by fused-rocket immunoelectrophoresis against a polyvalent precipitating rabbit immune

serum to chicken serum proteins, whereas the purity of the IgM preparations was checked by two-dimensional immunoelectrophoresis and by immunization of rabbits. Various IgM preparations obtained with the combinations of the above methods were found to be always contaminated with polymer IgA and aggregated IgG immunoglobulins and also with other serum globulin components, though in varying degree. Immunoaffinity chromatography on anti-μ heavy chain rabbit antibodies covalently coupled to CNBr-activated Sepharose 4B allowed to obtain polyclonal IgM through a one-step purification procedure. The IgM preparations obtained by this procedure fulfilled stringent criteria for protein purity.

APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DEMONSTRATION OF ANTIBODIES TO *PSEUDOMONAS AERUGINOSA*

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Using acetone-inactivated dried bacteria from Fisher's 7 *Pseudomonas aeruginosa* immunotype strains, we prepared several polyvalent bacterial antigens: (1) Boivin-type O-antigen (lipopolysaccharide, LPS); (2) whole-cell antigen containing all Fisher's immunotypes; (3) cell-free (distilled water-extracted) bacterial antigen. Rabbits were immunized according to various immunization schedules with whole-cell and cell-free antigens. Immune sera to *P. aeruginosa* were tested by passive haemagglutination (PHA). From the sera immunoglobulin fractions were also prepared. The results suggest that PHA is not sensitive enough to demonstrate differences in the titres of *P. aeruginosa* immune sera. According to the investigations the O-antigen and especially the acetone-inactivated whole-cell antigen (AWA) are suitable as antigens for the micro-ELISA test. The binding of these antigens to Hungarian polystyrol microtiter plates was successfully performed. Applying AWA in micro-ELISA test, there were significant differences between the titres of 5 different rabbit immune sera. Experiments were carried out for the determination of the quantitative IgG level of the sera, too.

THE EFFECT OF COMBINED TREATMENT WITH *HAEMOPHILUS INFLUENZAE* ENDOTOXIN PREPARATIONS IN MICE

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Detoxified endotoxin was prepared from *Haemophilus influenzae* endotoxin by ionizing radiation in order to study its protective effect against dyspnoeal attacks after endotoxin inhalation in mice. The detoxified endotoxin administered intravenously increased the aspecific immunoresponsiveness of the mice but could only partly prevent dyspnoeal attacks. The histological processing of the lungs also showed mild interstitial pneumonia. The most expressed protection was achieved by the intraperitoneal administration of 50 μg detoxified endotoxin after a 48 h incubation period. Only systemic administration was found capable of increasing aspecific resistance since the direct alterations in the lungs and the histological changes after the inhalation of the detoxified endotoxin has shown that the irradiation could not significantly change the direct effect of the endotoxin on the alveolar epithelium and the macrophages.

EFFECT OF LEVAMISOLE ON THE IMMUNE-RESPONSE OF LAMBS

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Levamisole is a widely used anthelmintic, but in addition this effect it also has an influence on the function of the immune-system. The effect of levamisole on the humoral and cellular immune-response of weaned lambs was examined. The animals were treated with the antigen (PI-3), levamisole (L) and/or exposed to heat stress in the following combinations: group 1 = PI-3; group 2 = PI-3 + L; group 3 = PI-3 + H; group 4 = PI-3 + L + H. Results for humoral immune-response: the highest antibody titres were recorded in the lambs treated with levamisole and exposed to heat stress; the antibody titre of the other three groups against PI-3 virus did not differ significantly. Cellular immune-response: the most expressed result was encountered in the test for blast-transformation to non-specific mitogens. These values

are significantly higher in the levamisole treated animals. There is not significant difference in the results of the lymphocyte stimulation tests, but the ratio of the immune rosette forming cells was higher in the levamisole treated groups.

ADJUVANTING EFFECT OF LIPOSOME-COMBINED IMMUNOMODULANTS

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The immunomodulant (immuno-adjuvant) activity of liposomes prepared in different manner was studied. As test antigen tetanus toxoid was applied. The changes in the antigenicity caused by the administration of immunomodulants were determined in mouse and guinea-pig immunization assays. Besides the liposomes supplementary adjuvants were applied. One year ago, we already reported on the immunomodulant activity of lipid A incorporated into liposomes. In the present studies liposome incorporated *Bordetella pertussis* and *Bordetella bronchiseptica* vaccines had an additional (supplementary) adjuvanting effect. Liposomes alone increased the antigenicity of the test-antigen 4 to 20 fold while the bacterial vaccine-liposome combination 30 to 300 times. The increase in the antigenicity exhibited dose-dependence. The adjuvanting effect of liposomes can be amplified by bacterins.

THE HOST RESISTANCE INCREASING ACTIVITY OF POLYSACCHARIDES PREPARED FROM *LENTINUS EDODES* and *LENTINUS CYATHIFORMIS*

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Raw polysaccharide (PS) preparations, extracted from 3 *Lentinus edodes* and 1 *Lentinus cyathiformis* strains were investigated in vitro. *L. edodes* strains were obtained from Vietnam, Korea and Japan, *L. cyathiformis* was harvested

in Hungary. The mycelia were cultured on the surface of biotin and malt extract agar medium. The raw PS preparations were applied as immunomodulators of the host resistance in mice infested with Ehrlich-ascites tumour and S-180 mouse fibrosarcoma. (i) It was unequivocally demonstrated that *L. cyathiformis* PS increased the host resistance in tumourous mice. (ii) The host resistance increasing activity of *L. cyathiformis* PS preparations seemed to be of higher degree than the PS made from *L. edodes*. This preliminary and tentative statement, however, needs further verification.

ROLE OF THE QUALITATIVE COMPONENTS OF MINERAL OILS IN THE ADJUVANCY

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The quality of oil plays an essential role in the development and stability of the emulsion and in its adjuvant effect. The hydrocarbon composition of Bayol F., Marcol 52 used generally in vaccine production and of certain white oils of pharmaceutic-cosmetic and technical grade were studied. Correlation has been established between the normal alcane content of oil and the adjuvanting effect exerted on several viruses (EDS, NDV, Aujeszky's, Gumboro). It seems that the presence of n-alcanes containing 12 to 20 carbon atoms is required, since the adjuvancy of oils containing the critical hydrocarbons in greater extent is higher than that of those containing n-alcanes of longer carbon chains or of those containing mainly i-alcanes.

POTENCY TESTING OF *BORDETELLA BRONCHISEPTICA* CONTAINING VACCINES IN A MOUSE MODEL SYSTEM

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As several bacterial components (capsular antigen, exotoxins, and a less identified cell wall antigen) are involved in protection against *Bordetella bronchiseptica* and these components vary in individual strains, in our view no vaccine of wide-ranging activity can be prepared from a single strain. Since at

present all commercially available *B. bronchiseptica* vaccines contain a single strain, we elaborated the following mouse model system for reliable potency testing. Tenfold serial dilutions of the vaccine are administered with an interval of 2 weeks to mouse groups of appropriate size. Ten days later 3 subgroups are formed and the animals are challenged with 10^9 organisms of 3 *B. bronchiseptica* test strains we elaborated the following mouse model system for reliable potency testing. Tenfold serial dilutions of the vaccine are administered with an interval of 2 weeks to mouse groups of appropriate size. Ten days later 3 subgroups are formed and the animals are challenged with 10^9 organisms of 3 *B. bronchiseptica* test strains, respectively, into the tail vein. The test strains are equally highly virulent, but are dissimilar in all other biological proterties. Deaths are recorded for 7 days after challenge and on the 7th day the survivors are examined for spleen weight related to 10 g body weight. The vaccine is regarded as highly active if the mice vaccinated with high dilutions survive the challenge, and as wide-range if there is little difference, if any, between the results of challenge with the 3 test strains.

EXPERIENCES OF VACCINATIONS AGAINST *HAEMOPHILUS PLEUROPNEUMONIAE*

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On the basis of our earlier results, a bivalent vaccine of *Haemophilus pleuropneumoniae* serotype 1 and 2 was produced and applied regularly on two swine farms from March 1982. (i) On one farm a total of 10 464 pigs were vaccinated at age of 50 and 70 days. Simultaneously 10 000 unvaccinated pigs were left for control. Neither illness nor death due to these microorganism occurred in vaccinated herds, whereas at the same time, 1540 and 2890 control pigs were treated for *H. pleuropneumoniae* infections on the batteries and in fattening groups, respectively, and 41 pigs died of such disease. Economic parameters also proved to be favourable for vaccinated pigs. (ii) On the other farm 11 592 pigs were vaccinated at 90 and 110th days of age and 3180 unvaccinated animals were left as controls. A loss of 0.32% of vaccinated pigs and that of 1.32% od unvaccinated controls were registered, meanwhile treatment rates stood at 9.24% and 27.92%, respectively. In May 1983 vaccinations were not carried out for technical reasons and 52 of 1200 swine died as a result of *H. pleuropneumoniae* infection over a 9-day period in spite of treatments.

MATHEMATICAL FORMULATION OF IMMUNOLOGICAL EVENTS CONNECTED WITH THE DEVELOPMENT OF THE PRIMARY ANTITOXIC IMMUNITY UNDER THE INFLUENCE OF IMMUNOMODULATION

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Killed whole-cell *Brucella abortus* and *Brucella suis* suspensions were applied as adjuvants in active mouse protection tests with tetanus and *Clostridium perfringens*-D-(epsilon) toxoids. (i) The *Brucella* suspensions exhibited high degree of adjuvancy in simultaneous application with the two toxoids. The increase in antigenicity ranged between 105–484%. (ii) To obtain more information about the characteristics of adjuvancy, the numerical data yielded by a polynomial equation were processed with an analysis of variance. (iii) The results unanimously showed the identity of results even in repeated tests. Concerning the correlations in the comparative assays, the value of the total-determination-coefficient ($R^2 = 0.91$ at p% 1.0) and the one of the determinant coefficient of linearity ($r^2_{lin} = 0.83$ at p% 1.0) unanimously show a strict parallel run of the two curves determined by the function of immunomodulant-induced alterations in immunogenecities of the two toxoids.

IMMUNOMODULATION IN IMMUNIZATION PRACTICE IN ELDERLY: EFFECT OF THE INCREASED ANTIGEN DOSE

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To avoid the unnecessary, mainly LPS-type immunomodulatns' introduction as amplifying agent of the hypofunctioning immune apparatus, the immuno-amplifying property of the antigen dose-increase was investigated with immunization of elderly people. (i) The dose-dependent increase in individual antitoxin titres showed a cause-effect correlation complying with our earlier findings concerning the determinative effect of age-dependent immune potential. The aged vaccinees, in the sequence of increasing antigen doses, exhibited increasing antitoxin production. The average of the antitoxin titres was always higher than the average titres after the usual immunization with conventional/routine vaccine. (ii) The proportionality of individuals with low

degree of immunity or of ones without any measurable antitoxin titres, significantly decreased in correlation with the deliberate (and well-defined) increase of the antigen dose applied. (iii) Generally speaking, we developed an adsorbed tetanus toxoid with the administration of which in aged people one can increase the probability of the safe protection, applying two-shot immunization method. (iv) With increasing the dose of the antigen, the ballast antigens' amount will be increased as well. This fact can be the limiting factor of free administration of this type of immunization.

POTENCY TESTING OF *CLOSTRIDIUM PERFRINGENS* C TOXOID BY ACTIVE MOUSE IMMUNIZATION – TOXIN-CHALLENGE TEST

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Adapting the method already established for the potency testing of tetanus toxoid by active mouse immunization–toxin-challenge test the following results were achieved with *Clostridium perfringens* C toxoid. (i) The optimum interval between immunization and toxin challenge was 3–5 weeks. (ii) Repeated tests performed with more than 6 months interval have shown the reproducibility of this standardization procedure. (iii) The optimum toxin challenge dose is not more than 10 DLM intravenously. (iv) Comparing the results of the mouse protection tests with the ones obtained in rabbit immunizations, the polynomial equation analysis showed a well defined parallelism between the results, and the subsequent analysis of variance exhibited a total determination coefficient of $R^2 = 0.87$ at p% 1.0 and a determinant coefficient of linearity of $r^2_{lin} = 0.79$ at p% 1.0. Considering the above results, the developed method may replace or substitute the officially prescribed method (rabbit immunization).

ESCHERICHIA COLI LPS EXHIBITS ANTIDIPSOGENIC EFFECT IN THE RAT

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Endogenous opiate systems have been reported to be involved in the pathophysiology of endotoxin shock as well as in the gulation of thirst. Drinking (measured as ml of tap water drunk/100 g of body weight) was induced in

adult male Sprague Dawley rats by: (a) 48 h water deprivation; (b) intravenous injection of either hypertonic NaCl solution (2.9%) or carbachol (250 μl); and (c) subcutaneous injection of isoprenaline (33.3 μg/kg). *Escherichia coli* LPS (640 μg/kg intravenously) showed powerful inhibitory effect on thirst stimulated by water deprivation (2.26±0.42 ml vs 5.83±0.51 ml in saline treated animals), isoprenaline injection (0.26±0.09 ml vs 0.63±0.15 ml in controls), NaCl hypertonic (0.45±0.06 ml vs 1.18±0.02 ml in controls) and carbachol injection (1.33±0.25 ml vs 2.98±0.24 ml in controls). The antidipsogenic effect was dose dependent LPS 320–640–1280 μg/kg intravenously and long-lasting (over 24 h after 1280 μg/kg) and result to be neither a mere consequence of behavioural alterations nor due to endotoxin peripheral vasodilatating properties. Naloxone (up to 240 μg/kg subcutaneously) did not influence endotoxin inhibition of water intake, while antidipsogenic effect of morphine was strongly reverted. On the contrary, aspirin pretreated animals were less sensitive to endotoxin effects on drinking behaviour. Data allow to postulate that LPS might act on circumventricular organs lying outside blood-brain barrier, where it induces a locally, prolonged synthesis of prostaglandins. These, in turn, might reduce thirst by vasodilatating the cavernous structures of those organs. Data also strongly exclude a role of endogenous opioids in endotoxin inhibition of drinking behaviour.

DEVELOPMENT AND POTENCY TESTING OF POLYVALENT *PSEUDOMONAS AERUGINOSA* VACCINES

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Using Fisher's *Pseudomonas aeruginosa* immunotype strains and cultures produced on agar medium and in a New Brunswick (MultiGen) fermenter, we have developed polyvalent vaccines: (1) acetone-inactivated, whole-cell freeze-dried and fluid vaccines; (2) cell-free vaccines prepared by extraction with distilled water of acetone-inactivated bacteria. The freeze-dried vaccine was considered as the reference preparation. In active mouse protection tests, both whole-cell and cell-free vaccines ensured satisfactory protection against challenge with Fisher's 7 immunotypes. Immune sera obtained in rabbits by immunization with polyvalent whole-cell vaccines showed excellent passive mouse-protective ability. Nitrogen, carbohydrate and lipid content of the vaccines was also determined.

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ASYMBIOTIC NITROGEN FIXATION AND SYMBIOTIC COMPETITION OF *RHIZOBIUM MELILOTI*

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The nitrogenase activity, independent from plant symbiosis was detected in several, mostly slow growing *Rhizobium* species. Such experimental system was worked out in our laboratory for the determination of non symbiotic nitrogen fixation in the fast growing *R. meliloti* 41. The method was used to detect the nitrogenase gene (nif) function in non nodulating (Nod^-) and symbiotic ineffective (Fix^-) mutants or to distinguish nif^- genotypes among Fix^- mutants. The efficiency of *Rhizobium* soil inoculants is not only depending on the nodulation and nitrogen fixation characters of the applied strain but also on its competition, the ability determining how much frequently it is forming symbiosis with the cultivated plant in the presence of soil habitat, natural population. Experimental system was worked out for the comparative determination of the vaguely known competition of *R. meliloti* strains. Competitive and non competitive Fix^- strains were selected by plant tests mixed inoculated in pairs with well characterized mutants, lysogenes and defective lysogenes. The competition of various strains was determined and compared by the nitrogenase activity of symbioses resulting from inoculation with Fix^+ and selected Fix^- populations mixed in different rations.

RNA-DEPENDENT RNA POLIMERASE FROM LEAVES OF *DATURA STRAMONIUM* INFECTED WITH POTATO VIRUS X

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RNA-dependent RNA polymerase (RNA-replicase-) was discovered in membrane (31 000 *g* pellet) and soluble (31 000 *g* supernatant) fractions of leaf homogenates of healthy and infected with potato virus X (PVX) datura

plants. The optimal conditions necessary for display of enzymatic activity and general properties of RNA- dependent RNA polymerase were studied. It was shown that enzymes of membrane and soluble fractions of homogenate differ from each other. The difference between them is expressed in different relation to Mg^{++} ions and buffer system used for obtaining enzymatic extracts. As a result of virus infection the activity of membrane-bound replicase increased, reaching maximum on the 4th day of infection, while the peak of activity of soluble RNA-replicase is revealed on the 6th day after inoculation with virus. The activity of membrane-bound RNA-replicase does not depend on exogenous template whereas the activity of soluble enzyme is stimulated in the presence of PVX RNA. Neither of the enzymes are sensitive to added actinomycine D and deoxyribonuclease. Enzyme activity was optimal at pH 8.0–8.2. The synthesis of viral RNA in vitro by membrane-bound replicase proceeded linearly for 30 min and required the four ribonucleoside triphosphates and Mg^{++} ions.

RHIZOBIUM MUTANTS OF HIGHER PRODUCTIVITY PRODUCED BY NEUTRON IRRADIATION

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On the basis of the plant test a lucerne *Rhizobium* strain which had proved to be of high productivity, was treated with a neutron generator ray source. The dose applied killed 80% of the cells. To cultivate the surviving cells, the culture was spread on the surface of bean agar and incubated at 28 °C. Two-day-old lucerne seedlings were inoculated with the bacterial cells of 300 cultures and of the control strain. Some bacterium lines which favourably influenced the symbiosis of rhizobia and higher plants could be produced.

STUDY OF mRNAs FROM *DATURA STRAMONIUM* LEAF TISSUE INFECTED WITH POTATO VIRUS X

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The fractions of mRNA with a molecular mass of 1.4×10^6, 0.34×10^6, 01.4×10^6, 0.85×10^5, 0.3×10^6 daltons were isolated by PAAG electrophoresis

technique from the total RNA preparations obtained from polysome-associated mRNP of datura leaves on the 4th day after the inoculation with potato virus X. The fractions of mRNA with a molecular mass of 1.4×10^6, 0.34×10^6, 0.14×10^6 and 0.85×10^5 daltons were isolated from the analogous preparations obtained from the healthy plants. In this connection the possibility of the translation from mRNA of PVX protein (mol.wt 0.3×10^6–0.51×10^6 daltons) may be assumed. This is found in mRNP from the infected plants and absent in mRNP from the healthy ones. The fact that mRNA, isolated from individual virus-specific polysomes obtained by indirect immunoprecipitation technique with the use of antiserum against PVX D-protein, has a molecular mass of 0.21–0.43×10^6 daltons speaks in favour of this suggestion.

HERBICIDE SENSITIVITY OF CORONILLA *RHIZOBIUM* AND *PSEUDOMONAS* RHIZOBACTERIUM STRAINS

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The effect of four herbicides recommended for weed control of crownvetch (*Coronilla* varia L.) was studied on 15 symbiotic, N_2-fixing *Rhizobium* and 15 *Pseudomonas* strains having antagonistic properties to the potential pathogenic microbes of crownvetch. *Pseudomonas* strains isolated from the ecologically more balanced rhizoplane of crownvetch originating from one site were less sensitive to the investigated herbicides than the *Rhizobium* strains isolated from nodules of crownvetch grown at ecologically different conditions. Among the four preparations tested in normal and super herbicide doses ($10^{-3}\,g \times dm^{-3}$, $10^{-4}\,g \times dm^{-3}$, $10^{-5}\,g \times dm^{-3}$, $10^{-6}\,g \times dm^{-3}$) the linuron and chlorobromuron applied preemergently proved to be the most toxic, the benefin and EPTC used in presowing were less inhibiting. Data established on the sensitivity of the investigated bacterial strains could be useful for the strain selection of seed inoculation.

SEROLOGICAL COMPARISON OF *FUSARIUM OXYSPORUM* RACES

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The fungus *Fusarium oxysporum* causes vascular wilt of cultivated plants. Many pathogenic strains designated as formae speciales (infecting different plant species) exist within *F. oxysporum* and some of these consist of several physiological races (infecting different plant cultivars). Since formae speciales and physiological races differ only in pathological characters, and not in their morphology, there are difficulties in their in vitro differentiation. Two races of *F. oxysporum* f.sp. *lupini* were compared serologically. Antisera against race 2 and race 3 of the agent were prepared in rabbits, using conidia and water-soluble mycelial extracts for immunization. When antigenic samples of the two races were analysed in tandem-crossed immunoelectrophoresis, 53 precipitin lines (antigens) were detected. The great majority of these antigens were common to both races, but one characteristic antigen was specific for race 3.

ISOLATION AND IDENTIFICATION OF *FUSARIUM* STRAINS ORIGINATING FROM CABBAGE

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Various *Fusarium* isolates were obtained from cabbage (*Brassica oleracea* var. *capitata* L.) by a single cell isolation method. Most of them proved to be *F. oxysporum*, while others were *F. fusaroides*, *F. moniliforme*, *F. equiseti*, *F. solani*, *F. moniliforme* and *F. semitectum*. The pathogenicity of isolates and physiological races originating from international collections of *F. oxysporum* Schl.f.sp. *conglutinans* (Wolenw) Snyder and Hansen were determined by artificial infection of plants in a greenhause maintained at approximately 26 °C; nearly all the members of the cabbage tribe and some other *Cruciferae* cultivated in Hungary were used. Most of the *F. oxysporum* isolates were pathogenic for these cultivars. All Hungarian cabbages were more or less sensitive to *F. oxysporum* f.sp. *conglutinans* race 1, but some of them seemed to yield resistant varieties by mass selection. These experiments were begun in a climatic chamber at 24 ± 0.5 °C to develop cultivars having monogenic homozygous dominant resistance (Foc/Foc).

ISOLATION OF VERRUCARIN J, SATRATOXINS G AND H FROM *STACHYBOTRYS ATRA* INFECTED BEDDING STRAW OF SPORTS HORSES WITH MASS DISEASE

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Stachybotryotoxicosis (lesions around the mouth and on the nose, nose-bleeding) occurs regularly in the stables of the Hungarian Horse-race Company. In May of this year, almost all animals of the riding-school, about 100 sports horses, showed symptoms of more or less severe disease. A couple of weeks earlier, horses had got ill at the trotting racecourse, and even the workers loading the mouldy straw had complained of nose-bleeding. We isolated verrucarin J, satratoxins G and H from the *Stachybotrys atra* infected bedding straw samples by methanol extraction, petroleum ether-water partition, silica gel liquid chromatography, then high-pressure liquid chromatography using reverse phase column. The chemical identity of these macrocyclic trichothecenes of the mould *S. atra* was verified also by capillary gas chromatography after their transesterification to verrucarol, and their toxicity was checked by brine shrimp test. This is the first diagnosis of horse stachybotryotoxicosis verified by analytical results, after stachybotryotoxicosis of sheep and calves had been verified in this way by us earlier.

SOME FACTORS AFFECTING GROWTH AND SYMBIOTIC PROPERTIES OF *RHIZOBIUM JAPONICUM* BACTERIA

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Growth behaviour of three effective bacterial strains of *Rhizobium japonicum* were tested against different temperatures, concentrations of two antibiotics and two fungicides. The sensitivity of these bacterial strains against 3 fungal and 3 actinomycetal strains were determined. In addition, the symbiotic properties of these bacterial strains with their soybean host plant (var. Clark)in presence and absence of different doses of NP fertilizers and molyb-

denum, boron and their mixtures were investigated. The bacterial strains were able to grow at 12 °C and 40 °C as well as a wide spectrum of neutral salt conditions. Alkaline salts were the most harmful to rhizobia. Rhizobial strains behaved differently against the two antibiotics and the two fungicides used. Inoculation of soybean plants, dressing with P and N fertilizers markedly benefited soybean but incubation only seemed sufficient to get high product of high protein content. Dressing with higher NP levels markedly decrease the free amino acid contents of soybean dry matter. Boron and molybdenum were of marked beneficial effect when added to inoculated plants at 0.5 and 4 ppm and their mixture at 1 ppm, respectively.

PSEUDOMONAS AERUGINOSA TEST IN THE QUALIFICATION OF DANGEROUS WASTES

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Besides the traditional methods (Daphnia, Guppy, Sinapis) for the toxicological examination of dangerous wastes containing heavy metals an increasing demand of the use of procaryotic organism has arisen. The new test system developed on the basis of in vitro growth inhibition by heavy metal ions (Pb^{++}, Cd^{++}, As^{+++}) of *Pseudomonas aeruginosa* is available to adjust the comprehensive toxicological examination of the industrial wastes containing the extract of the sample at different concentrations is incubated for 24 h at 37 °C with the cell-suspension of *P. aeruginosa*. Toxicity of the sample is expressed as the highest dilution inhibiting growth.

PROTEINS IDENTIFIED IN mRNP FROM *DATURA STRAMONIUM* LEAVES INFECTED WITH POTATO VIRUS X

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The proteins with the molecular mass of 90 000, 75 000, 57 000, 25 000 and 22 500 daltons were found by the PAAG electrophoresis methods in mRNP isolated from individual polysomes of dature leaf tissue obtained by

indirect immunoprecipitation technique with the use antiserum against D-protein of potato virus X. The proteins of 92 000, 78 000, 25 000, 23 000 daltons were found in cytoplasmic mRNP obtained by the same technique. The molecular mass PVX coat protein corresponds to 23 000–24 000 daltons according to PAAG electrophoresis data. The serological relationship of proteins with 22 500 and 23 000 daltons found in mRNP to PVX coat protein, identified by means of Ouchterlony double diffusion reaction, indicate that these proteins are analogous to structural virus protein.

UTILIZATION OF THE BIOMASS PRODUCED BY A *STREPTOMYCES* STRAIN FOR FEEDING PURPOSES

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The *Streptomyces* strain used in our experiments has been isolated from the soil of a young pine-forest and was identified according to the taxonomy of Hütter as *Streptomyces lavendulae*. The strain showed significant inhibitory effect of *Staphylococcus aureus* PCI 209 P; *Escherichia coli* B ATCC 9723; *Bacillus subtilis* 6633; *Bacillus radiobacter* ATCC 6466; *Sarcina lutea* ATCC 9341; *Serratia marcescens* NRLL–B 486; *Mycobacterium phlei* ATCC 10142; *Mycobacterium smegmatis* ATCC 607; *Streptomyces griseus* CBS 3496. Laboratory scale then pilot plant fermentation was performed for about 100 h in a nutrient medium composed of inexpensive ingredients. Spray-drying of the fermentation liquor yielded a yellowish powder of high protein content, about 4% lysine content and antibiotic activity. The biomass produced in this manner was added to the feedstuff at different rates and fed to pigs, hens and geese. Upon the effect of feeding, increased gain in weight, decreased mortality and better feedstuff utilization were observed as compared to the control.

ISOLATION AND GENETIC ANALYSIS OF THE 59 TEMPERATE PHAGE *ERWINIA CAROTOVORA* 268 CLEAR-PLAQUE MUTANTS

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The clear-plaque (c-) mutants of the temperate phage 59 of *Erwinia carotovora* 268 were able to lysogenize the sensitive *Erwinia horticola* 450 with a lowered lysogenization frequency. UV-light phage mutagenesis was carried out and mutants forming clear plaques on *E. horticola* 450 were picked. The frequency of c-mutants formation was not more than 3.5×10^{-5}. All c-mutants were divided into 5 complementation groups (C_1–C_5). All of the group C_3 and C_4 mutants complemented not only one another, but also every of the group C_1, C_2 and C_5 mutants. None of the group C_2 mutants complemented those of the group C_1 and C_5. Some of c-mutants complemented none of the mutants, but they were active on *E. horticola* 450 lysogenized with phage 59. This suggests that the mutation is located in the phage operator region(s). Physiologycal properties of certain mutants in each complementation group were studied. It may be concluded that establishment and maintainance of the lysogenic state in *E. carotovora* 268 phage 59 are probably more complicated than in other well investigated phages as χ, P_{22} etc.

A SIMPLIFIED SCHEME FOR IDENTIFICATION OF *BACILLUS* SPECIES IN ECOLOGICAL STUDIES

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In ecological surveys connected with food technologies or other practical applications it is often necessary to identify great number of strains. Hence it is practical to devise simplified identification schemes enabling quicker work and more facile handling of numerous isolates. For construction of our scheme we set out from the accepted characteristics of validly described species of the genus *Bacillus*. Out of the characters we selected those diagnostic tests which appeared most efficient for identification. The scheme applies 5 characters only. These are: growth anaerobially, hydrolysis of starch, oxidation or fermentation of glucose, Voges–Proskauer reaction and microscopic appearance

(e.g. swelling of sporangium). These tests can be performed by using 3 different media only. In a few cases, however, some additional tests are required to assign the final identity of a strain (e.g. reduction of nitrate, salt tolerance). By this simplified method we tested about 200 strains isolated from spices of different types and origin (paprika, black peppers) as well as from spoiled bread. The most frequently found species were as follows: *B. licheniformis*, *B. subtilis* and *B. pumilus*. Among 50 isolates from samples of soil and sewage the dominating species was *B. cereus* subsp. *mycoides* and in addition, *B. licheniformis*, *B. subtilis* and *B. polymyxa* were also frequent. In general, the spectrum of other species was broader than in spices.

CHARACTERISTICS OF *ERWINIA CAROTOVORA* 268 POLYLYSOGENY

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Erwinia carotovora 268 appeared to be polylysogenic. In the present work we showed that lysogenic induction had a seasonal character with a phage yield peak in April–May and September–November, whereas in other periods erwiniocines were produced. Lysogenic convertion was characterized by an inability to adsorb homoimmune phage and temperate virus clear-mutants on lysogenic cells. After lysogenic induction we detected the permanent presence of three viruses (49, 59 and E105). On *E. horticola* 450 we obtained a few transparent colonies called provisionally phage 69 in contrast to turbid colonies formed by phage 49. Studying interactions between phages on artificially lysogenized culture of *E. horticola* 60 and 450 (singular and double lysogens *E. horticola* 60/59, 450/59, 49), we have shown that phages 49 and 69 infected *E. horticola* lysogenic with phage 59 with the same effectiveness as they did it with the initial one (the percentage of lysogenized cells did not depend on the presence of prophage 59).

THE RESTRICTIONAL ANALYSIS OF TEMPERATE PHAGES DNA OF *ERWINIA*

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The effect of the restrictases on genomes of temperate phages 59, E105, 49 and 69 of the defective polylysogenic culture of L-asparaginase producing *Erwinia carotovora* 268 has been studied. By detergent-phenol method we isolated the DNA from the purified preparations of phages 59, E105, 49 and 69 grown on *Erwinia horticola* 60 and 450. The DNA preparations were subjected to hydrolysis by endonucleases Eco RI, Hind III and Cfr 61. Phage 59 DNA had 10 sensitive sites for restrictase Eco RI, 15 for Hind III and 21; fer Cfr 61 phage E105 DNA had no sensitive sites for Eco RI and Hind III, but was hydrolyzed by Cfr 61 at 21 fragment. The electrophoretic patterns for DNA preparations of the phages 49 and 69 digested with restrictases Eco RI, Hind III and Cfr 61 are identical (respective number of fragments, 10, 18 and 19). Restriction fragments electrophoresis in 1–1.2% agarose have shown that average DNA molecular weights are 28.14 and 31.00 MD for the phages 59 and 49/69, respectively. The value 25.84 MD for phage E105 DNA is not final, as it was obtained by hydrolysis with one enzyme only. As the restriction fragments of phage 49 and 69 DNAs are identical, phage 69 seems to be a clear-mutant of phage 49 with a site one mutation, not a deletion. The genome sequence colinearity is preserved.

PSEUDOMONAS SYRINGAE PV. *SYRINGAE* AS A NEW PATHOGENIC AGENT OF PEPPER PLANTS

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In 1982 and 1983 a chlorotical leaf-spot disease appeared at the South region of Hungary on pepper. The pathogenic agent *Pseudomonas syringae* pv. *syringae* van Hall also attacks veins, petiols, buds and top of shoots of pepper plants but does not attack the fruits. The pepper strains of the organism also infect leaves, apricot branches and fruitlets of cherry and apricot and cucumber plants. This pepper disease intensively spreads on field when the weather turns cold after a warmer period. The sensitivity of pepper cultivars were different, the antibiotic "Kasumin EC" (Japan) prevented the development of the disease in greenhouse experiments.

INFLUENCE OF SOME PESTICIDES ON THE BACTERIAL FLORA OF THE UPPER-TISZA AND SZAMOS

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The effect of ten pesticides (fungicides, insecticides and herbicides) used in high quantity in Szabolcs-Szatmár county in 1981 and 1982 was studied on the bacterial flora of the non-polluted region of the Upper-Tisza at Tivadar, its polluted region at Vásárosnamény and the polluted Szamos before flowing into the Tisza. All the tested pesticides decreased the "total" number of bacteria in all sampling places proportionally to the doses (10^{-6} g $\times$ dm^{-3}, 10^{-5} g $\times$ dm^{-3} and 10^{-4} g $\times$ dm^{-3}) and time of exposure (4, 8 and 12 h). Bacteria occuring in the Szamos river were the most resistant to the pesticides studied. At the Tivadar region the chlorbromuron, trifluralin, terbutryne, methyl parathion pesticides, at the Vásárosnamény region the benefine, aktinit PK, benomyl, mancozeb, DNOC, dimethoate were the most toxic to the bacteria.

THE COAT PROTEIN OF RED CLOVER MOTTLE VIRUS

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Three protein subunits with the molecular weights 39.2 ± 0.8, 20 ± 0.8 and 18.5 ± 0.4 kilodaltons were detected in the dissociated coat protein of red clover mottle virus strain U-1 by gradient polyacrylamide gel (15–30%) slab electrophoresis. Analysis of the protein of the separated electrophoretic forms of this strain revealed two protein subunits in the forms. They differ in the molecular weights of the second protein subunit: as a rule the slow-migrating form contains the 18.5 kilodaltons proteins and fast-migrating form 20 kilodaltons protein. The amount of the fast form decreased with age of infection considerably and that of the slow form increased correspondingly. The conversion of the fast to the slow forms seems likely due to proteolysis of the 20 kilodaltons protein resulting in the removal of the 1.5 kilodaltons oligopeptide.

