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THE DISTRIBUTION OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI IN INDIA

REKHA RANI and K. G. MUKERJI

Department of Botany, University of Delhi, Delhi, India

(Received May 29, 1987)

Vesicular-arbuscular mycorrhizal fungi are widely distributed throughout the area studied including different altitudes ranging from sea level to 2500 ft above sea level. VAM fungi were recorded from 88% of the sites examined with *Glomus fasciculatum* and *Glomus macrocarpum* being the most commonly recorded. Mean species diversity was found to be maximum in the areas thickly vegetated and undisturbed.

VAM fungi are known to be well distributed along both hemispheres. These fungi can be isolated from a wide variety of natural habitats and are particularly abundant in cultivated lands. Little work has been done regarding their distribution in India. During the present investigation we analysed soil samples from several parts of India, the eastern and western Himalayan region as well as several other parts of east and west India. VAM fungi were well represented with members of genera *Glomus* and *Gigaspora* being abundant. However, this synoptic survey indicated that although such fungi were abundant certain genera were conspicuously absent.

Materials and methods

During the period from March 1985 to March 1986 fifty soil samples were collected from different places in India. These collections were made from a variety of habitats covering the principal soil and vegetation types of the region. Full details of each site, location and predominant plant species are given in Table I. Three replicates of each sample were collected and stored in polythene bags for further studies at room temperature.

VAM spores were isolated from the soil samples by wet sieving and decanting technique [1]. The number of spores per 10 g of soil was determined for each sample. Results are expressed as mean of all the three replicates from each sample.

REKHA RANI, K. G. MUKERJI

Department of Botany, University of Delhi
Delhi 110007, India

Table I

Location and predominant plant species of sites of fungal isolates

Sample No.	Location	Dominant vegetation	VAM fungi isolated
1.	Old Delhi Ridge	*Azadirachta indica*	Ab, GF_1, GF_2, GiC_3, GM_3
2.	Old Delhi Ridge	*Prosopis juliflora*	GiC_3, GM_1, GM_2, GG, GF_2
3.	Old Delhi Ridge	*Cassia fistula*	GM_3, GL, GM_1, GiC_2, GF_2, Ab
4.	Old Delhi Ridge	*Adhatoda* sp.	Ab, GD, GiC_3, GF_2, GM_1, S
5.	Old Delhi Ridge	*Capparis decidua*	GiC_1, Ga, GP_2, GM_1, GM_3, GO
6.	Old Delhi Ridge	*Adhatoda*,Grasses	GiC_2, GC_1, GG, GM_1, GM_3, GR_2
7.	Old Delhi Ridge	*Prosopis spicigera*	GC_3, Ab, GiC_3, GF_2, GM_2, GM_4
8.	Old Delhi Ridge	*Zizyphus nummularia*	GiC_2, GC_4, Ab, GiC, GG, GR_1
9.	Old Delhi Ridge	*Grewia tenaz*	GiC_3, GC_1, GF_2, GM_1, GM_2
10.	Old Delhi Ridge	*Prosopis juliflora*	GP, Ab, GiC_2, GC_4, GF_2, GG, GR_1
11.	Samastipur, Bihar	*Cajanus cajan*	GC_1, GF_2
12.	Samastipur, Bihar	*Phaseolus* sp.	GF_2
13.	Samastipur, Bihar	*Brassica* sp.	GC_1, GM_1
14.	Samastipur, Bihar	Grasses	GF_2, GM_3
15.	Darbhanga, Bihar	*Dalbergia sissoo*	GF_2
16.	Darbhanga, Bihar	*Bamboo*	GF_2
17.	Darbhanga, Bihar	*Oryza sativa*	Nil
18.	Darbhanga, Bihar	*Artocarpus* sp.	GS, GD
19.	Muzaffarpur, Bihar	*Zea mays*	GF_2, GM_2
20.	Muzaffarpur, Bihar	*Brassica campestris*	GF_2
21.	Muzaffarpur, Bihar	*Triticum aestivum*	GL, GM_4
22.	Muzaffarpur, Bihar	*Nicotiana tobaccum*	GL
23.	Almohra, Gargar	*Marchantia* sp.	Ga, GC_3
24.	Almohra, Gargar	*Adiantum*, *Pteris*	Nil
25.	Almohra, Disaliyum	*Pinus* sp.	GC_3, GM_1, GF_2
26.	Almohra, Disaliyum	Gymnosperms	GM_1, GG
27.	Almohra	*Quercus* sp.	Nil
28.	Graminaceous Field	*Sorghum vulgare*	GiC_3, GG, GM_2
29.	Field outside Delhi	*Pennisetum typhoides*	Ab, GF_2, GL, GM_1
30.	Field outside Delhi	*Sorghum vulgare*	GiC_3, GF_2, GM_3, GR_1
31.	Field outside Delhi	*Triticum aestivum*	Ab, Ga, GF_2, GM_1
32.	Field outside Delhi	*Brassica* sp.	Ga, GL, GM_3, GR_3
33.	Delhi Zoo	*Bougainvilia* sp. I.	GM_1
34.	Delhi Zoo	*Azadirachta indica*	GF_2, GM_2
35.	Delhi Zoo	*Bougainvilia* sp. II	GM_1
36.	Bhagalpur, Bihar	*Cajanus cajan*	GF_2, GC_1
37.	Bhagalpur, Bihar	*Solanum tuberosum*	Nil
38.	Bhagalpur, Bihar	*Cicer arietinum*	GM_1
39.	Bhagalpur, Bihar	*Triticum aestivum*	GM_1, GF_2
40.	Budkhal lake, Haryana	*Albizzia lebbeck*	Nil
41.	Budkhal lake, Haryana	*Bougainvilia* sp.	GM_1
42.	Budkhal lake, Haryana	*Bougainvilia* sp.	GF_2
43.	Suraj Kund, Haryana	*Eucalyptus* sp.	GM_1
44.	Suraj Kund, Haryana	*Eucalyptus* sp.	Nil
45.	Suraj Kund, Haryana	Grasses	GG
46.	Goa	Grasses	GiC_2, GM_1
47.	Pune	Grasses	GiC_1, GiC_2, GM_1
48.	G. T. Road, Delhi	*Mangifera indica*	GF_2
49.	G. T. Road, Delhi	*Acyranthes*, *Withania*	GiC_3, Ga, S
50.	G. T. Road, Delhi	Grasses	GF_2, GM_1

Results and discussion

Our investigation has shown wide distribution of VAM fungi in the area studied ranging from longitude 14° E–85° 3″ E to 14° 20″ N–25° N latitude. All the VAM fungi isolated are commonly found and are equally abundant being recorded from 88% of the sites examined. Among the isolated VAM fungi were 19 species of *Glomus*, 4 species of *Gigaspora* and one each of *Acaulospora* and *Sclerocystis*.

Most of the VA mycorrhizal fungi were isolated as chlamydospores and some as sporocarps. They were well distributed in both cultivated and uncultivated soils, although their numbers were higher in cultivated areas. Similar results have also been reported by Mosse and Bowen [2], Hayman and Stovold [3]. Details of sample, location, habitat and the VAM fungi isolated are summarized in Table I. Since the plant growing in the vicinity of the site and soil type varied widely, all the fifty soil samples were classified into one of thirteen major habitat groups according to soil type and major site of collection (Table II). Maximum number of soil samples [10] were taken from the habitat relatively indisturbed and dominated by *Prosopis* sp. and *Adhatoda* sp. Maximum number of spores were isolated from these sites since it is a pocket of undisturbed natural vegetation. Five ot the samples had low frequency of occurrence of the VAM fungi. These sites were with saline sandy soils and one of the sample with clayey soils which might affect the frequency, since abiotic factors of the soil are known to influence the spore population quantitatively. Vegetation of the particular area is also determining factor and interaction of all these factors determines the spore population of a par-

Table II

Major habitat groups and sites of collection of fungal isolates

Habitat code	Place of collection	Soil type
A	Old Delhi Ridge	Sandy loam
B	Samastipur	Silty clay loam
C	Darbhanga	Saline silty clay
D	Muzaffarpur	Silty clay loam
E	Almohra	Coarse sandy loam
F	Cultivated fields of Delhi	Sandy clay loam
G	Delhi Zoo	Sandy loam
H	Bhagalpur	Clay loam
I	Budkhal lake	Saline sandy
J	Suraj Kund	Sandy soil
K	Goa	Coarse sandy
L	Pune	Clay loam
M	G. T. Road, Delhi	Sandy clay loam

ticular area. Eminent influence of host on population of *Endogonaceae* was also shown by Mukerji et al. [4].

Habitat A and F had the maximum number of records and also high species diversity. The mean species diversity of each habitat gives an indication of the species richness associated with habitat. However, species diversity gives a better indication of the difference in species diversity between the various habitat classification. It showed that only habitat A, F and L i.e. Delhi Ridge, Graminaceous fields from Delhi and Pune soil had 2.2 and more mean species diversities of VAM spores (Table III). Habitat I and J had extremely low frequency of occurrence of VAM spores. These were slopy sites having sandy loam located near a lake. That spores were few in number in these sites, may be due to the fact that they would have been washed away with water or might have settled down. Mukerji et al. [4] showed gradation in the density of the spore population at different horizons. The most abundant endophytes recorded were *G. fasciculatum* and *G. macrocarpum* having a frequency of 46% and 40%, respectively. followed by *G. mosseae* with 20% frequency of occurrence. *G. macrocarpum* and *G. fasciculatum* were found to be dominant species in both barren as well as cultivated areas. The percentage frequency of occurrence of individual VAM spore and their distribution in each habitat is given in Table IV. From the low number of samples examined and the subsequent low number of records, it is not possible to identify distinctive species-habitat association. However, it is found that more spore number and diversity were at the sites with undisturbed habitats having vegetation than disturbed habitats. The wet soils had less number of VAM spores than the dry

Table III

Species diversity of fungi according to habitat

Habitat	No. of sites sampled	No. of sites with fungi	% of sites with fungi	Total No. of records	Species diversity	Mean species diversity
A	10	10	100	837	22	2.2
B	4	4	100	72	4	1.0
C	4	3	75	83	3	1.0
D	4	4	100	102	5	1.25
E	5	3	60	122	5	1.6
F	5	5	100	212	12	2.2
G	3	3	100	48	3	1.0
H	4	3	75	72	3	1.0
I	3	2	67	34	2	1.0
J	3	2	67	18	2	1.0
K	1	1	100	40	2	2.0
L	1	1	100	104	3	3.0
M	3	3	100	70	4	1.3

Table IV

Frequency of VAM fungi according to habitat

	Name of the fungus	Number of records/habitat													Total	% Frequency
		A	B	C	D	E	F	G	H	I	J	K	L	M		
(Ab)	*Acaulospora bireticulata*	6	0	0	0	0	2	0	0	0	0	0	0	0	8	16
(GiC_1)	*Gigaspora corroloidea*	2	0	0	0	0	0	0	0	0	0	0	1	0	3	6
(GiC_2)	*Gi. calospora*	4	0	0	0	0	0	0	0	0	0	1	1	0	6	12
(GiP)	*Gi. pellucida*	0	0	0	0	0	2	0	0	0	0	0	0	0	2	4
(GiC_3)	*Gi. candida*	5	0	0	0	0	1	0	0	0	0	0	0	1	7	14
(Ga)	*Glomus albidum*	1	0	0	0	1	3	0	0	0	0	0	0	1	6	12
(GC_1)	*G. caledonicum*	2	2	0	0	0	0	0	1	0	0	0	0	0	5	10
(GC_2)	*G. clarum*	1	0	0	0	2	0	0	0	0	0	0	0	0	3	6
(GC_3)	*G. constrictum*	2	0	0	0	0	0	0	0	0	0	0	0	0	2	4
(GF_1)	*G. flavisporum*	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2
(GD)	*G. diaphanum*	1	0	1	0	0	0	0	0	0	0	0	0	0	2	4
(GF_2)	*G. fasciculatum*	8	3	2	2	1	3	1	1	1	0	0	0	1	23	46
(GG)	*G. geosporum*	5	0	0	1	1	1	0	0	0	1	0	0	0	9	18
(GL)	*G. leptotichum*	1	0	0	2	0	2	0	0	0	0	0	0	0	5	10
(GM_1)	*G. macrocarpum*	6	1	0	0	2	3	2	1	1	1	1	1	1	20	40
(GM_2)	*G. mosseae*	4	0	0	1	0	2	1	0	0	0	0	0	0	10	20
(GM_3)	*G. monosporum*	2	1	0	0	0	1	0	0	0	0	0	0	0	4	8
(GM_4)	*G. multicaule*	1	0	0	1	0	0	0	0	0	0	0	0	0	2	4
(GM_5)	*G. multisubstensum*	2	0	0	0	0	0	0	0	0	0	0	0	0	2	4
(GP)	*G. pulvinatum*	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2
(GO)	*G. occultum*	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2
(GR_1)	*G. radiatum*	2	0	0	0	0	1	0	0	0	0	0	0	0	3	6
(GS)	*G. scintillans*	0	0	1	0	0	0	0	0	0	0	0	0	0	1	2
(GR_2)	*G. reticulatum*	2	0	0	0	0	1	0	0	0	0	0	0	0	3	6
(S)	*Sclerocystis* sp.	1	0	0	0	0	0	0	0	0	0	0	0	1	2	4

ones. Earlier Readhead [5] and Khan [6] also reported a decrease in spore number in soils with high moisture content.

Although VAM fungi are well represented in the area studied but percentage frequency of occurrence differ with different areas having different types of soils. Similarly, species diversity varied considerably depending upon the different soil types and vegetation of that area.

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CITRIC ACID PRODUCTION WITH MIXED STRAINS OF *ASPERGILLUS NIGER IN* SUBMERGED CULTURE

KHALID HAFEEZ KHAN and S. SHAHID SHAUKAT

Department of Pharmaceutics, and Department of Botany, University of Karachi, Karachi, Pakistan

(Received July 21, 1987)

Citric acid yield of 8 different strains of *Aspergillus niger* in pure and mixed cultures was investigated. The yield varied remarkably with the strain and combination of strains. Generally, the yield of citric acid for a given strain was higher in pure than in mixed culture. However, low yielding strains in combination often augmented the yield under mixed culture. The possibility of an additive genic effect is discussed.

Many strains of *Aspergillus niger* are known to produce citric acid [1–5]. The capacity of citric acid production varies greatly with the strain [2, 6] and the high yielding strains are used in the commercial production of citric acid [7, 8]. One possible technique of improving the yield appears to be the use of mixed strains, particularly those that are high yielding. However, reports on citric acid production using mixed cultures are controversial. Yuill [9], Ciegler and Raper [10] and Chang et al. [11] obtained decreased yield in mixed cultures while Baracho and Monteiro [5] found at least one combination of strains where citric acid production exceeded that of the individual components. The objective of the present investigation was to evaluate the production of citric acid using single and mixed strains of *A. niger*.

Materials and methods

Media. Fermentation media used consisted of the following ingredients: sucrose, 140 g; NH_4NO_3, 2.5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7\,H_2O$, 0.25 g; Cu^{++}, 0.06 mg; Zn^{++}, 0.25 mg; Fe^{+++}, 1.3 mg; Mn, 1.0 μg. The volume of the medium was made up to 1 litre with distilled water and the pH was adjusted to 3.5 using diluted HCl.

Organisms. The organisms used were isolated by Khan [6] and were kept on potato dextrose agar slants, and identified as strains of *A. niger* in accordance with Raper and Fennel [12]. Eight strains used in this study were: A, B, C, D, E, F, G and H.

KHALID HAFEEZ KHAN,

Department of Pharmaceutics, University of Karachi
Karachi-32, Pakistan

S. SHAHID SHAUKAT

Department of Botany, University of Karachi
Karachi-32, Pakistan

Fermentation. Fifty ml of the fermentation medium were taken in each of the cotton-plugged 500 ml Erlenmeyer flasks. Media in each flask were inoculated with 1 ml of spore suspension containing 8×10^6 spores/ml of the respective strains. Mixed cultures of two strains were established in all possible combinations by inoculating the media with 0.5 ml of the above spore suspension of each of the two strains. The flasks were incubated at 30 °C in an orbital shaker incubator at 240 rpm for 240 h. Each treatment was replicated four times.

Estimation of citric acid. At 240 h of culture, citric acid in the filtrate was estimated by pyridine-acetic anhydride method developed by Maurier and Boulet [13].

Statistical analyses. The analysis of variance was performed on log-transformed data and pair-wise comparisons of means, and groups of means were performed using Scheffe's multiple contrasts [14]. All computations were performed on Apple IIe microcomputer.

Results

Table I shows the mean ($\pm$ standard error) of citric acid concentration produced by various strains and by each of the combination of strains. The production of citric acid varied significantly with the strain and combination of strains ($p < 0.001$) as demonstrated by the ANOVA table (Table II). Strain G gave the highest concentration (132 mg/ml) while strain C yielded the lowest concentration (1.802 mg/ml) which is about 70 times lower than that of strain G.

Table I

Mean citric acid yield ($\pm$ standard error) of single strains and strain combinations

Strain or strain combination	Citric acid mg/ml	Strain combination	Citric acid mg/ml
A	49.029±0.122	BF	2.023±0.088
B	2.219±0.040	BG	2.327±0.079
C	1.802±0.088	BH	1.759±0.065
D	8.415±0.017	CD	14.201±0.261
E	45.179±0.017	CE	7.036±0.369
F	2.745±0.077	CF	9.610±0.194
G	132.831±0.035	CG	0.925±0.006
H	8.888±0.0557	CH	1.551±0.022
AB	0.814±0.020	DE	2.785±0.074
AC	0.749±0.017	DF	13.923±0.428
AD	4.976±0.051	DG	4.627±0.168
AE	0.674±0.017	DH	2.375±0.082
AF	1.619±0.017	EF	0.757±0.02
AG	1.577±0.009	EG	37.381±0.334
AH	2.190±0.048	EH	1.037±0.029
BC	4.992±0.069	FG	1.775±0.041
BD	4.739±0.174	FH	4.963±0.209
BE	0.733±0.050	GH	5.368±0.214

Table II

*ANOVA table for citric acid yield of 8 different strains and strain combinations**

Source of variation	Sum of squares	d.f.	M.S.	F-ratio
Treatments	45.084	35	1.288	1502.768**
Residual	0.093	108	0.001	
Total	45.177	143		

* Log transformed data were used
** $p < 0.001$

In general, the yield of citric acid was lesser in the combinations compared with those of the single strains ($F = 49.3$; $p < 0.001$); the mean yield for single strains being 31.4 mg/ml while that of strain combinations being 4.9 mg/ml. Table III compares the yield of single strains and their combinations in terms of F-ratios obtained in Scheffe's multiple contrast tests. Combinations of strain A, D, E, G, and H gave a significantly lower citric acid yield than the strains alone ($p < 0.001$). Strain combinations involving B and F (both low yielding) did not differ on an average from their respective pure cultures. It is also evident from Table III that the combinations of high yielding strains, in general, gave a high average citric acid yield while the combinations involving low yielding strains (B, C and F) gave usually a poor yield in mixed cultures.

Table III

Comparison of citric acid yield of single strains with those of the corresponding strain combinations in terms of F-ratios obtained in Scheffe's multiple contrast tests

Strains		Strain combinations		F-Ratio
Code	Citric acid yield mg/ml	Codes	Average citric acid yield mg/ml*	
A	49.029	AB, AC, ... AH	1.796	278.10**
B	2.219	AB, BC, ... BH	2.480	0.353 NS
C	1.802	AC, BC, ... CH	5.580	6.668**
D	8.415	AD, BD, ... DH	6.804	4.178**
E	45.179	AE, BE, ... EH	7.200	199.099**
F	2.745	AF, BF, ... FH	4.953	0.169 N.S.
G	132.831	AG, BG, ... GH	7.711	294.304**
H	8.888	AH, BH, ... GH	2.749	39.101**

N.S. Non-significant
* Grand average of various combinations of a given strain
** $p < 0.001$

Discussion

The high order of variability in the ability of various *A. niger* strains to produce citric acid found in the present study accords well with the findings of Yuill [9], Mahmoud et al. [3] and Baracho and Monteiro [5]. The combinations of 5 out of 8 strains yielded lower concentrations of citric acid than did their corresponding pure cultures. Similarly, Yuill [9] and Baracho and Monteiro [5] found that generally the citric acid production of two different unrelated high acid strains of *A. niger* grown in mixed cultures was much lesser than that of obtained when either of the strain was grown separately. In the present study there was a tendency for the low yielding strains to augment the citric acid yield in mixed cultures over that of the respective pure cultures. This corrobotates the earlier findings of Baracho and Monteiro [5]. Furthermore, Ciegler and Raper [10] and Chang and Terry [1] reported that heterokaryons of *A. niger* produce intermediate or lesser amount of citric acid than either of the moulds alone, with the exception of low yielding strains in combination. The tendency to produce intermediate amounts of citric acid by the combinations of low and high yielding strain combinations and the augmented production in mixed cultures of low-yielding strains found here suggests the possibility of the formation of heterokaryons and thereby an additive genic effect with respect to yield. In the present work, however, formation of heterokaryons was not investigated and, therefore, the results can not be subjected to quantitative analysis of inheritance based on combining abilities of various strains. However, the possibility of an additive genic effect can not be ruled out since the formation of heterokaryons in mixed cultures of *A. niger* strains has been demonstrated by Chang et al. [11] and Baracho and Coelho [15]. The conidia of the different strains used in the present study were morphologically very similar. It would be interesting to use strains with readily identifiable contrasting conidial characteristics (e.g. Prasad [16] as this would permit the determination of the extent of heterokaryosis and the production of diploids that varies with the strains and the environmental conditions (Baracho and Coelho [15, 17]).

In brief, it may be concluded that strains or pairs of strains of *A. niger* can be found with enhanced citric acid yield and could be exploited for industrial production of citric acid.

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FACTORS INFLUENCING EXTRACELLULAR PROTEASE SYNTHESIS IN AN *ASPERGILLUS FLAVUS* ISOLATE

MALATHI SRINIVASAN and SUSIL C. DHAR

Department of Biochemistry, Central Leather Research Institute, Madras, India

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Our studies on the control of extracellular protease synthesis in an *Aspergillus flavus* strain isolated by us indicate that in a defined medium a protein must be present for enzyme to be produced. Soya bean protein and cotton seed protein were efficient inducers. The ability to induce enzyme synthesis was a characteristic property of the individual protein. Enzyme activity was not derepressed in the absence of a protein by limitation of nutrients. Cycloheximide blocked enzyme synthesis. Low levels (1%) of various carbohydrates did not repress enzyme synthesis, whereas most carbohydrates at 3% levels repressed enzyme synthesis. Addition of glucose to cultures actively producing the enzyme blocked further synthesis of enzyme. Addition of glucose to cultures producing the enzyme in the absence of sodium nitrate resulted in a decrease of enzyme activity.

Proteolytic enzymes are of great commercial importance contributing to more than 40% of the world's commercially produced enzymes [1]. Elaboration of extracellular proteases is characteristic of several species of microorganisms. Reports on the regulation of exocellular protease synthesis in various species of microorganisms are varied and there appears to be no common model applicable to this phenomenon. Exoprotease synthesis has been variably reported to be inducible, constitutive or subjected to catabolite repression [2].

In view of the considerable importance of the genus *Aspergillus* in the industrial production of enzymes, it was considered relevant to undertake a systematic study on the environmental factors influencing the extracellular protease synthesis by *Aspergillus*. In the present study, various factors that could increase enzyme synthesis and the other factors that could repress enzyme synthesis have been thoroughly investigated. Such a study will provide an understanding of the mechanism of control of exocellular protease synthesis in this organism.

MALATHI SRINIVASAN, SUSIL C. DHAR*

Department of Biochemistry, Central Leather Research Institute
Madras-600 020, India

* Corresponding author

Materials and methods

Organism. The organism used in the present study is an *Aspergillus flavus* Link strain isolated from soil in this laboratory.

Chemicals. Casein (Hammerstein) was obtained from SISCO Research Laboratories, Bombay, India. All other chemicals were of reagent grade.

Medium. The organism was routinely maintained on PDA (Potato Dextrose Agar) slants. The defined medium (DM) had the following composition: glucose, 1%; sodium nitrate, 0.2%; potassium dihydrogen phosphate, 0.1%; potassium chloride and magnesium sulphate, 0.05% each, ferrous sulphate and zinc sulphate traces. The pH was adjusted to 7.0 to 7.1. Additions or deletions to the DM were made as indicated in the text.

Culture conditions. Fifty ml of medium DM were dispensed into Erlenmeyer flasks (250 ml), sterilized and inoculated with 1 ml of a conidium suspension containing $5–10 \times 10^6$ conidia/ml. Conidia were harvested from agar slant cultures grown for 3–4 days at 37 °C on PDA. The flasks were incubated at 37 °C at 250 rpm in a New Brunswick Controlled Environmental Shaker.

Growth measurement. The rate of growth of the organism was measured by collecting the mycelial mats at regular interval on tared filter papers. The papers were dried at 80 °C in a vacuum oven to constant weight.

Assay for extracellular proteolytic activity. Culture filtrates were collected by filtration and assayed for activity according to the method described earlier [3]. To 1 ml of casein solution (2.5%), 1.9 ml of 0.1 M carbonate-bicarbonate buffer, pH 8.5 and 0.1 ml of a suitably diluted enzyme solution were added and the mixture was incubated at 42 °C for 30 min. After the period of incubation, 3 ml of 5% TCA was added to each tube and kept at room temperature for 30 min. The solution was then filtered through Whatman No. 1 filter paper. The absorbance of the TCA soluble fraction was read at 280 nm in a Shimadzu Model UV-260 Spectrophotometer. A control was run in an identical manner except that the enzyme solution was added after the addition of TCA to the substrate solution. The values obtained with respect to control were always deducted from their corresponding values obtained from experimental values. One unit of protease activity is the amount of enzyme causing an absorbance increase equal to that produced by 1 mg of tyrosine under assay conditions. Activity is expressed as units per ml of culture filtrate.

Table I

*Effect of various organic nitrogen sources on extracellular protease synthesis in A. flavus**

Organic nitrogen source	Protease activity (units/ml)
None	Nil
Casein	0.27
Peptone	1.39
Gelatin	0.56
Albumin	2.34
Beef extract	3.41
Meat extract	2.97
Yeast extract	0.76
Soya bean protein	8.04
Cotton seed protein	9.82
Corn steep liquor	0.23
Casitopeptone	0.40

* Values are the mean of two independent experiments

Results

Table I indicates the effect of various organic nitrogen sources (1% level with DM) on exocellular protease synthesis by *A. flavus*. It can be seen that among all the complex nitrogen sources evaluated, cotton seed protein and soya bean protein induce maximum enzyme synthesis. A large number of inorganic nitrogen salts tested could not promote significant enzyme synthesis (data not included).

Figure 1 illustrates the correlation between period of growth, enzyme activity and growth yields. It is observed that the enzyme production increases markedly during 48 h incubation, reaches a maximum by the 72nd h and thereafter remains constant up to 120 h. Maximum growth is reached by the 48th h, when the enzyme level begins to increase rapidly.

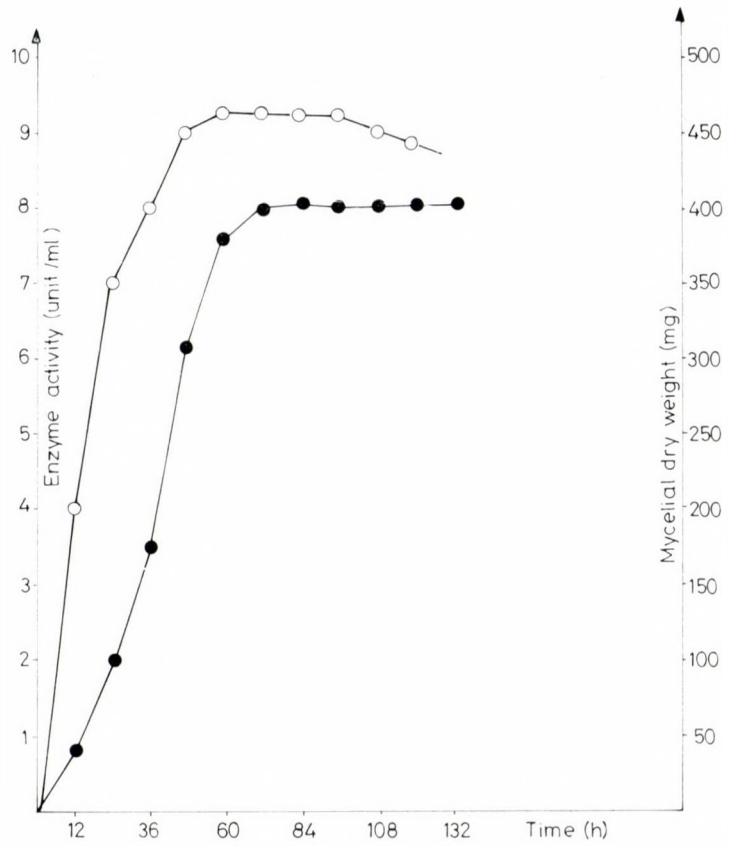

Fig. 1. Growth (dry weight of mycelia, mg/50 ml; ○——○) and extracellular protease activity in units/ml (×——×) of *A. flavus* grown in DM containing 1% soya bean protein

Table II

*Effect of different concentrations of soya bean protein on extracellular protease synthesis by A. flavus**

DM + soya bean protein (%)	Activity (units/ml) at growth period (h)			
	24	48	72	96
0	Nil	Nil	Nil	Nil
0.4	1.26	5.27	6.58	6.61
0.8	2.04	6.86	7.98	7.87
1.2	1.41	5.98	7.68	7.61
1.6	0.76	3.30	4.46	5.61
2.0	0.20	1.91	3.37	5.03

Soya bean protein was used in all our subsequent experiments as an inducer of the exocellular protease synthesis. Table II shows the effect of various concentrations of soya bean protein on exoprotease synthesis with respect to the period of growth. In the case of 0.4%, 0.8% and 1.2% concentrations of soya bean protein, maximum enzyme activity is observed after 72 h incubation. The enzyme activity induced is maximum at a concentration of 0.8% level. At 1.6% and 2% levels, the appearance of maximum activity is delayed and the total enzyme production is reduced.

Table III indicates that when soya bean protein is added to cultures grown in DM for 48 h, enzyme activity appears after 12 h and reaches a maximum at 48 h incubation. Maximum enzyme synthesis is induced by 0.8% soya bean protein.

Since the control of media components was impossible by addition experiments, a series of experiments were performed by transferring washed mycelium into the desired medium. The effect of limiting nitrogen, carbon or sulphur on enzyme activity in the presence or absence of soya bean protein was examined by this technique. Cultures were initially grown in DM for 48 h, filtered and washed with sterile distilled water. The washed mycelium was

Table III

*Effect of addition of varying concentrations of soya bean protein on extracellular protease synthesis by A. flavus grown for 48 h on DM**

Soya bean protein (%)	Activity (units/ml) at growth period (h) after the addition of soya bean protein					
	4	8	12	24	36	48
0	Nil	Nil	Nil	Nil	Nil	Nil
0.4	Nil	0.26	1.73	5.51	6.01	6.17
0.8	Nil	0.52	2.62	7.12	8.17	8.21
1.2	Nil	0.38	2.09	6.25	7.51	7.68

* Values are the mean of two indepentent experiments

Table IV

Effect of limiting carbon, nitrogen or sulphur on extracellular protease activity of A. flavus in the presence or absence of soya bean protein

Nature of medium	Activity (units/ml) at growth period (h) after transfer of mycelium			
	4	8	12	24
DM + 1% SBP	0.21	0.47	2.14	5.98
DM + 1% SBP — carbon	0.19	0.28	1.17	4.11
DM + 1% SBP — nitrogen	0.26	0.41	2.51	6.31
DM + 1% SBP — sulphur	0.23	0.32	1.76	4.99
DM	Nil	Nil	Nil	Nil
DM-carbon	Nil	Nil	Nil	Nil
DM-nitrogen	Nil	Nil	Nil	Nil
DM-sulphur	Nil	Nil	Nil	Nil

* Values are the mean of two independent experiments.
SBP = soya bean protein

transferred to the desired medium. Results presented in Table IV show that no derepression of proteolytic activity was observed under conditions of limiting nutrients in the absence of soya bean protein. In the presence of soya bean protein that also limits either nitrogen or sulphur or carbon in the medium, enzyme synthesis is not enhanced above control values.

Various carbohydrate substrated at 1% and 3% levels were studied for their effect on protease production in the presence of 1% soya bean protein (Table V). Several carbohydrate compounds tested, induce enzyme levels even

Table V

*Effect of different carbohydrates on extracellular protease synthesis by A. flavus in the presence of 1% soya bean protein**

Carbohydrate source	Activity (units/ml) at growth period (h)			
	48		72	
	1%	3%	1%	3%
Glucose	6.71	0.26	8.58	1.23
Fructose	7.80	0.88	8.40	1.55
Mannose	8.91	0.85	9.26	1.69
Sucrose	4.91	1.04	6.43	1.79
Maltose	6.63	1.71	8.33	2.61
Lactose	7.63	6.25	7.34	7.40
Sorbose	3.84	1.54	4.46	2.70
Starch	4.33	0.86	8.57	3.96
Dextrin	6.56	1.91	6.87	5.31
Glycerol	4.42	4.44	6,75	4.79
Sodium citrate	1.99	2.70	1.76	1.79
None	5.20		7.57	

* Values represent the mean of two independent experiments

greater than the control at 1% levels at the end of both 48 and 72 h of growth. At 3% levels all the carbon sources, except lactose, severely repress enzyme synthesis. In certain cases there is relief of repression at the end of 72 h.

The effect of varying concentrations of glucose on enzyme synthesis is presented in Table VI. It is observed that glucose at a concentration of 0.5% and 1% does not repress enzyme synthesis. At 2% levels, however, the repression of enzyme synthesis lasts up to 48 h. At 3%, 4% and 5% concentrations, the extent of repression is proportional to the concentration of glucose and the repression persists even at the end of 96 h of growth.

Table VI

*Effect of different concentrations of glucose on extracellular protease synthesis by A. flavus in the presence of 1% soya bean protein**

Glucose (%)	Activity (units/ml) at growth period (h)			
	24	48	72	96
0	2.46	4.70	7.96	7.82
0.5	2.16	5.78	8.06	8.03
1	2.20	6.71	8.58	8.34
2	0.98	3.11	8.92	8.63
3	0.46	0.81	1.23	5.52
4	0.42	0.19	0.89	1.73
5	0.01	0.06	0.08	0.12

* Values are the mean of two independent experiments

Catabolite repression of exoprotease synthesis in *A. flavus* was also studied by the addition of varying concentrations of glucose to cultures actively producing the enzyme (Table VII). It can be seen that the enzyme synthesis was completely blocked after initial synthesis at 4 h irrespective of the addition of different concentrations of glucose to the medium.

Table VII

*Effect of addition of varying concentrations of glucose on extracellular protease synthesis by A. flavus grown for 48 h in DM containing 1% soya bean protein**

Glucose (%)	Activity (units/ml) at growth period (h) after the addition of glucose			
	4	8	12	24
0	—	6.28	7.03	7.96
1	5.97	5.83	6.18	5.80
2	6.02	5.91	6.12	6.03
3	6.00	5.71	5.79	5.96
4	6.23	5.84	6.05	5.88
5	5.49	6.05	5.98	5.73

* Values are the mean of two independent experiments

Table VIII

Effect of addition of varying concentrations of glucose on extracellular protease synthesis by A. flavus grown for 48 h in DM containing 1% soya bean protein; $NaNO_3$ is not included in the medium

Glucose (%)	Activity (units/ml) at growth period (h) after the addition of glucose					
	4	8	12	24	36	48
0	6.66	7.88	10.13	10.06	10.17	9.88
1	5.82	5.47	5.32	5.78	6.01	6.17
3	3.36	2.75	1.54	0.66	0.52	0.51
5	2.11	1.88	0.83	0.46	0.41	0.39

* Values are the mean of two independent experiments

In experiments carried out under similar conditions sodium nitrate was omitted. It is seen from Table VIII that addition of glucose to culture medium lacking in sodium nitrate resulted in a reduction of enzyme activity. The decrease in enzyme activity paralleled the increase in the concentration of glucose added. When the glucose was exhausted from the medium enzyme synthesis began to increase.

Discussion

A. flavus is able to synthesise extracellular proteolytic enzymes when grown in a medium containing organic nitrogen sources (Table I) indicating that this is an inducible enzyme system in this organism. Extracellular protease synthesis has been reported to be inducible in some microorganisms [4] and constitutive in others [5].

It appears that in *A. flavus* induction involving macromolecular substrates occurs. An interesting observation is that the cells sense the presence of these macromolecules in the medium, although these cannot enter the cells as such due to permeability barriers.

It is of further interest to note that not all organic nitrogen sources are efficient inducers. The response of the cells to the presence of a protein source appears to be a characteristic property of the particular protein. There are two ways of looking at this. It is probable that the small peptides or mixtures of amino acids associated with the protein may be the actual inducers. The quantity and nature of these vary with the nature of the protein. But it has been indicated that protein hydrolysates or combinations of amino acids are ineffective as inducers [6]. Another hypothesis is that trace (undetectable) levels of enzyme be associated with the cells which hydrolyse macromolecules to produce low amounts of the actual inducers, which then trigger increased synthesis of enzyme.

Studies on the effect of different concentrations of soya bean protein (Table II) and the addition of different concentrations of this protein to cultures grown for 48 h on DM (Table III) confirm that exocellular protease synthesis in *A. flavus* is indeed an inducible enzyme system. The addition of cycloheximide at 5 μg/ml to cultures actively synthesizing the enzyme (data not shown) results in inhibition of further enzyme synthesis. This observation indicates that protein synthesis is essential for enzyme synthesis to occur and that it is not merely a release of the preformed enzyme molecules from the cell wall or membrane [7, 8].

Derepression of protease synthesis has been observed in other fungi when nutrients are limiting. In *Aspergillus nidulans* Eidam limitation of carbon, nitrogen or sulphur compounds in the medium results in exocellular protease synthesis [9], indicating that derepression is both necessary and sufficient for protease production to be initiated. In *Neurospora crassa* [4] and *Mucor miehei* [6] it is necessary but not sufficient; a proteinaceous substrate must also be present for induction of enzyme synthesis to occur. Cohen [9] has reported that in *Aspergillus* species derepression is a sufficient condition for enzyme synthesis. But our studies with *A. flavus* (Table IV) indicate that derepression is not essential. A protein must be present indicating that exocellular protease synthesis in *A. flavus* is an inducible phenomenon.

The amount of enzyme produced in a protein containing medium appears to be regulated by the composition of the medium with respect to the other substrates. The effect of carbohydrate sources on enzyme activity is a measure of both the nature and amount of the sugar in question. Our studies with *A. flavus* indicate that up to 1% levels, catabolite repression is not indicated but at higher concentrations, severe repression of enzyme synthesis takes place. In *M. miehei* it has been reported [6] that only at 16% levels, glucose completely represses exocellular protease synthesis. Catabolite repression of extracellular enzyme synthesis has also been reported [4]. In some instances addition of cAMP relieves the repression [10].

In cultures, actively synthesizing the enzyme, addition of glucose suppresses further enzyme synthesis. But when glucose is added to such cultures, growing in a medium lacking sodium nitrate, there is a decrease in enzyme activity (Table VIII). This would indicate that either the enzyme activity is inhibited by some factor or that it is being denatured. In *Cephalosporium* species a similar phenomenon has been observed [11], and it has been postulated that the enzyme protein may be used as the source of nitrogen in the absence of an inorganic nitrogen source.

Our studies, therefore, indicate that the regulation of the extracellular protease synthesis in the *A. flavus* isolate may involve several factors at the molecular level, some of which "switch on" the process, while others repress it.

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EFFECT OF AMINO ACIDS AND ITS ANALOGUES ON *GLOEOTRICHIA GHOSEI* AND ITS NON-NITROGEN FIXING MUTANTS

A. K. Mishra and D. N. Tiwari

Centre of Advanced Study in Botany, Banaras Hindu University Varnasi, India

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Mutants of *Gloeotrichia ghosei* fil^{1} het^{++} and pol^{-} het^{-} failed to grow on molecular nitrogen and required combined nitrogen sources. NO_3^- and NH_4^+ supported maximum growth of parent and mutant strains under aerobic conditions but not under reducing anaerobic or heterotrophic conditions. Glutamine and tryptophan were superior for the parent strain compared to inorganic nitrogen sources. By mutants only glutamine was used whereas tryptophan was inhibitory to them. Development of heterocyst and polarity in the parent and in fil^{1} het^{++} was inhibited in the presence of all combined nitrogen sources compared to molecular nitrogen which supported their formation. MSX and AZT (analogues of glutamine and tryptophan, respectively) completely inhibited the growth of all strains in the nitrogen-fixing and NH_4^+-medium. When glutamine and tryptophan were added to MSX- and AZT-containing medium, growth of parent strain was similar to that of the respective amino acids. The mutants grew only with glutamine + MSX, and tryptophan + AZT complex medium inhibited growth.

The developmental pattern encountered in the filaments of nostocacean cyanobacteria entirely differs from the whiplike revularian filaments of *Gloeotrichia ghosei*, which have certain advantage for developmental studies. It has fixed position of both heterocyst and akinete formation in the trichome (i.e., heterocyst always develops in the basal position of the filaments followed by an akinete), whose apical end is modified into a colourless hair and growth is restricted only to the cells near the basal heterocyst. These characteristics together with the prokaryotic nature make the cyanobacterium *G. ghosei* especially suitable for study of the genetic control of development. It has been mentioned earlier [1] that the whiplike morphology of the trichome of *Gloeotrichia* develops only under nitrogen-fixing conditions.

In order to understand the two processes viz. heterocyst differentiation and nitrogen fixation, it would be useful to isolate and characterize the mutants defective in either processes and to analyse these mutants by conventional microbial genetic methods. Mutants defective in heterocyst differentiation

Ashok Kumar Mishra, D. N. Tiwari

Department of Botany, Banaras Hindu University
Varanasi-221 005, India

(het⁻) and nitrogen fixation *(nif⁻)* have been reported to be sensitive to oxygen [2, 3]. The definition of auxotrophy in nitrogen fixing cyanobacteria was emphasized [4], in which the requirements for non-nitrogen fixing *(nif⁻)* mutants of *Nostoc linckia* were found to be either NO_3^- or NH_4^+ nitrogen. Eighty three auxotrophs of *Anabaena variabilis* have been reported [5], 65 of which required inorganic sources of combined nitrogen for growth, while others showed requirement for methionine, uracil, adenine, biotin and nicotinic acid.

Earlier studies conducted with amino acid utilization by cyanobacteria provide variable results and also none of the amino acids tested served as sole source of nitrogen [6]. The failure of amino acid utilization by the cyanobacteria is probably due to a preference of other inorganic nitrogen substances [7]. If such study is carried out with mutants defective in nitrogen fixation but capable of heterocyst formation *(nif⁻ het⁺)*, at least the assessment of the amino acid serving as nitrogen source may be clearly understood.

The present paper deals with the study of non-nitrogen-fixing mutants of *G. ghosei* isolated by us [8]. These mutants have been characterized with respect to their requirement of nitrogen sources along with nitrogen fixation. Interaction of amino acid analoges with amino acids has also been studied.

Materials and methods

Organisms and culture. The present study has been done on *G. ghosei* and its two *nif⁻* mutants *fil*[1] *het*$^{++}$, *pol⁻ het⁻)* isolated by us [8]. All these strains were routinely grown in modified chu-10 medium [9] with A. solution added for micronutrient [10], in a culture room illuminated with daylight fluorescent tube (light intensity approximately 2500 lux) with 14 h light and 10 h dark cycles at 24 ± 1 °C. The nitrogen-free medium contained $CaCl_2$ (0.0735 g l^{-1}) instead of $Ca(NO_3)_2$ (1 mM). The nitrogen-free medium to which NH_4Cl (0.5 mM) had been added was also used for growth experiments. Growth was measured in terms of increase in total protein of the culture by the method of Herbert et al. [11].

Nitrogen fixation study. Nitrogen fixed by the different strains of *G. ghosei* was studied by the estimation of increase in total nitrogen of the cultures grown for 25 days in nitrogen-fixing condition by microkjeldahl method. Since the mutant strains were unable to grow in aerobic nitrogen fixing medium, they were tried to grow in anaerobic/reducing nitrogen fixing conditions. The anaerobic/reducing condition in the medium was obtained by the addition of DCMU (3)3,4-dichlorophenyl)-1,1-dimethyl urea, (1 μg/ml)) along with either sodium sulphide (1 mM or glucose (1000 μg/ml). One set of such experimental flasks was kept in dark while the other grown in light.

Study with amino acids and their analogues. All the strains were grown in N_2-fixing medium with the addition of glutamine and tryptophan (each 50 μg/ml). Growth behaviour in thesemedia was compared with their growth behaviour in NH_4^+-medium. MSX (L-methionine-DL-sulphoximine, 2 μM) and AZT (7-azatryptophan, 20 μM) analogues of glutamine and tryptophan, respectively were added in the NH_4^+ as well respective amino acid containing medium separately. The growth behaviour in these media was also studied.

Developmental studies. The developmental behaviour of all the strains especially with respect to the formation of heterocyst and development of polarity in filaments was studied microscopically under the above growth conditions.

Results

Nitrogen fixation under aerobic and anaerobic/reducing condition of the different strains are shown in the Table I. None of the mutant strains showed fixation of nitrogen (measured in terms of increase in total nitrogen of the culture) under any growth condition (either aerobic or anaerobic/reducing), whereas the parent strain showed considerable increase in total nitrogen only under aerobic nitrogen fixing condition in light. But in the parent, similarly to mutants strains, no nitrogen fixation was recorded under aerobic dark as well as anaerobic/reducing conditions. Even the addition of glucose with DCMU did not support the growth and nitrogen fixation by the parent strain

Table I

*Nitrogen fixation (mg N/100 ml culture) by different strains of G. ghosei**

Medium**	Parent		*fil*^{1} *het*$^{++}$		*pol*$^-$ *het*$^-$	
	Light	Dark	Light	Dark	Light	Dark
C — N	1.005	—	—	—	—	—
C — N + glucose	1.065	—	—	—	—	—
C — N + DCMU	—	—	—	—	—	—
C — N + $Na_2S \cdot 9\,H_2O$	—	—	—	—	—	—
C — N + glucose + DCMU	—	—	—	—	—	—
C — N + $Na_2S \cdot 9\,H_2$ + DCMU	—	—	—	—	—	—

* After 25 days of growth; (data are means of three independent experiments)
** Concentration of glucose, DCMU and $Na_2S \cdot 9\,H_2O$ used were 1000 μg/ml, 1 μg/ml and 1 mM respectively

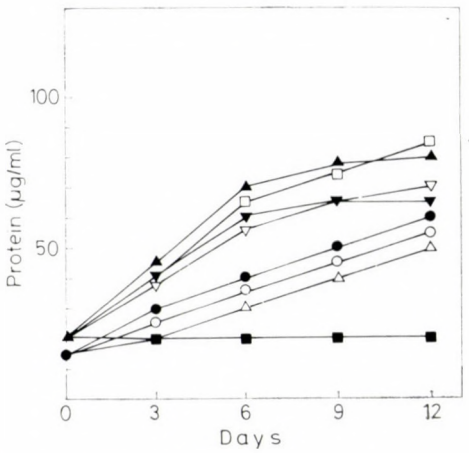

Fig. 1. Growth behaviour of different strains of *G. ghosei* in inorganic nitrogen sources. Parent in C—N (△———△), C + N (○———○), C + NH (●———●) media; mutant *fil*^{1} *het*$^{++}$ in C — N (■———■), C + N (□———□), C + NH (▲———▲) media; mutant *pol*$^-$*het*$^-$ in C — N (■———■), C + N (▽———▽), C + NH (▼———▼) media

either in dark or light. Whereas glucose alone was found to slightly increase the nitrogen fixation only in light for the parent strain.

Two inorganic nitrogen sources i.e. NO_3^- and NH_4^+, were used to study the growth characteristics of mutants. The nitrogen fixing medium (C–N medium) combined with nitrate (C+N medium) or ammonium (C+NH medium) supported growth of both mutants (Fig. 1). It was observed that there was some enhancement in growth of the parent strain under combined nitrogen sources (C+N and C+NH medium) over the growth in C—N medium. For all strains ammoniacal nitrogen was found to be a better nitrogen source than nitrate nitrogen (Fig. 1). It is apparent from the growth behaviour that only the parent was able to grow in C—N medium while the mutants, *fil*^{1} *het*$^{++}$ and *pol*$^-$ *het*$^-$ were unable to grow in C—N medium and required combined nitrogen sources e.g. NO_3^- or NH_4^+ for their growth (Fig. 1).

Amino acids, glutamine and tryptophan were used in the present study to see their effect on different strains of *G. ghosei*. Filter sterilized solution of these amino acids were added in the C—N medium. The growth behaviour of different strains was compared with their respective growth behaviour in C+NH medium. MSX (2 μM) was added in the C+NH and glutamine containing medium to see its effect on different strains. Similarly AZT (20 μM) was added in C+NH and tryptophan containing medium to see its effect. Figures 2, 3 and 4 show the growth behaviour of parent, *fil*^{1} *het*$^{++}$ and *pol*$^-$ *het*$^-$ strains respectively under the aforesaid growth conditions. Glutamine was found to increase the growth of all the strains compared to their growth

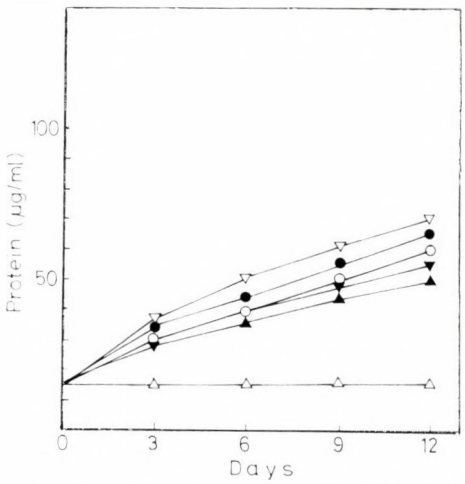

Fig. 2. Effect of amino acid and amino acid analoges on growth behaviour of parent strain of *G. ghosei*: C + NH (○———○), C + NH + (MSX or AZT) (△———△), C — N + glutamine (▽———▽), C — N + glutamine + MSX (●———●), C — N + tryptophan (▼———▼), C — N + tryptophan + AZT (▲———▲)

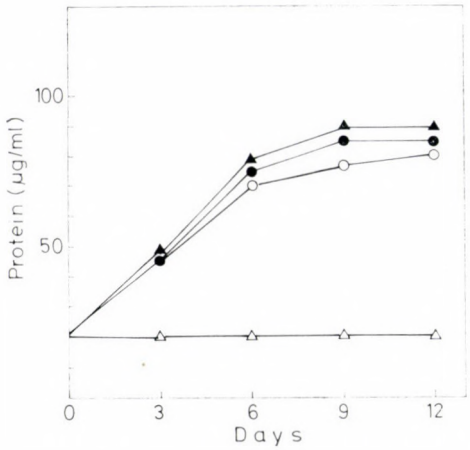

Fig. 3. Effect of amino acids and amino acid analoges on growth behaviour of mutant *fil*^{1} *het*$^{++}$ of *G. ghosei*: C + NH (○———○), C + NH + (MSX or AZT) (△———△), C − N + glutamine (▲———▲), C − N + glutamine + MSX (●———●), C − N + tryptophan and C − N + tryptophan + AZT (△———△)

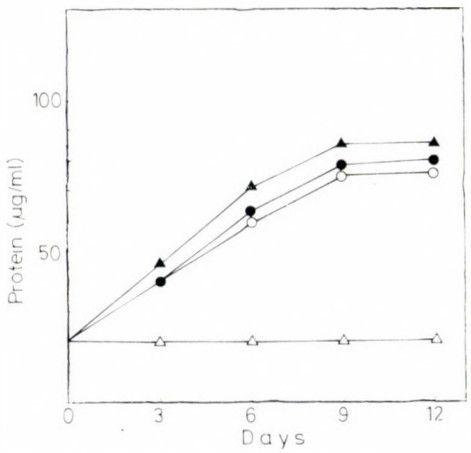

Fig. 4. Effect of amino acid and amino acid analoges on growth behaviour of mutant *pol*$^{-}$ *het*$^{-}$ of *G. ghosei*: C + NH (○———○), C + NH + (NSX or AZT) (△———△), C − N + glutamine (▲———▲), C − N + glutamine + MSX (●———●), C − N + tryptophan and C − N + tryptophan + AZT (△———△)

in C+NH medium. But in tryptophan containing medium only the parent was able to grow with slightly less growth than in C+NH medium, whereas both the mutants were unable to grow. Even the lower concentrations (5 and 10 µg/ml) of tryptophan tested for mutants (data not shown) failed to serve as combined nitrogen source for their growth.

Both the amino acid analogues (MSX and AZT) were found inhibitory for the growth of all the three strains in C+NH medium (Figs 2, 3, 4). But the

addition of glutamine and tryptophan in MSX and AZT containing medium respectively resulted in a more or less similar growth behaviour, for the parent strain only, as observed in the respective amino acid medium. In case of *nif*$^-$ mutants only with glutamine similar results (recovery of growth) was obtained when it was added in MSX medium whereas tryptophan was found unable to do so for mutants (Figs 2, 3, 4).

Filaments of the parent strain of *G. ghosei* showed normal rivularian pattern (having basal heterocyst and apical polarity) in C—N medium. Of the two mutants studied, *fil*^{1} *het*$^{++}$ showed an unique filamentous development. It showed the formation of intercalary chains of heterocysts and intercalary polarity in C—N medium. But the filaments of *pol*$^-$ *het*$^-$ never showed the formation of either heterocyst or polarity in any growth condition. When the parent and *fil*^{1} *het*$^{++}$ strain were grown in C+N or C+NH medium, loss of heterocyst and polarity was observed. Similarly, in the glutamine containing medium none of the strains showed formation of either heterocyst or polarity. The filaments of all the strains grown in glutamine medium were longer and healthier as compared to inorganic nitrogen media. In tryptophan medium only the parent strain grew and absence of heterocyst and polarity was observed as found in other combined nitrogen sources.

When amino acid analoges MSX and AZT were added in C—N or C+NH medium, filaments of the parent strain were observed with heterocyst and polarity. The vegetative cells of these filaments were found to be vacuolated and sometimes the filaments found to be fragmented into small pieces. But the addition of glutamine in MSX and tryptophan in AZT containing medium resulted in the recovery of growth and the filamentous development was found similar to that of respective amino acids.

In case of *fil*^{1} *het*$^{++}$ strains, growth inhibitory effect of MSX and AZT resulted in the morphological development of filaments as found in C—N medium. When addition of glutamine causes the recovery of growth in MSX medium, the developmental behaviour of filaments was as found in glutamine medium. Strain *pol*$^-$ *het*$^-$ never showed the formation of heterocyst or polarity in any medium. When tryptophan was added in AZT containing medium the lysis of filaments of both mutants was observed.

Discussion

Frequent occurrence of *het*$^-$ *nif*$^-$ mutants of *Nostoc* and *Anabaena* by mutagenic treatment strongly support the involvement of common genetic determinant in organization of nitrogen fixation apparatus in these cyanobacteria [4, 12–14], although several workers have reported mutants of these cyanobacteria which lack only nitrogen fixation but not heterocyst [2, 4, 5,

15]. These reports suggest that nitrogen fixation and heterocyst differentiation in these cyanobacteria are two distinct phenotypic traits which are controlled by independent genetic factors. The mutant strain *pol*$^-$ *het*$^-$ of *G. ghosei* described in the present study falls in the first category while the strain *fil*^{1} *het*$^{++}$ falls in the second category described above, and this supports the above idea of two distinct genetic factors controlling independently for nitrogen fixation and heterocyst differentiation. Although the heterocysts formed in the strain *fil*^{1} *het*$^{++}$ in C—N medium appear to be normal under light microscope, their structural defects at ultrastructural level could not be ruled out. Mutants defective in glycolipid component of envelope or membranes of heterocysts have been reported [3], which were unable to fix nitrogen aerobically, but showed high nitrogenase activity under anaerobic condition. The incapability of heterocystous mutant *(fil*^{1} *het*$^{++}$*)* of *G. ghosei* to fix nitrogen under anaerobic/reducing condition demonstrate that this mutant might be changed in some essential component of nitrogen fixation. Spontaneous mutants of *Anabaena variabilis* (strain 7118) have been reported [16] which lost the ability to form heterocyst and fix nitrogen aerobically but could synthesize nitrogenase anaerobically in the absence of molecular oxygen. Since there is no trace of nitrogen fixing growth or increase in total nitrogen of mutant *pol*$^-$ *het*$^-$ grown under reducing or anaerobic condition, it seems to have lost a common genetic determinant of both heterocyst and nitrogen fixation.