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Industrial microbiology

BIOTECHNOLOGICAL UTILIZATION OF MICROBIOLOGY IN DEVELOPING AND INDUSTRIALIZED COUNTRIES OF THE WORLD

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The interdisciplinary sphere of biotechnology is based on biochemistry, molecular biology and bioengineering (fermentation) and represents one of the dynamic developing, practical technical programmes in the field of research and development. In several countries of the world, traditional and new biotechnological processes are dealt with, and new scientific results, eminently gene techniques are expected to modernize them. Considering targets of the practice, five significant fields have been estabilished both in the developing and the industrialized developed countries: (i) In the field of public health, reducing genetic or widespread diseases (e. g. haemoglobin-damage, tropical diseases). (ii) New plant species in agriculture and food production, new technics of propagation in animal husbandry. (iii) Use of biomass as new raw material in the fied of industrial processing. (iv) Production of biogas and bioalcohol in the field of energy carries and fuels. (v) Biotechnological results obtained in environmental protection (e. g. nitrogen-fixing bacteria as substituents for chemical fertilizer). Today already about 15–20 genetic substances (DNA molecules) can be produced in a synthetic way and can be incorporated in microorganisms to exert their effect there.

PROTOPLAST FUSION AND PROTOPLAST TRANSFORMATION IN *MICROMONOSPORA* SP.

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Our strain improvement program of aminoglycoside antibiotics producing *Micromonospora* includes the use of recombinants, transformants, fused protoplasts and transformed protoplasts. Experiments to investigate the protoplast fusion in *Micromonospora* have been carried out with auxotrophic

and rifampicin resistant mutants of *Micromonospora olivoasterospora*. The frequency of protoplast fusion varied between 1.2×10^{-5} and 9.0×10^{-4} being calculated on regenerated protoplasts. The preliminary results given by interspecific protoplast fusion show that this technique is suitable to produce new hybrid strains being able to biosynthetise new antibiotics. In protoplast transformation experiments the optimal amount of DNA was found to be 2.0×10^{-7} mcg/c.f.u. Using DNA originating from wild type donor, the transformation frequency is between 1.5×10^{-7} and 4.8×10^{-5}. Interspecific protoplast transformation has also been carried out; this transformation frequency is lower than that of intraspecific one. The genetic analysis of the recombinants and transformants shows the majority of the clones to be recombinant phenotype but strains were also isolated having a segregation pattern typical of heterokaryons.

INVESTIGATION OF BETA-LACTAMASE INHIBITORY EFFECT OF PENAM SKELETON TYPE COMPOUNDS

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By the use of the in vitro section of a complex model system for researching beta-lactamase inhibitors we investigated the possible inhibitory action of penicillin derivatives on beta-lactamases of different origin. The following points of view were taken into consideration: (i) The beta-lactamase sensitive beta-lactams to be combined should be readily available in Hungary (ampicillin, cefalexin); (ii) the combination experiments are to be executed with bacteria carrying beta-lactamases which represent all the classes of the Richmond–Sykes classification system; (iii) the beta-lactamase inhibiting effects of the drugs to be combined are partly known from the references, partly they are new derivatives or known compounds but have new data of impact. The enzyme kinetical parameters (K_M, V_{max}, K_1) were assessed spectrophotometrically, using nitrocefin as substrate. With the in vitro bacterium systems a remarkable synergistic effect was found with clavulanic acid (which singly is the most effective with beta-lactamases); and with sublactam, when combined with ampicillin as substrate. All other combinations were unsuccessful. Based on our own estimations we set up a structure-activity relationship among compounds investigated in the in vitro systems.

THE EFFECT OF BIVALENT CATIONS ON ANTIBIOTIC SUSCEPTIBILITY TESTING

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We have examined the effect of calcium and magnesium ions on the susceptibility of bacteria to aminoglycosides (tobramycin, kanamycin, neomycin, gentamicin, streptomycin) and to tetracyclines. The calcium and magnesium concentration varies between 60 and 20 μg per ml of routinely applied Mueller–Hinton agar. Increasing the concentration of calcium and magnesium ions resulted in the decrease of inhibition zones for all antibiotics. In case of tetracyclin and streptomycin, an increase by only 10 μg/ml magnesium ions led to an apparent decrease of 70–80% in activity.

SIGNIFICANCE OF ENUMERATION AND IDENTIFICATION OF AEROBIC SPOREFORMING BACTERIA IN FOODS

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The aerobic mesophilic microbial count in paprika is mainly composed of aerobic sporeformers the dominantly species were *Bacillus licheniformis*, (46%), *Bacillus subtilis* (23%) and *Bacillus pumilus* (20%). No obligate thermophilic sporeformers were found. In flour samples *Bacillus cereus* which may cause food poisoning, was absent, however, *B. pumilus* mainly responsible for ropiness of bread occurred in 10^2/g in average. The QAL level of mesophilic aerobic sporeformers was 10^3/g. Sugars are generally considered as one of the source of thermophilic flat sour sporeformers causing the spoilage of preserved and heat-treated products. Monitoring of sugars produced in different factories showed that they meet the ICUMSA standards.

MICROBIOLOGICAL CONTROL OF INDUSTRIAL ETHYLENE OXIDE STERILIZATION OF DISPOSABLE MEDICAL DEVICES WITH *BACILLUS SUBTILIS* VAR. *NIGER* SPORE-TESTS

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In accordance with the 6th Hungarian Pharmacopoea, the effectiveness of any sterilizing process has to be tested through sterility controls. The effectivity of industrial ethylene oxide sterilization can also be tested by applying, so called, "master products". They are products that contain *Bacillus subtilis* var. *niger* spores and which are difficult for ethylene oxide to go through. We have tested the permeability of disposable medical devices made of PVC for the purpose of producing a "master product". In a gas sterilizer chamber at 800 mg/L ethylene oxide concentration at 58 °C we measured the time required for killing off *B. subtilis* var. *niger* spores without package and in 1×0.4 mm, 2×0.4 mm, 3×0.4 mm and 4×0.4 mm thick PVC packages. The corresponding LD_{50} values were 10.7, 24.8, 80.8, 151.4 and 213.4 min, the killing off times 11, 50, 100, 160 and 240 min.

THE EFFECT OF PHENOL ON THE RADIOSENSITIVITY OF *BACILLUS MEGATERIUM* SPORES

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Radiation modifying action of phenol was studied on *Bacillus megaterium* spores at different oxygen concentrations. Phenol was used at 10 mM with eight oxygen concentrations (100% N_2, 0.7% O_2, 1.2% O_2, 2.1% O_2, 3.1% O_2, 5.1% O_2, 21% O_2 in N_2 and 100% O_2). Gamma radiation facility was PX-gamma-30 ^{60}Co apparatus with 88.72 Gy/min dose rate. At increased oxygen concentration phenol protects spores to a small extent against lethal damage. It is due to the dydroxyl radical scavenging power of phenol.

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Mycology

EVALUATION AND APPLICATION THE REFERENCE VALUE OF YEAST COUNT AS MICROBIOLOGICAL CRITERION OF FOOD QUALITY

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Using glucose-yeast medium by addition of antibiotic or with a lower pH value (4.5) there is a good correlation between the yeast count and the sensorial test of soft drinks. The spoilage level (SL) of yeast in soft drinks is 10^5/ml. The reference value of yeasts is 10/ml in soft drinks, as described in the standard. The fermentation of apple-juice concentrates may occur when the yeast count is more than 5.10^4/g. The concentrate is sterile just after the processing, but it can be reinfected in tanks, or tank-trucks. Therefore the microbiological monitoring of hygienical practice of storage and transportation is of a great importance. The yeast count in corn-sugarsyrup (so called izo-syrup) is lower than 1/log in the majority of samples. As reference value 10/log is standardized. The yeast count was the same determined in OGA and osmophilic media (40 and 60%).

YEASTS IN FRESH WASTE WATERS

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During a five-year period the yeast flora of a surface water as well as of in- and effluents of four sewage treatment plants was studied. Altogether 865 strains from 352 samples, collected regularly from 24 places, have been isolated, out of which 296 representing 42 species of 14 genera have been identified. The studied stream showed the prevalence of candidae and cryptococci, viz. *Candida famata*, *Candida vulgaris*, *Cryptococcus curvatus* and *Cryptococcus humicolus*, with some seasonal fluctuations. Every incoming sewages, however, contained mainly candidae and cryptococci, viz. *Candida vulgaris*, *Candida*

vini and *Cryptococcus albidus* var. *albidus*, while in the effluents, besides a considerable decrease in population density candidae and saccharomycetes were the most frequent, viz. *Candida famata* and *Saccharomyces cerevisiae*. Although the contribution of excrements to sewage was considerable, *Candida albicans* failed to appear, while other potential pathogens were abundant in influents. In both of in- and effluents loose seasonal fluctuations were observed, referring to the development of a more or less stable ecosystem within the treatment plants. The interrelation between the yeast flora and the chemical properties of the samples requires more detailed discussion, but globally it can be stressed that the chemical (predominently chemical oxygen demand), and zymological data proved to be mutually indicative for each other.

MATING TYPES IN *SCHIZOSACCHAROMYCES POMBE* VAR. *MALIDEVORANS*

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The fission yeasts have a haplonic life cycle. The vegetatively propagating cells are predominantly haploid, and the diplophase is confined only to the zygotes. In *Schizosaccharomyces pombe* var. *pombe* the sexual behaviour of the cells is governed by the genetic information located in the mating type locus and can be influenced by certain auxiliary genes being outside this region. In tetrad analysis of randomly selected asci of a *S. pombe* var. *malidevorans* (CBS 5557) culture, spores of heterothallic (h^- and h^+) and homothallic ("spotty" and "grey") mating types were found. None of them proved completely stable, interconversions between them were frequent. Two directions of coversions predominated: cells of types h^- and "spotty" tended to change their type to h^+ and/or "grey" very intensively, while the changes to h^- and "spotty" were less frequent. This "spotty" type was so unstable that its iodine-positive colonies were never homogenious: they always contained numerous iodine-negative heterothallic (mostly h^+) or weakly iodine-positive "grey" spots. Occasionally, sterile cells also arose in the growig spore clones. Ten out of the 43 complete asci analysed contained 3 or 4 h^+ spores. The genetic analysis of these spores by crossing and protoplast fusion revealed that many of them were actually homothallic (being able to sporulate after protoplast fusion), and had lost the capability for expressing the h^- mating activity. Certain h^- spores from other asci have also proved to be criptically homothallic, but in reverse manner. The frequent occurrence of these "semiheterothallic" types can probably be attributed to changes of genes being located outside the mating type locus.

ARE CONJUGATION AND SPORULATION ALTERNATIVE POSSIBILITIES IN DIPLOID CELLS OF *SCHIZOSACCHAROMYCES POMBE?*

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In the life cycle of the haplontic yeast *Schizosaccharomyces pombe* var. *malidevorans*, zygotes produced by sexual fusion of haploid cells convert into four-spored asci shortly after conjugation, where by the haploid state is restored. Under special conditions, however, the zygotes can be prevented from sporulation (by being transferred onto a N-rich medium) and are forced to propagate mitotically. The diploid clones arising are unstable and undergo sporulation as soon as the medium becomes exhausted of nitrogen. Occasionally, two adjacent diploid cells conjugate instead of converting into asci, thereby forming a tetraploid zygote. Since both "developmental pathways" are triggered by the decrease of N-content under a critical value, one might believe them to be alternative possibilities controlled by the same mechanism in G1. But it has turned out that the sporulation has an advantage over conjugation (30–40% and 1–4%, respectively), which favours the supposition of a „'two-point-control" system. Both points should be in G1: the first one should switch on sporulation, while the second one might govern the turn towards conjugation. The tetrad analysis of the tetraploid zygotes has, however, raised an additional hypothesis. It has revealed that probably only one of the two mating type loci is active in the conjugating cells. If both loci are required to be activated during sporulation, indeed, one can assume that the number of active loci is the crucial factor: cells with one active matlocus might be conjugation-proficient, cells with two active loci might be sporulation-proficient.

POSSIBILITIES FOR THE DEVELOPMENT OF THE NETWORK OF HUMAN MYCOLOGY IN HUNGARY

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In our country the network of the human mycology has developed within the field of the dermato-venerological provision. For years the system of the mycological laboratories were created — mostly spontaneously — in the different kinds of medical institution because of the needs of different medical subsections. So the frames, which were made in the fifties turned into

insufficient and a new situation caused problems both in the field of the organization and profession. (i) In some parts of Hungary the diagnostic work is still unsolved. (ii) There is no homogenous professional direction all over the country. (iii) The mycological requirements of the dermatology and the other medical sections are different: these requirements should have been supplied by heterogenous units. (iv) In the present situation, the above mentioned units are not able to utilize even the decreased possibilities. (v) The teaching programme of mycology is unsolved or incomplete on the graduate, postgraduate and professional quality levels. For solving these problems, the county stations should be directed by the scientific-professional centre, and the diagnostic work should be controlled by the county stations.

SOME CONSEQUENCES OF THE ABSENCE OF ERGOSTEROL IN *CANDIDA ALBICANS*

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Various auxotrophic and nystatin-resistant ergosterol-less mutants of *Candida albicans* were isolated. The plasma membrane ultrastructure of a sterol mutant was not significantly different from that of the parental, ergosterol-producing strain. Significant alterations were observed in the phospholipid content of the sterol mutants, and the chitin synthase activity was also higher in comparison to the parental strains. Sterol mutants had increased plasma membrane order parameters, resulting in decreased levels of assimilation of carbon sources, and a significantly decreased uptake of 2-^{3}H glycerol. Intraspecific protoplast fusion was carried out and nutritionally-complemented hetero-zygous diploid hybrids were obtained, which proved to be sensitive or resistant to nystatin as a consequence of complementation or non-complementation for ergosterol biosynthesis.

KINETIC STUDIES OF GROWTH INHIBITION. EFFECT OF POLYENES ON *CANDIDA ALBICANS*

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In "growth inhibition" studies some kinetic problems are involved. (i) The virtual growth inhibition may be instead of real division inhibition due to cell killing (cide) effect. (ii) The effects may be combined (cide+static). (iii) The antimicrobial substance may be regarded in time. (i) Earlier published nystatin effect of *Candida albicans* is of this type. Our developed computer program discriminates between real growth (division) inhibition and decreased growth by killing. Thus the effect of nystatin, amphotericin B, their methylesters and candicidin methylester as well as primaricin on *C. albicans* proved to be of this type. (ii) Candicidin, however, showed an example of combined effect, which has been mathematically verified. (iii) On one hand upon comparison to the measured one of a simulated growth curve based on our growth equation containing the killing coefficient deduced from separate germicide experiments, as well as on the other hand upon direct kinetic analysis of growth curves, the significance and the profile of fungicid degradation were deduced.

THE EFFECT OF FUNGI ON CHANGES IN THE CHEMICAL COMPOSITION AND NUTRITIVE VALUE OF CORN GRAIN

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Corn grain of CORp 135/79 variety was ground, sterilized and inoculated with conidia suspension of the *Fusarium* sp. 72-T4, *Fusarium* sp. 72–76, *Aspergillus* sp. 72–72, *Aspergillus ochraceus* and *Penicillium rubrum* COR-25. All strains were isolated from maize corn. Moreover, natural maize ground populated with epiphytic microflora was used. The samples were incubated at 30 °C for 10 days, then dried for 48 h at 40–50 °C. Natural maize ground served as control. The fungi increased the fat (5.13–9.16%), total sugars (0.49–23%) and reducing sugars (0.17–5.61%), whereas decreased the starch content (70–30%). The major changes were caused by *A. ochraceus*. Changes in the total nitrogen and protein content were also found. The nutritive value of

the maize ground samples supplied with casein, soya oil, mineral salts and vitamins was examined by feeding rats of Wistar breed for 4 weeks. The body weight of the control rats (non-incubated ground) increased by 47% whereas of those receiving incubated samples from 2 to 13.8% only. With two samples it decreased (*A. ochraceus* by —14.4% and *P. rubrum* COR-25 by —8.5%. Haemoglobin levels in comparison with initial results decreased (control —0.5%, *P. rubrum* 23%) or increased *Fusarium* sp. 72–76 (2.4%). The number of erythrocytes decreased from —0.5% in the control to —31.7% (*P. rubrum*) and increased by 8.7% (*Fusarium* sp. 72–76). Sugar level in blood also varied (from +3.5 to +14.7% *Fusarium* and *P. rubrum*). A decrease of sugar (—2.3%) was caused by *A. ochraceus*. Kidney weight in all experiments was higher than in the control (by 0.8—1.06%) as well as the liver weight (3m39% in the control/4.25% *P. rubrum*).

In Memoriam Gyula Weiszfeiler

Professor Gyula Weiszfeiler, Member of the Hungarian Academy of Sciences died on April 9, 1984.

He was born in 1902 in Brassó, Transylvania. He completed his lower-grade studies in Budapest, then in 1920 he went abroad and pursued university studies in Paris, Brussels, Jena and Geneva. He won the title of "Doctor of Natural Sciences" in 1925 and he got his medical degree in 1928 in Geneva. Following this, he was working as microbiological researcher and tuberculosis specialist for 5 years in Switzerland and Germany and his keen interest in mycobacteria had remained throughout his life. He was expelled from Germany due to his political activity. From 1932 to 1958 he was working in the USSR in different research institutes of the Academy of Medical Sciences of the USSR, holding several high-ranking posts. Moscow, Sverdlovsk, Tashkent and again Moscow were his main working sites. In 1941, he won the title of "Doctor of Medical Sciences", in 1943 he became Professor of Medical Sciences. In the Great Patriotic War he was on voluntary service in the Red Army and got back to his scientific work as a major.

His main fields of research were the mycobacteria and above all the investigation of the biology and variability of *Mycobacterium tuberculosis*. His attenuated W 115 strain proved to be more efficient in immunizing activity even than the BCG vaccine, when tested in monkeys. He elaborated a gel-precipitation method for examining the antigenic structure of different mycobacterium species in order to study their degree of relationship. He isolated several atypical mycobacteria from monkeys, among them two, so far unknown species *(M. simiae, M. asiaticum)* which were later detected in patients in several countries.

He returned to Hungary in 1958 and worked first in the National Institute of Hygiene, then in the Experimental Research Institute of the Hungarian Academy of Sciences. From 1963 to 1972 he was director, and after retiring, scientific counsellor of the Microbiological Research Group of the Hungarian Academy of Sciences. Establishing and developing the Microbiological Research Group, Professor Weiszfeiler founded a high-level research basis.

In Hungary he was the first to deal with the examination of Sabin's living vaccina against poliomyelitis. He contributed to the introduction of the vaccine in Hungary in 1960. Anyhow, he remained faithful to his main field of research, the extensive examination of mycobacteria, though his interest and fruitful work covered several other important fields of microbiology. His scientific achievements won national and international tribute and his results are quoted in several publications and reference books. He published more than 160 scientific papers and was the author, co-author and editor of many successful monographs and books.

As an appreciation of his research work and scientific results, he was elected Corresponding Member of the Hungarian Academy of Sciences in 1960, he became university professor in 1964 and in 1982 he was elected ordinary Member of the Academy.

Besides his outstanding research work and versatile literary activity, Professor Weiszfeiler also worked actively in the organization of research and for the socialist society. He was secretary general of the Hungarian Microbiological Society from 1959 to 1966, then its honorary leading member; he was President of the Serum-Vaccine Committee of the Ministry of Health between 1959 and 1966. He was member of the Microbiological, Epidemiological and Vaccine Committee of the Hungarian Academy of Sciences and of the Ministry of Health; he acted as a member of the editorial board of Acta Microbiologica Hungarica, of the International Committee on Systematic Bacteriology and of the International Working Group on Taxonomy of Mycobacteria.

In 1964 he was elected member of the Cuban Microbiological and Sanitary Society and in 1972, honorary member of the Microbiological Society of the USSR.

As a tribute to his excellent activity, he won several Soviet and Hungarian honours: in 1945 he was awarded a Soviet medal for his outstanding work in the Great Patriotic War, also in 1947 on the occassion of the 800-year anniversity of Moscow's establishment. At home in 1959 he was awarded the partisan medal "In Arms for the Country", which was followed by the "Order of Labour" (1962), the "Gold Degree of the Order of Labour" (1972), the "Medal for Socialist Society" (1967) and the "Medal for Socialist Hungary" (1977). On his 80th birthday he was honoured with the "Flag Order of the Hungarian People's Republic".

Professor Weiszfeiler lived a rich, successful and laborious life. He experienced a great many storms and troubles but his career remained steady and unbroken. It can be seen maybe more clearly when his life and versatile activity are recalled in the memory of his admirers after the painful parting reminiscences.

We shall always remember him with honour and devotion.

István Nász

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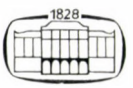

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PROTO-ONC GENE PRODUCTS AS DIFFERENTIATION ANTIGENS

(A REVIEW)

I. Földes and J. Minárovits

Microbiological Research Group of the National Institute of Hygiene, Budapest

(Received July 25, 1983)

Studies on oncogenic RNA viruses have resulted in most of the information referring to malignant transformation of cells. The life cycle of these viruses proved to be complex: a DNA copy is transcribed from their RNA genome by the enzyme reverse transcriptase (reverse transcription), which integrates into the host genome (provirus), then it is transcribed to RNA which is processed in the nucleus and transported to the cytoplasm where the viral proteins are manufactured [1–6]. In addition to the structural genes necessary for replication, oncogenic RNA viruses inducing acute malignant diseases (leukaemia, sarcoma, cancer) contain onc genes (v-onc genes) responsible for malignant transformation [1–4].

Experiments with nucleic acid hybridization have shown that (i) the genome of vertebrate cells contains sequences analogous to v-onc genes; (ii) most of these sequences appeared early during evolution; (iii) their majority was well preserved as it undervent no marked change during evolution [7–14].

On the basis of detailed analyses (mapping, in situ hybridization and DNA sequencing) it could be stated that (i) the cellular homologues of the viral onc-genes (the so-called c-onc genes) have a definite chromosomal position in a given species [see 15 for references], (e.g. c-myc gene is located in the human 8 chromosome [16]); (ii) with some exceptions [26, 27], similarly as the majority of the structural genes of vertebrates, most of the c-onc genes contain introns (non-coding sequences) [15–26], while v-onc genes have no introns [25, 28–40, 59]; (iii) some of the c-onc genes show structural polymorphism and can be regarded as members of a gene family (e.g. the human ras gene family) [19, 41].

As retroviral onc-genes have been derived by recombination from cellular onc-genes, the latter were called proto-onc genes [42]. This term has been accepted but may be misleading [43] since it refers only to the possible oncogenic capacity of these genes, which can be realized only after recombination

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Abbreviations used

AEV	Avian erythroblastosis virus
A-MuLV	Abelson murine leukaemia virus
AMV	Avian myeloblastosis virus
B77	B77 avian sarcoma virus
BALB MuSV	Balb murine sarcoma virus
CM II	Avian myelocytomatosis virus CM II
E26	Avian leukosis virus strain 26
ESV	Esh sarcoma virus
FSV	Fujinami sarcoma virus
Ga-FeSV	Gardner-Arnstein feline sarcoma virus
Gz-MuSV	Gazdar murine sarcoma virus
Ha-MuSV	Harvey murine sarcoma virus
Ki-MuSV	Kirsten murine sarcoma virus
MC29	Avian myelocytomatosis virus MC29
MH2	Avian myelocytomatosis and carcinoma virus MH2
Mo-MuSV	Moloney murine sarcoma virus
MS-FeSV	McDonough feline sarcoma virus
OKIO	Avian myelocytomatosis virus OKIO
PRC II	PRC II sarcoma virus
PR-RSV	Prague strain Rous sarcoma virus
Ra-RSV	Rasheed rat sarcoma virus
rASV	Recovered avian sarcoma virus
REV--T	Avian reticuloendotheliosis virus-T
RSV	Rous sarcoma virus
SSV	Simian sarcoma virus
ST-FeSV	Snyder-Theilen feline sarcoma virus
UR2	UR2 avian sarcoma virus
Y73	Y73 avian sarcoma virus

with a retroviral genome or through activation by viral regulatory elements (promoter insertion theory [44–50]), or by other processes [51–57], but does not refer to their possible role in normal (non-oncogenic) development. Thus, it has been hypothesized that proto-onc gene products are differentiation antigens [2, 42].

Antigens that are expressed on some but not all cells of an organism have been termed differentiation antigens. Their expression may be restricted to particular cell lineages, to determined stages of cell differentiation or other processes [58]. To prove that proto-onc gene products can be acknowledged as differentiation antigens, data referring to transcription and translation of proto-onc genes in normal (non-transformed) cells have to be studied. In this paper we shall therefore summarize the known retroviral onc-genes and their products, then data referring to transcription and translation of proto-onc genes.

Table I shows the known v-onc genes and their products. It can be seen that some of the onc-genes occur in more than one retrovirus genome, i.e. the number of onc-genes is less than the number of independently isolated retroviruses.

It is worth mentioning that in a number of acute retroviruses gag-genes (coding for core proteins of the virus) and onc-genes produce a fusion-protein.

Comparing the amino-acid sequences of the onc-gene products determined by the nucleic acid sequences of cloned onc-genes, a marked homology could

Table I

*V-onc genes and their protein products**

Virus	V-onc gene	Protein product	Known function of the protein product	References
		Avian sarcoma viruses		
RSV, B77, rASV, PR-RSV	src	$pp60^{src}$	protein-kinase	7, 8, 60–64, 66–70, 35–37
Y73	yes	$P90^{gag-yes}$		11, 38, 70, 81
ESV	yes	$P80^{gag-yes}$	protein-kinase	
FSV	fps	$P130^{gag-fps}$		11, 39, 70, 82, 84–87
PRC II	fps	$P105^{gag-fps}$, $P110^{gag-fps}$		
16L	fps	$P142^{gag-fps}$	protein-kinase	
UR1	fps	$P150^{gag-fps}$		
UR2	ros	$P68^{gag-ros}$	protein-kinase	11, 88–90
		Mammalian sarcoma viruses		
Ki-MuSV	kis	$p21^{kis}$		
Ha-MuSV	has	$p21^{has}$	guanine-nucleotide binding,	14, 31, 32,
Ra-RSV	ras	$P29^{gag-ras}$	autophosphorylation	73, 74, 91, 92
BALB MuSV	bas	$p21^{bas}$	guanine-nucleotide binding	74
FBJ murine osteosarc. v.	fos	$p41^{fos}$ (predicted) $p55^{fos}$ (observed)		25, 93
Mo-MuSV, Gz-MuSV	mos	$p37^{mos}$		28, 30, 94, 95, 98
ST-FeSV	fes	$P87^{gag-fes}$		17, 59, 65, 96
GA-FeSV	fes	$P108^{gag-fes}$	protein-kinase	97, 99
MS-FeSV	fms	$P180^{gag-fms}$, $gp140^{fms}$, $gp120^{fms}$		100, 101
SSV	sis	$p28^{sis}$		13, 18, 40, 76, 102
		Acute avian leucosis viruses		
AMV	myb	$p30^{myb}$ (predicted)		10, 20, 33
E26	myb			
AEV	erb	$P74^{gag-erbA}$, $p61^{erbB}$		10, 103–105
MC29	myc	$P110^{gag-myc}$	DNA-binding protein	
MH2	myc	$P100^{gag-myc}$		10, 34, 106–108
CM II	myc	$P90^{gag-myc}$		
OK10	myc	$P200^{gag-pol-myc}$		
REV-T	rel			12, 109
		Acute mammalian leucosis virus		
A-MuLV	abl	$P120^{gag-abl}$	protein-kinase	70, 110

* Names and spelling are in most cases in accordance with the guidelines proposed by Coffin et al. [111]

Table II

Transcription of c-onc genes in normal cells and tissues

C-onc gene	RNA copies per cell	Cell or tissue	Notes	References
src	0.5–1.5	14-day-old chicken brain, liver, bone-marrow, bursa, kidney, spleen, lung		10, 11, 47, 112, 113
	>0.3	10-day-old chicken thymus	Unaffected by cellular growth conditions	
	>0.3	14-day-old chicken heart and muscle		
	2–4	chicken and quail embryo fibroblasts		
	2–3	3–6-month-old chicken liver and bursa		
yes	1–5	In all chicken tissues examined except kidney		11
	26	14-day-old chicken kidney		
	>0.3	chicken and quail embryo fibroblasts		
fps	>0.3–1.1	in all chicken tissues examined except bone marrow		11, 47
		14-day-old chicken bone marrow		
	>0.3	chicken and quail embryo fibroblasts		
ros	>0.3–0.7	in all chicken tissues examined except kidney		11
	2.5	14-day-old chicken kidney		
kis		relatively high levels in a rat kidney cell line	110 times increased expression in a rat embryo cell line subjected to anaerobic stress	77, 128
has/bas		expressed at high but similar levels in all stages of prenatal development of the mouse		114, 115
		2 distinct bas-related RNA species were detected in normal human fibroblasts		
mos		transcriptionally silent in all rodent cells and tissues examined	c-mos sequences are hypermethylated	114, 116

fos		significant expression in mouse prenatal development especially in the placenta expressed at low levels in all postnatal tissues; 5–20 times higher levels in bone and skin		114
fes		not detectable in feline embryo cells		125
sis		not detectable in human embryonic fibroblasts		115
myb	1–2	15–19-day old chicken embryo and 1–3-day-old chicken muscle, kidney, liver, spleen and 3–6-month-old chicken liver		47, 117
	1–1.5	chicken embryo fibroblasts		
	9.6	15–19-day-old chicken embryo yolk sac		
	9.1	15–19-day-old chicken embryo bursa		
	21.3	15–19-day-old chicken embryo bone marrow		
	6.7	1–3-day-old chicken bursa		
	20.6	1–3-day-old chicken bone marrow		
	3	3–6-month-old chicken bursa		
erb	2–3	chicken and quail embryo fibroblasts	not detectable in chicken fibroblasts infected with a helper virus	10, 47, 118
	2–4	3–6-month-old chicken bursa and liver		
myc	3–10	chicken and quail embryo fibroblasts, expressed in duck embryos and in human fibroblasts as well		9, 10, 47, 115, 119
	2–5	3–6-month-old chicken liver and bursa		
rel		detectable in turkey, chicken, and quail embryo fibroblasts and in chicken muscle; 2–3 times higher levels in haemopoietic tissues		12, 122
abl		significant transcription during prenatal development of mouse increasing till day 10 and decreasing thereafter; expressed in all postnatal mouse tissues examined	3 times higher levels in spleen and thymus than in other organs, highest level in young adult testes. Normal human fibroblasts contain 2 distinct abl-related RNA species	114, 115

be found between $p60^{src}$ and $p90^{gag-yes}$ proteins, though this homology could not be detected by the commonly used nucleic acid hybridization method [38]. The amino-acid sequence of $p60^{src}$ shows also some relatedness to the $p108^{fes}$, $p130^{fps}$ and $p87^{fes}$ transforming proteins [39, 59]. The activities of these proteins show similarities, having a protein-kinase activity phosphorylating the tyrosine residues of common protein substrates [60–70]. Homology could be detected also between the carboxy-terminal part of $p60^{src}$ and $p37^{mos}$ coded for by Moloney MuSV, though this latter protein shows no protein-kinase activity and its function is not known [29]. It should, however, be noted that a variant of Mo-MuSV known as ts 110 MuSV codes for a gag-mos polyprotein ($p85^{gag-mos}$) which has an associated protein-kinase activity [71].

$p60^{src}$ and $p37^{mos}$ are related to the catalytic subunit of cyclic AMP-dependent protein-kinase isolated from bovine heart muscle [72]. Thus, a range of homology ($p108^{gag-fes}$-$p130^{fps}$-$p87^{gag-fes}$-$p90^{gag-yes}$-$p60^{src}$-$p37^{mos}$-bovine PK) can be constructed.

Similarly, the structurally related p21 proteins coded for by Ha^{ras} and Ki^{ras} onc-genes of the ras family also have similar activities; these are guanine-binding proteins [73, 74] showing a partial amino-acid sequence homology with the nucleotide-binding subunit of mitochondrial and bacterial H^+-ATPase [75].

The predicted sequence of $p28^{sis}$ is identical to the amino-acid sequence of human platelet-derived growth factor in a region of 104 contiguous amino-acids. The significance of this homology has not been clarified [76].

Data referring to transcription of proto-onc genes in normal (non-transformed) cells are summarized in Table II. Experiments were carried out by hybridization of H^3 or P^{32}-labelled cDNA onc probes or cloned c-onc and v-onc gene probes with RNA molecules extracted from cells or tissues. By this method one RNA molecule/cell can be detected (Table II).

On the basis of their transcription activity, proto-onc genes can be grouped as follows. (i) They are not transcribed in any of the examined normal cells (e.g. hypermethylated c-mos gene or probably fes, sis). (ii) They are transcribed at a similar level in all examined cells (e.g. src, myc, probably has). (iii) They show a cell-specific transcription activity (e.g. yes, ros, myb, abl, fos, fps).

It may be supposed that proto-onc genes of group (i) are also transcribed in a phase of development not yet examined.

Transcription of some proto-onc genes can be influenced in vitro (e.g. Ki^{ras} gene is activated by anaerobic stress [77]; c-myc gene is inhibited by DMSO [78]).

Translation of proto-onc genes in normal cells is summarized in Table III. It can be seen that only a few proto-onc genes have been examined in this respect. It is, however, expected that specific (monoclonal) antibodies against

Table III

C-onc gene related translation products in normal cells and tissues

C-onc gene	Protein product	Cell or tissue	Function	References
src	$pp60^{c-src}$	chicken and duck embryo fibroblast ring-necked pheasant cells quail, rat and human fibroblasts, Xiphophorus fish brain	protein-kinase	79, 80, 120
fps	NCP98 ($p98^{c-fps}$)	chicken bone marrow cells contain more than liver or lung	protein-kinase	121
has	$p21^{c-has}$	low levels in rat, mouse, mink, hamster, rabbit, turkey, bat, cat, dog, horse, monkey and human cells; 100 times higher level in 416B mouse haemopoietic precursor cell line	guanine-nucleotide binding protein	74, 123, 124
mos	?	not detectable in uninfected NIH/3T3 cells by anti-$p37^{mos}$ serum		94
sis	?	not detectable in uninfected marmoset cells by anti-$p28^{sis}$ serum		102
abl	NCP150 ($p150^{c-abl}$)	mouse thymus contains more than spleen and bone marrow; not detectable in liver and in mouse fibroblast cultures		126, 127

many viral „transforming proteins" will be produced shortly, and this will allow to detect the normal cell proteins cross-reacting with viral transforming proteins.

Expression of NCP 150^{c-abl}, $p21^{c-has}$, and $NCP98^{c-fps}$ proteins proved to be specific.

$p60^{src}$ protein has been detected in all the vertebrate cells examined except in those of the field vole and hamster; its expression is unaffected by cellular growth conditions [79, 80]. Proteins analogous to $p28^{sis}$ and $p37^{mos}$ could not be detected, in agreement with data of transcription studies.

Thus, some of the known proto-onc gene products can be accepted as differentiation antigens at least by definition. This does not yet mean that we have any knowledge about the mechanisms by which proto-onc gene products may influence cell differentiation. It, however, is supposed that studies referring to the role of proto-onc gene products represent one of the most promising areas not only in carcinogenesis, but also in cell differentiation.

REFERENCES

1. Tooze, J. (ed.): Molecular Biology of Tumour Viruses. Cold Spring Harbor Laboratory, 1973.
2. Graf, T., Beug, H.: Biochim Biophys Acta **516,** 269 (1978).

3. Bishop, J. M.: Ann Rev Biochem **47,** 35 (1978).
4. Klein G. (ed.): Viral Oncology. Raven Press, New York 1980.
5. Coffin, J.: J Gen Virol **42,** 1 (1979).
6. Junghans, R. P., Boone, L. R., Skalka, A. M.: J Virol **43,** 544 (1982).
7. Stehelin, D., Varmus, H. E., Bishop, J. M., Vogt, P. K.: Nature **260,** 170 (1976).
8. Spector, D. H., Varmus, H. E., Bishop, J. M.: Proc Natl Acad Sci USA **75,** 4102 (1978).
9. Sheiness, D., Bishop, J. M.: J Virol **31,** 514 (1979).
10. Roussel, M., Saule, S., Lagrou, C., Rommens, C., Beug, H., Graf, T., Stehelin, D.: Nature **281,** 452 (1979).
11. Shibuya, M., Hanafusa, H.., Balduzzi, P. C.: J Virol **42,** 143 (1982).
12. Wong, W. C., Lai, M. M. C.: Virology **111,** 289 (1981).
13. Wong-Staal, F., Dalla Favera, R., Gelmann, E., Manzari, V., Szala, S., Josephs, S. F., Gallo, R. C.: Nature **294,** 273 (1981).
14. Ellis, R. W., Defeo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R., Scolnick, E. M.: Nature **292,** 506 (1981).
15. Rowley, J. D.: Nature **301,** 290 (1983).
16. Neel, B. G., Jhanwar, S. C., Chaganti, R. S. K., Hayward, W. S.: Proc Natl Acad Sci USA **79,** 7842 (1982).
17. Franchini, G., Even, J., Sherr, C. J., Wong-Staal, F.: Nature **290,** 154 (1981).
18. Dalla Favera, R., Gelmann, E. P., Gallo, R. C., Wong-Staal, F.: Nature **292,** 31 (1981).
19. Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnik, E. M., Lowy, D. R.: Proc Natl Acad Sci USA **79,** 4848 (1982).
20. Klempnauer, K. H., Gonda, T. J., Bishop, M. J.: Cell **31,** 453 (1982).
21. Robins, T., Bister, K., Garon, C., Papas, T., Duesberg, P.: J Virol **41,** 635 (1982).
22. Vennstrom, B., Sheiness, D., Zabielski, J., Bishop, J. M.: J Virol **42,** 773 (1982).
23. Colby, W. W., Chen, E. Y., Smith, D. H., Levinson, A. D.: Nature **301,** 722 (1983).
24. Stanton, L., Watt, R., Marcu, K. B.: Nature **303,** 401 (1983).
25. Van Beveren, C., Van Straaten, F., Curran, T., Müller, R., Verma, I. M.: Cell **32,** 1241 (1983).
26. Chattopadhyay, S. K., Chang, E. H., Lander, M. R., Ellis, R. W., Scolnick, E. M., Lowy, D. R.: Nature **296,** 361 (1982).
27. Watson, R., Oskarsson, M., Vande Woude, G. F.: Proc Natl Acad Sci USA **79,** 4078 (1982).
28. Reddy, E. P., Smith, M. J., Canaani, E., Robbins, K. C., Tronick, S. R., Zain, S., Aaronson, S. A.: Proc Natl Acad Sci USA **77,** 5243 (1980).
29. Van Beveren, C., Galleshaw, J. A., Jonas, V., Berns, A. J. M., Doolittle, R. F., Donoghue, D. J., Verma, I. M.: Nature **289,** 258 (1981).
30. Van Beveren, C., Van Straaten, F., Galleshaw, J. A., Verma, I. M.: Cell **27,** 97 (1981).
31. Dhar, R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D. R., Scolnick, E. M.: Science **217,** 934 (1982).
32. Tsuchida, N., Ryder, T., Ohtsubo, E.: Science **217,** 937 (1982).
33. Rushlow, K. E., Lautenberger, J. A., Papas, T. S., Baluda, M. A., Perbal, B., Chirikjian, J. G., Reddi, E. P.: Science **216,** 1421 (1982).
34. Alitalo, K., Bishop, J. M., Smith, D. H., Chen, E. Y., Colby, W. W., Levinson, A. D.: Proc Natl Acad Sci USA **80,** 100 (1983).
35. Takeya, T., Feldman, R. A., Hanafusa, H.: J Virol **44,** 1 (1982).
36. Takeya, T., Hanafusa, H.: J Virol **44,** 12 (1982).
37. Czernilofsky, A. P., Levinson, A. D., Varmus, H. E., Bishop, J. M., Tischer, E., Goodman, H.: Nature **301,** 736 (1983).
38. Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y., Yoshida, M.: Nature **297,** 205 (1982).
39. Shibuya, M., Hanafusa, H.: Cell **30,** 787 (1982).
40. Devare, S. G., Reddy, E. P., Robbins, K. C., Andersen, P. R., Tronick, S. R., Aaronson, S. A.: Proc Natl Acad Sci USA **79,** 3179 (1982).
41. Hall, A., Marshall, C. J., Spurr, N. K., Weiss, R. A.: Nature **303,** 396 (1983).
42. Bishop, J. M.: Cell **23,** 5 (1981).
43. Weinberg, R. A.: Cell **30,** 3 (1982).
44. Robinson, H. L., Pearson, M. N., Desimone, D. W., Tsichlis, P. N., Coffin, J. M.: Cold Spring Harbor Symp Quant Biol **44,** 1133 (1980).
45. Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J., Astrin, S. M.: Cell **23,** 323 (1981).
46. Payne, G. S., Courteneidge, S. A., Crittenden, L. B., Faldy, A. M., Bishop, J. M., Varmus, H. E.: Cell **23,** 311 (1981).
47. Hayward, W. S., Neel, B. G., Astrin, S. M.: Nature **290,** 475 (1981).
48. Payne, G. S., Bishop, J. M., Varmus, H. E.: Nature **295,** 209 (1982).

49. Oskarsson, M., McClements, W. L., Blair, D., Maizel, J. V., Vande Woude, G. F.: Science **207,** 1222 (1980).
50. Chang, E. H., Furth, M. E., Scolnick, E. M., Lowy, D. R.: Nature **297,** 479 (1982).
51. Tabin C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R., Chang, E. H.: Nature **300,** 143 (1982).
52. Reddy, E. P., Reynolds, R. J., Santos, E., Barbacid, M.: Nature **300,** 149 (1982).
53. Cooper, G. M.: Science **217,** 801 (1982).
54. Rechavi, G., Givol, D., Canaani, E.: Nature **300,** 607 (1982).
55. Crews, S., Barth, R., Hood, L., Prehn, J., Calame, K.: Science **218,** 1319 (1982).
56. Ericson, J., Ar-Rushdi,, A., Drwinga, H. L., Nowell, P. C., Croce, C. M.: Proc Natl Acad Sci USA **80,** 820 (1983).
57. Schwab, M., Alitalo, K., Varmus, H. E., Bishop, J. M., George, D.: Nature **303,** 497 (1983).
58. Boyse, E. A., Old, L. J.: Ann Rev Genet **3,** 269 (1969).
59. Hampe, A., Laprevotte, I., Galibert, F., Fedele, L. A., Sherr, C. J.: Cell **30,** 775 (1982).
60. Collet, M. S., Erikson, R. L.: Proc Natl Acad Sci USA **75,** 2021 (1978).
61. Rübsamen, H., Friis, R. R., Bauer, H.: Proc Natl Acad Sci USA **76,** 967 (1979).
62. Collet, M. S., Purchio, A. F., Erikson, R. L.: Nature **285,** 167 (1980).
63. Hunter, T., Sefton, B. M.: Proc Natl Acad Sci USA **77,** 1311 (1980).
64. Levinson, A. D., Opperman, H., Varmus, H. E., Bishop, J. M.: J Biol Chem **255,** (1980).
65. Reynolds, F. H., Van de Ven, W. J. M., Stephenson, J. R.: Nature **286,** 409 (1980).
66. Levinson, A. D., Courtneidge, S. A., Bishop, J. M.: Proc Natl Acad Sci USA **78,** 1624 (1981).
67. Karess, R. E., Hanafusa, H.: Cell **24,** 155 (1981).
68. McGrath, J. P., Levinson, A. D.: Nature **295,** 423 (1982).
69. Cooper, J. A., Reiss, N. A., Schwartz, R. J., Hunter, T.: Nature **302,** 218 (1983).
70. Cooper, J. A., Hunter, T.: Mol Cell Biol **1,** 394 (1981).
71. Kloetzer, W. S., Maxwell, S. A., Arlinghaus, R. B.: Proc Natl Acad Sci USA **80,** 412 (1983).
72. Barker, W. C., Dayhoff, M. O.: Proc Natl Acad Sci USA **79,** 2836 (1982).
73. Shih, T. Y., Papageorge, A. G., Stokes, P. E., Weeks, M. O., Scolnick, E. M.: Nature **287,** 686 (1980).
74. Papageorge, A. G., Lowy, D. R., Scolnick, E. M.: J Virol **44,** 509 (1982).
75. Gay, N. J., Walker, J. E.: Nature **301,** 262 (1983).
76. Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C. H., Huang, J. S., Deuel, T. F.: Nature **304,** 35 (1983).
77. Anderson, G. R., Matovcik, L. M.: Science **197,** 1371 (1977).
78. Westin, E. H., Wong-Staal, F., Gelmann, E. P., Dalla Favera, R., Eva, A., Reddy, E. P., Tronick, S. R., Aaronson, S. A., Gallo, R. C.: Proc Natl Acad Sci USA **79,** 2490 (1982).
79. Opperman, H., Levinson, A. D., Varmus, H. E., Levintow, L., Bishop, J. M.: Proc Natl Acad Sci USA **76,** 1804 (1979).
80. Collett, M. S., Brugge, J. S., Erikson, R. L.: Cell **15,** 1363 (1978).
81. Kawai, S., Yoshida, M., Segawa, K., Sugiyama, H., Ishizaki, R., Toyoshima, K.: Proc Natl Acad Sci USA **77,** 6199 (1980).
82. Adkins, B., Hunter, T.: Mol Cell Biol **2,** 890 (1982).
83. Feldman, R. A., Hanafusa, T., Hanafusa, H.: Cell **22,** 757 (1980).
84. Neel, B. G., Wang, L. H., Mathey-Prevot, B., Hanafusa, T., Hanafusa, H., Hayward, W. S.: Proc Natl Acad Sci USA **79,** 5088 (1982).
85. Adkins, B., Hunter, T., Beemon, K.: J Virol **41,** 767 (1982).
86. Shibuya, M., Hanafusa, T., Hanafusa, H., Stephenson, J. R.: Proc Natl Acad Sci USA **77,** 5636 (1980).
87. Wang, L-H., Feldman, R., Shibuya, M., Hanafusa, H., Notter, M. F. D., Balduzzi, P. C.: J Virol **40,** 258 (1981).
88. Feldman, R. A., Wang, L. H., Hanafusa, H., Balduzzi, P. C.: J Virol **42,** 228 (1982).
89. Wang, L. H., Hanafusa, H., Notter, M. F. D., Balduzzi, P. C.: J Virol **41,** 833 (1982).
90. Adkins, B., Hunter, T., Sefton, B. M.: J Virol **43,** 448 (1982).
91. Shih, T. Y., Weeks, M. O., Young, H. A., Scolnick, E. M.: Virology **96,** 64 (1979).
92. Gonda, M., Young, H. A., Elser, J. E., Rasheed, S., Talmadge, C. B., Nagashima, K., Li, C-C., Gilden, R. V.: J Virol **44,** 520 (1982).
93. Curran, T., Peters, G., Van Beveren, C., Teich, N. M., Verma, I. M.: J Virol **44,** 674 (1982).
94. Papkoff, J., Verma, I. M., Hunter, T.: Cell **29,** 417 (1982).
95. Donoghue, D. J., Hunter, T.: Nuc Acids Res **10,** 2549 (1982).
96. Van de Ven, W. J. M., Reynolds, F. H., Stephenson, J. R.: Virology **101,** 185 (1980).
97. Barbacid, M., Beemon, K., Devare, S. G.: Proc Natl Acad Sci USA **77,** 5158 (1980).
98. Papkoff, J., Hunter, T.: J Virol **43,** 533 (1982).

99. Chen, A. P., Essex, M., Shadduck, J. A., Niederkorn, J. Y., Albert, D.: Proc Natl Acad Sci USA **78,** 3915 (1981).
100. Anderson, S. J., Furth, M., Wolff, L., Ruscetti, S. K., Sherr, C. J.: J Virol **44,** 696 (1982).
101. Donner, L., Fedele, L. A., Garon, C. F., Anderson, S. J., Sherr, C. J.: J Virol **41,** 489 (1982).
102. Robbins, K. C., Devare, S. G., Reddy, E. P., Aaronson S. A.: Science **218,** 1131 (1982).
103. Privalsky, M. L., Bishop, J. M.: Proc Natl Acad Sci USA **79,** 3958 (1982).
104. Anderson, S. M., Hanafusa, H.: Virology **121,** 32 (1982).
105. Bister, K., Duesberg, P. H.: Proc Natl Acad Sci USA **76,** 5023 (1979).
106. Donner, P., Greiser-Wilke, I., Moellig, K.: Nature **296,** 262 (1982).
107. Hayman, M. J., Kitchener, G., Graf, T.: Virology **98,** 191 (1979).
108. Ramsay, G., Hayman, M. J., Bister, K.: EMBO J **1,** 1111 (1982).
109. Chen, I. S. Y., Temin, H. M.: Cell **31,** 111 (1982).
110. Witte, O. N., Dasgupta, A., Baltimore, D.: Nature **283,** 826 (1980).
111. Coffin, J. M., Varmus, H. E., Bishop, J. M., Essex, M., Hardy, W. D., Martin, G. S., Rosenberg, N. E., Scolnick, E. M., Weinberg, R. A., Vogt, P. K.: J Virol **40,** 953 (1981).
112. Spector, D. H., Smith, K., Padgett, T., McCombe, P., Roulland-Dussoix, D., Moscovici, C., Varmus, H. E., Bishop, J. M.: Cell **13,** 371 (1978).
113. Spector, D., Baker, B., Varmus, H. E., Bishop, J. M.: Cell **13,** 381 (1978).
114. Müller, R., Slamon, D. J., Trembly, J. M., Cline, M. J., Verma, I. M.: Nature **299,** 640 (1982).
115. Eva, A., Robbins, K. C., Andersen, P. R., Srinivasan, A., Tronick, S. R., Reddy, E. P., Ellmore, N. W., Galen, A. T., Lautenberger, J. A., Papas, T. S., Westin, E. H., Wong-Staal, F., Gallo, R. C., Aaronson, S. A.: Nature **295,** 116 (1982).
116. Gattoni, S., Kirschmeier, P., Weinstein, B., Escobedo, J., Dina, D.: Mol Cell Biol **2,** 42 (1982).
117. Chen, J. H.: J Virol **36,** 162 (1980).
118. Sheiness, D., Vennstrom, B., Bishop, J. M.: Cell **23,** 291 (1981).
119. Sheiness, D. K., Hughes, S. H., Varmus, H. E., Stubblefield, E., Bishop, J. M.: Virology **105,** 415 (1980).
120. Schartl, M., Barnekow, A., Bauer, H., Anders, F.: Cancer Res **42,** 4222 (1982).
121. Mathey-Prevot, B., Hanafusa, H., Kawai, S.: Cell **28,** 897 (1982).
122. Chen, I. S. Y., Wilhelmsen, K. C., Temin, H. M.: J Virol **45,** 104 (1983).
123. Langbeheim, H., Shih, T. Y., Scolnick, E. M.: Virology **106,** 292 (1980).
124. Scolnick, E. M., Weeks, M. O., Shih, T. Y., Ruscetti, S. K., Dexter, T. M.: Mol Cell Biol **1,** 66 (1981).
125. Frankel, A. E., Gilbert, J. H., Porzig, K. J., Scolnick, E. M., Aaronson, S. A.: J Virol **30,** 821 (1979).
126. Witte, O. N., Rosenberg, N., Baltimore, D.: Nature **281,** 396 (1979).
127. Ponticelli, A. S., Whitlock, C. A., Rosenberg, N., Witte, O. N.: Cell **29,** 953 (1982).
128. Scolnick, E. M., Maryak, J. M., Parks, W. P.: J Virol **14,** 1435 (1974).

NOTE ADDED IN PROOF

Since the completion of the manuscript the following results have been published: v-erbB codes for a truncated epidermal growth factor receptor [Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., Waterfield, D. M.: Nature **307,** 521 (1984)] and the p21 proteins encoded by v-has, v-kis and c-has/bas genes show amino acid sequence homologies with elongation factor-Tu [Leberman, R., Egner, U.: EMBO J. **3,** 339 (1984)]; c-kis is expressed ubiquitously at similar level in all mouse tissues examined, while c-fms is expressed at elevated levels in the placenta and the extraembryonal membranes [Müller, R., Slamon, D. J., Adamson, E. D., Tremblay, J. M., Müller, D., Cline, M. J., Verma, I. M.: Molec Cell Biol **3,** 1062 (1983)]. Transcripts of c-ras increase concomitantly with the burst of DNA synthesis in regenerating rat liver and rapidly return to basal levels [Govette, M., Petropoulos, C. J., Shank, P. R., Fausto, N.: Science **219,** 510 (1983)]. Mitogen stimulation dramatically elevates c-myc mRNA levels in lymphocytes and fibroblasts [Kelly, K., Cochran, B. H., Stiles, C. D., Leder, P.: Cell **35,** 603 (1983)]; $pp60^{c\text{-}src}$ is expressed at high levels in neural tissues (Cotton, P. C., Brugge, J. S.: Molec Cell Biol **3,** 1157 (1983)] and its expression coincides with the onset of differentiation of neuronal cells [Sorge, L. K., Levy, B. T., Maness, P. F.: Cell **36,** 249 (1984)]. Normal mouse amnion cells contain high levels of $p55^{c\text{-}fos}$, a nuclear protein, the product of c-fos gene [Curran, T., Miller, A. D., Zokas, L., Verma, I. M.: Cell **36,** 259 (1984)].

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EFFECT OF THE CARBAMATE INSECTICIDE SEVIN® ON *ANABAENA* SP. AND *WESTIELLOPSIS PROLIFICA*

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Effect of the insecticide Sevin® (50% w/v) was examined on survival, growth and nitrogen fixation of two filamentous blue-green algae, *Anabaena* sp. and *Westiellopsis prolifica*. Lower concentration of the insecticide (10 μg/ml) increased survival, growth and nitrogen fixation while higher concentrations showed an inhibitory effect.

The increasing use of pesticides in agriculture is likely to have an impact on the soil microflora, particularly on blue-green algae whose role in soil fertility is well known [1, 2], consequently attention has turned to this aspect [3–8]. Blue-green algae in general have been found to show a high degree of tolerance to many of the agricultural chemicals although their tolerance limits [9, 10] vary widely. Sevin® (carbaryl-1-naphthyl methyl carbamate) is becoming of increasing importance as a possible replacement for DDT, since the latter tends to accumulate in the environment and along food chains. The present study deals with the effect of the carbamate insecticide Sevin® on the growth and nitrogen fixation of two rice field blue-green algae *Anabaena* sp. and *Westiellopsis prolifica*.

Materials and methods

Pure cultures of the blue-green algae *Anabaena* sp. and *W. prolifica* Janet were used as the experimental materials. *W. prolifica* was grown in Allen and Arnon's nitrogen free medium [11] with micro-nutrients at the concentration recommended by Fogg [12]. For *Anabaena* sp. the medium C of Kratz and Myers [13] was used. The cultures were maintained at 28 ± 2 °C under 2200 lux provided by daylight fluorescent tubes. Experiments were conducted by inoculation of actively growing algae into 10 ml of culture solution in 15 × 150 mm Corning test tubes. Commercial grade Sevin® (Union Carbazide Limited, India) containing 50% w/v active ingredients was used as the insecticide. Its stock solution was prepared freshly for experiments in sterilized media and added aseptically to the culture media to obtain the desired concentration. The pH of all the test media was adjusted to 7.8.

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Growth of algae was estimated in terms of the absorbancy of the acetone extract in an Erma photoelectric colorimeter at 660 nm. Heterocyst frequency of *Anabaena* sp. was expressed as the percentage of vegetative cells in the filaments. Total nitrogen was estimated at 3-day intervals during the 15 days of incubation. The cultures were subjected to Kjeldahl digestion and total nitrogen was estimated according to Friedrich as modified by Herbert et al [14]. Corrections were made for the nitrogen present in the insecticide.

Results and discussion

The effect of different concentrations of Sevin® (10–1500 μg/ml) on the survival of *Anabaena* sp. and *W. prolifica* shown in Table I indicates a progressive decrease in survival with increasing concentrations of the pesticide. At 500 μg/ml the insecticide could kill 70% and 90% of the population of *W. prolifica* and *Anabaena*, respectively; 1000 μg/ml of Sevin® was algistatic for *Anabaena* sp. but still higher concentrations (1500 μg/ml) were algicidal for *Anabaena* sp. and *W. prolifica*. The time course growth in different pesticide concentrations showed a prolongation of the lag phase particularly at higher concentrations (>50 μg/ml; Table II). There was also a perceptible inhibition of heterocysts in *Anabaena* with increasing concentrations of the insecticide (Table III). At 500 μg/ml heterocysts were completely suppressed. The trend in *W. prolifica* was similar. While higher concentrations of the pesticide (>50 μg/ml) inhibited the nitrogen-fixing potential of both organisms, it was hardly affected at 10 μg/ml (Table IV). These results were similar to those of other workers [8, 15].

The Sevin® dose recommended to control rice pests is 1.0–1.5 Kg/hectare which will be around 4–12 ppm in the rice fields. Since the toxic dose of Sevin® used in these experiments is >10 μg/ml, these concentrations may not affect the growth and nitrogen fixation of blue-green algae in field conditions.

Table I

Percent survival of Anabaena sp. and W. prolifica in the presence of different concentrations of Sevin® in the medium

Concentration of Sevin® μg/ml	Percent survival	
	Anabaena sp.	*W. prolifica*
10	100	100
20	85	90
50	55	75
100	26	52
200	20	45
500	10	30
1000	0.8	5
1500	0	0

Cultures were incubated at 28 ± 2 °C under 2200 lux for 15 days

Table II

Growth in terms of optical density of acetone dissolved pigments at 660 nm of Anabaena sp. and W. prolifica in the presence of various concentrations of Sevin®

Concentration of Sevin® μg/ml	*Anabaena* sp.					*W. prolifica*				
	days of incubation					days of incubation				
	3	6	9	12	15	3	6	9	12	15
0 (control)	0.08	0.15	0.3	0.4	0.44	0.04	0.1	0.13	0.15	0.17
10	0.1	0.2	0.31	0.42	0.46	0.05	0.12	0.135	0.145	0.16
50	0.02	0.11	0.27	0.35	0.39	0.025	0.065	0.1	0.12	0.13
100	0.01	0.03	0.04	0.15	0.22	0.02	0.03	0.075	0.095	0.11
500	0.01	0.01	0.01	0.015	0.018	0.01	0.01	0.03	0.05	0.06

Table III

Effect of different concentrations of Sevin® on heterocyst frequency (per cent of cells) of Anabaena sp.

Concentration of Sevin® μg/ml	Days of incubation				
	3	6	9	12	15
0 (control	6.2	6.5	5.4	5.0	5.3
10	6.5	6.7	5.9	5.85	5.7
50	5.8	5.0	4.4	3.9	3.2
100	4.0	1.9	1.7	1.0	0.8
500	3.0	0	0	0	0

Table IV

Effect of different concentrations of Sevin® on the total nitrogen content (mg/10 ml) of Anabaena sp. and W. prolifica cultures

Concentration of Sevin® μg/ml	*Anabaena* sp.					*W. prolifica*				
	days of incubation					days of incubation				
	3	6	9	12	15	3	6	9	12	15
0 (control)	0.7	1.3	1.8	2.4	3.8	0.3	0.45	0.7	1.2	1.5
10	0.5	1.0	1.6	2.5	4.0	0.25	0.5	0.8	1.25	1.35
50	0.2	0.25	0.6	1.2	2.0	0.2	0.4	0.5	0.6	0.75
100	0.2	0.22	0.25	0.5	1.3	0.15	0.2	0.4	0.5	0.6
500	0.18	0.2	0.2	0.22	0.22	0.1	0.1	0.12	0.12	0.12

At higher concentrations (>50 μg/ml), however, the nitrogen fixing ability of blue-green algae decreased considerably and thus excessive use of the insecticide may well affect the overall nitrogen economy of rice-field soils.

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REFERENCES

1. Singh, R. N.: The role of blue-green algae in nitrogen economy of Indian Agriculture. I. C. A. R. Monograph on Algae. New Delhi 1961.
2. Watanabe, A.: J Gen Appl Microbiol **8,** 85 (1962).
3. Lundqvist, I.: Svensk Bot Tidskr **64,** 460 (1970).
4. Batteton, J. C., Boush, G. M., Matsumura, F.: Bull Environ Cont Toxicol **6,** 589 (1971).
5. Singh, P. K.: Arch Microbiol **89,** 317 (1973).
6. Singh, P. K.: Arch Microbiol **97,** 69 (1974).
7. Da Silva, E. J., Henriksson, L. E., Henriksson, E.: 4th Int. Conf. Global Impacts Appl. Microbiol., São Paulo, 1973. Abstr. No. 14. pp. 3.
8. Da Silva, E. J., Henriksson, L. E., Henriksson, E.: Arch Environ Cont Toxicol **3,** 193 (1975).
9. Venkataraman, G. S.: Algal Bio-Fertilizer and Rice Cultivation, Today and Tomorrows. Printers and Publishers, New Delhi 1972.
10. Venkataraman, G. S., Rajyalakshmi: Ind J Agric Sci **42,** 119 (1972).
11. Allen, M. B., Arnon, D. I.: Plant Physiol **30,** 366 (1955).
12. Fogg, G. E.: Ann Bot N S **13,** 241 (1949).
13. Kratz, W. A., Myers, J.: Amer J Bot **42,** 282 (1955).
14. Herbert, D., Phipps, P. J., Strange, R. E.: *In* Norris, J. R., Ribbons, N. W. (eds): Methods in Microbiology. Vol. 5 B Academic Press, New York 1971. pp 210–344.
15. Kar, S., Singh, P. K.: Bull Environ Cont Toxicol **20,** 707 (1978).

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THE FAECAL ACTINOMYCETE FLORA OF *PROTRACHEONISCUS AMOENUS* (WOODLICE: *ISOPODA*)

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Following a marked order of preference, adults of *Protracheoniscus amoenus* consumed in preference tests the fallen, weathered leave species of forest litter much more selectively than those of the millipede *Chromatoiulus projectus*. Under natural circumstances, the individuals (prejuveniles, juveniles, young adults and adults) of these two species live frequently together in the forest litter-layer, feeding on weathered leaves of the very same tree species and placing their faecal pellets in the vicinity of each other. Accordingly, there is a high probability of mutual contamination with their faecal bacteria. In contrast, promicromonospora-type intestinal actinomycetes characterized by cell-wall composition type VI colonize only the faeces of *Ch. projectus*, and are completely lacking of the dropped pellets of woodlice. Diverse species of streptomycetes were detected in the fresh faecal matter of both litter-dwelling animal species, but presumably they were only survivors of gut passage and passive travellers through the digestive canal.

The genera *Oerskovia* and *Promicromonospora*, together with the non-motile oerskovia-like organisms may at present be regarded from both the taxonomic and evolutionary points of view, as one of the most problematic groups within the order *Actinomycetales* [1]. In older cultures, the substrate hyphae of these organisms break into bacterium-like cylindrical and spherical bodies producing, very rarely if at all, single spores of unknown structure and development, and diamino-pimelic acid is completely lacking in their cell-wall components (cell-wall type VI). These poorly known, partly facultatively anaerobic procaryotes might perhaps belong to a new family [1] of utmost taxonomic importance within the *Actinomycetales*, connecting evolutionally the true bacteria with the typical actinomycetes which produce branching hyphal filaments and conidiophores. Until recently, in culture collections and

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research laboratories there have been few available strains and those were without exception random, single laboratory and soil isolates. Consequently, nothing is known of their role and ecological importance in the biosphere and of their true natural habitats.

While studying the faecal actinomycetes of soil invertebrates feeding on forest litter [2–4], we have succeeded in isolating from freshly dropped pellets of millipedes *(Diplopoda)* large numbers of strains of a new species of *Promicromonospora (P. enterophyla)* [5]. This finding was the first one on the true natural habitats researched for a long time of oerskovia-promicromonospora-type microbes. The aim of the present work was to contribute to the ecology of these almost unknown organisms and to provide data on the microbiology of *Protracheoniscus amoenus*, an important woodlice species, which has a considerable part in the physical destruction of plant remainders in many forest ecosystems.

Materials and methods

Sampling site. Adults of *Protracheoniscus amoenus* and *Chromatoiulus projectus*, as well as fallen leaves of trees used for feeding experiments, were collected from the A_0- and A_F-horizons of a brown forest soil under a mixed forest stand, predominated by hornbeam and oak ssp., in the Vértes mountains in West Hungary.

Feeding experiments were carried out simultaneously with specimens of *P. amoenus* and *Ch. projectus*, under laboratory conditions, in the dark, with 3 parallels, in nylon mesh covered earthenware pots ($18 \times 18 \times 5$ cm) kept in moistened sand. In each pot 50 weighed animals were placed and fed one g air dry leaf matter moistened by spraying with distilled water. At the end of each feeding run of 10 days duration, the remained leaf matter was collected, weighed, and the amount of the consumed litter food was calculated. Results were expressed in mg of consumed leaf litter per day or month per g live body weight.

Isolation of strains. Freshly collected animal specimens were cleaned superficially from coarse soil particles and placed on a sterile wire screen, which allowed their faeces to fall into a sterile dish. From the fresh faecal pellets serial dilutions were prepared and spread in Petri dishes onto the surface of starch–casein (soluble starch, Difco, 1.0%; casein, 0.1%; Na_2HPO_4 0.05%; agar, 1.5%; pH 7.0) and starch–yeast extract (soluble starch, Difco, 2.0%; yeast extract, 2.0%; agar, 1.5%; pH 7.0) agar plates. After 17 days incubation at 28 °C, the number of colonies were counted and the total number of microbes (bacteria + actinomycetes) calculated in relation to 1 g fresh moist faecal matter. At the same time isolations were made on a random basis. Colonies of actinomycetes and also of bacteria the appearance of which showed a certain similarity to that of the bacteroid actinomycetes were transferred onto agar slopes for further studies.

Identification of strains. Streptomyces strains were taxonomically identified by using the methods and descriptive criteria of the International Streptomyces Project (ISP) and the determining key compiled by Szabó et al. [6] in relation to species redescribed by the collaborators of ISP. To identify promicromonospora-type isolates by direct comparison, the type strain (ATCC 15908 = USSR RIA-562) of *P. citrea* was used; the latter was received directly from the ATCC.

Results and discussion

Among the specimens collected the average live body weight of the adults of *P. amoenus* proved to be 21.9 ± 2.5 mg (Table I). It has to be noted that the range of weight distribution of *Isopoda* species varied considerably

according to habitat, time of sampling, etc. [7]. The mean weight of *Ch. projectus* adults collected at the same site was 108.9 ± 6.6 mg. In contrast, the amount of consumed mixed leaf-litter matter calculated for one woodlice specimen per month was comparatively higher (76.6 ± 2.7 mg) than that for a millipede (122.6 ± 7.4 mg, Table I). In preference tests, the specimens of *P. amoenus* were feeding more selectively than those of *Ch. projectus*, and consumed from the offered weathered Turkey-oak, beech and English oak litter very little or nothing at all (Table I).

At present we do not know how the particular orders of food preference of these animals demonstrated by different authors [8, 9] depend on the activities and composition of the gut flora. The role of the microbial factor in selecting leaf specimens might be important since apart from the fact that bacteria can multiply during passage of food through the gut e.g., the terrestrial isopod *Tracheoniscus rathkei* can intensively digest the resident gut microorganisms particularly under conditions of starvation [10]. Utilization of the microbial biomass may be important for woodlice, the less differentiated gut of which is simple tube-like in nature and not constantly anaerobic. It was suggested that the gut microbiota of woodlice competes for readily digestible substrates and does not significantly facilitate the digestion of more resistant plant constituents [11].

In general, aerobic and facultatively anaerobic bacteria are absolutely predominant elements in the gut flora of woodlice. Their total number including actinomycetes, calculated from plate count estimates related to starch yeast extract agar per one g moist, fresh faecal pellets of the adults of *P. amoenus*, averaged about 5×10^7. According to Reyes and Tiedje [10], in the gut contents of *T. rathkei* no obligate anaerobes were detected, since all organisms that grew during the initial anaerobic incubation also grew aerobically.

Although under laboratory conditions we found considerable differences in food selection of these two animal species, they feed under natural circumstances mostly on the same leaf-matter, namely on the locally common weathered hornbeam, and less frequently on sessile oak leaves, which are favourite food-sources for both of them. Consequently, in the litter layer of the sampling area, their feeding activities, regarding the qualitative composition of the consumed food-materials, seem to be similar. The composition of the actinomycete fraction of the faecal community found in the freshly dropped pellets showed, however, great qualitative and quantitative differences. While in the pellets of *Ch. projectus* the maximum number of actinomycetes counted on starch-casein agar plates approximated 34% of the total flora, these microbes occurred sporadically in the faeces of *P. amoenus*. The large actinomycete fraction in the millipede faeces consisted of a nearly homogeneous population of a single species of promicromonospora-type, true gut-inhabitants which were completely lacking in the faeces of the woodlice. Not one single strain

Table I

A comparison between selected feeding biological parameters and the composition of faecal

Species	Average live body weight of one adult specimen, mg	Feeding experiments			
		Linden-litter freshly fallen	Hornbeam-litter freshly fallen	Hornbeam-litter weathered	English-oak-litter weathered
		Amount of consumed leaf-litter in percent related the weathered			
P. *amoenus*	21.9 ± 2.5* (30.9)**	122.6	51.1	100	0.0
Ch. projectus	108.9 ± 6.6* (200.7)**	30,7	20,5	100	17,6

of the monospored, facultatively anaerobic, bacteroid-like, enteral actinomycetes characterized by a cell-wall composition of type VI was isolated from *P. amoenus* pellets. This finding was corroborated by Prof. J. M. Anderson (Exeter, England) who studied the intestinal tracts and contents of millipedes and woodlice by direct electronmicroscopy. He never found monospored branching actinomycete hyphae on the intestinal structures of woodlice, but did so frequently on those of millipedes (personal communication, Leuven-La-Neuve, 1982). Although further studies seem to be necessary, our present knowledge [2–5] allows to assume the promicromonospora-type gut actinomycetes have adapted themselves during their evolutionary history very closely to the intestinal milieu of millipedes, but do not occur either in true soil habitats or in the intestine of many litter dwelling invertebrates belonging to other large groups of the soil fauna.