Based on the negative nitrogen fixing growth response of different strains including parent under heterotrophic growth conditions (with glucose in dark), they seem to be exclusively dependent on light energy for the synthesis of cell materials, although some nitrogen fixing cyanobacteria have been reported to show heterotrophic growth [17, 18].

Both the mutants strains showed the requirement of combined nitrogen sources for their growth. Inability of tryptophan to act as nitrogen source for them showed that either tryptophan or its metabolic products are inhibitory for the growth of *nif*$^-$ mutants. Inhibition of heterocyst and polarity formation in parent as well as *fil*^{1} *het*$^{++}$ strain in the presence of combined nitrogen sources showed that these processes are linked with nitrogen fixation [1].

MSX, a known inhibitor of glutamine synthetase is, known to cause derepression of nitrogenase synthesis and heterocyst formation in the presence of NH_4^+-nitrogen [19]. The inhibition of growth by MSX with the formation of heterocyst and polarity in *fil*^{1} *het*$^{++}$ strain in NH_4^+-medium may be due to inhibition of NH_4^+-uptake which has already been reported for parent strain of *G. ghosei* [20]. The observations that amino acid glutamine prevent the effect of MSX on growth and heterocyst formation is consistent with earlier reports [21].

The action of AZT has been attributed its direct interference with diffusible inhibitor responsible for controlling the heterocyst spacing patterns [22], to its action on nitrogenase or the nitrogenase associated enzyme [23] or its action on an independent regulatory system other than controlled by glutamine synthethase [24]. Recovery of growth inhibitory effect in the parent strain in AZT medium by the addition of tryptophan was observed. It indicates that AZT may be incorporated in protein in place of tryptophan and the formation of defective protein might be responsible for such inhibitory effect. Addition of tryptophan may be inhibiting the uptake of AZT ultimately causing the recovery of growth. Both *nif*$^-$ mutants were unable to utilize tryptophan as source of combined nitrogen and lysis of cultures was observed both in tryptophan and tryptophan plus AZT medium. The metabolic defects of tryptophan catabolism in the mutant strains might be creating a condition for the inhibition of growth and ultimately lysis of filaments.

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THE COURSE OF LCMV INFECTION IN GNOTOBIOTIC AND CONVENTIONAL ADULT MICE PRETREATED WITH ATTENUATED NDV VACCINE

ILONA SZERI, L. K. CSATÁRY, PIROSKA ANDERLIK, ZSUZSANNA BÁNOS, I. NÁSZ and ZSUZSANNA BARNA

Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary and United Cancer Research Institute, Alexandria, Virginia, USA

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A single intraperitoneal treatment with two different doses of live Newcastle Disease Virus (NDV) containing attenuated NDV vaccine one day before intracerebral inoculation with lymphocytic choriomeningitis virus (LCMV) had no influence on the ratio and time of deaths after infection with a 100 LD_{50} dose of LCMV either in gnotobiotic or in conventional mice. There was no difference either in the LD_{50} values determined on the basis of three parallel LCMV titration performed on mice pretreated with two different doses of vaccine or untreated. NDV vaccine pretreatment thus did not influence the cellular immune response to LCMV infection either in gnotobiotic or in conventional adult mice. As the NDV vaccine increased the cellular immune response to LCMV infection in suckling mice according to earlier results, the present results reinforce our earlier statement that the direction of immunomodulatory effects can be influenced by age.

It has been shown that a single intraperitoneal treatment with live Newcastle Disease Virus (NDV) containing attenuated NDV vaccine enhanced the fatal outcome of lymphocytic choriomeningitis in suckling mice with undeveloped immune system, thus it proved to be immunostimulatory in terms of cellular immune response [1]. Our earlier experiences with mice similarly proved that the direction and degree of different immunomodulatory effects of microbial origin could be influenced — besides the dosing of substances — by the age of mice and the presence or absence of normal microbial flora [2–8]. In our present experiment, we examined whether pretreatment with NDV vaccine could have an influence on the course of LCMV infection in adult conventional mice with developed immune system and in adult germfree gnotobiotic mice with undeveloped immune system due to antigen-deficient environment.

ILONA SZERI, PIROSKA ANDERLIK, ZSUZSANNA BÁNOS, ISTVÁN NÁSZ, ZSUZSANNA BARNA

Institute of Microbiology, Semmelweis University Medical School
Nagyvárad tér 4, H-1089 Budapest, Hungary

LÁSZLÓ K. CSATÁRY

United Cancer Research Institute
1913 Windsor Rd. Alexandria, Virginia 22307, USA

Materials and methods

Experimental animals. Six-week-old gnotobiotic and conventional C3H mice of both sexes and conventional CFLP mice (LATI, Gödöllő) were used. Gnotobiotic state and its control were maintained according to methods described earlier [9].

NDV vaccine. The attenuated, mezogene variety (H strain) of the fowl plague virus (NDV) propagated in embryonated chicken egg was used (Phylaxia, Budapest). Mice were treated intraperitoneally with 0.2 and 1.0 ml of vaccine (HA titre: log 2^{16}/ml), one day before LCMV infection.

LCMV infection. The strain WE maintained in our laboratory by serial intracerebral passages in mice was used. For brain suspensions and virus dilutions, PBS solution was used. For titration, 0.03 ml amounts of LCMV diluted 1 : 10 were inoculated intracerebrally into 6-week-old adult mice. LD_{50} value of the virus was calculated according to the method of Reed and Muench [10]. Control mice were inoculated similarly to the virus infection with normal brain suspension. During the experiments, the development of neurological symptoms (tremor, spasm) characteristic of lymphocytic choriomeningitis were controlled twice a day and deaths were registered.

Experiments and results

The effect of pretreatment with different doses of NDV vaccine on the course of intracerebral LCMV infection in adult mice was examined in two experiments.

Experiment 1. Gnotobiotic (Gnt) and conventional (Cv) C3H mice were treated with 1 and 0.2 ml doses of NDV vaccine and with PBS. On the day following the treatments, two-third of the mice in each group were infected intracerebrally with 100 LD_{50} of the previously titrated LCMV. One third of the animals were inoculated similarly with virus-free, normal brain suspension. Experiments ended on the 21st day after LCMV infection. No deaths were recorded among mice not infected with LCMV. In groups infected with LCMV, mice showed the typical symptoms characteristic of lymphocytic choriomeningitis. The number of mice in groups infected with LCMV, the applied treatments, the ratio and time of deaths are shown in Table I.

Table I

Number and treatment of Gnt and Cv mice infected with 100 LD50 of LCMV. Ratio and time of deaths

Mice	Inoculation i.p.	Inoculation i. cer.	No. of mice	Mortality rate %	Time of death
Gnt	NDV 1 ml	LCM	16	100	8–14th day
	NDV 0.2 ml	LCM	16	100	8–14th day
	PBS	LCM	32	100	8–14th day
Cv	NDV 1 ml	LCM	14	100	7–8th day
	NDV 0.2 ml	LCM	14	100	7–8th day
	PBS	LCM	30	100	7–8th day

All Gnt and Cv mice infected with LCMV died of lymphocytic choriomeningitis. Cv mice died on the 7–8th day after LCMV infection, Gnt mice, due to their undeveloped immune system, died on the 8–14th day after infection, independently of being pretreated with vaccine or not. In case of both Gnt and Cv mice, the ratio and time of deaths were similar in NDV-pretreated and untreated animals.

This result shows that NDV pretreatment has no influence on the course of LCMV infection, either in Cv or in Gnt mice.

Experiment 2. Conventional adult CFLP mice were inoculated intraperitoneally with two different doses of NDV vaccine (1 ml/mouse and 0.2 ml/mouse) and with PBS solution as control. On the day after the treatments, three parallel virus titrations were performed at the same time and in similar circumstances using the animals pretreated with NDV vaccine or PBS. Titration was carried out by using tenfold dilutions (10^{-2}–10^{-6}) of the LCMV. Ten male and ten female mice were inoculated with each virus dilution. There were 10 male and 10 female control mice in the experiment that were inoculated with PBS solution or only with different doses of NDV. The experiment lasted 21 days after virus infection. Symptoms and deaths were registered.

There were no deaths among control mice not infected with LCMV. In groups infected with LCMV, the typical symptoms of lymphocytic choriomeningitis were responsible for the fatal outcome and deaths occurred on the 7–10th day after infection. The ratio of deaths observed during the three parallel virus titrations is shown in Table II. Here the LD_{50} values of the LCMV calculated on the basis of deaths are also shown.

The LD_{50} value regarding LCMV displays no difference during the three parallel titrations, which means that the applied doses of NDV vaccine do not influence the course of LCMV infection.

Table II

Death of LCMV infected conventional CFLP mice pretreated with NDV vaccine or PBS

LCM virus dilutions	Rate of deaths* Pretreatments i.p.		
	NDV vaccine 1 ml/mouse	NDV vaccine 0.2 ml/mouse	PBS solution
10^{-2}	10/10	10/10	10/10
10^{-3}	10/10	10/10	10/10
10^{-4}	10/10	9/10	10/10
10^{-5}	2/10	3/10	3/10
10^{-6}	0/10	1/10	0/10
LCM virus LD50 value	$10^{-4\,63}$	$10^{-4\,72}$	$10^{-4,72}$

* Number of deaths/Number of infected mice

Discussion

It is known that the outcome of intracerebral LCMV infection in mice is determined by the actual cellular immune response ability of the mice. The course of intracerebral LCMV infection in the form of fatal lymphocytic choriomeningitis is enhanced by effects increasing the cellular immune response and is hindered by effects decreasing them [2–8, 11–20]. Therefore, the ratio of deaths after the infection with the same dose of LCMV is increased by effects stimulating the cellular immune response and is decreased by effects suppressing it. The effects of different treatments influencing the cellular immune response can be detected in mice by observing the course of LCMV infection.

A single pretreatment with NDV vaccine in doses applied in our present experiments does not influence the course of LCMV infection either in Gnt mice with undeveloped immune system or in Cv adult mice with normal immune system. Deaths following infection with 100 LD_{50} dose of LCMV occurred in the same ratio and time in mice treated or not treated with NDV vaccine, both in Gnt and Cv groups. The result that deaths in the Gnt groups occurred later than in Cv groups is in accordance with our earlier results [14] and it can be explained by the undeveloped immune system of Gnt mice due to antigen-deficient environment. There was no valuable difference between the LD_{50} values determined in the three parallel virus titrations using NDV-pretreated and untreated animals. Thus, pretreatment with the applied two doses of NDV vaccine did not influence the cellular response to LCMV in Gnt or Cv adult mice. This experience is in accordance with data from our preliminary experiments, according to which no significant differences were observed as regards lymphocyte count determined in the peripheral blood and relative spleen weight in Gnt and Cv adult mice as compared to controls, on the 2nd, 8th and 14th days after treatment with NDV vaccine.

The present results reinforced our earlier observations that the direction of immunomodulatory effect can be influenced by age. In Table III, we compare our present results with earlier results attained in similar system with pretreatment with known microbial immunomodulants as *Bordetella pertussis* vaccine and radiodetoxified *Escherichia coli* lipopolysaccharide endotoxin (rdLPS).

According to these results, the cellular immune response of suckling mice with physiologically undeveloped immune system was stimulated alike by NDV vaccine, *B. pertussis* vaccine and rdLPS.

The effect of the same treatments on the cellular immune response was different in adult mice; the immune response to LCMV was stimulated by rdLPS and suppressed by *B. pertussis* vaccine both in Gnt mice with undeveloped immune system and in Cv mice with developed one, while it was not influenced by the NDV vaccine either in Gnt or in Cv animals. The result

Table III

The effect of microbial immunomodulants on the cellular immune response to LCMV

Mice	Immune system		Immunomodulatory effect of		
	State	Function	*B. pertussis*	rdLPS	NDV
Conventional suckling	undeveloped	insufficient	stimulation [3]	stimulation [4]	stimulation [1]
Conventional adult	developed	normal	suppression [2, 5] (delaying)	stimulation [8]	no effect
Gnotobiotic adult	undeveloped	insufficient	suppression [5] (inhibition)	stimulation [8]	no effect

of pretreatment with NDV vaccine was not dependent on the presence or absence of normal microbial flora, as was experienced in the case of *B. pertussis* vaccine.

The direction of immunomodulatory effects can be in connection — besides the actual state of the immune system — with the different mechanisms of the modulatory effects. The different immunomodulants can effect the immune system of similar state and function in a different way and degree, while the same effect on the immune system of definitely different state.

Our present results draw the attention to the fact that the actual state of the organism can influence the direction and degree of the effect of immunomodulatory treatments in several way, and it has to be taken into account during immunomodulatory treatments in the medical practice.

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EXTRACELLULAR ENZYMES OF PULMONARY FLUID AND THEIR BACTERICIDAL EFFECTS ON *MYCOBACTERIUM TUBERCULOSIS*

P. KUMAR and SAROJ CHANDRASEKHAR

Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India

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The enzyme activity and bacillary content of cell free pulmonary lavage fluid has been compared in infected and immunized infected animals. Increased enzyme production was found as a result of macrophage activation. Marked decrease in the number of tubercle bacilli was observed in immunized infected animals. The active enzyme production is considered to be the impact of availability of enzyme specific substrate, particularly cell wall components of tubercle bacilli viz. arabinogalactan, arabinomannan and sulphatides. These macromolecules are considered as active substrates for the action of arabinosidase (EC:3.2.1.55), mannosidase (EC:3.2.1.24) and sulphatase-C (EC:3.1.6.1).

The participation of hydrolytic enzymes in defence against the invading organism is well recognized [1]. Studies on biochemical properties of pulmonary fluid clearly indicate the presence of such enzymes. The nature of these enzymes and their specificity towards the structural components of the invading organism are important factors in revealing their bactericidal effects on extracellular tubercle bacilli in pulmonary fluid. Several workers have elucidated biochemical and functional activities of cultured mouse peritoneal macrophages [2–4]. Presence and distribution of acid hydrolases in mouse and rabbit macrophages and their secretory potential has been extensively studied by several previous workers [1, 3, 5–9]. Lysosomal association and secretory potential of both peritoneal and alveolar macrophages has been extensively studied [1, 2, 6, 8].

The present investigation is an attempt to delineate the bactericidal effects of extracellular enzymes and also to demonstrate the exact sequelae of enzyme action on tubercle bacilli present in the pulmonary fluid of infected guinea pigs.

P. KUMAR

Division of Microbiology, Defence R and D Establishment
Tansen Road, Gwalior-474 002, India

SAROJ CHANDRASEKHAR

Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi
Delhi-110 007, India

Materials and methods

Animals. Randomly bred albino guinea pigs weighing 300–400 g were obtained from the animal house of the institute. They were kept in aluminium cages, fed pelled diet (Hindustan Lever Limited, Bombay) and given water ad libitum.

Immunization. Guinea pigs were divided into four groups. Animals of group A were included as normal controls. Animals of group C and D were immunized intramuscularly with BCG suspension ($5–6 \times 10^6$ c.f.u.) in 0.1 ml of HBSS (Hanks Balanced Salt Solution. CSIR Centre for Biochemicals, Delhi). Three weeks after the first injection a booster dose of one tenth c.f.u. of the first injection was given to group C and D animals. Five weeks after the first injection all guinea pigs underwent Mantoux test to assess their immunological status and to group them as normal and immunized.

Intratracheal challenge. Guinea pigs of groups B and D were challenged with 0.1 ml homogenous suspension containing approximately 4×10^5 c.f.u. of *Mycobacterium tuberculosis* ($H_{37}Rv$). Guinea pigs were anaesthetized with anaesthetic ether (Sarabhai Chemicals Ltd., India). An incision was made along the ventral side of the neck, the trachea was exposed and lifted up. A homogenous suspension of the challenge organism was injected directly into the lungs of the animal through the trachea with a sterile syringe. Thereafter, the guinea pigs per group were sacrificed on days 1, 7, 14, 21, and 28 following intratracheal challenge to study extracellular enzyme and tubercle bacilli in cell free pulmonary lavage fluid (CFPLF).

Enzyme assay and bacillary content of CFPLF. The lungs were lavaged as described previously [10]. The bronchoalveolar lavage was made cell free by centrifugation for 10 min (200 g). The supernatant was used for the study of extracellular enzyme and bacillary content. Protein content of CFPLF was determined prior to the enzyme assay [11]. Three enzymes,

Table I

Extracellular enzymes of pulmonary lavage and

Days	Enzyme	Normal control group		Normal infected group	
		Enzyme activity		Enzyme activity	
		Specific*	Total	Specific	Total
1	Arabinosidase	2.40±0.19	3.44	1.44±0.17	1.49
	Mannosidase	1.75±0.17	2.51	1.92±0.19	2.01
	Sulphatase-C	3.11±0.33	4.45	3.79±0.28	3.96
7	Arabinosidase	1.55±0.19	2.06	1.42±0.15	2.15
	Mannosidase	1.44±0.16	1.92	1.31±0.13	1.98
	Sulphatase-C	2.65±0.24	3.54	3.79±0.27	4.70
14	Arabinosidase	1.78±0.16	2.05	3.11±0.52	4.72
	Mannosidase	1.90±0.10	2.18	6.28±1.04	9.53
	Sulphatase-C	2.78±0.21	3.19	5.06±0.33	7.67
21	Arabinosidase	1.64±0.32	1.93	3.11±0.28	3.47
	Mannosidase	1.41±0.19	1.66	4.71±0.73	5.26
	Sulphatase-C	2.44±0.29	2.88	6.30±0.47	7.03
28	Arabinosidase	0.70±0.09	0.92		
	Mannosidase	1.92±0.36	2.30	AD	AD
	Sulphatase-C	1.89±0.36	2.27		

* One unit of specific activity of enzyme corresponds to the μmol of substrate hydrolyzed per min, per mg of protein

AD = Animal died

BA = Bacilli absent

namely alpha-L-arabinosidase, alpha-D-mannosidase and sulphatase-C were studied using 4-Methyl Umbelliferyl (4-MU)-alpha-L-arabinoside (Lot 32F-078), 4-MU-alpha-D-mannoside (Lot 72F-5020) and 4-MU-sulphate (Lot 20F-502), Sigma Chemicals Coy., USA.

Substrate solution for the respective enzyme of 100 μmol strength was prepared separately. The substrate was dissolved in 2 ml dimethyl sulphoxide (DMSO, Sigma) and stored at 4 °C as stock. To prepare 100 μmol of substrate, before use 0.2 ml stock was diluted to 10 ml in 0.1 M-acetate buffer (pH 4.5). Enzyme assay was performed separately for each enzyme as described previously [2]. Briefly, in a screw capped tube 0.1 ml CFPLF was incubated at 37 °C for 60 min with 0.1 ml of 100 μmol buffered substrate for the assay of arabinosidase and mannosidase separately.

The assay of sulphatase-C was performed by sequential addition of 0.05 ml of 0.5 M immidazole-HCl buffer (pH 7.5), 0.05 ml of 50 mM barium acetate followed by 0.1 ml of a solution consisting of 400 mM Na_2SO_4 and 400 mM NaCl to 0.1 ml sample. This condition minimized the interference by other sulphatases [2]. Substrate 4-MU-sulphate (100 μmol in DMSO) 0.1 ml was added and mixture incubated at 37 °C for 1 h. These substrates were checked for homogeneity before addition and were found to contain no free 4-MU. The reaction was terminated by the addition of 3 ml of 0.5 M sodium carbonate-bicarbonate buffer (pH 10.2–10.4). The liberated 4-MU was assayed in a fluorometer (Kontron Instrument LTD., UK) at excitation 360 nm and emission 460 nm. Suitable enzyme and substrate blank assays were performed.

Standardization was carried out by mixing different concentrations of 4-MU in 3 ml carbonate-bicarbonate buffer and the amount of fluorescence was measured. For all enzymes, one unit activity corresponds to μmole of substrate hydrolyzed per min at 37 °C.

The extracellular tubercle bacilli in CFPLF were determined by standard c.f.u. count on Middlebrook 7H10 medium as described previously [12]. Data were analyzed using standard statistical formulae and represented as mean $\pm$ standard error of mean (X $\pm$ SEM).

their effects on extracellular tubercle bacilli

Normal infected group	Immunized group		Immunized infected group		
Bacterial content	Enzyme activity		Enzyme activity		Bacterial content
c.f.u./ml $\times 10^3$	Specific	Total	Specific	Total	c.f.u./ml $\times 10^3$
	1.24 $\pm$ 0.04	2.40	1.47 $\pm$ 0.14	2.12	
9.7 $\pm$ 0.176	1.65 $\pm$ 0.09	3.18	2.09 $\pm$ 0.27	3.01	5.8 $\pm$ 0.055
	3.58 $\pm$ 0.12	6.91	5.59 $\pm$ 0.39	8.05	
	2.55 $\pm$ 0.21	3.79	7.13 $\pm$ 0.42	15.58	2.7 $\pm$ 0.058
9.9 $\pm$ 0.08	2.47 $\pm$ 0.15	3.67	4.86 $\pm$ 0.09	10.64	
	3.66 $\pm$ 0.43	5.44	8.39 $\pm$ 1.64	18.34	
	2.56 $\pm$ 0.08	4.46	5.75 $\pm$ 0.41	10.75	
7.7 $\pm$ 0.12	1.95 $\pm$ 0.23	3.40	21.38 $\pm$ 1.22	39.99	1.76 $\pm$ 0.049
	4.59 $\pm$ 0.54	8.01	10.77 $\pm$ 1.03	20.14	
	4.07 $\pm$ 0.33	7.18	11.31 $\pm$ 0.20	40.14	
8.5 $\pm$ 0.23	2.29 $\pm$ 0.16	4.03	9.15 $\pm$ 1.51	32.47	1.63 $\pm$ 0.028
	4.83 $\pm$ 0.71	8.52	20.25 $\pm$ 2.11	45.95	
	2.81 $\pm$ 0.32	4.94	12.95 $\pm$ 1.80	45.91	
AD	2.40 $\pm$ 0.23	4.22	7.68 $\pm$ 1.07	27.23	BA
	2.61 $\pm$ 0.29	4.59	8.28 $\pm$ 0.52	29.36	

Results and discussion

Relative recovery of extracellular bacilli in pulmonary lavage fluid, one day after challenge was 5 to 6% in normal infected animals (unimmunized infected) and 2 to 3% in immunized infected animals. This was manifested by the increased phagocytic and bactericidal activity of alveolar macrophages obtained from immunized infected animals.

In a previous study slightly acidic pH in tubercle homogenates was reported and this was shown to be mainly confined to the granulation tissue and in densely packed epithelioid granuloma [13]. This acidic environment favoured the action of certain hydrolases active under acidic conditions. Another report [14] revealed appreciable amount of lysosomal hydrolases in extracellular fluid of granulomatous tissue. Our observations substantiated the presence of hydrolytic enzyme like alpha-L-arabinosidase, alpha-D-mannosidase and sulphatase-C apart from those reported by several previous workers (Table I).

The capacity of alveolar macrophages to secrete enzymes was found to be comparable among normal, immunized and normal infected guinea pigs. Analysis of cell-free pulmonary lavage fluid indicated the presence of these enzymes with minor variations in all the animal groups excepting the immunized infected animals. However, the level was found to be inadequate to restrict tubercle bacilli in the normal infected animals (Table I). The level of these enzymes at different intervals varied considerably, especially in the normal infected and immunized infected animals. It was observed that arabinosidase in the lavage of normal infected animals did not increase appreciably in comparison to mannosidase and sulphatase-C (Table I). The marked increase of mannosidase was observed during the second week after challenge in both normal infected and immunized infected animals (Table I). Vaccination had enhancing effects on the production and secretion of arabinosidase and mannosidase, the extent of their production and secretion was further boosted by challenge with tubercle bacilli in the immunized infected group. It may be inferred from these observations that peak level of these enzymes were attained only in immunized infected animals.

Belated appearance (21 days after challenge) of all the enzymes except mannosidase (14 days) in the lavage fluid of immunized infected animals and the gradual disappearance of extracellular bacilli reflected an inverse relationship between the enzyme and the tubercle bacilli. Sulphatase-C level in the lavage fluid was not much affected in normal, immunized and normal infected animals. Elevated levels of this enzyme were recorded only in the lavage fluid of immunized infected animals. This may be the effect of increased synthesis of the enzyme or enzyme leakage. Some investigators have also reported similar changes in the activated macrophages which rendered

them more susceptible to the injury than normal cells, when they become involved in phagocytosis, leading to subsequent release of hydrolytic enzymes [15].

In the present investigation three enzymes were studied in the pulmonary lavage of normal infected (unimmunized infected) and immunized infected guinea pigs. In the former group the cidal concentrations of the enzymes showed a late appearance as compared to latter group. The cidal concentration in the normal infected group remained for a short period. The bactericidal effect of these enzymes was therefore inadequate to correlate in the host defence of unimmunized infected animals. It seems probable that in immunized infected animals the peak enzyme levels were achieved faster than those observed in normal animals and remained in cidal concentrations for a prolonged period of time thus resulting in fast reduction of extracellular bacilli from the pulmonary lavage fluid. It is indicated that, while these observations are confirmed in situ, the bactericidal activity of these enzymes (purified preparations) specifically on tubercle bacilli in vitro needs to be established.

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BINDING OF ENTERIC BACTERIA TO HOG GASTRIC MUCIN*

I. KÉTYI

Institute of Microbiology, University Medical School, Pécs

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The binding features of enteric bacteria were studied using a model mucin of hog gastric origin. The time requirement of binding is short, it is temperature-independent, but dose-dependent. The binding effectiveness of *Escherichia coli*, *Shigella sonnei* and *Shigella flexneri*, as well as *Salmonella minnesota* had a narrow range: 1.5–9 germs pro pg of mucin. The bacterial ligand of the binding is certainly not a polysaccharide as proved by the uniform binding of the R-mutant series of *S. sonnei* and *S. minnesota*. On the basis of inhibition tests by an outer membrane protein fraction, the ligand may be a common outer membrane protein of the enteric bacteria. The outer membrane proteins encoded by the Shigella-EIEC invasivity plasmids do not take part in this binding. The inhibition by killed bacteria or by their culture supernatants of mucin binding of heterologous species may suggest a non-species specific common ligand, too. Similarly to the mucin utilization, the binding ability also seems to be a general phenomenon among the enteric bacteria.

The mucous membranes of the body, including the intestinal tract, are covered by mucus, which in case of the intestine is about a 400 nm thick layer [1]. The invasion by enteric pathogens, as well as the presence of bacteria belonging to the normal, or accidental bowel-flora cannot exist without some interaction with this mucous material. According to Freter [1] this interaction is a multifaceted process. In our previous experiments [2] it was shown that the utilization of mucin as a source of energy is a general character of the enteric bacteria. Furthermore, we demonstrated [3] that in vitro, in a mucin-minimal medium, strains of *Escherichia* and *Shigella* show a "quasi-biofilm" character during their growth. This observation in itself suggested a step of binding to the mucin receptors as a possible requirement for the phenomenon. In this paper we show the results of experiments concerning the mucin binding characteristics of some selected strains of enteric bacteria.

IVÁN KÉTYI

Institute of Microbiology, University Medical School, Szigeti út 12, H-7643 Pécs, Hungary

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Materials and methods

Strains used are listed in Table I. The plasmid mutant strain of *E. coli* 0143, No. 2/33 was selected and mobilized transfer of the invasivity plasmid into *E. coli* K-12 was performed in our institute [4, 5]. The R-mutant series of *S. minnesota* was kindly sent by O. Lüderitz [6], while the R-mutant series of *S. sonnei* was isolated and characterized by Kontrohr and Kocsis [7] in our institute.

Antiserum against Form 2 of *S. sonnei* was prepared in rabbits.

Media. For complete media Luria broth (LB) and LB-agar were used. A minimal medium was prepared according to Rothman and Corwin [8].

Mucin. As a model mucin a hog gastric preparation (Granular Mucin, Type 1701-W, Wilson Laboratories, Chicago, Ill.) was used in a concentration of 5%, heated at 100 °C for 1 h.

Testing the bacterial binding to mucin. Appropriate dilutions of overnight LB cultures were added to glucose-free minimal media containing different concentrations of mucin. After incubation (usually 30 min) at 37 °C the mixtures were centrifuged in a refrigerator centrifuge. The sediments were washed twice with saline and for the determination of the germ count the washed sediments and their supernatants were plated in appropriate dilutions on LB-agar. The rates of adsorption were calculated by comparing the germ counts of supernatants and sediments. A preliminary comparison of the direct platings and the platings treated with Triton X-100 showed no differences, therefore no Triton treatment was used in the experiments.

Inhibition of the bacterial binding to mucin. An effective concentration of the inhibitory material determined in preliminary experiments was added to glucose-free minimal media containing a low concentration of mucin (usually 0.005%). After incubation at 37 °C for 30 min the bacterial culture was added and incubated for another 30 min at 37 °C. Further procedures: centrifugation, washing and plating were identical with the method described above. The estimation of the rates of inhibition was based on a comparison to a control incubated in the same manner but using saline only.

As inhibitors killed bacteria and culture supernatants of the murine strain of *E. coli* were used. Killing was made by heat (100 °C, 30 min), or by formalin (0.3% overnight), or by streptomycin (500 μg/ml overnight). The supernatants in the two latter cases were dialysed and the bacteria washed by saline twice.

Inhibition of binding was tested also by an outer membrane protein fraction prepared from the murine strain of *E. coli.*

Outer membrane protein fraction was prepared according to Schnaitman [9] from the murine strain of *E. coli* with the only modification of using ultrasonic treatment instead of French Press. Its protein content was determined by the method of Lowry et al. [10]. Our preparation had 250 μg/ml of protein. In mucin binding inhibition tests bovine serum albumin (250 μg/ml) and saline were used as control to compare the mucin adsorption to the former.

Results

1. *General characteristics of the binding and the binding effectiveness of some selected strains of enteric bacteria.* The influence of incubation time, incubation temperature, as well as concentration of mucin is shown in Table II. whe binding seems to be rather rapid and the prolongation of the incubation time over 10 min does not result in higher adsorption rates. Similarly the temperatures applied: 23, 30, 37 and 43 °C do not influence the effectivenes of binding. On the other hand, the binding ratio seems to be dose-dependent. The 50% end point of binding was expressed with the model strain of *E. coli* "murine" at the concentration of mucin of 0.0031% — using a culture with a germ count of about 10^7/ml. Comparing these data: about 1 pg of this purified hog gastric mucin seems to bind 2 germs (Table II).

Table I

Strains used

Species	Designation	Characteristics
	K-12 (J53)	
Escherichia coli	J53 (pSP1)	Carries the invasivity plasmid and expresses about 7–16 outer membrane proteins (OMPs)
	murine	Serologically not determined strain isolated from a healthy mouse
	EIEC O143, No. 2	Invasivity plasmid carrier expressing the plasmid encoded OMPs
	No. 2/33	A derivative of No. 2, IpaB and C are not expressed
	No. 31	With the loss of the invasivity plasmid and loss of the plasmid-encoded OMPs
Shigella flexneri	2a, No. 20780	Avirulent strain
Shigella sonnei	Form 1	O-specific side chain positive
	"Kiss" Form 2	R-core: KDO-LD-Hep-glc_3-gal_2
	R86	KDO-LD-Hep-glc_3
	R41	KDO-LD-Hep_3
	R562 H	KDO-DD-Hep
	R562 A	KDO-
Salmonella minnesota	1114 S	O-specific side chain positive
	1112 R60	R-core: KDO-Hep-glc_2-gal_2-NAcglc
	1113 R345	KDO-Hep-glc_2-gal_2
	1119 R5	KDO-Hep-glc_2
	1167 R595	KDO-

KDO = keto-deoxy-octonic acid; Hep = heptoses; glc = glucose; gal = galactose; NAcglc = N-acetyl-glucosamine

Table II

Some characteristics of the binding of a "normal", murine E. coli strain to hog gastric mucin (rates of adsorption)

Incubation period*		Influences of			
		Incubation temperature*		Concentration of mucin	
min	adsorbed, %	°C	adsorbed, %	vol/vol, %	adsorbed, %
10	99.5	23	98.6	0.0500	> 99.9
30	99.8	30	96.6	0.0250	> 99.9
				0.0125	90.0
60	> 99.9	37	96.1	0.0062	72.1
120	98.0	42	97.6	0.0031	50.0
				0.0015	21.1
< 10 min		no influence		End point of 50% = 0.0031% about 1 pg adsorbs 2 germs	

* Hog gastric mucin concentration, 0.0125%

Table III

Binding effectiveness of the representative strains to hog gastric mucin

Strains	Designation	Binding values (c.f.u. to 1 pg of mucin)
E. coli	"murine"	2.0
	K-12, J53	5.1
	O143, No. 2	3.2
S. flexneri	2a, No. 20780	1.5
S. sonnei	"Kiss", Form 1	9.0
S. minnesota	S, No. 1114	6.0
	$\bar{x} \pm SE = 4.4 \pm 1.1$	

In the next series of experiments some representative strains of enteric bacteria were compared concerning their binding effectiveness (Table III). whese were *E. coli* O143 (No. 2), K-12 (J53), *S. sonnei* (Form 1), *S. flexneri* 2a (20 780), and *S. minnesota* (S, 1114). whe result of these experiments is showing a very narrow scale of mucin-binding effectiveness, ranging from 1.5 to 9.0 bacterial cells pro 1 pg of mucin (Table III).

2. *The role of (lipo)polysaccharide in binding to mucin.* In proving or disproving the role of the lipopolysaccharide (LPS) or its polysaccharide (PS) chain in the binding to mucin it seemed to be optimal to use R-mutant series, including backbone defective mutants, too. There is a well known mutant series from a strain of *S. minnesota* and we have a similar well characterized R-series from the strain "Kiss" of *S. sonnei*. The results of these experiments using R-mutant cultures standardized to a germ count of about 10^6/ml and

Table IV

Role of the (lipo)polysaccharide in the binding to mucin in cases of S. sonnei and S. minnesota R-series

Common S—R	*S. sonnei* "Kiss" R-series*		*S. minnesota* R-series**	
	designation	adsorbed, %	designation	adsorbed, %
S	Form 1	92.9	1114—S	90.3
Ra	Form 2	94.3	1112—R60	81.3
Rb	R86	94.3	111—R345	89.7
Rc			1119—R5	81.9
Rd_1	R41	89.2		
Rd_3	R562 H	90.0		
Re	R562 A	96.2	1167—R595	84.7
	$\bar{x} \pm SE = 92.8 \pm 1.05$		$\bar{x} \pm SE = 85.6 \pm 1.9$	

* Mucin concentration, 0.0025%
** Mucin concentration, 0.0005%

mucin in concentrations of 0.0025% *(S. sonnei)*, or 0.005% *(S. minnesota)* are summarized in Table IV.

The presented data show equivocally that the PS chain has no role in the binding of *S. sonnei* or *S. minnesota* to mucin (Table IV).

3. *The role of the outer membrane proteins in the binding to mucin.* There is an easy way to investigate the specific outer membrane proteins (OMPs) concerning their role in the mucin binding by using strains and their derivatives carrying the invasivity plasmid of Shigella-EIEC and expressing the plasmid-encoded OMPs. The following strains offered us a good opportunity: the virulent, plasmid carrier strain of *E. coli* O143, No. 2, expressing 7 to 16 plasmid-encoded OMPs, its derivative No. 2/33 which is supposed to be a regulatory mutant do not expressing the OMPs B and C (Ipa B, C), furthermore the derivative No. 31 with the loss of plasmid together with the loss of expression of the plasmid-encoded OMPs. The *E. coli* K-12 (J53) and its invasivity plasmid carrying derivative [J53(pSP1)] were also included in these experiments (Table V).

Table V

Role of the invasivity plasmid-encoded outer membrane proteins in the binding to mucin

Strains and designations	Plasmid-encoded outer membrane proteins expressed	Adsorption %
E. coli O143, No. 2	7–16 polypeptides	88.1
O 143, No. 2/33	2 of them is missing*	91.9
O143, No. 31	none of them is expressed	88.8
K-12, J53	none	92.9
J53 (pSP1)	7–16 polypeptides	85.4

* Ipa B and C

The data express a negative finding: the plasmid-encoded OMPs are not involved in the mucin binding of these strains of *E. coli*. Taking into account that there is no difference in the binding effectiveness between Form 1 (carrying also the invasivity plasmid) and Form 2 of *S. sonnei*, this is true also for *S. sonnei*.

In the next series of experiments an OMP fraction prepared from the murine strain of *E. coli* was tested in mucin binding inhibition test. This OMP fraction showed marked and dose-dependent inhibition in homologous system (murine *E. coli*) and inhibited the mucin binding of the heterologous strain of *S. minnesota* S, too. The protein control, using bovine serum albumin had no inhibiting effect. The non-inhibited (saline control) strains showed an effective binding (99.7%, 98.4%) to mucin (Table VI).

Table VI

Inhibition of enteric bacterial binding to mucin by an outer membrane protein fraction from the murine strain of E. coli

Outer membrane protein fraction (protein content)	Inhibition of the binding of	
	"murine" *E. coli*	*S. minnesota* S
25 μg/ml	96.8%	.
5 μg/ml	84.7%	98.2%
1 μg/ml	53.0%	.
200 pg/ml	14.3%	.
BSA* control		
25 μg/ml	< 20%	< 20%
5 μg/ml	< 20%	< 20%
	Binding effectivity (rates of adsorption)	
Saline control	99.7%	98.4%

* BSA = Bovine Serum Albumin

NB. The 50% end point of binding inhibition of outer membrane protein fraction in homologous system is about 900 pg/ml

4. *The inhibition of binding by killed bacteria and culture supernatants.* The murine strain of *E. coli* was killed by heat, formalin, or streptomycin. Washed bacteria exerted an inhibition of the living homologous strain with 96, 67 and 50% effectiveness in the above order. Their dialysed supernatants produced also a binding inhibition of 97.5, 91.9 and 99.8%. The most effective material, the culture supernatant of streptomycin-killed bacteria exerted an effectiveness of 35.6% even in a dilution of 1 : 25 and the binding inhibition was dose dependent (data not shown). This culture supernatant was used for heterologous binding inhibition experiments in a dilution of 1 : 5 (Table VII).

Table VII

Inhibition of the mucin binding of some enteric bacteria by the culture supernatant of the streptomycin-killed murine strain of E. coli

Strains	Inhibition %	Rate of adsorption (% in controls)
E. coli "murine" homologous	96.2	96.3
E. coli O143, No. 2	95.5	95.6
S. sonnei "Kiss", Form 1	96.2	96.4
S. minnesota S	88.7	93.6

Mucin concentration 0.005%. The dilution of the inhibitory supernatant was 1 : 5. The rates of inhibition was calculated by comparing the c.f.u. values which bind to mucin with and without inhibition

The results show that the mucin binding inhibition of the tested representative strains were of the same effectiveness between 88.7 and 96.2%, while the uniformized binding rate of the controls was between 93.6 and 96.4% (Table VII).

5. *Inhibition of the mucin binding by* D-*galactose.* No significant inhibition of binding of the murine strain of *E. coli* to 0.005% mucin happened by using D-galactose (10, 2 and 0.4%) (data not shown).

Discussion

Freter [1] listed many steps in the enteric bacterial-mucin interaction. Some of them may be important in the pathomechanism of enteric pathogens, and some of them are assumed to take part in the maintaining mechanism of the normal and accidental bowel flora in the coeco-colonic tract.

One of these steps, the utilization of the intestinal mucin permitting the bacterial growth in the intestinal canal has already been studied in our previous paper [2] showing that it is a general character of the enteric bacteria. On the basis that the gene of the alpha-glucosidase and the genes of permeases, partly at this and partly at the *lac*-operon may suggest that this ability is a conserved one [11, 12].

The presented experiments concerning the binding ability to mucin similarly support the hypothesis that this is also one of the general characteristics of the enteric bacteria. In the course of our earlier, as well as of the present experiments a purified hog gastric mucin was used as a "model mucin". Its advantage was the purified and standardized character with the disadvantage of not being an intestinal mucin of a certain animal species. In a preliminary experiment Dinari et al. [13] showed that the HeLa invasivity of *S. flexneri* was inhibited by the intestinal mucin of guinea pigs, but not of monkeys. Therefore we suppose only that some general phenomena, including the binding may be modeled also by hog gastric mucin. On the other hand, in a concrete animal experiment the mucin binding step could be proved only using the intestinal mucin of the same species.

The very first question emerging at the beginning of our experiments was that whether the interaction between bacteria and mucin is a simple physical adsorption or a ligand-receptor interaction. The presented data, showing the short time requirement, the temperature independency together with the dose-dependent characters of the process all point to a ligand-receptor interaction.

Testing the binding effectiveness of other strains of *E. coli* (O143, K-12), *S. sonnei*, *S. flexneri* and *S. minnesota* resulted a surprisingly narrow scale: 1.5 to 9.0 germs per pg of mucin. This findings favours the conception that

a common mechanism exists among the different species of enteric bacteria.

The next series of experiments was dealing with the nature of the ligand. There are data in the literature about the possible role of the polysaccharides: tests on *Salmonella typhi-murium* mucin binding assumed the requirement of intact LPS for its effectiveness [14, 15], likewise as in the case of *Pseudomonas aeruginosa* and the respiratory tract mucus [16, 17]. On the other hand, Cohen's team [18–20] working with a wild-type strain of *E. coli* and its Col^- derivative, attributed the lower binding effectiveness of the latter to a relatively low level of its outer membrane proteins, and a weaker motility.

The role of the PS chain of LPS in mucin binding was tested with R-mutant series of *S. sonnei* and *S. minnesota*. R-mutants with the loss of heptoses from the backbone were also included. The presented results did not show differences in the binding effectiveness therefore we rejected the PS chain as a ligand in this phenomenon.

Concerning the role of the OMPs and based on the data of the Cohen-team of col plasmid-encoded OMPs, we tried in this aspect the polypeptides encoded by the invasivity plasmid of Shigella-EIEC. Having the virulent strain O143 expressing 7–16 plasmid encoded polypeptides, the derivative No. 31 and strain J53 of *E. coli* K-12 without these polypeptides, as well as the strain J53(pSP1) carrying this plasmid or the supposed plasmid mutant strain of O143, No. 2/33 with the loss of expression of polypeptides "B" and "C" [21], this possibility was tested, too. The uniform binding effectiveness of all these strains shows that the specific, plasmid-encoded proteins have no role in the formation of the ligand.

The fraction of OMPs prepared from the murine strain of *E. coli* showed a marked inhibition in the mucin binding of the homologous strain and also the heterologous one proving the protein nature of the ligand. Further purification may be assumed to show only that one or more of them are responsible for the binding to mucin receptor(s).

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IN VITRO AND IN VIVO (LD_{50}) EFFECTS OF HUMAN LACTOFERRIN ON BACTERIA

ÉVA CZIRÓK, HEDDA MILCH, KATALIN NÉMETH and I. GADÓ

National Institute of Hygiene, and National Institute of Haematology and Blood Transfusion, Budapest

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The in vitro and in vivo effects of human lactoferrin (LF), apoLF, iron saturated LF and of different iron containing compounds (ferric chloride, ferric sodium citrate) were studied on *Escherichia coli*, *Salmonella typhi-murium* and *Pseudomonas aeruginosa* reference and wild-type strains with well-defined virulence markers (i.e. enterochelin, aerobactin production). LF exert in vitro antibacterial effect, and iron-free Vogel-Bonner medium proved to be suitable for its determination. The effect of intraperitoneally administered LF could not be evaluated because of its aspecificity, as any treatment (e.g. saline, Ringer solution) before bacterial challenge activated macrophages. In contrast to the in vitro results, intramuscular challenge failed to inhibit bacterial growth in vivo, as siderophores produced by bacteria were able to acquire lactoferrin-bound iron. LF treatment, like iron addition, enhanced the virulence of bacteria in mice, whereas apoLF — using iron present in the body fluids — turned to LF being unable to acquire siderophore-bound iron from bacteria. These findings do not support the literary view that LF would be useful as an antimicrobial drug.

Findings in the last 50 years have indicated that there is a competition for the iron essential for growth between bacteria and their mammalian hosts [1]. Though Fe^{++} ions occur in nature in soluble form, Fe^{+++} compounds are insoluble aggregates. The solubility of Fe^{+++} salts depends on the pH: they are hundred-fold less soluble at neutral pH than at pH 6 [2].

Although body-fluids contain plenty of iron, the amount of free iron available for bacteria is extremely small. Most of the bound iron is found intracellularly and the extracellular iron is bound to high affinity iron-binding proteins [3]. The iron transporting proteins in the human organism are transferrin (TF) found in blood [4], and lactoferrin (LF) found in milk, tear and polymorphonuclear leukocytes [5–7]. Competiting for the biologically inaccessible iron, the microorganisms secrete ironchelating compounds of low molecular weight, known as siderophores: enterochelin [8], aerobactin [9], ferrichrome [10], ferricitrate [11] and mycobactin [12]. The two most important siderophores, from the aspect of bacteria, are classified into two chemical groups: phenolates (enterochelin), and hydroxamates (aerobactin).

ÉVA CZIRÓK, HEDDA MILCH, ISTVÁN GADÓ

National Institute of Hygiene
Gyáli út 2—6, H-1966, Budapest, Hungary

KATALIN NÉMETH

National Institute of Haematology and Blood Transfusion
Daróczi út 24, H-1113 Budapest, Hungary

During the infection, such defensive changes occur in the iron metabolism by the human organism, which produce a decreased iron content in the circulating blood [13, 14]. In the process the circulating iron accumulates in the iron stocks (RES) in the form of ferritin [14], and as a consequence the iron content and the iron-binding capacity of the serum decreases [14]. Accordingly, the infecting microorganism has to survive and multiply under iron-poor conditions in the body-fluids of its host. As a protective mechanism, the microorganism starts to produce a chelator that helps to bind and utilize the limited iron [15].

The iron-binding protein of human blood-plasm (TF) was described by Shade and Caroline in 1944 [16], the iron-binding protein found in human milk (LF) was detected by Sørenson and Sørenson in 1939 [17]. The antibacterial effect of LF on the mucous membrane of the respiratory tract was observed by Masson et al. [18] and in bovine milk by Oram and Reiter [19]. The iron-binding property of LF is active under acidic conditions (pH 4.0) too, while the other iron-binding proteins are not active and do not bind protein.

Numerous experiments proved the in vitro bacteriostatic effect of LF being present in the milk. The human milk contains beside the 10% iron-saturated LF also citrate and bicarbonate, taking part in the iron-uptake system. The antibacterial effect of LF is highly influenced by these and by the pH of the environment. Arnold et al. [20] reported the bactericidal effect of purified, iron-free "apoLF" on *Streptococcus mutans*, *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Candida albicans*. However, since such effect on enteropathogenic *Escherichia coli* was not demonstrated [21], it was suggested that these bacteria secreted siderophores. Finkelstein et al. [22] found that iron-saturated LF had no antibacterial effect. They observed bacteriostatical effect only of apoLF on *V. cholerae*, *Salmonella typhi-murium*, *Shigella flexneri*, *P. aeruginosa* and *Staphylococcus aureus* strains. In vivo experiments were reported first by Bullen et al. [23], Who found in guinea pig experiments that in the small intestine the bacteriostatic effect of human milk is mainly due to LF.

Examination of the siderophores secreted by the microorganisms have shown that the iron-binding system is mediated by certain virulence plasmids [24].

The strict correlation existing between iron content and virulence was described first by Jackson and Burrows [25] in 1956. They showed that *Yersinia pestis* became virulent for mice after the addition of iron. Payne and Finkelstein [26] found increased virulence of *Neisseria gonorrhoeae* in chicken embryo after adding iron.

The in vitro effect of LF on bacteria was recognized by several authors. Some of them believed that LF would be the antimicrobial drug of the future,

especially in reduced immunological status of the organism (organ transplantation, infection of infants and aged people) deterioration of condition because of other disease (tumour) or multiple drug resistance of bacteria, when good results were hardly expected from antibacterial treatment.

On the basis of these findings we undertook to examine the iron-uptake system of bacteria and the in vitro and in vivo effectiveness of LF. We aimed to determine the in vitro and in vivo effects of human LF on bacteria with well-defined virulence markers.

Materials and methods

Bacterial strains. To test the in vitro and in vivo effect of LF on bacteria the following enterochelin and aerobactin producing and transferring *E. coli* and *S. typhi-murium* reference strains were used (abbreviation in parentheses indicate their enterochelin (ent) and aerobactin (aer) production (p) and transport (t) capacity): K12 W0987 (ent$^-$, aer$^+$), K311, KH576 (ent: P$^+$T$^+$, aer: P$^+$T$^+$), CA7S, 1, 7a Col V (ent$^+$, aer$^+$), enb 7 (ent$^-$, aer$^-$), LT2 (ent$^+$, aer$^+$), LG1522 (ent$^-$, aer$^-$). Wild type strains, isolated in our laboratory were also used: 21 *E. coli* and 4 *P. aeruginosa* strains. The strains were different according to their virulence markers (MRHA, Hly, antigens K1 and K5, Col) LD_{50} values and origin (22 strains of extraintestinal and 3 of enteric source).

Detection of siderophore production. To detect enterochelin secretion, an Ent$^-$ *S. typhi-murium* enb 7 culture was mixed into iron-free Vogel-Bonner medium and plates were poured [27]. The strains to be tested were precultivated in iron-free liquid medium, then spot inoculated onto the plates and incubated at 37 °C for 24 h and 48 h. As controls, $FeCl_3$ and iron-free media were used. Strain *S. typhi-murium* enb 7 served for negative control, and the enterochelin producer strain LT2 for positive control (Fig. 1). The growth of the tested strain without the appearance of a surrounding growth-zone of strain enb 7 in the medium showed that the strain produced a siderophore other than enterochelin (W0987).

The qualitative determination of aerobactin was carried out by bioassay, using strain LG1522 ara, azi, fepA403, lac, leu, mte, proC, rps, L, supE, ton A, tsx, thi, Col V-K30 iuc as indicator. The medium was Simon–Tessman agar, complemented with alpha, alpha'-dipyridyl [28].

Detection of the effect of LF. Wells were cut with iron-free glass tubes into iron-free Vogel–Bonner medium containing strain enb 7 and different concentrations of LF were dropped into the wells. The tested strain was precultivated in iron-free medium for 3 h, then streak-inoculated on both sides of the wells (Fig. 2).

Preparation of human lactoferrin (LF). LF was isolated from human mother's milk using Johansson's [29] modified method. Five litres of milk were centrifuged for 1 h at +4 °C at 1000 rpm. The fat was removed from the surface, 3 g of CM-Sephadex C50 was added to the clear sediment and it was mixed for 1 h at 4 °C. The wet gel was allowed to deposit and it was handled with 2 g of CM-Sephadex C50, twice. The gels were united after the process and washed with 10 mM (pH 7.4) phosphate buffer 5–6 times, and with 20 mM (pH 7.4) phosphate buffer twice. The gel-bound proteins were eluated with saline solution of gradually increasing concentrations (0.25, 0.5 and 0.7 M NaCl in 20 mM phosphate buffer). The LF was present in the eluate obtained with the 0.5 M and 0.7 M saline solution. The solutions were concentrated by ultrafiltration, the salt was removed by dialysis, LF was lyophilized and stored at +4 °C. The purity of LF was checked by polyacrylamide gel electrophoresis containing sodium-dodecylsulphate (SDS) and by immuno electrophoresis. The LF produced this way contained 8–10% of protein-bound iron.

Apo lactoferrin (ApoLF) was produced from LF by the method of Mazurier and Spik [30]. The water solution of LF was dialyzed for 48 h to 40 mM EDTA and 0.2 M Na_2HPO_4 containing sodium acetate buffer (final pH 4.0) changing the buffer once. The salt-free, pure protein was lyophilized.

Labelling of LF by radio-isotope ^{125}I. The labelling of the pure LF prepared from mother's milk by ^{125}I was carried out with Enzymobead (Bio.Rad.Labs, Richmond, Ca, USA), according to the instructions of the manufacturer.

Infection of animals. To determine the in vivo effect of LF, four groups of mice were examined, each containing ten mice of 16 g. They were challenged intraperitoneally with 0.5 ml bacterial suspension incubated for 4–6 h at 37 °C and serially diluted tenfold in physiological saline. The 50% of lethal dose (LD_{50}), lethality and significance were calculated as described previously [31].

Virulence markers [mannose resistant haemagglutinating capacity (MRHA), haemolysin production (Hly), antigens K1 and K5, colicin production (Col)] and LD_{50} values were determined as described previously [31, 32].

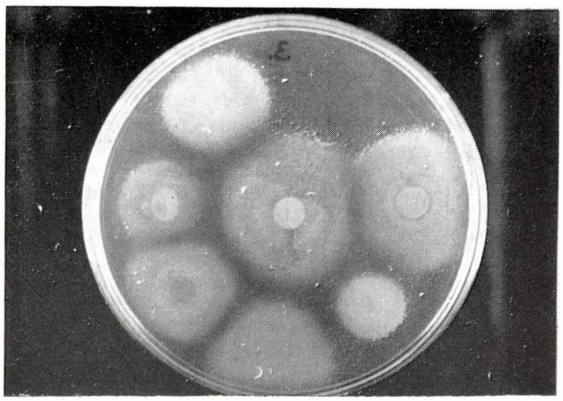

Fig. 1. Strain enb 7 (ent^-, aer^-) was mixed in iron-free medium and plated. Wells clockwise: strains enb 7, Col V, Wo 987, CA 7S, KH576, *E. coli* Nos 1 and 2. Centre well: LT 2

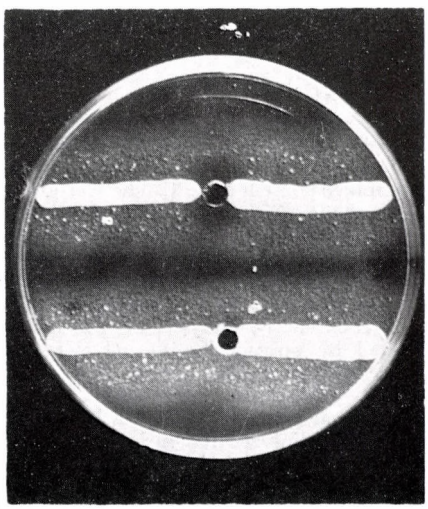

Fig. 2. Upper well: lactoferrin (LF-40 mg); lower well: iron-free water. Streaks: strain LT 2. Strain enb 7 was mixed in iron-free medium

Results

Examination of the in vitro effect of LF. Figure 2 shows the growth of strain *S. typhi-murium* LT2 on iron-free medium plated with strain enb 7. Strain LT2 grew well along the streak on the iron-free medium and enb 7 also grew abundantly around the well filled with iron-free water, due to the chelating effect of Ent produced by LT2. That the chelating effect of LT2 was inhibited by LF, was evident from the lack of growth of strain enb 7 around the well containing 40 mg LF. Inhibition around the 10- and 100-fold dilution of LF was weaker. Figure 3 shows a weak growth inhibition of another ent$^+$

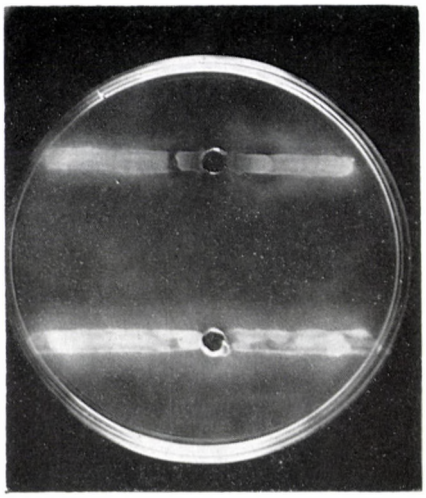

Fig. 3. Arrangement as in Fig. 2. Streaks: strain Col V

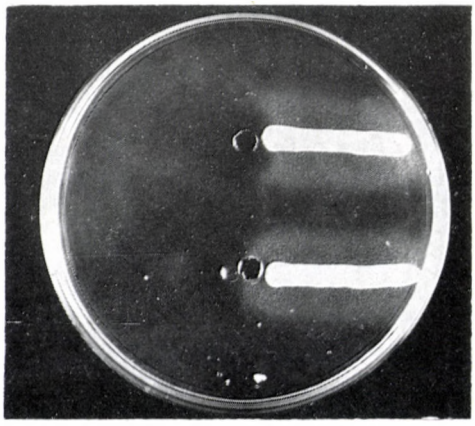

Fig. 4. Arrangement as in Fig. 2. Left hand streaks: strain enb 7, right hand streaks: strain *E. coli* No. 2

Col V producer strain as an effect of LF. Figure 4 shows that the chelating effect of a wild type ent$^+$ aer$^+$ *E. coli* strain is inhibited by LF (right hand streak, upper well); the negative control strain (enb 7) failed to grow in the medium (left hand streaks).