A few streptomycete strains were isolated from both millipede and woodlice pellets. At our sampling site, the faecal streptomycete flora of *P. amoenus* was composed mostly of strains of *Streptomyces pluricolorescens*, *S. moderatus*, *S. spadicis*, *S. humidus* and *S. nodosus*. The total number of these species and that of the total actinomycete fraction in the microbial community of the woodlice faeces were practically inestimable. The faecal *S. spadicis* strains produced grey spore-mass colour, rectus-type sporophores, smooth spores (observed in electronmicroscope), melanoid pigments only in pepton-iron agar, and except saccharose, they utilized all diagnostic carbon sources (D-glucose, L-arabinose, D-xylose, i-inositol, D-mannitol, D-fructose, rhamnose and raffinose). Strains of *S. pluricolorescens* produced red aerial mycelium, rectus-type sporophores, red endopigment in substrate mycelium, no melanoid pigments, and they utilized D-glucose, L-arabinose, D-xylose, D-mannitol and

actinomycete floras of adults of P. amoenus and Ch. projectus

(preference tests)				Actinomycetes in fresh faecal pellets	
Sessile oak-litter weathered	Turkey oak-litter weathered	Beech-litter weathered	Consumed mixed litter in mg/one animal/month (September)	Promicromonospora type organisms characterized by cell-wall composition type VI	Streptomycetes
hornbeam litter consumed as 100 percent					
49.4	1.7	0.3	76.6 ± 2.7	Completely lacking	Sporadically occurring organisms
17.0	14.8	15.9	122.6 ± 7.4	Common, codominant organisms	Rare elements of the faecal community

* Average body weight of specimens involved in feeding experiments
** Average body weight of adults calculated on basis of Hungarian data

rhamnose. In the presence of i-inositol, raffinose and sucrose, they did not show any development. In contrast to the typical-strains of this species, our faecal isolates were unable to utilize D-fructose. Only one *S. moderatus* strain (No. Ta-A-10) obtained from woodlice faeces could utilize except sucrose all diagnostic C-sources, and produced red spore-mass, rectus-flexibilis type sporophores, smooth spores, red endopigment in substrate hyphae and no melanoid pigments. Similarly, we isolated only a single *S. humidus* strain (Ta-A-14) which showed in many respects a similarity to strain Ta-A-10 of *S. moderatus*. These two species are perhaps very closely related organisms. Finally, the only *S. nodosus* faecal strain (Ta-A-18) proved, as did many strains of this species, to be D-glucose, D-xylose, i-inositol, D-mannitol, D-fructose, and rhamnose positive and L-arabinose, raffinose, sucrose, and melanoid negative. It developed grey aerial mycelium, spiral sopophores, smooth spores and yellow-brown substrate mycelium. These *Streptomyces* species are widely distributed soil inhabiting organisms which have never been observed in large numbers in animal droppings. This fact, together with their observed low population density in the fresh faecal matter of woodlice make it probable that the streptomycetes do not belong to the resident gut bacterial population of *P. amoenus*. A similar statement was also drafted by us [2] on the relation between streptomycetes and millipedes. In contrast to millipedes in the intestine of *P. amoenus*, neither streptomycetes nor other actinomycetes can form an active, multiplying population. The isolated faecal *Streptomyces* strains can only be considered passive survivors of the passage of food through the intestine.

Adults of *P. amoenus* and *Ch. projectus* live and feed together in the very same forest litter habitat. Fresh faecal pellets of *Ch. projectus* containing

promicromonospora-type organisms frequently occur in large masses in the proximity of moving and feeding specimens of *P. amoenus*. Therefore, there is a high probability of rapid contamination of the woodlice and its food with cells of promicromonosporae. The gut content of *P. amoenus* remains, however, completely free from this type of true gut actinomycetes.

REFERENCES

1. Lechevalier, H. A., Lechevalier, M. P.: *In* Starr, M. P., Stolp, H., Trüger, H. G., Balows, A., Schlegel, H. G. (eds): The Prokaryotes. A Handbook on Habitats, Isolation and Identification of Bacteria. Vol. II. Springer Verlag, Berlin—Heidelberg—New York 1981. pp. 2118–2123.
2. Dzingov, A., Márialigeti, K., Jáger, K., Contreras, E., Kondics, L., Szabó, I. M.: Pedobiologia **24,** 1 (1982).
3. Contreras, E.: The intestinal microflora of millipedes. Thesis, Budapest 1983.
4. Szabó, I. M., Jáger, K., Contreras, E., Márialigeti, K., Dzingov, A., Barabás, Gy., Pobozsny, M.: In Lebrun, Ph., André, H. M., De Medts, A., Grégoire-Wibo, C., Wauthy, G. (eds): New Trends in Soil Biology. Ottignies-Louvain-La-Neuve (Belgium)1983. pp. 197–206.
5. Jáger, K., Márialigeti, K., Hauck, M., Barabás, Gy.: Int J Syst Bact **33,** 525 (1983).
6. Szabó, I. M., Marton, M., Buti, I., Fernandez, C.: Acta Botan Acad Sci Hung **21,** 387 (1975).
7. Snider, R., Shaddy, J. H.: Pedobiologia **20,** 394 (1980).
8. Pobozsny, M.: The influence of soil fauna on the decomposition of litter in two deciduous forest-stand. Thesis, Budapest 1980.
9. Neuhauser, E. F., Hartenstein, R.: Pedobiologia **18,** 99 (1978).
10. Reyes, V. G., Tiedje, J. M.: Pedobiologia **16,** 67 (1976).
11. Reyes, V. G., Tiedje, J. M.: Soil Biol Biochem **8,** 103 (1976).

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ETHANOL-SOLUBLE SUBSTANCES ISOLATED FROM *ESCHERICHIA COLI* CULTURE FILTRATE INDUCE POLYMORPHONUCLEAR CHEMOTAXIS

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Substances chemotactic (CT) for human PMN were isolated from the culture medium of *Escherichia coli* O2 : K1 grown to stationary phase. The ethanol soluble fraction of the lyophilized culture filtrate, which contained the bulk of CT activity, was gel filtrated on Sephadex G-10. The 5×10^3 molecular weight fraction showed the highest CT activity. On the basis of partial chemical characterization the active CT substances probably consist of glycopeptides and lipids originating from the cell envelope.

Chemotactic substances inducing the directed migration of leukocytes in the absence of serum, have been isolated from the filtrates of a variety of bacterial culture media [1–12]. These behaved as low molecular weight (in the range of 10^2–10^3) substances. Though these agents have not been purified to homogeneity, some of them have been found to be proteins, peptides, or lipids.

Considering the complex nature of bacterial cells, it is probably simplistic to think that the chemoattractant effect is due to a single compound in each case. Furthermore, it is known that under various conditions Gram-negative bacteria shed amphiphile structures (e.g. lipopolysaccharides, lipoproteins, enterobacterial common antigens) from their envelope into the surrounding medium during division [13–21].

The above data are consistent with the supposition that the chemotactic substances derive from the bacterial envelope (membrane and/or wall).

Attempts to isolate and characterize the low-molecular weight, chemoattractant material present in the culture medium of *Escherichia coli* are the subject of this report.

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Materials and methods

Isolation of chemotactic factors from the culture medium. E. coli 02 : K1, NCTC 9002 (kindly supplied by Dr. B. Lányi, National Institute of Hygiene, Budapest) was cultured for 16 h to stationary phase in a minimal medium [22]. On every occasion, 20 litres of culture was centrifuged to remove cells. The supernatant was filtered by passage through a membrane filter (0.45 μm pore size, Sartorius, Göttingen, GFR). The filtrate was lyophilized (this preparation will be referred to as supernatant, SN). A part of the lyophilized powder was extracted twice with 80% ethanol (24 °C, 16 h). The extract was concentrated by rotary evaporation (at max. 40 °C) and the residue was dissolved in distilled water (ethanol extract, ESN). Desalting and fractionation of ESN was performed by gel filtration on Sephadex G-10 column with distilled water as eluant. The fractions were lyophilized. In chemotaxis assays the lyophilized powder of each preparation was freshly dissolved in Gey's solution.

Isolation of polymorphonuclear (PMN) leukocytes. Human PMN cells were obtained by 6% dextran sedimentation (at 37 °C for 30 min) of heparinized samples of venous blood. The cells of the upper layer were collected and washed with Gey's solution. Remaining red cells were removed by NH_4Cl–EDTA lysis. PMN cells were suspended in Gey's solution (10^7 cells/ml containing over 95% neutrophils).

Measurement of chemotaxis. Chemotaxis was studied by the method of Boyden [23]. as modified by Lukács et al. [24]. PMN suspension containing 1.0% bovine serum albumin was placed into the upper chamber and the chemoattractant was filled into the lower chamber. They were separated by a membrane filter (3 μm pore diameter, Sartorium, Göttingen, GFR). After incubation for 45 min at 37 °C in humidified air containing 5% CO_2, the filters were removed, fixed, stained and mounted on microscopic slides. Duplicate samples were run in each case and the distance (in μm) of the leading front of cells was measured at 50fold magnification. Ten randomly chosen fields were read for each filter.

Analytical methods. Protein, carbohydrate (hexose) and lipid contents were measured by standard methods [25–27]. Amino acid analysis of acid hydrolysates was performed using a Durrum D-500 amino acid analyser. Thin-layer chromatography was performed on Kieselgel 60 precoated sheets (Merck). The developing solvent 1 for one dimensional chromatography was chloroform-methanol-water (65 : 35 : 1, v/v). The two dimensional chromatogram was developed first in solvent 2 (chloroform-methanol-water-ammonia, 130 : 70 : 8 : 0.5, v/v), then in the second direction in solvent 3 (chloroform-methanol-acetic acid-acetone-water, 100 : 20 : 20 : 40 : : 10, v/v). The chromatograms were visualized by iodine vapour and ninhydrin or phosphomolybdenic acid spray.

Results

Chemotactic activity of the materials from the bacterial supernatant. Cell-free SN of stationary cultures of *E. coli* 02 : K1 contained 75 μg protein/ml (average of 5 experiments). Since the filtrate did not exhibit significant UV-light absorption around 270 nm, cell lysis did not occur. This material showed chemotactic activity for human PMN.

Ethanol extraction of the lyophilized filtrate was performed in order to remove high molecular weight substances, extracellular particles and salts. This procedure considerably reduced the salt content (ash in Table II). In our experience high salt concentration reduced chemotaxis, therefore removal of salts was necessary. Further, we wanted to examine the molecular weight distribution of the chemotactic agents. These aims were achieved by Sephadex G-10 gel filtration of the ESN. After elution with distilled water three main fractions (f_1–f_3) were obtained as shown in Fig. 1. The molecular weight of these peaks were estimated by comparison with the elution of markers of known molecular weight (Fig. 1 and Table II).

The chemotactic activities for PMN of the different preparations (SN, ESN, f_1–f_3) are shown in Table I. Ethanol extraction removed practically all CT activity of SN and the residue was inactive. The most active was fraction 2 of Sephadex gel filtration. Dose response curves (Fig. 2) indicated that f_1

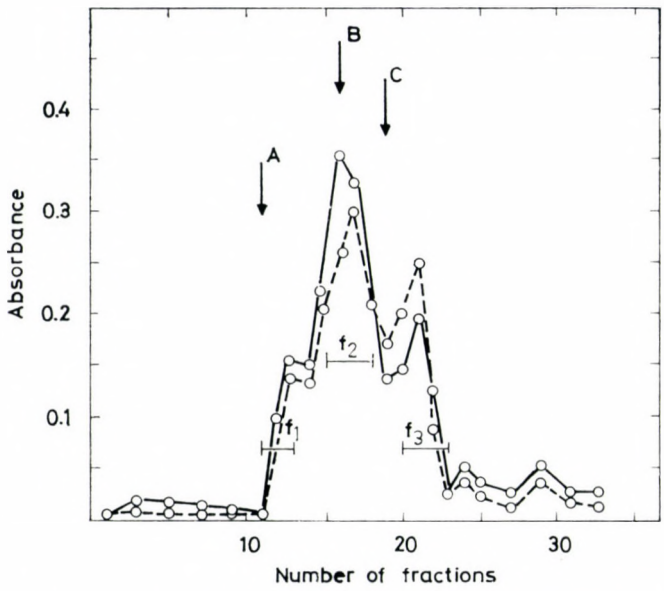

Fig. 1. Gel filtration on Sephadex G-10 (1.8×48 cm) of ethanol extract of the supernatant of *E. coli* culture (ESN) containing 3 mg protein. Eluant: distilled water. Flow rate 0.75 ml/min. Fraction size: 3 ml. Letters indicate the elution position of blue dextran (A), ACTH (B, 3700 mol. weight) and vitamin B_{12} (C, 1579 mol. weight); ○– – –○, OD 260 nm; ○—○, OD 280 nm

Table I

Effect of various fractions of E. coli extracellular substances on PMN migration

Materials*	Chemotaxis	
	in μg ± SEM	SD
Gey's solution	36 ±0.31	4.07 (n = 87)
Total lyophilized culture filtrate	43 ±1.27	3.6 (n = 16)
Ethanol extract	42 ±1.86	6.3 (n = 41)
Residue (after ethanol extraction)	37 ±0.6	
Sephadex G-10		
fraction 1	37 ±0.62	1.2 (n = 58)
fraction 2	54 ±0.66	1.0 (n = 57)
fraction 3	49 ±1.03	1.3 (n = 38)

* 10 μg protein/ml
± SEM, standard error
SD, standard deviation
n, number of experiments

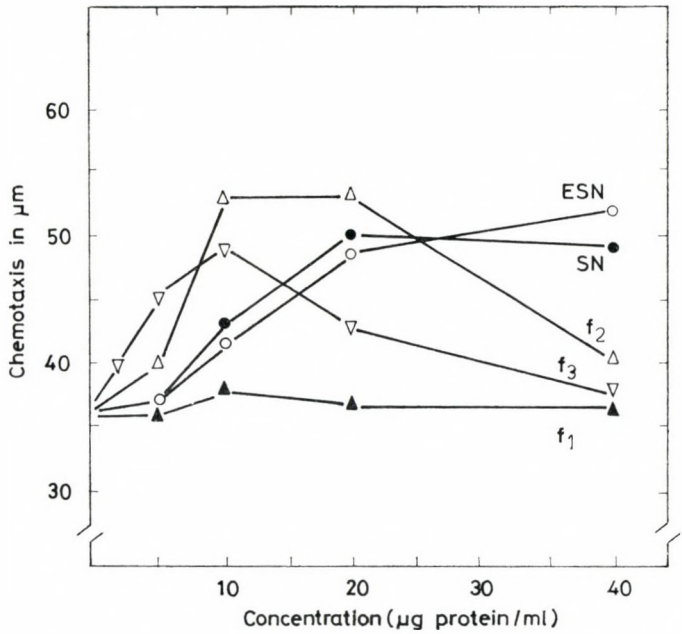

Fig. 2. Effect of *E. coli* chemotactic material on human PMN. Letters at the curves denote different fractions described in Methods

was devoid of chemotactic activity. In the concentration range examined the chemotactic effects of ESN and SN increased, while optimum concentrations were found for f_2 and f_3.

Beside human PMN we also investigated the chemotactic activity of f_2 for mouse peritoneal exudate cells which were induced by casein for 66 h. In the presence of f_2 (10 μg protein/ml) the migration increased from the control value of 44 μm to 59 μm. Since f_2 contained 55% of ESN as calculated from Fig. 1, this fraction was subjected to further analysis by ion-exchange

Table II

Characterization of bacterial preparations

	OD 260/280 ratio	Peptide	carbohydrate	lipid	ash	molecular weight, % (w/w)
Total lyophilized culture filtrate	1.2	2	11	1.3	54	—
Ethanol extract	1.05	8	35	7.5	31	—
Sephadex G-10						
f_1	1.16	n.d.	n.d.	n.d.	n.d.	10^4
f_2	1.03	13	62	7.7	11	5×10^3
f_3	1.36	1	4	n.d.	n.d.	10^3

n.d., not determined

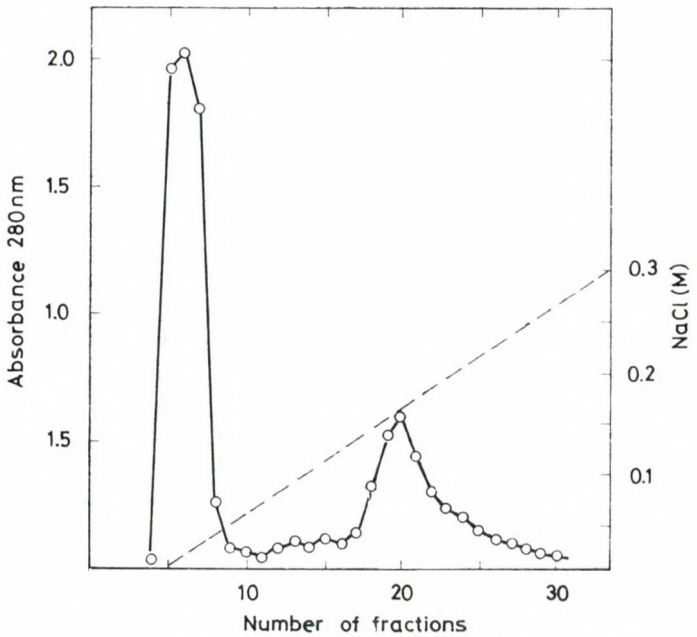

Fig. 3. DEAE-cellulose chromatography of f_2. The material (500 μg protein) was eluted by a 0.001–0.3 μ linear gradient of NaCl at pH 7.4

chromatography. No binding to a cation exchanger (Cellulose Phosphate, P-11), was observed, while a minor part was bound to anion exchanger (DEAE-Sephacell, Fig. 3). There was, however, no significant difference in CT activity between the neutral and anionic components of f_2.

CT activity of f_2 was abolished by chloroform-methanol extraction by the method of Folch et al. [27]. PMN migration was not influenced by the extracted lipid substances. The CT activity was apparently due to a complex of hydrophilic and lipophilic substances, which were not active in themselves.

Since some of the chemotactic factors of bacterial origin are active in the presence of serum only, we tested the effect of both native and heat (56 °C, 30 min) inactivated sera. Addition of 10% serum abolished the chemotactic activity of the preparations, even if serum was present only in the upper or lower compartments. Since preincubation of f_2 with human IgG (1 mg/ml) did not affect the CT activity, the inactivating effect of serum was not due to binding to IgG.

Heat (80 °C, 45 min) treatment of the preparations did not decrease their chemotactic activity. When stored at 4 °C, the aqueous solutions of the preparations remained active for several months.

Chemical characterization of the preparations. The preparations did not exhibit characteristic light absorption between 220–700 nm. We investigated the peptide, carbohydrate (hexose), lipid and ash (salt) contents (Table II).

The purification procedure yielding f_2 decreased the salt, and increased the peptide and hexose, content. Correspondingly, chemotactic activity was the highest in f_2 (cf. Table I).

The peptides were investigated by amino acid analysis; the results are shown in Table III. Six amino acids were detected in ESN which were unevenly distributed in the gel filtration fractions.

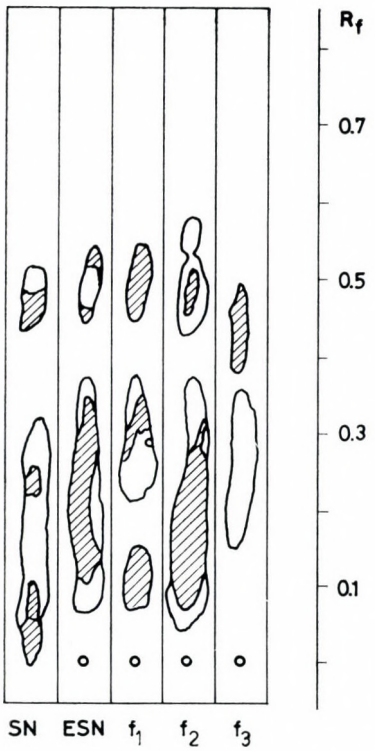

Fig. 4. Thin-layer chromatography of *E. coli* chemotactic material. Development: iodine vapour (shaded) and ninhydrin (open areas)

Table III

Amino acid composition of bacterial preparations. Relative molar amounts (Ala: 1.00)

Amino acid	Ethanol extraction	Sephadex G-10	
		f_2	f_3
Ser	traces	0.0	0.62
Glu	0.82	0.64	0.0
Gly	0.17	0.0	0.38
Ala	1.00	1.00	1.00
Val	0.42	0.26	0.0
Lys	0.44	0.58	0.0

Thin-layer chromatogram of the preparations is shown in Fig. 4. The various preparations were analysed by one dimensional chromatography. The best resolution was obtained using a solvent system generally applied for the analysis of lipids. After developing by iodine vapour and ninhydrin several

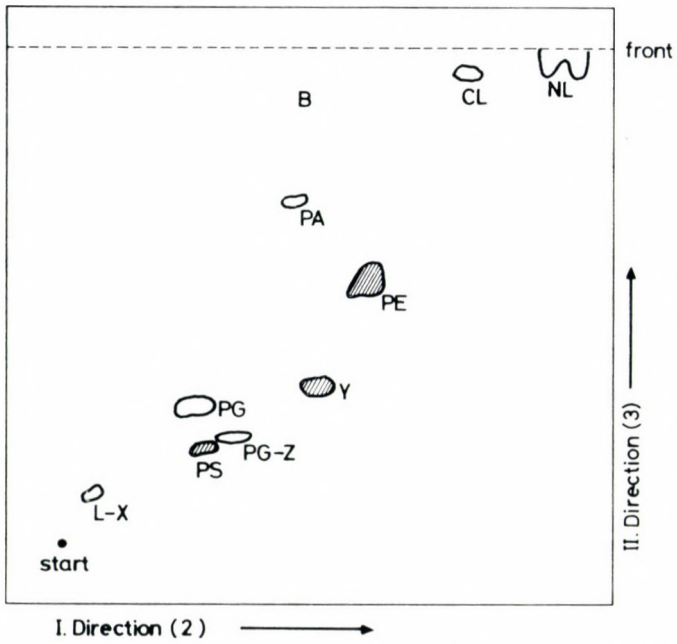

Fig. 5. Two dimensional thin-layer chromatogram of total lipids of f_2. Development was first in the horizontal direction in solvent 2 chloroform-methanol-water-ammonia (130 : 70 : 8 : 0.5 v/v), then in the vertical direction in solvent 3 chloroform-methanol-acetic acid-acetone-water (100 : 20 : 20 : 40 : 10, v/v). Approximately 1 mg of lipid was applied at the origin. All spots were visualized by idine-vapour and phosphomolybdenic acid; ninhydrin positive spots are shaded. The spots are neutral lipid (NL), cardiolipin (CL), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), lysophosphatide material (L—X), derivative of PG (PG-Z). Spot Y has not been identified

spots were found in the preparations (Fig. 4). The lipid components of f_2 were extracted by the method of Folch et al. [27], then subjected to two dimensional thin layer chromatography. As shown in Fig. 5, nine spots were detected. By the use of lipid standards 8 substances were identified, including neutral lipids, phospholipids and their derivatives (see legends to Fig. 5).

Materials gained from whole E. coli cells. Attempts were made to isolate the CT active substances directly from whole bacteria, from which a greater yield could be expected. Two methods were used.

When LPS was prepared from washed stationary *E. coli* cells by heat-treatment (100 °C, one hour [28]), the resulting material itself did not exhibit CT activity, though it was effective in the presence of 10% serum (data not

shown). Similarly, commercially available LPS (Difco) exhibited CT activity in the presence of serum only, in agreement with earlier observations [28].

According to the other method *E. coli* cells were treated with 80% ethanol (24 °C, 16 h), since the majority of CT activity of SN was soluble in ethanol, but the ethanolic supernatant of the cells did not show CT activity.

Discussion

Bacterial supernatants are known to contain CT factors in extremely low concentration [3, 6, 10–12]. Isolation of CT material from large volumes of *E. coli* culture medium (up to 140 litres) was possible by ethanol (80%) extraction of lyophilized medium. This procedure is less time consuming and more effective than earlier methods [3, 6, 8]. The presence of high amounts of salts and UV-absorbing materials (nucleotides, tryptophan, pigments, etc., excreted by the bacteria during growth) caused difficulties in the isolation of low molecular weight CT substances. Fractionation of the low molecular weight material obtained by ethanol extraction was performed by gel filtration applying Sephadex G-10. This proved useful since most of the CT activity could be recovered in a fraction (f_2) of low salt concentration. Although this fraction contained about 50% of the UV absorbing material present in the ethanol extract, the ratio of 260/280 nm absorption (1.03) was low in f_2.

Where do the CT substances come from? As regards the origin of bacterial CT substances several suggestions have been published. According to Bennett et al. [29], bacterial CT may correspond to the signal peptides derived from bacterial membrane proteins and secretory proteins which could be released into the medium. Schiffmann et al. [30] considered the formyl-methionyl-peptides, which are formed during protein synthesis, to be chemotaxins. Russel et al. [3] suggested that lipid-like CT material is loosely attached to the bacterial surface and is released during growth. The composition of this substance has not been elucidated [31].

Normally growing bacteria release outer membrane fragments [32] of variable composition. They contain 60–90% lipopolysaccharide and 5–26% phospholipid by weight, while their phospholipid/protein ratio varies between 1–2.5 [15, 16, 18–20, 33, 34]. The carbohydrate, lipid and amino acid building blocks have been identified. This is a population of characteristically high-molecular weight molecules (up to 5×10^7, depending on the method of isolation). Their release is due to imperfect coordination between outer membrane assembly and linkage to the underlying peptidoglycan layer. This type of bacterial material was, however, absent from the CT substances isolated by us, since ethanol did not extract high-molecular weight complexes. The residue of lyophilized SN after ethanol extraction (containing ethanol insoluble macro-

molecules) was not active, which seems to rule out the CT property of large outer membrane fragments. Further, outer membrane components (mostly LPS) released by heat-treatment were not chemotactic in themselves.

The CT material isolated by us proved to be chemically complex. Its qualitative composition (UV-absorption, carbohydrate, amino acid and lipid composition) indicates that the CT materials originate from the bacterial envelope. Amphiphile structures can be released from the wall and the outer membrane, and they form LPS-phospholipid-protein complexes in the environment of the bacteria [32]. Thus, the CT substances are considered to be complexes of this kind. Therefore, they are probably extracellular products which were excreted by the bacteria. There is a possibility that they are transformed in the medium.

Which components of the complex originate from the wall? The amino acid composition of ESN (Table III) closely resembled that of peptidoglycan [35]. Iodine- and ninhydrin-positive spots of the TLC chromatogram may be identical with amino-sugar intermediates and amino acids. Further, stationary bacteria have been shown to contain low-molecular weight cell wall precursor compounds which are formed during wall synthesis [36]. These data suggest that the CT material contains peptidoglycans originating from the wall. It was, however, shown earlier that the CT activity of peptidoglycans manifests itself exclusively in the presence of serum (due to the activating effect of the fifth component of complement). The size of effective wall-fragments has been found to have a molecular weight of around 3000. However, without serum the tetrasaccharide-peptide fragment inhibited leukocyte migration while both the peptide and tetrasaccharide were ineffective [37–40].

The outer membrane may be the source of the other components detected in the CT material. Phospholipids and neutral fat identified by TLC (Fig. 5) correspond to outer membrane lipid of *E. coli* [41, 42].

In conclusion, it is logical to suppose that high molecular weight complexes are shed from the cell envelope. They are transformed in the culture medium, resulting in low molecular weight CT substances. The exact mechanism of the release and the nature of these transformations awaits elucidation.

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REFERENCES

1. Adamu, S. A., Sperry, J. F.: Infect Immun **33,** 806 (1981).
2. Keller, H. U., Sorkin, E.: Int Arch Allergy Appl Immun **31,** 505 (1967).
3. Russel, R. J., McInroy, R. J., Wilkinson, P. C., White, R. G.: Immunology **30,** 935 (1976).
4. Sahu, S., Lynn, W. S.: Inflammation **2,** 47 (1977).
5. Schiffman, E., Showell, H. V., Corcoran, B. A., Smith, E., Ward, P. A., Becker, E. L.: Fed Proc **33,** 631 (1974).

6. Schiffmann, E., Showell, H. V., Corcoran, B. A., Ward, P. A., Smith, E., Becker, E. L.: J Immunol **114,** 1831 (1975).
7. Spagnuolo, P. J., Ellner, J. J.: Infect Immun **27,** 519 (1980).
8. Tainer, J. A., Turner, S. R., Lynn, W. S.: Am J Pathol **81,** 401 (1975).
9. Temple, T. R., Snyderman, R., Jordan, H. V., Mergenhagen, S. E.: J Periodontol **41,** 3/71
1 (1970).
10. Walker, W. S., Barlet, R. L., Kurtz, H. M.: J Bacteriol **97,** 1005 (1969).
11. Ward, P. A., Lepow, I. H., Newman, L. J.: Am J Pathol **52,** 725 (1968).
12. Yoshida, T., Cohen, S., Bigazzi, P. E., Kurasuji, T., Amsden, A.: Am J Pathol **81,** 389 (1975).
13. Hoekstra, D., van der Laan, J. W., De Leij, L., Witholt, B.: Biochim Biophys Acta **455,** 889 (1976).
14. Leive, L., Shovlin, V. K., Mergenhagen, S. E.: J Biol Chem **243,** 6384 (1968).
15. Loeb, M.: J Virol **13,** 631 (1974).
16. Mug-Opstelten, D., Witholt, B.: Biochem Biophys Acta **508,** 287 (1978).
17. Rothfield, L., Pearlman-Kothencz, M.: J Molec Biol **44,** 477 (1969).
8. Gankema, H., Wensink, J., Guinée, P. A. M., Jansen, W. H., Witholt, B.: Infect Immun **29,** 704 (1980).
19. Knox, K. W., Cullen, J., Work, E.: Biochem J **103,** 192 (1967).
20. Knox, K. W., Vesk, M., Work, E.: J Bacteriol **92,** 1206 (1966).
21. Weckesser, J., Drews, G., Ladwig, R.: J Bacteriol **110,** 346 (1972).
22. Person, S., Bockrath, R. C.: Biophys J **4,** 355 (1964).
23. Boyden, S.: J Exp Med **115,** 453 (1962).
24. Lukács, K., Szabó, G., Kávai, M., Szegedi, Gy.: Orv Hetil **120,** 657 (1979).
25. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J.: J Biol Chem **193,** 265 (1951).
26. Jawahr, S., Sawardeker, J. H., Sloneker, A. J.: Anal Chem **37,** 1602 (1965).
27. Folch, J., Lees, M., Sloane Stanley, G. H.: J Biol Chem **226,** 497 (1957).
28. Gnarpe, H., Belsheim, J., Persson, S.: Infection **6,** 98 (1978).
29. Bennett, J. P., Hirth, K. P., Fuchs, E., Sarvas, M., Warren, G. B.: FEBS Letters **116,** 453 (1980).
30. Schiffmann, E., Corcoran, B. A., Wahl, S. M.: Proc Natl Acad Sci USA **72,** 1059 (1975).
31. Becker, E. L., Ward, P. A.: In Parker, C. W. (ed.): Clinical Immunology. Vol 1. W. B. Saunders Co., Philadelphia 1980. p. 272.
32. Wicken, A. J., Knox, K. W.: Biochim Biophys Acta **604,** 1 (1980).
33. Inouye, M., Shaw, J., Shen, C.: J Biol Chem **247,** 8154 (1972).
34. Stinnett, J. D., Gilleland, H. E., Eagon, R. G.: J Bacteriol **114,** 399 (1973).
35. Schleifer, K. H., Kandler, O.: Bacteriol Rev **36,** 407 (1972).
36. Cziharz, B., Schleifer, K. H., Kandler, O.: Biochemistry **10,** 3574 (1971).
37. Grov, A., Helgeland, S., Endersen, C.: In Jeljaszewicz, J. (ed.): Zentralblatt für Bakteriologie, Parasitenkunde, Infections-Krankheiten und Hygiene. I. Abt. Suppl. 5. Stuttgart 1976. p. 389.
38. Kelly, M. T.: Infect Immun **15,** 180 (1977).
39. Schmeling, D. J., Peterson, P. K., Hammerschmidt, D. E., Kim, Y., Verhoef, J., Wilkinson, B. J., Quie, P. G.: Infect Immun **26,** 57 (1979).
40. Grov, A., Sveen, K.: Acta Path Microbiol Scand **B86,** 375 (1978).
41. Ames, G. F.: J Bacteriol **95,** 833 (1968).
42. Higashi, Y., Strominger, J. L.: J Biol Chem **245,** 3691 (1970).

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EFFECT OF ZINC ON THE LOCAL SHWARTZMAN PHENOMENON

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Local Shwartzman reaction is substantially reduced in intensity by zinc administered locally before and after preparation of the reaction. The endotoxin-induced accumulation of PMN leukocytes is inhibited by zinc rubbed in the skin.

We have reported recently [1] that the release of procoagulant tissue factor from PMN leukocytes is considerably reduced by zinc in endotoxin-pretreated rabbits and that this inhibitory action is dose-dependent. In earlier investigations we [2, 3] found that PMN leukocytes had a decisive role in the local Shwartzman phenomenon. It seemed therefore justified to examine the effect of zinc on the development of this phenomenon.

Materials and methods

Animals. Male NZ albino rabbits of 2300 to 2500 g body weight were used. The back of the animals was depilated 24 h before the experiment.

Endotoxin. Bacto LPS *Escherichia coli* O111 : B4 was used.

Preparatory injection. Endotoxin (125 μg) was injected intradermally into both sides of the back.

Challenge. Endotoxin (125 μg/kg) was injected intravenously 24 h after the intradermal injection. The reactions were read on the inner surface of the stripped-off skin after another 24 h.

$ZnSO_4$ treatment. An ointment containing 0.2 g $ZnSO_4$ in non-ionic emollient cream as vehicle was applied percutaneously on the right side of the back. Each rabbit was treated with zinc in four times, viz., rabbits Nos. 1–5 6 and 3 h before and 3 and 6 h after intravenous challenge and rabbits Nos. 6–10, 6 and 3 h before and 3 and 6 h after the intradermal injection of endotoxin.

Determination of Zn in cutaneous samples. The skin of the back of rabbits Nos. 11–13 was rubbed in with the ointment containing zinc on the one side and with the vehicle devoid of zinc on the other side. Six hours later, the treated skin surfaces were defatted with zinc-free alcohol and samples of 200–300 mg were taken, each in an incinerating dish which had been

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rinsed consecutively with a 2% solution of detergent DECON 90, N HCl solution and de-ionized water and dried at 125 °C for 24 h. Then the dishes were kept in an electric furnace at 600 °C for 6–8 h. The ash was dissolved in 20 μl of a mixture of equal volumes of pure nitric acid (Merck) and de-ionized water and diluted to 2 ml by adding de-ionized water. The zinc content of the solutions was assayed in an atom-absorption spectrophotometer Zeiss AASI at 213.9 nm wavelength.

Histological method. Samples from cutaneous areas pretreated with endotoxin or endotoxin and zinc were stained according to Masson–Goldner.

Results and discussion

The macroscopic observations are presented in Table I. It is clear that the zinc rubbed in on the day of challenge did not influence the Shwartzman reaction. On the other hand, the haemorrhagic area was considerably reduced and necrosis was practically prevented by zinc administered before and after the preparatory injection.

Table I

Effect of zinc on local Shwartzman phenomenon

Rabbit No.	Local Shwartzman reaction without zinc treatment maximum/minimum diameters of		Zinc treatment before and after		Maximum/minimum diameter of haemorrhagic necrosis on the zinc-treated side	
			challenge	preparation		
	haemorrhage	necrosis	with endotoxin			
1	19/16	10/6	+	—	18/16	10/6
2	20/15	9/6	+	—	21/15	9/6
3	16/12	8/4	+	—	14/12	8/4
4	17/12	8/4	+	—	15/13	7/4
5	16/14	7/5	+	—	16/12	7/5
6	15/10	6/4	—	+	8/3	0
7	20/17	12/8	—	+	12/8	3/2
8	17/14	10/6	—	+	6/4	0
9	18/14	12/8	—	+	6/4	0
10	18/14	10/6	—	+	6/4	0

Table II

Zinc concentration (μg/g) in rabbit skin samples

Rabbit No	Untreated area		Zinc-treated area	
	a	b	a	b
11	274	88.5	528	170
12	307	99	686	221
13	310	100	899	290
Means	299	95.8	704	227

[a] = calculated for skin samples dried at 125 °C
[b] = calculated for fresh samples
Mean dry content was 32.3% in freshly measured samples; mean water content, 67.7%

The histological pictures (Figs 1 and 2) show that the accumulation of leukocytes was much more intense in the untreated area than in the areas treated with zinc before and after the preparatory dose of endotoxin.

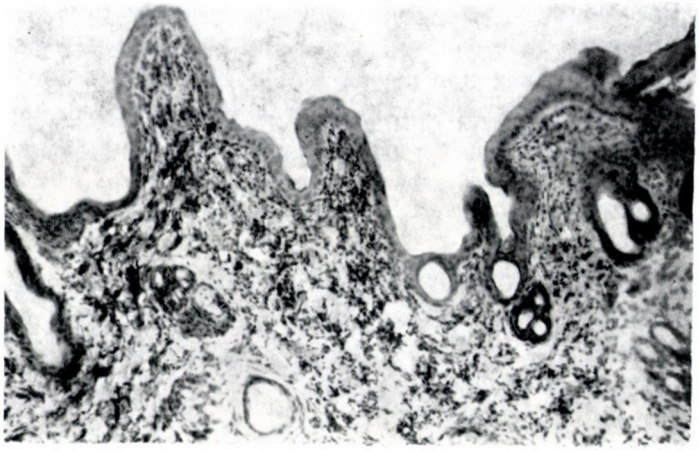

Fig. 1. Cutaneous section. Masson–Goldner trichrome × 140. Treatment with zinc-free ointment before and after the preparatory endotoxin injection. Intensive leukocyte infiltration

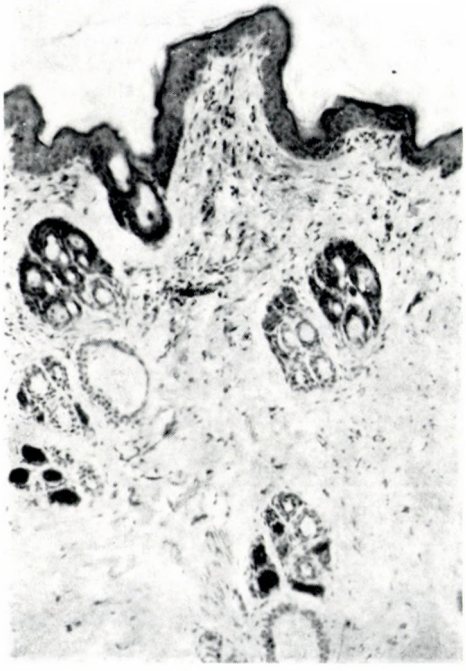

Fig. 2. Cutaneous section. Masson–Goldner trichrome × 140. Treatment with ointment containing zinc before and after the preparatory endotoxin injection. Slight leukocytic infiltration

The zinc contents of the cutaneous samples are presented in Table II. The zinc level increased to its double or more in the treated areas as compared to the untreated areas.

In the course of the Shwartzman reaction the preparative dose of endotoxin drives PMN leukocytes together, and the endotoxin given subsequently intravenously liberates lysosomal enzymes from the leukocytes. Consequently, a thrombohaemorrhagic reaction develops [4]. According to our results, zinc treatment fails to influence the mechanism of induction, while percutaneous zinc treatment is successful in the preparatory phase, viz., the accumulation of PMN leukocytes i.e., the chemotaxis due to the endotoxin is considerably inhibited by zinc. Chvapil et al. [5] reported that in their in vivo experiments the chemotactic stimulus was strongly inhibited by zinc. It deserves attention that in the zinc-treated areas of our rabbits the haemorrhage was inhibited only partially while necrosis was practically absent. This agrees well with the fact [1] that in case of a slight accumulation of PMN leukocytes the release of procoagulant factor is inhibited by zinc.

REFERENCES

1. Gazdy, E., Csernyánszky, H., Szilágyi, T.: Acta Physiol Acad Sci Hung **57,** 29 (1981).
2. Tóth, S., Szilágyi, T., Went, M., Lévai, G.: Acta Physiol Acad Sci Hung **42,** 163 (1972).
3. Szilágyi, T., Tóth, S., Deseő, G., Benkő, K.: Beitr Path **153,** 395 (1974.)
4. Szilágyi, T., Tóth, S., Csernyánszky, H., Sümegi, J.: Agents and Actions **6,** 721 (1976).
5. Chvapil, M., Stankova, L., Bartos, Z., Cox, T., Nichols, W.: J Reticuloend Soc **25,** 345 (1979).

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PROPHAGE INDUCTION BY LIVER MICROSOMAL METABOLITES OF AFLATOXIN B1 IN LYSOGENIC *PSEUDOMONAS AERUGINOSA*

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Microsomal metabolites of aflatoxin B1 (AFB1) causing induction of prophage in lysogenic strain of *Pseudomonas aeruginosa* SM was studied. Reduction of culture turbidity was determined at various concentrations of toxin. The effect of the toxin was also studied on deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis. AFB1 at the concentration of 50 μg/ml reduced initial turbidity to approximately 90% in 4 h. DNA synthesis stopped completely in the first hour but reappeared due to induction of the temperate phage. Soon after induction both RNA and protein synthesis continued but later little or no net synthesis of these macromolecules occurred. Plaque forming units (pfu) were increased approximately 90 times at 2 h as compared to the control. Testing of the effect of AFB1 on the non-lysogenic, sensitive strain demonstrated that although there was no significant decrease in culture turbidity at 50 μg/ml concentration of AFB1, DNA synthesis stopped completely within 1 h, while RNA and protein synthesis were increasing throughout the test interval. It has been concluded that the liver microsomal fraction of AFB1 caused induction of prophage in lysogenic cells and inhibited DNA synthesis significantly in non-lysogenic cells.

Most *Pseudomonas* strains are known to harbour a variety of latent particles ranging in complexity from temperate phages [1, 2] to pyocin [3–5].

Several physical and chemical agents induce bacteriophage development in lysogenic bacteria. The induction ability attributed to a particular substance has been related to carcinogenic, carcinostatic, or mutagenic properties of the agent [6, 7]. Aflatoxin (AFBl) has been regarded as the most potent hepatocarcinogen, a powerful mutagen and also a teratogen [8, 9]. Several reports have described procedures involving production of infective phage from lysogenic bacteria as a screening mechanism for compounds that cause tumours or mutation, or are tumouricidal [10–12]. AFBl has been shown to induce phage production in lysogenic bacteria [13, 14]. The present work described the effect of rat liver microsomal metabolite of AFBl on lysogenic and non-lysogenic *P. aeruginosa*, and especially DNA, RNA and protein synthesis in both systems.

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Materials and methods

Bacterial strains. The lysogenic strain of *P. aeruginosa* SM was obtained from Dr. Shriniwas, AIIMS, New Delhi, India; and the indicator *P. aeruginosa* PAO1 was a kind gift from Dr. B. W. Holloway, Monash University, Clayton, Victoria, Australia.

Culture media. Basic medium was tryptic soy broth (TSB, Difco). Plates and overlays contained 1.5 and 0.7% (w/v) agar, respectively.

Chemicals. Aflatoxin B1 (Sigma Chemical Co., USA), NADP (Sigma Chemical Co., USA), glucose-6-phosphate (Sigma Chemical Co., USA), dimethyl sulfoxide (Sarabhai M. Chemicals, India) and magnesium chloride (Sarabhai M. Chemicals, India).

Liver homogenate fraction and the metabolizing mixture. Rat liver homogenate fraction S-9 (AFB1) prepared by the method of Garner et al. [15] was obtained from Dr. J. K. Buch, Department of Microbiology, M. S. University of Baroda, Baroda, India. The metabolizing mixture of the carcinogen consisted of 1 volume of the post-mitochondrial fraction and 6 volumes of NADPH generating system consisting of 150 mM phosphate buffer pH 7.5, 3 mM NADP, 10 mM glucose 6-phosphate and 12 mM $MgCl_2$, sterilized by filtration and stored at 4 °C. The final concentration of AFB1 in the above metabolizing mixture ranged from 1–50 μg/ml and was dissolved in dimethyl sulfoxide (DMSO). AFB1 means liver microsomal metabolite of AFB1 throughout the text.

Turbidity determination. Different concentrations of AFB1 dissolved in DMSO containing NADPH generating system was added to 50 ml of the exponentially growing bacteria 5×10^8 cells/ml (Lysogenic as well as non-lysogenic). The flasks were incubated at 37 °C on a rotary shaker (150 r.p.m.). Growth was measured at 550 nm at various intervals.

Induction of lysis. Plaque forming units (pfu) were determined at 2 h and 3 h after induction with AFB1. Cellular components were cleared by centrifugation. 0.1 ml of appropriately diluted supernatant was added to 0.9 ml of indicator bacteria (5×10^8 cells/ml) followed by incubation at 37 °C for 30 min and subsequently plated on TSB agar plates. The number of pfu were determined after 18 h incubation at 37 °C.

DNA, RNA and protein determinations. The DNA of the cells was determined by a combination of DNA extraction as described by Sekiguchi and Takagi [16] and the diphenylamine reaction described by Burton [17].

RNA was determined using the procedure of Mejbaum [18]. Protein was determined using the Folin-phenol method of Lowry et al. [19].

The amounts of DNA, RNA and protein were calculated from standard curves.

Results

AFB1 induced lysis of the lysogenic *P. aeruginosa* SM, as shown by turbidity variation of the growth medium containing various levels of AFB1 and NADPH generating system (Fig. 1).

At 25 μg^8ml of AFB1 a decrease of approximately 70% was observed but a dramatic decrease in turbidity occurred only at 50 μg/ml concentration of AFB1. At this concentration the growth of lysogenic bacteria was inhibited to approximately 90% after 4 h exposure.

Turbidity curves indicated that bacterial lysis in the presence of AFB1 caused induction of phage development in the lysogenic strain. This was examined by plating the supernatants of 2 and 3 h cultures of the indicator strain (Table I). The bacterial lysate obtained after 2h exposure to 10 μg/ml contained 54×10^6 pfu/ml, as compared to 5×10^6 infectious centres in the control. Although growth determination (Fig. 1) of the lysogenic strain in 10 μg/ml of AFB1 displayed no reduction as compared to the initial level, plaque count showed a toxin-induced release of plaque forming units from

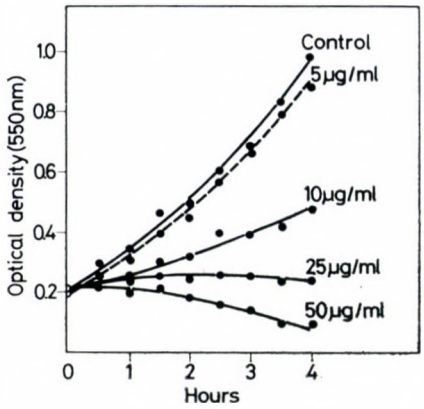

Fig. 1. Turbidometric determination of the effect of aflatoxin B1 on the development of a lysogenic strain of *P. aeruginosa* SM

lysogenic bacteria. Incubation for 2 h in 50 µg/ml of AFB1 increased the yield of infectious centres 90 times, whereas a 3 h culture at the same concentration of toxin increased the plaque forming units about 120 times.

The effect of AFB1 on the non-lysogenic indicator strain *P. aeruginosa* PAO1 is presented in Fig. 2. Although 10 µg/ml of AFB1 modified the growth only slightly, 25 and 50 µg of toxin per ml inhibited development to about 50 and 70%, respectively, after 4 h of incubation. No plaque forming unit was observed in the supernatant of cells when grown even in the presence of 50 µg of AFB1 per ml.

The effect of AFB1 on DNA, RNA and protein synthesis in lysogenic and in non-lysogenic bacteria are presented in Table II. After addition of AFB1 at a concentration of 50 µg/ml, the synthesis of host DNA was inhibited

Table I

Aflatoxin B1 (Liver microsomal fraction) induction of plaque forming units in P. aeruginosa SM

Aflatoxin B1 µg/ml	Plaque forming units/ml	
	2 h	3 h
0	5.0×10^6	1×10^7
1	5.2×10^6	1×10^7
5	10.0×10^6	3.0×10^7
10	54.0×10^6	15×10^7
25	30.0×10^6	74×10^7
50	450×10^6	120×10^7

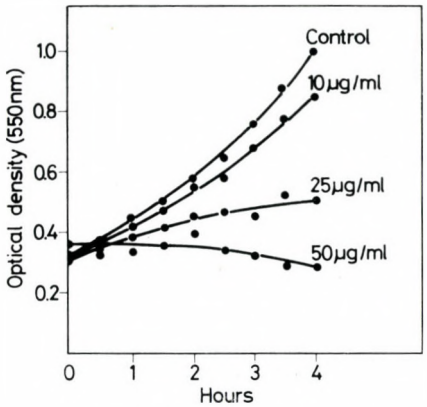

Fig. 2. Turbidometric determination of the effect of aflatoxin B1 on development of the indicator strain *P. aeruginosa* PAO1

completely for a period of 1 h, which was then followed by a sudden rise in the lysogenic bacteria, while in the non-lysogenic indicator bacteria DNA synthesis was inhibited completely in the first hour and failed to reappear thereafter. In contrast, after the addition of AFB1 neither RNA nor protein synthesis was markedly affected until 90 min and 120 min, respectively, and after that time they remained at a constant level. On the other hand in non-lysogenic bacteria RNA and protein synthesis continued to increase throughout the test interval (Table II).

Table II

Effect of Aflatoxin B1 (Liver microsomal fraction) on DNA, RNA and protein synthesis in lysogenic P. aeruginosa SM and nonlysogenic P. aeruginosa PAO1

Time, Hours	Lysogenic culture			Non-lysogenic culture			Control[c]		
	aflatoxin B1 (50 µg/ml)			aflatoxin B1 50 µg/ml)			none		
	DNA[a]	RNA[a]	Protein[b]	DNA	RNA	Protein	DNA	RNA	Protein
0	35	60	120	26	57	123	38	63	130
1	32	78	220	22	73	246	45	76	255
1.5	48	97	342	23	98	385	60	103	410
2	69	101	425	25	119	535	78	123	576
3	90	98	470	22	138	597	97	139	605
4	110	99	485	21	150	680	108	163	695

[a] DNA and RNA, given in µg per 10 ml of cells
[b] Protein was estimated as µg per one ml of cells
[c] Since no significant difference was found in the levels of DNA, RNA and protein synthesis in either lysogenic or nonlysogenic cells, the control was kept with nonlysogenic cells

Discussion

Many agents which affect DNA synthesis have been shown to have the ability to induce lysogenic bacteria [20]. As reviewed by Magee [21], carcinogens are known to require metabolic activation to transform the inactive molecules into mutagenic and/or carcinogenic species. In vivo studies do not demonstrate clearly the metabolic requirement for aflatoxins, but microsomal enzyme activation is an absolute requirement for mutagenicity [22]. Although not explicitly mentioned, AFB1 can induce lysogenic bacteria to enter the replicative stage [13, 14]. At the present moment the mechanism of prophage induction by AFB1 is obscure. Recent evidence suggests that in bacteria treated with AFB1, the DNA lesion, which persists after pyrimidine dimer excision repair, induces a metabolic pathway leading to mutagenesis, lysis induction and cell filamentation [22].

Measurement of turbidity, both in lysogenic and non-lysogenic bacteria indicates that it decreases markedly at 50 μg/ml concentration of AFB1 in lysogenic bacteria, nearly inhibiting 90% of cells within 4 h of incubation. The decrease in non-lysogenic bacteria is 20% less, indicating that lysogenic bacteria are more sensitive to AFB1 and that the dramatic decrease in turbidity is attributed to the induction of prophage.

Determination of DNA, RNA and protein synthesis in lysogenic bacteria after the addition of AFB1 (50 μg/ml) indicates that both RNA and protein synthesis increases up to 90 and 120 min, respectively, but then remain at a constant level. DNA synthesis is almost completely inhibited within 1 h but increases rapidly thereafter. This increase is due to the synthesis of new phage DNA which is not inhibited by AFB1 at the concentration used.

Determination of DNA, RNA and protein synthesis in non-lysogenic bacteria after the addition of 50 μg/ml AFB1 indicates that DNA synthesis stops completely in the first hour and does not increase thereafter, thus confirming that the rise of the DNA level in lysogenic bacteria after 1 h is due to phage DNA synthesis. In contrast, both RNA and protein synthesis continue to increase during the test interval. This observation in *P. aeruginosa* coincides with an earlier observation made by Lillehoj and Ciegler [23] in *Flavobacterium aurantiacum*. No significant increase in RNA and protein synthesis occurred after 90 and 120 min in lysogenic bacteria. This would indicate either their partial inhibition by 50 μg/ml of AFB1, or it might be due to a partial involvement of both in the synthesis of phage components.

Previous reports indicates that aflatoxin reacts with DNA in vitro and this reaction has been suggested to be responsible for the toxin-induced inhibition of RNA synthesis in liver cells [24, 25]. Since RNA synthesis in *P. aeruginosa* seems to be less sensitive to aflatoxin, the toxin may not be initiating the same process in bacterial and liver cells. Besides induction of the prophage by

AFB1, studies are being continued to elucidate the mode of action of aflatoxin on lysogenic and non-lysogenic bacterial systems.

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REFERENCES

1. Holloway, B. W., Egan, J. B., Monk, M.: Aust J Exp Biol **38,** 321 (1960).
2. Patterson, A. C.: J Gen Microbiol **39,** 295 (1965).
3. Ishii, S. I., Nishi, Y., Egami, F.: J Molec Biol **13,** 428 (1965).
4. Kageyama, M., Egami, F.: Life Sci **9,** 471 (1962).
5. Kageyama, M.: J Biochem **55,** 49 (1964).
6. Endo, H., Ishizawa, M., Kamiya, T., Sonoda, S.: Nature **198,** 258 (1963).
7. Gesaler, E.: Neoplasma **9,** 113 (1962).
8. Shank, R. C., Wogan, G. N., Gibson, D. B.: Fd Cosmet Toxicol **10,** 51 (1972).
9. Garner, R. C., Wright, G. M.: Br J Cancer **28,** 544 (1973).
10. Gadó, I., Savtchenko, G., Horváth, I.: Acta Microbiol Acad Sci Hung **12,** 363 (1965/66.)
11. Gots, J. S., Bird, T. J., Mudd, S.: Biochim Biophys Acta **17,** 449 (1955).
12. Heinemann, B., Howard, A. J.: Appl Microbiol **12,** 234 (1964).
13. Lillehoj, E. B., Ciegler, A.: Appl Microbiol **20,** 782 (1970).
14. Buch, J. K., Dave, P. J.: Indian J Exp Biol **18,** 1162 (1980).
15. Garner, R. C., Miller, E. C., Miller, J. A.: Cancer Res **32,** 2058 (1972).
16. Sekiguchi, M., Takagi, Y.: Biochim Biophys Acta **41,** 434 (1960).
17. Burton, K.: Biochem J **62,** 315 (1956).
18. Mejbaum, W.: Z Physiol Chem **258,** 117 (1939).
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: J Biol Chem **193,** 265 (1951).
20. Lwoff, A.: Bact Rev **17,** 269 (1953).
21. Magee, P. N.: Essays Biochem **19,** 105 (1974).
22. Goze, A., Sarasin, A., Moule, Y., Devoret, R.: Mutation Res **28,** 1 (1975).
23. Lillehoj, E. B., Ciegler, A.: J Bacteriol **94,** 787 (1967).
24. Clifford, J. I., Rees, K. R.: Nature **209,** 312 (1966).
25. Sporn, M. B., Dingman, C. W., Phelps, H. L.: Science **151,** 1539 (1966).

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CELLULAR AND HUMORAL CYTOTOXICITY IN PATIENTS WITH LEUKAEMIA AND PRELEUKAEMIA

II. ONCOVIRUS SPECIFICITY OF THE REACTION

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Cytotoxic activities of lymphocytes and antibodies against autologous tumour cells detected by ^{51}Cr release technique were frequent in patients with chronic granulocytic leukaemia, but infrequent in patients with blastosis of acute myeloid leukaemia. Among subjects with potentially preleukaemic haematological disorders autologous cytotoxic activity was observed only in cases of cytopenia, while samples from patients with polycythaemia vera proved to be devoid of cellular and humoral cytotoxicity. In the majority of cases the cytotoxic activity of lymphocytes and antibodies could be blocked by gp70 antigens of baboon endogenous virus (BaEV) and gibbon ape leukaemia virus (GaLV). p15(E) antigens of BaEV and GaLV showed blocking activity less frequently. Digestion by glycosidase of the carbohydrate of gp70 antigens reduced their blocking activity.

There have been reports on the isolation from cultured human cells of viruses very similar to primate C-type viruses [1—4] and certain nucleic acids and proteins related to these viruses have been found in human myeloid leukaemic cells [5, 6]. Other laboratories succeeded in detecting immune reactivity to animal oncovirus glycoproteins [7–9].

We have assumed that expression of oncovirus antigens during leukaemogenesis in humans might lead to a similar immune response. In a previous report it was shown that lymphocytes and blood plasma samples from patients with myeloproliferative disorders exhibited a cytotoxic effect against autologous tumour cells [10], and the cytotoxicity of lymphocytes and antibodies was inhibited by the envelope glycoprotein (gp70) of primate C-type viruses. It is, however, difficult to interpret the virus-specificity of these reactions, because lymphocytes and antibodies reactive with the viral gp70-s may be directed against carbohydrate determinants specified by both viral genes and the host cell [11, 12].

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The presence of antibodies reactive with p15 (E), a polypeptide envelope antigen of C-type oncoviruses, has not been examined in humans. The present studies were undertaken in an effort to detect cytotoxic lymphocytes and antibodies reacting with native and carbohydrate-free gp70 and p15 (E) antigens in samples from healthy humans, and patients with myeloid leukaemias and potentially preleukaemic disorders.

Materials and methods

Separation of cells from human blood. Separation of cells from heparinized blood samples was carried out by Ficoll-Urumiro medium (Flow Laboratories Ltd., Irvine, U. K.) in siliconised glass centrifuge tubes as described by Boyum [13]. After layering the diluted blood sample above the separation medium, samples were centrifuged for 30 min at 400 *g* at room temperature. After centrifugation, the lymphocytes formed a layer grey in colour at the interface of the supernatant blood plasma and the separating medium. The pathological myeloid elements and/or granulocytes were suspended in the separating medium while the erythrocytes formed a pellet at the bottom of the tube. Blood plasma, lymphocytes and myeloid elements were separately aspirated by Pasteur pipettes. Then the cells were washed three times with PBS of pH 7.2. The content of samples taken from each fraction was checked after May–Grünwald–Giemsa staining. The purity of cell fractions proved to be 95–100% as revealed by haematological investigation.

Cytotoxicity test. For study of cell-mediated and complement-dependent antibody cytotoxicity, the ^{51}Cr-release technique [14] was used [10]. The per cent cytotoxicity for each sample was then calculated as described by Oren et al. [15].

Purification of gp70 and p15 antigens. The baboon endogenous virus (BaEV) propagated on A 204 cells (Lot No 990A) was from the Frederick Cancer Research Center (Frederick, MD, USA) and the gibbon ape leukaemia virus (GaLV) Propagated on NC 37 cells (Lot No 18–76) was from the Pfizer Laboratory (Maywood, NJ, USA). Both viruses were provided in purified form through the Office of Resources and Logistics, N. C. I. (Bethesda, MD, USA).

A method for isolating pure viral envelopes from C-type RNA viruses [16] was combined with methods for preparation of envelope glycoproteins from Rauscher virus [17, 18] and from endogenous primate retroviruses [19].

Sodium dodecyl sulphate (SDS) 200 μg was added to 10 ml of virus suspension containing 20 mg of GaLV in TNE buffer (10 mM Tris–HCl pH 7.4, 100 mM NaCl, and 1 mM EDTA). After incubation at 4 °C for 5 min, 2 volumes of cold ether were added and the suspension was shaken for 10 min. The aqueous phase was centrifuged at 110 000 g for 90 min in a SW 27 rotor (Beckman) and the supernatant was then dialysed against TNE buffer. Solubilized gp70 and p15(E) were isolated by chromatography on 1.5×6 cm phosphocellulose (Whatman P-11) columns under conditions described by Strand and August [17].

The gp70 fractions dialysed against Con-A buffer containing 0.05 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM mercaptoethanol and 2 mM Tris–HCl pH 7.4 were further purified by affinity chromatography on 0.9×10 cm Con-A Sepharose column [19]. The absorbed glycoproteins were eluted with 0.1 M alpha-methyl mannoside. Molecular weight of gp70 and p15(E) was determined by SDS-PAGE [20]. Gels were stained for protein with Coomassie brilliant blue and for carbohydrate by the PAS procedure [21]. Protein quantitation was according to Lowry et al. [22].

Purification of BaEV gp70 and p15(E) was essentially that described for GaLV antigens except that the glycoprotein was further purified on Lens culinaris Sepharose column and eluted with 1 M alpha-methyl-mannoside [19].

Glycosidase treatment of viral gp70-s. The procedure described by Ohno et al. [23] was followed: 40 μg of a glycosidase mixture (Miles, Frankfurt, FRG) was added to 400 μg of BaEV or GaLV gp70 in 50 mM sodium citrate buffer pH 4.0. The efficacy of glycosidase treatment was controlled by SDS-PAGE; the glycosidase treated antigens proved to be free of carbohydrate.

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

Results

Data of 18 patients in the quiescent phase of chronic granulocytic leukaemia (CGL) are presented in Table I. Both cellular and humoral cytotoxicity could be demonstrated in 6 patients. In 8 patients only cellular cytotoxicity, and in 4 patients only humoral cytotoxicity was found. The native gp70 antigen of GaLV and BaEV inhibited both cellular and humoral cytotoxicity in each case. The glycosidase-treated gp70 antigens, free of carbohydrate, showed a weak inhibitory activity as compared to that of the native gp70 antigens. In the last four patients the humoral cytotoxicity to BaEV gp70-like antigens could not be blocked by previous incubation with glycosidase-treated gp70 antigen of BaEV. Blocking activity of p15 (E) antigens was less frequent than of native or glycosidase-treated gp70 antigens. Such activities were found approximately in half of the cases.

All patients listed in Table II were in the blastoid phase of acute myeloid leukaemia (AML) at the time of investigation. Cytotoxic effect of lymphocytes

Table I

Autologous cytotoxicity in patients in the quiescent phase of CGL

Patient No.	Cytotoxic activity													
	CMC							CDA						
	per cent cytotoxicity	per cent inhibition						per cent cytotoxicity	per cent inhibition					
		GaLV			BaEV				GaLV			BaEV		
		gp70		p15(E)	gp70		p15(E)		gp70		p15(E)	gp70		p15(E)
		native	glyc.		native	glyc.			native	glyc.		native	glyc.	
1	55	80	67	8	78	70	60	40	100	100	40	35	30	8
2	80	75	60	7	60	62	10	29	100	100	8	90	38	7
3	78	95	80	16	70	48	50	30	89	75	15	41	40	15
4	58	75	48	40	70	25	10	55	40	36	10	50	36	0
5	60	85	50	40	48	20	0	50	85	60	10	50	30	0
6	48	68	45	10	98	47	0	61	48	30	11	38	34	0
7	52	88	40	15	100	58	20	0	—	—	—	—	—	—
8	80	45	40	10	100	45	0	0	—	—	—	—	—	—
9	78	70	52	42	98	15	0	0	—	—	—	—	—	—
10	55	82	40	15	100	25	0	0	—	—	—	—	—	—
11	48	55	12	0	88	26	0	0	—	—	—	—	—	—
12	62	85	88	0	95	17	0	0	—	—	—	—	—	—
13	48	62	18	0	100	80	0	0	—	—	—	—	—	—
14	52	18	10	0	98	75	0	0	—	—	—	—	—	—
15	0	—	—	—	—	—	—	58	40	12	12	48	0	0
16	0	—	—	—	—	—	—	63	34	15	0	18	0	0
17	0	—	—	—	—	—	—	22	85	30	0	16	0	0
18	0	—	—	—	—	—	—	28	36	20	0	12	0	0

CMC = cell mediated cytotoxicity
CDA = complement-dependent antibody cytotoxicity

Table II

Cellular and humoral cytotoxicity in patients in the blastoid phase of acute myeloid leukaemia

Patient No.	Diagnosis	Leukocyte		Cytotoxic activity													
				CMC							CDA						
				per cent inhibition							per cent inhibition						
					GaLV			BaEV				GaLV			BaEV		
		G/l	blast per cent	per cent cytoto-xicity	gp70		p15(E)	gp70		p15(E)	percent cytoto-xicity	gp70		p15(E)	gp70		p15(E)
					native	glyc.		native	glyc.			native	glyc.		native	glyc.	
1	AML	12.8	42	15	35	89	80	86	60	51	8	100	85	60	100	95	16
2	AML	0.9	80	75	0	0	0	0	0	0	80	0	0	0	0	0	0
3	AML	7.4	80	7	0	0	0	0	0	0	6	0	0	0	0	0	0
4	AML	8	75	0	—	—	—	—	—	—	0	—	—	—	—	—	—
5	AML	9	48	0	—	—	—	—	—	—	0	—	—	—	—	—	—
6	AML	6.2	25	0	—	—	—	—	—	—	0	—	—	—	—	—	—
7	AML	20	25	0	—	—	—	—	—	—	0	—	—	—	—	—	—
8	AML	6	25	0	—	—	—	—	—	—	0	—	—	—	—	—	— —
9	AML	2	42	0	—	—	—	—	—	—	0	—	—	—	—	—	
10	AMMoL	7	22	0	—	—	—	—	—	—	0	—	—	—	—	—	
11	AMMoL	9.3	49	0	—	—	—	—	—	—	0	—	—	—	—	—	— —

CMC = cell mediated cytotoxicity
CDA = complement-dependent antibody cytotoxicity
AML = acute myeloid leukaemia
AMMoL = acute myelomonocytic leukaemia

Table III

Cytotoxicity of lymphocytes and antibodies against autologous granulocytes in patients with potentially preleukaemic disorders

Patient No	Diagnosis	Cytotoxic activity													
		CMC							CDA						
		per cent cytotoxicity	per cent inhibition						per cent cytotoxycity	per cent inhibition					
			GaLV			BaEV				GaLV			BaEV		
			gp70		p15(E)	gp70		p15(E)		gp70		p15(E)	gp70		p15(E)
			native	glyc.		native	glyc.			native	glyc.		native	glyc.	
1	IRSA	18	97	50	30	80	77	45	22	70	65	15	19	16	10
2	IRSA	50	40	35	27	20	20	18	0	—	—	—	—	—	—
3	Pancytopenia	45	22	20	0	40	31	0	35	29	10	0	19	14	0
4	Pancytopenia	30	42	38	0	50	41	0	0	—	—	—	—	—	—
5	Pancytopenia	18	92	70	0	80	37	0	0	—	—	—	—	—	—
6	PV	0	—	—	—		—	—	0	—	—	—	—	—	—
7	PV	0	—	—	—	—	—	—	0	—	—	—	—	—	—
8	PV	0	—	—	—	—	—	—	0	—	—	—	—	—	—
9	PV	0	—	—	—	—	—	—	0	—	—	—	—	—	—
10	PV	0	—	—	—	—	—	—	0	—	—	—	—	—	—
11	PV	0	—	—	—	—	—	—	0	—	—	—	—	—	—
12	PV	0	—	—	—	—	—	—	0	—	—	—	—	—	—

IRSA = idiopathic refractory sideroblastic anaemia
CMC = cell mediated cytotoxicity
PV = polycythaemia vera
CDA = complement-dependent antibody cytotoxicity

and antibodies against autologous tumour cells was detected in 3 patients. Blocking effect of envelope antigens of C-type viruses could be observed only in patient No. 1.

Table III shows the results of studies on 12 patients with potentially preleukaemic haematological disorders. Cytotoxic activity of lymphocytes or antibodies against autologous granulocytes was detected only in patients with cytopenia, whereas those with polycythaemia vera proved negative for the presence of cytotoxic lymphocytes and antibodies. Cellular cytotoxicity was found in each cytopenic patient, while humoral cytotoxicity could only be detected in two of them. The cellular and humoral cytotoxicity could be inhibited by native or glycosidase-treated gp70 antigens in each case. On the contrary, blocking activity of p15 (E) antigens was observed less frequently. Cellular cytotoxicity was blocked in 2 cases while blocking of humoral cytotoxicity was detected only in 1 case.

In 9 patients with non-preleukaemic cytopenia and in 5 with eosinophilia, cytotoxic activity of lymphocytes or antibodies against autologous granulocytes was not found. Studies of 20 healthy humans gave similarly negative results.

Discussion

Results showed that lymphocytes and antibodies cytotoxic against autologous tumour cells were detected frequently in patients with the quiescent phase of CGL but rarely in those being in the blastoid phase of AML. In patients with potentially preleukaemic haematological diseases cytotoxic activity was found only in those with cytopenia, but not in patients with polycythaemia vera. The presence of cytotoxic lymphocytes or antibodies may be connected with a favourable prognosis, due to the elimination of tumour cells and virus-infected ones. Results of testing of patients with AML and potential preleukaemia suggested a prognostically favourable role of the presence of antibodies reacting with envelope glycoproteins of oncoviruses [24, 25]. This seems to be supported by the low frequency or absence of autologous cytotoxic activity in patients with the blastoid phase of AML and potentially preleukaemic disorders, respectively. On the other hand, a relationship was found between the expression of oncoviral genome and the development of overt leukaemic disease in preleukaemic children [26]. The presence of C-type particles in bone marrow smears of preleukaemic patients was connected with development of leukaemia and malignant histiocytosis [27]. The biological significance of anti-oncoviral immune response detected in our studies is not clear, but one has to keep in mind that such lymphocytes and antibodies may possess both virus neutralizing [28] and cytotoxic [29] activity.