The effect of iron saturated LF was examined using strains LT2, Col V, (ent$^+$, aer$^+$) enb 7 (ent$^-$ aer$^-$) and a wild type strain (ent$^+$ aer$^+$). The inhibition zone obtained around iron saturated LF was smaller, than the inhibition zone of LF shown in Fig. 2, and the effect of enterochelin produced by the tested strains was only weakly inhibited. The growth and chelating effect of the wild-type strain were not inhibited.

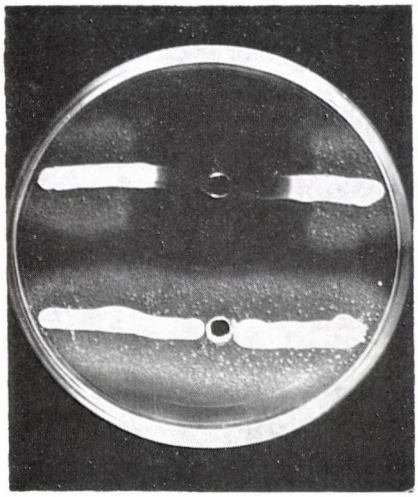

Fig. 5. Upper well: apoLF (40 mg); lower well and medium: see Fig. 2. Streaks: strain LT 2

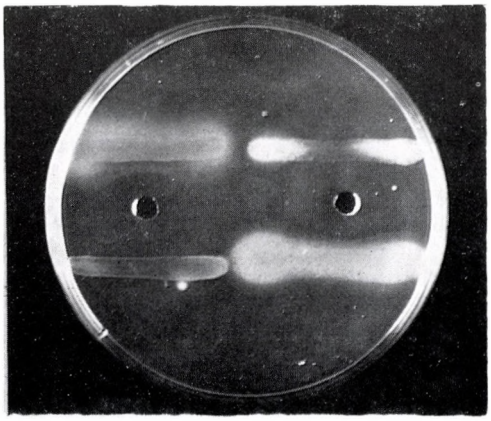

Fig. 6. In well LF (40 mg). Upper left hand streak: strain KH 576, upper right hand streak: strain *E. coli* No. 1; lower left hand streak: strain WO 987; lower right hand streak: strain LG 1522

The growth of strain LT2 (Fig. 5) and the wild-type strain was highly inhibited by 40 mg/ml iron-free apoLF. The inhibiting effect of the 10- or 100-fold diluted apoLF was weaker than the effect of the concentrated apoLF. The growth of the reference strains (KH576, WO987, LG1522) and the wild-type strain (ent$^+$ aer$^+$) inoculated along the side of the well containing LF was also inhibited (Fig. 6).

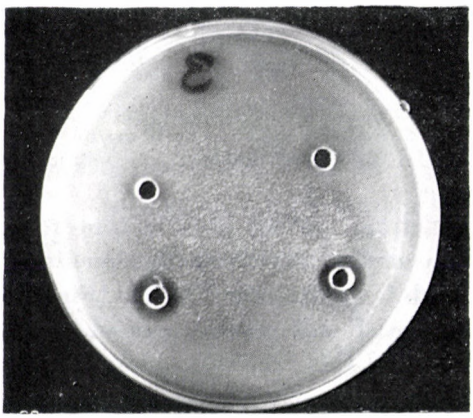

Fig. 7. Strain enb 7 was mixed in agar medium and plated. Upper wells: PBS; lower left well: LF; lower right well: apoLF

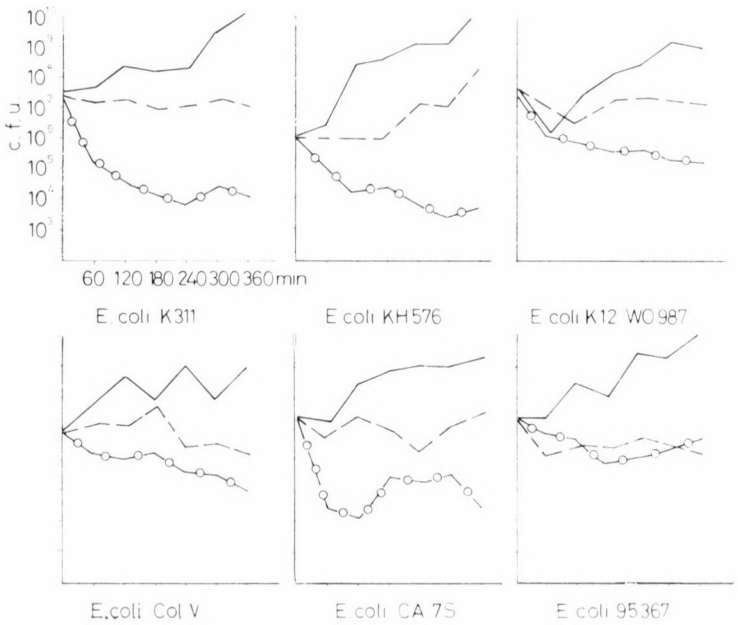

Fig. 8. Bacteriostatic effect of lactoferrin and apo-lactoferrin on different reference strains. ——— control; – – – LF; ○——○ apoLF

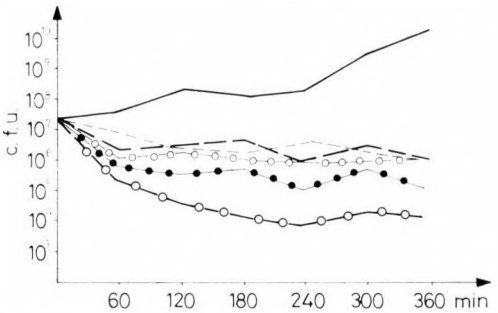

Fig. 9. Effect of different concentrations of apolactoferrin on the multiplication of *E. coli* K311. ——— control; – – – 2 mg/ml apoLF; – – – 4 mg/ml apo LF; ○——○ 8 mg/ml apo LF; ○–○–○ 16 mg/ml apoLF; ○–○–○ 40 mg/ml apoLF

Because the ent$^-$ strain did not grow in iron-free medium, to examine the effect of LF only complete (not iron-free) medium could be used. The growth of strain ent$^-$ enb 7 was inhibited by LF (Fig. 7) in this medium, and the inhibiting effect of apoLF was more expressed.

The in vitro effects of LF (40 mg/ml) and apoLF (40 mg/ml) were examined on the growth of different test and wild-type strains. In case of every *E. coli* strain, a growth inhibiting effect was observed compared with the control viable bacterial count between 60 to 360 min, either ent$^+$ or ent$^-$ strains, LF or apoLF were used (Fig. 8). The dose dependent effect of apoLF was demonstrated when apoLF was tested between 2 mg/ml and 40 mg/ml (Fig. 9).

Examination of in vivo effect of LF. To examine the effect of LF, mouse experiments were carried out to determine the mode of injection and the effective dose of LF (Tables I and II). "Protective" effect was observed after intraperitoneal injection of 1 mg LF when it was given 4 days to 3 h prior to challenging. However, LF had no "protective" effect when applied one

Table I

The effect of time of LF administration of the virulence of bacteria
(LF: 1 mg i.p. injection; challenging: *E. coli*, LD_{50})

	Control		LF administration as compared to infection						
			—4 days	—3 days	—6 h	—3 h	—1 h	—5 min	0*
Infection	+	−	+	+	+	+	+	+	+
LF	−	+	+	+	+	+	+	+	+
No of killed/infected mice	30/37	0/20	3/10	2/10	4/28	3/10	9/10	7/10	47/50

LF = lactoferrin
* Simultaneous LF injection and infection

Table II

The effect of the quantity and mode of introduction of LF on the virulence of bacteria
(Administration of LF 6 h prior to infection, challenging: *E. coli*, LD_{50})

Mode of introduction	LF (mg)							
	0	0.0005	0.005	0.05	0.1	1.0	10	20
i.p.	4/10*	3/10	2/10	1/10	2/10	2/10	5/10	4/10
i.m.	16/30	•	•	3/20	7/30	4/40	16/20	•

LF = lactoferrin
* No. of killed/infected mice
• not examined

hour before, or simultaneously with the infection; on the contrary, the lethality of the experimental animals increased.

Examining the effect of the quantity of LF (Table II), no significant difference was found in the "protective" effect changing the dose between 1 to 0.0005 mg of LF and total protection was never observed. When the dose of LF was increased higher than 10 mg, the lethality of mice increased a bit compared with lower doses.

The experiments were repeated using sodium bicarbonate added to LF (because the presence of sodium bicarbonate has been assumed favourable for the production of iron chelator complex [23, 33, 34]). In our experiments, the mouse killing effect of LF was independent of the presence of sodium bicarbonate.

On the basis of the above findings ("protective" effect of LF given 4 days earlier and even in 0.0005 mg quantity) we examined how long was present LF in the organism and in which organs did it accumulate.

For these experiments ^{125}I-labelled LF was used. The results are summarized in Table III. Radioactivity in the blood was detectable 1 h after the injection and it was present in a high level after 6 h and decreased to minimum after 24 h. Within one hour radioactivity appeared in the organs (liver, spleen)

Table III

Clearence of lactoferrin in different organs

Time h	Blood	Radioactivity of organs, cpm/g organ			
		Peritoneum	Liver	Spleen	Faeces
0	990	•	•	•	24
1	19 225	90 100	59 422	41 746	64 762
6	23 350	17 200	21 131	17 946	51 808
24	2 900	800	1 456	1 585	22 222

Administered: 0.1 mg ^{125}I-labelled lactoferrin
• not examined

Table IV

The effect of vehicle substances used for LF dilution on the virulence of bacteria

Vehicle substance	Not treated	Treated 6 h prior infection		Treated simultaneously with infection	
		Vehicle substance	Vehicle substance + LF	Vehicle substance	Vehicle substance + LF
Distilled water	•	4/10*	•	•	•
Physiological saline	6/10	10/20	•	10/10	10/10
PBS	15/20	4/10	5/10	•	•
Ringer solution	6/10	16/40	18/30	•	20/20

LF = lactoferrin
PBS = phosphate buffered saline
* No of killed/infected mice
• not examined

and in the peritoneal fluid and after 24 h it diminished significantly, similarly to that in blood. The isotope labelled LF appeared after 1 h in the stools and urine, too (not shown in the Table) and it was detectable in high amounts after 24 h. It was excreted with the stools even on the fourth day. Radioactivity of the intestinal wall was not higher than that of the other organs, and it was minimal in the kidney after 24 h.

On the basis of these data it appeared to us that LF had an aspecific "protective" effect, therefore the animals were pretreated 6 h prior to infection with phosphate buffered saline (PBS) used for dissolving the lyophilized LF (Table IV). Indeed, PBS exerted a "protective" effect as compared with the controls that received no injection. Other vehicle materials (physiological saline, Ringer-solution, distilled water) were also tested. The results were similar to those for PBS, including the observation that when the vehicle substances were simultaneously administered with challenging, the "protective" effect failed to appear independently of the presence or absence of LF.

Further examinations were carried out with 14 *E. coli* strains to compare the effect of LF given intraperitoneally 2 h before and after infection. These results are presented in Table V. The animals were "protected" compared with the control, when LF was given before infection. When LF was given after infection, the number of the killed animals was higher than of the controls. The physiological saline given 2 h before infection had a "protective" effect but if it was given after infection the number of the killed animals was higher in the test group than in the control group. There was no statistically significant difference in the protective effect observed in the group pretreated with physiological saline and in the control group ($p > 0.3$). There was no significant difference in the "protective" effect between the group pretreated

with LF and the group treated with physiological saline ($p > 0.05$). The "protective" effect of the standard LF (Sigma No. L8881) did not differ significantly from the effect of LF produced by us.

The mode of administration was changed and LF was given intramuscularly 2 h before infection (Table V). In this manner "protection" was

Table V

The effect of mode and time of administration of LF on the virulence of bacteria

No. of examined *E. coli* strains	Administered material, mode of introduction	Control	Treatment	
			before 2 h of infection	after 2 h
14	LF NIHBT 0.5 mg i.p.	107/200**	38/200	125/200
2	LF Standard* 0.5 mg i.p.	14/20	2/20	18/20
3	Physiological NaCl i.p.	15/40	12/40	22/40
2	LF NIHBT 1 mg i.m.	8/20	6/20	10/20
			before 6 h	
6	LF NIHBT 1 mg i.m.	43/60	12/60	
6	Physiological NaCl i.m.	43/60	37/60	
			before for 3 days	
2	LF NIHBT 10 mg per os	8/20	10/20	

LF = lactoferrin
NIHBT = National Institute of Haematology and Blood Transfusion, Budapest
* Sigma No L 4881
** No. of killed/infected mice

Table VI

Effect of iron-compounds administered simultaneously with infection and after standing in vitro for 3 h, on the deaths of infected mice
(Challenging: *E. coli* i.p. LD_{50})

Administered material (i.p.)	Administration	
	simultaneously with infection	after standing in vitro for 3 h
Physiological NaCl	5/10*	12/20
Ferric chloride (100 μM)	5/10	12/20
Sodium citrate (200 mM)	5/10	8/20
Ferric ammonium citrate (100 μM)	9/10	15/20
LF (5 mg) + physiological NaCl	8/10	16/30
LF + ferric chloride	8/10	12/20
LF + sodium citrate	10/10	9/20
LF + ferric ammonium citrate	9/10	11/20

LF = lactoferrin
* No. of killed/infected mice

observed and when LF was given after infection, the killing effect was higher, than in the control group.

In further experiments 1 mg LF and physiological saline were given to two groups of mice intramuscularly, 6 h before infection. The aspecific protective effect of LF administered in this way could be excluded. There was no significant difference between the lethality of the group pretreated with physiological saline and that of the control group ($p > 0.2$), while the protective effect of LF compared with the group treated with physiological saline was significant ($p < 0.001$).

Protective effect was not demonstrated in per os experiments.

In further experiments LF was injected intramuscularly, 6 h before infection.

Table VII

Effect of LF and apo LF produced at different times on the deaths of infected mice
(Administration of LF: i.m.; challenging: i.p. with various *E. coli* and *P. aeruginosa* strains)

Designation of LF	Administered (mg) occasion	*E. coli*	*P. aeruginosa*
Control	—	71/100*	45/50
6	1×0.1	9/20	•
	1×1.0	3/50	•
	1×10.0	7/10	•
7	1×0.01	8/10	10/10
	1×0.1	11/20	•
	1×1.0	16/30	34/40
	1×10.0	3/20	10/10
ApoLF	1×1.0	2/10	10/10
Control	—	42/80	27/50
7	1×10.0	5/54	4/12
	2×10.0	0/19	0/2
8	1×1.0	•	4/26
	2×1.0	•	4/27
	1×10.0	7/30	7/16
	2×10.0	•	1/16
	1×100.0	2/20	•
9	1×1.0	•	2/14
	2×1.0	•	1/14
	1×10.0	12/30	8/10
	1×100.0	2/20	•
ApoLF	1×1.0	2/25	2/16
	2×1.0	0/20	3/16

LF lactoferrin
* No. of killed/infected mice
• not examined

Examination of the in vivo effect of iron. The effect of different iron containing (ferric chloride, sodium citrate, ferric ammonium citrate) and chelating compounds was examined by injecting them intraperitoneally immediately after having been mixed with the bacteria and after leaving to stand the mixture for 3 h (Table VI). The virulence in mice was not increased either by ferric chloride or by sodium citrate but ferric ammonium citrate or LF injected immediately after being mixed with the bacteria increased the mouse killing effect. When the mixtures were left to stand at room-temperature for 3 h, the virulence decreased in the case of the two latter substances compared with the control administered without standing.

Comparison of the effect of different batches of LF-s. It was advisable to compare different batches of LF-s, because each of them could be produced in small quantities. Consistent, unambigous results were not obtained when the experiments were repeated with different LF-s, high number of strains, different injection schedules and doses (Tables VII and VIII); in the case of *E. coli* strains 1 mg dose of LF batch "6" and 10 mg of LF batch "7" proved to be better after intramuscular injection, while in case of *P. aeruginosa* 1 mg LF batch "7" gave some weak protection and in 10 mg quantity it was ineffective. Mice infected with *E. coli* were protected the best by 100 mg LF batch "8", but when they were infected with *P. aeruginosa* the protection was better using doses 1×1 mg and 2×1 mg, than 1×10 mg. There was no

Table VIII

Effect on LD_{50} of different amounts of different batches of LF and apoLF

Batch of LF	Quantity (mg)	LD_{50}
Control	—	3.1×10^6
10	10	9.5×10^6
	20	9.7×10^6
	40	1.1×10^7
11	10	6.5×10^7
	20	6.5×10^6
12	10	2.0×10^7
	20	1.0×10^7
13	10	1.0×10^7
	20	2.0×10^7
14	10	8.0×10^6
	20	9.0×10^6
apoLF	10	2.0×10^7
	20	1.0×10^7

LF = lactoferrin

significant difference in the protective effect of apoLF and LF. It would appear that the contradictory results are due to the biologic system and not to the different quality of LF batches.

Because of the errors of the biologic method it was not possible to determine the exact LD_{50} values in each experiment, the effect of LF on the infection with lower and higher cell counts was examined, e.g. its effect on the LD_{50} of bacteria (Table VIII). It was found that the LD_{50}-s were not changed significantly by either sort of LF-s (they varied between 7×10^6 and 6×10^7) and the changes were not due to the circumstances of the production or to the dose of LF. The results obtained with apoLF and with LF did not differ from each other.

Discussion

The in vitro antibacterial effect of LF is apparent from literary data [35–37]. These were confirmed by our present examinations, since the growth of *E. coli* and *S. typhi-murium* strains carrying different virulence markers and producing enterochelin were inhibited by LF used in high concentration (40 mg/ml). The growth of the strains was inhibited by iron-saturated LF in a small extent and by apoLF in a greater extent than by LF, possibly by inhibiting the iron uptake of the strains.

The growth of the strains producing no enterochelin was inhibited both by LF and apoLF in complete medium.

On the basis of these experiments it was established that the bacteriostatic effect of LF can only be determined by test strains with well-defined enterochelin and aerobactin production.

The Vogel-Bonner medium seemed to be suitable to determine the growth inhibiting effect of LF. The effect of LF can be tested in iron-free medium and the decrease of enterochelin effect by LF can be determined only in this medium.

Martin et al. [38] found a slight protective effect when human TF was injected intraperitoneally immediately before infection to rats (LD_{50} values increased by 0.5–1 exponent). A protective effect was also found when young mice were treated with TF before intraperitoneal infection.

These results influenced our experiments, in the beginning we used LF in intraperitoneal injection. This mode of introduction was described in several papers [38–40] as a generally accepted method.

In the course of our examinations we found that the "protective" effect of the intraperitoneal injected LF was not due to the specific action of LF. This was demonstrated by observations that PBS, Ringer solution and physiological saline had some "protective" effect if given intraperitoneally 3–6 h

before infection. Accordingly, the observation of Martin et al. [39] concerning the effect of intraperitoneally given TF, seems to be due to this aspecific effect.

The aspecificity of the protective effect was supported by the results of the LF clearence examinations: LF was not detectable in considerable amount in the organs 24 h after injection. Our results were confirmed by Bennet and Kokicinski [41] and Karle et al. [42] who administered LF intravenously. The fact that there was no dose-response refers also to the aspecificity of the protective effect.

The aspecific "protective" effect may be due to a macrophage activation by the intraperitoneally administered LF, PBS, Ringer solution and other substances, so that an aspecific resistance develops in a few hours, before the challenging organisms are injected.

To eliminate the aspecific effect observed in our first experiments we tried to find another mode of introduction. The oral administration had no protective effect in our examinations. A similar observation was published by Moreau et al. [43], who gave feed supplemented with LF to mice, but failed to demonstrate any effect of LF.

When injection LF intramuscularly, a specific, though not total protective effect was demonstrated, but we failed to show a dose-response. Similar results were published by Martin et al. [38], who described a weak protection after intramuscular injection of TF in suckling mice. In comparative examinations the weak protective effect of the standard LF was the same as obtained with the LF (NIHBT).

To examine the cause of the lack of in vivo bacteriostatic effect of LF, parallel experiments were carried out using ferric chloride, sodium citrate, ferric ammonium citrate, LF and apoLF. The iron-compound accessible to bacteria (ferric ammonium citrate) enhanced the mouse-killing effect of bacteria and the multiplying ability on the peritoneum, in accordance with literary data [2, 22, 39, 44–47]. The effect of sodium citrate was detectable only when iron was added to the system, so that the in vivo induction of citrate dependent system described by Frost and Rosenberg [48] and Reiter [33], was not reproducible in our experiments.

On the basis of our results the LF in a well-chosen in vivo system (administered in a high quantity simultaneously with the infection) acts like iron was given to the experimental animal: it increases the death-rate of mice. The in vitro bacteriostatic effect of LF does not exist in vivo. It is caused by the iron uptake of LF, till it is saturated, in the living organism. According to the examinations of Arnold et al. [49] iron-saturated LF serves as a source of iron in iron-free medium.

Decrease of the virulence of bacteria by LF could not be demonstrated in LD_{50} assay using low cell count (Kochan et al. [50]).

On the basis of the above findings, the enhanced virulence of bacteria caused by LF may be explained so that LF fails to deprive its environment of iron (stability $K = 10^{36}$ [2]), but one of the siderophores produced by the bacteria can deprive LF of iron (enterochelin $K = 10^{52}$ [3], aerobactin $K = 10^{23}$ [15]).

Thus LF, by supporting bacteria in the host with iron, enhances their virulence, similarly to iron. This is the reason why the mouse killing effect of iron was not prevented by LF, subsequently given to iron pretreated animals. Our results were reproducible, the virulence increased by the effect of LF in repeated experiments, using different strains. It has to be mentioned that enterochelin was produced by all *E. coli* strains tested in vivo and the majority of them (except two strains) produced aerobactin. Our suggestion is confirmed by the results of Moreau et al. [43], who also failed to demonstrate bacteriostatic effect of LF in vivo.

The effect of apoLF was not different from that of LF. This can be explained by that, apoLF in biologic system (living organism) can get enough iron to bind it. This is supported by reports [49, 51].

Several authors have referred to the fact that TF and LF can be deprived of iron [2, 7, 22, 40, 46, 48, 52–56]. In the case of a variety of microorganisms *(N. meningitidis, N. gonorrhoeae, Trichomonas vaginalis, S. typhimurium, S. paratyphi-B, E. coli)* this effect was demonstrated in vitro and in vivo.

Our results obtained with several bacterial strains and with more than 5000 mice demonstrated, that the idea, met with often in the last 50 years, that LF can be used as an antibacterial drug, was a mistake. LF cannot be used in the therapy of bacterial infections, moreover, its administration may lead to an enhanced virulence of bacteria. We agree with the opinion of Williams and Carbonetti [57], that bacteria may be supported with iron by aerobactin in the presence of LF. This virulence increasing effect of LF is not altered by apoLF, because iron is present in sufficient quantity in the living organism.

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DETECTION OF HIV IN THE PERIPHERAL MONONUCLEAR CELLS OF ASYMPTOMATIC HAEMOPHILIACS IN HUNGARY

B. Szabó, F. D. Tóth, J. Laczkó, Jolán Kiss, Eszter Újhelyi, G. Füst and Zsuzsa R. Hollán

Institute of Microbiology, and Central Research Laboratory, University Medical School, Debrecen, and National Institute of Haematology and Blood Transfusion, Budapest

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The presence of virus in peripheral blood mononuclear cells of asymptomatic antibody positive haemophiliacs was detected by assaying for reverse transcriptase and confirmed by electron microscopy and immunofluorescence. HIV has been detected in 5 out of 7 individuals. In order to investigate strain variation, supernatant fluids of cultures were added to H9 and MT-4 cells. Virus was recovered in MT-4 cells in 3 cases, whereas the H9 cells only supported the replication of 2 strains. Viruses isolated from asymptomatic haemophiliacs have a narrower range of infectivity than HTLV-III$_B$.

Since the isolation of the aetiological virus of AIDS by Barré-Sinoussi et al. [1] and by Popovic et al. [2], evidence is accumulating of variation among HIV isolates [3, 4]. Variations were found not only in restriction patterns or nucleotide sequences, but also in biological properties of the viruses, like replication rate and cytopathic effect on lymphocytes [5]. In view of this striking heterogeneity of HIV it may be assumed that the wide spectrum of incubation times and clinical manifestations of HIV infections may be related to the particular virus strain infecting a patient and/or arising within a patient during persistent infection. Hence, it would seem important to examine HIV isolates from different countries to evaluate the extent of strain variation. Here we report HIV-1 isolations from virus-exposed asymptomatic persons in Hungary. Furthermore, we investigate the difference in the susceptibility of H9 and MT-4 cells to the isolates.

Béla Szabó, Ferenc D. Tóth, Jolán Kiss

Institute of Microbiology, University Medical School
4012 Debrecen, P.O.B. 17, Hungary

Jenő Laczkó

Central Research Laboratory, University Medical School
4012 Debrecen, P.O.B. 3, Hungary

Eszter Újhelyi, György Füst, Zsuzsa R. Hollán

National Institute for Haematology and Blood Transfusion
1502 Budapest, P.O.B. 44, Hungary

Materials and methods

Cell cultures. The established human cell line H9 and the H9 cells infected with the HTLV-III$_B$ strain of HIV-1 were kindly supplied by R. C. Gallo (National Cancer Institute, Bethesda, MD, USA) and maintained in RPMI-1640 medium containing 20% fetal calf serum (Gibco, Paisley, Scotland) and T-cell growth factor (Sigma, St. Louis, MO, USA). The MT-4 cell line, carrying human T-lymphotropic virus type I (HTLV-I) was kindly provided by G. Szücs (Public Healths Station, Pécs, Hungary). The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum.

Recovery of virus from peripheral blood leukocytes. Virus isolation was attempted with single blood specimens collected from 7 antibody positive haemophiliacs, who were apparently healthy. Virus isolation was based on the technique described by Feorino et al. [6]. Separation of the peripheral blood mononuclear cells (PMBC) was performed as previously described [7]. In brief, aliquots of heparinized venous blood were underlayed with Ficoll-Uromiro (Pharmacia, Uppsala, Sweden) and centrifuged at 700 *g* for 20 min. PBMC present at the interface with the Ficoll-Uromiro were recovered, washed twice and resuspended in tissue culture flasks (Greiner No. 690 160, Nürtingen, FRG), at approximately 2×10^6 cells/ml in 5 ml RPMI-1640 medium with phytohaemagglutinin (10 μg/ml; Sigma, St. Louis, MO, USA), 5% T-cell growth factor, and 500 IU/ml antiserum to human alpha-interferon (Boehringer, Mannheim, FRG). After 3 days, the culture was continued in the same medium without phytohaemagglutinin. PMBC cultures were monitored for virus replication with reverse transcriptase assay. When evidence of infection was noted by this method, cells were examined by electron microscopy and immunofluorescence.

Reverse transcriptase (RT) assay. RT activity in culture supernatant fluids was assayed by the method described by Popovic et al. [2]. Briefly, virus was precipitated from 1 ml of cell-free culture supernatant, using 30% PEG 6000 (Fluka, Buchs, Switzerland) in 0.5 M NaCl, and pelleted at 10 000 rpm for 10 min. Pelleted virus was disrupted by incubating on ice for 30 min in 100 μl of solubilization buffer (50 mM Tris pH 7.8, 20% glycerol, 0.5 mM PMSF, 800 mM NaCl, 0.5 Triton X-100). 20 μl samples were assayed for RT activity using poly (rA) dT_{12-18} as a template in 50 mM Tris pH 7.8, 10 mM $MgCl_2$, 5 mM dithiothreitol, 80 μg/ml dATP, and 50 μCi/ml ^{3}H-thymidine triphosphate (specific activity 100 mCi/ml; Amersham, Buckinghamshire, England). Samples were incubated at 37 °C for 2 h. The reaction was stopped and radiolabelled material precipitated by the addition of 50 μl trichloracetic acid and incubation at 4 °C for 30 min. Each sample was then filtered on a 0.45 μm Millipore filter (Millipore, Bedford, MA, USA) and processed for beta-emission counting. Supernatants with RT activity 5 times higher than the background were considered positive. All chemical compounds, except ^{3}H-thymidine triphosphate and Millipore filter, originated from Sigma Chemical Company (St. Louis, MO, USA).

Immunofluorescence assay (IFA). Immunofluorescence was used to demonstrate viral antigen in infected cells. Briefly, washed cell suspension (10^5 cells) was spotted onto slides and air-dried at room temperature. The cells were fixed for 10 min in cold acetone, air-dried, and used immediately. For the assay, anti-HIV-1 reference serum was added to the cell spots, and the slides were then incubated in a humidified atmosphere at 37 °C for 30 min. The slides were washed three times in PBS and rinsed in distilled water. FITC-conjugated sheep anti-human IgG serum (Hyland, Costa Mesa, VA, USA) was diluted 1 in 40 in PBS and added to each spot. The slides were reincubated for 30 min and washed again. Appropriate cell and serum controls were included in each assay. The slides were examined with the fluorescence microscope.

Electron microscopy. For transmission electron microscopy, cells were embedded in Durscupan ACM (Fluka, Buchs, Switzerland) resin after conventional glutaraldehyde-osmium-tetroxide fixation. Ultrathin sections were cut on an ultramicrotome with glass or diamond (Balzers/WR, Liechtenstein) knives, then stained with lead citrate and uranyl acetate. Preparations were viewed and photographed with a JEOL JEM 100B transmission electron microscope at 80 kV accelerating voltage.

Infection of H9 and MT-4 cells with retroviral isolates. For the cell-free transmission assay, supernatant fluids were removed from the patient's primary lymphocyte cultures and added to cultures of H9 and MT-4 cells. Target cells were used as pretreated or not with 2 μg/ml Polybrene (Sigma, St. Louis, MO, USA). Targets were pretreated with Polybrene for one to three days prior to infection. Approximately 2×10^6 cells were pelleted and resuspended in 1 ml supernatant. After adsorption for 1 h at 37 °C, the cells were diluted to 10 ml with culture medium and incubated at 37 °C in flasks. Cells were diluted to $1–2 \times 10^5$/ml every three to four days and assayed for presence of retrovirus by cytopathic effect [8, 9] and IFA

on acetone fixed cells. Aliquots of H9 and MT-4 cells were mock-inoculated with complete medium or the HIV reference strain, HTLV-III_B and cultured in parallel as negative or positive controls.

Results

1. *Recovery of virus from PBMC samples.* The results of virus isolation experiments are summarized in Table I. Virus was primarily detected by the RT activity. Significant RT activity was produced by five PBMC cultures from patients 1, 4, 5, 6, and 7 during a period of 14 to 18 days of cultivation.

Intracellular rounded viral particles measuring between 90 and 130 nm in diameter were seen in ultrathin sections of these five PBMC cultures around the periphery of the lymphocytes (Fig. 1). Accumulation of virus was sometimes found within cytoplasmic vacuoles (Fig. 2), where the inner viral structures were seen as different shapes, depending upon the plane of section viewed and stage of virus maturation.

Studies by IFA on PBMC samples from patients 1, 4, 5, 6, and 7 gave positive results, whereas samples from patients 2 and 3 as well as control PBMC-s proved to be negative (Table I). The comparison of our isolates with reference strains from the USA and France is in progress.

2. *Characterization of infectivity of the isolated viruses on H9 and MT-4 cell lines.* We compared the replication ability of HIV strains recovered from PBMC cultures on H9 and MT-4 cells. Supernatant fluids from cultures showing RT activity were inoculated into these two cell lines. Supernatant of HTLV-III_B-infected H9 cells was used as positive control, and medium of

Table I

Detection of HIV in PBMC samples of antibody-positive haemophiliacs

Sample	RT activity in supernatant (cmp $\times 10^3$)			EM*	IFA
	Day 11	Day 14	Day 18		
1.	4.1	11.6	61.4	+	+
2.	2.1	0.7	2.4	N.D.	—
3.	2.3	1.7	2.2	N.D.	—
4.	5.7	10.9	91.7	+	+
5.	7.3	18.8	140.0	+	+
6.	6.1	11.6	145.4	+	+
7.	11.0	28.2	182.6	+	+
Negative control**	1.7	1.4	2.7	N.D.	—

* Electron microscopy

** The sample used was supernatant fluid from the PBMC culture of a seronegative, healthy, low risk donor

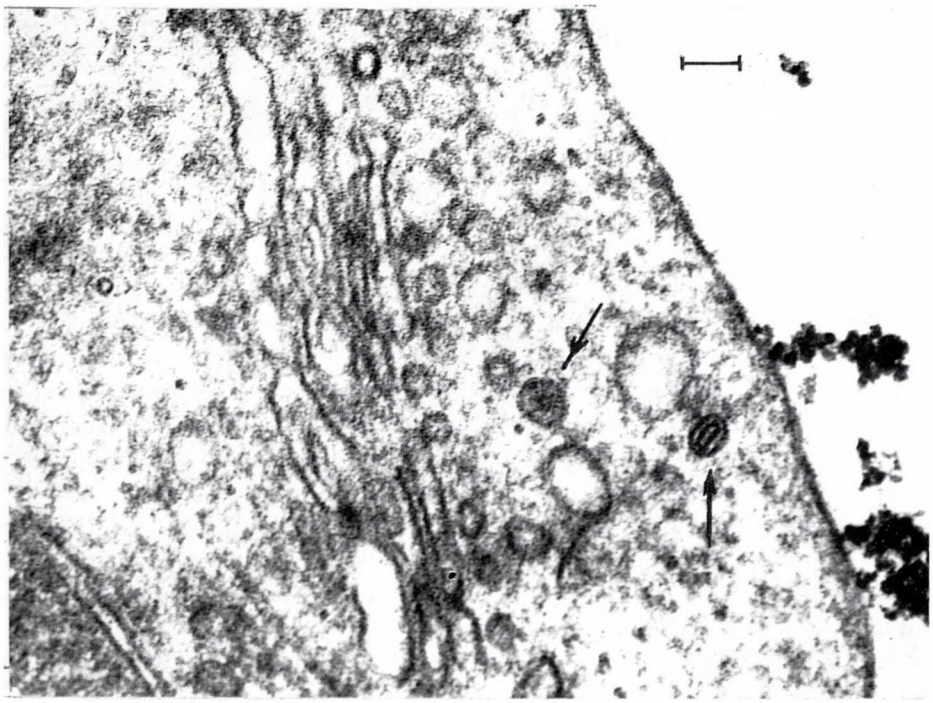

Fig. 1. Premature viral particles with typical cylindrical nucleoid around the periphery of the lymphocyte (arrows). Bar indicates 100 nm

uninfected H9 cells served as negative control. Evidence of infection was obtained by cytopathogenic effect and IFA.

HIV-infected H9 cultures showed the appearance of foci consisting of multinucleated giant cells, followed by cell damage. Clusters of mock-infected MT-4 cells, when dissociated by pipetting, reclustered after 3 h of incubation at 37 °C (Fig. 3). On the contrary, HIV-infected cells did not recluster. Many infected cells developed balloon like and then most of them diminished in size (Fig. 4). Finally, many dead single cells remained in the culture (Fig. 5).

The HTLV-III_B strain of HIV-1 could infect both H9 and MT-4 cells with high efficiency, not depending on the Polybrene pretreatment (Table II). Viral isolates from PBMC cultures of patients 4 and 6 were able to infect H9 cells and replicate in them only after pretreatment of the appropriate cells with Polybrene. HIV replication was demonstrated by cytopathogenecity and IFA in MT-4 cells, inoculated with supernatants from PBMC cultures Nos 4, 6 and 7. More interestingly, this phenomenon was not dependent on Polybrene pretreatment.

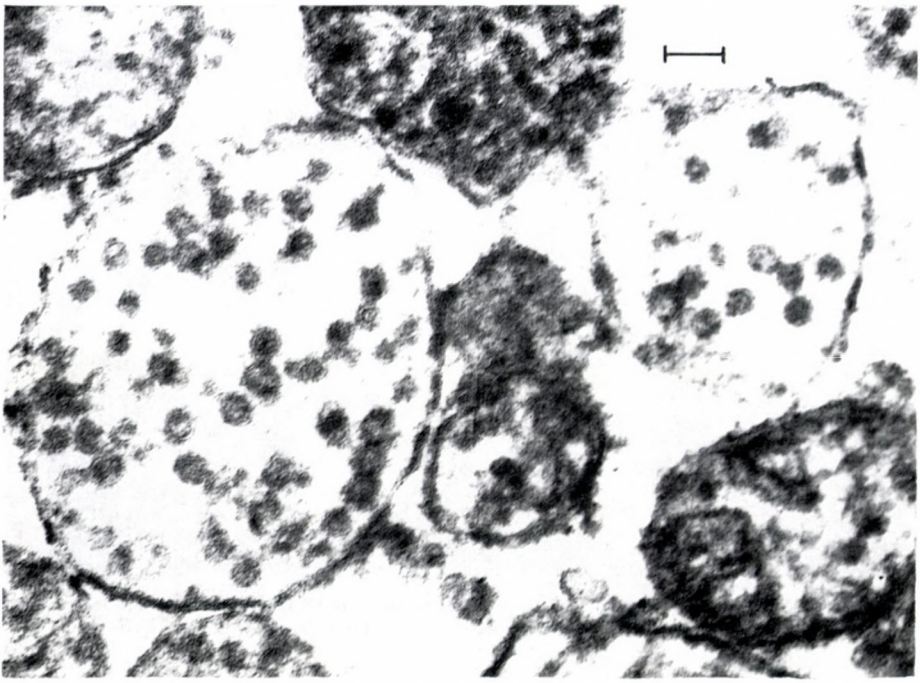

Fig. 2. Cytoplasmic vacuoles which contain a number of viral particles. Intermediates forms as "empty" (no-nucleoid) ring-shaped particles are also seen. Bar indicates 100 nm

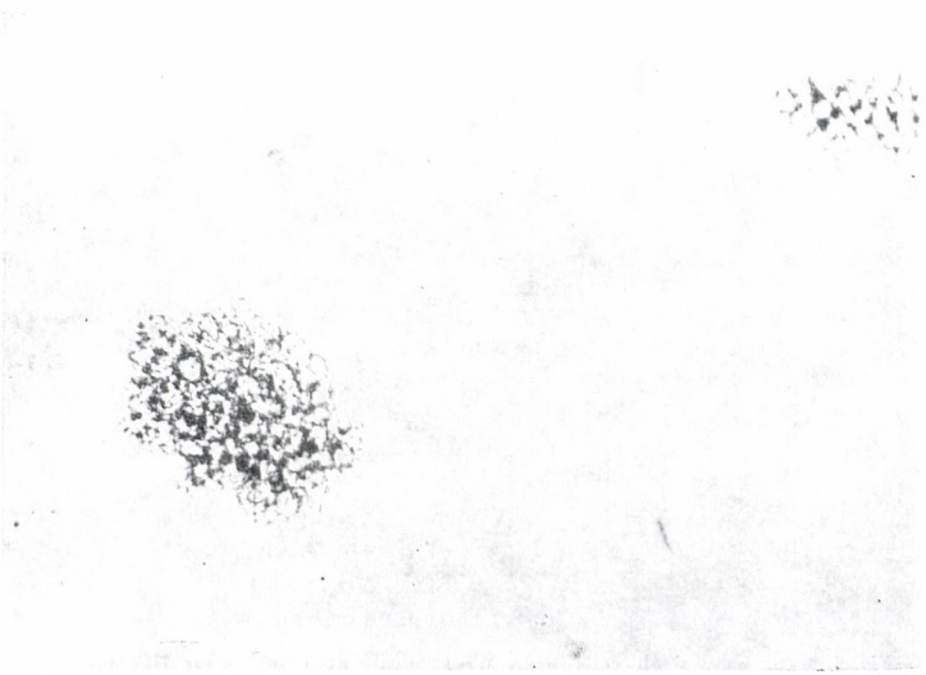

Fig. 3. Mock-infected MT-4 cells incubated for 2 h at 37 °C reform clusters after dissociating them by pipetting

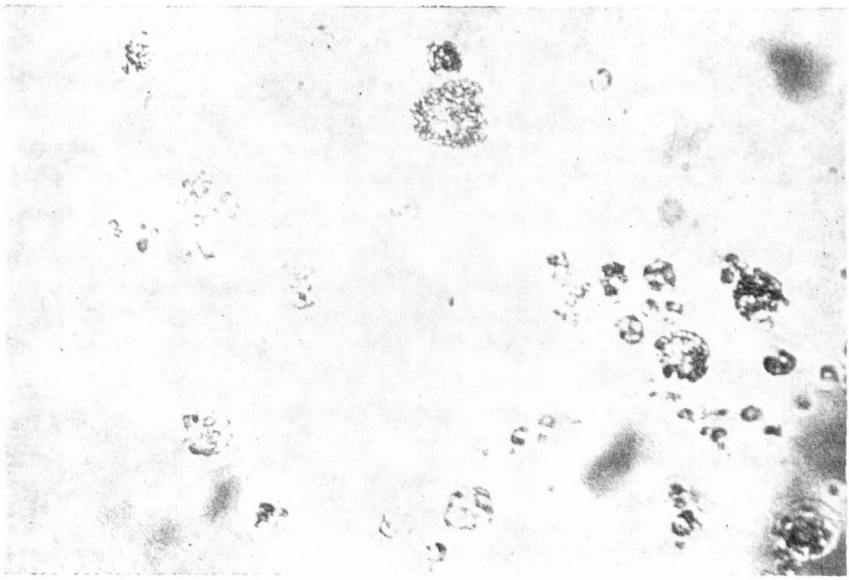

Fig. 4. Phase-contrast microscopic pictures of MT-4 cells after HIV infection at 3 days post-infection. Some giant cells can be observed among the remaining live cells

Fig. 5. Many dead single cells in an MT-4 culture at 5 days after HIV infection

Table II

Host cell range of retroviruses isolated from asymptomatic haemophiliacs

Sample	Replication of virus on			
	H9		MT-4	
	without	after	without	after
	Polybrene treatment			
1.	—	—	—	—
4.	—	+	+	+
5.	—	—	—	—
6.	—	+	+	+
7.	—	—	+	+
HTLV-III_B	+	+	+	+
Negative control	—	—	—	—

Discussion

HIV-1 has been detected in PBMC samples of 5 asymptomatic antibody positive individuals. When specimens from 2 other antibody positive haemophiliacs were tested, virus was not recovered. Whether this represents infection with levels of circulating virus undetectable by our culture technique, predominant infection of organs other than peripheral blood or immunization by repeated exposure to viral antigens, remains to be determined.

We compared the sensitivity of H9 cells to MT-4, by inoculating them in parallel with HIV isolates. Virus was recovered in MT-4 cells in 3 cases, whereas the H9 cells only supported the replication of 2 strains. The failure of recovery from HIV-positive PBMC samples in H9 cells in 3 cases, and in 2 cases in MT-4 cells cannot be explained by the small amount of virus inoculum. This assumption is supported by the fact that HIV was recovered from sample No. 4 with RT activity of 91.7×10^3 cpm, but not from sample No. 5 with RT activity of 140.0×10^3 cmp (Tables I and II). Moreover, both H9 and MT-4 cells were susceptible to HIV isolate No. 4, while the isolate No. 7 with higher RT activity replicated only in MT-4 cells. Thus, the differences in replication capability of various isolates reflect the different susceptibility of target cells. Isolates from PBMC cultures could infect H9 cells only after Polybrene treatment of the targets. On the other hand, successful infection of MT-4 cells with any of the isolates did not depend on the Polybrene pretreatment. Our results confirm the observation that MT-4 cells carrying the HTLV-I genome are highly susceptible to HIV infection [10]. The HTLV-III_B strain of HIV, isolated in H9 cells [2], was able to infect both kinds of target cells without any pretreatment.

The results presented here indicate that there exist biological differences among AIDS retroviruses. Viruses isolated from asymptomatic haemophiliacs have a narrower range of infectivity than HTLV-III_B. This may be related to the fact that all of them were derived from so-called "healthy virus carriers". At the moment, the basis of virulence of HIV is poorly understood. For clarification of the virulence of the virus, it is important to analyze the relationship between the characters of the virus isolated and the stage of the disease. Such experiments are going on in our laboratory.

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CHEMOTAXIS IN *LISTERIA MONOCYTOGENES**

S. B. GALSWORTHY, S. GIRDLER and S. F. KOVAL

Department of Microbiology and Immunology,
University of Western Ontario, London, Ontario, Canada

(Received September 16, 1988)

Listeria monocytogenes is a flagellated bacterium with a characteristic tumbling motion. It is intriguing to speculate that directional motility might facilitate penetration of the intestinal epithelium or selective colonization of the central nervous system or gravid uterus. There are conflicting reports on the extent of flagellation and degree of motility at temperatures corresponding to the internal environment of the mammalian host. No studies of chemotaxis in *Listeria* have been reported. We examined *Listeria* for flagella, motility, and chemotaxis after growth at 10°, 24°, 30°, 37° and 40 °C. *Listeria* grown at all temperatures possessed flagella and were motile to at least some degree. Those grown at 24° or 30 °C were the most abundantly flagellated and the most vigorously motile. The bacteria were able to swim towards tryptose at all temperatures and toward glucose at all temperatures except 40 °C.

Listeria monocytogenes resides in soils, water, and on plants world-wide. Listeriosis among many animals is common. The usual human victim is immuno-compromised: pregnant, newborn, alcoholic, or a transplant recipient. Severity of infection ranges from a mild influenza-like illness to septicaemia, meningitis, and granulomatosis infantiseptica. Most human infections are sporadic and of unknown etiology. Food has been implicated in several outbreaks. In order to induce severe illness the ingested bacteria must invade the epithelium of the gut and enter the blood or lymphatic system.

L. monocytogenes is peritrichously flagellated and exhibits a peculiar tumbling motility interspersed with spurts of rapid motion in one direction for 10–20 cell lengths. However, the degree of flagellation and extent of motility of this organism at 37 °C have been disputed [1–3]. It is tempting to speculate that directional motility could facilitate penetration of the intestinal epithelium or the selective colonization of the central nervous system or the gravid uterus.

These studies were designed to compare motility and flagellation at several temperatures and to determine whether *L. monocytogenes* would move toward glucose or tryptose.

S. B. GALSWORTHY, S. GIRDLER, S. F. KOVAL

Department of Microbiology and Immunology, University of Western Ontario
London, Ontario, Canada N6A 5C1

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

Materials and methods

Media and chemicals. Medium contained (per litre distilled water) 23 g tryptose, 5 g NaCl, and 3.2 g Na_2HPO_4. Complete medium used for propagation of bacteria contained, in addition, glucose and thiamine, separately sterilized, at final concentrations of 2 g/l and 5 mg/l, respectively. The latter were omitted from samples of tryptose used as chemo-attractant. Agar and soft agar contained, in addition to complete medium, agar at concentrations of 1.5% and 0.75%, respectively. Wash fluid contained potassium phosphate buffer (10^{-2} M, pH 7.0) and potassium EDTA (10^{-4} M). Glucose solutions used in the chemotaxis assays were sterilized by filtration.

Growth of bacteria. L. monocytogenes, strain 42 (serotype 1/2a), was obtained from the culture collection of the late E. G. D. Murray and propagated on blood agar plates. For motility studies, a colony from the plate was transferred to 10 ml of complete medium described above and incubated overnight at 24 °C. Aliquots of this culture (1 ml) were used to inoculate 50 ml of the same medium in 250 ml Klett flasks. These cultures were shaken at the indicated temperature at 100 cycles per minute until the mid-logarithmic stage of growth was attained. Cells at that stage were examined for presence of flagella, motility, and ability to undergo chemotaxis as described below.

Electron microscopy. Samples (1 ml) were centrifuged and the cells resuspended in 0.2 ml of medium. A carbon-Formvar-coated copper grid was inverted over a drop of the sample and allowed to float for 30 s. The grid was then washed on 2 drops of water and negatively stained with 0.5% uranyl acetate (pH 4.4) containing bacitracin. Specimens were examined with a Philips EM 300 electron microscope operated at 60 KV.

Motility. A drop of the culture was placed on a glass slide, covered with a coverslip and examined under ×400 magnification with a light microscope.

Chemotaxis. Cultures were washed twice by centrifugation (6500 *g*, 10 min) and gently resuspended in buffer to make a final concentration of 7.5×10^6 bacteria/ml. The suspension was examined under the microscope before each assay to assess motility. Aliquots (2 ml) of the suspension were placed in 12 × 75 mm sterile plastic tubes. Sterile capillary tubes were filled to the 20 µl mark with attractant, sealed in a flame and the open end inserted through the aseptically punctured cap into the tube containing the bacterial suspension.

Tubes were allowed to incubate at the desired temperature in a water bath. At the appropriate time, the capillary was removed and rinsed 3 times with sterile distilled water. The top was snipped off with forceps and the contents of the capillary blown into 10 ml chilled tryptose. The interior of the capillary was rinsed 3 times with medium. Tubes were held at 4 °C until plating. Samples (1 ml) of this broth or of a suitable dilution of it were added to soft agar, and poured onto an agar plate.

Cultures were incubated for 48 h at 37 °C and colonies were counted. Assay points were duplicates for all experiments reported here. Averages are reported for all points shown graphically (maximum range for average 20%).

Results

Cells grown at all temperatures possessed flagella (Fig. 1), but the degree of flagellation varied with the temperature. The highest number of flagella per cell was seen on cells grown at 24 °C. Cells grown at 10° and 40 °C had very few flagella. In all cultures there were some cells with no flagella. The extent and quality of motility also varied with temperature (Table I). The samples which showed the most vigorous tumbling and running had been grown at 24° or 30 °C.

Bacteria accumulated in capillaries in response to tryptose medium without glucose and to pure glucose (Table II) at 24°, 30°, and 37 °C. Accumulation towards glucose was most rapid at 30 °C. Tryptose medium more efficiently attracted bacteria than did any concentration of glucose at all

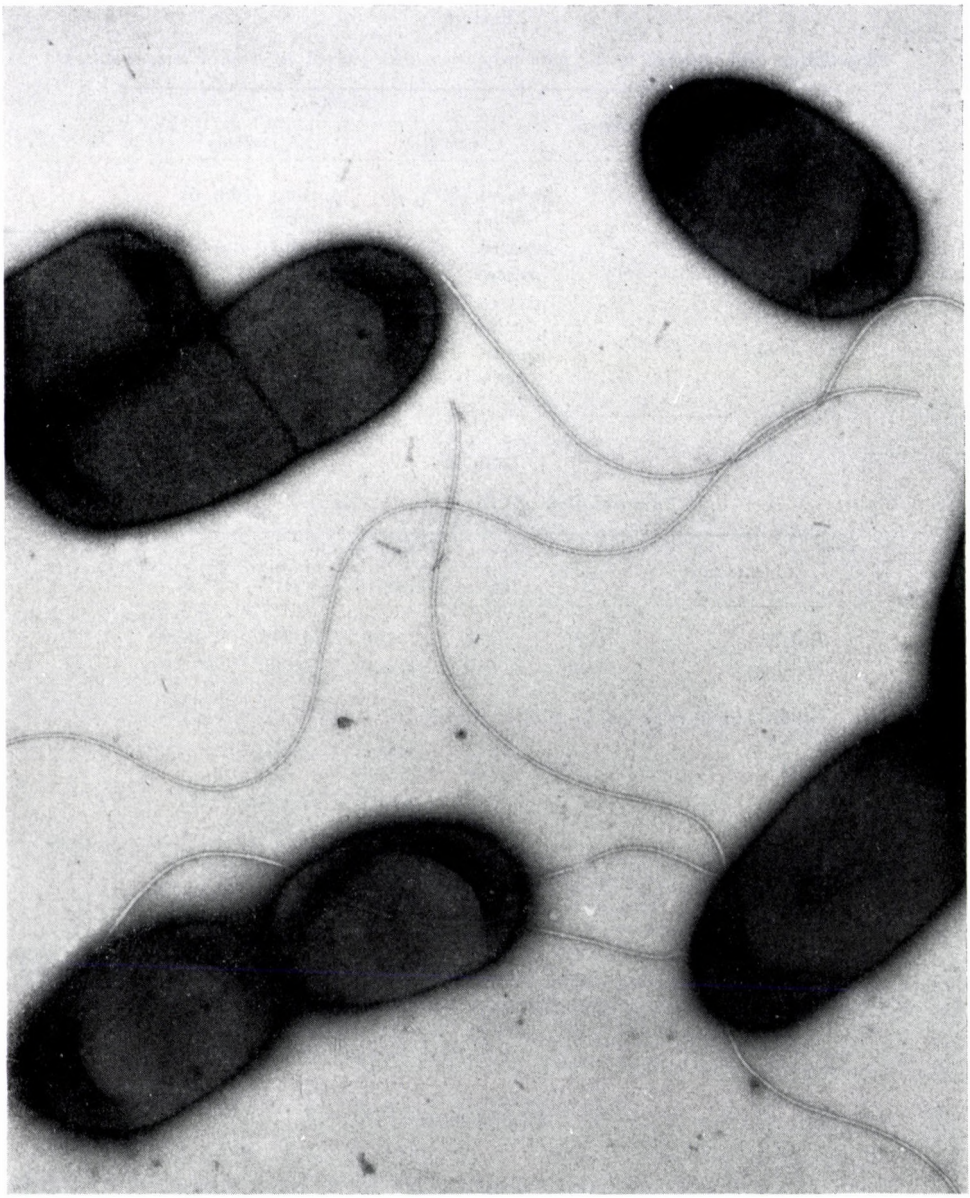

Fig. 1. Electron micrograph of *L. monocytogenes* grown at 24 °C

temperatures tested. A preliminary evaluation of chemotaxis was done at the extreme temperatures 4° and 40 °C. In this case, bacteria were grown at 24 °C to ensure that they possessed flagella. They were allowed to equilibrate at the extreme temperature for 15 min and exposed to the chemoattractant for 90 min.

Table I

Flagellation and motility in L. monocytogenes after growth at several temperatures

Temperature, °C	Flagellation	Motility	
		tumbling	running
10	+	present (20% of cells)	present (10% of cells)
24	+	present	present
30	ND	present	present
37	+	present (20% of cells)	present (10% of cells)
40	+	present (10% of cells)	virtually absent

Table II

*Accumulation of Listeria in Capillaries**

Attractant	Temperature, °C				
	4	24	30	37	40
Buffer	1100	1 130	100	1 375	850
Medium	7100	15 950	56 000	73 500	30 240
Glucose (molarity)					
10^{-6}	•	1 620	ND	516	•
10^{-5}	•	660	7 500	650	•
10^{-4}	•	880	14 000	1 200	•
10^{-3}	•	2 690	20 500	9 000	•
10^{-2}	•	11 240	15 050	6 510	•
10^{-1}	7280	8 840	10 000	15 300	1 200

* Bacteria were incubated with capillaries containing 20 μl of each attractant. After 90 min at the specified temperature the number of bacteria which had migrated into the capillary was determined. Results represent the average of 2 determinations

Discussion

Both glucose and elevated temperature have been reported to inhibit synthesis of flagella in *Escherichia coli* [4] and in *Listeria* [1]. Therefore, we determined whether our standard conditions of cultivation permitted motility in *Listeria*.

Since flagella were present and the bacteria were motile after growth in complete medium we evaluated chemotaxis at 24° and 30 °C, where the bacteria showed greatest motility and at 37 °C which would show whether chemotaxis could occur at mammalian body temperature.

Although the absolute number of bacteria accumulating in the capillaries is considerably smaller than the number reported by Templeton and Adler [4], the 48-fold increase (Table II) compares well with the optimum accumulation reported by these workers. The bacteria accumulated more rapidly in tubes containing tryptose (sans glucose) than they did in ones containing any concentration of glucose. This was true at all temperatures where a comparison was done. Since this medium is a complex one, the role of individual amino acids needs to be evaluated in future experiments. The response toward tryptose increased markedly with temperature (up to 37 °C). The response toward glucose inproved only marginally with temperature, was poor at 37 °C and absent at 40 °C. The optimum concentration was 10^{-2} M at 24°, 10^{-3} M at 30° and 10^{-1} M at 37 °C. The relevance of a chemotaxis toward the latter unphysiologically high concentration of glucose is unclear.

The existence of directional motility in *Listeria* at 37 °C encourages us to examine its response to concentration gradients of other defined substances and ultimately to substances which may be present in tissues such as the intestinal mucosa, fetal membranes, and the meninges.

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REVISION OF THE O ANTIGENIC SCHEME OF *LISTERIA**

J. A. García Cabrera, L. Dominguez Rodriguez, V. Briones Dieste, J. F. Fernandez Garayzabal, J. A. Vazquez-Boland and G. Suarez Fernandez

Department of Animal Pathology I, Department of Microbiology and Immunology, Veterinary Faculty, Compultense University, Madrid, Spain

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Factor sera against *Listeria* O antigens (serologic reference strains) were produced. The results confirm the validity of the O antigenic scheme established for *Listeria*, but show slight discrepancies in strains of serovars 4b, 4ab and 6b.