Blocking of cytotoxic activity by the native envelope glycoproteins of BaEV and GaLV could be observed in a considerable part of cases while the inhibitory effect of p15 (E) envelope polypeptides was found rarely. Digestion of the carbohydrate part of gp70 antigens did not result in disappearance of the blocking activity, but glycosidase treatment caused a weakening of the inhibitory effect. The immune response raised against the carbohydrate part of gp70-s may have a Forssman-like character, while the virus-specific nature of lymphocytes and antibodies reactive with polypeptide epitopes of carbohydrate-free gp70 and p15(E) antigens cannot be excluded. Their virus-specificity is supported by the presence of antigens closely related to structural polypeptides of primate C-type viruses in human cells [30]. The results of Hehlmann et al. [31] seem further to strengthen this assumption as they have succeeded in detecting antigens cross-reactive with gp70 of primate C-type viruses in the sera of leukaemic patients. The reaction was not due to recognition of carbohydrate moieties as demonstrated by prior treatment with glycosidases.

The present results indicate that the virus specific cytotoxic activity of lymphocytes and antibodies may be quite different in different groups of patients. Though those activities might have a prognostic importance, their biological significance is not clear. Further cytotoxic studies are done in our laboratory to compare the active and remission phase of AML as well as the quiescent and blastoid phase of CGL.

REFERENCES

1. Gallagher, R. E., Gallo, R. C.: Science **187,** 350 (1975).
2. Panem, S., Prochownik, E. V., Reale, F. R., Kirsten, W. H.: Science **189,** 297 (1975).
3. Nooter, K., Aarssen, A. M., Bentvelzen, P., De Groot, F., Van Pelt, F. G.: Nature **256,** 595 (1975).
4. Kaplan, H. S., Goodenow, R. S., Epstein, A. L., Gartner, S., Decléve, A., Rosenthal, P. N.: Proc Natl Acad Sci USA **74,** 2564 (1977).
5. Campbell, W. F., Hoagland, H. G., Smith, D. M.: In Bentvelzen, P., Hilgers, J., Yohn, D. S. (eds): Advances in Comparative Leukemia Research 1977. Elsevier-North Holland Biomedical Press, Amsterdam—New York 1978. p. 150.
6. Tóth, F. D., Váczi, L., Kiss, J., Réthy, A., Kiss, A., Rák, K.: Acta Virol **25,** 376 (1981).
7. Kurth, R., Teich, N. M., Weiss, R. A., Oliver, R. T. D.: Proc Natl Acad Sci USA **74,** 1237 (1977).
8. Hirsch, M. S., Kelly, A. P., Chapin, D. S., Fuller, T. C., Black, P. H., Kurth, R.: Science **199,** 1337 (1978).
9. Thiry, L., Sprecher-Goldberger, S., Bossens, M., Neuray, F.: J Natl Cancer Inst **60,** 527 (1978).
10. Szabó, B., Tóth, F. D., Váczi, L., Réthy, A., Kiss, A., Rák, K.: Neoplasma **30,** 129 (1983).
11. Barbacid, M., Bolognesi, D., Aaronson, S. A.: Proc Natl Acad Sci USA **77,** 1617 (1980).
12. Löwer, J., Davidson, E. A., Teich, N. M., Weiss, R. A., Joseph, A. P., Kurth, R.: Virology **109,** 409 (1981).
13. Boyum, A.: Tissue Antigens **4,** 269 (1979).
14. Goodman, H. S.: Nature **190,** 269 (1961).
15. Oren, M. E., Heberman, R. B., Canty, T. G.: J Natl Cancer Inst **46,** 621 (1971).
16. Van de Ven, W. J. M., Vermorken, A. J. M., Onnekink, C., Bloemers, H. P. J., Bloemendal, H.: J Virol **27,** 595 (1978).

17. Strand, M., August, J. T.: J Biol Chem **248,** 5627 (1973).
18. Strand, M., August, J. T.: J Biol Chem **251,** 559 (1976).
19. Fine, D. L., Arthur, L. D., Schochetman, G.: Virology **101,** 176 (1980).
20. Segrest, J. P., Jackson, R. L., Andrews, E. P., Marchesi, V. T.: Biochem Biophys Res Commun **44,** 390 (1971.)
21. Glossmann, H., Neville, D. M.: J Biol Chem **246,** 6339 (1971).
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: J Biol Chem **193,** 265 (1951).
23. Ohno, T., Mesa-Tejada, R., Keydar, J., Ramanarayanan, M., Bausch, J., Spiegelman, S.: Proc Natl Acad Sci USA **76,** 2460 (1979).
24. Tóth, F. D., Váczi, L., Réthy, A., Szabó, B., Kiss, J., Kiss, A., Rák, K.: Neoplasma **29,** 397 (1982).
25. Tóth, F. D., Kiss, J., Váczi, L., Madár, Zs., Jakó, J., Rák, K.: Acta Microbiol Acad Sci Hung **27,** 147 (1980).
26. Chandra, P., Steel, L. K., Laube, H., Kornhuber, B.: FEBS Letters **58,** 71 (1975).
27. Vosika, C. J., Krivit, W., Carrard, J. M., Coccia, P. F., Nosbit, M. E., Coalson, J. I., Kennedy, B. J.: Proc Natl Acad Sci USA **72,** 2804 (1975).
28. Bendinelli, M., Toniolo, A., Mattencci, T., Celeste, F., Revoltella, R.: J Gen Virol **46,** 311 (1980).
29. Portetelle, D., Druck, C., Mammericka, M., Burny, A.: Virology **105,** 222 (1980).
30. Suni, J., Wahlström, T., Vaheri, A.: Int J Cancer **28,** 559 (1981).
31. Hehlmann, R., Schetters, M., Erfle, V.: In Neth, R., Gallo, R. C., Graf, T., Mannweiler, K., Winkler, K. (eds): Modern Trends in Human Leukaemia IV. Springer Verlag, Berlin—Heidelberg—New York 1981. p. 530.

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IN VITRO CULTIVATION OF *MYCOBACTERIUM* X FROM *MYCOBACTERIUM LEPRAE* INFECTED TISSUES IN PROPANE-TETRADECANE MEDIUM

(A PRELIMINARY COMMUNICATION)

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Host grown *Mycobacterium leprae* and cultures of *Mycobacterium* X, cultivated from *M. leprae* infected armadillo and human specimens, were inoculated into propane and propane–tetradecane media. The media contained in one litre distilled water KH_2PO_4, 7 g; Na_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4$, 0.1 g; ferric ammonium citrate, 20 mg and yeast extract (Difco), 0.1 g. Twenty ml media, distributed into each of 50 ml screw cap tubes, were inoculated with the bacilli and bubbled aseptically for 10 s with 99% purity propane gas. Tetradecane–propane media were prepared by adding 0.1 ml tetradecane to each of the tubes containing 20 ml propane medium. When incubated at 32 °C a logarithmic growth rate was counted in the propane–tetradecane media following a one to two week latency period. The time of division was estimated at seven days. In the propane–tetradecane medium, growth occurred at the interface of the tetradecane oil and water as a thin veil developing into a 1 to 3 mm thick emulsion in two to three months. No growth occurred in the propane medium and growth was extremely slow in the tetradecane medium. When added to the tetradecane medium, propane considerably shortened the latency period and the generation time, resulting in increased bacterial yield. Bacilli were strongly acid-fast; the culture did not grow on Löwenstein–Jensen or in Dubos media, but produced the localized disease typical of *M. leprae* in the foot pads of mice.

Mycobacterium X was cultivable from human and armadillo *M. leprae* infected tissues in media containing tetradecane [1–3]. The growth was extremely slow and difficult to quantitate. *Mycobacterium* X was the first strain cultivable from *M. leprae* infected tissues which did not grow on Löwenstein–Jensen or in Dubos media, but produced the disease typical of *M. leprae* in the foot pads of mice.

The terminal methyl bindings of tetradecane and other medium length straight chain hydrocarbons are known carbon and energy sources for in vitro growth of most species of mycobacteria [3–7]. Propane, a short chain water soluble hydrocarbon gas, served as sole source of carbon and energy for a few saprophytic strains of mycobacteria [4, 7]. As an attempt to replace the oily tetradecane in the medium, water soluble propane was an obvious candidate to support growth or improve yield of *Mycobacterium* X.

Cultivation of *Mycobacterium* X in liquid media containing dissolved propane gas will be reported in this communication.

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Materials and methods

All containers, pipettes, centrifuge tubes and syringes were made of glass, because hydrocarbons react with plastic materials.

Basal medium. KH_2PO_4, 7.0 g, Na_2HPO_4, 0.5 g, $(NH_4)_2SO_4$, 2 g, $MgSO_4$, 0.1 g and yeast extract (Difco), 0.1 g were dissolved in one litre distilled water. The pH was 5.8. Twenty ml of basal medium was distributed into each of the 50 ml screw cap tubes and autoclaved for 40 min.

Propane–tetradecane. Ten ml tetradecane (USP grade) in a 50 ml screw cap tube was autoclaved for 40 min. This was bubbled with 99% purity propane gas for one minute under one lb/in^2 pressure at room temperature through a one ml serological pipette under aseptic conditions. (Propane gas is highly soluble in tetradecane).

Propane media. Two kinds of propane media were used. All operations were performed under aseptic conditions.

(a) Basal medium — propane: Compressed propane gas in a one liter container was of 99% purity grade and was purchased from Union Carbide Ltd., Canada. According to the supplier, the impurities contained ethane and methane. The container was provided with a safety valve with two manometers attached, one measuring gas pressure and another measuring the gas flow. A further valve served to open the gas flow into the outlet tube. To this an interchangeable sterile cotton packed glass tube was attached by connecting Nalgene tubes. Disposable 1 ml sterile pipettes were used to introduce the propane gas into the bottom of the liquid basal medium in the screw cap tube. The pipette outlet was 0.5 mm in diameter. Separate pipettes were used for each tube of culture medium.

Propane gas was introduced into the media after they were inoculated with the bacilli. The gas outflow was adjusted to approximately 1 lb/in^2 pressure. The medium was bubbled for 10 s. Propane was added to the cultures at 3 week intervals.

Following gassing of the inoculated media, the screw cap tubes were tightly closed. It is known that 6.5% propane gas is dissolved in water. Propane is heavier than air. An unquantified but adequate supply of propane was therefore ensured for the bacilli in the above experimental conditions.

(b) Basal medium — propane-tetradecane: by adding autoclaved 0.1 ml propane–tetradecane to each of the screw cap tubes containing 20 ml basal medium. The inoculated cultures were again bubbled with propane for 10 s.

Sources of M. leprae. Armadillo spleen specimens were received from Melbourne, Florida. The animals were killed 12 to 18 months following intravenous injection with *M. leprae* suspensions isolated from skin biopsies of human lepromatous leprosy cases. The spleens were heavily infected with acid-fast bacilli. The tissue specimens were transported without refrigeration in the basal medium. Each specimen was washed with distilled water and cut with scissors into small pieces. These were homogenized in a Potter–Elvehjem homogenizer to obtain a 10% (w/v) suspension in the basal solution. The suspension was diluted with an equal volume of 4% NaOH solution and kept for 20 min at 37 °C. The suspension was centrifuged for two minutes at 1000 rpm and the sediment was discarded. The supernatant was centrifuged for 10 min at 6000 rpm. The obtained sediment was washed twice with 30 ml basal medium. The sediment so obtained was used without further purification for inoculation of the media.

Mycobacterium X strains were previously cultivated [3] from *M. leprae* infected armadillo spleens or from human lepromata. The strains were transferred at three month intervals into acetone–DMSO–tetradecane medium. The fifth subculture was diluted 1 : 1 v/v with acetone, shaken for 10 s with a Vortex homogenizer and centrifuged for 20 min at 10 000 rpm. The sediment was washed three times with 20 ml basal medium.

Inoculation of the media. The obtained sediments were suspended in the basal medium, to contain 5×10^7 acid-fast rods per one ml suspension; 0.4 ml was added to each of the tubes containing 20 ml medium. A 2 mm loopful of the sediment was spread on the surface of the Löwenstein–Jensen medium. Five ml Dubos liquid medium was inoculated with 0.2 ml suspension containing 5×10^7/ml acid-fast rods.

Incubation and estimation of growth. The cultures were incubated at 32 °C. They were shaken manually twice weekly. All cultures were observed during at least two months of incubation. Multiplication of acid-fast bacilli in the cultures was taken as the criterion of growth.

The multiplication of acid-fast bacilli in some of the media was so obvious that actual counting of bacilli was not necessary. However, counting of acid-fast bacilli was carried out to estimate the relative growth rate of cells in the cultures. It was necessary to dilute the cultures with acetone 1 : 1 v/v in order to obtain a homogeneous suspension for counting. A 5 mm diameter loopful of culture or its dilution was spread evenly on a 2 cm^2 surface of siliconized slides. The smears on the slides were allowed to dry overnight. Fixation was achieved over flame from a gas burner. The preparations were stained by the Ziehl–Neelsen method.

The number of cells was counted in twenty 1 × 1 cm microscopic fields using a Zeiss net micrometer under 1000 × magnification. The average number of acid-fast rods per microscopic field was multiplied by the dilution factor of the cultures when serial dilutions of the cultures were necessary for counting.

Results

Primary cultures in the propane medium from M. leprae-infected armadillo spleen. Turbidity of inoculated media did not increase after a latency period of one to two months. Microscopic examination showed no evidence of multiplication of cells in the cultures.

Growth in the propane–tetradecane medium. The inoculated bacilli accumulated within a few days at the interface of the tetradecane oil floating on the water phase of the medium. Within a week or two this became increasingly visible as a whitish veil floating at the interface of the oil in water system. Then the veil thickened, developing into a disklike dense emulsion one to three mm high. Complete homogenization was not achieved even after vigorous shaking with the Vortex homogenizer. Maximal growth developed in one to three months, depending on the size of the inoculum. Microscopic examination of the described growth left no doubt that the veil, which was growing progressively in size, consisted of masses of acid-fast bacilli.

The growth characteristics were the same whether media were inoculated with host grown *M. leprae* or with any of the four *Mycobacterium* X cultures.

Subcultures in the propane–tetradecane media. The primary cultures were transferred into the propane–tetradecane medium when the disklike growth was one to two millimeters thick. It was not possible to transfer a uniform size of transfer inoculum, since uniform homogenization was not fessible. After the cultures were shaken for 15 s with the Vortex homogenizer, 2 ml culture was transferred into 20 ml sterile medium. After the transfer, bubbling with propane gas was performed. Growth characteristics in the subcultures were the same as in the primary cultures. It seemed that the latency growth period was considerably shorter in the subcultures. All cultures are now regularly transferred at two to three month intervals.

Evidence of growth in the propane–tetradecane medium. Figure 1 shows logarithmic rate of multiplication plotted against time in weeks of one of the primary cultures as well as the fourth subculture in the propane–tetradecane medium. Theoretical latency periods of growth were estimated by extrapolating the logarithmic line to the level of inoculated cells per ml at t_0 of incubation. The latency period was twelve to seventeen days for the four primary cultures as shown in Table I. The latency period was considerably shorter in the subcultures as compared to the primary cultures.

Table I also shows the rate of multiplication expressed as generation time. This was calculated at the level of half the time of the logarithmic growth pe-

riod. The time of division in the propane–tetradecane medium was close to seven days in the primary cultures and subcultures.

Table I also shows that generation time was approximately twice as long in the tetradecane medium as compared to the same parameter in the propane–tetradecane media.

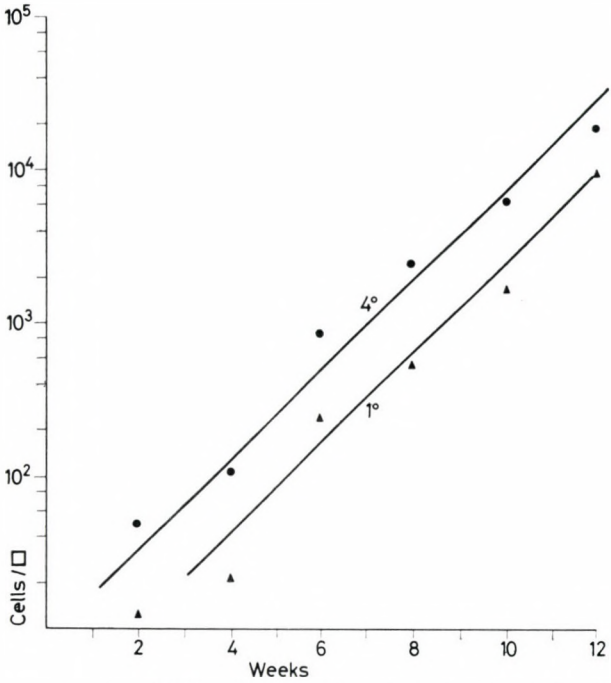

Fig. 1. Logarithmic rate of multiplication plotted against time in weeks of a primary culture (1°) and the fourth subculture (4°) of *Mycobacterium* X in propane–tetradecane medium. Number of cells is expressed as average number of acid-fast rods in 1 cm² field of a net micrometer eyepiece

Table I

Latency period and generation time in days of four strains of Mycobacterium X grown in propane tetradecane medium from M. leprae infected armadillo spleens

Mycobacterium X	Latency period in days		Generation time (cell division) in days	
	primary culture	4th subculture	primary culture	4th subculture
In propane-tetradecane medium				
1	12	5	5	6
2	17	9	6	8
3	14	9	7	7
4	16	6	7	6
In tetradecane medium				
1	17		15	

Adaptation to propane in subcultures. Four *Mycobycterium* X cultures [3] grown in acetone–DMSO–tetradecane medium, obtained from subcutaneous human lepromata or from the spleens of *M. leprae* infected armadillos, were transferred (1 : 10 v/v) into fresh homologous media at three month intervals. The three months old subcultures were transferred (1 : 10/v/v) into propane-

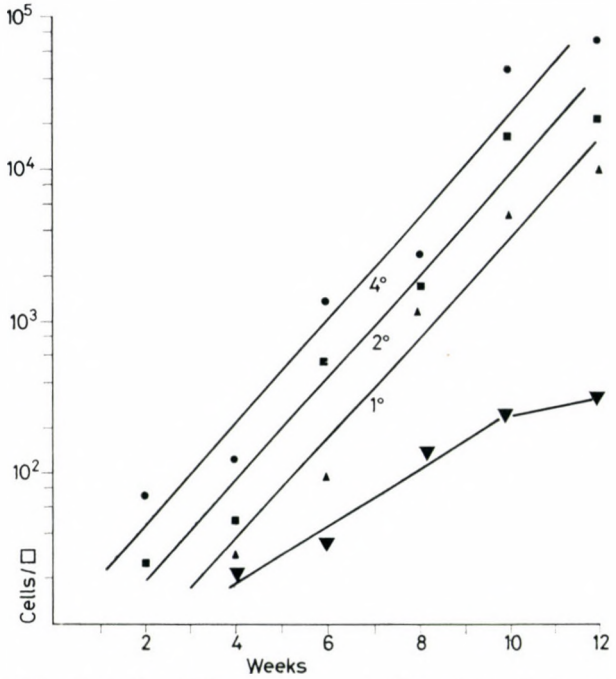

Fig. 2. Growth of *Mycobacterium* X in tetradecane medium (▲) and adaptation of *Mycobacterium* X grown on tetradecane medium to propane–tetradecane medium. The logarithmic growth rate is indistinguishable in the 1°, 2° and 4° subcultures, but the latency period is progressively shortened. Evidence of cell multiplication as in Fig. 1

tetradecane media. After a 10 to 12 day latency period bacilli multiplied at a logarithmic rate. Figure 2 shows that in the first subculture in the propane–tetradecane medium there was already a shortened latency period of growth and practically no measurable latency period in the fourth in vitro subculture in the propane media. Only one of the propane grown cultures is shown in Fig. 2. The growth rate of two cultures isolated from human tissues and of two cultures from armadillo *M. leprae* infected tissues was practically indistinguishable.

Löwenstein–Jensen and Dubos media. M. leprae suspensions from armadillo spleen and the *Mycobacterium* X cultures grown in acetone–DMSO–tetradecane, as well as *Mycobacterium* X grown in propane–tetradecane medium, were inoculated into Löwenstein–Jensen and Dubos media. These were incubated at 37 °C. No growth was observed in any of the inoculated cultures during a 12 week incubation period.

The number of acid-fast rods did not increase in the propane–tetradecane media when inoculated with in vitro or in vivo grown but heat killed cells (autoclaved one hour) when incubated for 12 weeks at 32 and 37 °C, respectively.

Animal inoculation. Three of the four cultures grown in the propane–tetradecane media were injected into the foot pads of mice. In each case the fourth subculture was diluted with equal volumes of acetone, centrifuged and the sediment was suspended in physiological saline solution to obtain 5×10^3 acid-fast bacilli per 0.05 ml. Using a tuberculine syringe, this amount was injected from each culture respectively into the hind foot pads of 10 Swiss albino mice. Five mice were killed four months later and the surviving mice were killed six months after infection. The number of acid-fast rods was counted in the pooled homogenized foot pad connective tissues. Depending on the strains, the number of acid-fast bacilli in the foot pads increased from 5×10^3 to 1.2 to 2.1×10^4 in four months and to 1.4 to 3.2×10^5 in six months.

Discussion

Most strains of mycobacteria grow on medium length straight chain liquid hydrocarbons as the sole source of carbon and energy (3–7). Identical strains of acid-fast cultures, tentatively named *Mycobacterium* X, werecultivated from human and armadillo *M. leprae* infected tissues in a liquid medium containing an oily liquid hydrocarbon, tetradecane [1–3]. The slow growing cultures did not grow on Löwenstein–Jensen or in Dubos medium. Attempts were made to improve the cultivation techniques, increase bacterial yield, avoid use of the disadvantageous two —phase growth system, thus permitting accurate measurements of growth and facilitate separation of bacilli from the cultures. A simplified one-phase system medium, using a water soluble carbon and energy source, was also expected to permit to obtain easily reproducible results.

These expectations were only partly realized. Bacilli did not grow in the one phase propane medium; the oily tetradecane was necessary for growth. However, propane considerably shortened the latency period and the generation time, thus leading to increased bacterial yield.

While nearly all species of mycobacteria grow on liquid oily hydrocarbons, there are only a very few which utilize water soluble hydrocarbon gases for multiplication. Lukins and Foster [7] reported that among many cultures of mycobacteria tested, one strain of *Mycobacterium smegmatis* and three strains of *Mycobacterium rhodochrous* were the only organisms to grow abundantly at 30 °C at the expense of propane. The strains are saprophytic and also grow on Löwenstein–Jensen medium.

Mycobacterium X, cultivated from *M. leprae* infected tissues, is so far the only known strain of mycobacteria which grows in propane–tetradecane media as the sole source of carbon, does not grow on Löwenstein–Jensen or in Dubos media, but produces disease in the foot pads of mice. It is known that bacteria utilizing hydrocarbon gas oxidize propane to n-propanone, or propionic acid and acetone, which are the oxygenated products of the bacterial oxidation of propane [5]. It was reported previously that acetone, an enzymatic oxidation product of propane, was a growth-promoting nutrient for *Mycobacterium* X [3].

Lukins showed that a *M. smegmatis* strain grown at the expense of propane did not grow on methane or ethane. The propane gas used to cultivate *Mycobacterium* X contained 1% methane and ethane as impurities. In the light of the results of Lukins [6], it is safe to assume that for the observed effect on growth of *Mycobacterium* X propane was the factor responsible rather than the minute amounts of methane and ethane impurities.

The strains of mycobacteria which were grown on propane also grow on tetradecane, as reported by Lukins and Foster [7]. *Mycobacterium* X grows preferentially on tetradecane enriched with propane.

Lukins and Foster [7] stated that "the evidence is overwhelming that an affinity for hydrocarbons is a generic property of the mycobacteria regardless of origin or cultivation history". The cultivation of *Mycobacterium* X from *M. leprae* infected tissues in a medium enriched with propane and tetradecane is further experimental evidence regarding the special appetite of mycobacteria for hydrocarbons.

Several strains of mycobacteria which grow on hydrocarbons also grow autotrophically on H_2 and CO_2 [7]. Host grown *M. leprae* also has some autotrophic characteristics, although not an autotroph per se [8].

It is known that growth of bacteria occurs in mineral salt solutions in the presence of minute concentrations of gaseous hydrocarbons. For practical purposes the exact concentration of propane dissolved in the propane medium was not estimated in the experiments presented. The propane concentration was, however, sufficient to stimulate growth of *Mycobacterium* X. Results not presented in this communication have shown that for cultivation of *Mycobacterium* X in the subcultures simple commercial "propane" fuel containing over 15% impurities can be used just as well as the 99% pure propane gas.

Since the discovery of the leprosy bacillus a great number of strains of mycobacteria have been cultivated from leprosy specimens. Without exception all of them grow on Löwenstein–Jensen and in Dubos media. *Mycobacterium* X is the only one that does not grow on media which support growth of cultivable mycobacteria. Although *Mycobacterium* X was cultivated from leprosy infected tissues in special media and under special physical conditions, and the cultures produce disease identical to *M. leprae* in the foot pads of mice, the results are not convincing enough to claim the successful cultivation of *M. leprae*.

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REFERENCES

1. Kato, L.: Abstracts of the International Symposium on *Mycobacterium leprae*. Bombay 1981. pp. 9 10.
2. Kato, L.: Int J Lepr **49,** 93 (1981).
3. Kato, L.: Int J Lepr (In press)
4. Foster, J. W.: In Hayaishi, O. (ed.): Oxygenases. Academic Press, New York 1962. pp. 241 274.
5. Leadbetter, E. R., Foster, J. W.: Nature **184,** 1428 (1959).
6. Lukins, H. B.: Ph. D. Dissertation. University of Texas 1962.
7. Lukins, H. B., Foster, J. W.: Zschr Allg Mikrobiol **3 4,** 251 (1963).
8. Kato, L., Ajdukovic, D., Donawa, A.: Nature New Biol. **242,** 179 (1973).

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PROPANE AND TETRADECANE AS CARBON SOURCES FOR IN VITRO CULTIVATION OF *MYCOBACTERIUM LEPRAEMURIUM* IN A LIQUID MEDIUM

(A PRELIMINARY COMMUNICATION)

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(Received July 22, 1983)

Three strains of host grown *Mycobacterium lepraemurium* and five strains of *Mycobacterium lepraemurium*, grown on egg yolk medium, were inoculated into propane–tetradecane media. The media contained in one litre distilled water: KH_2PO_4, 7 g, Na_2HPO_4, 0.5 g, $(NH_4)_2SO_4$, 2 g, $MgSO_4$, 0.1 g, ferric ammonium citrate, 20 mg, and yeast extract (Difco), 0.1 g. Tetradecane 0.1 ml was added to each tube containing 20 ml of the medium. Media were sterilized in the autoclave. Following inoculation with the bacilli, the cultures were bubbled aseptically with 99% purity propane gas for 10 s. When incubated at 32 °C, logarithmic growth rate was counted in the cultures. Bacilli were strongly acid-fast. Growth occurred at the interface of the tetradecane oil and water as a thin weil, developing into a one to three millimeter thick emulsion in two to three months. Cultures were transferred into fresh media at two to three month intervals. Growth pattern in the subcultures were indistinguishable from the growth in the primary cultures. The cultures did not grow on Löwenstein–Jensen or in Dubos media, but produced the characteristic disease of murine leprosy when injected subcutaneously into mice. Bacilli isolated from the subcutaneous lepromas of mice were again cultivable in the propane–tetradecane medium, but not on Löwenstein–Jensen or in Dubos.

Ogawa and Motomura [1] reported the cultivation of *Mycobacterium lepraemurium* on egg yolk medium. This finding was confirmed by Koseki et al. [2], Mori [3], Kato et al. [4] and by Pattyn and Portaels [5]. Attempts were made to cultivate *M. lepraemurium* in a chemically well-defined medium. Cultivation of *M. lepraemurium* in a liquid medium containing propane and tetradecane will be reported in this paper.

Materials and methods

Microorganisms. The following in vivo grown *M. lepraemurium* strains were used: 1. the Hawaiian strain was obtained from Dr. O. K. Skinsnes, University of Hawaii; 2. the Kurume strain and 3. the Odessa strain were obtained from Dr. K. Kohsaka, Osaka University, Japan.

These were maintained by serial transmission in Swiss albino mice. The aseptically removed subcutaneous leproma was washed in distilled water and homogenized in a Potter–Elvehjem homogenizer to obtain a 10% (w/v) suspension. This was centrifuged for one minute at 1000 rpm. The sediment was discarded. The supernatant was centrifuged for 10 min at 6000 rpm. The supernatant was discarded and the sediment was diluted with the basal medium to obtain 5×10^7 acid-fast bacilli per one ml suspension.

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The following in vitro grown strains of *M. lepraemurium* were used: 1. the Kurume strain 3rd subculture; 2. the Odessa strain 7th subculture; 3. the Manitoba cat strain 7th subculture; 1–3 were cultivated in our laboratory on Ogawa egg yolk medium from subcutaneous murine lepromata from mice; 4. Hawaiian strain 5th subculture; 5. Fukuoka strain 23th subculture; 4–5 were obtained from Dr. K. Kohsaka on egg yolk medium.

A loopful of the four-month-old heavy growth was removed from the surface of the egg yolk medium and homogenized with a Potter–Elvehjem homogenizer in the basal medium. The suspension was diluted with the same medium to contain 5×10^7 cells per one ml suspension.

Culture media. A liquid medium containing dissolved propane gas and tetradecane was used. Preparation of the medium and estimation of growth were described in a previous communication [6].

Incubation and criteria for growth. Each tube containing 20 ml media was inoculated with 0.5 ml of the suspension containing in vivo or in vitro grown *M. lepraemurium*, respectively. Löwenstein–Jensen and Dubos media were similarly inoculated. Cultures were incubated at 32 °C. Cultures in liquid media were shaken with a Vortex homogenized for 10 s twice weekly.

Multiplication of acid-fast bacilli in the cultures was taken as the criterion of growth. Smears on siliconized slides were dried at least 20 h. Fixation was achieved over flame. Preparations were stained by the Ziehl–Neelsen method.

Animal inoculation. Adult female Swiss albino mice weighing 25 g were inoculated subcutaneously with suspensions of in vitro grown cultures. Each mouse received 10^6 acid-fast cells in 0.5 ml basal medium.

Results

Primary cultures in the propane–tetradecane medium. The in vivo grown *M. lepraemurium* bacilli, inoculated into the liquid propane-tetradecane media, accumulated within a few days at the surface of the medium. Within one to three weeks a whitish veil developed close to the surface of the medium, giving the impression of a fine emulsion with the small amount of tetradecane floating on the surface. With the progression of the age of the cultures, this whitish veil increased in volume into a disklike dense emulsion reaching maximal growth in one to three months. Complete homogenization of the surface growth was not achieved even after vigorous shaking with the Vortex homogenizer. Microscopic examination of the cultures confirmed that the progressively advancing surface growth consisted of masses of acid-fast bacilli. Figure 1 shows evidence of growth in the primary cultures of the Fokuoka, Kurume, Ogawa and Hawaiian strains of *M. lepraemurium* in propane–tetradecane medium. As previously described, a simplified counting method was used, showing number of cells per square millimeter of microscopic field. After a latency period of less than eight days, the growth rate of multiplication was logarithmic.

There were slight differences in the latency period and growth rate of the different strains. It did not, however, become evident whether this was due to the percentage of viability of the inoculated cells or a genetic characteristic of the different strains.

Figure 1 shows that the fastest growth rate was counted with the Hawaiian strain, while the Fokuoka strain showed a considerably slower growth rate with the Odessa and Kurume strains in order of increasing growth rate. No counting was performed with the Manitoba cat leprosy strain because of heavy clumping of the cells with growing cultures.

Subcultures in the propane–tetradecane media. In two to three months, the surface growth of the primary cultures reached a thickness of 1 to 2 mm. At this time the primary cultures were transferred into fresh propane–tetra-

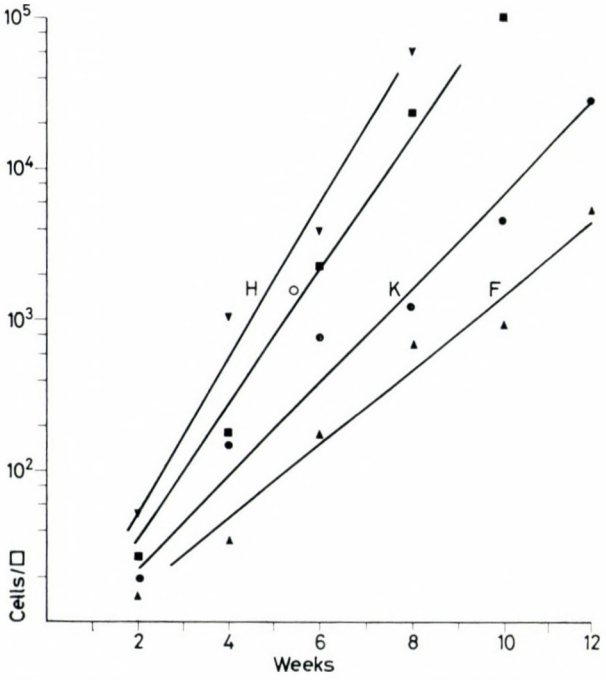

Fig. 1. Growth of primary cultures of the Fokuoka, Kurume, Ogawa and the Hawaiian strains of *M. lepraemurium* in propane–tetradecane medium. Number of cells is expressed as average number of acid-fast bacilli in a 1 cm^2 field of a Zeiss net micrometer eyepiece

decane medium. The primary cultures were shaken for 15 s with the Vortex homogenizer, then 2 ml of the culture was transferred into 20 ml of fresh sterile medium. After the transfer bubbling with propane gas was performed. The growth characteristics in the subcultures were the same as in the primary cultures; however, the latency growth period was considerably shorter in the subcultures. Figure 2 illustrates the growth rate of the primary and fourth subcultures of the Hawaiian strain of *M. lepraemurium*. It seems that with adaptation to the propane–tetradecane medium the growth rate of the fourth subculture is somewhat faster than of the primary culture.

Differences in the gowth rate of the in vitro grown *M. lepraemurium* strains remained characteristically different for the four in vitro grown strains in which counting was made. All strains enumerated in the Materials section are now regularly transferred at two to three month intervals.

Adaptation from egg yolk medium to propane–tetradecane medium. The five in vitro grown *M. lepraemurium* cultures were transferred from the Ogawa egg yolk media into the propane–tetradecane media as described in the Materials

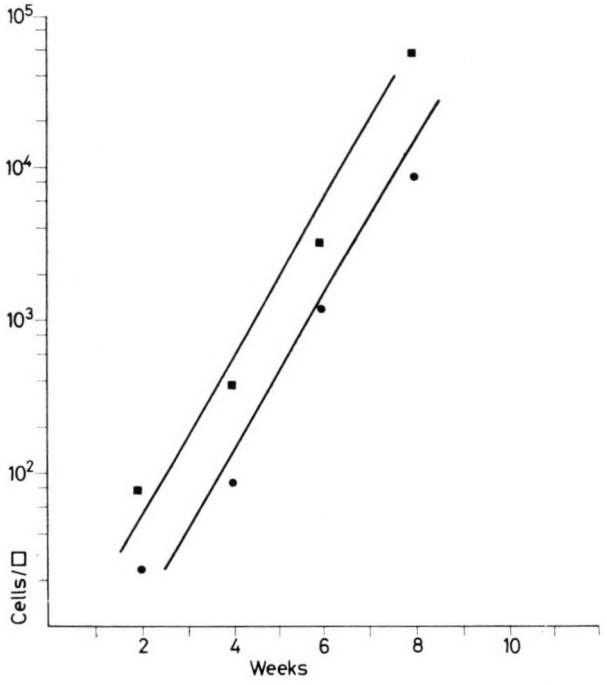

Fig. 2. Logarithmic growth rate of the primary (●) and the fourth (■) subcultures plotted against time in weeks of the Hawaiian strain of *M. lepraemurium* in propane–tetradecane medium

section. As in the cultures obtained from in vivo grown bacilli, growth developed on the surface of the propane–tetradecane media when bacilli were transferred from egg yolk media into the liquid propane–tetradecane medium. Depending on the strains, the latency period ranged from one to two weeks and variations occurred in the growth rats. The Ogawa grown cultures, adapted to the propane–tetradecane medium, are now transferred at two to three month intervals into fresh propane–tetradecane media. The growth characteristics remained constant in the fifth subcultures, which are now being grown.

No growth in the control cultures. None of the primary cultures or subcultures grew on Löwenstein–Jensen or Dubos media during the three-month incubation period at 32 ° or 37 °C. No growth was observed in propane–tetradecane media inoculated with heat-killed bacilli.

Animal inoculations. One month after subcutaneous inoculation with suspensions of 10^6 acid-fast bacilli prepared from the fifth subculture of the Hawaiian strain, a small growth was palpable at the site of the inoculation. This increased in size and reached an average of $12 \times 12 \times 6$ mm in nine out of ten mice, when removed at necropsy three months following inoculation. Microscopic examination of the granuloma showed histiocytic invasion, loaded with acid-fast bacilli. Few acid-fast bacilli were found in the liver, spleen and adjacent lymph nodes. The host response to the injected culture was indistinguishable from the changes observed following injection of *M. lepraemurium* transferred from mice to mice. Bacilli separated by partial purification from mice at necropsy were inoculated on Löwenstein–Jensen and in Dubos media. No growth was observed during two months of incubation at 37 °C. In the propane–tetradecane media the bacilli separated from mice multiplied at 32 °C at a rate comparable to those shown in Fig. 1.

Discussion

The special appetite of mycobacteria for hydrocarbons [7–9] and the theoretical background of the experiments presented in this communication has been described recently [6, 10–12]. Propane was incorporated into the medium used for cultivation of *M. lepraemurium* because Lukins and Foster [9] have shown that some mycobacteria grow on propane as sole source of carbon. Further, it was found that in media containing propane, strains of mycobacteria were regularly cultivable from *M. leprae* infected human and armadillo tissues [6].

The following experimental evidence is offered to support the designation of the cultivated strains as *M. lepraemurium.*

From host-grown and authentic in vitro grown suspensions of *M. lepraemurium* cultures of identical growth pattern were obtained in a chemically well-defined liquid medium. A special medium with the dimethylated carbon sources propane and tetradecane, was required to achieve logarithmic multiplication of the inoculated cells. The cultures did not grow on Löwenstein–Jensen or in Dubos media. Three months after subcutaneous inoculation of a late subculture, mice developed lesions which were identical to those obtained originally from the host-grown and in vitro grown suspensions of *M. lepraemurium.* Bacilli separated from the murine lepromata were again cultivable in propane–tetradecane media, but not on Löwenstein or in Dubos media.

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REFERENCES

1. Ogawa, T., Motomura, M.: Leprologia **38,** 246 (1969).
2. Koseki, Y., Anchi, T., Okamoto, S.: Leprologia **41,** 127 (1972).
3. Mori, T.: Leprologia **43,** 226 (1974).
4. Kato, L., Kim, S. J., Ishaque, M.: Int J Lepr **46,** 376 (1978).
5. Pattyn, S. R., Portaels, F.: Int J Lepr **48,** 7 (1980).
6. Kato, L.: Acta Microbiol Acad Sci Hung **31,** 373 (1984).
7. Foster, J. W.: In Hayaishi, O. (ed.): Oxygenases. Academic Press, New York 1962. pp. 241 274.
8. Lukins, H. B.: Ph. D. Dissertation. University of Texas 1962.
9. Lukins, H. B., Foster, J. W.: Zschr Allg Mikrobiol **3 4,** (1963).
10. Kato, L.: Int J Lepr **49,** 93 (1981).
11. Kato, L.: Int J Lepr (In press).
12. Kato, L., de Thököly, I.: Acta Leprol **78,** 13 (1980).

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NATURAL ANTIBODIES TO SIMIAN TYPE-C VIRUSES AND HUMAN RETROVIRUS HTLV IN PATIENTS WITH LYMPHOID MALIGNANCIES

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Blood plasma samples from adult patients and children with malignant lymphoproliferative diseases were studied for presence of antibodies to primate type-C viruses by membrane immunofluorescence. Antibodies to baboon endogenous virus could be detected in various types of lymphoid leukaemias and lymphomas. Gibbon ape leukaemia virus-specific antibodies were mainly found in B- and O-cell leukaemias and lymphomas. Anti-HTLV antibodies could be detected only in a few cases of T-cell malignancy. There was no evidence of a horizontal transmission of such viruses.

The recent development of continuously growing cell lines from patients with leukaemia and lymphoma has led to reports on the occurrence of retroviruses in some of them [1–3]. Most of these isolates were closely related to previously isolated type-C primate viruses [1, 2]. However, retrovirus particles detected from cell lines from patients with histiocytic lymphoma [3] and type-C simian viruses showed significant differences [4]. Human T-cell leukaemia/lymphoma virus (HTLV) was the first retrovirus consistently to be isolated from patients with a specific disease [5, 6]. HTLV is not related to any of the known animal retroviruses by antigenic test of structural proteins [7] or by nucleotide sequence homology [8]. HTLV is an exogenous retrovirus, but serum antibodies apparently directed against HTLV have been demonstrated in normal subjects in the endemic regions [9, 10].

In the present work we report the results of a serological study on the presence and distribution of antibodies directed to simian type-C viruses and HTLV in Hungarian patients with malignant lymphoproliferative disease.

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Materials and methods

Cell cultures and their maintenance. The cell cultures used originated from the following sources. The established human cell line NC-37 and the NC-37 cells infected with gibbon ape leukaemia virus (GaLV), the human cell line A-204 and the A-204 cells infected with the M7 strain of baboon endogenous virus (BaEV) were kindly supplied by the Office of Logistics and Resources of the National Cancer Institute (Bethesda, MD, USA). The HTLV-producing HUT102 cell line [5] has been established in the Laboratory of Tumor Cell Biology (National Cancer Institute, Bethesda, MD, USA). The cell cultures were maintained in RPMI 1640 medium containing 10% fetal calf serum (Gibco Bio-Cult. Ltd., Paisley, Scotland). The suspended cultures were incubated at 37 °C in an atmosphere containing 5% CO_2 at 80% relative humidity.

Blood plasma samples. Fresh heparinized blood was centrifuged and the plasma decanted and stored at −70 °C until used.

Indirect membrane immunofluorescence. The cell suspension to be used contained at least 90% viable cells, as determined by the trypan blue exclusion test. As target cells, BaEV-infected A-204, GaLV-infected NC-37 and HUT 102 cells were used. The uninfected A-204 and NC-37 cells and con-A stimulated normal human T lymphocytes [11] were used as controls. For the assay, 50 μl of each plasma sample diluted in PBS were incubated with 10^6 target cells. After incubation at 37 °C for 30 min the cells were washed three times with PBS. 50 μl of goat anti-human IgG conjugated with FITC (Hyland, Costa Mesa, CA, USA) and appropriately diluted in PBS were added and the cells were incubated again at 37 °C for 30 min. The cells were washed three times in PBS and suspended in 50 μl of PBS for microscopic examination. Titres reflect the plasma dilution at which 50% of target cells fluoresced markedly.

Results

1. *Antibodies to BaEV, GaLV and HTLV in plasma samples of adults with acute lymphoid leukaemia (ALL).* Plasma samples from 18 patients with progressive stage ALL were tested (Table I). Anti-BaEV antibodies could be demonstrated in samples of seven patients with T-cell ALL and of one patient with O-cell ALL. Anti-GaLV antibodies were present in the plasma of one patient with T-cell ALL and of two patients with O-cell ALL. Antibodies to HTLV were found in one sample from a patient with T-cell ALL.

2. *Anti-BaEV, anti-GaLV and anti-HTLV antibodies in plasma samples from adults with chronic lymphoid leukaemia (CLL) or lymphoma.* The results for 4 patients with CLL and 8 patients with lymphoma are summarized in Table II. All the patients were in the progressive stage of the disease at the time of investigation. In four lymphoma patients tumour cells have appeared in the peripheral blood, while in the others such cells were absent. Anti-BaEV antibodies could not be detected in any of the patients. Anti-GaLV antibodies were demonstrated in the plasma of two patients with CLL and of one patient with B-cell lymphoma. Anti-HTLV antibodies were found in one patient with T-cell lymphoma.

In the control group of 87 healthy adults antibodies to BaEV were detected in two cases, in very low (1 : 4) titre while antibodies to GaLV and HTLV were not found in any. Plasma samples from close family members of 5 patients with lymphoid leukaemia or lymphoma proved to be consequently negative for presence of antibodies to simian oncoviruses and HTLV. Control samples with

uninfected A-204 and NC-37 cells and con-A stimulated normal human T lymphocytes also gave negative results.

3. *Antibodies to BaEV, GaLV and HTLV in plasma samples of children with progressive stage ALL or lymphoma.* Table III shows the results for 16 children. Among those with ALL, antibodies to BaEV were found in two chil-

Table I

Anti-BaEV, anti-GaLV and anti-HTLV antibodies in the blood plasma of adult patients with acute lymphoid leukaemia

Patient		Cell type	Peripheral leukocytes		Antibody titre		
No.	age (yrs)		G/l	blast %	anti-BaEV	anti-GaLV	anti-HTLV
1	19	T	3.7	0	0	0	0
2	29	T	4.3	0	8	0	0
3	34	T	4.7	4	0	16	0
4	25	T	3.8	0	8	0	0
5	16	T	300	95	32	0	0
6	18	T	16	24	0	0	0
7	14	T	3.6	0	0	0	32
8	29	T	100	0	32	0	0
9	47	T	25	96	16	0	0
10	38	T	18	4	8	0	0
11	42	T	3.2	0	0	0	0
12	52	T	34	78	0	0	0
13	42	T	12	2	0	0	0
14	16	T	4.8	14	16	0	0
15	16	0	100	89	32	16	0
16	32	0	25	43	0	0	0
17	17	0	18	80	0	0	0
18	32	0	32	64	0	16	0

Table II

Anti-BaEV, anti-GaLV and anti-HTLV antibodies in the blood plasma of adult patients with chronic lymphoid leukaemia (CLL) and lymphoma

Patient		Diagnosis	Cell type	Peripheral leukocytes		Antibody titre		
No.	age (yrs)			G/l	blast %	anti-BaEV	ant-GaLV	anti-HTLV
1	76	CLL	B	46	1	0	16	0
2	56	CLL	B	30	15	0	8	0
3	78	CLL	B	52	2	0	0	0
4	61	CLL	B	39	3	0	0	0
5	49	lymphoma	B		normal	0	0	0
6	46	lymphoma	T	3.5	0	0	0	32
7	48	lymphoma	B		normal	0	0	0
8	52	lymphoma	B		normal	0	0	0
9	50	lymphoma	B	4.8	1	0	0	0
10	55	lymphoma	Lennert	6	14	0	0	0
11	46	lymphoma	B	5.6	0	0	8	0
12	47	lymphoma	Hodgkin		normal	0	0	0

Table III

Anti-BaEV, anti-GaLV and anti-HTLV antibodies in the blood plasma of children with progressive stage acute lymphoid leukaemia (ALL) and lymphoma

Patient		Diagnosis	Cell type	Peripheral leukocytes		Antibody titre		
No.	age (yrs)			G/l	blast %	anti-BaEV	anti-GaLV	anti-HTLV
1	8	ALL	T	4.5	0	4	0	16
2	5	ALL	T	245	90	0	0	0
3	12	ALL	T	5.6	34	8	0	0
4	7	ALL	0	2.8	10	0	0	0
5	5	ALL	0	1.8	2	8	0	0
6	9	ALL	0	5.6	34	8	4	0
7	9	ALL	0	4.3	15	0	8	0
8	11	ALL	0	26.4	96	0	0	0
9	14	ALL	0	4.8	30	0	0	0
10	13	ALL	0	1.6	0	0	0	0
11	5	ALL	0	5	2	0	0	0
12	12	ALL	0	4	0	16	4	0
13	7	NHL	n.c.	3.4	0	0	0	0
14	10	NHL	B	3	0	16	8	0
15	4	HL	—	4.5	0	0	0	0
16	12	HL	—	5.2	0	0	0	0

NHL = non-Hodgkin lymphoma
HL = Hodgkin lymphoma
n.c. = not characterized

Table IV

Anti-BaEV, anti-GaLV and anti-HTLV antibodies in the blood plasma of children in remission of acute lymphoid leukaemia and lymphoma

Patient		Diagnosis	Cell type	Peripheral leukocytes		Antibody titre		
No.	age (yrs)			G/l	blast %	anti-BaEV	anti-GaLV	anti-HTLV
1	4	ALL	T	4.5	0	0	0	8
2	3	ALL	T	4.8	0	0	0	0
3	5	ALL	T	3	0	0	0	0
4	4	ALL	0	3	0	0	4	0
5	15	ALL	0	7.4	0	4	0	0
6	10	NHL	n.c.	5.6	0	8	0	0
7	14	HL	—	8	0	4	0	0

NHL = non-Hodgkin lymphoma
HL = Hodgkin lymphoma
n.c. = not characterized

dren with T-cell ALL and three with O-cell ALL. The plasma of three children with O-cell ALL contained anti-GaLV antibodies. Antibodies to HTLV could be detected in a single of T-cell ALL. The plasma sample of one child with B-cell lymphoma contained antibodies to both BaEV and GaLV.

4. *Anti-BaEV, anti-GaLV and anti-HTLV antibodies in plasma samples of children in remission of ALL or lymphoma.* Antibodies to BaEV were found in one case of O-cell ALL and in two cases of lymphoma (Table IV). The plasma sample of one child with O-cell ALL contained anti-GaLV antibodies. Anti-HTLV antibodies could be detected in the plasma of one child suffering from T-cell ALL.

Plasma samples from 24 healthy children and close family members of 3 children with malignant lymphoproliferative disease did not contain antibodies to BaEV, GaLV or HTLV. Control samples proved to be negative.

Discussion

The present results indicate that antibodies to type-C primate retroviruses may be present in the blood plasma of patients with lymphoid malignancy. There was, however, a difference between the different cell types in the specificity of antibodies. Antibodies to BaEV could be detected in various types of lymphoid leukaemia, lymphoma and rarely in healthy subjects. GaLV-specific antibodies were found mainly in B- and O-cell leukaemia and lymphoma. Anti-HTLV antibodies were detected only in a few cases of T-cell malignancy. Reliable judgement of the frequency of antibodies in progression versus remission would have required to study more patients in remission.

Interpretation of immunological and biochemical markers of animal retroviruses found in human neoplastic tissues and sera has some difficulties [12, 13]. Recently nucleic acid sequences related to the proviruses of AKR murine leukaemia virus and BaEV have been detected in preparations of human DNA fragments, by a highly sensitive and specific method [14]. Presence of antigens cross-reacting with p30 and carbohydrate-free gp70 of BaEV and simian sarcoma virus was demonstrated in human leukaemias by the ELISA technique [15]. These results are supported by the present findings from another point of view, viz. many plasma samples exhibited a strong precipitating activity on p15 and carbohydrate-free gp70 of BaEV and GaLV [16]. High-titre antibodies were detected especially in patients with potentially preleukaemic disorders.

HTLV has been isolated from several cases of cutaneous T-cell lymphoma and leukaemia in the United Sates [5, 6] and subsequently from several cases of adult T-cell leukaemia in Japan [9, 17]. Nucleic acid hybridization studies have identified HTLV-related sequences in the DNA of fresh lymphoblasts from a

child with T-cell ALL [18]. Anti-HTLV antibodies were found in patients with T-cell lymphoma and leukaemia and in normal subjects in the virus-endemic areas [9, 10]. In a survey of normal donors from other regions, only two sera reacted positively with HTLV. Both of them were close relatives of patients positive for HTLV [19, 20]. Our finding of HTLV antibodies in some T-cell malignancies suggests that HTLV infection may be associated with the occurrence of leukaemias and lymphomas. Normal donors and close family members of patients had no antibodies to HTLV. We suspect that future seroepidemiologic studies by highly sensitive radioimmunologic methods will clarify the origin of these HTLV infections.

REFERENCES

1. Gallagher, R. E., Gallo, R. C.: Sciences **187,** 350 (1975).
2. Nooter, K., Aaronson, A. M., Bentvelzen, P., de Groot, F. G., Pelt, F. G.: Nature **256,** 595 (1975).
3. Kaplan, H. S., Goodenow, R. S., Epstein, A. L. Gartner, S., Decleve, A., Rosenthal, P. N.: Proc Natl Acad Sci USA **74,** 2564 (1977).
4. Goodenow, R. S., Kaplan, H. S.: Proc Natl Acad Sci USA **76,** 4971 (1979).
5. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., Gallo, R. C.: Proc Natl Acad Sci USA **77,** 7415 (1980).
6. Poiesz, B. J., Ruscetti, F. W., Reitz, M. S., Kalyaranaman, V. S., Gallo, R. C.: Nature **294,** 268 (1981).
7. Rho, H. M., Poiesz, B. J., Ruscetti, F. W., Gallo, R. C.: Virology **112,** 355 (1981).
8. Reitz, M. S., Poiesz, B. J., Ruscetti, F. W., Gallo, R. C.: Proc Natl Acad Sci USA **78,** 1887 (1981).
9. Mioyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K., Hinuma, Y.: Nature **294,** 770 (1981).
10. Blattner, W. A., Kalyaranaman, V. S., Robert-Guroff, M., Lister, T. A., Galton, D. A. G., Sarin, P. S., Crawford, M. H., Catovsky, D., Greaves, M., Gallo, R. C.: Int J Cancer **30,** 257 (1982).
11. Oppenheim, J. J., Schecter, B.: In Rose, N. R., Friedman, H. (eds): Manual of Clinical Immunology. American Society for Microbiology, Washington DC, 1976. pp 81 94.
12. Barbacid, M., Bolognesi, D., Aaronson, S. A.: Proc Natl Acad Sci USA **77,** 1617 (1980).
13. Snyder, H. W., Fleissner, E.: Proc Natl Acad Sci USA **77,** 1622 (1980).
14. Martin, M. A., Bryan, T., Rasheed, S., Khan, A. S.: Proc Natl Acad Sci USA **78,** 4892 (1981).
15. Hehlmann, R., Schetters, H., Erfle, V.: In Neth, R., Gallo, R. C., Graf, T., Mannweiler, K., Winkler, K. (eds): Modern Trends in Human Leukaemia IV. Springer Verlag, Berlin 1981. pp 530 536.
16. Tóth, F. D., Váczi, L., Szabó, B., Réthy, A., Rák, K., Kiss, J., Kiss, A.: Acta Virol **28,** 191 (1984).
17. Yoshida, M., Miyoshi, I., Hinuma, Y.: Proc Natl Acad Sci USA **79,** 2031 (1982).
18. Gallo, R. C., Poiesz, B. J., Ruscetti, F. W.: In Neth, R., Gallo, R. C., Graf, T., Mannweiler, K., Winkler, K. (eds): Modern Trends in Human Leukaemia IV. Springer Verlag, Berlin 1981. pp 502 514.
19. Posner, L. E., Robert-Guroff, M., Kalyaranaman, V. S., Poiesz, B. J., Ruscetti, F. W., Fossieck, B., Bunn, P. A., Minna, J. D., Gallo, R. C.: J Exp Med **154,** 333 (1981).
20. Kalyaranaman, V. S., Sarngadharan, M. G., Bunn, P. A., Minna, J. D., Gallo, R. C.: Nature **294,** 271 (1981).

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TAKE AND GROWTH OF THE TRANSPLANTABLE MC29 HEPATOMA IN ALLOGENEIC AND XENOGENEIC HOSTS

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We report here the unexpected biological behaviour of the transplantable MC29 virus-induced hepatoma. This neoplasm, originating from an inbred white Leghorn (Duke) chicken, is maintained in our laboratory by serial in vivo passages in Hunnia hybrid chickens allogeneic to the original host. More than 80% of the tumours developing after subcutaneous inoculation of 3×10^6 hepatoma cells into newly hatched chickens grew progressively, while after injection of the same number of cells into 7 days old birds regressive tumour growth was observed. Transplantation from the allogeneic hosts into 7 days old inbred white Leghorn (Duke) chickens also resulted in regression of tumours in the great majority of cases. After inoculation to xenogeneic Japanese quails, progressor tumours developed in both two weeks old and adult birds with a dramatic increase of the frequency of liver metastases. Transplantation to another xenogeneic host, the turkey, revealed an age-related resistance similar to that of Hunnia hybrid chickens.

According to the genetic theory of transplantation formulated by Little and Strong, the outcome of animal-to-animal tumour transplantation depends upon the degree of genetic similarity between the grafted tissue and the host [1]. Although the genetic rules of transplantation are well-established, there are some exceptions: allogeneic as well as xenogeneic tumour transplants can be successful under certain conditions. Implantation to the chorioallantoic membrane of chick embryos, inoculation into immunologically incompetent (fetus, newborn) or immune-depressed, immune-deficient, immune-tolerant animals, as well as injection into immunologically privileged sites (brain, anterior chamber of eye, hamster cheek pouch, etc.) may also allow the growth of transplants [2]. We report here on the progressive growth of a chicken hepatoma of viral origin in xenogeneic Japanese quails not affected by any immunological manipulation. The factors permitting this unusual biological behaviour are unknown at present.

Materials and methods

The transplantable, MC29 virus-induced chicken hepatoma established by Langlois et al. [3] was maintained by serial in vivo passages in Hunnia hybrid chickens.

The aseptically removed tumours were homogenized with Potter in PBS and the number of cells was counted in a haemocytometer. 3×10^6 cells were inoculated under the skin of the

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neck or into the wing web of newly hatched and 7 days old chickens. White Leghorn (Duke) chickens originating from an inbred line established at Duke University Medical Center and randomly bred Japanese quails and turkeys were transplanted similarly.

Tumour development was monitored by palpation twice weekly. The time taken for palpable growth to appear determined the latency period. The tumours were measured in two dimensions with calipers. Growth rates are expressed as the increase of the mean tumour diameter per week.

Birds were examined post mortem for overt metastases. Suspected metastatic foci were further examined histologically.

Results

Results are summarized in Tables I and II. Inoculation of 3×10^6 MC29 hepatoma cells into newly hatched Hunnia hybrid chickens (allogeneic hosts) resulted in a 95% take after 7 days latency and 86.4% of the tumours grew progressively. A rapid local growth (mean growth rate: 32 mm per week), development of large necrotic tumours and a short mean survival time (12 days) could be observed.

The course of the disease was profoundly altered if 7 days old Hunnia hybrid chickens were inoculated with the same number of MC29 hepatoma cells. After a 7 days period of latency the take was 78%, but the mean growth rate (6.7 mm per week) was significantly slower than that of the tumours of chickens inoculated on the day of hatching. The tumours regressed as fastly as they had developed.

Table I

Growth characteristics of MC29 hepatoma transplanted from Hunnia hybrid chickens into various hosts

Host	Take	Latency, days	Mean growth rate (mm/week)	Course of the disease	Mean survival time, days	Frequency of liver metastases
1 day old Hunnia hybrid chickens	22/23 (95%)	7	32	19 progressor 3 regressor	12 —	2/22 (9%)
7 days old Hunnia hybrid chickens	18/23 (78%)	7	6.7	18 regressor	—	—
7 days old white Leghorn (Duke) chickens	17/20 (85%)	7	4.3	1 progressor 16 regressor	19 —	—
4 days old Duke chickens	11/11	7	16	9 progressor 2 regressor	—	—
14 days old Japanese quails	38/45 (84%)	17	11.6	38 progressor	39	20/38 (52.6%)
1 year old Japanese quails	13/17 (76.4%)	10	3.2	6 progressor 7 regressor	35 —	2/6 (33%)
1 day old turkeys	10/10 (100%)	7	13.8	5 progressor 5 regressor	12 —	—
7 days old turkeys	0/5	—	—	—	—	—

A similar take (85%) and course could be observed in the great majority of cases after inoculation of 7 day old inbred white Leghorn (Duke) chickens, assumed to be the closest to the MC29 hepatoma genetically. Progressor tumour developed only in one out of the 20 inoculated birds.

Inoculation of 14 days old Japanese quails (xenogeneic hosts) resulted in a 84.4% take after a prolonged latency period (17 days). The mean growth (11.6 mm per week) was slower than that of the progressor tumours developing in Hunnia hybrid chickens inoculated on the day of hatching, but faster than the growth of regressor Hunnia hybrid tumours. The mean survival time was 39 days and a marked increase in the frequency of liver metastases (52.6%) compared to the allogeneic hosts (9%) could be registered.

Transplantation of MC29 hepatoma from Hunnia hybrid chickens to 17 adult (1 year old) Japanese quails resulted in 7 regressor tumours and 6 slowly growing progressor tumours.

Inoculation into another xenogeneic host, the turkey, revealed an age-related resistance similar to that observed in Hunnia hybrid chickens. Progressor tumours developed in 5 out of 10 turkeys transplanted on the first day after hatching, while in 7 day old birds the tumour did not take. The histological picture of regressor MC29 hepatomas in Hunnia hybrid chickens and turkeys resembled that of the regressor line 1 guinea pig hepatocarcinoma described by Dvorak et al. [4]. Necrotic tumour masses were surrounded by connective tissue consisting mostly of fibroblasts. At the periphery of the tumours hepatoma cell islands were interspersed in the fibrous stroma.

Table II shows the results of transplantation to various hosts of MC29 hepatoma grown in xenogeneic birds. Inoculation of 1 day old Hunnia hybrid

Table II

Growth characteristics of MC29 hepatoma transplanted from Japanese quails or turkeys into variou s hosts

Source of transplanted cells	Host	Take	Latency, days	Mean growth rate, mm/week	Course of the disease	Mean survival time, days	Frequency of liver metastases
Sc. tumour of a Japanese quail	1 day old Hunnia hybrid chickens	0/16	—	—	—	—	—
Liver metastasis of a Japenese quail	1 day old Hunnia hybrid chickens	0/5	—	—	—	—	—
Sc. tumour of a Japanese quail	2 weeks old Japanese quails	13/18	12	8.2	13 progressor	25	8/13 (61.5%)
Sc. tumour of a turkey	1 day old Hunnia hybrid chickens	15/15 (100%)	7	24	15 progressor	18	0/14

chickens with 3×10^6 cells of MC29 hepatoma grown in the neck of a Japanese quail, or with the same number of cells from the liver metastasis of another MC29 hepatoma bearing Japanese quail failed to take. If, however, the hepatomas developing at the site of inoculation in Japanese quails were transplanted into two week old Japanese quails, progressor tumours developed after 12 days latency in 72.2% of the birds, with a high frequency (61.5%) of liver metastases.

Retransplantation of turkey-grown hepatoma cells to 1 day old Hunnia hybrid chickens produced progressor tumours in 15 out of 15 cases.

Discussion

After transplantation of virus-producing transformed cells, the tumour developing at the site of inoculation may grow by the multiplication of transplanted cells and/or by growth of the host cells transformed by the oncogenic virus released by the grafted neoplasm. Thus, several amphibian lymphoid tumours proved to be readily transferable to adults of a number of different amphibian species, both urodele and anuran. The tumours resulting from inter-order grafts were, however, composed of transformed host cells [4]. Such a mechanism did not operate in case of the MC29 virus-producer chicken hepatoma investigated in this study, as inoculation of MC29 virus per se in the wing web or intramuscularly did not induce rapid-growing tumours [3], and according to the histological picture the tumours developing at the site of inoculation in chickens, Japanese quails and turkeys were composed of hepatoma cells. Still, secondary infection by MC29 virus released from the tumour cells may have contributed to the spread of the disease into the liver.

The factors permitting progressive growth of MC29 hepatoma in xenogeneic hosts are unknown. Immunological immaturity or deficiency can be excluded in the Japanese quail. Assuming a tumour-induced immunodepression and/or loss of various antigens from the hepatoma cells, the age-related resistance of Hunnia hybrid chickens and turkeys remains unexplained. Furthermore, the subcutis of the neck and the wing web are not immunologically privileged sites. Development of immunological tolerance is a possible explanation, but why should some adult quails be tolerant and others resistant? One may suppose the existence of some para-immunological defence mechanisms recognizing neoplastic cells by markers other than classical surface antigenic determinants, developing soon after hatching in chickens and turkeys but slowly maturing and less efficient against the MC29 hepatoma cells in Japanese quails. Such a mechanism could account for the data summarized in Table I. Cook et al. [6] demonstrated that the capability of SV40 transformed hamster cells to induce tumours in histoincompatible hamsters is due to their resistance

to macrophage-mediated cytolysis. Macrophages were postulated to play a surveillance function against transformed cells [7], and monocytes collaborating with immune T cells inhibit the growth of carcinogen-induced chicken fibrosarcoma [8]. Besides, natural killer (NK) cells and thrombocytes should also be considered as potential antitumour effector cells. Since NK activity occurs only sporadically in the spleen of 1–3 week old chickens [9], NK cells cannot play a significant role in the development of the age-related resistance of chickens and turkeys against the MC29 hepatoma. The avian thrombocyte is a phagocytic cell and responds to lymphokines [10], but there are no data about its interaction with tumour cells.

Inoculation of MC29 hepatoma grown in xenogeneic Japanese quails or turkeys into newly hatched Hunnia hybrid chickens led to fundamentally different results (see Table II). In the former case no tumour growth could be observed, though progressor tumours arose after transferring tumour cells from quails to quails. Back-inoculation of turkey-grown hepatoma cells to 1 day old Hunnia hybrid chickens produced progressor tumours in 15 out of 15 cases.

These results suggest that the chicken hepatoma cells selected or adapted to grow in the Japanese quail hav echanged irreversibly, or cannot reaccommodate quickly enough to the chicken microenvironment. On the contrary, the milieu provided by turkeys for the MC29 hepatoma cells seems to be similar to that of Hunnia hybrid chickens.

Microenvironmental signals induce reversible shifts in tumour cell phenotypes and influence the in vivo behaviour of neoplastic cells [11–13]. Inductive interactions may result in irreversible changes of gene expression [14]. It would be difficult to distinguish this latter mechanism from the selection of newly mutated cells by various host microenvironments. Characterization of the mechanism permitting or inhibiting the growth of MC29 hepatoma in various hosts would help to explain the unexpected biological behaviour of the investigated tumour.

REFERENCES

1. Little, C. C., Strong, L. C.: J Exp Zool **41,** 93 (1924).
2. Friedman, M., Moldovanu, G., Moore, A. E., Miller, D. G.: Progr Exp Tumor Res **10,** 1 (1968).
3. Langlois, A. J., Lapis, K., Ishizaki, R., Beard, J. W., Bolognesi, D. P.: Cancer Res **34,** 1457 (1974).
4. Dvorak, H. F., Dvorak, A. M., Manseau, E. J., Wiberg, L., Churchill, W. H.: J Nat Cancer Inst **69,** 1459 (1979).
5. Balls, M., Ruben, L. N.: Progr Exp Tumor Res **10,** 238 (1968).
6. Cook, J. L., Hibbs, J. B., Lewis, A. M.: Proc Natl Acad Sci USA **77,** 6773 (1980).
7. Adams, D. O., Snyderman, R.: J Nat Cancer Inst **62,** 1341 (1979).
8. Palladino, M. A., Thorbecke, G. J.: Cell Immunol **38,** 350 (1978).
9. Sharma, J. M., Coulson, B. D.: J Nat Cancer Inst **63,** 527 (1979).
10. Stinson, R. S., Mashaly, M. M., Glick, B.: Immunology **36,** 769 (1979).
11. Diamandopoulos, G. T.: Proc Natl Acad Sci USA **76,** 6456 (1979).
12. Schirrmacher, V.: Immunobiology **157,** 89 (1980).
13. Portis, J. L.: J Nat Cancer Inst **62,** 611 (1979).
14. Solter, D., Shevinsky, L., Knowles, B. B., Strickland, S.: Develop Biol **70,** 515 (1979).

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HYDROXYLAMINE INACTIVATION OF CEPHALOSPORINS: NUCLEOPHILIC ATTACK ON BETA-LACTAM STRUCTURES

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Cephalosporins are not degraded by hydroxylamine (NH_2OH) in neutral and acidic solutions. Their reaction with NH_2OH in slightly alkaline solutions leads to microbiological inactivation which seems to be a structure dependent phenomenon. In these experiments the mandelic acid-type compounds appear to be quite stable to the effect of NH_2OH, whereas, cefazolin is gradually degraded and the straight chain-containing cephalosporins are variably inactivated. The phenylglycine-type oral cephalosporins were generally sensitive to the alkaline conditions used in these tests and apparently are not inactivated by NH_2OH. On the contrary, the phenylglycine-type cephalosporins seem to be somewhat stabilized in the presence of NH_2OH.

Hydroxylamine, a nucleophilic reagent, rapidly reacts with penicillins with the formation of the microbiologically inactive hydroxamic acid [1, 2] which forms a purple complex with ferric ion. This well-studied reaction is usually pH dependent, proceeds more readily in alkaline medium and is the basis of colorimetric determination of penicillins. Although the penicillin and cephalosporin molecules are similar, they show striking differences in their chemical and biological behaviour. Therefore, it was undertaken in this experiment to study the reaction of cephalosporins with NH_2OH. Hydroxylamine itself (NH_2OH) and many oxime ($=C=NOH$) derivatives are used, in addition to the above mentioned color-reaction formation, in research and therapy as enzyme-reactivators or restorators. Recently NH_2OH is used in the study of the ultimate mode of action of beta-lactam antibiotics.