The first valid serologic classification of *Listeria* is due to Paterson [1]. By serological analysis of somatic and flagellar antigens following the method of White and Kauffmann for *Salmonella* typing, this author recognized four distinct serovars (1 to 4) in *Listeria monocytogenes*.

On the basis of the Paterson work, serological analyses performed by different authors (Seeliger, Donker-Voet, Ivanov, Stuart and Welshimer) with *Listeria* strains isolated for over 30 years demonstrating new antigenic factors have extended the original antigenic scheme [2]. Further research led to successive modifications [3–5].

As a first step of a research work we have produced factor sera against *Listeria* O antigens. The results of the serological analysis are reported in this paper.

Materials and methods

Bacterial strains. The following serological reference strains were used. *L. monocytogenes* NCTC 7973 serovar (sv) 1/2a, NCTC 5105 sv 3a, NCTC 5214 sv 4a, NCTC 10528 sv 4ab, NCTC 10527 sv 4b, ATCC 19116 sv 4c, NCTC 10888 sv 4d, ATCC 19118 sv 4e, SLCC (Special Listeria Culture Collection, Institut für Hygiene und Mikrobiologie, Würzburg University, FRG)

J. A. García Cabrera, L. Dominguez Rodriguez

Department of Animal Pathology I,
Veterinary Faculty, Compultense University
28040 Madrid, Spain

V. Briones Dieste, J. F. Fernandez Garayzabal,

J. A. Vazquez-Boland, G. Suarez Fernandez
Department of Microbiology and Immunology,
Veterinary Faculty, Compultense University
28040 Madrid, Spain

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

2482 sv 7; *Listeria ivanovii* ATCC 19119 sv 5, C-664 sv 5 (Leicester University Department of Microbiology Collection, UK); *Listeria innocua* NCTC 11288 sv 6a, NCTC 11289 sv 6b, C-645 sv 6b; *Listeria welshimeri* SLCC sv 6a; *Listeria seeligeri* CIP (Collection de l'Institut Pasteur, Paris, France) 100/100 sv 1/2b; *Listeria grayi* SLCC.

The strains were grown at 37 °C for 18–24 h on a common use medium: tryptone (Difco), 10g; Lab-Lemco powder (Oxoid), 10 g; yeast extract (Oxoid), 3 g; NaCl, 2.5 g; glucose, 5 g; NaH_2PO_4, 7.12 g; K_2HPO_4, 2 g; agar, 15 g in 1 litre.

O antigens. O suspensions were prepared as described [2]. After heating, they were treated with trypsin 1% [6].

Agglutination tests. The quantitative determination of agglutinins was performed using a microtechnique [7]. The O antigens were titrated to determine their optimal concentration, a critical factor for obtaining reproducible results.

For routine titrations 100 µl of serum diluted 1 : 10 was placed in the first well of a row of V-type microtitre plates, while the rest were filled with 50 µl of diluent. Serial dilutions of the sera were made using a micropipette (Titertek) adjusted to 50 µl. After adding 50 µl of the antigen to each well the final dilutions were twofold, starting from 1 : 20. The plates were incubated overnight at 37 °C, and then placed at 4 °C for at least 2 h before reading. The titres were expressed as the reciprocal of the highest dilution showing agglutination.

The diluent for sera and antigens was PBS 0.1 M pH 7.2 with 0.005% 0-safranin (Sigma) dye to enhance visibility of the sedimented antigen.

Antisera. Antisera were obtained by intravenous injection of New Zealand white rabbits with two doses given 10 days apart. The suspensions of the organisms were prepared from 18–24h plate cultures.

Factor sera. Factor sera were obtained as previously described [2], with the following modifications (performed to reduce cross-reactivity): for preparing O VI factor serum, the sv 4b antiserum was absorbed with sv 4c and 4a O antigens; for O VII, with sv 4b 3a, 4d, 4e and 5; for O VIII, with sv 4b, 4ab and 6b; for O X, with sv 4e. We were not able to produce O XI factor serum, in spite of multiple assays with different O antigens. Absorptions were carried out as described [8].

Results and discussion

The titres obtained with whole antisera and after absorptions (factor sera) against different *Listeria* serovar O antigens are shown in Table I and II, respectively.

These results are in general accordance with the reported O-antigenic formulas for *Listeria* serovars [3]. However, we have observed some discrepancies concerning the O antigen composition of the serologic reference strains of serovars 4b, 4ab and 6b.

The results obtained with O IX factor serum allow us to conclude that the strains NCTC 10528 sv 4ab and NCTC 11289 sv 6b do not posses this O-antigenic factor, since the titres against O antigens from these strains were as weak as those obtained with other O-antigenically unrelated serovars (Table II). In consequence, from our point of view, the O IX factor should appear in brackets in serovars 4ab and 6b.

On the other hand, the O IX factor serum reacted with significant titres with the O antigen from serovar 4b, and the adsorption of this factor serum with serovar 4b antigen led to the reduction of the titres against the serovars that would share O IX factor. These results strongly suggest that strain NCTC 10527 sv 4b possess also the O IX factor, then it should appear at least in brackets in the O-formulas.

Table I

Cross-titres of rabbit antisera

Sera \ Antigens	1/2a	1/2b	3a	4a	4b	4ab	4c	4d	4e	5	6a	w6a*	6b	7	G
1/2a	10 240	20 480	10 240	10	40	20	20	80	160	40	160	160	80	40	80
1/2b	5 120	20 480	1 280	40	40	20	20	40	40	20	80	80	20	5 120	20
3a	10 240	10 240	2 560	20	80	80	80	320	320	160	320	80	320	160	160
4a	80	160	160	2 480	1 280	1 280	5 120	1 280	20 480	1 280	2 560	10 240	1 280	160	80
4b	80	40	160	640	20 480	5 120	320	20 480	20 480	5 120	320	1 280	10 240	160	80
4ab	80	160	160	1 280	10 240	20 480	1 280	10 240	20 480	10 240	640	1 280	20 480	40	320
4c	80	80	640	2 560	1 280	2 560	10 240	1 280	5 120	2 560	160	5 120	10 240	160	320
4d	40	80	160	160	20 480	10 240	320	20 480	20 480	10 240	80	320	20 480	160	80
5	160	160	10 240	160	1 280	1 280	320	2 560	1 280	5 120	80	160	2 560	80	40
6a	2 560	20 480	5 120	5 120	5 120	640	640	5 120	560	640	20 480	20 480	1 280	1 280	320
w6a*	80	1 280	160	5 120	5 120	640	1 280	80	320	1 280	10 240	5 120	640	20	20
6b	80	320	80	2 560	2 560	10 240	1 280	5 120	20 480	10 240	320	1 280	20 480	80	80
7	80	40	320	640	40	160	20	160	160	160	20	80	20	20 480	2 560
G	40	160	40	40	40	20	40	40	40	20	40	80	40	20	5 120

* O antigen and antiserum of *L. welshimeri* serovar 6a

Table II

Titres of factor-sera against different Listeria O antigens

Factor sera \ Antigens	1/2a	1/2b	3a	4a	4ab	4b	4c	4d	4e	5	6a	w*	6b	7	G
I	2 560	20 480	40	10	20	20	20	20	20	20	40	20	40	20	80
IV	40	80	2 560	20	80	80	20	80	20	160	80	80	20	80	40
V + VI	80	40	160	640	5 120	20 480	320	20480	20 480	5 120	320	1 280	10 240	160	80
VI	20	40	40	20	5 120	10 240	20	10 240	10 240	2 560	80	20	5 120	20	80
VII	20	40	160	2 560	320	160	2 560	160	160	160	20	640	1 280	20	80
VIII	20	40	80	10	160	160	10	5 120	5 120	320	20	10	320	40	80
IX	20	40	40	2 560	20	640	80	20	20	20	160	1 240	20	20	20
X	40	160	80	80	640	160	160	160	160	640	80	160	640	80	20
XIII	40	80	160	160	80	40	80	40	40	20	10	40	80	2 560	80
XIV	20	80	40	80	20	40	20	40	40	20	80	80	20	40	2 560
XV	80	40	40	40	20	80	20	40	40	20	20 480	10 240	40	20	40

* O antigen from *L. welshimeri* serovar 6a

Table III

O antigenic formulas of Listeria serovars, including proposed modifications

Serovar/Serogroup	O antigens																	
1/2	I	II	(III)															
3		II	(III)	IV														
4a			(III)		(V)		VII		IX									
4ab			(III)		V	VI	VII		(IX)*	X								
4b			(III)		V	VI			(IX)*									
4c			(III)		V		VII											
4d			(III)		(V)	VI		VIII										
4e			(III)		V	VI		(VIII)	(IX)									
5			(III)		(V)	VI		(VIII)		X								
6a			(III)		V)	(VI)	(VII)		(IX)							XV		
6b			(III)		(V)	(VI)	(VII)	(VIII)*	(IX)*	X	XI							
7			(III)									XII	XIII					
L. grayi			(III)									XII		XIV		XVI**		
L. murrayi			(III)									XII		XIV			XVII**	

* Modifications
** Vázquez-Boland et al. [5]

Finally, the O VIII factor serum reacted significantly with the O antigen from strain NCTC 10289 sv 6b (titre 320, the same obtained with O antigen from strain ATCC 19119 sv 5, that would possess the O VIII factor). Then, we consider that this O VIII factor should appear (in brackets) in the antigenic formulation of serovar 6b.

Taking into consideration these results, and other from our laboratory concerning *Listeria grayi* and *Listeria murrayi* [5], the O-antigenic formulation for *Listeria* serovars may be that set out in Table III.

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O ANTIGENIC STRUCTURE OF *LISTERIA GRAYI* AND *LISTERIA MURRAYI**

J. A. Vazquez-Boland, L. Dominguez Rodriguez, J. F. Fernandez Garayzabal, V. Briones Dieste, J. A. García Cabrera and G. Suarez Fernandez

Department of Animal Pathology I, (Animal Health) Veterinary Faculty, Complutense University, Madrid, Spain

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Cross-agglutination and absorption studies indicated that two serological reference strains of *Listeria grayi* and *Listeria murrayi* differed in at least one O factor. The serological analysis revealed that the unshared O factors are not present in other *Listeria* serovars. These results are not in accordance with the antigenic structure previously reported for *L. grayi* and *L. murrayi*, then a new O antigen formulation is proposed for these species: *L. grayi* (III), XII, XIV and XVI; *L. murrayi* (III), XII, XIV and XVII.

The O antigens formulation established for *Listeria grayi* and *Listeria murrayi*, i.e. (III), XII and XIV for both [1, 2], was considered "sub judice" by Seeliger in the VI ISOPOL (Nottingham 1974) [3], but seemed to be validated in successive publications [4–6] in spite of some reported discrepancies with respect to O antigen composition [7].

The results obtained by us in a previous serological study indicating that *L. grayi* and *L. murrayi* differed in O antigen composition [8], led us to investigate the presence of undescribed O factors in these species.

Materials and methods

Bacterial strains. The bacteria used in the experiments, shown in Table II, are serological reference strains from the Special Listeria Culture Collection (SLCC) of the Institut für Hygiene und Mikrobiologie of Würzburg University, FRG.

O antigens. Suspensions were prepared as previously described [4]. The O suspensions from serovars 3a and 5 were trypsinized [9] to avoid autoagglutination.

J. A. Vazquez-Boland, L. Dominguez Rodriguez, J. F. Fernandez Garayzabal, G. Suarez Fernandez

Department of Microbiology and Immunology
Veterinary Faculty, Compultense University
28040 Madrid, Spain

V. Briones Dieste, J. A. García Cabrera

Infectious Diseases Unit
Veterinary Faculty, Complutense University
28040 Madrid, Spain

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

Antisera against L. grayi and L. murrayi. New Zealand white rabbits (2 for each strain) received four intravenous doses at 15-day intervals of suspensions prepared from 24 h cultures on blood agar. The animals were bled at 2, 5–6, 9–10 and 13–14 days after injections and weekly until the 131st day.

Agglutination and absorption tests. For the quantitative determinations of agglutinins a microtitre method was used [10]. The O suspensions were titrated to determine their optimal concentration, a critical factor for obtaining reproducible results. Absorption tests were performed as described [11].

Results and discussion

The results obtained in cross-agglutination tests [8] are summarized in Table I. *L. grayi* antisera reacted with *L. murrayi* O antigens at a very low level, the cross-reactivity titres being of similar value, and even lower, than those obtained against control O antigens of serovars 1/2 and 4ab (serologically

Table I

Geometric mean titers to the homologous and heterologous antigens in L. grayi and L. murrayi antisera

Antisera	Test antigen			
	L. grayi	*L. murrayi*	1/2a	4ab
L. grayi	2552.90	11.19	2.00	15.21
L. murrayi	86.30	3338.93	16.31	14.59
Uninoculated	9.73	1.41	1.11	1.83

related with *L. grayi* and *L. murrayi* only by the *Listeria* common OIII factor [5]). The cross-reactions with *L. grayi* antigen in *L. murrayi* antisera were more significant, but also weak: the maximum heterologous titres were sharply lower than the homologous ones. These results provide evidence for major antigenic differences between *L. grayi* and *L. murrayi*

These results were confirmed by cross-absorption tests performed with *L. grayi* and *L. murrayi* antisera as the homologous titres remained after absorption with the heterologous antigen (Table II). The O factors responsible for the specific titres against *L. grayi* and *L. murrayi* remaining after cross-absorptions appeared to be distinct from all the O factors described up to date in *Listeria:* absorptions performed with O antigens from serovars 1/2a, 3a, 3b, 4e, 4ab, 5, 6a, 6b and 7, that should have removed O factors I to XIII and XV agglutinins, failed to reduce significantly the titres against *L. grayi* and *L. murrayi* (Table II).

The results, that are not in accordance with the O-antigenic structure classically reported for *L. grayi* and *L. murrayi* [3–6] led us to propose new O-formulas for these species:

L. grayi: (III) XII, XIV, XVI

L. murrayi: (III), XII, XIV, XVII

Table II

Absorption tests with 2 L. grayi and 2 L. murrayi rabbit antisera. Titres before and after absorption

Antisera	Rabbit No.	Absorption	Test antigens									
			L. grayi	*L. murrayi*	1/2a	3a	3b	4e	4ab	5	6a	6b	7
L. grayi	1A	Unabsorbed	5120	20	—	~20	~20	20	20	20	~20	20	20
		Pool[a]	5120	—[b]	—	—	—	—	—	—	—	—	—
		Serovar 7	2560	—									
		L. murrayi	~ 2560[c]	—									
	1B	Unabsorbed	20480	80	—	160	40	20	20	40	~20	~20	~320
		Pool	20480	20	—	—	—	—	—	—	—	—	—
		Serovar 7	20480	—									
		L. murrayi	10240	—									
L. murrayi	2A	Unabsorbed	80	10240	~320	80	40	20	20	20	20	~40	80
		Pool	40	~10240	—	—	—	—	—	—	—	—	—
		Serovar 7	40	~ 5120									
		L. grayi	—	~ 2560									
	2B	Unabsorbed	160	10240	~80	160	40	20	40	20	~20	~40	80
		Pool	80	5120	—	—	—	—	—	—	—	—	—
		Serovar 7	40	5120									
		L. grayi	—	~ 2560									

[a] Antigenic Pool composed by O-suspensions of serovars 1/2a, 3a, 3b, 4a, 4e, 4ab, 5, 6a and 6b.
[b] Reaction not detected at serum dilution 1/20.
[c] The symbol "~" means partial agglutination reaction.

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MONOCYTOSIS PRODUCING ACTIVITY FROM VIRULENT AND AVIRULENT STRAINS OF *LISTERIA**

S. B. GALSWORTHY

Department of Microbiology and Immunology,
University of Western Ontario, London, Ontario, Canada

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Monocytosis is a hallmark of listeriosis in many species. A similar phenomenon is induced by a monocytosis-producing activity (MPA) purified from the organism. The relationship between MPA production and virulence is unclear. The purpose of this study was to measure MPA in extracts prepared from strains with known degrees of virulence and haemolysin production. Differential leukocyte counts on peripheral blood cells were performed 24, 48 and 72 h after injection of 0.5 mg MPA. In the strains we tested those known to be virulent produced monocytosis; those which were avirulent did not.

Interactions between mononuclear phagocyte and *Listeria* determine the outcome of infection. Survival requires the ability of *Listeria* to exist within macrophages and intracellular multiplication is required both for virulence and for development of cell-mediated immunity [1]. Resident macrophages contain the bacteria during early stages of infection [2]. Efficient augmentation of monocytopoiesis is required for superior resistance [3]. Final clearance of *Listeria* requires activation of macrophages by antigen-specific T cells [4]. Not surprisingly, monocytosis is a hallmark of *Listeria* infection in some species [5]. A monocytosis of similar degree and duration can be induced by a monocytosis-producing activity (MPA) from the organism [6, 7]. The relationship between MPA and virulence is unclear. The purpose of this study was to measure MPA in extracts prepared from strains with known degree of virulence.

Materials and methods

Bacteria. Strain 42 was obtained from the culture collection of the late E. G. D. Murray at the University of Western Ontario. The other strains were kindly supplied by Professor H. Hof from the Special Listeria Culture Collection of the University of Würzburg.

Preparation of MPA. Bacteria were grown in 2 litre batch cultures at 30 °C as described [6] and harvested and washed with distilled water using a Pellicon HVLP membrane cassette system (pore size 0.45 μm, Millipore Corporation, Bedford, MA). The bacteria were suspended in distilled water (200 ml for an 8 litre harvest) and killed by heating at 80 °C for 30 min.

S. B. GALSWORTHY

Department of Microbiology and Immunology, University of Western Ontario
London, Ontario, Canada N6A 5C1

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

The suspension of killed cells was sonicated using five 1 min bursts. The sonicate was centrifuged at 20 000 *g* for 20 min and the supernatant filtered through a DPO45 membrane (pore size 0.45 μm, Amicon Corporation, Lexington, MA) to remove debris and bacteria. Inactive material was removed from the filtrate by passing it through an Amicon PM10 membrane (molecular weight cut off 10 000 daltons) (Amicon Corporation). This filtrate, designated crude MPA, was further purified by adjusting it to pH 2 using 2 N HCl and applying it to cartridges (C-18 Sep Pak, Waters Associates, Milford, MA). Inactive material was eluted with distilled water and MPA was eluted from the columns with CH_3CN : H_2O (1 : 1). Solvent was removed in vacuo and water by lyophilization. MPA was reconstituted at 5 mg/ml in distilled water and sterilized by filtration through a 0.45 μm filter unit (Millex-HV, Millipore Corporation) and stored in 1 ml aliquots at −20 °C.

Differential leukocyte counts. Blood samples were taken from the retro-orbital sinus into EDTA-coated capillary tubes to prepare blood smears and to obtain total leukocyte counts. Total leukocyte counts were determined by diluting blood in 3% acetic acid (1 : 20 dilution) (Unopette test 5856; Becton-Dickinson, Rutherford, N. J.) and counting the cells in a haemocytometer. Blood smears were fixed for 15 min in absolute methanol and stained for 30 min with Giemsa stain. Differential cell counts were obtained by examination of Giemsa-stained smears under the light microscope. At least 200 leukocytes were counted on each side.

Results

Extracts from strains known to be virulent were able to stimulate monocytosis, while extracts from the avirulent strains failed to do so (Table I).

Discussion

These results suggest that MPA can be derived from virulent but not from avirulent strains of *Listeria*. These results are the reverse of those we had expected. One would expect that MPA would decrease virulence by improving recruitment of protective macrophages. Others reported that MPA content varies with growth conditions and correlates inversely with patho-

Table I

Comparisan of MPA from strains of Listeria

Serovar	Strain	Haemolysis	Virulence	MPA[1]
1/2a	UWO (EGDM-42)	+	+	950
1/2a	NCTC 7973	+	+	817
1/2a	SLCC 5850	−	−	110
3a	SLCC 5224	−	−	89
3a	NCTC 5105	+	int	106
3b	SLCC 5543	+	+	645
6a	NCTC 11288	−	−	160
PBS injected control				104

[1] Number of monocytes/mm^3 peripheral blood 48 h after injection. Results represent the average from determinations on 4 mice

genicity [8]. The propensity of an organism to make a substance which will promote the production of cells destined to destroy it is indeed puzzling. Since macrophages are a site of multiplication for *Listeria* it is possible that MPA would confer some advantage by stimulating monocyte production. If MPA were subtly toxic to macrophages, it would be quite advantageous to the bacteria producing it.

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SENSITIVITY OF *LISTERIA MONOCYTOGENES* TO IRRADIATION*

VERONIKA TARJÁN

Central Food Research Institute, Budapest, Hungary

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Irradiation of *Listeria monocytogenes* was carried out in culture media and pork meat paste at room temperature with ^{60}Co radiation source of 6.6 kGy.h^{-1} dose rate. The employed doses were 0, 0.5, 1, 2, 3, 4 and 6 kGy. Because of the expected great number of injured cells, parallel to the direct counting at selective media, liquid as well as solid resuscitation methods were used. One strain of out 3 survived as high as 4 kGy irradiation. Radiation with 2 kGy resulted 7 log cycles reduction of cell count. After lower irradiation doses the *L. monocytogenes* count decreased in proportion to increasing doses. It has been concluded that *L. monocytogenes*, compared with Gram-negative pathogens, are less sensitive to irradiation.

Listeria monocytogenes is an important pathogen causing listeriosis in both domestic and wild animals and man. In recent years there has been an increase in the number of outbreaks of listeriosis in which contaminated foods, such as raw milk [1], cheese [2], coleslaw [3] have been considered to be the source of infection. Nicolas [4] isolated *Listeria* from meat and meat products. Dijkstra [5] found that in The Netherlands 23.7% of the examined broiler carcasses were positive for *L. monocytogenes*.

There are several data about the presence of *L. monocytogenes* in foodstuffs. The behaviour of the bacteria during food processing (e.g. heating, cooling, fermentation, addition of chemicals: H_2O_2, NaCl or chlorine) and storage has also been widely examined. Our aim was to study the resistance of *L. monocytogenes* to irradiation.

Materials and methods

Bacterial strains. ATCC 1911, Hungarian National Collection of Medical Bacteria (HNCMB) 135001 and a strain from sheep brain isolated freshly by Dr. F. Kemenes (Phylaxia, Budapest).

Media. The selective plating medium was that of Ralovich et al. [6]. For enumeration of injured microorganisms the following repair methods were used.

Solid medium repair (SMR) on tryptone soya agar (Oxoid CM 131) for 1 h at 25 °C, followed by overlaying with the selective medium. Liquid medium repar (LMR) in tryptone

VERONIKA TARJÁN

Central Food Research Institute
Herman Ottó út 15, H-1022 Budapest, Hungary

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

soya broth (Oxoid CM 129) for 30 min at 25 °C followed by spreading to the surface of the selective medium. All plates were incubated at 37 °C for 24–48 h.

Irradiation was carried out at room temperature with an RH-gamma- 30 type, self-shielded ^{60}Co gamma radiation source of 6.6 kGy · h^{-1} dose rate. The employed doses were: 0, 0.5, 1, 2, 3, 4 and 6 KGy.

Results

One strain out of three survived as high as 4 kGy irradiation. Radiation with 2 kGy resulted 7 log cycles reduction of cell count. As the influence of lower radiation doses the *Listeria* count decreased in proportion to increasing doses (Fig. 1).

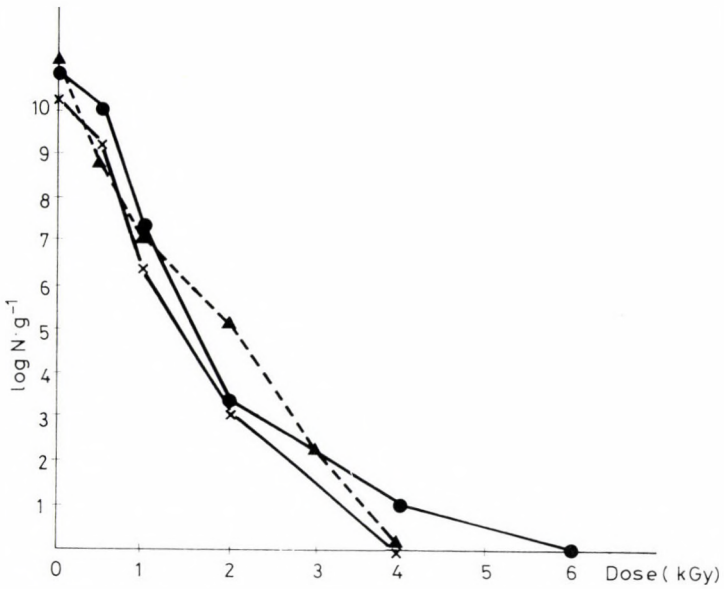

Fig. 1. Radiosensitivity of different *L. monocytogenes* strains. ×———× ATCC 1911, ●———● HNCMB 135001 (from human cerebrospinal meningitis), ▲———▲ freshly isolated from sheep brain

When the bacteria were irradiated in TSB, the SMR technique was more effective than the LMR, and the colony count was nearly the same order of magnitude either non-selective agar or repair methods were used (Fig. 2). In pork pulp the result of direct plating to non-selective agar was equal to the LMR or SMR methods (Fig. 3).

After 12 weeks storage at 4 °C, there was no change in the count of listeriae that survived the 0.5 and 1 kGy irradiation. The number of the 2 kGy-

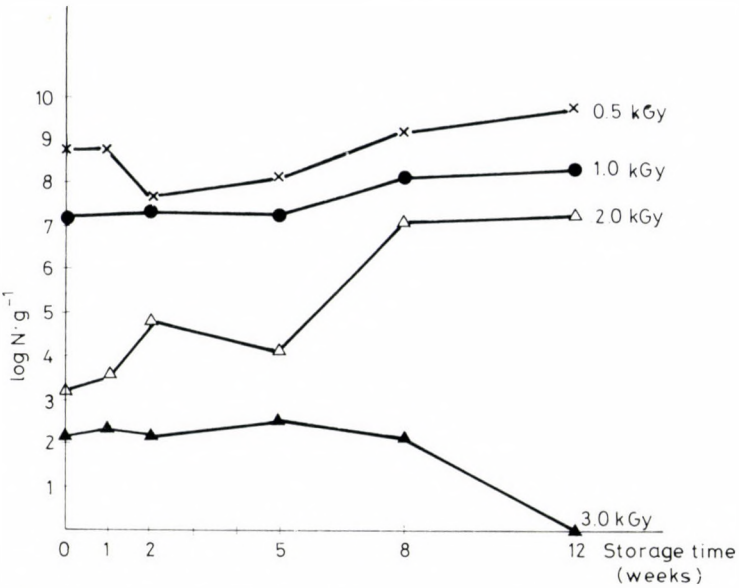

Fig. 4. Cell counts of irradiated *L. monocytogenes* stored at 4 °C in TS-broth

irradiated cells increased from 3 to 7 exponents. As the effect of the 3 kGy irradiation combined with 4 °C storage, there was no viable cell at the end of the 12th week of the experiment (Fig. 4).

Conclusions

To eliminate certain pathogenic microorganisms from foodstuffs the irradiation pasteurization is widely used. The radurization doses (3–5 kGy) are sufficient to destroy the Gram-negative pathogens, such as *Shigella*, *Salmonella*, *Campylobacter* or *Yersinia*, but some of the Gram-positive organisms like *L. monocytogenes* are less sensitive to irradiation, some strains can survive as high as 4 kGy irradiation. Cells that survived the lower radiation doses (0.5–2 kGy) can multiply during storage in refrigerator.

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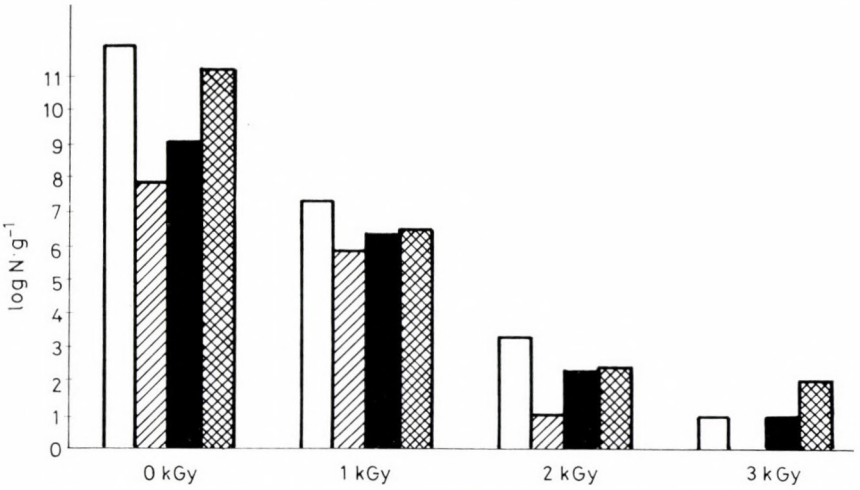

Fig. 2. The effect of different plating methods on the enumeration of irradiated *L. monocytogenes*. Open columns: nutrient agar; shaded columns: listeria selective agar; solid columns: LMR; hatched columns: SMR

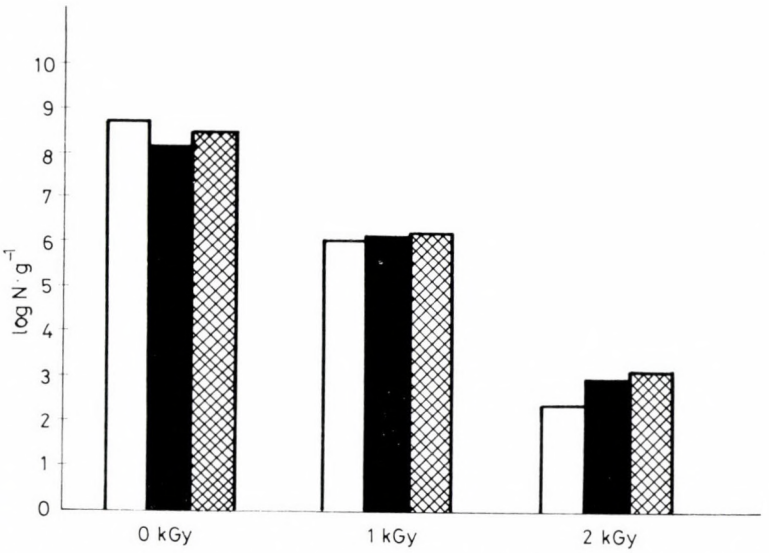

Fig. 3. The effect of repair methods on the enumeration of *Listeria* from artificially infected then irradiated ground pork meat. Open columns: nutrient agar; solid columns: LMR; hatched columns: SMR

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ANTIBODY RESPONSE OF DAIRY CATTLE EXPERIMENTALLY INFECTED WITH *LISTERIA MONOCYTOGENES**

IRENE VARELAS WESLEY, M. VAN DER MAATEN and J. BRYNER

Agricultural Research Services,
National Animal Disease Center, Ames, Iowa, USA

(Received September 16, 1988)

The purpose of our study was to monitor the humoral immune response of 34 lactating dairy cattle experimentally infected with *Listeria monocytogenes* (strain Scott A, serotype 4b). Twenty-seven animals were inoculated with 10^6 to 10^7 c.f.u./ml of *L. monocytogenes* via the intramammary route at ten day intervals for one month and 7 were inoculated with 4×10^2 to 4×10^3 c.f.u./ml. Preinfection and weekly-post infection serum and whey samples were prepared during acute infection. Antibody titres of serum and whey were evaluated in agglutination tests using whole-cell formalin-fixed *L. monocytogenes* suspensions. Preinfection agglutination titres ranged from 1 : 20 to 1 : 320. At least four-dilution increase over pre-infection titres were apparent by the 8th week of infection. Eighty per cent of the bovine sera (26/34) exhibited titres of $\geq$ 1 : 20 480. Whey titres, which reflected the local humoral response to *L. monocytogenes*, rarely exceeded 1 : 256 which may be ascribed in part to the lower immunoglobulin concentration in milk vs. blood. Antibody levels indicate active listeriosis but could not be used to predict the magnitude of the recovery of *Listeria* (c.f.u./ml) in milk.

Listeria monocytogenes [1] has been incriminated in deaths in high-risk groups such as pregnant women, neonates and patients on immunosuppressive drugs [2]. Few attempts have been made to correlate the serological response with the severity of infection as measured by the recovery of *Listeria* in clinical specimens. The purpose of this study was to monitor the antibody response with the recovery of *Listeria* (c.f.u./ml milk) in dairy cattle experimentally infected via the intramammary route with high (10^7 to 10^8 c.f.u./ml) and low (10^2 and 10^3 c.f.u./ml) doses of *L. monocytogenes*.

Materials and methods

Bacteria. L. monocytogenes (Scott A strain; serotype 4b) was used throughout this study.

Cows. Thirty-four lactating Holstein dairy cows (5–7 years old) were divided into 4 groups and inoculated via the intramammary route as follows: 27 animals (groups I, II, III) received 10^6 to 10^7 c.f.u./ml at ten-day intervals for one month; 7 cows (group IV) were inoculated twice with 4×10^2 and twice with 4×10^3 c.f.u./ml.

IRENE VARELAS WESLEY, MARTIN VAN DER MAATEN, JOHN BRYNER

United States Department of Agriculture, Agricultural
Research Services, National Animal Disease Center
P.O.B. 70, Ames, Iowa 50010 USA

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Isolation of L. monocytogenes. Bulk milk samples were plated (100 μl samples) to McBrides or BHIA containing 5% defibrinated bovine blood. Later in the course of this project, foremilk was collected and sonicated to maximize the recovery of *Listeria.*

Serology. Pre- and post-infection serum samples were obtained during acute experimental infection. Whey was prepared from a random selection of experimentally infected cows. In addition, 55 human serum samples of laboratory personnel and secretarial staff at this Institute, and 82 bovine sera, which were obtained from local dairies on 4 occasions and were taken as representative of non-infected animals in Iowa, were tested. Serum samples and whey samples were evaluated in a microtiter agglutination test using formalin-fixed whole cell suspension of *L. monocytogenes* ($OD_{540} = 0.27$) as follows. Two-fold serial dilutions (1 : 20 through 1 : 40 960) were made in 50 μl of PBS-0.1% BSA. To each well was added 50 μl of antigen. Samples were incubated (37 °C, overnight) and titres read. The reciprocal of the highest serum dilution without any evidence of a button was taken as the titre. Results are given as the number of dilutions required to achieve the end point: (e.g., 1 : 20 = 1, 1 : 40 = 2, ... 1 : 2560 = 8, etc.).

Results

Bovine serum samples (79 out of 82) obtained from Iowa dairies on 4 occasions (I, II, III, IV) exhibited titres of $\leq$ 1 : 160 (Table I). In contrast, the majority (53/55) of human sera tested had titres of $\leq$ 1 : 20 (Table I). Titres achieved 1–3 months post infection from experimentally infected dairy cattle are shown in Table II. Titres of cows inoculated with high or low doses of *Listeria* were comparable.

Group I (N = 9). Cow 1033. The humoral immune response following 9 inoculations (arrows) increased from a preinfection titre (1 : 160) to a final titre (1 : $\geq$ 40 960) nine months after the last inoculation. Recovery of *Listeria* exceeded 10^3 c.f.u./ml milk (Fig. 1a).

Cow 1177. The antibody response rose from a preinfection level (1 : 160) to final titre (1 : 81 920) 8 months after the last intramammary inoculation.

Table I

Serum-antibody titres to Listeria monocytogenes (Type 4b)

	Bovine sera*				Human sera**
	I	II	III	IV	
< 1 : 20	7	10	8	0	53
1 : 20	4	2	3	1	2
1 : 40	6	1	4	0	0
1 : 80	7	1	9	3	0
1 : 160	1	3	2	7	0
1 : 320	0	2	0	0	0
1 : 640	0	0	0	0	0
1 : 1280	0	0	1	0	0
Total	25	19	27	11	55

* 82 Bovine sera from 4 herds (I—IV)
** 55 human sera from NADC personnel

Table II

Agglutination titres of Listeria-infected cows

Cow	1st injection	Titre	
		pre-infection	post-infection
1033	3/22/87	160	40 960**
1177	3/2/87	1280	40 960
Chancey	3/2/87	0	640
9236	4/30/87	160	1 280
9378	4/30/87	160	5 120
0014	5/5/87	80	1 280
D1	5/19/87	0	5 120
D10	5/19/87	0	5 120
D14	5/19/87	0	5 120
D29	5/19/87	0	2 560
D35	5/19/87	0	2 560
D3	6/2/87	80	5 120
D22	6/2/87	40	5 120
D23	6/2/87	40	2 560
D27	6/2/87	80	1 280
D30	6/16/87	80	5 120
0062	8/4/87	160	320
S10	9/29/87	160	2 560
S52	9/29/87	80	40 960
S88	9/29/87	20	40 960
S198	9/29/87	80	40 960
S223	9/29/87	160	40 960
S49	10/14/87	160	20 480
S60	10/14/87	160	20 480
S73	10/14/87	160	20 480
S228	10/14/87	160	40 960
S227	1/5/88	80	40 960
W1125	12/15/87	40	40 960
W1309	12/15/87	160	40 960
W1323	12/15/87	80	40 960
W1417	12/15/87	80	40 960
W1518	12/15/87	20	5 120

* Maximum titre (1—3 months post-infection)
** Highest titre tested = 1 : 40 960

Shedding rates in excess of 10^3 c.f.u./ml reflect active infection of the mammary gland. Administration of dexamethasone (week 26) significantly enhanced shedding rates (Fig. 1b).

Cow Chancey. Preinfection titres ($<$ 1 : 20) contrast with the final titres (1 : 640) achieved two weeks after the last of 9 inoculations. The peak shedding rate exceeded 5×10^3 c.f.u./ml immediately after the sixth inoculation into the teat canal (week 10). Despite the shedding rate of this cow, no *Listeria* were

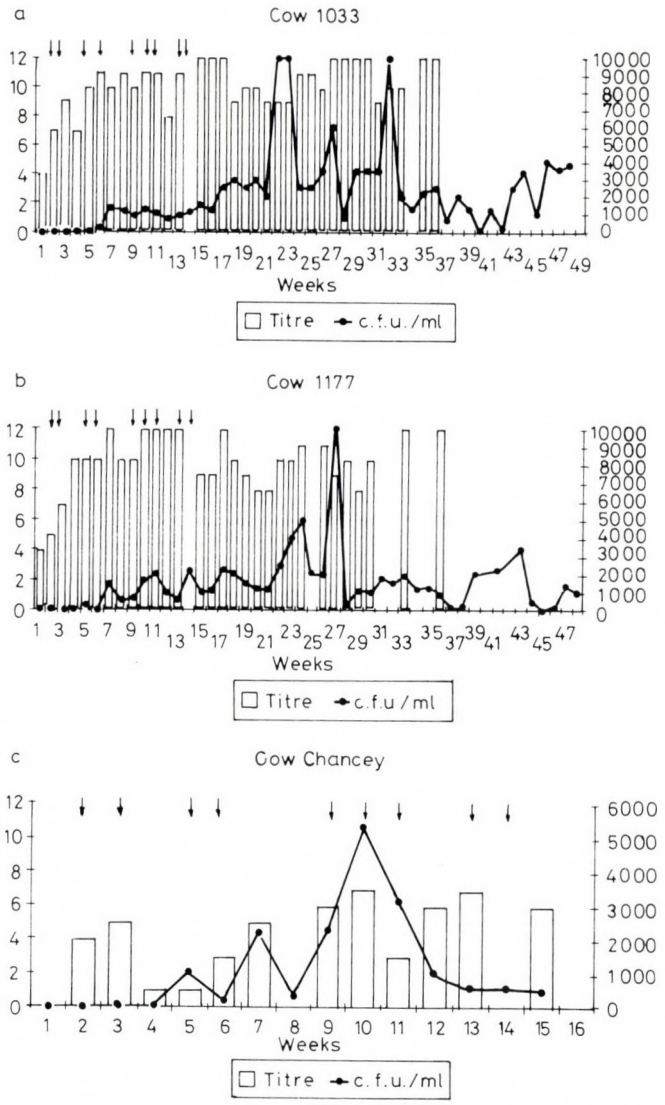

Fig. 1. Correlation of antibody titres (given as number of dilutions) and number (c.f.u./ml milk) of *Listeria* recovered from cow (a) 1033; (b) 1177; (c) Chancey. Arrows indicate inoculation

cultured at necropsy from muscle meat, brain, spleen and liver. This animal delivered an apparently healthy calf one day prior to necropsy which may suggest that the hormones associated with pregnancy predisposed her to listeriosis (Fig. 1c).

Group III (N = 10). Cow S198. Following two inoculations, this animal consistently shed *Listeria* in the milk. Sonication of foremilk samples (week

25) enhanced recovery of *Listeria*. Co-infection with bovine immune deficiency virus may have predisposed to listeriosis (Fig. 2a).

Group IV (N = 6). Group IV cows were infected four times via the intramammary route with relatively low doses of *Listeria* as follows: 4×10^2 c.f.u./ml ($2\times$) and 4×10^3 c.f.u./ml ($2\times$). Foremilk samples were sonicated (beginning week 16) in attempts to release intracellular bacteria, to disperse bacterial clumps, and thus, to better approximate the status of bovine listeriosis.

Cow 1125. Low shedding rates of *Listeria* were accompanied by a rise in antibody titre indicating acute systemic auto-immunization. A three-fold increase in the number of *Listeria* was seen after sonication of foremilk sample (Fig. 2b, week 16).

Cow 1477. The increase in antibody titre seen during the four-month interval indicates active infection. Sonication of foremilk samples (week 16) increased the numbers of detectable *Listeria* (Fig. 2c).

Discussion

Concerns over the use of a serological test for *L. monocytogenes* center on (a) lack of a uniform method which precludes comparison between laboratories; (b) inability to correlate the state of infection with serological titre; (c) lack of adequate testing with pair (pre- and postinfection) sera; and (d) cross-reactivity of sera with other Gram-positive bacteria including *Streptococcus*, *Corynebacterium* and *Bacillus* (reviewed in [2]). We have developed a simple agglutination test which utilizes a formalin-fixed antigen to monitor experimental bovine listeriosis. We examined serum obtained from employees at NADC including veterinarians, office personnel and laboratory scienctists as well as from culture negative and from experimentally infected cows. All of human serum samples (N = 55) representing 47 employees, showed titres of $< 1 : 20$. Of bovine sera from a random sampling of 82 cows from local more dairies, 79 showed titres of $\leq 1 : 160$ which may in part reflect their frequent exposure to cross-reactive Gram-positive microbes. In contrast, titres of $\geq 1 : 1280$ were achieved in 94% of the 32 infected cows 1–3 months post initial infection. Despite experimental infection and relatively high recovery rates ($\geq 10^3$ c.f.u./ml), low titres ($\leq 1 : 640$) developed in two cows. One of the cows (Chancey) was pregnant throughout the experimental period which suggests that the hormones associated with gestation may have dampened the immune response. Cow 62 exhibited general wasting syndrome, developed neurological signs suggestive of listeriosis and was euthanitized by the tenth week of infection.

Previous studies have attempted to correlate serological titre with bovine listeriosis. In a study involving a single naturally infected cow [3], agglutina-

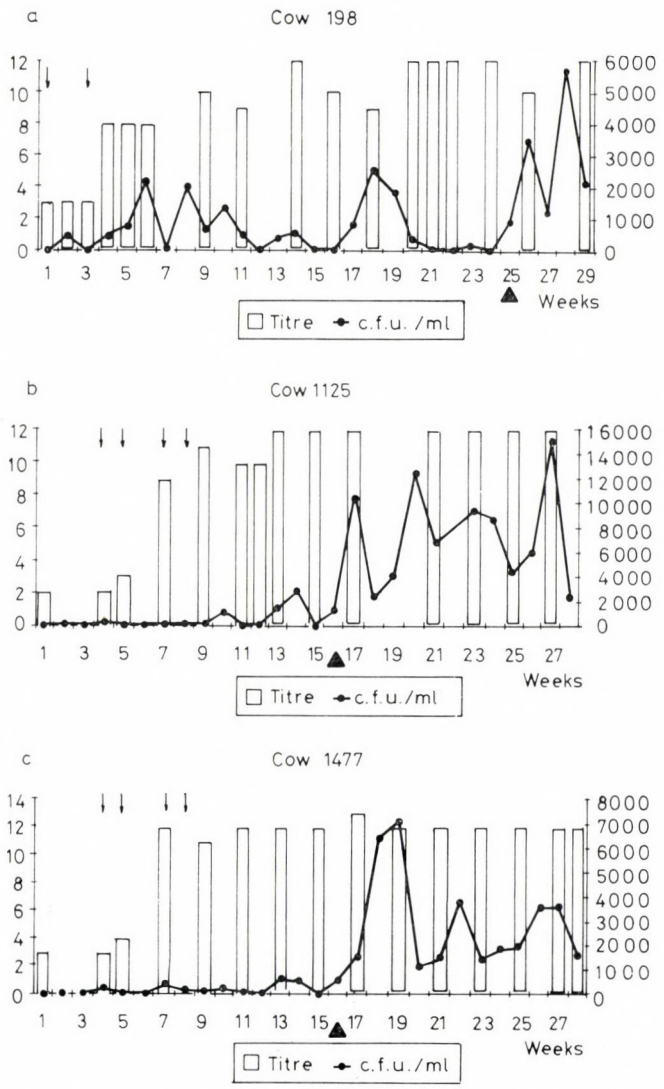

Fig. 2. Correlation of antibody titres (given as number of dilutions) and number (c.f.u./ml milk) of *Listeria* recovered from cow (a) 198; (b) 1125; (c) 1477. Upper arrows indicate inoculation. Bottom arrow indicates initial recovery of *Listeria* from sonicated foremilk

tion titres to the O antigen ranged from 1 : 320 to 1 : 2560 over the 6-month interval, but no information is provided on the recovery of *Listeria* in milk. In a more extensive study [4] including 110 bacteriologically positive naturally infected cows, agglutination titres of $\geq$320 were compatible with the diagnosis of listeriosis. As in the earlier study, the number of *Listeria* recovered from these cows was not provided. In our experimental setting, four-fold increases in antibody titres from pre-infection levels followed intramammary inocula-

tions and indicated the generalized systemic response prior to detecting *Listeria* in milk. The magnitude of the antibody response was the same following multiple inoculations of either high or low inoculating doses.

During the early stages of this project daily milk samples were enumerated. Ten-fold variation in the recovery of *Listeria* in milk samples over a seven day interval occurred in 31 (97%) of the animals. Later in the course of this study in order to better approximate the infection rate and to maximize recovery of *Listeria* in poor or low shedders, foremilk samples were collected and sonicated. This procedure yielded at least a two-fold increase in the recovery of *Listeria* in 8 of 9 cows. In contrast to the daily fluctuations in the recovery of *Listeria* in milk, serum antibody levels were relatively unchanged once high titres were achieved. While serum antibody titres may reflect a generalized systemic response whey antibody titres were evaluated in order to monitor the localized response of the mammary gland to infection. The lower immunoglobulin levels in milk contributed to the generally lower titres achieved in whey (maximum = 1 : 256). In conclusion, generally high antibody titres followed intramammary inoculations and could be achieved in animals despite the levels of *Listeria* recovered in milk. This suggests that although serological testing may be strongly suggestive of infection, bacteriological isolation, preferably from foremilk samples, confirms bovine listeriosis.

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EFFECT OF LISTERIA INFECTION ON THE ALLOTRANSPLANTATION REACTION IN CYCLOSPORIN A TREATED MICE*

MARIA WALENCKA and TERESA GOŚCICKA

Department of Immunology, Institute for Microbiology, University of Łódź, Łódź, Poland

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Allogeneic reaction of listeria-infected Balb/c mice to C3H skin graft was examined. The recipients were infected with *Listeria innocua* and treated with Cyclosporin A. Listeria infection in the treated mice enhanced skin rejection reaction reduced by the immunosuppressor.

In previous paper it was described that *Listeria innocua* infection can intensify the allotransplantation reaction in mice [1]. Hence, it was of interest to observe the development of rejection reaction to allogeneic skin graft in mice treated with the very effective immunosuppressor Cyclosporin A (CsA) and infected with *Listeria* in different time after transplantation.

Materials and methods

Skin transplantation. Inbred male Balb/c mice (haplotype H-2d) were grafted with the ear skin of inbred male C3H mice (haplotype H-2k). Each experimental group contained at least 6 animals. Intensity of allotransplantation reaction was expressed as a survival time of the graft in days.

Listeria infection. The skin graft recipient Balb/c mice were injected once intraperitoneally with 10^7 live cells of nonpathogenic *L. innocua* serovar 6a [2] kindly supplied by Dr. E. Menčiková. The bacteria were suspended in 0.5 ml of saline. The animals were injected on the 1st, 3rd, 5th, 7th, 9th, 11th or 13th day after skin implantation.

Application of Cyclosporin A. Cyclosporin A (Sandimmun) dissolved in 95% ethanol and diluted with PBS + 20% Tween 80 to 5 mg/ml, was given subcutaneously in daily doses of 50 μg/g during 8 days, starting 1 day before and continuing 7 days after skin transplantation (total dose 400 μg/g) [3].

Cytotoxic test. Lymphocytes from draining lymph nodes, isolated on the 8th day after skin implantation i.e. 1, 3, 5 days after listeria injection, were suspended in Eagle medium containing 10% calf serum, glutamine (200 μmol/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml), and incubated with ^{51}Cr-labeled fibroblasts of line 929 in ratio 50 : 1 at 37 °C in humified air atmosphere with 5% CO_2 for 36 h [4].

Blast transformation assay. Lymph node cells (10^7) were suspended in Parker medium buffered with 0.5 mg/ml Hepes (Calbiochem) and enriched in 8% FCS, 2% mouse serum,

MARIA WALENCKA, TERESA GOŚCICKA

Department of Immunology, Institute for Microbiology, University of Łódź
Łódź, Poland

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

glutamine (200 μmol/ml) and the antibiotics were incubated with 0.5 μg/ml of PHA (Wellcome) or 2.5 μg/ml of cancanavalin A (ConA, Calbiochem) or 20 μg/ml of a crude antigen isolated from *L. innocua* (LA) [1] at 37 °C in humidified air atmosphere with 5% CO_2 for 72 h. The cell proliferation was assessed after 18 h incorporation of ^{3}H-thymidine (methyl-^{3}H-thymidine, Amersham) [1]. The test was always made on the 8th day after skin implantation but at different times of infection: 1, 3 or 5 days after injection of bacteria.

Results

The best suppressory effect of CsA on the survival time of C3H skin in Balb/c mice was observed after 8 days application to recipients, in daily dose 50 μg/g, starting 1 day before grafting. The average survival time was 16 (±0.5) days, while in all normal control animals 12 days (Fig. 1).

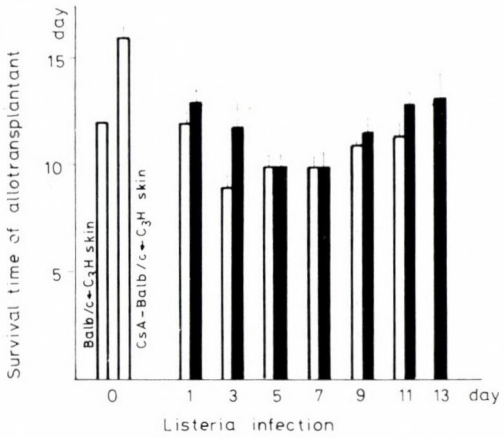

Fig. 1. Allotransplantation reaction in Balb/c mice treated with CsA and infected with *L. innocua* (solid columns) compared with the reaction in only infected animals (open columns)

Listeria infection started 3 days after transplantation enhanced in 25% allogenic reaction and then the survival time of skin graft was only 9 (±0.5) days. When it began later, after 5 or 7 days, an average survival time was 10 (±0.6) days. In CsA treated mice, listeriae intensified the rejection reaction previously suppressed in 33%. It was most demonstrative in mice infected 5 or 7 days after skin grafting. In these animals listeria infection completely abolished CsA effect.

In order to check if an intensification of rejection reaction in listeria-infected organism was connected with enhanced cytotoxicity of lymphocytes draining skin allograft, lymph node cells were examined in direct cytotoxic test using allogeneic fibroblasts as target cells (Fig. 2).

It was found that CsA suppressed the cytotoxicity of lymph node cells in recipients. Specific ^{51}Cr-release induced by effector cells dropped from 40.2%

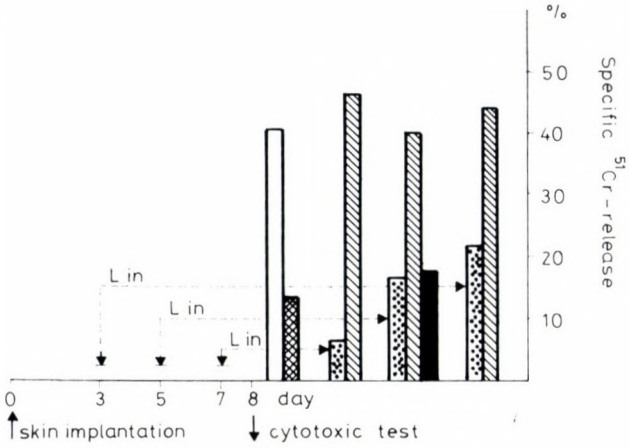

Fig. 2. Cytotoxicity of lymph node cells. Balb/c mice grafted with C3H skin (open columns), CsA treated mice grafted with C3H skin (hatched column), listeria infected mice (stripped columns), skin grafted mice infected with listeriae (shaded columns), CsA treated mice grafted with skin and infected with listeriae (solid column). L in = listeria infection

to 13.5%. The injection of listeriae on the 5th day after skin implantation did not affect this activity of lymphocytes at all. In *L. innocua* infected organism a specific delayed hypersensitivity developes which can be determined by blast transformation test [1]. The results of the reaction of lymph node cells to PHA, ConA and LA from infected and grafted with allogeneic skin mice are gathered in Table I.

Listeria infection lasting 5 days increased 103%, 224.2% and 107.4% of cell reactivity to PHA, ConA and LA, respectively. It was interesting that lymphocytes from allografts exhibited a lower ability to blast transformation on the 8th day than the cells of normal animals. However, also in this case *L. innocua* enhanced the reaction to PHA (+58.0%) and ConA (+91.4%). When a complete inhibition of CsA effect by listeriae was observed i.e. when the infection lasted 5 or 3 days, starting from the 3rd or 5th day after skin grafting, the draining lymph node cells exhibited a strongly intensified reaction to PHA (+166.3%) and ConA (+196.2%).

Discussion

Immunosuppressory effect of CsA was found to be a result of disturbance of normal lymphokine cascade essential to the development of effector cells responsible for graft rejection and cell hypersensitivity [5–7]. This drug is also able to suppress the defence mechanism of organism against different pathogens, particularly *L. monocytogenes* [8, 9]. Listeria infection triggering

Table 1

Blast transformation reaction of lymph node cells to PHA, ConA and LA

Group of Balb/c mice	Mitogen	Incorporation of ^{3}H-thymidine							
		Duration of *L. innocua* infection (days)							
		0		1		3		5	
		cpm	index	cpm	index	cpm	index	cpm	index
Infected with listeriae	PHA	157 466 ±5235	197.8	196 449 ±524	244.6	167 626 ±6531	201.7	167 573 ±8122	401.6
	ConA	140 730 ±6219	176.8	161 209 ±6466	200.7	175 077 ±4838	210.0	239 030 ±5529	573.2
	LA	2 180 ±191	2.7	1 021 ±28	1.3	1 361 ±290	1.6	2 361 ±893	5.6
Grafted with C3H skin and infected with listeriae	PHA	109 673 ±6562	40.5	124 471 ±3195	64.4	147 427 ±6554	71.0	156 098 ±4129	64.0
	ConA	120 669 ±7651	44.5	163 843 ±5541	84.8	148 647 ±4301	71.6	207 700 ±3350	85.2
	LA	3 669 ±994	1.3	5 104 ±438	2.6	3 181 ±574	1.5	3 457 ±538	1.4
Treated with CsA, grafted with C3H skin and infected with listeriae	PHA	218 200 ±5913	65.1	n.t.		246 252 ±5630	173.4	n.t.	
	ConA	150 208 ±6223	44.8	n.t.		188 550 ±2508	132.7	n.t.	
	LA	5 737 ±864	1.7	n.t.		4 143 ±5	2.9	n.t.	

index = a mean value of radioactivity (cpm) of lymphocytes cultured with mitogen divided by; a mean value of radioac tivity (cpm) of lymphocytes cultured without mitogen
± standard deviation
n.t. not tested

T cell mediated immunity can strongly modify an allogeneic reaction to tissue graft [1]. In our experiments we observed that *L. innocua* was able to abolish the immunosuppressory effect of CsA not affecting the cytotoxicity of draining lymph node cells, impaired by CsA. However, the activity of cells to PHA and ConA was clearly intensified at that time. It may be supposed that listeriae being able to stimulate the secretion of IL 1 and IL 2 [10] can effect organism quite the contrary to CsA which inhibits the production of lymphokines and suppresses many activities of lymphocytes [5–7].