Materials and methods

The concentration of cephalosporins or penicillins selected for assay was 4 μg/ml, and for cephalexin and SKF 60524 with poorer intrinsic in vitro activity, it was 8 μg/ml (Table I). All antibiotics were dissolved in deionized water. The final concentrations of NH_2OH, sodium bicarbonate ($NaHCO_3$) or citric acid, as used in these tests alone or in combination with antibiotics, were 100 μg/ml. These concentrations of chemicals did not produce inhibition zones in the bioassay procedure used throughout this experiment. The compounds were examined for their NH_2OH sensitivity in neutral, acidic (pH $\sim$ 3–4, with citric acid) and

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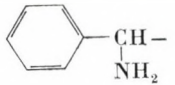
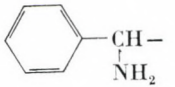
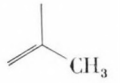
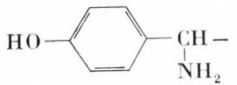
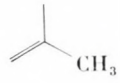
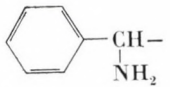
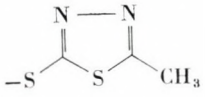
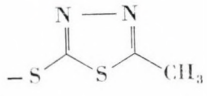
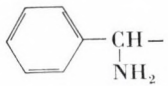
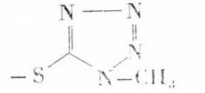
Table I

Effect of hydroxylamine on the degradation of cephalosporins with diverse structures in neutral, acidic and alkaline solutions

Name and/or SK & F number (drug concentration)	Substituents at		Sampling time, h	Mean diameters (mm) of inhibition zones with added reagents					
	7-position	3-position		Dist. H_2O	+ NH_2OH	+ $NaHCO_3$	+ $NaHCO_3$ + NH_2OH	+ Citric acid	+ Citric acid + NH_2OH
Cephaloglycin (4 μg/ml)			0	26	28	12	±	27	28
			3	25	28	0	0	28	28
			5	23	28	0	0	28	27
			24	10	26	0	0	25	26
Cephalexin (8 μg/ml)			0	19	18	16	17	19	20
			3	19	18	0	17	20	20
			5	19	18	0	17	20	20
			24	19	18	0	0	20	19
60524 (8 μg/ml)			0	17	17	0	12	18	18
			3	17	17	0	±	17	17
			5	17	18	0	9	17	17
			24	15	16	0	0	17	16
44065 (4 μg/ml)			0	23	25	18	20	26	26
			3	21	25	13	17	26	26
			5	16	24	±	17	26	26
			24	0	24	0	0	20	21
59589 (4 μg/ml)			0	23	24	21	22	24	24
			3	22	24	16	19	24	24
			5	21	24	14	18	24	24
			24	9	22	0	0	23	23
46029 (4 μg/ml)			0	24	27	14	12	27	28
			3	22	26	0	±	27	28
			5	18	27	0	±	28	27
			24	0	26	0	0	26	28

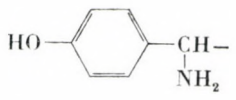
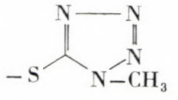
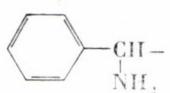
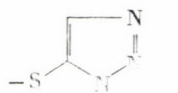
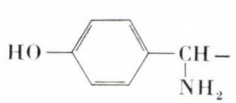
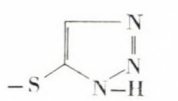
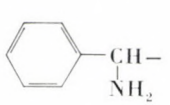
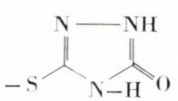
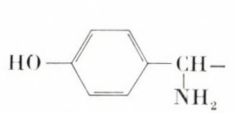
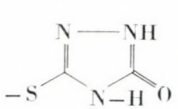
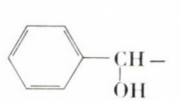
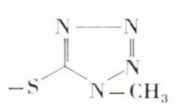
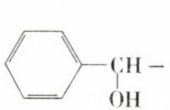
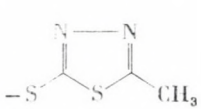
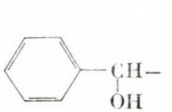
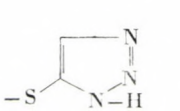
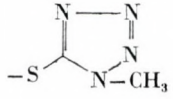
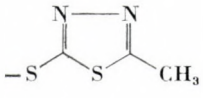
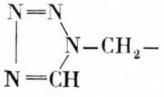
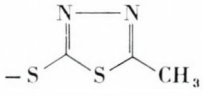
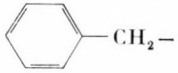
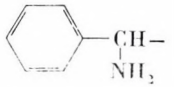
60701 (4 μg/ml			0	24	26	0	±	26	26
			3	24	26	0	0	26	26
			5	24	26	0	0	26	26
			24	10	24	0	0	24	24
60222 (4 μg/ml)			0	22	25	0	17	25	25
			3	23	25	0	8	24	25
			5	23	24	0	±	25	26
			24	12	23	0	0	23	23
Cefatrizine (60771) (4 μg/ml)			0	25	27	9	21	26	27
			3	24	26	0	14	26	26
			5	24	26	0	14	26	27
			24	17	24	0	0	24	24
58891 (4 μg/ml)			0	20	21	10	15	21	22
			3	19	21	0	9	21	21
			5	20	22	0	±	23	23
			24	11	20	0	0	20	20
60542 (4 μg/ml)			0	19	21	0	12	20	20
			3	20	21	0	12	21	21
			5	20	21	0	0	21	21
			24	13	18	0	0	19	19
Cefamandole (46028) (4 μg/ml)			0	27	28	27	19	28	26
			3	29	29	28	24	28	28
			5	29	28	28	25	29	28
			24	26	26	23	19	27	27
42669 (4 μg/ml			0	29	29	29	28	28	28
			3	29	29	29	28	28	28
			5	28	28	28	28	28	28
			24	27	27	25	23	25	24
59667 (4 μg/ml)			0	23	23	20	19	23	23
			3	22	23	20	19	23	23
			5	21	22	21	19	23	22
			24	22	22	17	13	23	22

Table I (continued)

Name and/or SK & F number (drug concentration)	Substituents at		Sampling time, h	Mean diameters (mm) of inhibition zones with added reagents					
	7-position	3-position		Dist. H_2O	+ NH_2OH	+ $NaHCO_3$	+ $NaHCO_3$ + NH_2OH	+ Citric acid	+ Citric acid + NH_2OH
Cefazaflur (59962) (4 μg/ml)	CF_3SCH_2-		0	27	25	26	18	28	27
			3	27	27	26	22	27	26
			5	28	27	27	25	27	26
			24	27	24	24	16	25	25
60451	$nC_3H_7SO_2CH_2-$		0	21	21	15	0	21	21
			3	21	20	15	0	21	21
			5	21	21	15	0	21	21
			24	19	19	13	0	19	18
Cefazolin			0	23	23	17	10	23	22
			3	22	23	19	14	22	22
			5	23	22	22	22	22	22
			24	22	21	19	9	21	19
Benzyl-Penicillin	6-position		0	27	22	27	0	27	22
			3	27	16	27	0	25	18
		6-APA	5	27	±	27	0	16	±
			24	26	0	21	0	0	0
Ampicillin		6-APA	0	23	16	22	0	23	21
			3	23	0	21	0	24	19
			5	23	0	16	0	21	16
			24	20	0	12	0	21	0

alkaline (pH ~ 8, with $NaHCO_3$) solutions. The solutions and/or reaction mixtures were kept at 37 °C and samples were taken at 0. 3, 5 and 24 h for microbiological assay. The antibiotic activity was determined by the disc agar-diffusion method with *Bacillus subtilis* ATCC 6633 as indicator organism using unbuffered Trypticase soy agar [3]. The discs absorb about 20 μl of the solutions; consequently, the antibiotic concentrations were approx. 0.1 μg or 0.2 μg per discs, respectively. On all assays 3 discs were used and the inhibition zone diameters in Table I represent the average values of the three discs [4].

The names and/or Smith Kline and French Laboratories (SK & F) designations, as well as the structural features of the cephalosporins and penicillins employed in the study are shown in Table I. The cephalosporins are grouped according to their chemical structures, particularly substituents at 7-position of the aminocephalosporin molecule. Eleven phenylglycine or substituted phenylglycine-type cephalosporins, three of them, cephaloglycin, cephalexin and cefatrizine, are commercially available, are followed by three mandelic acid-type preparations, of which cefamandole is a marketed drug, and two compounds containing straight chains at 7-position were included in the study. In addition, cefazolin with a quite different chemical structure was also included. Penicillins examined to date behaved similarly, therefore, results obtained with two representative ones, namely benzyl-penicillin and ampicillin, are presented in Table I.

Results and discussion

The nucleophilic attack of NH_2OH on the sensitive penicillin nucleus and the subsequent molecular rearrangement is depicted in Fig. 1.

Cephalosporins, while still prone to react with most of the nucleophilic reagents and other beta-lactam degrading agents [5–8], are not as readily attacked by NH_2OH as are penicillins under the described experimental conditions. Some cephalosporins appear to be attacked only slightly and after a latent period. The reason for this may be that the primary degradation product of cephalosporins corresponding to penicilloic acid apparently is short-lived, most probably because of its instability [2].

It can clearly be seen from the data of Table I that the nucleophilic attack by NH_2OH on the semisynthetic cephalosporins as measured by the loss of microbiologic activity is strongly structure, time and pH dependent. The mandelic acid-type compounds are rather stable to NH_2OH attack at neutral, acidic and alkaline milieu. The phenylglycine-type compounds not only resist to the nucleophilic attack by NH_2OH, but even are stabilized by it in alkaline reaction, where usually they tend to decompose with loss of biological activity. Cefazolin and the straight chain-containing cephalosporins behave variably. Penicillins, as expected, are sensitive to the attack by NH_2OH especially in neutral and alkaline solutions.

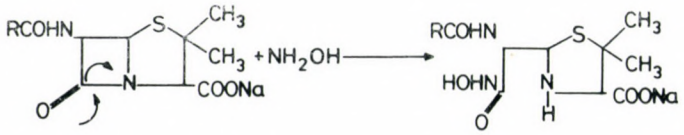


Fig. 1. Nucleophilic attack of NH_2OH on the penicillin beta-lactam ring (left) and the subsequent molecular rearrangement into hydroxamic acid (right)

From the results of these experiments, a number of important conclusions can be drawn. They supply additional confirmative material to the knowledge that the two fused beta-lactam-rings (beta-lactam-thiazolidine and beta-lactam-dihydrothiazine), despite their similarities, are quite different. They behave differently not only to the nucleophilic attack by NH_2OH, but also they are variably sensitive to beta-lactamases of diverse origin and the cephems are more resistant to the ambient acidity or alkalinity. The differences can even further be emphasized by the versatile possibilities offered for substitutions of the beta-lactam-dihydrothiazine ring system (positions 7 and 3 and others). Our observations as to the influence of NH_2OH attack on semisynthetic cephalosporins should be borne in mind whenever the hydroxylamine method [9–12] is to be adopted as a method for determination of cephalosporins in solutions and/or body fluids [13]. It is also important when examining the role of the nucleophilic properties of NH_2OH in the beta-lactam release reactions related to the binding of beta-lactam antibiotics to the penicillin-binding membrane proteins of bacteria [14].

REFERENCES

1. Ford, J. H.: Ind Eng Chem Anal Ed **19,** 1004 (1947).
2. Hou, J. P., Poole, J. W.: J Pharm Sci **60,** 503 (1971).
3. Uri, J. V., Actor, P., Phillips, L., Weisbach, J. A.: Experientia **31,** 54 (1975).
4. Uri, J. V.: Experientia **39,** 1085 (1983).
5. Abraham, E. P.: Quart Rev Chem Soc **21,** 231 (1967).
6. Spencer, J. L., Siu, F. Y., Flynn, E. H., Jackson, B. G., Sigal, M. V., Higgins, H. M., Chauvette, R. R., Andrews, S. L., Bloch, D. E.: Antimicrob Agents Chemother. 1966, p. 573.
7. Indelicato, J. M., Norvalis, T. T., Weehler, W. J., Wilham, W. L.: J Med Chem **17,** 523 (1974).
8. Yamana, T., Tsuji, A., Kanayama, K., Nakano, O.: J Antib **27,** 1000 (1974).
9. Boxer, G. E., Everett, P. M.: Anal Chem **21,** 670 (1949).
10. Flynn, E. H.: In Flynn, E. H. (ed.): Cephalosporins and Penicillins. Academic Press, New York—London 1972. p. 680.
11. Holl, W. W., O'Brien, M., Filan, J., Mazeika, T. R., Post, A., Pitkin, D., Actor, P.: J Pharm Sci **64,** 1232 (1975).
12. Dash, C. H.: J Antimicrob Chemother **1,** (Suppl) 107 (1975).
13. Mays, D. L., Bangert, F. K., Cantrell, W. C., Evans, W. G.: Anal Chem **47,** 2229 (1975).
14. Waxman, D. J., Strominger, J. L.: Ann Rev Biochem **52,** 825 (1983).

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CHARACTERIZATION OF ADENOVIRUS HEXON EPITOPES BY MONOCLONAL ANTIBODIES

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Three-thousand hybridoma clones were produced using crystallized hexon of human adenovirus type 1 for immunization. ELISA revealed 69 homospecific antibody producing clones. The monoclonals were reactive with both crystallized and non-crystallized purified soluble hexon preparations, as well as with the hexons derived from purified virions. Hexon preparations of 10 different human adenovirus species (members of 4 different subgenera) were used for the characterization of 33 monoclonal antibodies. ELISA and passive haemagglutination (HA) tests revealed 6 and 15 distinct reactivity patterns (RP) i.e. intertype cross-reaction of a given antibody with the panel of test-antigens, respectively. Four of these RP-s were identical by both techniques. Significant quantitative differences existed between extinction values in ELISA and HA-titres. The results indicate the presence of identical epitopes as well as epitypic relationships among the hexons. The common epitopes and the epitypic relationship seem to be characterized by 16 different interspecies specificities under the experimental conditions, which are independent of subgenus boundaries and are demonstrable in different combinations on the hexons of adenovirus species belonging either to the same or to different subgenera.

Antiviral immune sera are essential tools in the study of the antigenic structure of viruses and of individual viral components. Since viruses and viral protein components are often antigenically complex, the use of multireacting antisera limits the appropriate antigenic analysis to considerations of the total antigenicity of a complex viral protein and precludes the characterization of individual epitopes of a protein. The introduction of the use of monoclonal antibodies has added a powerful tool for the antigenic analysis of the antigenically complex viral proteins [1–4].

The capsomeric form of the adenovirus hexon is a trimer which consists of three identical polypeptide subunits of 105 000–120 000 M_r each [5–8]. The hexon is known to contain both species-specific (type-specific) and genus-specific antigenic determinants (epitopes) [8–10]. By absorption of antihexon sera with purified heterologous hexons and with the help of monoclonal antibodies, subgenus- and intersubgenus antigenic specificities have been demon-

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strated [8, 9, 11, 12], but actual distinctions in antibody populations have not yet been demonstrated [8].

In the present study, by employing monoclonal antibodies against crystallized hexons of human adenovirus type 1, we were able to discriminate — in the form of various types of cross-reactions — 16 determinants that were expressed on a various number of heterologous hexons in different combinations.

Materials and methods

Hybridoma-production. Animals and immunizations. Conventionally bred, 8–12 weeks old Balb/c female mice were used for immunization. The animals were injected twice intraperitoneally with 5 μg of Alum-precipitated antigen, three weeks apart. Four weeks after the last immunization and three days prior to the fusion, spleen cell donors received an intravenous injection of 20 μg antigen in PBS.

Antigen-preparation for the hybridoma. Hexons from HEp-2 cells infected with human adenovirus type 1 were separated and purified as described earlier [13]. The highly purified and concentrated hexon preparation was crystallized according to the method described by Ádám and Nász [14], then the crystals were sedimented with 2000 r.p.m. The crystals were washed by centrifugation in acetate buffer two times, then dissolved in 0.01 M (pH 7) phosphate buffer. To dissolve the crystals, the phosphate buffer was slightly alkalized by the addition of Na_2CO_3. The redissolved material from the crystals was used for immunization of the spleen cell donor mice.

Cells, media and culture conditions. All cell lines used for producing hybridomas were cultured in RPMI 1640 (Flow Laboratories), supplemented with 10% fetal calf serum (FCS). Culturing was carried out at 37 °C, in 5% CO_2. The nonsecreting parental cell line, Sp2/O originated from G. Köhler [15].

Cell fusion, selection and cloning of hybrids. Cell fusion procedure was carried out at room temperature. The donor lymphocyte preparation was washed by centrifugation in PBS. Parallel, the myeloma cells (at exponential growth phase) were spun out of their culture medium and washed with PBS. The washed lymphocytes and myeloma cells were counted, mixed at a ratio of 5 : 1–10 : 1 and were spun down together. The cell pellet was resuspended in 0.5–1 ml of 50% (v/v) PEG-6000 (Fluka). After incubation for one minute, the cell suspension was gradually filled up with serum-free RPMI to 20 ml, the cells were spun down and gently resuspended in FCS-supplemented RPMI to give a final concentration of 0.4 to 2×10^6 lymphocytes/ml. Then 0.1 ml aliquots of the cell suspension were distributed to 96-well flat-bottom tissue culture plates (Nunc). The day after the fusion, approximately half of the cell supernatants was sucked off and the wells were refilled with RPMI containing hypoxanthine (13.6 μg/ml), aminopterine (0.186 μg/ml) and thymidine (15.5 μg/ml) (HAT). Feeding of cells with half volume of the HAT medium was repeated 2–3 times, depending on cell growth.

The culture fluid of individual cultures was tested by ELISA for the presence of antibodies (positive clones).

Positive clones were transferred to 24-well flat-bottom tissue culture plates (Nunc), cultured to appropriate cell density, cryopreserved at —192 °C and cloned.

Cloning of hybrid cell lines was performed by the limiting dilution procedure, mouse peritoneal exsudate cells were used as filler cells (10^5/ml) in 96-well plates.

Antigen-production for the analysis of monoclonal antibodies. In order to produce purified hexon antigens, HEp-2 cells were infected with the following adenovirus species: type 1, 2, 5 and 6 (subgenus C), type 8, 9, 10 and 13 (subgenus D), type 7 (subgenus B) and type 12 (subgenus A). All the virus strains used were the prototype strains except type 7 and 8. The type 7 strain was the Pinkney isolate [16]; the type 8 strain has been isolated and characterized in detail by us [17, 18]. The maintenance and infection of the cells and purification of hexon proteins by chromatography were carried out according to the method described earlier [13, 18, 19].

ELISA procedure. 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 carbonate–bicarbonate buffer (pH 9.6) at 10 μg/ml final concentration. The plates were incubated overnight at 4 °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 h incubation at 37 °C,

the wells were washed twice and then 50 μl of hybridoma supernatant were added per well and incubated at 37 °C for 2 h. 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 h 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 nm wavelength filter. Polyclonal serum of mice immunized with the purified hexon of adenovirus type 1 and the culture media used for the hybridoma cells were applied as positive and negative controls, respectively. Hybridoma supernatants displaying at least the double absorbance of negative controls were considered to be positive.

Haemagglutination (HA). According to the method of Ross and Ginsberg [20], a 2.5% suspension of washed sheep red blood cells (SRBC) was prepared in phosphate-buffered saline (pH 7.2) and an equal volume of tannic acid, 1 : 20 000 in the same buffered saline was added. The suspension was incubated for 10 min at 37 °C, then centrifuged and washed in the above solution. The SRBC were then resuspended as a 5% suspension in the solution of purified adenovirus hexon preparation containing 150–200 μg protein in phosphate-buffered (pH 6.4) saline. After 30 min incubation, the SRBC were washed twice and resuspended as 0.5% suspension in the same buffered saline containing 1 : 400 bovine serum albumin (Fluka). HA test was carried out in Takátsy's microtitrator. Agglutination patterns were read after 1 h incubation at 37 °C. Antibodies were tested from a 1 : 50 or 1 : 30 dilution and the resulting titres are expressed as $\log_2 \times 10^{-2}$.

Results

Spleen cells of Balb/c mice immunized with crystallized human adenovirus type 1 hexon were fused with Sp2/O myeloma cells. The supernatant of 3000 hybridoma clones were examined by ELISA and 69 clones were found to produce specific monoclonal antibodies when tested with adenovirus type 1 hexon. The monoclonal antibodies were reactive both with the crystallized and non-crystallized purified soluble hexon preparations as well as with hexons derived from purified complete virions.

In order to examine the antigenic relationship among different hexons, 33 monoclonal antibodies were tested by both ELISA and passive haemagglutination methods with hexon preparations of 10 different human adenovirus species (serological types) belonging to 4 different subgenera. Table I shows the results of the ELISA experiments with the 10 different purified hexons, among which the A and B subgenera are each represented by one, the D subgenus is represented 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 [11]. The sections of Table I separated by horizontal intermissions indicated distinct reactivity patterns (RP), i.e. type of cross-reaction of a given hybridoma antibody with the panel of the test antigens. The number of reactive hexon types giving positive results varied between five and ten. These belonged mainly to members of subgenus C and to types 8 and 9 of subgenus D. Only 3 to 24% of the antibodies reacted with the hexons of other types. One hybridoma antibody (32D3) did not react with type 2 of subgenus C, and one single antibody (31A3) reacted with hexons of all ten types examined.

Table I

ELISA reactions of 33 monoclonal antibodies tested with adenovirus hexons

Designation of hybridoma antibody	Human adenovirus types according to subgenera									
	A	B	C				D			
	12	7	1	2	5	6	8	9	10	13
31A3	+	+	+	+	+	+	+	+	+	+
32C2	+	+	+	+	+	+	+	+	+	−
2H12	+	+	+	+	+	+	+	+	+	−
2A10	+	+	+	+	+	+	+	+	+	−
32A1	−	+	+	+	+	+	+	+	+	−
32A6	−	+	+	+	+	+	+	+	+	−
32D6	−	+	+	+	+	+	+	+	+	−
2E3	−	+	+	+	+	+	+	+	+	−
32A4	+	−	+	+	+	+	+	+	−	−
32A3	+	−	+	+	+	+	+	+	−	−
31D3	+	−	+	+	+	+	+	+	−	−
31B5	−	−	+	+	+	+	+	+	−	−
32C4	−	−	+	+	+	+	+	+	−	−
31C5	−	−	+	+	+	+	+	+	−	−
31A2	−	−	+	+	+	+	+	+	−	−
32A5	−	−	+	+	+	+	+	+	−	−
32C3	−	−	+	+	+	+	+	+	−	−
31A5	−	−	+	+	+	+	+	+	−	−
31A4	−	−	+	+	+	+	+	+	−	−
31A6	−	−	+	+	+	+	+	+	−	−
31D4	−	−	+	+	+	+	+	+	−	−
32B1	−	−	+	+	+	+	+	+	−	−
32B2	−	−	+	+	+	+	+	+	−	−
32B3	−	−	+	+	+	+	+	+	−	−
32B5	−	−	+	+	+	+	+	+	−	−
32C1	−	−	+	+	+	+	+	+	−	−
32D4	−	−	+	+	+	+	+	+	−	−
32C5	−	−	+	+	+	+	+	+	−	−
32C6	−	−	+	+	+	+	+	+	−	−
32D1	−	−	+	+	+	+	+	+	−	−
32D2	−	−	+	+	+	+	+	+	−	−
32A2	−	−	+	+	+	+	+	+	−	−
32D3	−	−	+	−	+	+	+	+	−	−
Number of positives	7	8	33	32	33	33	33	33	8	1

HA titres of the hybridoma supernatants are summarized in Table II. The number of reactive hexon types varied between four and ten. These also belonged mainly to members of subgenus C and to types 8 and 9 of subgenus D. In HA test only 21 to 42% of the samples reacted with other types. Also in HA, only one single hybridoma antibody failed to react with type 2 of subgenus C; this one, however, was not identical with the antibody which did not react with

Table II

*Haemagglutination titres of 33 monoclonal antibodies with adenovirus hexons**

Designation of hybridoma antibody	Human adenovirus types according to subgenera									
	A	B	C				D			
	12	7	1	2	5	6	8	9	10	13
31A3	7	7	9	2	10	9	10	7	6	9
32C2	7	4	7	2	7	6	6	4	5	7
2H12	4	6	6	3	7	4	2	2	4	1
2A10	3	1	5	3	6	4	3	4	3	—**
32A1	—	2	7	3	7	6	1	1	1	1
32A6	—	2	7	1	7	6	1	1	1	1
32D6	—	3	7	2	7	7	2	2	3	3
32A4	3	—	6	3	7	6	2	3	1	3
32A3	1	—	6	6	6	6	0.3	1	—	1
2E3	—	1	6	3	6	4	1	1	2	—
31B5	—	—	7	5	6	5	0.3	0.3	3	1
32C4	—	—	5	0.3	7	5	0.3	3	3	1
32D3	—	2	6	1	7	6	0.3	0.3	—	—
31C5	—	—	4	3	6	3	0.3	0.3	3	—
31A2	—	—	7	6	7	3	4	2	—	1
32A5	—	—	6	2	7	6	0.3	1	—	1
32C3	—	—	7	3	7	5	1	3	—	1
31D3	0.3	—	6	6	6	6	—	0.3	—	—
31A5	—	—	7	—	7	7	1	1	—	1
31A4	—	—	6	5	7	5	0.3	1	—	—
31A6	—	—	6	2	7	4	1	1	—	—
31D4	—	—	6	3	5	4	0.3	1	—	—
32B1	—	—	6	3	7	5	0.3	1	—	—
32B2	—	—	7	3	7	6	1	2	—	—
32D4	—	—	6	3	6	4	0.3	1	—	—
32B3	—	—	6	0.3	7	6	1	0.3	—	—
32B5	—	—	7	0.3	7	6	0.3	1	—	—
32C1	—	—	5	0.3	6	3	0.3	0.3	—	—
32C5	—	—	4	3	6	4	—	1	—	—
32C6	—	—	4	3	6	4	—	1	—	—
32D1	—	—	4	2	4	3	—	0.3	—	—
32D2	—	—	4	3	4	3	—	2	—	—
32A2	—	—	3	3	4	3	—	—	—	—
Number of positives	7	9	33	32	33	33	28	32	12	14
Mean titre	3.7	3.1	5.9	2.8	6.5	5.0	1.6	1.6	2.9	2.3

* $\log_2 \times 10^{-2}$
** < 0.3 ($< 1 : 120$)

Table III

Reactivity patterns of 33 monoclonal antibodies and their frequency tested by ELISA and haemagglutination

Number of hybridoma antibodies showing identical reactivity pattern		Human adenovirus types according to subgenera									
		A	B	C				D			
		12	7	1	2	5	6	8	9	10	13
ELISA	1	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	−
	4	−	+	+	+	+	+	+	+	+	−
	3	+	−	+	+	+	+	+	+	−	−
	21	−	−	+	+	+	+	+	+	−	−
	1	−	−	+	−	+	+	+	+	−	−
Haemagglutination	3	+	+	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+	+	−
	3	−	+	+	+	+	+	+	+	+	+
	1	+	−	+	+	+	+	+	+	+	+
	1	+	−	+	+	+	+	+	+	−	+
	1	−	+	+	+	+	+	+	+	+	−
	2	−	−	+	+	+	+	+	+	+	+
	1	−	+	+	+	+	+	+	+	−	−
	1	−	−	+	+	+	+	+	+	+	−
	3	−	−	+	+	+	+	+	+	−	+
	1	+	−	+	+	+	+	−	+	−	−
	1	−	−	+	−	+	+	+	+	−	+
	9	−	−	+	+	+	+	+	+	−	−
	4	−	−	+	+	+	+	−	+	−	−
	1	−	−	+	+	+	+	−	−	−	−

type 2 in ELISA. Three antibodies reacted with all ten types in HA, and one of these (31A3) was the same which displayed general positivity in ELISA. One hybridoma antibody (32A2) reacted only with the four members of subgenus C and not with types of other subgenera. The highest HA titres were obtained with type 5 hexon. Only two antibodies exhibited titres below the values obtained with the homologous type 1, while a considerable part of the antibodies exceeded these values and the geometric mean titre was even higher than that of type 1. With type 6, which belongs to the same subgenus as type 1, titres approximated the values with the homologous type, while in the case of the fourth member of subgenus C, type 2, the titres of only a few antibodies were at similar levels and the geometric mean titre remained below the values obtained even with types 12 and 7 of subgenus A and B, respectively. The lowest incidence of positive results was found with type 12 and type 7 hexons, however, the geometric mean titre with these types surpassed that with types belonging to subgenus D.

The reactivity patterns of the 33 antibodies and their frequency in the two assay systems are demonstrated in Table III. Six RPs were observed with ELISA and fifteen ones with HA. Four RPs were identical in the two assays. The same RP was recorded usually for only one or a few hybridoma antibodies.

Table IV

Cumulated results obtained by haemagglutination (H) and ELISA (E) for the 33 antibodies

Designation of hybridoma antibodies	Human adenovirus types according to subgenera									
	A	B	C				D			
	12 HE	7 HE	1 HE	2 HE	5 HE	6 HE	8 HE	9 HE	10 HE	13 HE
31A3	++	++	++	++	++	++	++	++	++	++
32C2, 2H12	++	++	++	++	++	++	++	++	++	+−
2A10	++	++	++	++	++	++	++	++	++	−−
32A1, 32A6, 32D6	−−	++	++	++	++	++	++	++	++	+−
32A4	++	−−	++	++	++	++	++	++	+−	+−
32A3	++	−−	++	++	++	++	++	++	−−	+−
2E3	−−	++	++	++	++	++	++	++	++	−−
31B5, 32C4	−−	−−	++	++	++	++	++	++	+−	+−
32D3	−−	+−	++	+−	++	++	++	++	−−	−−
31C5	−−	−−	++	++	++	++	++	++	+−	−−
31A2, 32A5, 32C3	−−	−−	++	++	++	++	++	++	−−	+−
31D3	++	−−	++	++	++	++	−+	++	−−	−−
31A5	−−	−−	++	−+	++	++	++	++	−−	+−
31A4, 31A6, 31D4, 32B1, 32B2, 32B3, 32B5, 32C1, 32D4	−−	−−	++	++	++	++	++	++	−−	−−
32C5, 32C6, 32D1, 32D2	−−	−−	++	++	++	++	−+	++	−−	−−
32A2	−−	−−	++	++	++	++	−+	−+	−−	−−

The most frequent pattern shown in the fifth line of Table III, was characteristic of 21 clones in ELISA, whereas in HA 9 clones displayed a similar activity.

Combining the results of the two methods (Table IV), 16 different RPs have been distinguished. Twelve RPs represented by 21 antibodies were characterized with certain hexon types by divergent results between HA and ELISA. With two types 5 RPs, and with one type 7 RPs exhibited positive HA and negative ELISA tests or vice versa. Considering the individual types of hexons, it may be summarized that each clone reacted similarly by the two methods with types 1, 5 and 6 of subgenus C and with type 12 of subgenus A. With the hexon of the fourth member of subgenus C, type 2, two clones while with type 7 of subgenus B, one clone reacted differently. Diverse results between the two methods were the most frequent for members of subgenus D. Different reactions were recorded with one antibody for type 9, four antibodies for type 10, six antibodies for type 8 and thirteen antibodies for type 13. In about one third of the sixteen different interspecies specificities one or two clones exhibited the same reactivity pattern; in nearly two-third of the clones, however, 3–4 or even 9 clones displayed identical patterns.

Discussion

The number of antibodies giving positive or negative ELISA and haemagglutination results with distinct hexon types, and the values of the HA titres may indicate different degrees of relationship between the hexons, provided that the concentration of the purified hexon preparations used as test antigen is the same. On this basis, the presence of identical epitopes as well as epitypic relationships [1] can be assumed among the different hexons. In the case of the species examined in this work, antigenic relationship seems to be characterized by 16 distinct interspecies specificities, which are independent of subgenus boundaries and are demonstrable, in different combinations on the hexons of adenovirus species belonging to either the same or different subgenera. Only one monoclonal antibody (32A2) and only with one of the methods (HA) showed a subgenus specificity. However, increasing the number of the different test antigens, i.e. the different hexon types, this antibody may give a positive reaction with hexons of one or more species belonging to other subgenera; the more it is probable because antibody 32A2 definitely reacted in ELISA with hexons of two species belonging to an other subgenus. Considering the grouping of the 16 different interspecies specificities, the closest antigenic relationship seems to exist among types 1, 5 and 6 belonging to subgenus C. Hexons of type 2 of subgenus C and of types 8 and 9 of subgenus D join with this group in a somewhat looser degree of relationship. An even lower degree of antigenic connection can be assumed for hexons of the other species.

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REFERENCES

1. Yewdell, J. W., Gerhardt, W.: Ann Rev Microbiol **35,** 185 (1981).
2. Sinkovics, J. G.: Cancer Research **41,** 1246 (1981).
3. Köhler, G., Milstein, C.: Nature **256,** 495 (1975).
4. Evans, D. M. A., Minor, P. D., Schild, G. S., Almond, J. W.: Nature **304,** 459 (1983).
5. Ádám, É., Nász, I.: Intervirology **15,** 37 (1981).
6. Ádám, É., Nász, I.: Virology **114,** 265 (1981).
7. Ádám, É., Nász, I.: Arch Virol **79,** 299 (1984).
8. Ginsberg, H. S.: Comp Virol **13,** 409 (1979).
9. Russel, W. C., Patel, G., Precious, B., Sharp, I., Gardner, P. S.: J Gen Virol **56,** 393 (1981).
10. Cepko, C. L., Whetstone, C. A., Sharp, P. A.: J Clin Microbiol **17,** 360 (1983).
11. Wigand, R., Bartha, A., Dreizin, R. S., Esche, H., Ginsberg, H. S., Green, M., Hierholzer, J. C., Kalter, S. S., McFerran, J. B., Petterson, U., Russell, W. C., Wadell, G.: Intervirology **18,** 169 (1982).
12. Norrby, E., Wadell, G.: J Virol **4,** 663 (1969).
13. Ádám, É., Nász, I., Medveczky, P.: Acta Microbiol Acad Sci Hung **24,** 181 (1977).
14. Ádám, É., Nász, I.: Intervirology **13,** 1 (1980).
15. Shulman, M., Wilde, C. D., Köhler, G.: Nature **276,** 269 (1978).
16. Nász, I., Pereira, M. G.: Proc IV. Congress Hung Assoc Microbiol 205 (1964).
17. Takács, M., Berencsi, Gy., Lengyel, A., Nász, I.: Acta Virol **27,** 289 (1983).
18. Lengyel, A., Nász, I.: J Virol **6,** 406 (1970).
19. Nász, I., Lengyel, A., Cserba, I.: Arch Ges Virusforsch **36,** 80 (1972).
20. Ross, E., Ginsberg, H. S.: Proc Soc Exp Biol Med **98,** 501 (1958).

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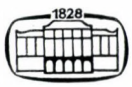

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