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STUDY OF *LISTERIA MONOCYTOGENES* SURVIVAL DURING THE PREPARATION AND THE CONSERVATION OF TWO KINDS OF DAIRY PRODUCT*

J. COTTIN, F. PICARD-BONNAUD and B. CARBONNELLE

Bacteriology–Virology Laboratory C.H.R.U. and University Technology Institute, Angers, France

(Received September 16, 1988)

We tested yoghurts and soft cheeses for survival of *Listeria monocytogenes* during their manufacture and their storage at 4 °C. In yoghurt, even when the concentration of germs is high, the bacterial population decreases rapidly and the life of time of *L. monocytogenes* in this product depends on the sample acidity. The microorganisms disappear when the pH falls to 3.5. In soft white cheeses, only a fabrication with chemical and bacterial ferments allows an acidity which is compatible with destruction of majority of *L. monocytogenes*. Under these conditions, the pH decreases to 4–4.5 according to the series of production.

The presence of *Listeria monocytogenes* has been revealed for a long time in different food stuffs destined for human consumption, particularly in milk [1–5] and in meat [6, 7]. Since 1979, several human epidemic infections due to *L. monocytogenes* have implicated the ingestion of contaminated food and more especially of dairy products [1, 2, 7–10]. *L. monocytogenes* can contaminate these products either by the intermediary of raw material (milk), or at some stages of manufacture. We intended to study the future of *L. monocytogenes* conveyed by the milk, when yoghurts and soft white cheeses are manufactured and preserved.

Materials and methods

Materials. Sterilized milk (U.H.T. = Ultra High] Temperature) half skimmed milk (the pH changing from 6.1 to 7 according to samples). Yoghurts used as a source of lactic ferments (whole milk with a pH varying from 3.5 to 4.1). Soft white cheese, containing 40% of fats, used as source of lactic ferments (pH 4.4 to 4.6).

L. monocytogenes serovar 4b, isolated from a human infection, maintained as strain No. 96 of our collection.

Chemical ferments. Presure (Cooper Melun) with active chymosine $\geq$ 500 mg/l.

J. COTTIN, B. CARBONNELLE

Bactériologie Virologie, Centre Hospitalier-Régional-Universitaire
4 rue Larrey, 49040, Angers cedex, France

F. PICARD-BONNAUD

Institut Universitaire de Technologie
4 boulevard Lavoisier, 49045, Angers cedex, France

* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

Indicators. pH indicator papers Merck: neutralit (pH 5 to 10), acilit (pH 0 to 6) and spezialindikator (pH 6.5 to 10 and pH 4 to 7).

Manufacturing of yoghurts. After 18 h of culture in brain heart broth, *L. monocytogenes* was added to milk in different proportions. Yoghurt was made using 100 ml of yoghurt per litre of milk as a source of lactic ferments (corresponding to 3×10^{10} of *Lactobacillus bulgaricus* and 10^9 *Streptococcus acidominimus*). This preparation was kept at 37 °C for 16 h. Then the flasks were placed at 4 °C. Three series of fabrication were made and each series was divided into 3 groups.

Manufacturing of soft white cheeses. The first method consisted of adding to the milk *L. monocytogenes* and chemical ferments (at the rate of 0.1 ml of chymosine per litre of milk).

The second method used two sorts of ferments. Thus, in the milk already contaminated with *L. monocytogenes* were added active chymosine (0.1 mg/l) and 100 ml/l of soft white cheese as a source of lactic ferments (corresponding to 10^{10} of *S. lactis* per litre of milk).

In both methods, the milk was left to curdle for 16 h at 37 °C. Then the cheeses were slightly pressed, the lactoserum discarded, and kept at 4 °C. Four series were made using the first method and 2 series for the second one. Each series was divided into three groups.

Search of L. monocytogenes in the dairy product. The presence of *L. monocytogenes* was investigated on the day of the manufacturing and on days 2, 7, 14, 21 and 28 after incubation of the yoghurts and soft white cheese at 4 °C.

After homogenization, 100 μl aliquots of different dilutions of each sample (10^{-2}, 10^{-4}, 10^{-6}) were inoculated on blood-agar supplemented with nalidixic acid (40 μg/ml). Counting of *L. monocytogenes* was performed in 3 parallel experiments.

Thirty grams of dairy product were also inoculated into 500 ml of a selective enrichment broth for *Listeria* (20 g/l Bacto Trypto Agar, 20 mg/l acriflavine, 40 mg/l nalidixic acid and 3 mg/l colistine). The cultures were incubated at 37 °C for 48 h, then kept at 4 °C. Every week, 100 μl portions of each broth were cultured on blood agar with nalidixic acid. Colonies suspected to be *L. monocytogenes* were identified using standard methods [11].

Results

The results are shown in Tables I, II and III. Upon incubation at 4 °C, *L. monocytogenes* disappears from yoghurts, but the speed of its elimination depends on the pH of the product. In case of the two series of preparations which had pH 3.5, 48 h after manufacturing *L. monocytogenes* was absent.

Soft white cheese prepared by the first method, had a pH $\geq$ 6 and allowed a good rate of multiplication of *Listeria*, even when the number of the inoculated bacteria was very low (12/litre). Using both chemical and lactic ferments (second method), the pH decreased to 4–4.5 according to the series of production.

It seems that *L. monocytogenes* disappears from the first series whereas after enrichment it is still present in the second series of preparation which contains a high rate of *L. monocytogenes* inoculated to the milk, and has not a pH lower than 4.5.

Discussion

A pH of 3.5 to 4 leads to the elimination of *L. monocytogenes* from dairy products, whereas pH 4.5 does not allow a total disappearance of the inoculated germs. The different results show the important role played by the pH

Table I

L. monocytogenes counts and pH in yoghurts
Average of results obtained for each lot of yoghurts

	Series of fabrication	Day of fabrication	Time of incubation of yoghurts, at 4 °C, in days				
			2	7	14	21	28
I	pH	7	4.5	4.5	4.2	3.5	3.5
	Listeria concentration c.f.u./ml .	1.8×10^7	5.2×10^5	1.3×10^5	5.3×10	—	—
II	pH	7	4.5	4.5	4	3.7	3.5
	Listeria concentration c.f.u./ml .	1.8×10^6	5.5×10^4	1.7×10^4	3.3×10^2	—	—
III	pH	6.5	3.5	3.5	3.5	3.5	3.5
	Listeria concentration c.f.u./ml	6×10^4	—	—	—	—	—
IV	pH	6.1	3.5	3.5	3.5	3.5	3.5
	Listeria concentration c.f.u./ml	3.1×10^7	—	—	—	—	—

c.f.u. = colony forming units
+ presence of *Listeria* after enrichment, — absence of *Listeria* after enrichment

Table II

L. monocytogenes counts and pH in cheeses
Average of results obtained for each lot of soft white cheeses prepared with chymosine

	Series of fabrication	Day of fabrication	Time of incubation (soft white cheese) at 4 °C in days				
			2	7	14	21	28
I	pH	6.4	6.2	6.2	6.2	6	6
	Listeria concentration c.f.u./ml	2.3×10^4	1.3×10^8	1.7×10^8	1.8×10^8	1.8×10^8	1.6×10^8
II	pH	6.4	6.2	6	6	6	6
	Listeria concentration c.f.u./ml	8.8	1.9×10^8	1.4×10^8	1.3×10^8	1.2×10^8	1.8×10^8
III	pH	6.6	6.2	6.2	6	6	6
	Listeria concentration c.f.u./ml	0.67	1.7×10^8	1.9×10^8	1.2×10^8	1.4×10^8	1.8×10^8
IV	pH	6.5	6.2	6	6	6	6
	Listeria concentration c.f.u./ml	0.012	1.8×10^8	1.6×10^8	1.2×10^8	2.9×10^8	1.9×10^8

c.f.u. = colony forming units

Table III

L. monocytogenes counts and pH in cheeses
Average of results obtained for each lot of soft white cheeses prepared with chymosine and lactic ferments

	Series of fabrication	Day of fabrication	Time of incubation (soft white cheese) at 4 °C in days				
			2	7	14	21	28
I	pH	6.5	4.5	4.5	4.2	4.2	4.2
	Listeria concentration c.f.u./ml	11	—	—	—	—	—
II	pH	6.5	4.5	4.5	4.5	4.5	4.5
	Listeria concentration c.f.u./ml	2×10^4	+	+	—	+	—

c.f.u. = colony units
+ presence of *Listeria* after enrichment, — absence of *Listeria* after enrichment

in the survival of *L. monocytogenes* in foods especially in dairy products. Ryser [12, 13] has already described this phenomenon. He has shown that *L. monocytogenes* can survive in some cheeses where the pH is around 5. According to Beuchat [14] and Conner [15] *L. monocytogenes* disappears from cabbages juice when the pH is ≤ 4.6.

As a conclusion, we can say that yoghurts which usually have a pH of 3.5 allow a good elimination of *L. monocytogenes* and may be classified into the less contaminated dairy products for patients with high risk of infection, such as pregnant women.

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IDENTIFICATION OF SPECIES OF THE GENUS *LISTERIA* BY FERMENTATION OF CARBOHYDRATES AND ENZYMATIC PATTERNS*

J. Mira-Gutiérrez, C. Pérez de Lara and M. A. Rodriguez-Iglesias

Department of Microbiology, Faculty of Medicine, University of Cádiz, Cádiz, Spain

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Patterns of carbohydrates and determination of API ZYM and API oxidases can be considered a useful way to differentiate the strains of *Listeria*. With all this information it is possible to work out a schematic table that allows the identification of *Listeria* strains with a remarkable certainty. By numerical analysis four differentiated clusters have been demonstrated.

In recent years, the use of new taxonomic techniques has permitted the reclassification of the species within the genus *Listeria*. Rocourt et al. [1] divide the genus into five genomic groups composed of *Listeria monocytogenes*, *Listeria bulgarica* (serovar 5 of *L. monocytogenes)*, described later as *Listeria ivanovii* [2], *Listeria innocua*, and groups 4 and 5 which correspond to the species *Listeria welshimeri* and *Listeria seeligeri*, respectively [3]. Finally, *Listeria denitrificans* whose inclusion in the genus was never completely admitted, has been reclassified in a new genus as *Jonesia denitrificans*.

In this work previous studies of a biochemical character have been completed [4], using enzymatic microtests and fermentation of carbohydrates with a collection of strains which included all the species of the genus.

Materials and methods

Thirty strains corresponding to the following species have been studied: *L. monocytogenes* (20), *L. welshimeri* (1), *L. seeligeri* (1), *L. innocua* (1), *L. murrayi* (2), *L. grayi* (2), *L. ivanovii* (2) and *J. denitrificans* (1). Among these are included the strain-types of each of the species studied.

The micromethod API 50CH has been used to observe the fermentation of carbohydrates, employing phenol red broth base and incubating for three days, whilst evaluating the reaction every 24 h. The enzymatic profiles were obtained by the methods API ZYM and LRA

J. Mira-Gutiérrez, C. Pérez de Lara, M. A. Rodriguez-Iglesias

Department of Microbiology, Faculty of Medicine, University of Cádiz
Cádiz, Spain

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Table I

Carbohydrate fermen

	L. m. 1/2ba	*L. m.* 1/2a	*L. m.* sv 1/2a 7973	*L. m.* 1/2b	*L. m.*. 1/2c	*L. m.* c	*L. m.* c 19 116	*L. m.* 1e	*L. m.* 2	*L. m.* 3a	*L. m.* 4ab	*L. m.* 4a	*L. m.* 4b
Glycerol	+	+	−	−	−	−	−	−	−	−	−	−	−
Erythritol	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−
Ribose	−	+	−	+	−	−	−	−	−	+	+	±	−
D-Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−
Adonitol	−	−	−	−	−	−	−	−	−	−	−	−	−
Beta-methyl-xyloside	−	−	−	−	−	−	−	−	−	−	−	−	−
Galactose	−	+	−	−	−	−	+	+	+	+	+	−	−
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	−	±	−	−	−	−	−	−	−	+	−	−	−
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−
Manitol	−	+	−	±	−	−	−	+	−	+	−	−	−
Sorbitol	−	+	−	+	−	−	−	−	−	+	−	−	−
Alpha-methyl-D--mannoside	+	+	+	+	+	+	+	+	+	+	+	+	+
Alpha-methyl-D--glucoside	+	+	+	+	+	−	+	+	+	+	+	+	+
N-Acetyl-glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	−	±	+	±	−	−	−	−	+	+	−	−	−
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−
Melezitose	−	±	−	−	−	−	−	+	+	−	+	−	−
D-Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−
Starch	+	+	±	±	+	+	+	±	+	+	+	+	+
Glycogen	−	−	−	−	−	−	−	−	−	+	−	−	−
Xylitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Beta-gentibiose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	+	+	±	±	−	−	−	−	−	+	−	−	−
D-Lyxose	−	−	−	−	−	−	±	−	−	−	−	−	−
D-Tagatose	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Fucose	−	−	−	−	−	−	−	−	−	±	−	−	−
L-Fucose	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Arabitol	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabitol	−	−	−	−	−	−	−	−	−	−	−	−	−
Gluconate	−	−	−	−	−	−	−	−	−	±	−	−	−
2-Keto-gluconate	−	−	−	−	−	−	−	−	−	−	−	−	−
5-Keto-gluconate	−	−	−	−	−	−	−	−	−	−	−	−	−

tation patterns

L. m. 4d	*L. m.* 4d 19 117	*L. m.* 4f	*L. m.* 4g	*L. m.* 5 19 119	*L. bulgarica*	*L. m.* 6a	*L. innocua* 11 288	*L. m.* 6b	*L. m.* 7	*L. grayi*	*L. grayi* 19 120	*L. murrayi*	*L. murrayi* 25 501	*L. denitrificans*	*L. seeligeri* sv 1/2b	*L. welshimeri* sv 6a
+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
+	+	−	−	+	+	−	−	±	−	+	+	+	+	±	−	−
−	−	−	−	+	+	−	−	−	−	+	−	−	−	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
-	+	−	−	+	+	−	−	−	+	+	+	+	+	+	−	−
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	+	−	−	−	−	−	+	+	+	+	−	−	−
−	−	−	−	−	−	−	−	−	+	−	−	±	−	−	−	−
+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+
+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	±
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	−	−	−	−	−	−	+	±	−	−	−	−	±	±	±
−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
+	−	+	+	−	−	+	+	+	+	+	+	+	−	+	±	±
−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−
+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+
±	−	−	±	−	−	+	±	+	−	−	−	−	−	+	±	±
−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−
−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	±	+	±	+	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

ZYM oxidases, incubating for 4 h, and interpreting the reaction immediately after the reagents were added.

The results obtained were submitted to a numerical analysis, using the similarity coefficients of Sokal and Michener (S_{sm}) and of Jaccard (S_j), and employing the UPGMA as technique of clustering.

Results

The pattern of fermentation of carbohydrates permits the clear recognition of the species of the genus *Listeria*. Thus, whereas a series of substrates were fermented usually by all strains (glucose, fructose, mannose, amigdalin, esculin, salicin, celobiose, maltose, lactose, gentibiose, and others, Table I), some others were fermented by specific strains; differential patterns of special interest have been found. *L. murrayi* is distinguished by the fermentation

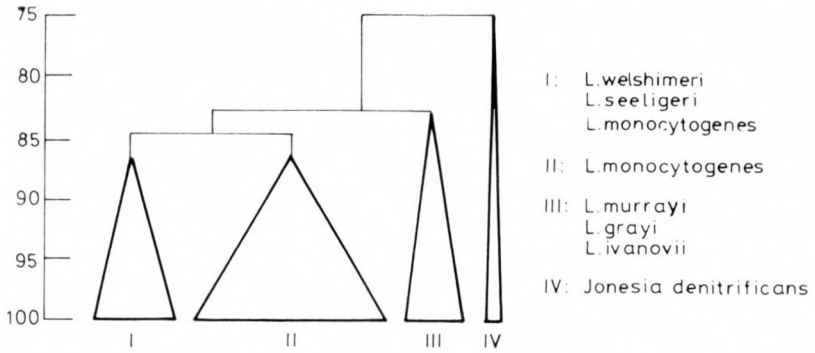

Fig. 1. Dendogram of clustering in phenons of *Listeria* spp

of raffinose and gluconate, sharing this characteristic with *L. grayi*. *J. denitrificans* maintains important differences from the rest of the strains studied, such as the fermentation of L-arabinose and melibiose. Some substrates, such as alpha-methyl-mannoside allow the recognition of very closely related species such as *L. seeligeri* and *L. welshimeri*. The serovars of *L. monocytogenes* present very variable results which do not allow a determined biochemical pattern to be assigned to each one of them.

The API ZYM (Table II) show the presence of phosphohydrolase and acid but not alkaline phosphatase. There are esterases from fatty acids of short chain, leucine-aryl-amidase and alpha- and beta-glucosidases (using substrates with a beta-naphthyl group conjugated). With the LRA ZYM osidases (Table III) the sensitivity of the substrates conjugated with p-nitrophenol, is superior and a greater activity can be detected, outstanding in the case of the hydrolysis of alpha- and beta-glucosidase, N-acetyl-beta-D-glucosaminidase, alpha-maltosidase, and alpha- and beta-mannosidase.

Table II

Enzymatic profiles (API ZYM)

	L. m. 1/2ba	*L. m.* 1/2a	*L. m.* sv 1/2a 7973	*L. m.* 1/2b	*L. m.* 1/2c	*L. m.* e	*L. m.* e 19 116	*L. m.* 1e	*L. m.* 2	*L. m.* 3a	*L. m.* 4ab	*L. m.* 4a	*L. m.* 4b	*L. m.* 4d	*L. m.* 4d 19 117	*L. m.* 4f	*L. m.* 4g	*L. m.* 5 19 119	*L. bulgarica*	*L. m.* 6a	*L. innocua* 11 288	*L. m.* 6b	*L. m.* 7	*L. grayi*	*L. grayi* 19 120	*L. murrayi*	*L. murrayi* 25 401	*L. denitrificans*	*L. seeligeri* sv 1/2b	*L. welshimeri* sv 6a
Alkaline phosphatase	1	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0
Esterase (c4)	3	3	1	5	3	5	3	5	5	5	5	5	3	3	3	3	3	3	3	5	3	5	3	5	1	5	0	0	3	0
Lipase esterase (c8)	1	1	1	3	1	3	1	3	3	3	1	3	1	5	1	1	1	1	1	3	3	3	1	3	1	1	0	1	3	1
Lipase (c14)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Leucine aryl-amidase	1	1	0	3	3	3	1	5	5	1	1	1	1	1	1	1	1	3	3	3	1	1	1	1	0	3	5	5	3	1
Valine aryl-amidase	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Cystine aryl-amidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alpha-chemotrypsin	1	0	0	3	3	1	0	5	5	1	0	0	0	0	0	1	3	1	0	1	0	0	5	0	0	0	0	0	1	1
Acid-phosphatase	3	1	1	5	3	3	5	5	3	1	1	1	0	1	1	1	3	5	3	3	3	1	3	0	0	0	0	0	3	1
Phosphohydrolase	3	3	3	5	5	1	3	5	0	3	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	3	3
Alpha-galactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Beta-galactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alpha-glucosidase	0	5	5	3	0	0	3	0	5	0	1	3	5	5	3	0	0	1	0	1	0	1	5	1	0	3	0	5	5	0
Beta-glucosidase	3	3	3	1	3	5	5	1	0	5	5	5	0	3	5	0	3	5	0	3	1	3	3	5	1	5	0	1	3	3
N-Acetyl-beta-glucosaminidase	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	3	0	1	0	1	0	0
Alpha-mannosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alpha-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = 0 nanomol; 1 = 5 nanomoles; 3 = 20 nanomoles; 5 = 40 nanomoles

Table III

Enzymatic profiles (LRA ZYM oxidases)

	L. m. 1/2na	*L. m.* 1/2a	*L. m.* sv 1/2a 7973	*L. m.* 1/2b	*L. m.* 1/2c	*L. m.* c	*L. m.* c 19 116	*L. m.* 1e	*L. m.* 2	*L. m.* 3a	*L. m.* 4ab	*L. m.* 4a	*L. m.* 4b	*L. m.* 4d	*L. m.* 4d 19 117	*L. m.* 4f	*L. m.* 4g	*L. m.* 5 19 119	*L. bulgarica*	*L. m.* 6a	*L. innocua* 11 288	*L. m.* 6b	*L. m.* 7	*L. grayi*	*L. grayi* 19 120	*L. murrayi*	*L. murrayi* 25 401	*L. denitrificans*	*L. seeligeri* sv 1/2*b*	*L. welshimeri* sv 6a
Alpha-D-galactosidase	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Beta-D-galactosidase	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phospho-beta-D-galactosidase	3	3	1	1	1	1	5	3	5	3	0	0	5	3	1	3	3	1	3	0	1	3	5	1	0	0	1	0	3	1
Alpha-L-arabinosidase	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	5	0	0
Alpha-D-glucosidase	3	5	3	5	0	0	5	1	5	0	5	1	5	5	3	5	3	1	0	5	0	3	5	3	1	1	3	5	5	0
Beta-D-glucosidase	5	5	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	3	5	5	5	3	5	5	5	5	3
Beta-D-galacturonohydrolase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-D-glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alpha-maltosidase	3	5	0	5	0	1	3	3	3	3	5	1	3	5	3	5	5	1	0	5	0	0	5	5	3	5	3	3	0	0
Beta-maltosidase	1	5	0	3	0	0	0	0	1	0	3	0	1	3	0	1	1	0	0	3	0	1	3	1	0	0	0	0	1	0
N-Acetyl-alpha-D-glucosaminidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-Acetyl-beta-D-glucosaminidase	5	5	1	5	3	3	5	1	5	5	3	1	5	5	5	5	5	3	3	5	1	5	5	5	1	5	3	0	3	1
Alpha-L-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-D-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
Beta-L-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-D-lactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alpha-D-mannosidase	5	5	0	5	0	0	1	1	0	5	5	0	0	5	1	0	0	0	0	5	0	5	5	3	0	0	1	0	0	0
Beta-D-mannosidase	5	5	0	5	1	1	3	3	3	5	3	0	5	5	3	5	5	1	1	5	0	5	5	5	0	1	3	0	1	0
Alpha-D-xylosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Beta-D-xylosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0

For explanation see Table II

The processing of the data in order to obtain the coefficients of similarity and the clustering by means of UPGMA demonstrates the formation of four differentiated clusters (Fig. 1). Three, with a similarity of 85% (S_{sm}) groups the phenon I *(L. welshimeri, L. seeligeri, L. innocua* and three strains accepted as *L. monocytogenes)*; the phenon II, which includes the rest of the strains of *L. monocytogenes* together with the strain type; and the phenon III composed of *L. murrayi, L. grayi* and *L. ivanovii*. Further away, with 75% similarity, one finds the phenon IV which includes as only strain *Jonesia denitrificans*.

Discussion

The patterns of fermentation of carbohydrates can be considered as a useful method in the differentiation of strains of *Listeria*, and has thus been recognized in previous works [4, 5]. API ZYM and API oxidases bring important data. With all this information it is possible to elaborate reduced tables which permit the identification of *Listeria* with a notable level of reliability.

In the same way, the numerical analysis of these data shows us the homogeneity of the genus, once *J. denitrificans* has been separated, also the convenience of maintaining *L. grayi* and *L. murrayi* in the genus, in agreement with data of other authors [3, 6].

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COMPARISON OF ALLERGENIC SKIN TEST WITH SEROLOGICAL AND BACTERIOLOGICAL EXAMINATIONS OF CATTLE FOR *LISTERIA MONOCYTOGENES**

I. Kovinčić, B. Štajner, I. F. Vujičić,
M. Švabić-Vlahović, M. Mrdjen, M. Galić,
M. Gagić and J. H. Bryner

*Faculty of Agriculture, Veterinary Science Institute.
and Livestock Research Institute, Novi Sad, Yugoslavia,
Faculty of Medicine, Belgrade, Yugoslavia,
and National Animal Disease Center, Ames Iowa, USA*

(Received September 16, 1988)

Five herds, each containing 200 to 300 cows, were surveyed for listeriosis. Since listeriosis is predominately linked to reproductive diseases in the surveyed area, suspected cows were first selected according to the records on their previous reproductive disorders such as abortion, endometritis and sterility. By simultaneous serological, bacteriological and skin allergenic testing 40 cows suffering from listeriosis were detected. But, only seven of those cows were occasionally shedding listeriae in milk. The specific agglutinins for *Listeria monocytogenes* were detected in blood sera of all seven cows and the titre was from 1 : 20 to 1 : 160. The difference of skin thickness induced by allergenic test was from 1 to 2 mm. *L. monocytogenes* was isolated from milk of all seven cows and indirectly through the inoculation in enrichment broth, and directly only from milk of two cows. The results indicate that the positive skin allergenic and serological tests do not necessarily mean the shedding of listeriae in milk. However, the listeria-shedding in milk was always accompanied by a positive skin allergenic test and the presence of agglutinins in blood sera.

Diagnosis of sporadic cases of bovine listeriosis represents a complex problem. Clinical signs may be mild and atypical and when they appear as a reproductive disorder there are no special signs. Similar disorders in reproduction may also be caused by other agents [1–4]. In the framework of regular

I. Kovinčić, B. Štajner, M. Mrdjen, M. Galić, M. Gagić

Faculty of Agriculture, Veterinary Science Institute, Novi Sad, Yugoslavia

I. F. Vujičić

Faculty of Agriculture, Livestock Research Institute
Novi Šad, Yugoslavia

M. Svabić-Vlahović

Faculty of Medicine
Belgrade, Yugoslavia

J. H. Bryner

National Animal Disease Center,
Ames, Iowa, USA

* Presented at the 10th International Symposium on Liesteriosis, Pécs, Hungary, August 22–26, 1988. Investigations were supported by the Yugoslavia–US Joint Board for scientific and technological cooperation, Project YO-ARS-90-JB-103, PP636.

health control of herds it is necessary to affirm, beside other diseases, the presence and frequency of listeriosis. Classical methods which are being used are not the most favourable in field work. Isolation of agents, especially in the case of agents with mixed bacterial flora, requires the application of a great number of selective and enrichment media. There are no unique opinions about the titre of antibodies for *L. monocytogenes* in blood sera, which is the most decisive thing for serodiagnosis of listeriosis [5]. Therefore, the objectives of our investigation were to try to apply allergenic skin test together with serological and bacteriological examinations [6].

Materials and methods

Examinations were performed at 5 cattle herds in Vojvodina, each containing about 200 cows. Forty heads were chosen, which, by the previous examinations were proven to have different reproductive disorders (abortion, endometritis and other). Allergen was prepared from *L. monocytogenes* 4b-1071 strain, by chloroform extraction [6]. Skin allergenic test was performed by inoculation of 0.5 ml of allergen into the tail wrinkle intracutaneously. The reaction was judged after 24 and 48 h taking into the account the skin thickness at the spot of inoculation and the quality of change. Beside, the skin test, repeated serological examinations and bacteriological checks of uterus secretion and milk for the presence of listeriae were performed for these 40 cows.

Results

All 40 cows with reproductive disorders had positive skin allergenic test. Blood sera of these animals had specific antibodies for *Listeria monocytogenes* and by bacteriological examinations listeriae were isolated from the uterus mucus. *L. monocytogenes* was isolated from milk of these cows only in 7 cases (Table I).

At relatively low and mean titre values found for antibodies for *L. monocytogenes* in blood sera (1 : 20 up to 1 : 160) at 7) cows shedding listeriae in milk, the positive skin allergenic test was confirmed (1–2 mm difference in

Table I

Comparative analysis for L. monocytogenes of 40 cows with reproductive disorders

	No. of cases	Percentage of positive findings
No. of cows examined	40	100
Positive skin allergenic test	40	100
Positive serological test	40	100
Bacteriological findings in uterine mucose	40	100
Bacteriological findings in milk	7	17.5

skin thickness). At the same time, bacteriological finding from uterine mucus was positive, and by direct plating in two cases *L. monocytogenes* was isolated in milk of these cows and in 5 cases it was proved through enrichment broth (Table II).

Table II

Comparative results of serological, allergenic and bacteriological examinations at cows shedding L. monocytogenes in milk

No. of cows	Titre of specific agglutinins for *L. monocytogenes* (days)			Allergenic test of difference in skin thickness in mm	*L. monocytogenes* in uterine mucose	*L. monocytogenes* in milk	
	1	30	60			direct plating	through the enrichment broth
1	1 : 160	1 : 80	1 : 80	2	+	$+4.8\times10^2$	+**
2	1 : 20	1 : 40	1 : 40	1	+	−*	+
3	1 : 40	1 : 80	1 : 80	1	+	−	+
4	1 : 40	1 : 40	1 : 40	2	+	−	+
5	1 : 160	1 : 80	1 : 80	1.5	+	−	+
6	1 : 40	1 : 40	1 : 40	1.5	+	−	+
7	1 : 80	1 : 40	1 : 40	2	+	3.1×10^2	+

* Listeriae were not isolated in direct plating
** Listeriae isolated through enrichment broth

Discussion

Enough or even high specific quality of skin allergenic test in diagnosis of bovine listeriosis follows from the results of preliminary comparative allergenic, serological and bacteriological examinations. The specific quality of allergenic test points out that the test could be applied at field work, i.e. for detection of listeriosis in infected herds, but, it should be justified by additional serological and bacteriological examinations.

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HAEMATOLOGICAL REACTIONS OF RABBITS INFECTED INTRAVENOUSLY WITH LISTERIA STRAINS OF DIFFERENT VIRULENCE*

YU. A. MAZING, M. A. DANILOVA, V. N. KOKRYAKOV, V. G. SELIVERSTOVA, V. E. PIGAREVSKII, S. VÖRÖS, M. KERÉNYI and B. RALOVICH

Institute of Experimental Medicine of Academy of Medical Science of USSR, Leningrad, USSR,
Institute of Microbiology, and Institute of Public Healths and Epidemiology, University Medical School, Pécs, Hungary

(Received May 23, 1989)

Listeria strains of different virulence were injected intravenously into rabbits of both sexes (2–4 kg). The infectious dose was 10^8 cells/kg. Blood samples were taken from the ear wein one day and immediately before the infection, then 3 h, 1, 2, 3, 6 and 10 days after it. Total white blood cell counts and their changes were determined and the number of lysosomal granules in neutrophils were counted. A marked monocytic reaction was observed after the injection of virulent *Listeria monocytogenes* 18 and 87/5467, partly virulent *Listeria ivanovii*, non-pathogenic *Listeria seeligeri* 87/5626 and 87/5575, and *Listeria murrayi* G44 strains. A late slow growth was provoked by *Listeria innocua* C644 and a very slight reaction by *Listeria welshimeri* 1830 strains. There was no change after injection of *L. innocua* strain 10. The number of lysosomal granules decreased significantly and remained at a low level for 6 days after the infection of *L. monocytogenes* strain 18 and for 3 days after the injection of *L. innocua* strain 10.

Murray, Webb and Swann [1] described that listeria infection caused a considerable mononuclear reaction in rabbit. Nyfeldt [2, 3] observed that *Listeria monocytogenes* could produce a clinical syndrome similar to infectious mononucleosis in man but in this case the Paul-Bunnel reaction was negative.

Now it is clear that *Listeria* strains can provoke monocytosis in most species of monogastric animals and in men (Seeliger [4]).

YU. A. MAZING, M. A. DANILOVA, V. N. KOKRYAKOV, V. G. SELIVERSTOVA, V. E. PIGAREVSKII,

Institute of Experimental Medicine of Academy of Medical Science of USSR
Leningrad, USSR

SÁNDOR VÖRÖS, MIKLÓS KERÉNYI

Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

BÉLA RALOVICH (corresponding author)

Hungarian Meat Research Institute
H-1453 Budapest, P.O.B. 17, Hungary

* Part of this paper was presented as a poster on the 10th International Symposium on Listeriosis, 22—26, August, 1988. Pécs, Hungary.

Stanley [5] isolated the Monocytosis Producing Agent (MPA) and stated that "R" strains yielded only a small amount of MPA. Muenker and Roots [6] studied the effect of incubation temperature (20 °C and 37 °C) and that of the CO_2 content of air on the virulence and monocytosis-producing ability of *L. monocytogenes* and found that cultivation at 37 °C resulted in a higher virulence and lower monocyte-producing ability than cultivation at room temperature. Virulence was increased andmonocyte-producing ability decreased when the cultures were grown under 10% CO_2. They concluded that the influence of incubation temperature was greater than that of the CO_2 content. Galsworthy and Fewster [7] observed different responsiveness to MPA in genetically susceptible or resistant mice.

The relationship between MPA production and virulence of *Listeria* strains is unclear at present. The aim of our study was to examine haematological reactions provoked by living *Listeria* strains of different virulence in rabbits after intravenous injection.

Materials and methods

Listeria strains of different virulence were used in the experiments. Data for the strains are listed in Table I. Biological and pathogenic properties of the strains were studied using the methods published by Ralovich [8].

Animals. Thirty two rabbits (2–4 kg) of both sexes were used.

Infection. The bacteria were cultured in nutrient broth at 37 °C for 18 h. After centrifugation they were suspended in physiological saline solution. Bacterial counts were controlled with a Specol photometer at 690 nm and with the plate count method. The infectious dose was 10^8 cells/kg body weight. The suspension was injected in the peripheral vein of the ear.

Sampling. Blood samples were taken from the peripheral vein of ear one day and immediately before, then 3 h, 1, 2, 3, 6 and 10 days after the injection of bacteria.

Haematological examinations. Quantitative and qualitative white blood cell counts were determined. Blood smears were stained with Giemsa for determination of monocytes. Lysosomal granules of neutrophils were counted after staining with Fast Green and Asure A as described by Pigarevskii. Haematological changes were presented as relative values in Figs 1–10. Results received before the injection were considered as a basis of comparison (100%).

Results

Figures 1–4 show haematological reactions of rabbits after injection of more or less virulent *L. monocytogenes* or *L. ivanovii* strains. All strains were beta-haemolytic on sheep blood agar, 3 out of them caused keratoconjunctivitis and 2 of them killed mice after intraperitoneal injection (Table I).

All strains provoked strong monocytosis. At the same time, the number of leukocytes was practically unchanged in 3 experiments (Figs 1, 3 and 4). When *L. monocytogenes* strain 87/5467 was used for the infection, one animal died on the 3rd day. A slight increase could be observed in the number of leukocytes in the surviving animal.

Table I

Characterization of Listeria strains

Strains	Properties														
	Pathogenicity for mice	Anton's test	Beta-haemolysis on sheep blood agar	Motility	Catalase	Arginine hydrolysis	Nitrate reduction	Methyl red test	Voges-Proskauer test	Mannitol	D-Xylose	L-Rhamnose	α-Methyl-D-mannoside	CAMP-test (*S. aureus*)	Serotype
L. monocytogenes 18	+	+	+	+	+	−	−	+	+	−	−	+	+	+	1/2a
L. monocytogenes 87/5467[1]	+	+	+	+	+	−	−	+	+	−	−	+	+	+	1/2b
L. ivanovii 4535	−	+	+	+	+	−	−	−	+	−	+	−	−	+	5
L. ivanovii non-motile strain[2]	−	±	+	−	+	−	−	−	−	−	+	−	−	+	5
L. innocua 10	−	−	−	+	+	−	−	+	+	−	−	+	+	W	4ab
L. innocua C644[2]	−	−	−	+	+	−	−	−	+	−	−	+	+	W	6a
L. murrayi G44[2]	−	−	−	+	+	−	+	+	+	+	−	+	+	−	
L. seeligeri 87/5575[1]	−	−	−	+	+	−	−	±	−	−	+	−	−	+	1/2b
L. seeligeri 87/5626[1]	−	−	−	+	+	−	−	−	−	−	+	−	−	+	6b
L. welshimeri 1830	−	−	−	+	+	−	−	+	+	−	+	−	−	−	1830

W = weak
[1] Strains sent by A. L. Courtieu from Nantes (Franze)
[2] Strains offered by H. P. R. Seeliger from Würzburg (FRG)

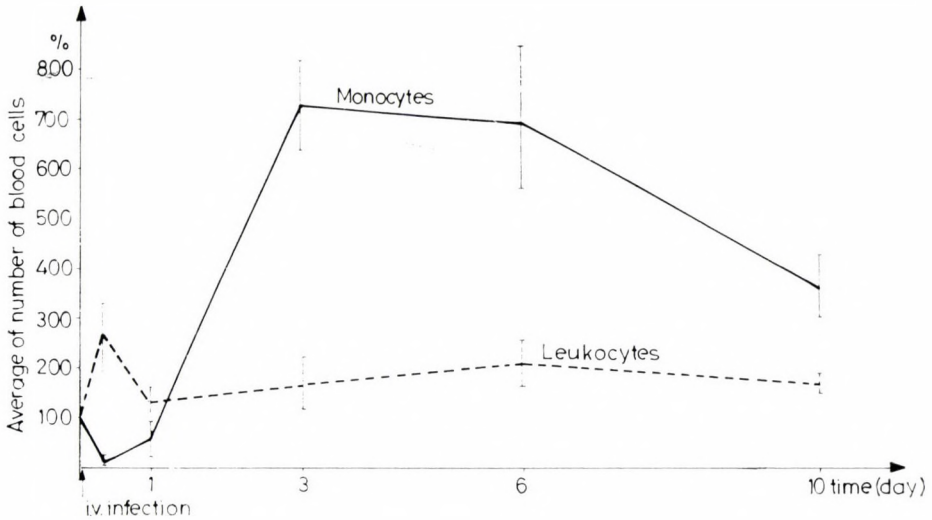

Fig. 1. Results of experiments with *L. monocytogenes* strain 18. Number of rabbits tested, 5

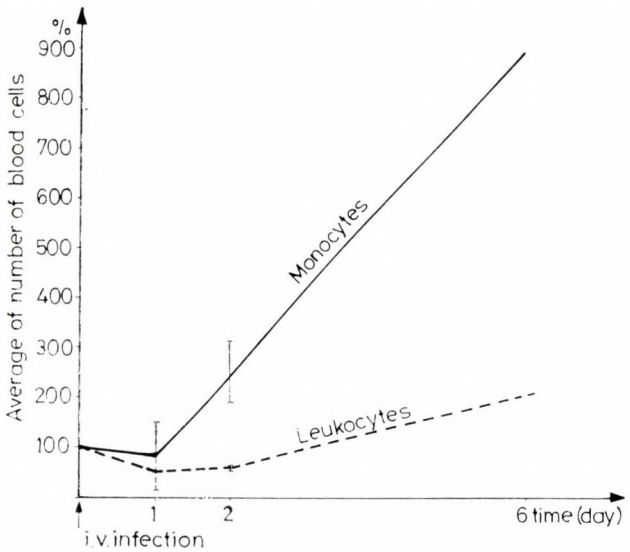

Fig. 2. Results of experiments with *L. monocytogenes* strain 87/5467. Number of rabbits tested, 2

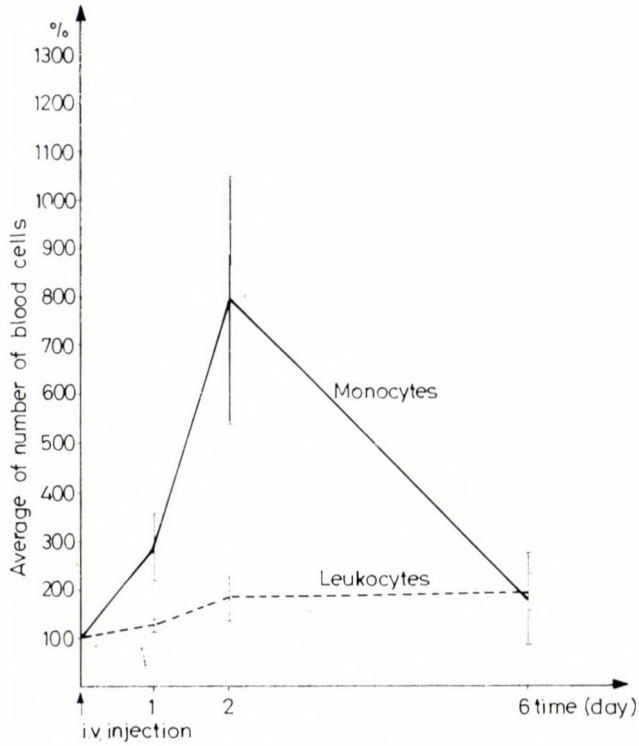

Fig. 3. Results of experiments with *L. ivanovii* non-motile strain. Number of rabbits tested, 2

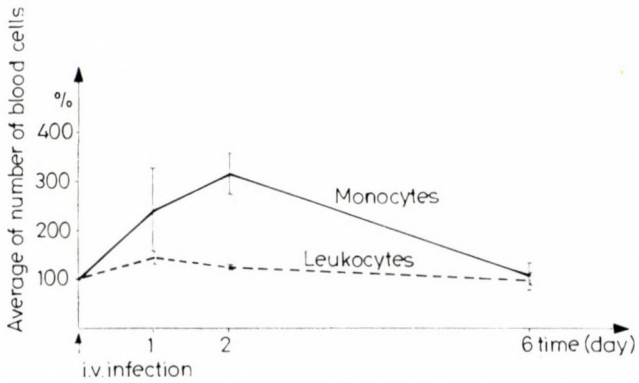

Fig. 4. Results of experiments with *L. ivanovii* strain 4535. Number of rabbits tested, 2

When the cell count was controlled 3 h after the infection (Fig. 1), an important increase of leukocytes and a marked decrease of monocytes were observed.

The effect of two *L. seeligerii* strains was also studied. Results of the animal tests can be found in Figs 5 and 6 as well as in Table I. *L. seeligerii* strains proved to be non-beta-haemolytic, they did not cause keratoconjunctivitis and could not kill mice, however, they produced monocytosis and even a slight leukocytosis.

Figures 7, 8, 9 and 10 show changes which occurred in the animals after injection of non-pathogenic *Listeria* strains. Inoculation of *L. innocua* strain C 644 resulted in some weak and late monocytosis as well as leukocytosis (Fig. 7). In case of *L. innocua* strain 10 only the very early (non-specific?) changes in the blood cell counts could be observed (Fig. 8). The *L. welshimeri* strain produced a light monocytic reaction for some days (Fig. 9). The *L. murrayi* strain produced an early leukocytosis and a late monocytosis (Fig. 10)

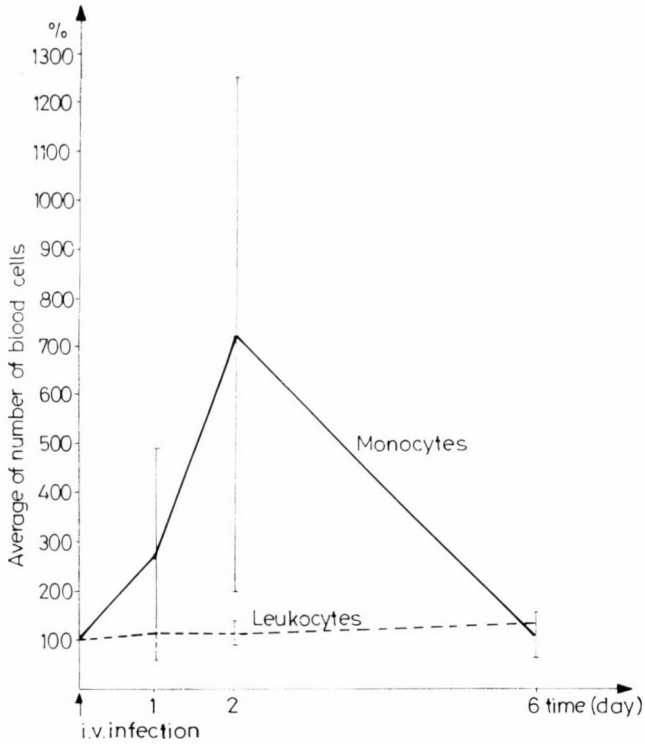

Fig. 5. Results of experiments with *L. seeligeri* strain 87/5626. Number of rabbits tested, 2

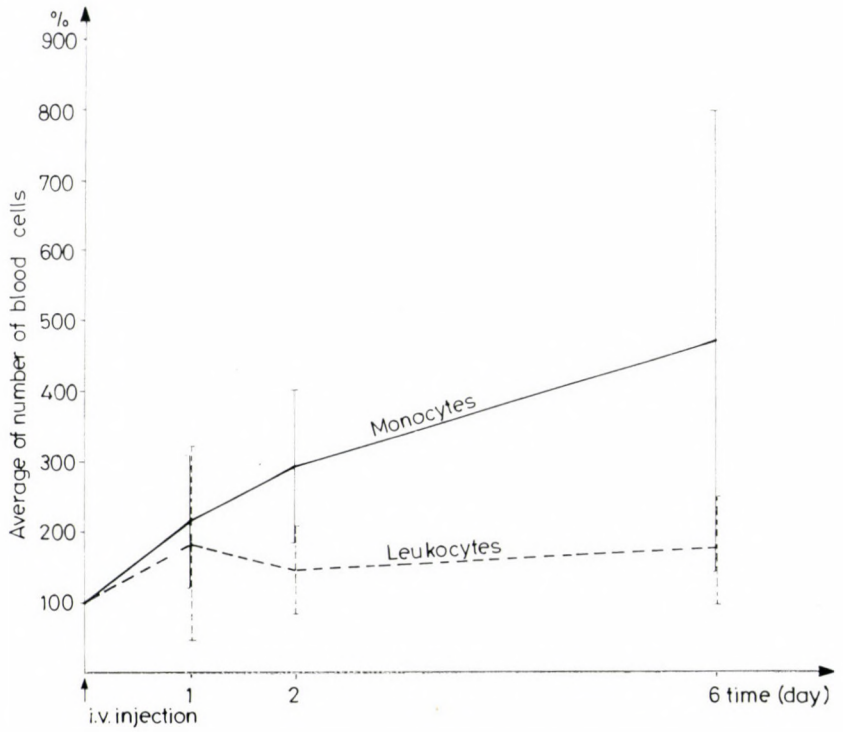

Fig. 6. Results of experiments with *L. seeligeri* strain 87/5575. Number of rabbits tested,4

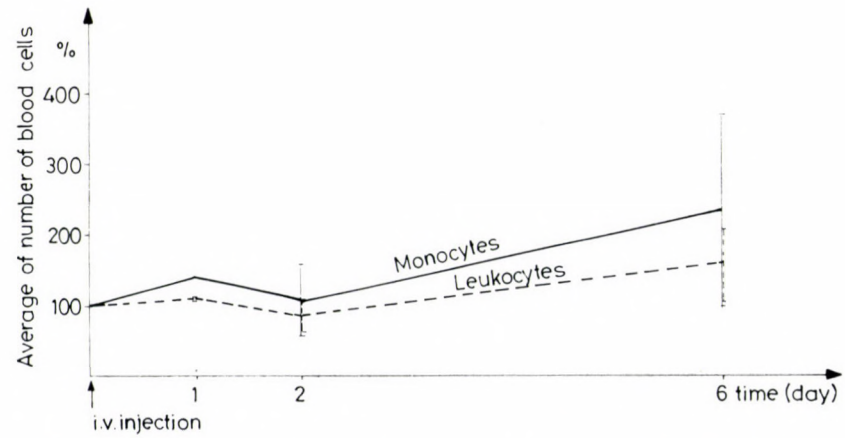

Fig. 7. Results of experiments with *L. innocua* strain C644. Number of rabbits tested, 4

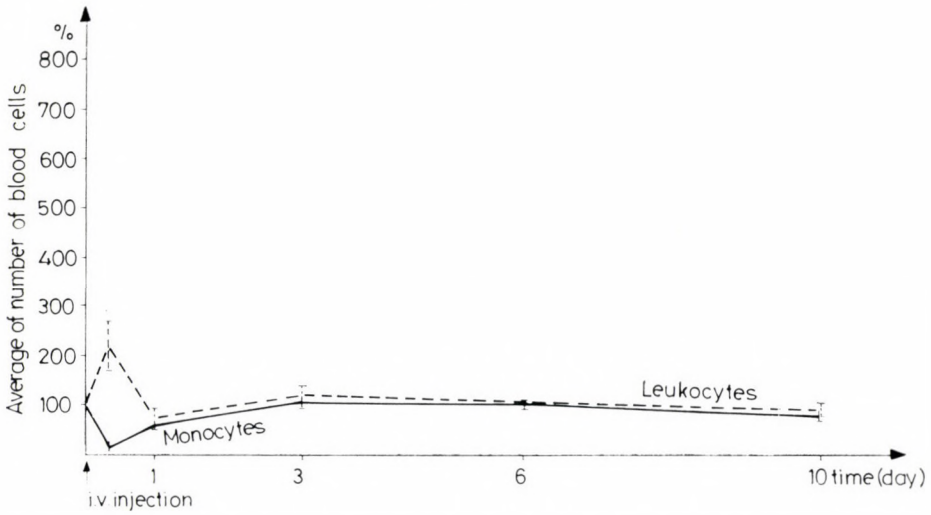

Fig. 8. Results of experiments with *L. innocua* strain 10. Number of rabbits tested, 5

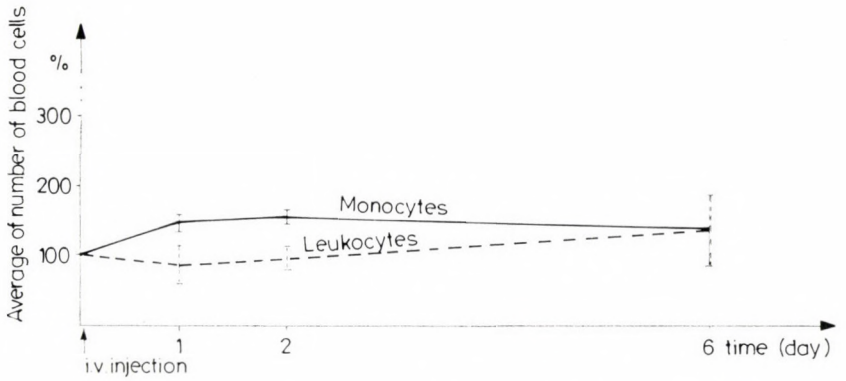

Fig. 9. Results of experiments with *L. welshimeri* strain 1830. Number of rabbits tested, 2

Table II shows the change in the number of lysosomal granules of leukocytes. A marked decrease was observed only on the first day in the case of control rabbits injected with physiological saline solution. When the non-pathogenic *L. innocua* strain 10 was injected, the decrease lasted for 3 days. By infection with the virulent *L. monocytogenes* strain 18 the same was observed during 6 days.

Discussion

On the basis of literary data, the relationship between monocytosis-producing activity and virulence of *Listeria* strains is not well understood.

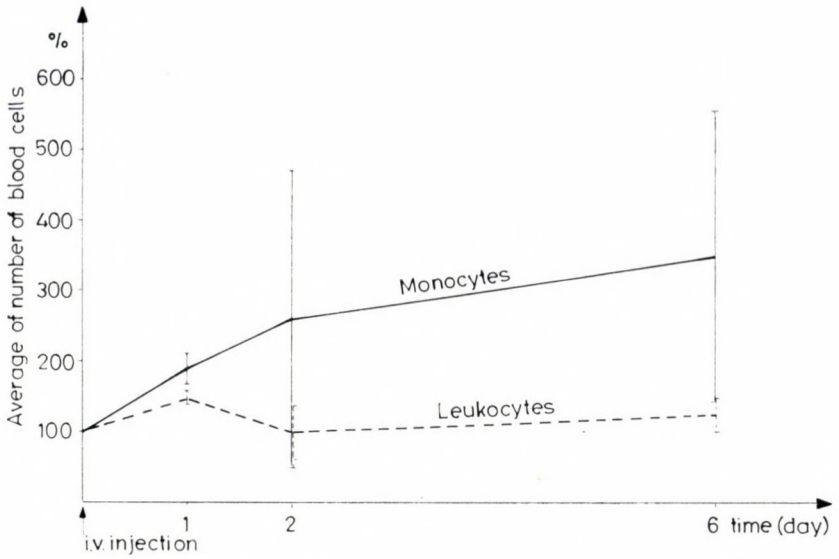

Fig. 10. Results of experiments with *L. murrayi* strain G44. Number of rabbits tested, 4

Table II

Effect of different treatments on the number of lysosomal granules in rabbit neutrophils

Treatment	Time of sampling and average number of lysosomal granules					
	Before the treatment	After the treatment				
		3 h	1 day	3 days	6 days	10 days
Injection with saline	67.7±0.95	58.0±1.23	60.5±n.c.	64.6±0.89	68.1±1.18	68.9±1.57
Injection with non-pathogenic *L. innocua* strain 10	63.6±1.13	53.9±1.02	49.1±n.c.	56.2±n.c.	59.8±0.73	62.3±n.c.
Infection with virulent *L. monocytogenes* strain 18	62.7±1.20	56.3±2.28	51.2±n.c.	51.4±n.c.	51.6±1.54	55.6±n.c.

Note: the number of lysosomal granules was counted in 100 single cells for each average
n.c. = not calculated

Stanley [5] observed that smaller quantity of MPA could be extracted from an "R" variant than from an "S" strain. In contrast, Muenker and Roots [6] found that MPA production and virulence were inversely related. In our experimental conditions all fully or partly virulent *L. monocytogenes* and *L. ivanovii* strains induced strong monocytosis in the infected rabbits. Besides these the two non-pathogenic *L. seeligeri* strains as well as the non-pathogenic

L. innocua C644 and *L. murrayi* strains also increased the number of monocytes in the animals. No changes occurred after the injection of *L. innocua* strain 10, and *L. welshimeri* strain 1830 caused only a weak reaction.

Our results are partly in accordance with Stanley's observation but it is also clear that there is no strong relationship among monocytosis producing activity and presence of other virulence markers (beta-haemolysis, keratoconjunctivitis causing ability, killing of mice) of *Listeria* strains. Recently Galsworthy [10] stated that "in the strains we tested those known to be virulent had MPA; those which were avirulent did not".

Changes observed in the number of granulocytes were only moderate except the early leukocytic reactions. The number of lysosomal granules of neutrophils decreased by each treatment. Nevertheless, a longlasting effect (6 days) was only observed in the animals injected with virulent *L. monocytogenes*. For the time being there is no explanation of this finding.

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MODULATING THE MICROBIAL COLONIZATION OF THE GASTROINTESTINAL TRACT BY ORAL ADMINISTRATION OF DEFINED *ESCHERICHIA COLI* STRAINS

I. INFLUENCING THE BIOTOPE BY MEANS OF METABOLIC DRIFT MUTANTS OF *ESSHERICHIA COLI*

R. Blatz aod K. Linde

Institute of Medical Microbiology, Karl Marx University, Leipzig, GDR

(Received January 22, 1988)

Through the selection of spontaneous metabolic drift mutants (selection marker Rif^R) of an *Escherichia coli* strain (O6:H2 Sm^R) of known settling capacity in conventional mice, it was attempted to obtain clones with positively optimized in vitro characteristics, which may exert a promotive influence upon the in vivo colonizing behaviour. Using 512 drift mutant strains (*E. coli* O6:H2 Sm^R Rif^R) we were able to establish positive optimizations, at an over-aleatory rate, for each of the in vitro characters tested (haemagglutination capacity of rabbit, guinea pig, and fowl erythrocytes; overgrowing power of mouse-adapted wild-type *E.coli* strains; formation of biomass with exclusive utilization of dextrose, lactose, fructose, adonitol, salicin, rhamnose, mannose; multiplying power; quantitative motility; and capacity to synthesize mucus). A higher settling rate (larger number of animals in which the test strain shared $\geq 50\%$ of the *Enterobacteriaceae* population than that obtained with the initial strain) could be established for one out of the 62 clones tested in vivo (a rise from 20% to 55%). The higher settling rate was associated with combinations of various functional parameters and not with an improvement of any of the individual functions. Despite the increase in settling rate relative to the number of experimental animals used in these studies, it was not generally possible to obtain a more than 3 days' dominance of the test strain within the lac^+ *Enterobacteriaceae*. This is considered o be due primarily to the incipient synthesis of secr etory IgA.

Initial recommendations for therapeutically and prophylactically influencing the microbial biotope ("intestinal flora") through the oral administration of living *Escherichia coli* were made by Nissle [1] as early as 1914. Quantitative aspects were not included in his concept. It was not until the mid-1950s that the previously purely empirical experience with the oral administration of living *E. coli*, lactobacilli, of bifidobacteria was assimilated, by Baumgartel and Zahn [2], to the present fund of knowledge. They were the first to include the host organism as a dialectic principle in approaches adopted to influence biotopes.

Since that time, results of research carried out into molecular biology, immunochemistry, and morphology enabled a large range of detailed knowledge to be acquired, which provides a better understanding of the unity of regulatory

R. Blatz, K. Linde
Institute of Medical Microbiology, Karl Marx University
Liebigstr. 24, Leipzig 7010, GDR

mechanisms of the host and of microbial capabilities with the resultant colonization of the biotope.

Basically, three factors can be considered to be essential, namely, first, competition in the nutrient substrate; second, competition in the binding receptor; and, third, specific immunoglobulins A that tend to regulate microbial colonization. Details are given in references [3–6].

In contrast, local defensive mechanisms such as phagocytosis, bactericidal complement action on the part of the host or colicin synthesis or pH sensitivity on the part of the microorganism probably play a secondary role in the colonization of intestinal mucosal surfaces. However, many questions remain to be answered. With few exceptions, the large number of previous publications reporting the results of experimental studies on colonization failed to functionally characterize the strains used (with respect to adhesiveness, capability of displacement and colonization), with the host's own regulatory factors also being ignored. A paper published by Freter [3] discusses possible advantages of selection for the colonizing strain — for example, increased adhesiveness — in connection with problems associated with the successful establishment of an exogenous strain in a settled biotope.

Internationally, there are no experimental studies on the specific optimization of biological parameters by genetic drift. From extensive studies undertaken by workers at this Institute [7–9] it is evident that the selection of metabolic drift mutants causes clones with an optimization of specific parameters to occur at an overaleatory rate. The term "metabolic drift" implies the mutative change in essential enzymes and functionally significant structures such as, for example, RNA-polymerase (transcription), gyrase (DNA replication), or ribosomal proteins (translation). Such metabolic drift mutants were obtained in the laboratory (by avoiding the natural process of evolution) via spontaneous or mutagen-induced, chromosomal antibiotic-resistance mutants [8]. Each of these resistance phenotypes splits up into a wide spectrum of clones. Of these, thirty to eighty per cent show a reduced degree of virulence. Depending on the particular genotype involved, this attenuation may vary between low and high levels. At the same time, there are strains with optimized biological performance parameters (in an order of about 1 in 1000 clones), e.g., reduced generation time [10] or increased haemolysin production in staphylococci [8]. On the whole, this so-called metabolic drift involves the systematic utilization of a natural phenomenon which serves the adaptation of micro-organisms to the changing environment.

Accordingly, strains that are potentially capable of setting up colonies might, despite the regulatory mechanisms of both microbes and hosts (receptor and substrate competition, induction of regulative s-IgA), be expected to show an improvement in their capability of colonization through the optimization of biological factors affecting the capacity to form colonies (e.g., fimbrial ex-

pression). Starting with this proposition, a strain of known colonizing behaviour (*E. coli* BL2 O6:H2) was used to determine the extent to which such parameters as quantitative haemagglutination capacity, generation time, formation of biomass with limited substrate supply, degree of motility, capability to synthesize mucus, and in vitro overgrowth of wild-type *E. coli* strain mixtures [1] may be optimized through drift mutation and [1,2] ascertain the in vivo effects of these optimized characteristics upon the colonizing capability of the drift mutants.

Materials and methods

Strains. Parent strain *E. coli* BL2 Sm^R (Sm^R spontaneous mutant) was selected at this Institute from a patient strain (*E. coli* O6:H2) obtained from Dr. H. Steinrück (Test Laboratory of Escherichia coli Infections, Institute of Infectious Diseases of Childhood, Buch Clinical Centre). After one single oral dose the strain remains detectable in the faecal flora of mice for a period of three months, but with a share of only 15% in *Enterobacteriaceae*. A share in the autochthonous population of *Enterobacteriaceae*, which is greater than or equal to 50%, is obtained for up to three days in 20% of the experimental animals.

E. coli clones BL2 Sm^R Rif^R (C1–C512) were spontaneous mutants obtained on nutrient agar with 100 μg of rifampicin/ml.

Parameters tested. Haemagglutination. Guinea pig, rabbit and fowl erythrocytes were used for semiquantitative slide test; quantitative testing was carried out with strains showing haemagglutination equal to or stronger than that of the parent strain.

Extinction measurement as generation-time equivalent. Starting with 18-h broth cultures, broth suspensions containing 10^7 cells per ml were made. After 2–3 h incubation at 37 °C, measurement of the clones to be tested was done when the parent strain *E. coli* BL2 Sm^R had reached an extinction of 0.1 in the "Spekol" spectrophotometer (VEB Carl Zeiss, Jena) with ER1 test-tube attachment at 560 nm.

Utilization of substrate with respect to formation of biomass. After 3 h incubation at 37 °C in a minimal medium supplemented with the respective test substrate (dextrose, lactose, fructose at a final concentration of 0.5%; adonitol, mannose, rhamnose, salicin at a final concentration of 0.1%). Comparative extinction measurements (650 nm) were made between the initial strain and the clones derived therefrom.

In vitro overgrowth power. In 1 ml aliquots of 1% peptone water 2×10^7 germs of the clone to be tested and 2×10^7 germs of a wild-type *E. coli* strain mixture obtained from different mice were suspended and incubated at 37 °C. After 5 and 24 h, separate determinations of the number of live germs were made for clone and wild-type strain moieties. A strain-specific multiplication factor is obtained by dividing the 24-h value by the 5-h value. By relating the multiplication factors of the individual clones to that of the wild-type strain mixture, it is possible to obtain a quotient that shows whether the clone tends to multiply more rapidly ($Q \geq 1$) or more slowly ($Q \leq 1$) than the wild-type strain mixture.

Quantitative motility. Starting with 18-h broth cultures, U tubes were inoculated. After 4 and 6 h incubation, the motility fronts were marked and the difference (in mm) was recorded. The distance covered by the clones in 2 h was related as percentage of the parent strain's performance.

Capability of in vitro formation of mucus. All strains were placed on a maltose-containing medium [11]. After 14 days storage at room temperature, they were qualitatively checked for formation of mucus.

In vivo testing of selected clones. To groups of two animals (male ICR mice weighing 18–22 g, kept under conventional conditions and supplied with standard feed), the test strains were administered once in drinking water (2×10^9 germs/ml at a daily dose of approximately 1×10^{10} germs/animal). The numbers of live germs/g of freshly excreted faeces were separately determined for each animal on the third, seventh, and tenth days of experiment for the antibiotic-resistant test strain and the antibiotic-sensitive flora of lac^+ *Enterobacteriaceae*. To evaluate the results, a quotient was calculated from the number of animals in which the test strain reached a 50% share in the lac^+ *Enterobacteriaceae* population in 1–3 days and from the total number of animals tested.

Results

All of the 512 clones selected according to size of colony were tested for their haemagglutination capacity (guinea pig, rabbit, fowl erythrocytes), multiplication power, and capacity for mucus formation. The best and worst clones were selected for each character in accordance with these results. These 62 strains were additionally characterized for the remaining three parameters and compared in vivo with the initial *E. coli* BL2 Sm^R strain. In this manner functional improvements could be selected for the individual test parameters at an overaleatory rate (see Tables I and II).

Table I

Functional changes in 512 E. coli BL2 Sm^R Rif^R drift mutants in comparison with the initial E. coli BL2 Sm^R strain

Function	Percentage of functional changes compared to the initial *E. coli* BL2 Sm^R strain		
	Improvement of function	Unchanged function	Deterioration of function
Haemagglutination			
GPE	1	2	97
RE	1	13	86
FE	2	17	81
Formation of mucus	41	59	—

GPE, guinea pig erythrocytes; RE, rabbit erythrocytes; FE, fowl erythrocytes

Table II

Functional changes in 62 selected E. coli BL2 Sm^R Rif^R drift mutants in comparison with the initial E. coli BL2 Sm^R strain

Function	Percentage of function changes compared to the initial *E. coli* BL2 Sm^R strain		
	Improvement of function	Unchanged function	Deterioration of function
Overgrowing power	52	3	45
Formation of biomass with utilization of			
Dextrose	29	10	61
Lactose	14	6	80
Fructose	16	24	60
Raffinose	64	18	18
Salicin	37	35	27
Adonitol	47	43	10
Mannose	42	18	40
Motility	53	3	44

As it might be expected, not only clones with improved functions (positive optimization), and with unaltered functions (neutral optimization), but also clones showing deteriorations of biological parameters (negative optimization) could be obtained.

The in vivo results are given in Tables III and IV. Table III shows all those clones that reached, in at least one experimental animal, a short-term share of $\geq 50\%$ in the population of *Enterobacteriaceae*. The remaining strains were not considered. The clones were ranked in accordance with the in vivo results and complemented with their in vitro parameters. Independently of the frequent in vitro improvements in the functions of all test strains, optimization

Table III

Comparison of parent strain with its drift mutants which after one single oral administration temporarily shared more than 50% of the intestinal lac⁺ Enterobacteriaceae population

Strain	Share of reactive animals*	HA			Generation time	Overgrowth	Formation of biomass with utilization of							S	Motility
		GPE	RE	FE			D	L	F	R	S	M	A		
C48	*0.55*	+	+	=	−	+	−	−	−	+	+	+	+	=	−
C50	0.33	−	−	−	−	−	−	−	−	+	+	+	+	=	+
C59	0.33	=	=	=	−	+	−	−	−	+	+	=	+	=	+
C65	0.23	−	+	−	−	+	−	−	−	+	+	−	=	+	−
C33	0.2	−	−	−	+	+	−	−	−	+	+	+	+	=	+
C37	0.19	−	−	−	−	−	+	−	+	=	=	+	+	=	+
C57	0.18	−	−	−	−	+	−	−	−	+	+	=	=	+	−
C30	0.17	−	−	−	−	+	−	−	=	−	−	−	=	=	−
C34	0.17	−	−	−	+	+	−	−	−	−	=	+	=	+	+
C39	0.17	−	−	−	−	+	+	−	+	−	+	−	=	+	−
C54	0.17	−	−	−	−	+	+	=	−	=	=	=	=	=	+
C55	0.17	−	−	−	−	+	=	=	−	=	=	=	=	=	+
C58	0.17	=	=	=	+	+	−	−	−	+	+	=	+	=	+
C63	0.17	−	=	+	+	−	−	−	−	+	+	+	+	=	−
C67	0.17	−	+	=	+	+	=	+	=	+	+	−	+	=	+
C53	0.16	−	−	=	−	−	=	−	−	=	−	−	=	+	−
C38	0.12	−	−	−	−	+	+	−	+	+	+	+	+	+	−
C13	0.1	+	−	−	−	−	−	−	=	−	+	=	−	+	−
C40	0.1	−	−	=	−	+	+	−	−	+	+	+	+	+	−
C45	0.1	+	−	=	−	+	+	−	−	+	+	+	+	+	−
C46	0.1	−	−	−	+	+	+	−	=	+	+	+	+	=	−
C52	0.1	−	=	=	−	+	=	−	−	=	−	−	−	+	+
Parent**	0.21	=	=	=	=	=	=	=	=	=	=	=	=	=	=

⁺ Improvement of function; − deterioration of function; = unchanged function; HA, haemagglutination; S, synthesis of mucus; GPE, guinea pig erythrocytes; RE, rabbit erythrocytes; FE, fowl erythrocytes; D, dextrose; L, lactose; F, fructose; R, raffinose; S, salicin; M, mannose; A, adonitol.

* Reactive animals carried the test strain for up to 3 days sharing $\geq 50\%$ of the lumenal lac⁺ *Enterobacteriaceae* population.

** Parent strain: *E. coli* BL2 Sm^R

of the limited colonizing power is clearly perceptible for one clone (C48) only. While the parent strain (*E. coli* BL2 Sm^R) reaches, in about one fifth of the animals, a share of $\geq 50\%$ in the flora of lac$^+$ *Enterobacteriaceae* for a period of 1 to 3 days, this is about one half in the case of C48 (see Table III, figure printed in italics).

According to Table III the result of in vivo colonizing seems to be independent of the in vitro improvement in individual characters. For each of the individual characters, it is possible to find examples of positive optimization which have no influence whatsoever upon animal experiments. The combination of the parameters improved for C48 is not repeated within the clones undergoing testing.

In Table IV an attempt was made to establish a rank order of the significance of different in vitro improvements in functions for the in vivo test result. All of the clones with improved in vitro parameters were evaluated using a quotient of the number of samples with a test strain moiety greater than or equal to 50% relative to the quantity of all faecal samples tested (on the third, seventh, and tenth days of experiment) for each strain. These results were arranged in groups according to the improved individual in vitro functions. Accordingly, haemagglutination of rabbit erythrocytes would take first place in significance for the quality of the in vivo result to be expected,

Table IV

Number of samples with a share of more than 50% of the drift mutant strain in the lac$^+$ Enterobacteriaceae population relative to the total number of samples of all drift mutant strains with improved in vitro functional parameters in comparison with the initial E. coli BL2 Sm^R strain*

No. of strains with improved in vitro functions	In vitro functions with improvements over the initial strain	Samples with a share of more than 50% of the drift mutant strain in the population of lac+ *Enterobacteriaceae*		
		No. of samples with more then 50%	Total No. of samples tested	Share of samples with more than 50%
5	Haemagglutination of RE	10	40 △	25%**
5	Haemagglutination of GPE	7	43 △	16%**
23	Salicin splitting	30	213 △	14%**
34	Overgrowth	35	294 △	12%**
26	Mannose splitting	24	216 △	11%**
29	Adonitol splitting	25	235 △	11%**
40	Raffinose splitting	31	295 △	10%**
27	Formation of mucus	22	249 △	9%
33	Motility	21	246 △	8%
18	Dextrose splitting	12	145 △	8%
	the intial strain			
26	Multiplying power in comparison with	8	172 △	5%
	the initial strain			
5	Haemagglutination of FE	1	30 △	3%

* Samples taken on the 3rd, 7th and 10th days
** Containing drift mutant C48

followed by guinea pig erythrocytes, salicin splitting, and overgrowth capacity in vitro. The influence of an increased rate of multiplication upon the result of colonizing is surprisingly small. The present results (increase in the rate of colonization — share of $\geq 50\%$ in the population of lac$^+$ *Enterobacteriaceae* for one to three days — from 20 to 55% of the experimental animals) show that it is, in principle, possible to use metabolic drift and screening-like selection of suitable clones in order to find strains with an optimization of the biologically multifactorially controlled capacity for successful colonization.

Discussion

As is apparent from Tables I and II that metabolic drift mutations can be used to optimize various biological functions occurring at an overaleatory rate, it being possible for the positive optimization of a particular parameter to be simultaneously correlated with the negative optimization of another parameter. However, it is important to note here that the true order of functional optimization — which is 1 in 1000 as reported in the literature [12] — cannot be derived from our material inasmuch as only colonies equal in size or larger than those of the initial *E. coli* BL2 Sm^R strain (about 10% of all strains that occurred after spontaneous mutation) were selectively tested for the parameters referred to above. It is only the overaleatory frequency and, thus, the confirmation of the working hypothesis "metabolic drift improvement" that can be demonstrated convincingly.

Proceeding from this possibility of selecting strains of positively optimized functional parameters with potential effects upon colonizing results, we anticipated an improvement of in vivo results compared with the parent strain *E. coli* BL2 Sm^R. However, the relatively large number of optimized individual in vitro characters contrasts with a rather small number of clones having a comparable colonizing power, or only with one clone having an improved short-term colonization capacity.

Colonization is — on the part of the microorganism alone — to be considered a multifactorially influenced variable. It results from the germ's capability to reach an adequate receptor, adhere by agents known as adhesins, multiply, and supplant, through advantages of selection, the original local flora until the specific immune response by the host organism begins to occur. It follows from this that the result of colonizing is affected by a combination of essential characteristics rather than an individual character. It is at best possible to set up a list showing the rank order of the potential significance of the individual parameters tested by us for the capacity to form colonies (see Table IV).

The extent to which this is a random coincidence of characters is an open question inasmuch as the parameters tested by us (and which are referred

to above) represent only a limited number of functions that might have an effect upon the result of colonizing.

Aside from a few exceptions [13–15], the haemagglutination capacity is regarded as a fimbrial and, therefore, attachment equivalent. Accordingly, strains sharing $\geq 50\%$ of the lac$^+$ *Enterobacteriaceae* population had frequently a positively optimized, mannose-sensitive haemagglutination capacity of rabbit erythrocytes (and guinea pig erythrocytes).

In spite of the widely recognized significance of substrate competition for the makeup of a microbial biotope [3, 16, 17], there is a lack of studies into the species-related importance of individual substrates to the colonization of the gastrointestinal tract. It is clear from our results that a potential influence of in vitro salicin utilization upon the in vivo colonizing result cannot be ruled out. On the whole, the relations between substrate utilization and colonizing capacity should be considered to be dependent upon the dietetics of the particular experimental conditions.

For a determination of the in vitro overgrowth capacity it is necessary to include several functional parameters (substrate utilization, substrate competition, multiplying power) in a test system. Nevertheless, the promotive influence of a positively optimized overgrowth power of our in vitro system on the in vivo result (Table IV) was negligible.

In the literature, the in vivo generation time of idigenous intestinal flora is reported to be in the order of 10 h [18]. We considered the necessity of a reduced generation time to be prerequisite to a rapid settlement of orally administered colonizing strains, with no consideration being given to the multiplication-controlling regulatory mechanisms in the settled biotope. Although the gene expression under in vivo conditions is, in general, different from that under in vitro conditions, yet the present results showed the influence of a high in vitro multiplication rate upon the colonizing capacity of drift mutants to be surprisingly small. Evaluation of animal experiments by the relation of "in vitro multiplication rate to in vivo colonizing result" (unpublished) alone even suggests a negative correlation. There is a possibility that, in the microorganism, restrictions of other functions relating to the colonizing capacity tend to favour a reduction in generation time. This assumption may be supported by information learned from the literature [3, 19].

The shorter the generation time of a colonizing strain, the earlier is, in general, the time at which the strain reaches concentrations that carry an immune stimulus for the synthesis of regulative s-IgA and, consequently, its biotope elimination (or reduction). Persistence over weeks and months is possible only if an exogenous germ multiplies at a rate similar to that of the local flora, stimulating a roughly identical antibody synthesis [20].

Analogous to the fundamental investigations conducted by Costerton et al. [21, 22], we expected an in-vitro-detectable synthesis of mucus and an

increased in vitro mobility to have an at least promotive influence upon the result of colonizing. However, both characters are of secondary importance in our results.

The results obtained show that there is a possibility of optimizing the strain with respect to its colonizing potential — measured by the lumenal flora — through the selection of metabolic drift mutants. An isolated improvement of the individual functions considered in these studies has no influence whatsoever upon the result of colonizing since this is determined multifactorially. It was not possible to convincingly demonstrate a several days' dominance of the clones within the population of lac$^+$ *Enterobacteriaceae*. It is extremely probable that the induction of regulative secretory IgA is responsible for this [23].

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MODULATING THE MICROBIAL COLONIZATION OF THE GASTROINTESTINAL TRACT BY ORAL ADMINISTRATION OF DEFINED *ESCHERICHIA COLI* STRAINS

II. INFLUENCING THE BIOTOPE BY MEANS OF GEOGRAPHICALLY UNRELATED *ESCHERICHIA COLI* STRAINS

R. Blatz, K. Linde and R. Schröder

Institute of Medical Microbiology, Karl Marx University, Leipzig, GDR

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An effective increase in the rate of colonization can be accomplished, in analogy with the pathogenetic mechanism of what is known as travelers' disease, through the administration of geographically unrelated strains. As compared to *Escherichia coli* strains isolated in the GDR, metabolic drift-optimized and Ethiopian strains showed an increase from 20 to 50%. It was not, however, possible to maintain a $\geq 50\%$ share in the population of *Enterobacteriaceae* over a period of more than up to three days. Immunologic mechanisms are considered to be responsible for this.

The previous paper in this series [1] presented studies on improving in vivo colonization through metabolic drift mutations by optimization of particular in vitro parameters with potential effects upon in vivo results. Compared with the parent strain (*Escherichia coli* BL2 Sm^R), an increase in the rate of colonization (percentage of animals in which the strain reached $\geq 50\%$ of the lac^+ *Enterobacteriaceae* relative to the total number of animals tested could be demonstrated. After one single oral administration (1×10^{10}), in 1 out of the 62 selected metabolic clones (*E. coli* BL2 Sm^R Rif^R C1–C62) the rate of colonization rose from 20 to 50%. Despite the improved rate of colonization, predominance of the orally administered strain in the *Enterobacteriaceae* population lasted not longer than op to three days.

Since so-called natural antibodies have been observed to occur in the intestinal mucus [2], an inhibitory influence upon an increase of particular moieties of the microbial population cannot be ruled out. The higher the rate of additional specific antigen contacts, the higher will be the effectiveness of this immunological barrier.

In travelers' disease it was observed that tourists from industrialized countries often became ill from the same enteropathogens that cause childhood

R. Blatz, K. Linde, R. Schröder
Institute of Medical Microbiology, Karl Marx University
Liebigstr. 24, Leipzig 7010, GDR

diarrhoea in developing countries [3]. From this observation it is possible to suggest, that persons with prolonged exposure (esp. the natives) acquire immunity and are at lower risk of developing travelers' diarrhoea [4]. Further it seems likely that in analogy to humans also conventional mice in permanent contacts with *E. coli* of our latitudes should be more susceptible to implantation of Ethiopian than of German *E. coli* strains. This preconception was to be examined.

Materials and methods

Strains. A total of 38 Ethiopian *E. coli* strains (G1–16; H1–22) obtained from different patient materials were used. The strains were made available by Dr. Höhne, Institute of Medical Microbiology, Martin-Luther-University in Halle. All of the strains showed consistent biochemical reactions. Serotype classification of 32 strains was not possible by the use of commercially available diagnostic sera. Six strains were typable as follows: O6:H$^-$ (strain H4), O8:H spont.aggl. (strain H14), O59:H19 (strain H15), O91:H17 (strain G12) and two of them were serotypes O125:K11. Serotyping was performed by Dr. H. Steinrück, Reference Laboratory of Escherichia Coli Infections, Institute of Childhood Infections, Berlin-Buch Clinical Centre.

Before use is animal experiments, the strains were selected for Sm^R or Rif^R (streptomycin and rifampicin resistance) in order to obtain identification and metabolic drift markers.

Colonization experiments. To groups of three male ICR mice each of the Ethiopian *E. coli* strains were intragastrically administered using one dose of 1×10^{10} live germs per probe. On the third, seventh, and tenth days of experiment, faecal samples were examined for the presence of the administered strain and of other *Enterobacteriaceae* per gram of excreted material. The number of animals in which colonizing strain reached $\geq 50\%$ of the lac$^+$ *Enterobacteriaceae* population was recorded. All strains that reached or exceeded this value were retested using the same experimental setup. Also, the mean shares in the lac$^+$ *Enterobacteriaceae* were determined on the third and tenth days of experiment for all those strains that temporarily exceeded the 50% population moiety. (Individual results of all faecal samples that were examined on the third and tenth days of experiment were entered into the calculations.)

Results

Out of the 38 Ethiopian strains examined, 21 reached temporarily, after one single oral administration a share of $\geq 50\%$ in the lumenal lac$^+$ *Enterobacteriaceae* population. For each of these strains, the said population moiety was reached in at least one third of the experimental animals. In one case (*E. coli* G12 Rif^R), it was reached in all of the experimental animals tested. These results contrast with those that were obtained using selected native strains (unpublished) and metabolic drift mutants [1]: strains with an optimum rate of colonization reached dominant shares in the biotope ($\geq 50\%$ of lac$^+$ *Enterobacteriaceae*) max. in one fifth (*E. coli* BL2 Sm^R) or in one half (*E. coli* C48 Sm^R Rif^R) of the experimental animals, respectively. That is, an increase in the rate of colonization had already been obtained through metabolic drift optimization, then this could be improved further through the use of geographically unrelated *E. coli* strains (Table I). The observation on the third day of ex- periment that the share in the population of the Ethiopian strains was greater than that of the na itve strains was also attributable to this increased

Table I

Comparison of the colonization activities of native (GDR), metabolic drift-optimized and geographically unrelated (Ethiopian) E. coli in conventional mice

	Source of strains		
	GDR	GDR, metabolic drift-optimized	Ethiopia
Total No. of strains	12	62	38
Rate of colonization* of the top strains	20%	55%	100%
No. of mice	33	11	10
Percentage and number of strains reaching a colonization rate 50% over up to 3 days	17% (n=2)	1,6% (n=1)	26% (n=10)
Colonization rate for all strains at intervals after oral administration	3 days 21%	23%	36%
	7 days 14%	7%	13%
	10 days 11%	4%	4%

* Percentage of animals in which the administered strain reached 50% of the lac$^+$ *Enterobacteriaceae*

rate of colonization. On the tenth day of experiment, however, differences were hardly detectable. As in the case of native or metabolic drift-optimized *E. coli* strains, it was not possible for an up- to three-day dominance to be detected within the *Enterobacteriaceae* population.

Discussion

That the colonization of geographically foreign *E. coli* takes place at a higher rate than that of local strains has been supported by experimental evidence. Especially strain G12 showed a marked improvement of the rate of colonization over that of both native and metabolic drift-optimized strains. This may be explained immunologically. The lipopolysaccharide of serogroup O91 obviously plays no role in our latitude and has never been obtained from clinical specimens as a relevant causative agent: nothing is known about cross antigenicity with frequently occurring native serotypes [5].

It is acceptable to assume that in a distinct geographic area, the *E. coli* population both in mice and in man is recruited from bacteria different in characteristics and frequency of serotypes. For human beings travelling in geographically foreign areas, first contacts with ETEC strains, nonendemic in their home countries, are believed to be one of reasons for the so-called travellers' disease [3, 4]. A low level of naturally acquired immunity to endogenous serotypes will prevent diseases in natives. In foreigners without previous immunostimulation by these serotypes the first contacts may lead to intra-

intestinal accumulation, a short time colonization and furthermore, clinical disease.

Accordingly it is likely to assume that the experimental animals had less likely acquired any local immune protection to Ethiopian strains than to *E. coli* strains isolated in GDR. It may be assumed that in primary contacts a colonization would give rise to a synthesis of specific s-IgA during the time that is required generally to obtain an effective IgA concentration on the mucous membrane.

Although we were able to convincingly demonstrate a marked increase in the rate of colonization as compared with the metabolic drift-optimized mutants (see Table I), yet it was not, in accordance with all of the previous results [1], possible to realize in individual experimental animals more than a three-day dominance and, consequently, an effective displacement by the administered strain of the local lac$^+$ *Enterobacteriaceae* population. We assume that this phenomenon is caused by the induction of secretory IgA: immune response counteracts adherence — a prestep for colonization — as an antagonistic principle. The incipient synthesis of s-IgA counteracts further colonization through receptor blocking [6] and detachment of already closely attached microorganisms [7]. Accordingly, the foreign germs are either inhibited in their multiplication or eliminated completely. The fact that shares in the biotope are markedly greater on the third than on the tenth day of experiment may also indicate effective immunological defenses.

Accordingly, a longer-term displacement of the local flora would not occur by the settlement of a live strain that is capable of colonizing, but perhaps by the successive aquiring of immunologically unrelated, different serotypes, when the respective immune response to the previously settled strain becomes effective.

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BIOSYNTHESIS, PURIFICATION AND SOME PROPERTIES OF EXTRACELLULAR PHYTASE FROM *ASPERGILLUS CARNEUS*

M. GHAREIB

Biology Department, Faculty of Education, Ain Shams University, Roxy, Cairo, Egypt

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Aspergillus carneus (Van Tiegh) Blockwitz synthesized moderate quantities of phytase in culture filtrate. Maximal enzyme yield was obtained after eight days incubation statically in a medium containing sucrose and K_2HPO_4 in a C/P ratio of 591.8/1 with 0.1% corn steep liquor (CSL) as the sole source of nitrogen. Substitution of $(NH_4)_2SO_4$ with certain amino acids decreased phytase yields significantly. Addition of fish or soybean meal to the nitrogen-free medium failed to enhance phytase production. The enzyme was purified about 43-fold from the culture filtrate by precipitation with acetone, gel filtration through Sephadex G–75 and ion exchange chromatography on DEAE-cellulose. Activity of both crude and purified phytase was influenced greatly by changing of pH and reaction temperature: maximum activity of the crude enzyme occurred at 35 °C and pH 5.6, whereas that of the purified preparation at 40 °C and pH 5.6. The pure enzyme was found stable between pH 5.6–6.2. About 68% of the enzyme activity was lost by heating at 45 °C for 60 min. The pure phytase retained its activity over a long period when stored at 4 °C.

Phytases are a group of enzymes of prime importance in converting phytate-phosphorus to a form available by monogastric animals [1, 2]. Production of phytase by fungi dates back for few decades ago. Different species of *Aspergillus* were the most studied organisms in this respect [3–10].

This work was initiated to study phytase production by a species of *Aspergillus*, to my knowledge not recorded in this regard before.

Materials and methods

Organism and cultivation. Aspergillus carneus (Van Tiegh) Blockwitz used in this work was isolated from Egyptian soil and identified by the Commonwealth Mycological Institute. The organism was grown on Czapek agar medium and monthly subcultured. Inoculum was a spore suspension obtained from 7-day-old cultures. The fermentation medium had the following composition: sucrose, 5 g; $(NH_4)_2SO_4$, 0.3 g; $Na_2HPO_4 \cdot 12H_2O$, 0.03 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeCl_3 \cdot 6H_2O$, 0.0002 g and $ZnSO_4 \cdot 7H_2O$, 0.0044 g per 100 ml. Erlenmeyer flasks of 250 ml capacity each charged with 50 ml of the medium were used throughout this work. The flasks were sterilized, left to cool and the pH was initially adjusted to 6. The basal medium was modified by replacing $Na_2HPO_4 \cdot 12H_2O$ with equimolecular weights of various phosphorous sources. When the effects of nitrogen sources other than $(NH_4)_2SO_4$ were studied, these were used in those amounts necessary to supply the same weight of nitrogen as was contained in

MOHAMED GHAREIB
Biology Department, Faculty of Education, Ain Shams University
Roxy, Cairo, Egypt

$(NH_4)_2SO_4$. After inoculation, the cultures were incubated statically at 30 °C. Different estimations were carried out at the end of incubation period.

Phytase activity was determined as described [11] using calcium phytate as a substrate. Liberated orthophosphate was determined by the method described in reference [12]. One unit of enzyme activity is tentatively defined as the amoung of the enzyme which liberates one μg of inorganic phosphorous per minute.

Protein content of the cell-free culture filtrate was determined by the method of Lowry et al. [13].

The procedure of enzyme purification was as follows. Pulverized ammonium sulphate was slowly added to the crude cell-free filtrate to obtain 0.1–1 saturation. In another trial different cooled volumes of acetone and low molecular weight alcohols were separately added to the cell-free culture filtrate. Materials which precipitate when using acetone in a volume of 5:1 contained most of the extracellular phytase activity. The resulting precipitate was suspended in 0.05 M Tris–HCl buffer (pH 8.2) and applied to a Sephadex G–75 column. The packed column was equiliberated with the previous buffer. Further purification of the enzyme was obtained by ion-exchange column chromatography. Diethylaminoethyl (DEAE)-cellulose was prepared according to the procedure of Peterson and Sober [14]. The enzyme was eluted by stepwise addition of 5 ml portions of increasing molarities (0.1–1 M) of NaCl in 0.05 M Tris–HCl buffer. The active fractions were pooled, concentrated by lyophilization, stored at 0 °C, and used throughout the work on the properties of the enzyme.

Results and discussion

Many species of *Aspergillus* were recorded as a good producers of phytase [3–10]. *A. carneus* was found suitable for biosynthesis of phytase extracellulary. The effects of some culture conditions were studied in an attempt to develop a medium yielding the maximum phytase production. The results (Table I) show that of the studied phosphorus compounds only K_2HPO_4 produced a high stimulating effect on phytase production. The ratio of carbon to phosphorus (C/P) affects the enzyme biosynthesis considerably (Table II). Maximum enzyme content amounting to 136 units/100 ml was determined at a moderately low C/P ratio. This is in coincidence with the previous findings of Youssef et al. [9] and Shieh and Ware [11]. Data represented in Fig .1 reveal that the enzyme reached its maximum accumulation after 8 days of incubation at 30 °C. Substi-

Table I

Effect of different phosphorus sources on phytase biosynthesis by A. carneus

Phosphorus sources	Protein (mg/100 ml)	Enzyme activity (units/100 ml)	Specific activity
$NH_4H_2PO_4$	351.5	75.2	0.21
$(NH_4)_2HPO_4$	484.8	73.6	0.15
KH_2PO_4	448.4	67.6	0.15
K_2HPO_4	533.3	104.0	0.19
NaH_2PO_4	642.4	76.8	0.12
$Na_2HPO_4 \cdot 12H_2O$	375.7	74.4	0.19
Sodium phytate	424.2	75.2	0.18

Incubation period, 7 days

Table II

Phytase biosynthesis by A. carneus as influenced by different C/P ratios

K_2HPO_4 concentration (g/100 ml)	Sucrose concentration			
	5%		7%	
	C/P	units/100 ml	C/P	units/100 ml
0.005	2367.3	88.2	3314.2	64.3
0.010	1183.6	116.8	1657.1	81.6
0.015	789.1	127.2	1104.7	96.6
0.020	591.8	136.0	828.6	108.8
0.025	473.5	119.4	662.8	84.6
0.030	394.5	105.6	552.4	70.7

Table III

Phytase biosynthesis by A. carneus in the presence of different nitrogen sources

Nitrogen source	Protein (mg/100 ml)	Enzyme activity (units/100 ml)	Specific activity
$(NH_4)_2SO_4$	945.4	130.1	0.14
NH_4NO_3	327.2	121.6	0.37
$NaNO_3$	521.2	98.0	0.19
Urea	702.9	83.2	0.19
Peptone	509.0	84.8	0.17
Asparagine	469.9	89.6	0.19
Arginine	727.2	86.4	0.12
Histidine	581.8	86.4	0.15
Glycine	557.5	88.0	0.16
Lysine	399.9	86.4	0.22
Methionine	462.4	84.8	0.18
Valine	290.9	91.2	0.31
Corn steep liquor	606.0	206.2	0.34
Fish meal	206.0	131.2	0.64
Soybean meal	339.4	126.4	0.37

Incubation period, 8 days

tution of $(NH_4)_2SO_4$ with different inorganic and organic sources (Table III) produced fluctuating effects towards synthesis of the extracellular phytase of *A. carneus*. With the exception of CSL, all the investigated sources failed to sustain the enzyme productivity as $(NH_4)_2SO_4$. On using 0.1% (w/v) CSL as a sole nitrogen source, the mould could accumulate about 1.5-fold of the content obtained in $(NH_4)_2SO_4$ experiment.

The enzyme was isolated from the culture filtrate by precipitation using different saturations of $(NH_4)_2SO_4$, acetone and low molecular weight alcohols at different concentrations. The accumulated results confirmed that the organic

Table IV

Scheme of treatments used for the purification of A. carneus extracellular phytase

Treatment	Total protein (mg)	Total activity (units)	Specific activity	Recovery (%)	Purification (fold)
1. Cell-free filtrate	606.0	202.2	0.33	100	1.00
2. Precipitation with acetone (5:1)	266.6	148.4	0.56	73.39	1.69
3. Gel filtration through Sephadex G-75	13.3	85.6	6.44	42.33	19.52
4. Ion exchange chromatography on DEAE-cellulose	2.3	32.4	14.09	16.02	42.69

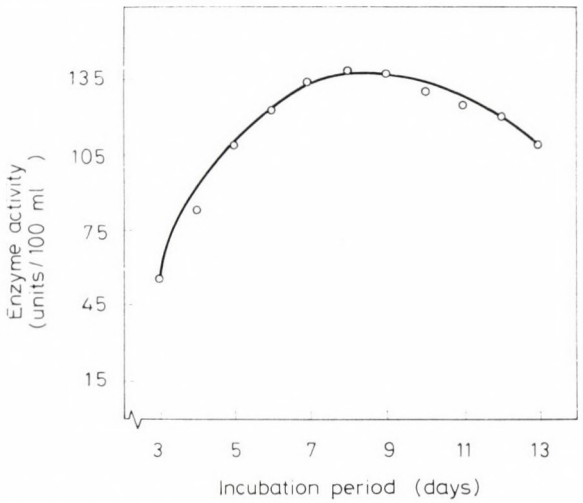

Fig. 1. Biosynthesis of phytase by *A. carneus* at different periods of incubation

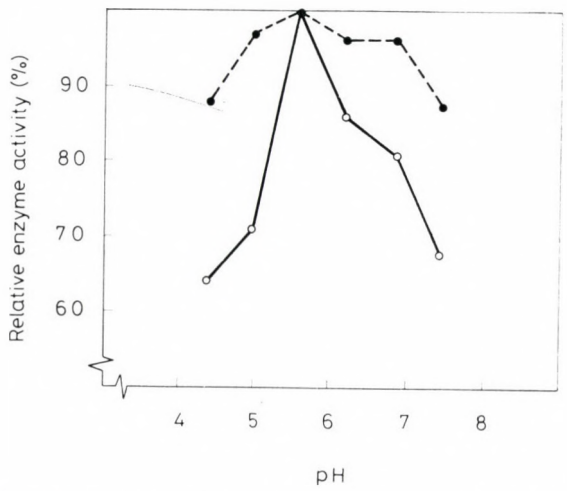

Fig. 2. Effect of pH value on the activity of crude (●——●) and purified (○——○) phytase

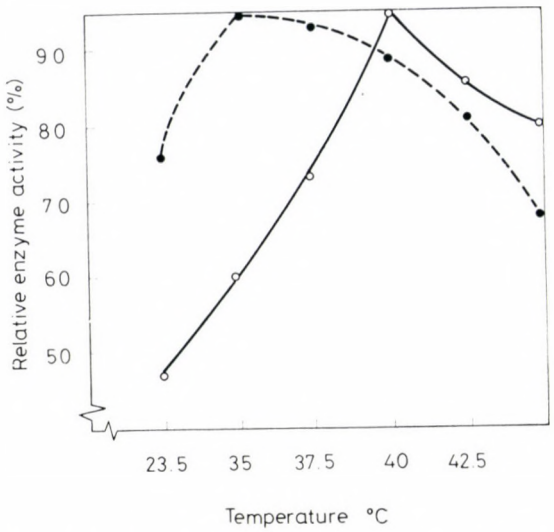

Fig. 3. Effect of temperature on the activity of crude (●——●) and purified (○——○) enzyme

solvents were superior to $(NH_4)_2SO_4$ in this regard. This finding confirmed that this enzyme has a particular structure which makes it resist to the denaturating effect of organic solvents. The precipitate obtained when using acetone in a ratio of 5:1 was redissolved in Tris–HCl buffer (pH 8.2) and applied to a column of Sephadex G-75. Elution was achieved using NaCl in Tris–HCl buffer of different molarities. Fraction 3 contained the highest activity, thus it was used to inoculate a column of DEAE-cellulose. Two fractions, i. e. 20 and 21 were found to contain the maximum enzyme activity. A summary of treatments used for purification of the extracellular phytase of *A. carneus* is demonstrated in Table IV. The purified enzyme gave 14.09 units/mg protein with a purification of 42.69-fold.

Effects of pH and temperature on the activity of phytase in both crude and pure states are illustrated in Figs 2 and 3, respectively. The data show that the enzyme in crude state had a maximum activity at pH 5.6 and 35 °C. Moreover, the purified enzyme had a pH maximum of 5.6 also but at 40 °C. These results accord with the general properties recorded for phytases of different aspergilli [7, 8, 15]. The purified enzyme was found stable between pH 5.6–6.2 and lost a great part of its activity on either side of pH 4.4 and 6.8. Concerning the thermal stability of the purified enzyme, it was found that the enzyme lost 68.3% of the original activity by exposing its preparation to 45 °C for 60 min.

On storage in the pure state for prolonged periods at 4 °C the enzyme retained about 74% of its activity for 27 weeks when stored in 0.2 M acetate buffer (pH 5.6).

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NEW METHOD FOR THE ISOLATION OF *LISTERIA MONOCYTOGENES* FROM CONTAMINATED SAMPLES*

GY. SZEMERÉDI

Veterinary and Food Control Service of the Ministry of Agriculture and Food, Central Veterinary Institute, Budapest, Hungary

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A new method for the isolation of *Listeria monocytogenes* from samples contaminated with other bacteria is described. The method requires 10 to 11 days and is based on the motility of listeriae in semisolid selective medium. The new method was compared with isolation techniques described by Füzi and Pilis [1] and by Ralovich et al. [2]. All 30 faecal samples containing 100 listeriae per g of faeces were shown positive by the new method. Using the method of Ralovich et al. [2] the same positivity ratio could be obtained only with samples containing 1000 listeriae per g of faeces. At a contamination rate of 10000 listeriae per g of faeces 28 out of 30 faecal samples were positive by the method of Füzi and Pilis [1]. The new method gave 10 to 86% positivity when 775 faecal samples from 10 animal species were tested.

Isolation of listeriae from materials such as pus and silo samples, where significant incidental flora might occur, has always been a difficult task.

In 1963, Murray tried to cultivate listeriae from faeces. However, these attempts rarely succeeded. In 1960 Jentzsch [3] reported that *Listeria monocytogenes* displayed significant resistance against high concentrations of saline and was able to proliferate even in a rather alkaline medium (pH 8–9). These characteristics and the long-known proliferating ability of these bacteria at 4 °C were first used in the processing of samples where, apart from *L. monocytogenes*, a significant incidental flora occurred.

In most laboratories potassium tellurite broth, first described by Gray in 1950 [4], was applied for selective enrichment. However, as some *Listeria* strains are sensitive to potassium tellurite, in Hungary Füzi and Pilis [1] suggested that nutrient agar prepared with 3% potassium rhodanide and 20 μg/ml guanofuracine was the most reliable medium for the selective cultivation of *L. monocytogenes*. Ralovich et al. [2] reported that this selective enrichment medium was unsuitable because the proliferation of Gram-positive cocci and some other aerobic saprophytic bacilli was not restrained. Therefore, parallel with the finding of Ortel [5], they used tripaflavine and nalidixic acid for restraining the incidental flora and the TNSA agar prepared by them

GYULA SZEMERÉDI
Veterinary and Food Control Service, Central Veterinary Institute
Tábornok u. 2, H-1149 Budapest, Hungary

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was used for the isolation of listeriae. According to their method the material containing mixed bacterium flora was inoculated into Holman chopped meat broth, incubated at 4 °C for several weeks, then streaked onto TNSA agar. The cultures were incubated at 37 °C for 48 h and the colonies were examined under microscope, using oblique illumination of the agar.

The use of a shortened version of the method to isolate listeriae from incidental flora is reported in this paper.

Materials and methods

Ten faecal samples each from pigs, cattle and rabbits (30 samples altogether) were examined. Faecal samples were processed only if their freedom from listeriae was certain. One g each of the faecal samples was mixed with 0.1 ml of the dilution of a 24-hour *Listeria* culture so that 1 g of each sample contained 1×10^1–1×10^6 germs.

Method 1. The enrichment medium prepared as described by Füzi and Pilis [1] contained 3% potassium rhodanide and 20 μg/ml guanofuracine in 1% Witte (peptone) broth at pH 7.4. Inoculations were made into 5 ml aliquots of broth and incubation lasted for 48 h. Seedings were performed onto blood agar.

Method 2 involved the use of two agars. The artificially contaminated faecal samples were first inoculated into Holman broth, incubated at 4 °C for 28 days and inoculated onto TNSA agar plates. These agar plates were incubated at 37 °C for 48 h and examined under oblique illumination. *Listeria* colonies were characterized by a yellowish-greenish, somewhat bluish iridescence.

Method 3 consisted of two phases. The artificially contaminated faeces was inoculated into Holman broth. Dextrose (1%) broth could be used with good results as a substitute to this medium. The liquid cultures were incubated at 4 °C for 6 days. Then 0.1 ml culture was layered onto the surface of a semisolid medium in a U-tube 2 cm in diameter. The components of the medium in the U-tube were the same as that of the TNSA agar, but it contained only 0.1–0.2% agar. The U-tubes were incubated at 20–25 °C for 48 h. After that time a subculture was made from the opposite arm of the U-tube onto blood agar. The blood agar plates were incubated at 37 °C for 24 h, then beta-haemolysing colonies were stained by Gram and identified according to the characteristics of listeriae, including the checking of catalase activity, mannitol-negativity and aesculin-positivity. Colonies regarded as *L. monocytogenes* were agglutinated in 1/2 and 4 type sera (Fig. 1).

Using Method 3, samples taken from the faeces of various animal species and humans, from silage, fetal membranes and organs of dead animals were examined.

Results

The results obtained for a total of 30 samples taken from the faeces of various animals (pigs, cattle, and rabbits) unanimously proved that the U-tube method was the most reliable for isolating *L. monocytogenes*. When 100 listeriae were present in 1 g of faeces, 21 out of 30 samples (70%) were positive. Using the method of Füzi and Pilis [1] only faecal samples containing 10 000 listeriae per g gave a similar positivity rate. By the method of Ralovich et al. [2] 7 out of 30 samples (23.3%) containing 1000 listeriae/1 g of faeces, and 25 out of 30 samples (83%) containing 10 000 listeriae/1 g of faeces proved positive. The higher efficiency of our method may be due to the fact that listeriae multiply further in the U-tube at 20–25 °C, and growth seems to be quicker

at that temperature. Another disadvantage of the method of Ralovich et al. [2] is that it cannot be used as a rapid diagnostic test.

From this point of view the method of Füzi and Pilis [1] seems to be the quickest because by their technique results can be obtained in 3 to 5 days. However, another disadvantage is that *Streptococcus faecalis* and Gram-positive intestinal bacteria as well as aerobic spore-forming bacteria develop

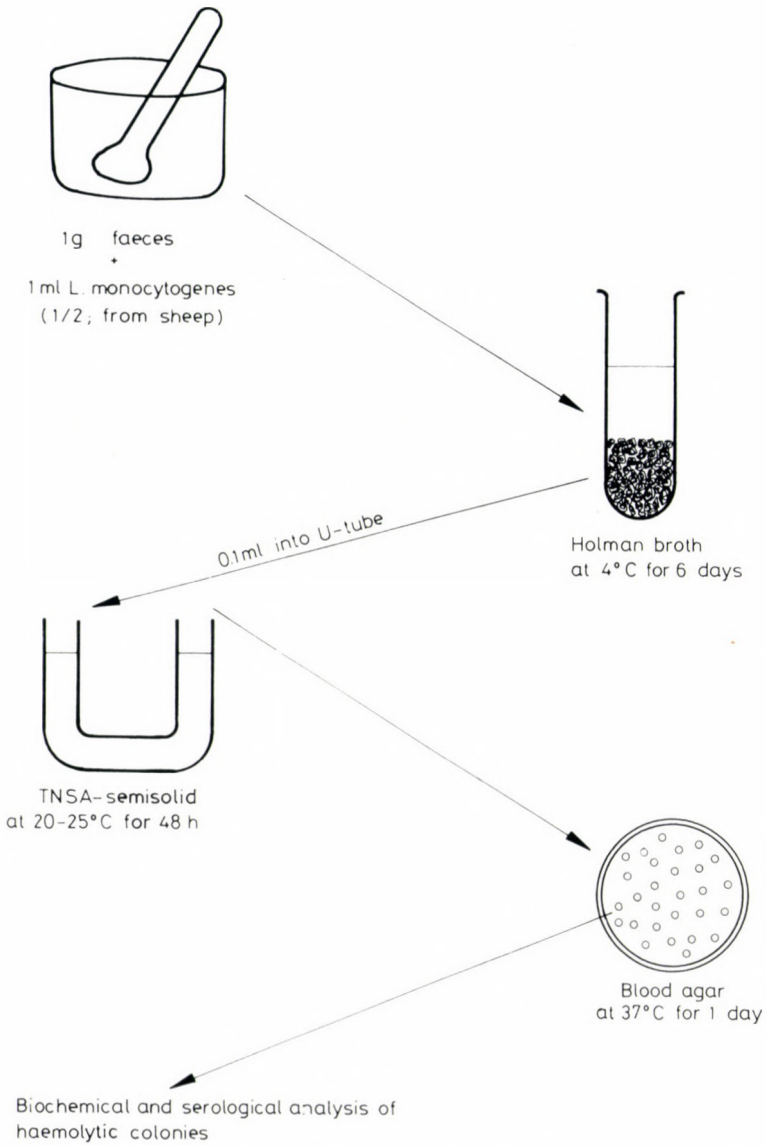

Fig. 1. Processing of model experiments for the recovery of *L. monocytogenes*

well in the enrichment agar which makes the isolation of *L. monocytogenes* rather difficult.

Our method is especially good for processing intensely contaminated samples. The development of *Pseudomonas* species occurring in the samples cannot be blocked by trypaflavine and nalidixic acid, therefore they develop well enough in the agar together with listeriae. However, the colony morphology of *Pseudomonas* differs from that of *Listeria*. Therefore, the isolation of *L. monosytogenes* involves no diagnostic problem. As isolation and identification of *L. monocytogenes* take about 10 to 11 days, this method cannot be considered a rapid one. However, it is essentially quicker than that of Ralovich et al. [2] in which results are obtained in 2 months. The agar, which is a modified TNSA, can be stored in the U-tube at 4 °C, for no longer than a week.

Conclusions

The cultivation of listeriae is absolutely necessary for the safe diagnosis of listeriosis and for identifying the bacterium carriers. Since recently potassium tellurite sensitive strains of *L. monocytogenes* have been reported to exist, Gray's [4] medium is not suitable for selective enrichment of listeriae.

In the method of Füzi and Pilis [1], the use of potassium rhodanide and guanofuracine in the agar ensures intensive selection. Unfortunately, it does not inhibit the growth of Gram-positive cocci and aerobic spore-forming bacilli. It may be assumed that this medium somewhat hinders the development of listeriae because *L. monocytogenes* could be isolated only when the number of these bacteria was high in the samples. The great advantage of this method is its rapidity. It can be evaluated in a few days.

Table I

Incidence of listeria excreters among animals

Animals	Number of faecal samples	Number of positive samples
Sheep	110	77
Cattle	172	35
Goat	110	36
Big	88	8
Poultry (hen)	97	84
Rabbit	40	3
Hare	66	13
Deer	11	6
Pheasant, partridge	21	15
Zoo wild animals	60	5
Total	775	282

It was proved by Hungarian [2] and foreign researchers that the best selectivity is granted by TNSA agar. If the amount of agar is decreased in the medium (semi-solid agar), with the help of their flagella the bacteria can move to the opposite arm of the U-tube and, thus, another characteristic of listeriae improves the specificity of the method.

In addition to increasing the reliability of the method, the time needed for isolating the bacteria can be reduced to 10–11 days. This procedure seems to be reliable also when rather few listeriae are present.

Finally, it can be stated that the efficiency of detecting listeriae has markedly increased and this offers good opportunity for studying the epidemiology and for increasing the reliability of therapy.

Using the new method, a 10 to 86% listeria positivity rate was found for a total of 775 faecal samples from 10 animal species (Table I).

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EFFECT OF CHLORPROMAZINE (CPZ) ON THE COURSE OF LCM VIRUS INFECTION IN MICE WITH DEVELOPED AND UNDEVELOPED IMMUNE SYSTEM

ILONA SZERI, PIROSKA ANDERLIK, ZSUZSANNA BÁNOS, L. K. CSATÁRY, I. NÁSZ and ZSUZSANNA BARNA

Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary, and United Cancer Research Institute, Alexandria, Virginia, USA

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The cellular immune response to lymphocytic choriomeningitis (LCM) virus in germfree adult and conventional (Cv) suckling mice with undeveloped immune systems and in Cv adult mice with deve oped immune systems was suppressed by a single large, sublethal dose of the calmoduline antagonistic chlorpromazine and stimulated by a 100-times smaller dose administered intraperitoneally one day before the intracerebral virus infection. CPZ thus exerted a two-directional dose-dependent immunomodulatory effect in mice with both undeveloped and developed immune system.

Chlorpromazine (CPZ), usually applied as an antipsychotic agent, has also a calmoduline antagonistic effect [1, 2]. Calmoduline is a regulator protein controlling the calcium pump and has also an important role in the regulation of several cell functions, such as cell metabolism, cell division, processing of hormonal effects and regulating of immunological functions [3–7]. Calmoduline-antagonistic agents, like CPZ, can have an immunomodulatory effect as well.

The fatal lymphocytic choriomeningitis following intracerebral (i. cer.) lymphocytic choriomeningitis (LCM) virus infection is the consequence of the cytotoxic reaction of LCM virus antigen specific T lymphocytes to cells expressing viral antigens on the leptomeninx [8–10]. The course of virus infection thus greatly depends on the cellular immune responsiveness of the animal. Acute lymphocytic choriomeningitis develops in adult mice with intact, developed immune system and the animals die on the 6–8th day following virus infection. In mice with insufficient T lymphocyte function, like in adult germfree (Gf) and in conventional (Cv) suckling mice both with undeveloped immune system, lymphocytic choriomeningitis fails to develop and the mice surviving the infection become virus carriers [11–13]. Manifestation of LCM virus infection in the form of fatal meningitis is enhanced by effects stimu-

LÁSZLÓ K. CSATÁRY
United Cancer Research Institute
1913 Windsor Rd, Alexandria, Virginia 22307, USA

ILONA SZERI, PIROSKA ANDERLIK, ZSUZSANNA BÁNOS, ISTVÁN NÁSZ, ZSUZSANNA BARNA
Institute of Microbiology, Semmelweis University Medical School
Nagyvárad tér 4, H-1089 Budapest, Hungary

lating the cellular immune response [14–19], and is hindered by immunosuppressive effects [13, 20–23].

It has been examined whether CPZ pretreatment can influence the cellular immune response to LCM virus infection in Gf adult mice with undeveloped immune system due to antigen-deficient environment, in Cv suckling mice with undeveloped immune systems due to age, and in Cv adult mice with developed immune system.

Materials and methods

Experimental animals. Gj and Cv 6-week-old C3H mice and Cv 6-week-old young adult and 2-week-old suckling CFLP mice (LATI, Gödöllő, Hungary) of both sexes were used. Germfree state was maintained and controlled as published earlier [24]. In the experiment with suckling mice, the number of mice was decreased to 10 per litter.

Chlorpromazine treatment. A 2.5% solution of chlorpromazine hydrochloricum (EGIS, Budapest) was used. Further dilutions for different doses were prepared with PBS directly before application.

LCM virus infection. The strain WE maintained in this laboratory by serial intracerebral passages in mice was used. For titration it was administered intracerebrally (i.cer.) to 6-week-old mice. LD_{50} value of the virus was determined by Reed and Muench's method (25).

Recovery of LCM virus. Young adult mice were inoculated i. cer. with 1:10 dilutions of brain suspensions prepared from mice surviving LCM virus infection and sacrificed on the 21st day of the experiment. Presence of LCM virus was confirmed by the typical neurological symptoms and deaths of mice.

Experiments and results

The animals were treated intraperitoneally with a single large, sublethal or with a 100 times smaller dose of CPZ. The doses were chosen on the basis of LD_{50} values. There were significant differences among the LD_{50} values for different types of mice (Table I) and the applied CPZ doses in the three experiments differed accordingly.

Following treatments with large doses of CPZ, all animals were sleeping for two days. There was no difference in the duration of sleep between Gf and Cv mice. Small CPZ doses did not cause sleeping. The mice were infected i. cer.

Table I

LD_{50} values of CPZ in mice of different types

Mice	Chlorpromazine LD_{50} mg/kg
Gf adult (C3H)	160
Cv adult (C3H)	75
Cv adult (CFLP)	75
Cv suckling (CFLP)	50

Table II

Germfree adult mouse groups and their treatments in Experiment I

Groups	Inoculation		No. of mice
	i.p.	i.cer.	
CPZ_{100}–LCM	CPZ 100 mg/kg	LCM virus	20
CPZ_1–LCM	CPZ 1 mg/kg	LCM virus	20
LCM	PBS	LCM virus	20
CPZ_{100}	CPZ 100 mg/kg	X	10
CPZ_1	CPZ 1 mg/kg	X	10
C	PBS	X	10

X virus free mouse brain suspension

with LCM virus on the day after CPZ treatment in all experiments. Experiments were concluded on the 21st day after LCM virus infection. There were no deaths among mice not infected with LCM virus.

Experiment I. Germfree adult mice were treated i. p. with 100 mg/kg or 1 mg/kg doses of CPZ and with PBS. On the day after treatment, two-third of mice in each group were infected i. cer. with 100 LD_{50} of LCM virus, one-third of the animals were inoculated i. cer. with virus-free, normal brain suspension. Groups, treatments and number of mice are shown in Table II.

In groups infected with LCM virus, deaths occurred with neurological symptoms characteristic of LCM. Rate and time of death in the virus infected groups are shown in Fig. 1.

Of mice infected with LCM virus but not treated with CPZ 85% died on the 7–10th day after virus infection (LCM group). Rate and time of deaths were changed by CPZ pretreatment. In the CPZ_{100}–LCM group deaths occurred later, on the 9–11th day after virus infection and only 65% of mice died. In the CPZ_1–LCM group deaths occurred earlier, on the 7–9th day and rate of deaths was 100%.

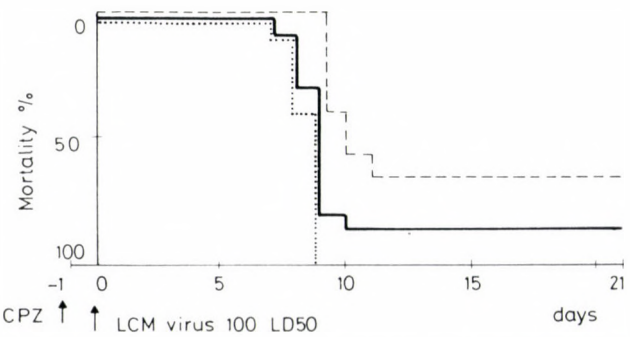

Fig. 1. Rate and time of deaths after LCM virus infection in adult germfree mice (Experiment I). Groups: CPZ_{100}–LCM – – – –; CPZ_1–LCM · · · · ·; LCM ———

Table III

Conventional suckling mouse groups and their treatments in Experiment II

Groups	Inoculation		No. of mice
	i.p.	i.cer.	
CPZ_{25}–LCM	CPZ 25 mg/kg	LCM virus	30
$CPZ_{0.25}$–LCM	CPZ 0.25 mg/kg	LCM virus	30
LCM	PBS	LCM virus	60
CPZ_{25}	CPZ 25 mg/kg	X	10
$CPZ_{0.25}$	CPZ 0.25 mg/kg	X	10
C	PBS	X	20

X, virus free mouse brain suspension

From the brain of mice surviving the infection by 21 days, LCM virus could be isolated, thus these mice were symptom-free virus-carriers.

Experiment II. Sixteen litters of 2-week-old suckling mice were included in this experiment. Half of the mice were treated i. p. with 25 mg/kg and 0.25 mg/kg doses of CPZ in each of 8 litters. The other half of the mice in all litters were treated in the same way with PBS solution. On the day after treatments, in each of 6 litters mice that had been treated with different doses of CPZ were infected i. cer. with 100 LD_{50} dose of LCM virus, and mice in each of the remaining 2 litters were inoculated in a similar way with normal mouse brain suspension. Mouse-groups, number of mice in each group and treatments are shown in Table III. Rate and time of death in the virus infected groups are shown in Fig. 2.

Rate and time of deaths were different in the three virus-infected groups. Mice not treated with CPZ died in 43% on 8–11th day after virus infection (LCM group), while mice pretreated with large doses of CPZ died only in 30% and later, on 9–11th day (CPZ_{25}–LCM group). Those treated with small CPZ doses died in larger proportion (67%) and earlier, on 8–10th day ($CPZ_{0,25}$–

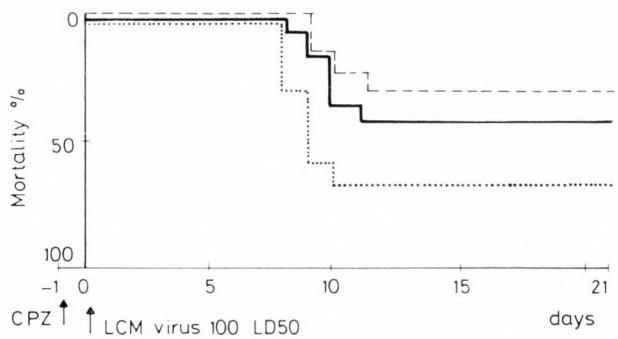

Fig. 2. Rate and time of deaths after LCM virus infection in conventional suckling mice (Experiment II). Groups: CPZ_{25}–LCM · · · · ·; $CPZ_{0.25}$–LCM – – – –; LCM ———

Table IV

LD_{50} values of LCM virus in conventional adult mouse groups treated with different doses of CPZ

Pretreatments	LCM virus LD_{50} value
CPZ 50 mg/kg	10^{-3}
CPZ 0.5 mg/kg	$10^{-4.5}$
PBS solution	$10^{-3.63}$

LCM group). LCM virus could be isolated from the brain of mice surviving the virus infection by 21 days thus these animals were symptom-free virus-carriers.

Experiment III. Conventional adult CFLP mouse groups were inoculated i. p. with 50 mg/kg or 0.5 mg/kg of CPZ and with PBS as control. On the day after treatments, three parallel virus titrations were performed under identical conditions simultaneously on animals pretreated with two different doses of CPZ and PBS. Titration was carried out by tenfold dilutions (10^{-2}–10^{-6}) of LCM virus. Ten male and 10 female mice were inoculated with each dilution. Ten mice inoculated only with PBS solution and 10 mice given different doses of CPZ served as controls. The experiment lasted 21 days after virus infection.

Deaths of virus infected animals occurred on the 7–10th day after infection, with characteristic symptoms of lymphocytic choriomeningitis. The time of deaths was not influenced by CPZ treatments. LD_{50} values calculated on the basis of death rate during the three parallel titrations of LCM virus are shown in Table IV.

LD_{50} value of the virus was smaller in mice pretreated with a large dose of CPZ and it was larger in mice pretreated with a small dose of CPZ, than in mice not treated with CPZ. CPZ pretreated and untreated animals thus displayed different response to the same quantity of virus.

Discussion

CPZ doses applied in the three experiments were chosen on the basis of LD_{50} values determined previously. Significant differences among the LD_{50} of CPZ was observed in different types of mice. Cv suckling mice were more sensitive to CPZ than adult ones and adult Gf mice were less sensitive than conventionals of the same age. It is well known that there are differences in sensitivity towards different drugs depending on age, newborn and suckling animals being more sensitive to drugs than adult ones. In the case of Cv adult and suckling mice the definite LD_{50} values of CPZ are in accordance with this. It is less known, however, that Gf mice display less sensitivity to certain drugs

than Cv mice of the same age. Results with cytostatic agents and gamma ray have already been published [26–29]. In the present experiment, a significant difference was observed in sensitivity to CPZ between adult Gf and Cv mice. As for our present knowledge no data concerning this is known in the literature. It may be supposed that the tolerance of Gf mice to CPZ can be due to the absence of microbial flora.

In Experiments I and II the effect of a single pretreatment with different doses of CPZ on the course of i. cer. LCM virus infection was studied in Gf adult and Cv suckling mice both with undeveloped immune systems. The results showed that in virus infected and large dose treated mice the death started later and its rate was lower, whereas in small dose treated mice the death occurred earlier, and its rate was higher than in virus infected but not pretreated mice. Thus the large doses of CPZ hindered, small doses enhanced the development of fatal lymphocytic choriomeningitis, i. e. in mice with undeveloped immune system the cellular immune response to LCM virus was modulated by CPZ pretreatment. The immunomodulatory effect — depending on CPZ doses — was two-directional; large doses were suppressive, small doses were stimulatory, independently of the fact, whether the immune systems of mice were undeveloped due to their age or environment poor in antigen-stimulus.

In Experiment III the immunomodulatory effect of CPZ on the cellular immune response to LCM virus was studied in Cv adult mice with developed immune system on the basis of comparing the LD_{50} values of virus determined by the results of the three parallel virus titration carried out on CPZ pretreated and untreated animals.

The LD_{50} of LCM virus was smaller in large dose treated mice and it was larger in small dose treated ones than that in mice not treated with CPZ. Thus the large dose of CPZ decreased, the small dose of the drug increased the rate of death in LCM. CPZ thus proved to be of immunomodulatory effect in Cv adult mice with developed immune system, and this effect, similarly to the cases of the mice with undeveloped immune system, was two-directional depending on the dose, large dose was suppressive, small dose stimulatory.

Data have been published about the dose-dependent two-directional immunomodulatory effects and the mechanisms of calmoduline antagonistic neuropeptides [30]. Reports and experiences are not known in connection with CPZ which is also calmoduline antagonistic substance.

It cannot be determined yet, whether the immunomodulatory effect of CPZ is connected to its calmoduline antagonistic effect. There are other possibilities as well. Different mechanisms can be responsible for the two-directional immunomodulatory effect triggerred by two different doses of CPZ. Sleeping state was induced only by large doses. Data about decreased immune response in persistent sleep have already been published. For example, allergic reactions

based on cellular immune reactions in case of tuberculosis [31] and brucellosis [32] are suppressed by medicated sleep. Suppression of the cellular immune response on LCM virus infection due to large doses of CPZ can be connected to the sleep-inducing effect of large doses. No sleep was induced with small doses of CPZ, thus other mechanisms can be valid in the immunostimulatory effect. CPZ — which was earlier known only as an antipsychotic drug — has several other biological, therapeutical effects. In case of some tumours, for example, CPZ is of regressive effect [33–38], where the role of CPZ is attributed to its lysosomal membrane stabilizing, DNA synthesis hindering [39], or to antihistamine effects [40]. Recently CPZ has been applied in the therapy of cutaneous leishmaniasis [41, 42]. The effect mechanism in this case is not known. It can be supposed that the therapeutical value of CPZ in different clinical cases may be in connection with its immunomodulatory effect.

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THE EFFECT OF METHICILLIN ON THE FATTY ACID COMPOSITION OF TOTAL POLAR LIPID IN METHICILIN RESISTANT *STAPHYLOCOCCUS AUREUS**

F. Rozgonyi, P. Biacs, Jolán Kiss, Katalin R. Szitha and I. Andirkó

Department of Microbiology, University Medical School, Debrecen, and Central Food Research Institute, Budapest, Hungary

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The effect of two concentrations of methicillin on the fatty acid (FA) distribution in intracellular total polar lipid (TPL) of the log-phase cultures of a methicillin resistant *Staphylococcus aureus* strain No. 5814R was studied during a period of 2 h. Half the MIC of methicillin (= 1000 μg/ml) caused 18.6% increase in branched-FAs and a same decrease in straight-FAs, while one MIC (= 2000 μg/ml) of the drug induced a moderate change in those of TPL. The ratio of branched-FAs to straight-FAs increased from 1.24 to 1.56 in the presence of 1/2 × MIC of methicillin and reduced from 1.24 to 0.87 in the presence of 1 × MIC of the antibiotic. In TPL of the control cultures it gradually decreased from 1.24 to 0.77. It is concluded that under the effect of methicillin, FA composition of TPL in methicillin resistant cocci does not change as dramatically as in methicillin sensitive ones indicating lipid synthesis in methicillin resistant *S. aureus* to be less sensitive to the action of methicillin than in methicillin susceptible strains. This may contribute to the resistance against the lytic effect of the drug. Membrane lipid properties seem to be involved in the mechanisms of methicillin resistance.

One of the probable mechanisms of methicillin resistance in *Staphylococcus aureus* seems to be a permeability barrier [1] coupling with a decreased affinity for beta-lactams [2]. Polar lipids of membranes play probably key roles in the impermeability to these drugs [3–5].

Lipid compositions of staphylococcal membranes vary to considerable extent under different cultural conditions [6–10]. The permeability of bacterial membranes basically depends on their protein and lipid composition, on their charge, and on protein-lipid interactions [11]. As to lipids, they are not only structural parts of the membrane maintaining its fluidity [12] but take part in vital functions of the bacteria such as enzyme activation and/or inhibition, transports, etc. [11–15]. Changes in the acyl moiety of the membrane lipids

Ferenc Rozgonyi, Jolán Kiss, Katalin R. Szitha, István Andirkó
Department of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

Péter Biacs
Central Food Research Institute
H-1525 Budapest, Hungary

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may strongly influence the permeability rate of non-electrolytes and the facilitated diffusion processes across the biological membranes [11–13].

Beta-lactam antibiotics have been reported to affect the lipid synthesis, composition and secretion in a variety of bacterial species [7, 14–19]. Therefore, it has seemed to be reasonable to study the effect of various concentrations of methicillin on the FA distribution in TPL of a highly methicillin resistant *S. aureus* strain possessing a big amount of TPL [8].

Materials and methods

Cultures. The experiments were carried out with the widely studied methicillin resistant *S. aureus* 5814R strain [2, 7, 8, 20]. In a 10-litre flask 7 litres of lipid-free minimal medium broth was inoculated with $1.2–1.5 \times 10^7$ bacteria per ml of stationary phase. The broth consisted of 10 g Bacto Casitone (Difco Laboratories, Detroit, Michigan, USA), 5 g NaCl, 5 g $Na_2HPO_4 \cdot 12H_2O$, 5 g KH_2PO_4, 0.03 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.01 g $MgSO_4 \cdot 4H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$ and distilled water up to 1000 ml, pH 7.2. It was sterilized by autoclaving at 121 °C for 20 min. Immediately before use, the broth was supplemented with 1% glucose. The culture was incubated at 37 °C and stirred with a Teflon-covered magnetic stirrer for 5 min at half hour intervals. Reaching the middle-log phase (5×10^8 organisms per ml) 1000 μg/ml (= 1/2 × × MIC) or 2000 μg/ml (= 1 × MIC) of methicillin (Chinoin, Budapest) was added to the culture. This time was noted as 0 h. Further incubation lasted at 37 °C for 2 h after methicillin treatment.

Lipid analysis. At 0, 1 and 2 h of the exposure 2-litre samples were withdrawn for the lipid extraction. Cocci were harvested by centrifugation and their lipids were extracted with chloroform-methanol-water (2:1:0.2) according to Gould and Lennarz modified by us as previously described [21]. Polar lipids were separated from neutrals on Silica gel H (Merck Type 60) by one-dimensional thinlayer chromatography in petroleum ether-diethylether-glacial acetic acid (82:18:1) and, after elution from the chromatoplates, were purified on Sephadex G-25 fine columns (Pharmacia, Uppsala, Sweden). Methylation of FAs was performed with 5% HCl in methanol at 80 °C for 2 h. Mixtures of FA methyl esters were resolved by a capillary column gas chromatography using columns coated with 12.5% diethylene-glycolsuccinate. Isotherm temperature was 180 °C. The relative retention times of all FAs were confirmed with available Serva Kit standards and with a wool-wax of known composition for anteiso- and iso-acids. The other methods used for the complete analysis of FA methyl esters were essentially the same as described by Christie [22]. Each type of experiments was at least twice repeated.

Results

Figure 1 shows the effect of methicillin on the viable counts in cultures of methicillin resistant *S. aureus* 5814R. Prior to the methicillin additions the cells were growing exponentially. After adding 1000 μg/ml (= 1/2 × MIC) and 2000 μg/ml (= 1 × MIC) multiplication continued and a significant gain in viable count occurred. Between the 1st and 2nd hour of methicillin exposure, however, the viable count in the presence of 2000 μg/ml failed to increase substantially.

The effects of two concentrations of methicillin on the iso-FA distribution in TPL of logarithmically growing cells of *S. aureus* 5814R are shown in Table I. Under the conditions used it was possible to separate and measure i-C15 : 0

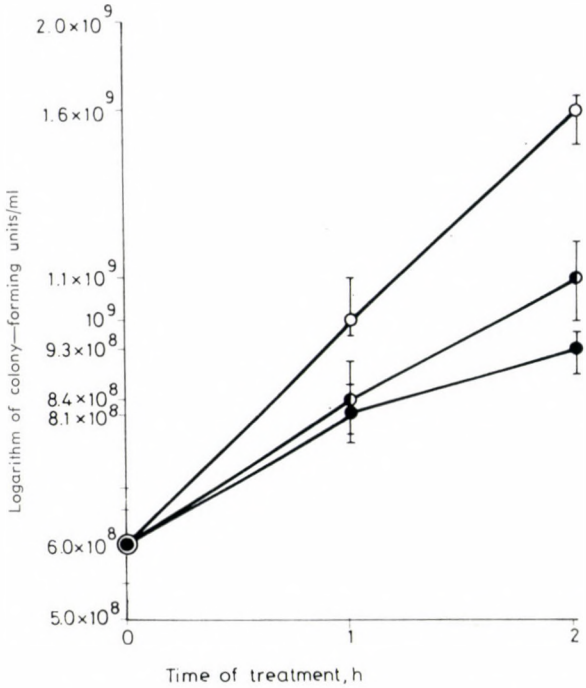

Fig. 1. Semilogarithmic representation of the effect of methicillin on the growth kinetics in the exponential phase of methicillin resistant *S. aureus* 5814R. ○——○ control [6]; ◐——◐ 1000 μg methicillin per ml = 1/2×MIC [7]; ●——● 2000 μg methicillin per ml = 1×MIC [6]; figures in parentheses indicate the number of experiments

and ai-C15 : 0 FAs which had previously given only one peak on the chromatograms and had been evaluated as ai-C15 : 0 [7, 8, 17]. The predominant iso-FA was i-C15 : 0 in TPL of both the untreated and the treated cocci during a 2 h period of the log-phase studied. The addition of 1/2×MIC of methicillin to the cultures brought about an increase in the proportion of iso-FAs attributable mainly to the enhancement of i-C15 : 0 and i-C17 : 0. Administering 1×MIC of methicillin to the cultures resulted in an almost entire fixation of the iso-FA composition of TPL at the 0-time (middle-log) distribution level. As a total, 1/2×MIC of methicillin caused a considerable, while 1×MIC did a slight increase in the proportion of iso-FAs.

Table II demonstrates that the main anteiso-FA in both the control and the methicillin-exposed cocci was ai-C15 : 0. On the whole, the anteiso-FA proportion of TPL in the bacteria of the untreated cultures decreased with the preceeding of the growth phase due mainly to the fall of ai-C15 : 0 and ai-C17 : 0. Under the influence of methicillin the cocci enhanced the amount of ai-C15 : 0 and ai-C17 : 0 in TPL resulting in a substantial accumulation of anteiso-

Table I

Iso-fatty acid composition of total polar lipid in exponentially growing methicillin resistant cells of S. aureus 5814R treated with methicillin

Iso-fatty acid	Methicillin added (×MIC)	Per cent iso-fatty acid in total polar lipid from cells treated for		
		0 h	1 h	2 h
i-C13 : 0	0	0.1	0.1	0.2
	1/2	0.1	0.1	0.1
	1	0.1	0.2	0.2
i-C14 : 0	0	1.8	1.3	2.3
	1/2	1.8	1.2	1.3
	1	1.8	1.5	1.6
i-C15 : 0	0	24.0	23.3	22.2
	1/2	24.0	23.3	27.2
	1	24.0	23.0	25.9
i-C16 : 0	0	0.4	0.2	0.2
	1/2	0.4	0.4	0.3
	1	0.4	0.2	0.4
i-C17 : 0	0	2.9	2.2	1.1
	1/2	2.9	3.8	5.5
	1	2.9	3.1	1.4
i-C19 : 0	0	0.6	0.2	trace
	1/2	0.6	1.4	0.9
	1	0.6	0.7	trace
Total iso	0	29.8 ± 1.2	27.4 ± 1.3	26.0 ± 1.2
	1/2	29.8 ± 1.2	31.2 ± 1.5	35.4 ± 1.8
	1	29.8 ± 1.2	28.7 ± 1.4	29.5 ± 2.0

1/2×MIC is equivalent to 1000 μg/ml methicillin
1×MIC is equivalent to 2000 μg/ml methicillin

FAs. One MIC of methicillin did the same as 1/2×MIC in the first hour of the exposure, then the content of anteiso-FAs of TPL fell to the control value. As a total, methicillin at 1/2×MIC caused 18.6% increase in the branched-FA content of TPL in the culture, whereas 1×MIC of the drug induced a moderate change.

As can be seen from Table III the predominant straight-chain FAs in both the untreated and the treated cocci were in the order of n-C20 : 0, n-C18 : 0 and n-C16 : 0. In the TPL of the control cocci the proportion of straight-FAs increased with the proceeding of the log-phase at the expense of anteiso-FAs and to a less extent of iso-FAs. The addition of 1/2×MIC of methicillin to the cultures caused a considerable reduction in the proportion of n-C14 : 0, n-C15 : 0, n-C17 : 0, n-C19 : 0 and n-C20 : 0 compared to that of the 2 h control. Surpris-

Table II

The effect of mehicillin on the anteiso-fatty acid composition of total polar lipid in exponentially growing methicillin resistant cells of S. aureus 5814R

Anteiso-fatty acid	Methicillin added (×MIC)	Per cent iso-fatty acid in total polar lipid from cells treated for		
		0 h	1 h	2 h
ai-C13 : 0	0	0.9	1.4	1.2
	1/2	0.9	0.8	0.5
	1	0.9	1.3	0.6
ai-C15 : 0	0	20.9	18.0	15.0
	1/2	20.9	21.0	20.0
	1	20.9	21.0	15.0
ai-C17 : 0	0	2.6	2.4	1.2
	1/2	2.6	3.2	4.0
	1	2.6	2.9	1.4
ai-C19 : 0	0	1.0	0.8	trace
	1/2	1.0	1.8	1.1
	1	1.0	0.8	trace
Total anteiso	0	25.4 ± 1.3	22.6 ± 1.1	17.4 ± 1.2
	1/2	25.4 ± 1.3	26.8 ± 1.3	25.6 ± 1.9
	1	25.4 ± 1.3	26.0 ± 1.4	17.0 ± 1.8
Total branched	0	55.2 ± 1.3	50.0 ± 1.2	43.4 ± 1.2
	1/2	55.2 ± 1.3	58.0 ± 1.4	61.0 ± 1.9
	1	55.2 ± 1.3	54.7 ± 1.4	46.5 ± 1.9

1/2×MIC is equivalent to 1000 μg/ml methicillin
1×MIC is equivalent to 2000 μg/ml methicillin

ingly, no substantial change was revealed in the proportion of n-C16 : 0 and n-C18 : 0. Adding 1×MIC of methicillin induced a moderate decrease in the straight-FA proportion of TPL.

A calculation was made on the alteration in the relationship of different chain type FAs during the courses of the methicillin exposures. As Table IV displays, the ratio of iso-FAs to anteiso-FAs increased whereas those of iso-FAs to straight-FAs, anteiso-FAs to straight-FAs and, of course, the total branched-FAs to straights decreased during the 2 h period of the log-phase examined. Under the influence of 1/2×MIC of methicillin the ratio of iso-FAs to anteiso-FAs became smaller while the ratio of both the iso- to straight-FAs and anteiso- to straight-FAs and, of course, total branched to straights significantly increased. Exposing the cocci to 1×MIC of methicillin brought about a moderate increase in all the ratios listed relative to the control values of the same ages except the ratio of iso- to anteiso-FAs after 1 h exposure.

Table III

Effect of methicillin on the straight-fatty acid distribution in total polar lipid of exponentially growing methicillin resistant cell of S. aureus 5814R

Straight fatty acid	Methicillin added (×MIC)	Per cent iso-fatty acid in total polar lipid from cells treated for		
		0 h	1 h	2 h
n–C14 : 0	0	2.4	3.3	5.6
	1/2	2.4	2.0	1.1
	1	2.4	2.6	2.9
n–C15 : 0	0	0.5	0.2	0.4
	1/2	0.5	0.3	0.2
	1	0.5	0.3	0.5
n–C16 : 0	0	5.4	4.6	5.9
	1/2	5.4	4.7	4.6
	1	5.4	5.2	6.2
n–C17 : 0	0	1.1	1.1	1.2
	1/2	1.1	1.3	0.4
	1	1.1	0.9	0.9
n–C18 : 0	0	13.9	15.4	15.3
	1/2	13.9	12.8	15.5
	1	13.9	13.8	16.6
n–C19 : 0	0	4.6	3.9	4.0
	1/2	4.6	3.2	2.4
	1	4.6	3.7	3.1
n–C20 : 0	0	16.2	21.3	24.2
	1/2	16.2	17.2	14.3
	1	16.2	18.7	23.2
Total straight	0	44.7 ± 0.8	50.3 ± 1.0	56.6 ± 0.9
	1/2	44.7 ± 0.8	41.8 ± 1.2	39.0 ± 1.3
	1	44.7 ± 0.8	45.2 ± 1.5	53.5 ± 1.6

1/2×MIC is equivalent to 1000 μg/ml methicillin
1×MIC is equivalent to 2000 μg/ml methicillin

Discussion

These results are in agreement with previous findings of a number of investigators [7, 14–19], and confirm our earlier observations that methicillin acts on the polar lipid composition in both the methicillin resistant [7] and in the methicillin susceptible *S. aureus* [17]. Nevertheless, the actions of methicillin on the lipid composition seem to be different according to the sensitivity of the strains, the growth phases, the methicillin concentrations and the FA classes.

Previously we have shown methicillin to exert a dramatic effect on the lipid composition of the methicillin susceptible *S. aureus* 5814S which is a congenic counterpart of the highly resistant stabile mutant 5814R studied

Table IV

Changes in the ratios of different chain type fatty acids in total polar lipid of logarithmically growing methicillin resistant cells of S. aureus 5814R treated with methicillin

Chain type comparison	Methicillin added (×MIC)	Ratios of fatty acids after incubation period		
		0 h	1 h	2 h
Iso to anteiso	0	1.17	1.21	1.49
	1/2	1.17	1.16	1.38
	1	1.17	1.10	1.74
Iso to straight	0	0.67	0.55	0.46
	1/2	0.67	0.75	0.91
	1	0.67	0.63	0.55
Anteiso to straight	0	0.57	0.45	0.31
	1/2	0.57	0.64	0.66
	1	0.57	0.58	0.32
Total branched to straights	0	1.24	1.00	0.77
	1/2	1.24	1.38	1.56
	1	1.24	1.21	0.87

1/2×MIC is equivalent to 1000 μg/ml methicillin
1×MIC is equivalent to 2000 μg/ml methicillin

here. In the sensitive cocci 1000 times less methicillin ($^1/_2$ μg/ml = 1×MIC, and 2 μg/ml = 1×MIC) caused a decrease in the proportion of branched-FAs and an increase in that of straight-FAs resulting in a significant fall in the ratio of branched- to straight-FAs. Parallel to these alterations a gradual and significant decrease in viability and a considerable cell lysis were observed in the sensitive culture [17].

On the contrary, in the culture of the methicillin resistant *S. aureus* 5814R, as it is demonstrated here, 1000 μg/ml (= 1/2×MIC!) of methicillin induces only a relative accumulation of branched-FAs bringing forth an increase in the ratio of branched to straight acids and consequently a more fluid TPL in the membranes of the treated cocci. This change may be advantageous for the methicillin resistant cocci in partially preventing the access of methicillin to its targets since polar lipids have been shown to interact with beta-lactams when they penetrate membranes [3–5]. It is well known that penicillin binding proteins are located in the membrane of *S. aureus* [23] and this is the place of most of TPL as well [13]. So, at 1/2×MIC of methicillin when the cocci have the possibility to survival and multiply the shift to a higher proportion of branched-FAs in TPL, may be a part of the defense mechanisms of the cells. Interestingly, the cell walls of these cocci become significantly thicker, there are an enhanced septation but a retarded separation of the daughters, and an accumulation of nucleotide-bound peptidoglycan precursors [Rozgonyi et al. unpublished observations].

On the action of 1 × MIC (= 2000 μg/ml !) of methicillin these events also occur but the retention of branched-FAs disappears. The cause of it may be the well-known phenomenon that certain bacteria excrete lipids into the surroundings under the effect of beta-lactams without any detectable lysis [15, 16, 19]. The other possibility may be that the susceptibility to methicillin of synthetic pathways of various FA classes are different in methicillin resistant *S. aureus*. Furthermore, a complete rest of metabolism may also occur under the effect of 1 × MIC of methicillin. These assumptions are to be proven in further study. Comparing the present results with our earlier findings in methicillin sensitive and resistant *S. aureus* [7, 17], it seems obvious that methicillin cannot induce such dramatic changes in the intracellular lipid composition of methicillin resistant staphylococci as in that of susceptible ones [17, 18]. On the other hand, the alterations caused in resistant cocci are opposite. This, and the relative stability of FA composition in TPL of methicillin resistant *S. aureus* may be parts of the mechanism of methicillin resistance.

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NONSPECIFIC RESISTANCE-ENHANCING ACTIVITY OF ZYMOSAN IN EXPERIMENTAL BACTERIAL INFECTIONS

J. Emőd and I. Joó

WHO Collaborating Centre for Reference and Research on Bacterial Vaccines, Human Institute for Serobacteriological Production and Research, Budapest, Hungary

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The nonspecific resistance-enhancing activity of zymosan on experimental bacterial *(Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Serratia marcescens* and *Staphylococcus aureus)* infections was investigated. Zymosan, being of very low toxicity, significantly enhanced nonspecific resistance to the above bacteria. The enhancement was the most pronounced 2 to 5 days after zymosan treatment.

It is well known that the immune system is considerably damaged by tumours, severe injuries, burns and immunosuppressive drugs, thus making the organism more susceptible to infections which may lead often to septicaemia. Besides, the pathogen, developing resistant, may diminish the success of antibiotic therapy. For this purpose, it is justified to enhance the organism's nonspecific resistance with immunostimulants, like zymosan (Z). Z is a water-insoluble polymannan-polyglucan polysaccharide complex, first isolated from the cell wall of baker's yeast *(Saccharomyces cerevisiae)* by Pillemer et al. [1]. The name Z was introduced into the literature by the same authors. The molecular structure of Z was revealed by Hassid et al. [2]. The literature on the infection-inhibiting effect of Z is poor. Kiser et al. [3], using *Bacillus anthracis, Klebsiella pneumoniae, Staphylococcus aureus, Pasteurella multocida, Proteus vulgaris* and *Pseudomonas fluorescens* as test organisms, observed the stimulating effect of Z in mouse experiments. Similar results were published by v. Baehr et al. [4], who worked with *Escherichia coli, Bordetella bronchiseptica, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* also in mice. Joyce et al. [5] proved the resistance-enhancing activity of Z in experiments performed in rats suffering from *E. coli* peritonitis.

In the present work, we studied the resistance-enhancing effect of Z in experimental infections produced in mice with opportunistic — facultative pathogenic — bacteria that may cause severe infections mainly in immunocompromised patients.

János Emőd, István Joó
Human Institute for Serobacteriological Production and Research
Szállás utca 5, H-1107 Budapest, Hungary

Materials and methods

Bacterial strains. E. coli O18a, c:K1:H– and O111:B4, *K. pneumoniae* capsular types K3, K9 and K10, *P. mirabilis* 033:H1, *P. aeruginosa* immunotype 5, *S. marcescens* O6/014:H12 and the Smith strain of *S. aureus*.

Mice. Animals of the outbred strain OF1; both sexes were used in an equal number. Mice of 16 to 18 g body weight were used throughout.

Zymosan. Z prepared from baker's yeast by the method of Cseh et al. [6, 7] was used. This preparation has been issued by our Institute under the name Mannozym^R for more than 25 years. The individual dose of Z was suspended in 0.5 ml of isotonic saline throughout.

Examination of the toxicity of Z. (i) *Test for mouse body-weight-decreasing toxicity.* Twelve numbered groups of mice were treated intraperitoneally (i.p.) with different doses of Z. The mice were observed for seven days: the changes in average body weight and the average deviations from the initial body weights were recorded.

(ii) *Testing of the lethal effect of Z.* Groups of mice were inoculated i.p. with rising doses of Z. Deaths were registered during an observation period of 72 h.

Dose-dependence of the nonspecific resistance-enhancing effect of Z. Groups of mice were injected i.p. with various doses of Z. Forty-eight hours later the animals were challenged by a suspension of *P. aeruginosa* immunotype 5 prepared from a 6-h slant agar culture of the bacterium. The inoculum contained 20×10^6 viable cells. The challenge suspension was prepared in phosphate gelatine buffer (PGB pH 7.2). Deaths occurring in the subsequent 72 h were recorded. Groups of mice not treated with Z were used as control. Observation lasted for 72 h.

Nonspecific resistance-enhancing effect of Z in the function of time. Groups of mice were inoculated i.p. with 1 mg of Z (1 mg is the usual single human dose of Mannozym^R) and challenged on the day of Z treatment and 12, 8, 5, 2 and 1 day before Z treatment with 20×10^6 bacteria of the *P. aeruginosa* immunotype strain 5. Animals were observed for 72 h.

Nonspecific resistance-enhancing effect of Z against various bacteria. Groups of mice received i.p. 1 mg of Z and were challenged 5 days after Z treatment with graded doses of the various bacterial strains, depending on their virulence. Groups of mice without pretreatment with Z served as controls. Observation lasted for 72 h.

Statistical analysis. The LD_{50} and ED_{50} values were calculated by the method of Reed and Muench [8]. Significance between results was calculated by variance analysis.

Results

Examination of the toxicity of Z

1. *Test for mouse body-weight-decreasing toxicity.* This method is used world-wide, in agreement with the WHO requirements for assessing the toxicity of pertussis vaccines [9]. Table I shows that a 3 mg/0.5 ml dose of Z caused no body loss, not even on the first day following treatment.

2. *Testing of the lethal effect of Z.* The LD_{50} value of Z was 13.65 mg/0.5 ml, indicating a very low toxicity (Table II).

Dose-dependence of the nonspecific resistance-enhancing effect of Z. The dose-dependence and the ED_{50} value of Z are presented in Table III. The ED_{50} for the mice used in the experiment proved to be 3.19 mg/0.5 ml. The data agree with the results obtained by Kiser et al. [3] with *K. pneumoniae*.

Nonspecific resistance-enhancing effect of Z in function of time. The effect was the most pronounced when Z was administered 2 to 5 days before challenge (Table IV). Though the effect gradually diminished later, the LD_{50} calculated for the 12th day still significantly surpassed the LD_{50} for the control group. However, given on the day of challenge, Z hardly increased resistance.

Table I

Toxicity of zymosan

Zymosan dose, mg	Mean body weight (g) before treatment	difference from the pre-treatment body weight after days						
		1	2	3	4	5	6	7
9	27.52	26.81 −0.71	27.76 +0.24	28.07 +0.55	—	—	—	28.39 +0.87
3	23.00	23.01 +0.01	24.18 +1.18	24.32 +1.32	—	—	—	25.26 +2.26
1	26.12	26.18 +0.06	26.31 +0.19	26.57 +0.45	—	—	—	27.59 +1.47
0.33	23.67	23.30 −0.37	23.72 +0.05	24.01 +0.34	—	—	—	25.08 +1.41
0.11	24.23	24.42 +0.19	24.54 +0.31	25.00 +0.77	—	—	—	25.43 +1.20
Control (saline)	25.17	25.85 +0.68	26.34 +1.17	26.38 +1.21	—	—	—	27.49 +2.32

Table II

Lethal effect of zymosan

Zymosan dose, mg	mg/kg	Mortality in %	LD_{50}
9	500	27	
15	800	58	13.65 mg/0.5 ml
21	1100	75	SD = 1.48
27	1500	81	

Experiments were repeated twice

Table III

Dose-dependence of the nonspecific resistance-enhancing effect of zymosan

Zymosan dose, mg	Survivors %	LD_{50}
9	67	
3	35	
1	29	3.19 mg/0.5 ml
0.33	17	SD = 0.78
0.11	8	
Control (saline)	0	

Challenge with *P. aeruginosa* immunotype 5 strain 48 h after Z treatment
Experiments were repeated 3 times

Table IV

Nonspecific resistance-increasing effect of zymosan in function of time

	Zymosan treatment on day						Untreated mice
	0	—1	—2	—5	—8	—12	
LD_{50} (10^6 viable cells)	1.91	13.99	23.73	16.61	5.79	2.78	1.28
RE_{LD50}	1.40	10.93	18.54	12.98	4.52	2.17	1.00

Challenge strain: *P. aeruginosa* immunotype 5
Experiments were repeated 6 times

Variance table

Factors	SQ	FG	MQ
Total	1755.17	43	40.81
Treatment	725.45	6	120.90
Error	1029.72	35	29.42

F = 4.1094
Significant difference 5% = 6.36

Table V

Nonspecific resistance-enhancing effect of zymosan against various bacteria

Bacterium	Treatment	LD_{50}	Difference
E. coli O18	nil	0.35×10^6	
	ZY	2.85×10^6	8.16×
E. coli O111	nil	9.70	
	ZY	1.28×10^3	132.0 ×
P. aeruginosa 5	nil	1.21×10^6	
	ZY	6.29×10^6	5.20×
K. pneumoniae K3	nil	5.01	
	ZY	1.19×10^2	23.90×
K. pneumoniae K9	nil	1.26×10^3	
	ZY	4.10×10^3	3.16×
K. pneumoniae K10	nil	1.00×10^5	
	ZY	10.00×10^6	100.0 ×
P. mirabilis O33	nil	1.10×10^5	
	ZY	1.88×10^6	17.10×
S. marcescens O6/O14	nil	3.50×10^5	
	ZY	20.40×10^6	58.29×
S. aureus Smith	nil	1.20	
	ZY	1.20×10^3	1000.0 ×

ZY = zymosan-treated

Nonspecific resistance-enhancing effect of Z against various bacteria. Taking into account that in the experiments performed with *P. aeruginosa* Z was very effective when administered 5 days before challenge, we chose this interval for further experiments. As shown in Table V, the protective effect of Z was consistently significant, irrespective of the challenge strain.

Discussion

The present results indicate that Z is of very low toxicity. Its nonspecific resistance-enhancing effect significantly depends on the time interval between Z treatment and challenge. A nonspecific resistance was induced against all bacterial strains tested, even by a dose as small as 1 mg of Z.

The mechanism of action of Z as well as the resistance-enhancing effect of glucan and mannan prepared from Z or baker's yeast will be dealth with in our next paper. Now we mention briefly that presumably the RES is stimulated and phagocytosis is increased, accelerated by Z [10–12]. The resistance-enhancing effect of glucan and mannan will also be dealt with in our next paper.

In the last years various immunostimulants, including synthetic substances, as well as studies with such agents have considerably increased in number. We refer to reports and monographs [13–25]. However, only very few of the substances have been studied in man. The experiences obtained with Z in the course of more than 25 years have proved the full innocuity of Z. Doses much higher (3 mg) than the dose recommended at present for man (1 mg) did not induce undesirable effects. We suggest that Z is suitable as a supplementary immunostimulant to be used for prevention of infections with opportunistic bacteria.

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INFLUENCE OF THE MEASLES VIRUS ON THE PROLIFERATION AND PROTEIN SYNTHESIS OF AORTIC ENDOTHELIAL AND SMOOTH MUSCLE CELLS

ÉVA CSONKA, P. I. BAYER, K. BÜKI and GY. VÁRADY

Second Department of Pathology, and Second Department of Biochemistry, Semmelweis University Medical School, Budapest

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To clarify whether some viruses could influence the different functions and membrane permeability of the aortic cells, we have examined in a model experiment the in vitro effect of the measles virus on the aortic endothelial and smooth muscle cells. The aortic cells infected with the virus failed to reveal gross cythopathic effect. Occasionally, however, syncytium formation and nuclear inclusions were observed. In infected endothelial cells lysosome containing viral nucleocapsids were seen. The early phase of measles virus replication inhibited the proliferation of endothelial cells of all species tested, while uniformly stimulated the replication of the smooth muscle cells relative to the control. In bovine aortic endothelial and smooth muscle cells the protein synthesis had been suppressed by the 4th to 6th hours postinfection. The results indicate that measles virus infection may be among the risk factors of atherosclerosis. It may damage endothelial cells by altering the cell membrane permeability and could induce proliferation of aortic smooth muscle cells.

The cells of the vessel wall are exposed not only to the normal components of the blood but also anything (different molecules, particle, viruses, etc.) that may be present in the circulation under pathological conditions. Many viruses are known to produce viraemia in the host. Some of them have also been supposed as playing role — among other agents — in the pathogenesis of atherosclerosis, by altering the fine structure of the cell membrane and hence the permeability [1, 2]. Fabricant et al. [3] succeeded in producing severe atherosclerosis in chicken by a strain (MDV) of herpes virus, and the same virus affected also the cholesterol metabolism of the medial smooth muscle cells [4]. It was also found that herpes virus suppressed the protein synthesis of cultured endothelial cells [5]. In aortic biopsy samples of atherosclerotic patients the presence of herpes virions in the endothelium as well as in the smooth muscle cells was frequently demonstrable [6].

The examinations listed above were done mostly with different herpes virus (DNA) strains. Relatively few data are, however, available on the effect

ÉVA CSONKA
Second Department of Pathology, Semmelweis University Medical School
Üllői út 93, H-1091 Budapest, Hungary

PÁL I. BAUER, KÁLMÁN BÜKI, GYÖRGY VÁRADY
Second Department of Biochemistry, Semmelweis University Medical School
Puskin u. 9, H-1081 Budapest, Hungary

of RNA viruses on the vessel wall cells. Kimura et al. [7, 8] succeeded to demonstrate measles virus antigen in the endothelium of dermal capillaries of patients with measles. Several RNA viruses (measles, parainfluenza, coxsackie 3, 4, etc.) were successfully grown in cultured endothelial cells [9]. Increased granulocyte adherence was observed in measles virus infected cells [10]. Endothelial cells are thought to be involved in the mechanisms of defence against viral infections. Cultured bovine aortic endothelial cells after infection with paramyxoviruses (NDV or Sendai) produced interferon [11]. It is known, that some enveloped non-oncogenic viruses modify the cytoplasmic membrane of the cell so as to develop a lectin-binding capability similar to the malignant transformed cells [12]. Using this phenomenon as an indicator in our previous experiments we have demonstrated that cultured cells of vessel wall origin (endothelium, smooth muscle, adventitial fibroblasts) developed lectin-binding after infection with measles virus. The endothelial cells were the most sensitive ones. The lectin-binding developed as early as 6 h after virus infection. Our observations may contribute to the better understanding of the capillary lesions in virus-induced eruption and in some accidental, local permeability changes in the vessel of the patients with unidentified infections caused by some enveloped viruses [13].

In the present study we examined the replication of measles virus in different cells, cultured from the vessel wall and we made attempts to clarify whether the measles virus has any other effect on in vitro cultivated aortic cells in respect of proliferation, protein synthesis and cell morphology.

Materials and methods

Cell cultures. The BAEC (Budapest Aortic Endothelial Cell-culture)-806 bovine aortic endothelial; the BASC (Budapest Aortic Smooth muscle Cell-culture)–809 bovine aortic smooth muscle; the BAEC-812 mini-pig aortic endothelium; the BASC-813 mini-pig aortic smooth muscle; the BASC-76 transformed mini-pig aortic smooth muscle; the 10HUAS human fetal aortic smooth muscle and the 10HUAF human fetal aortic adventitial fibroblast were used. All cell cultures were established in our laboratory and were maintained in modified Dulbecco MEM with 10% fetal calf serum. The cells used in the experiments — except the BASC-76 transformed cell line — were in the first to sixth passage in vitro.

Virus. The vaccinal strain Leningrad-16 (Edmonston origin) Batch No. 021–1969 of measles virus, maintained in our transformed permanent virus sensitive BASC-76 cell line [14] was used as inoculum in all experiments.

Virus replication in aortic cell cultures. For these experiments confluent monolayers of bovine aortic endothelial, bovine aortic smooth muscle or the virus sensitive cell line as control were infected with measles virus at a multiplicity of infection (MOI) 5×10^{-3}, 5×10^{-4}, 5×10^{-5} and 5×10^{-6}/ml. Cultures in tubes were harvested for virus titrations on days 2, 4, 8, 10 and 16 after infection. Samples of the cell monolayers with the supernatant fluids were frozen and thawed, and stored at −70 °C. To measure the virus yields serial 10-fold dilutions of these harvests were made and inoculated onto the virus sensitive cell line.

Pulse labelling. Monolayers of the different aortic cell types of different species, growing directly on the bottom of the counting vials were infected at a multiplicity of infection of 5×10^{-2} MOI. At different times after infection, infected and control cultures were labeled either with 1 μCi/ml ^{3}H-thymidine or with 0.5 μCi/ml ^{14}C-proline. Four samples were prepared

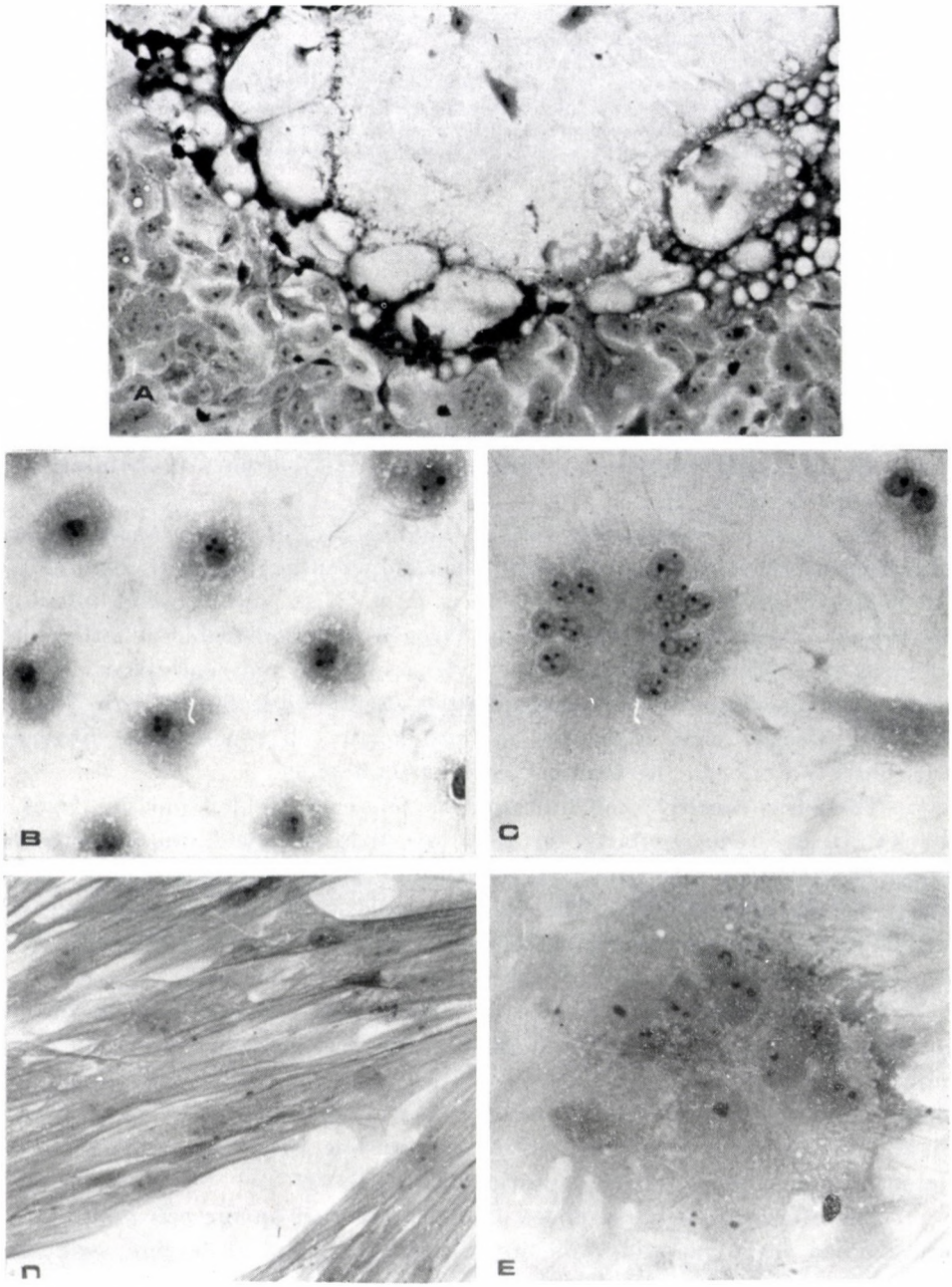

Fig. 1. Morphological examinations of the virus infected aortic cell cultures. (A) Syncytium formation after measles virus infection in the virus sensitive BASC-76 cell line. (B) Uninfected bovine aortic endothelial cells 9 days after seeding. (C) Bovine aortic endothelial cells 7 days after measles virus infection. Syncytium formation and nuclear inclusions are present. (D) Uninfected bovine aortic smooth muscle cells 9 days after seeding. (E) Bovine aortic smooth muscle cells 7 days after measles virus infection. Syncytium formation without nuclear inclusions. Haematoxylin-eosin $\times 630$

1 h after labelling the cell monolayers as described previously [15], and the radioactivity was determined with a Beckman M-25 spectrometer.

Morphological examinations. For light microscopy, the cells were grown on coverslips in Leighton tubes. After fixation in 4% neutral formaldehyde they were stained with haematoxylin-eosin. For transmission electron microscopy the cells were grown in Microplates on collagen membranes [16]. The cell sheet was prepared for electron microscopy as described earlier [17]. The preparations were examined in a JEM 100 B electron microscope.

Results

Endothelial or smooth muscle cells infected with measles virus mostly failed to reveal any conspicous cythopatic effect, in spite of measurable replication of the virus. In some cases, however, some syncytium formation and in endothelial cells nuclear inclusions were observed (Fig. 1). In infected endothelial cells we could demonstrate the presence of lysosomes containing viral nucleocapsides (Fig. 2).

The decisive evidence for viral replication in cells of the vessel wall was obtained by titrating the total virus yield (supernatant and cells together) of the infected aortic cell cultures on days 2, 4, 8, 10 and 16 after infection. As shown in Fig. 3, replication of the virus was demonstrable as an increase of virus titre. To distinguish between virus survival and replication, measles virus was inoculated at low multiplicities of infection, 5×10^{-3} to 5×10^{-6} MOI/ml. Measles virus replicated in both aortic cell types, some difference was observed only in the kinetics of virus replication.

The virus sensitive cell line used as reference yielded measles virus of 10^5 $TCID_{50}$/ml titre regularly. At very low MOI the yield was considerably lower. Endothelial cells produced measurable amounts of virus only on the 4th to 8th day after infection. The yield produced on the 16th day was, however, similar to that obtained in the susceptible cell line. At very low MOI the yields in endothelium was superior to those in the susceptible cells.

The virus production of smooth muscle cells was maximal on the 8th to 10th day after infection. The yield was, however, always lower than in the endothelial or in the susceptible cell lines. In contrast to the endothelial cells, smooth muscle cells started to produce measurable amounts of virus already on the 4th day at a MOI of 5×10^{-3}. At very low MOI virus replication was detectable only after the 10th postinfectional day.

The effect of the early phase of virus infection on the cell proliferation was studied on bovine and swine aortic endothelial and on bovine, swine and human fetal aortic smooth muscle cells. For reference there was also studied the postinfectional proliferation of the virus sensitive BASC-76 and primary human fibroblast cells (Fig. 4).

On the 1st and 2nd day measles virus infection was found to inhibit the proliferation of aortic endothelial cells in both species. The proliferation of

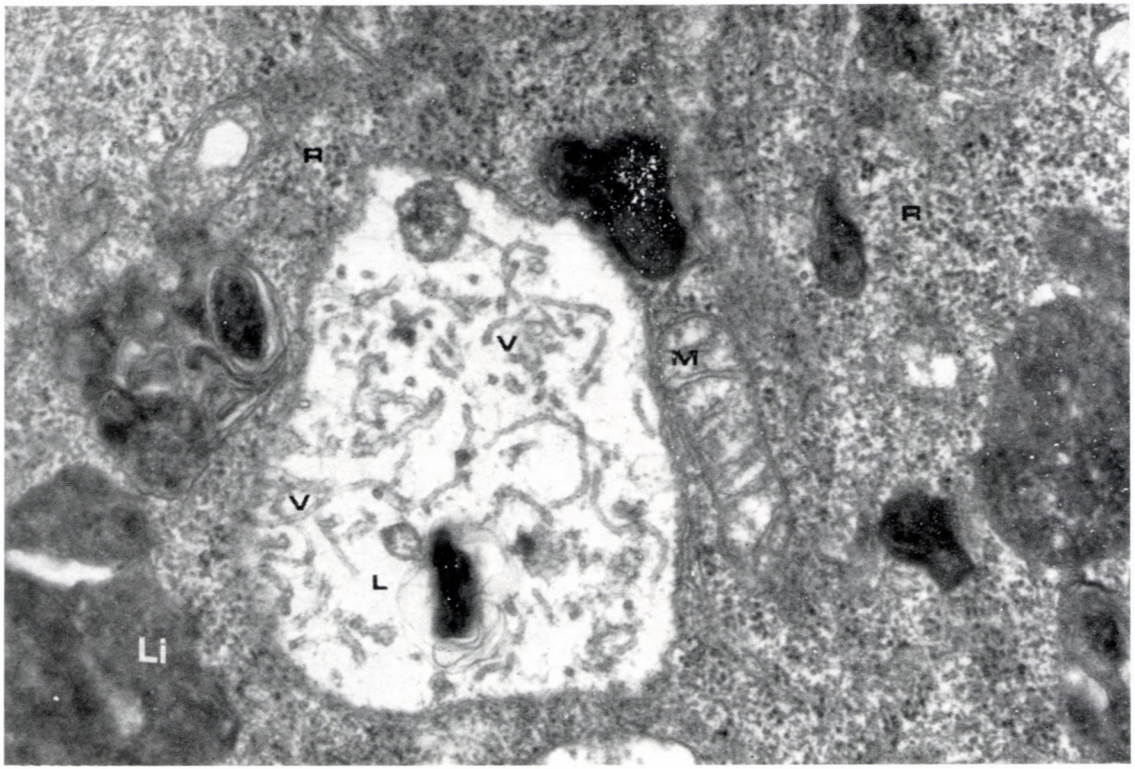

Fig. 2. Measles virus nucleocapsids in the lysosome of a virus infected bovine aortic endothelial cell. L: lysosome; V: viral nucleocapsid; M: mitochondrium; R: ribosome; Li: lipid droplet. ×30 000

smooth muscle cells was, on the contrary, stimulated on the same days in both species. A moderate stimulation by measles virus infection of proliferation was registered in both BASC-76 and human fibroblast cells.

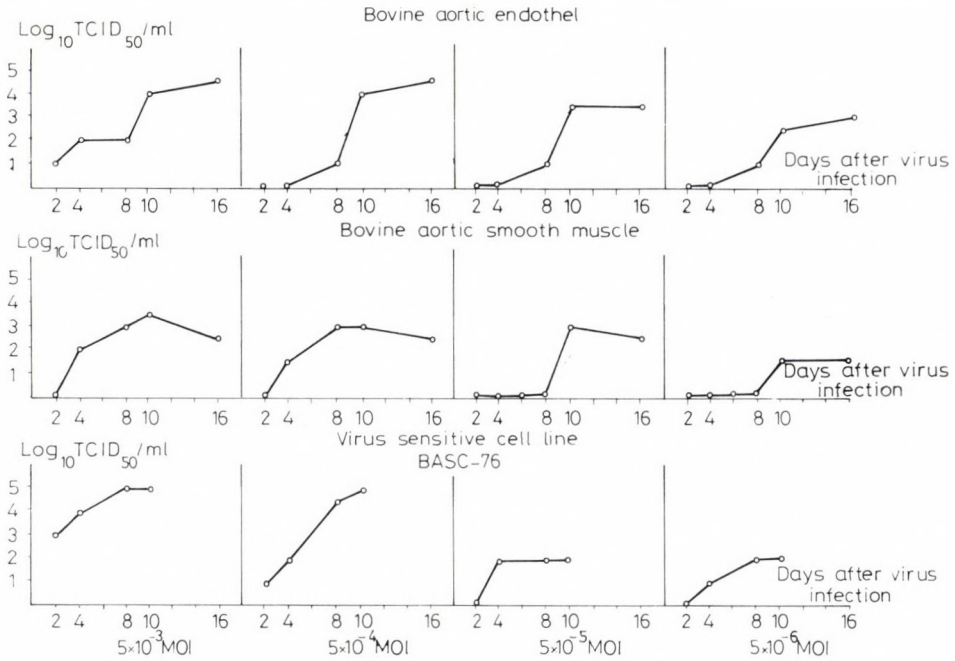

Fig. 3. Titration of measles virus yield in aortic cell cultures

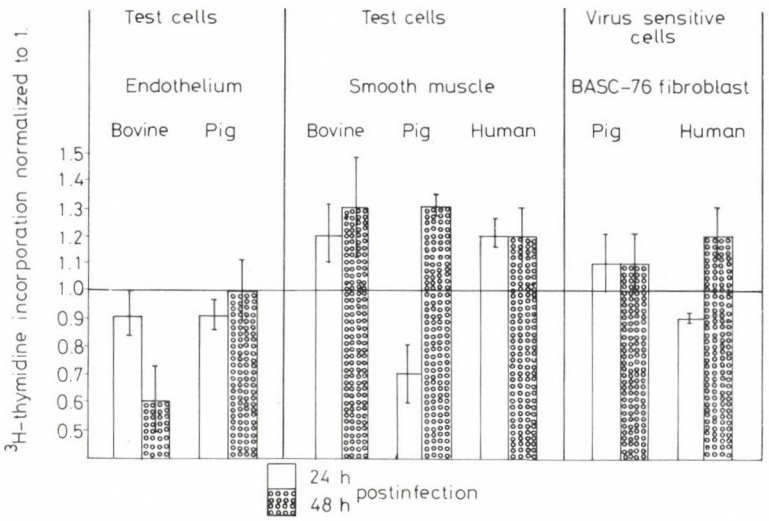

Fig. 4. Effect of measles virus on the proliferation of different aortic cells

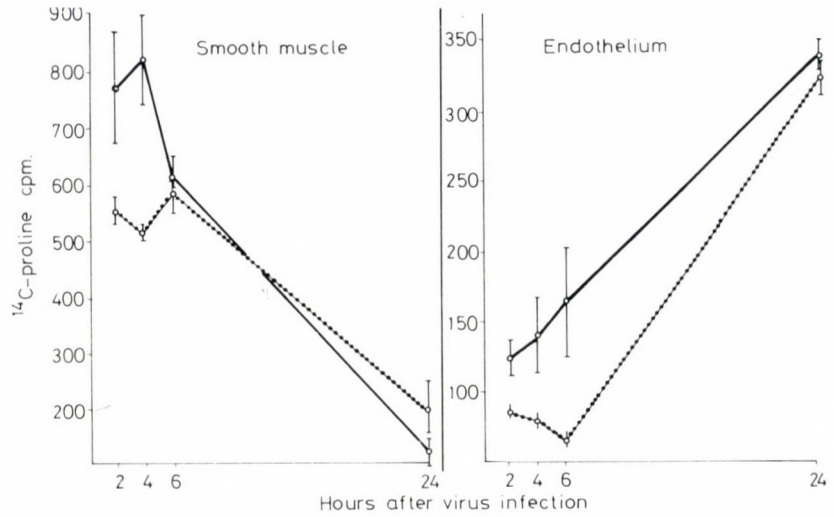

Fig. 5. Effect of measles virus on the protein synthesis of bovine aortic cells; ——— control, ····· infected cells

The effect of measles virus infection on protein synthesis of endothelial and smooth muscle cells was examined at hours 2, 4, 6 and 24 postinfection. Control and infected cultures were pulsed for 1 h with ^{14}C-proline. The protein synthesis of bovine endothelial cells was reduced for 6 h after infection but had returned to normal by the 24th h (Fig. 5).

The protein synthesis of smooth muscle cells was significantly inhibited at 2–4 h after virus infection. From the 6th to 24th h the inhibition disappeared smoothly and the synthesis reached the value of the controls.

Discussion

Measles is contracted by practically everybody and is characterized by a viraemic phase during which the virus can be recovered from the leukocytes. In this viraemic phase the possibility exist that the virus may infect also cells of the vessel wall. Therefore this virus may serve as a model for studying the effect of enveloped RNA viruses (RS, parainfluenza, etc.) on the vessel wall cells.

No marked cytopathic effect was seen in the endothelial or smooth muscle cells infected with measles virus. In some cultures, however, typical syncytia and nuclear inclusion were seen. In the lysosomes of some infected endothelial cells viral nucleocapsids were detected by transmission electron microscopy. Total destruction of the endothelial or smooth muscle cell monolayers was never seen as a result of virus infection.

Replication of the virus in infected cells was demonstrable above any doubt by means of titration on susceptible cell line. The lag phase appeared to be longer in the endothelial than in the smooth muscle cells. The final yield was, however, always higher (even with very low MOI) in the endothelial than in the smooth muscle cells.

It seemed to be of some interest that measles virus infection inhibited endothelial cell proliferation through 2 days independently of the species examined. Proliferation of smooth muscle cells of all species studied was invariably stimulated suggesting a possible smooth muscle cell proliferation enhancing effect of measles virus.

Cellular protein synthesis was inhibited during the initial phase of measles virus infection in both the endothelial and smooth muscle cells. All these results favour the idea, that measles virus may survive and replicate in the cells of the vessel wall thus affecting their rates of proliferation, their metabolism and membrane structure. As a consequence also the permeability conditions may change and open a way to the transport of various damaging factors, playing role in the pathomechanism of atherosclerosis.

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REVISION OF THE VALIDITY OF CAMP TESTS FOR *LISTERIA* IDENTIFICATION. PROPOSAL OF AN ALTERNATIVE METHOD FOR THE DETERMINATION OF HAEMOLYTIC ACTIVITY BY *LISTERIA* STRAINS*

J. A. Vazquez-Boland, L. Dominguez, J. F. Fernandez, E. F. Rodriguez-Ferri, V. Briones, M. Blanco and G. Suarez

Department of Animal Pathology I. Institute of Microbiology and Immunology, Veterinary Faculty, University of León, León, and Complutense University, Madrid, Spain

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The validity of CAMP tests with *Staphylococcus aureus* and *Rhodococcus (Corynebacterium) equi* as defined for *Listeria* identification was revised. This characterization method appeared to be unreliable for two reasons: first, a positive CAMP test with *R. equi* is not specific for *Listeria ivanovii* as *Listeria monocytogenes* (and *Listeria seeligeri*) give also a clear positive reaction; second, doubtful reactions could be observed with *S. aureus* when assaying haemolytic and non-haemolytic *Listeria* strains (possibility of false negative and false positive results; subjectivity of the interpretation). The use of a Microplate technique previously described [1] instead of CAMP tests is proposed for the reliable demonstration of the haemolytic character of *Listeria* in the routine identification of these organisms.

The *Listeria* species recently described *(Listeria monocytogenes, Listeria innocua, Listeria welshimeri, Listeria seeligeri* and *Listeria ivanovii)* [2–4] from *Listeria monocytogenes* "sensu lato" (as defined in the 8th edition of "Bergey's Manual" [5]) are differentiated by two main characters: haemolytic activity and acid production from D-xylose [6].

Due to the problematic interpretation of the haemolytic activity of *Listeria* on blood agar plates, a synergistic haemolysis assay with beta-haemolytic *Staphylococcus aureus* and *Rhodococcus (Corynebacterium) equi* (described as CAMP test [7] with *S. aureus* or *R. equi)* was proposed [6, 8–10] and accepted as a fundamental criterion for identification [3].

It was stated that the haemolysis produced on blood agar by *L. monocytogenes* and *L. seeligeri*, which is frequently so weak that it is difficult to determine, is enhanced when this species have grown on a beta-haemolysis

J. A. Vazquez-Boland, E. F. Rodriguez-Ferri
Departamento de Patologia Animal I (Sanidad Animal), U. D.
Microbiologia e Immunologia, Facultad de Veterinaria, Univ. de León, León, Spain

L. Dominguez, J. F. Fernandez, V. Briones, M. Blanco, G. Suarez
Univ. Complutense, Madrid, Spain

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zone produced by *S. aureus* ("CAMP test positive with *S. aureus*") but not when grown in the vicinity of *R. equi* ("CAMP test negative with *R. equi*"). On the other hand, it was also stated that *L. ivanovii*, a species easily recognizable by the strong haemolysis (often bizonal) produced on sheep blood agar, show a positive reaction in the CAMP test with *R. equi* but not with *S. aureus*. The species *L. innocua* and *L. welshimeri*, as well as *Listeria grayi* and *Listeria murrayi*, which are non-haemolytic, are considered as CAMP test negative with *S. aureus* and *R. equi* [3].

However, reports suggesting the possibility of false negative results [11] or difficulties in the interpretation of *S. aureus* CAMP test [12], that could be subjective or inconclusive [13], as well as reports showing discrepancies with the standard results in *R. equi* CAMP test [14–16], all of them corroborating our own experience [1, 17], led us to revise the validity of this identification method.

Materials and methods

We have tested 43 *Listeria* strains (Table I). *Listeria* from SLCC (Special *Listeria* Culture Collection) were reference strains for serovar determination, sent by H. P. R. Seeliger (Institut für Hygiene und Mikrobiologie, Würzburg University, Würzburg, FRG). Strains of milk or dairy origin were sent by F. Blanchard (Soredab et Cie., France) except P- and L- strains, that were isolated by us. Milk and dairy strains were lysotyped by J. Rocourt (Unité d'Ecologie Bactérienne, Institut Pasteur, Paris, France) and serovar or serogroup was determined by A.-L. Courtieu (Centre National de Listeria, U. F. R. de Médicine, Nantes, France) except strains P- and L-, for which the serogroup was determined in our laboratory. For the CAMP test *S. aureus* CIP 5710 and *R. equi* CIP 5869 obtained from the Pasteur Institute Collection, Paris, were used as described [3, 6, 8], on sheep blood agar (5% v/v) with Brain Heart Infusion (Difco) as base medium. The plates were incubated aerobically at 37 °C and were read at 18, 24 and 48 h. All these strains were tested also by a Microplate technique previously described by us for the determination of the haemolytic activity [1].

Results and discussion

All the strains of *L. monocytogenes* tested gave a clear positive CAMP test with *R. equi*. In the confluence of the cultures of *L. monocytogenes* and *R. equi* a well-defined round-shaped zone of complete haemolysis appeared after 24 h incubation, developping in width with time. Some strains presented a more marked reaction, i. e. NCTC 7973 (serovar 1/2a) and NCTC 5105 (serovar 3a), being the halo of complete haemolysis in these cases at least as large as that observed with *L. ivanovii* ATCC 19119. In this sense we have to point out that *L. ivanovii* produces itself a large zone of complete haemolysis surrounding the colonies, and that the enhancement of haemolytic activity showed by this species in the CAMP test with *R. equi* is due to the clearing of the outer halo of incomplete haemolysis exposed to *R. equi* exosubstances, giving a typical large shovel-shaped lytic phenomenon (Fig. 1). In contrast, *L. mono-*

Table I

Listeria strains tested in the CAMP tests with S. aureus CIP 5710 and R. equi CIP 5869

Strain		Serovar (or serogroup)	Origin
L. monocytogenes	SLCC 2371 (NCTC 7973^{T*})	1/2a	collection
	SLCC 2755	1/2b	collection
	SLCC 2372 (NCTC 5348)	1/2c	collection
	SLCC 2373 (NCTC 5105)	3a	collection
	SLCC 2540	3b	collection
	SLCC 2479	3c	collection
	SLCC 2374 (NCTC 5214)	4a	collection
	SLCC 2375 (NCTC 10527)	4b	collection
	SLCC 2376 (ATCC 19116)	4c	collection
	SLCC 2377 (NCTC 10888)	4d	collection
	SLCC 2778 (ATCC 19118)	4e	collection
	SLCC 2482	7	collection
	LB7	1/2	milk
	LID	1/2	milk
	LI5	1/2	environmental (dairy)
	L2A	1/2	milk
	LI1	1/2	milk
	LT1	4	milk
	LP1	1/2	environmental (dairy)
	LJG151	4	environmental (dairy)
	P–1	4	sheep encephalitis
	P–2	4	sheep encephalitis
	L–488a	4	milk
	L–973	4	milk
	L–1284	1/2	milk
L. innocua	SLCC 3379^{T} (ATCC 33090)	6a	collection
	SLCC 3423	6b	collection
	L1M	6b	environmental (dairy)
	L4F	6a	milk
	LP7	6b	milk
	LB1	6b	milk
	LHG1612	6b	milk
	L–157	—	milk
L. welshimeri	SLCC 5334 (CIP 8149^{T})	6a	collection
L. seeligeri	SLCC 3954 (CIP 100100^{T})	1/2b	collection
	SLCC 5921	1/2b	collection
	LS1	6b	milk
	LT3	1/2b	environmental (dairy)
	L–16	1/2	milk
	L–123	1/2	milk
	L–1453	1/2	milk
L. ivanovii	SLCC 2379^{T} (ATCC 19119)	5	collection
	L1PE	5	milk

^{T}: type strain.
^{T*}: proposed type strain, in replacement of ATCC 15313, which is non-haemolytic

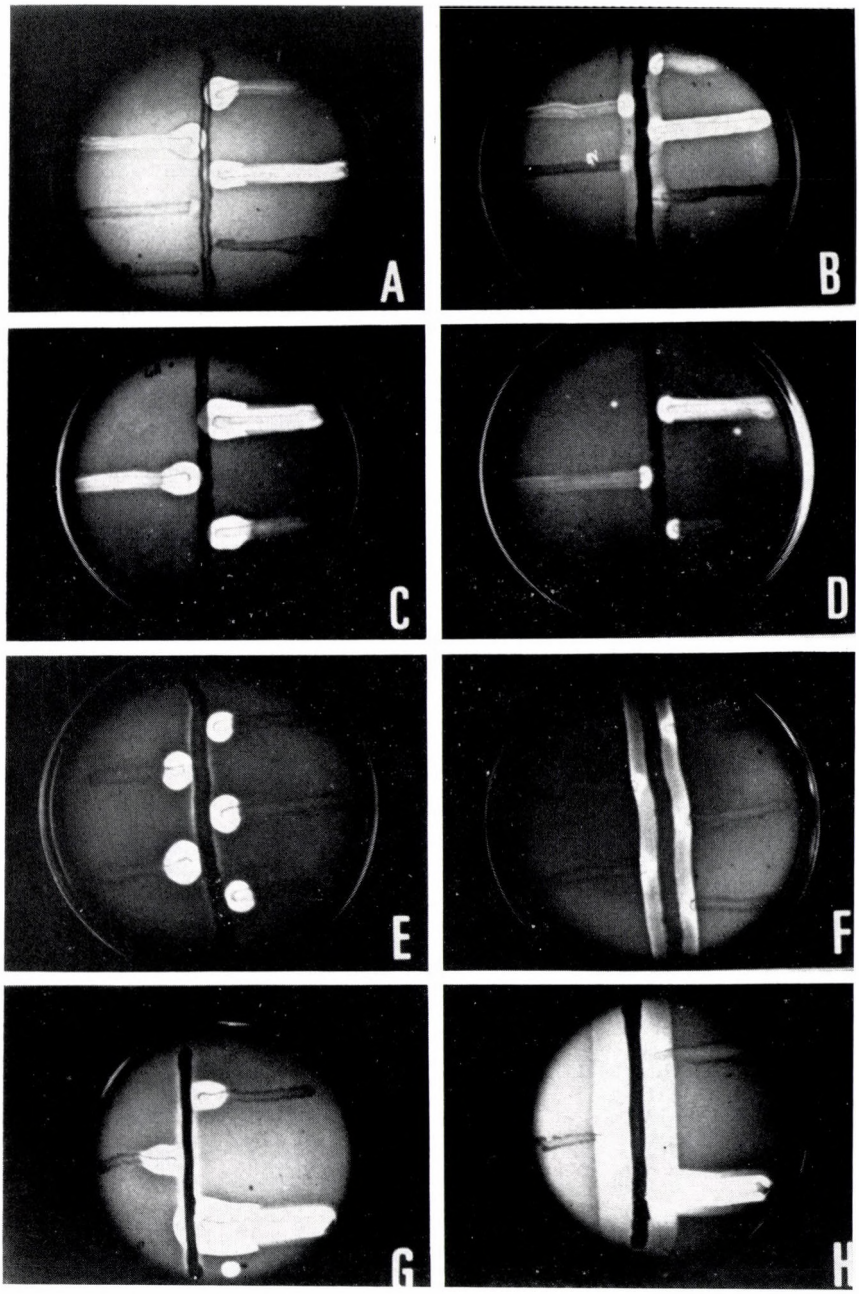

Fig. 1. Listeria strains in the CAMP tests with *R. equi* CIP 5869 (A, C, E, G; vertical streak of growth) and *S. aureus* CIP 5710 (B, D, F, H; vertical streak of growth). A and B: from up to down, *L. monocytogenes* SLCC 2371 sv 1/2a, *L. monocytogenes* SLCC 2373 sv 3a, *L. ivanovii* SLCC 2379 sv 5, *L. seeligeri* SLCC 3954 sv 1/2b, *L. innocua* SLCC 3379 sv 6a and *L.welshimeri* SLCC 5334 sv 6a; 24 h culture. C and D: from up to down, *L. ivanovii* SLCC 2379 sv 5, *L. monocytogenes* SLCC 2373 sv 3a and *L. monocytogenes* SLCC 2371 sv 1/2a; 18 h culture. E and F: from up to down, *L. monocytogenes* strains SLCC 2540 sv 3b, SLCC 2778 sv 4e, SLCC 2376 sv 4c, SLCC 2482 sv 7 and SLCC 2374 sv 4a; 48 h culture. G and H: from up to down, *L. monocytogenes* L-488a, *L. monocytogenes* L-1284 and *L. ivanovii* L1PE; 48 h culture

cytogenes strains always produce a weak beta-haemolysis that is difficult to determine (haemolytic character of 58.4% of the strains tested was doubtful on blood agar), and in proportion a dramatic synergic haemolysis reaction when grown close to *R. equi*. Therefore, the *R. equi* CAMP phenomenon could be considered even more remarkable with *L. monocytogenes* (a species defined as CAMP negative with *R. equi* [3]) then with *L. ivanovii*.

L. seeligeri strains presented variable results in the CAMP test with *R. equi*. While CIP 100/100 and SLCC 5921 gave a positive reaction (these strains being defined as CAMP negative with *R. equi* [3]), the remaining were negative. *L. innocua* and *L. welshimeri*, which are non-haemolytic, were always negative.

With respect to the CAMP test with *S. aureus*, *L. monocytogenes* strains were positive but the reactions were not as clear as those obtained with *R. equi*. At 24 h incubation, in the zone of beta-haemolysis a weak halo of complete haemolysis appeared (not always present) and an external one, arrow-head shaped, incomplete haemolysis. After 48 h incubation the reactions were doubtful due to the lytic effect of the beta-haemolysin from *S. aureus*. The results of the CAMP test with *S. aureus* were more difficult to interpret when compared to those obtained with *R. equi* because in certain cases *L. innocua* strains produced a clearing reaction (Fig. 1) very similar to that observed with some *L. monocytogenes* strains. The results were also doubtful with all *L. seeligeri* strains. *L. ivanovii* presented sometimes a weak synergistic haemolysis reaction (Fig. 1).

In accordance with these results it could be considered that the CAMP test as defined for *Listeria* is not a reliable tool for the characterization of these organisms, for two reasons: first, doubtful reactions could be observed in the *S. aureus* CAMP test when assaying haemolytic and non-haemolytic *Listeria* strains, i. e. the possibility of false-negative and false-positive results; and second, a positive CAMP test with *R. equi* is not specific for *L. ivanovii* as *L. monocytogenes* give also a clear positive reaction (this was demonstrated previously by other authors [14—16]), i. e. this method is not as discriminative as considered.

Taking into consideration these facts we propose the use of a reliable Microplate Technique previously described by us [1], instead of CAMP test with *S. aureus* and *R. equi*, for the determination of the haemolytic activity in the routine typing of *Listeria*. The Microplate Technique proposed appeared to be a simple and reliable method of a clear and unmistakable differentiation between haemolytic and non-haemolytic *Listeria* strains, that is, of the fundamental purpose of the CAMP test for which it seems not to be valid enough. Furthermore, the Microhaemolysis technique allows the quantitation of the haemolytic activity, by means of CHU (Complete Haemolysis Units) and MHU (Minimal Haemolysis Units [1]), a character that should be considered

of taxonomic interest according to our results, especially in the case of *L. ivanovii* and *L. seeligeri*, that present the same sugar acidification pattern but a characteristic differential haemolytic one [1].

Employing the Microhaemolysis technique, the five *Listeria* species referred to are differentiated as follows: *L. monocytogenes*, haemolytic (variable intensity [1]) and D-xylose-negative; *L. innocua*, non-haemolytic and D-xylose-negative; *L. welshimeri*, non-haemolytic and D-xylose-positive; *L. seeligeri*, weakly haemolytic (0 CHU, 3–6 MHU) and D-xylose-positive; and *L. ivanovii*, highly haemolytic (24 CHU, 384 MHU) and D-xylose-positive [1].

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ASSOCIATION OF VIRULENCE MARKERS WITH ANIMAL PATHOGENICITY OF *ESCHERICHIA COLI* IN DIFFERENT MODELS

ÉVA CZIRÓK, MARYVONNE DHO, MARIA HERPAY, I. GADÓ and HEDDA MILCH

National Institute of Hygiene, Budapest, Hungary, and National Agricultural Research Centre, Research Centres of Tours-Nouzilly, Station of Avian Pathology and Parasitology, Monnaie, France

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Employing chicken and several strains of mice, different routes (intraperitoneal, subcutaneous) of infections and isogenic pairs of strains, association of virulence markers with animal pathogenicity was studied in *Escherichia coli*. Mouse virulence of avian strains was less significant than the lethality for chicks of human strains. LD_{50} in various animals did not differ significantly. Strains with antigen K1 were more virulent for mice than their $K1^-$ derivatives. Loss of haemolysin (Hly), mannose resistant haemagglutinating capacity or antigen K5 less markedly decreased the virulence. As opposed to other virulence factors, increased virulence of $K1^+$ strains could also be demonstrated in mouse sepsis assay based on bacterial counts in the liver. Loss of Hly alone did not influence the persistence in the liver, however, these strains killed less mice. Aerobactin acts together with other factors, it is not per se a virulence factor. In organotropic experiments 19 strains out of 36 belonging to serotypes O7:K1:H-, O18:K1:H-, O78:H- and spontaneously agglutinable $K1^+$ cultures, caused ophthalmitis with purulent discharge, and 4 out of 22 strains that belonged to serotype O78:H- induced incoordinated movement of mice. Because of its special organotropic affinity to the brain and as it caused two epidemics of meningitis among newborns in Hungary, serotype O78:H- has a special pathogenic property and differs from other O78 strains that were isolated in other countries.

Since Theodor Escherich described "Bacterium coli commune" 100 years ago, important advances have been made on *Escherichia coli* to establish the difference between non-pathogenic organisms (as believed at that time) and pathogenic ones (that were later discovered [1]). As early as in 1907 Winslow and Walker [2], later on Goldschmidt [3] differentiated the harmful organisms from commensal strains by means of fermentation reactions and even by serological techniques. Owing to the serological typing scheme for *E. coli* worked out by Kauffmann in the early 1940's [4] it was possible to establish that differences exist between normal faecal strains and strains isolated from pathological material [5]. Several other properties of *E. coli* have been recognized to be often associated in strains isolated from infections and relatively

ÉVA CZIRÓK, MARIA HERPAY, ISTVÁN GADÓ, HEDDA MILCH
National Institute of Hygiene
Gyáli út 2–6, H-1966 Budapest, Hungary

MARYVONNE DHO
Institut National de la Recherche Agronomique, Centre de Recherches de Tours, Station de Pathologie, Aviaire et de Parasitologie
Nouzilly-37380 Monnaie, France

uncommon in *E. coli* of the normal enteric flora. In 1921 Dudgeon et al. [6] first reported that *E. coli* strains from extraintestinal sources were very often haemolytic compared with those strains isolated from the faecal flora. In several other publications [5, 7–9] one also meets the conception that haemolysin production may be a virulence factor. From his investigation Kauffmann [5] concluded that O group, O inagglutinability, origin, toxicity, haemolysis and ability to cause necrosis were correlated. Adhesion of bacteria to the host's epithelium has been recognized, too, as an important virulence factor in many bacterial infections [8–11]. According to other investigations colicin V production is a common property of *E. coli* strains isolated from bacteraemias of humans [11, 12].

Various animal models have been employed, which confirmed the pathogenic role of *E. coli*. Kauffmann [5] demonstrated that strains isolated from pathological material were toxic for mice. Powell and Finkelstein [13] found that differences in virulence among *E. coli* strains could be demonstrated in 13-day-old chicken embryos inoculated allantoically. In these tests different routes of infection were employed: intranasal installation lung oedema [14], intraperitoneal injection [12], intravenous [7] and subcutaneous [9] infections. Van den Bosch et al. [7] investigated the kinetics of the viable count in the mouse kidney. Hacker et al. [9] noticed mouse lethality (i. e. LD_{50}) after intraperitoneal infection comparing with chicken embryo assay, mouse nephropathogenicity test, mouse respiratory infection model and mouse sepsis system.

In our former paper [15] studying association in *E. coli* of LD_{50} with haemolysin production, haemagglutinating capacity, antigens K1, K5, colicinogenicity and pathogenicity, 663 *E. coli* strains of human origin were examined. As a result, it was concluded that: (i) strains of the most frequently occurring serogroups were more pathogenic for mice than those of belonging to the others; (ii) the outcome of an *E. coli* infection depended not on one virulence factor but on a combination of these determinants acting together, however, possession of antigen K1 played an important role in pathogenicity of mice, other virulence markers could be arranged in an order of importance, and could strengthen the effect of each other; (iii) *E. coli* with different virulence factors could be classified into different pathogenetical groups (i. e. meningitis in newborns and septicaemia with organotropism in adults).

It appeared pertinent to ask whether results of statistical analysis on a large number of strains, or of intraperitoneal infections (i. p.) of one (CFLP) animal line, or of LD_{50} mice would be of universal validity? Thus in the present study we employed different animals (chicken and mice), several strains of mice beside CFLP (NMRI, OF1), i. p. as well as subcutaneous (s.c.) routes of infections, and we compared results of LD_{50} with sepsis assay. The effect of virulence markers on animal experiments was examined by using isogenic pairs of strains (e. g. wild-type strains and their virulence factor defective

spontaneous mutants). Our aim was to answer the question whether virulence factors of *E. coli* would be factors contributing to the specific pathogenicity of *E. coli* causing septic infection and meningitis.

Materials and methods

Bacterial strains were isolated from different human (19) and avian (28) sources. After characterizing *E. coli* strains by determination of their several virulence factors, the next pairs of isogenic strains were employed: 3 strains that possessed antigen K1 and their $K1^-$ spontaneous mutants; one Hly^+/Hly^- pair (isolated by L. Emődy, Pécs, Hungary); strain 119 ($MRHA^+$, $K5^+$, Hly^+) and its $MRHA^-$ pair (119/1, isolated by Kuch et al. [16]), as well as their spontaneous $K5^-$ mutants; strain 536 (serum resistant, S fimbria$^+$, Hly^+) and its derivatives 536–21 (having lost all these properties) and 536–31 (Hly^- derivative of 536) were kindly provided by Hacker et al. [9]. Data of *E. coli* strains and their variants are listed in Tables I–III.

Classification according to serological examination of O and H antigens, mannose resistant haemagglutination (MRHA), haemolysin production (Hly), detection of K1 and K5 antigens, determination of colicinogenicity (Col), examination for aerobactin (Aer) production were carried out as described previously [17–20]. Presence or absence of antigen K1 was controlled by Wellcogen *N. meningitidis* B/*E. coli* K1 monoclonal sera.

Animal experiments. Assay for lethality in mice after i.p. infection were carried out in CFLP (Carworth Farm Lane Petter), NMRI (originated from Naval Medical Research Institute, USA) and OFI (Oncius France 1) lines of mice. The lethality of bacteria for mice and 50% lethal dose (LD_{50}) were assessed as described earlier [15]. *Lethality test in chicken* was conducted by inoculating undiluted overnight broth cultures s.c. into groups of one-day-old chicks, as described previously [21]. *Mouse sepsis assay* was performed according to Hacker et al. [9]. The experiments were done repeatedly from time to time (as indicated in the tables). *Examination of organotropism in mice.* Groups of 20–20 CFLP mice weighing 14–16 g were inoculated i.p. with 0.5 ml of bacteria grown for 4–6 h in broth and diluted in physiological saline so that the viable number of bacteria corresponded to LD_{50}.

Results

Differences in virulence of human and avian E. coli strains for chickens and mice. E. coli strains of human (19) and avian (28) origin were tested for mouse as well as for chicken lethality. Results of human strains with well-defined virulence markers are shown in Table I. Thirteen out of 19 strains proved to be virulent (e. g. LD_{50} was less than 1×10^6 [15]) both for mice and chickens. Further 3 strains (840, 990 and 52 771) were virulent only for chickens. In case of strain 840, LD_{50} was 3.1×10^5, accordingly, it was near to the highest value of lethal class 1 (e. g. 10^3 to 10^6 [22]) for chicken. Meanwhile LD_{50} in mice of strains 52 771 and 990 (2.7×10^6 and 2.1×10^6) was just over the highest value of virulent group for mice. LD_{50} values for chicks proved to be at a lowest level (LD_{50} of 13 out of 15 virulent strains fell between 10^2–10^3) as compared to those of mice (7 out 13 strains with 10^3–10^4, another 6 strains with 10^5 LD_{50}).

As to virulence markers of human strains, we failed to show correlation between MRHA positivity and animal virulence of strains either in mice or in chicken (e. g. strains 847, 047, 97358 were $MRHA^+$ and avirulent for ani-

Table I

LD_{50} values of human E. coli strains in mice and day-old-chicks

Strain designation	Serotype	Origin	Virulence markers					LD_{50}	
			Hly	MRHA	Col	Enterochelin	Aerobactin	Mice	Chiks
847	O1:K1:H–	Autopsy material	–	+	–	+	–	1.5×10^{7}	1.6×10^{7}
990	O1:K1:H7	U, Pn	–	+	Nc	+	+	2.1×10^{6}	3×10^{3}
505	O2:K·:H–	U, Cyst.	–	–	+	+	+	6.3×10^{4}	5.2×10^{2}
840	O2:K·:H–	lochia	+	–	+	+	–	1.10^{7}	3.1×10^{5}
180	O7:K1:H–	CSF, M	–	–	E1	+	+	1.7×10^{5}	2.4×10^{6}
074	O7:K1:H–	CSF, M	–	+	E1	+	+	7.1×10^{6}	3.1×10^{7}
916	O18ac:K1:HNT	B, S	–	–	Ib	+	+	2.2×10^{4}	$<1.8\times10^{2}$
895	O18ac:K1:H–	CSF, M	–	–	–	+	–	1.5×10^{5}	$<1.4\times10^{2}$
896	O18ac:K1:H–	CSF, M	–	–	Nc	+	+	2.2×10^{4}	$<1.6\times10^{2}$
072	O18ac:K1:H7	cervix	–	–	–	+	+	1×10^{5}	1.4×10^{3}
321	O18ac:K1:H7	CSF, M	–	–	–	+	+	3.2×10^{5}	$<1.3\times10^{2}$
899	O18ac:K1:H7	B, S	–	–	Nc	+	+	6.3×10^{4}	$<1.8\times10^{2}$
918	O18ac:K1:H–	B, S	–	–	E1	+	+	6.3×10^{3}	$<2.5\times10^{2}$
561	O18ac:K1:H7	cervix	–	–	Nc	+	+	3.3×10^{4}	1.4×10^{3}
920	O18ac:K1:H7	CSF, M	–	–	Nc	+	+	6.3×10^{3}	$<1.2\times10^{2}$
97358	O18ac:K5:H–	F, H	+	+	–	+	+	$>1.5\times10^{7}$	9.2×10^{6}
249	O18ac:K5:H–	CSF, M	+	+	Nc	+	+	3×10^{5}	2.9×10^{5}
97978	O78:K·:H–	F, H	–	–	–	+	+	1.9×10^{5}	8.1×10^{3}
52771	O78:K·:H–	CSF, M	–	–	V	+	+	2.7×10^{6}	8.2×10^{3}

Hly = Haemelysin; MRHA = mannose resistant haemagglutination; Ib = type of colicin; Nc = not classified, NT = not typable; U = urine; CSF = cerebrospinal fluid; B = blood; F = faeces; Pn = pyelonephritis, Cyst = cystitis, M = meningitis; S = sepsis; H = healthy; K· = antigen K is not determined

mals). One out of the 3 Aer^{-} strains (847) proved to be avirulent in both animal models, another (895) having antigen K1, was virulent for both kinds of animals, the third (840) was virulent only for chicks.

Examining the 28 strains of avian origin (Table II) it was found that 10 strains belonged to lethal class 1 for chicks, and 4 of them were virulent also for mice. Thus, the difference between the susceptibility of two animal models was higher in case of avian strains than of human ones. This is partly due to the selection of human strains, as they were chosen on the basis of the results of former examinations in which 13 out of 19 strains had belonged to the virulent group. At the same time, main part of avian strains (18 out of 28) belonged to the less pathogenic group (i. e. LD_{50} was over 10^{6}) even in the more sensitive chicken test. However, one cannot leave out to consider the differences in sensitivity between the two animal models. Since LD_{50} of strains in lethal class 1 fell between 10^{3}–10^{6}, those 6 strains that belonged to lethal

Table II

Lethality of avian E.coli strains for mice and one-day-old chicks

Strain designation	O antigen	Origin	Adhesiveness*	Colicinogeny	Syntrophism of strain WO987**	LD_{50} for mice	LD_{50} class for chicks
1	O2	trachea, ch	+	V	+	7.1×10^5	1
2	O2	F, ch	—	—	—	1.2×10^7	NL
3	O2	salpinx, ch	—	V	+	5.3×10^5	1
5	O78	lung, ch	+	V, nd	+	9.1×10^6	1
9	O2***	trachea, ch	+	V	+	$>1.6\times10^7$	1
10	O78	liver, ch	—	V, nd	+	2.7×10^7	1
12	O1	liver or vitellus	+	V, nd	+	9×10^6	2
13	O2	turkey poults	—	V, nd	+	3.4×10^6	2
14	O2	turkey poults	—	V, nd	+	4.2×10^6	2
15	O2	turkey poults	—	V, nd	+	1.0×10^7	2
16	O1	turkey poults	—	V, nd	+	1.2×10^7	2
20	O1	turkey poults	+	V, nd	+	$>2.4\times10^7$	2
22	O141	turkey poults	+	V, Ib	+	$>2.1\times10^7$	3
24	O1	turkey poults	+	V, nd	+	$>2.2\times10^7$	1
26	O141	turkey poults	—	—	—	$>2.7\times10^7$	3
27	O2	turkey poults	+	V	+	5.6×10^5	1
28	O1	turkey poults	—	V, nd	+	$>2.7\times10^7$	2
31	O1	turkey poults	+	V, nd	+	$>3.0\times10^7$	2
33	O2	turkey poults	—	V, nd	+	8.4×10^6	3
36	O141	turkey poults	—	—	—	1.8×10^7	NL
38	O141	turkey poults	—	V, nd	+	1.5×10^7	3
39	O141	turkey poults	—	—	—	2.8×10^7	3
43	O141	turkey poults	—	V, Ia	+	1.5×10^7	2
49	O2	turkey poults	+	V, Ib	+	1.4×10^5	1
50	O141	turkey poults	—	V, nd	+	9.4×10^6	3
51	O2	turkey poults	+	V	+	1.2×10^6	1
56	O1	turkey poults	+	—	+	$>2.8\times10^7$	1
57	O1	turkey poults	—	V, nd	+	1.2×10^7	2

* Examined on chicken epithelial cells
** See Reference 22
*** Antigen $K1^+$

V, Ia etc = type of colicin; nd = presence of another undetermined colicin; ch = chicken; LD_{50} class 1 = 10^3 to 10^6 organisms; LD_{50} class 2 = 10^6 to 10^8 organisms; LD_{50} class 3 = 10^8 to 10^{10} organisms; NL = nonlethal

class 1 in chicken and were avirulent for mice, might have had a high value of LD_{50} in chicks (e. g. near to 10^6). Anyhow, mouse virulence of avian strains was less significant than lethality for chicks of human strains.

The 4 Aer^- strains (see syntrophism of strain WO987 in Table II [22]) proved to be avirulent in both models. It was not possible to show any correlation between adhesiveness (see Table II) and animal virulence in avian group of strains either, as 4 adhesive strains were avirulent in both test; 5 strains

Table III

LD_{50} values of pairs of isogenic strains on CFLP, OF1, NMRI mice and one-day-old chicks

Designation of strains	Antigenic structure	Virulence factors	LD_{50} values			
			Chicken	CFLP	OF1	NMRI
317	O18ac:K1:H7	K1, ColNc	$<5.5\times10^2$	2.2×10^3	5×10^3	4.7×10^4
317/3	O18ac: :H7	ColNc	4.5×10^7	$>1.7\times10^7$	$>1.7\times10^7$	$>1.7\times10^7$
895	O18ac:K1:H–	K1	$<7.6\times10^2$	7.4×10^4	2.6×10^5	7.4×10^4
895/4	O18ac: :H–		1.6×10^7	$>2.5\times10^7$	$>2.5\times10^7$	$>2.5\times10^7$
896	O18ac:K1:H–	K1, ColNc	$<2.6\times10^2$	2.2×10^3	6.3×10^4	1×10^4
896/4	O18ac: :H–	ColNc	6.0×10^7	$>1.9\times10^7$	$>1.9\times10^7$	$>1.9\times10^7$
009	O4: :H5	Hly, MRHA	2.9×10^5	5.3×10^6	5.3×10^6	5.3×10^6
010	O4: :H5	MRHA	4.8×10^5	2.8×10^6	2.8×10^6	2.8×10^6
119	O18ac:K5:H–	K5, MRHA, Hly	1.3×10^8	5.6×10^6	7.5×10^6	7.5×10^6
119K5$^-$	O18ac: :H–	MRHA, Hly	2.0×10^8	$>1.7\times10^7$	.	.
119/1	O18ac:K5:H–	K5	.	$>1.6\times10^7$	1.6×10^7	5×10^6
119/1/K5$^-$	O18ac: :H–		.	1×10^7	.	.
536	O6: :H31	S, Hly, Sre	.	1.6×10^5	1.6×10^5	1.2×10^5
536/21	O6: :H31		.	$>0.7\times10^7$	$>0.7\times10^7$	$>0.7\times10^7$
536/31	O6: :H31	S, Sre	.	8.4×10^6	6.3×10^6	8.4×10^6

Not examined; Hly = haemolysin production; MRHA = mannose resistant haemagglutination; S = S fimbria; Sre = serum resistance, Nc = not classified

Strains: 536, 536/21, 536/31 = derived from Hacker et al. [9]; 119, 119/1 = isolated by Kuch et al. [16], 009,010 = provided by L. Emődy

only for chicks and another 4 in both models were virulent. Association of Col V plasmid and animal virulence could not be proved either, as 24 out of 28 strains with different virulence were Col V$^+$. Avian strains were MRHA$^-$ and all but one Hly$^-$. Only 1 strain (No. 9) had antigen K1.

Comparison of the lethal effect of E. coli strains with different virulence factors in various animal lines. One-day-old chicks and 3 different lines (CFLP, NMRI, OF1) of mice were tested in parallel by infecting them with isogenic pairs of strains (see Table III). LD_{50} given in various animals did not differ significantly from each other, thus our earlier results obtained by the analysis of LD_{50} on CFLP mice [15] are acceptable. It can also be stated that results of statistical analysis on 663 strains comparing the effect of different virulence markers with LD_{50} in mice are acceptable as strains with antigen K1 showed virulence higher by 3–4 log for mice than those that lacked K1. The Hly$^-$ variants were not less virulent than Hly$^+$ wild type strains since they showed no increase in LD_{50} values (see strains O09, O10) or it was increased only by one exponent (see strains 536, 536–31). Also loss of MRHA and K5 properties decreased the virulence of strains by only one exponent (see strains 119, 119/1, 119K5$^-$ etc.).

Table IV

Mouse sepsis assay by isogenic pairs of E. coli strains with different virulence factors

Designation of strains	Virulence factors	Killed*	Cell number/liver on		
			1st day	2nd day	3nd day
317	K1,ColNc	9, 10, 14	8×10^5	2.3×10^7	9×10^3
317/3	ColNc	1, 7, 0	1.2×10^1	4.5×10^3	7×10^2
895	K1	8	$2.5\times10t$	6×10^7	4.8×10^7
895/4		0	4.6×10^3	2.6×10^5	9×10^3
896	K1, ColNc	9	1×10^7	1.6×10^8	+
896/4	ColNc	0	4×10^2	1.2×10^4	1.3×10^2
119	K5, MRHA, Hly	7, 3	$2\times10:$	3×10^4	1.3×10^5
119K5$^-$	MRHA, Hly	0	8×10^1	4×10^3	1.2×10^4
119/1	K5	0	2×10^0	2.3×10^2	1.3×10^3
119/1K5$^-$		0	2×10^0	$<2\times10^0$	8×10^0
42541	K5, MRHA, Hly	11	1.4×10^4	3.1×10^4	1×10^5
42541K5$^-$	MRHA, Hly	0	5×10^2	1.4×10^1	2.7×10^1
009	Hly, MRHA	9, 8	3.3×10^3	1.4×10^3	4.6×10^4
010	MRHA	1, 2	1.2×10^3	1.6×10^4	5.4×10^4
536	S, Sre, Hly	11, 10	1×10^5	5×10^4	3.4×10^3
536–21		0, 1	2×10^5	1.3×10^1	3×10^0
536–31	S, Sre	0, 3	8×10^1	1.1×10^4	1.3×10^3

* In each assay 15 mice were included and each was infected with 10^4 bacteria s. c. The figures indicate the number of mice killed in one assay. S — S fimbria, Col = colicin production, Nc = not classified, MRHA = mannose resistant haemagglutinating capacity, Hly = haemolysin production, Sre = serum resistance

Results of mouse sepsis assay. For challenge isogenic pairs of strains with well-defined virulence markers were employed (Table IV). In mouse sepsis assay, an increased virulence of K1$^+$ strains could be demonstrated, as their bacterial counts in the liver were higher than those of the K1$^-$ derivatives, although the cell number increased during the 3 days following subcutaneous injection (Table IV). There was a parallelism between lethality for mice and bacterial counts in the liver. Fourteen out of 15 isolates originating from the liver of infected mice, retained their K1 positivity. Similarly, 13 out of 17 cultures recovered from the liver of mice infected by K1$^-$ mutants, were K1$^-$, whereas the remaining 4 were K1$^+$ (revertants?). Simultaneous loss of MRHA and Hly properties resulted in a more reduced number of bacteria in the liver, compared to the lack of antigen K5 alone (Table IV). However, loss of K5 decreased both cell counts in the liver and lethality rate, although the strain kept its MRHA and Hly properties. Loosing all of MRHA, Hly, K5 properties, the number of cell counts in the liver decreased further.

Loss of Hly activity (Table IV) did not influence the bacterial counts in the liver in case of strains O09 and O10 retaining their MRHA properties, but Hly$^-$ mutants killed less mice. In case of strains originating from Hacker et al.

Table V

Organotropic effect and LD_{50} values in mice of E. coli strains with different serogroups and virulence factors

Age of patients	Origin	Diagnosis	Serogroup	Virulence factors			LD_{50}	Ophthalmopathy	Dyskinesis
				Hly	MRHA	Col			
1 w	CSF	M, Ex	O7:K1:H–	–	–	E1	7×10^5	+	–
1 w	CSF	M, Ex	O78:H–	–	–	V	9×10^5	+	+*
34 y	CSF	M	Sp:HNT	+	–	NC	2×10^6	+	+
1 w	CSF	M, Ex	Sp:K1:H7	–	+	V	6×10^6	+	–
50 y	CSF	S	O6:H1	+	+	–	1×10^6	–	–
1 m	CSF	M	ONT:K5:H–	–	+	–	6×10^6	–	–
5 d	CSF	M, Ex	O7:K1:H–	–	+	E1	7×10^6	+	–
5 d	CSF	M, Ex	O4:H1	+	+	+	4×10^6	–	–
30 y	CSF	M	O7:K1:H–	–	+	E1	2×10^5	+	–
?	CSF	M	O18ac:K5:H–	+	+	NC	3×10^5	–	–
?	CSF	M	O4:H5	+	+	–	7×10^6	–	–
3 d	CSF	M	Sp:K1:H7	–	–	NC	2×10^5	–	–
2 w	CSF	M	Sp:K1:H7	–	–	–	3×10^6	+	–
3 d	CSF	M	O18ac:K1:H–	–	–	E1	2×10^4	+	–
1 w	CSF	M, Ex	O4:H5	+	+	–	6×10^6	–	–
3 w	B	S	O78:H–	–	+	V	2.4×10^5	+	–
1 w	B	S	O78:H	–	+	+	6.9×10^5	+	–
5 d	CSF	M	O78:H–	–	+	V	7.5×10^5	+	–
1 y	F	Ent	O78:HNT	–	–	–	$>2\times10^7$	–	–
3 y	F	H	O78:HNT	–	–	–	$>2\times10^7$	+	–
2 m	F	H	O78:H–	–	–	E1	3.6×10^6	+	–
2 m	F	H	O78:H–	–	–	–	1.9×10^5	+	–
2 w	CSF	M	O78:H–	–	–	+	1×10^7	+	–
2 w	CSF	M, S	O78:H–	–	–	+	1.7×10^6	–	–
7 d	Nose	?	O78:H–	–	+	Ib	9.2×10^6	+	–
	Tape		O78:H–	–	+	V	2.3×10^6	+	+*
7 d	Nose	?	O78:H–	–	+	V, Ib	5.9×10^5	+	+*
7 d	CSF	M	O78:H–	–	–	V	2.7×10^6	–	+*
3 m	F	Ent	O78:H–	–	–	V	1.2×10^6	–	–
3 y	F	Ent	O78:HNT	–	–	E, I	2.8×10^7	–	–
3 m	F	Ent	O78:H–	–	–	–	3.4×10^5	–	–
6 m	CSF	M	O78:H–	–	–	V	1.4×10^6	+	–
3 m	F	Ent	O78:HNT	–	–	V	$>3\times10^7$	–	–
30 y	F	H	O78:H–	–	–	V	1×10^7	–	–
1 w	Umbilicus, S		O78:H–	–	–	V	2.1×10^6	–	+
3 m	F	H	O78:HNT	+	–	I	1.1×10^7	–	–

* Challenge strain could be reisolated from the brain of mice on the 4th, 7th, 11th days following infection, M = meningitis; S = sepsis; Ex = fatal; B = blood; CSF = cerebrospinal fluid; F = faeces; Ent = enteritis; H = healthy; Sp = spontaneously agglutinable; V, E1 etc. = type of colicin; Hly = haemolysin production; MRHA = mannose resistant haemagglutinating activity; Col = colicin production; d = day; w = week; m = month; y = year; ? = not known; NC = not characterized

[9] 536–21 had exhibited a decreased number of bacterial counts in the liver by the second and third days, and the lethality rate was also lower than that of wild-type strain 536. In contrast to the low level of mouse lethality, bacteria persisted in the liver when infected by strain 536–31 (S fimbria$^+$, serum resistant mutant of 536). All the wild-type strains produced aerobactin and they retained Aer positivity loosing their other virulence factors. All of them had their original properties and virulence markers when they were isolated from the liver of mice, with the exception of the above-mentioned K1 strains.

Organotropism of E. coli. In examinations of organotropic effect of *E. coli*, at first 15 strains from meningitis were included. Results are shown in Table V. Eight strains caused ophthalmitis with purulent discharge in 58 out of 100 infected mice. Challenge strains could be isolated from the eyes of diseased animals. Lesions were induced by strains belonging to serotypes O7:K1:H–(3), O18ac:K1:H–(1), O78:H–(1) and to spontaneously agglutinable K1$^+$ strains (3). This effect in other strains (e. g. O4:H5, O6:H1, O18ac:K5:H–, O non-typable K5$^+$) could not be demonstrated. There was no association between pathogenic markers of strains and occurrence of lesions. Beside ophthalmopathy, dystaxia could be observed, if the mice were infected by serotype O78:H– strains. Mice showed dyskinesia and sometimes a circular-fashion movement. The challenge strain could be isolated also from the brain of infected mice, even on the 11th day after i. p. infection. Thus, 21 additional strains belonging to serogroup O78 were included in further examinations (Table V). Four of them also caused incoordinated movement of mice. It is remarkable that 11 strains out of 21 induced ophthalmopathy, too. Strains of serogroup O78 were reisolated from the brain of mice on the 4th, 7th and 11th days following infection. This dyskinesia of mice was associated with neither Hly or MRHA, nor Col V positivity of the strains.

There was another observation, characteristic only for mice that were infected by strains of serogroup O78. In our previous work on 663 *E. coli* strains belonging to serogroups other than O78, it was found that only 5 strains — altogether in 6 mice — killed the animals not earlier than 3 days. In contrast, 4 out of 7 recently examined serogroup O78 strains killed the mice after 3 days (mainly on the 6th or 7th days).

Discussion

Results of various assays gave clear out evidence that antigen K1 is most discriminative in the in vivo virulence of *E. coli*. It can also be concluded that other virulence factors (MRHA, K5, Hly) must be involved in the determination of a strain's virulence level, too, as elimination of any of these factors resulted in a decrease of bacterial counts in the liver and in a decrease of lethality rate on various animal models. Findings on K1 virulence correlate

with those of Smith and Huggins [12] as their K1$^-$ mutants were less virulent for mice after i. p. inoculation than the parent strain.

Dho and Lafont [21] found a close correlation between lethality and an increased ability of the strains to acquire iron from transferrin. They assumed that iron sequestering abilities could be a major determinant of virulence in avian strains. On the basis of our present study we can emphasize that antigen K1 is a more definite virulence factor than siderophores even in day-old chicks (see Tables I and III), supporting the results of Smith and Huggins [12], we examined their K1$^+$ strains in 1 to 25 days old chicken.

The animal assays used, did only reflect one particular part of the infection. For example, in case of i. p. or s. c. infection, fimbriae of *E. coli* seem to be rather a disadvantage than a virulence factor for the bacterium because of phagocytosis. Receptor specificity plays an important role in virulence of such bacteria. In agreement with Milch et al. [23] it should be mentioned that elimination of Col V plasmid could influence the virulence of a strain. Summarizing, different virulence factors can contribute to the effect of each other, but they never approach that of antigen K1. Aer negative strains can be avirulent if the strain lacks other virulence factors (e. g. Hly, K1), too. Thus Aer is not a virulence factor per se, but it acts together with the other factors. Hacker et al. [25] observed in their mouse sepsis assay that there were no differences between Hly$^+$ and Hly$^-$ variants of their strains. They supposed that haemolysin of *E. coli* was not an important factor in s. c. infection resulting in a mouse sepsis. In contrast to their findings, in our experiments we could demonstrate the influence of haemolysin on the lethality rate of mice even after s. c. infection. Namely, elimination of haemolysin production of a strain (strain 536–31, O10) resulted in a decrease of lethality rate of mice though persistence in the liver failed to change. Accordingly, the behaviour of Hly determinant is different from other virulence markers.

Emődy et al. [24] observed that strain 536, isolated by Hacker et al. [9] caused organotropism in mice after intravenous injection, giving rise to abscesses and purulent endophthalmitis. The lesions were not produced if animals were infected by strain 536–21 (e.g. S fimbria and Hly negative mutant). From their results, they have assumed that this fimbria, beside UTI, must play a pathogenetic role in septic infections, too. We also noticed ophthalmopathy of infected mice in certain cases during our earlier experiments. Consequently, setting out from the observation of Emődy et al., we have undertaken a detailed investigation. The results were in good agreement with those of Emődy et al. [24]. Besides, we have shown that strains of serogroup O78 have a special organotropic affinity to the brain of animals. This finding is all the more interesting as in 1974 [26] and in 1987 (unpublished) there were two epidemics of meningitis in Hungary among newborns caused by *E. coli* belonging to serotype O78:H–. According to literary data [27–29] and also of our experiences

[15, 19] *E. coli* belonging to serotype O7:K1:H–, O18:K1:H– (and also to serotype O18:K1:H7), or at least those that possess antigen K1 are the main causative agents of meningitis among newborns. Pathogenic properties of *E. coli* serotype O78:H– strains in Hungary might be different from those that were isolated in other countries, as so far they have been described to cause meningitis among newborns in epidemic form only in this country. All the 4 strains that induced incoordinated movement in animals and 8 out of 11 strains that caused ophthalmopathy, were associated with outbreaks of meningitis among newborns. Epidemiological data and results of the animal tests (e. g. dyskinesis and late death of mice) may permit to assume that this serotype possesses a special pathogenic property.

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PARATUBERCULOSIS IN A CATTLE HERD: COMPARISON OF ALLERGIC, SEROLOGIC AND FAECAL MICROSCOPIC TESTS

B. KÖRMENDY

Veterinary and Food Control Service, Central Veterinary Institute, Budapest, Hungary

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In a beef herd of 230 cows kept on pasture and infected with *Mycobacterium paratuberculosis*, faecal samples were tested microscopically and the blood serum by the complement fixation and the immundiffusion test. Intradermal tests were carried out simultaneously with bovine and avian tuberculin as well as with two dilutions (0.86 mg/ml and 0.50 mg/ml tuberculoprotein) of paratuberculin. In the tested herd 35.2% of the cows gave positive faecal tests while 10 and 9.1% were positive in the agar gel immunodiffusion (AGID) and complement fixation test (CFT), respectively. The allergens showed wide variations, but as a whole they were positive in 58.1% of the faecal positive, and in 60.9 and 76.2% of the AGID and CFT positive ones, respectively. The allergic tests were positive in 54.4, 53.7 and 52.2% of the faecal, AGID and CFT negative cows, respectively. The prognosis of such herds is disadvantageous. Elimination of infection can only be expected if after improvement of hygienic circumstances the diseased animals are continuously culled and a new breeding stock reared in isolation.

Mycobacterium paratuberculosis is the aetiological agent of paratuberculosis or Johne's disease in chronic enteritis of ruminant animals with consequently fatal outcome. Infection and mainly the clinically manifest disease causes important losses to cattle production [1–4].

Infection of an animal with *M. paratuberculosis* is unequivocally proved by the isolation of the agent. In the initial, so called tuberculoid phase of infection allergic reactions and cellular tests, but in the later lepromatous phase observation of intermittant diarrhoea as well as demonstration of antibodies in the blood serum and of the causal agent in the faeces by direct microscopy can contribute to the diagnosis.

Due to the very slow growth of the agent on culture media, veterinary practice prefers quicker indirect diagnosis methods, mainly serological tests, such as the complement fixation test (CFT) agar gel immunodiffusion (AGID) and the enzyme linked immunosorbent assay (ELISA) to reveal infection of animals [5]. These tests however, like microscopic investigation of the faeces are reliable only in animals affected with the clinical form of the disease.

In a cow herd in which animals in various phases of the disease were kept together were investigated to compare the effectiveness of allergic tests, the CFT and of the AGID, with the microscopic investigation of the faeces.

BÉLA KÖRMENDY
Veterinary and Food Control Service, Central Veterinary Institute
H-1581 Budapest, P. O. B. 2, Hungary

Materials and methods

The tested herd contained 230 Limousin beef cows kept in open farms all over the year. Four years before the present investigation bacterial strains were isolated from four cows of this herd, which proved to be mycobactin dependent *M. paratuberculosis*. For the present investigations samples were collected at the same time when also allergic tests were carried out.

Allergic tests. Intradermal allergic tests were simultaneously carried out using bovine tuberculin (50000 IU/ml), avian tuberculin (25 000 IU/ml) and two paratuberculin preparations corresponding to PPD quality requirements. All preparations were used in 0.1 ml individual doses and the tests were red 72 h post application. The test was qualified 1. as positive if the skin fold swoll by at least 3 mm; 2. doubtful if by 2–3 mm and as "moved" if swelling of the skin fold could be observed but did not reach 2 mm.

Serologic tests. Complement fixation and AGID tests were carried out as described earlier [6].

Faeces microscopic test. One g faeces was homogenized in 4 ml saline and shaken with 1 ml diethylaether. The drops of the diethylaether fraction were placed on a microscopic slide. Smears were stained by the Ziehl–Neelsen's method. At last one hundred microscopic fields were investigated and only those were regarded as positive in which red stained organisms 1–2 μm in size were demonstrated in characteristic clumps. The serological and allergic tests of cattle showing positive faecal microscopic results were compared and the diagnostic value of the tests were evaluated in per cents of infected animals.

Table I

Results of allergic, serologic and faecal microscopic tests in a paratuberculosis infected herd of 230 cattle

Tests	No. of animals	Per cent of herd	B+/d,m	A+/d,m	P1+/d,m	P2+/d,m	B, A, P1, P2 negative (X)	$\frac{X}{S}$ %	Value of infection index of allergens	
Fm +	81	35.2	2/31	11/16	1/8	7/13	34	41.9	58.1	
AGID +	23	10.0	2/9	6/3	2/2	2/6	9	39.1	60.9	clinical
CFT +	21	9.1	1/6	3/5	2/2	4/1	5	23.8	76.2	phase
Total:			5/46	20/24	5/12	13/20				
Fm —	149	64.7	7/45	19/38	6/2	11/33	68	45.6	54.4	
AGID —	207	90.0	10/63	25/48	4/27	15/41	96	46.3	53.7	tuber-
CFT —	209	90.8	13/59	31/43	6/25	17/39	100	47.8	52.2	culoid
Total:			35/213	95/153	21/66	56/133				phase

Relation of tests:	Animal		Animal	
Fm+ AGID+ CFT+	3	Fm— AGID— CFT—	131	B = bovine tuberculin
Fm+ AGID— CFT+	9	Fm— AGID+ CFT+	2	A = avian tuberculin
Fm+ AGID+ CFT—	9	Fm— AGID— CFT+	7	
Fm+ AGID— CFT—	60	Fm— AGID+ CFT—	9	P1 = paratuberculin (0.86 mg/ml)
Total:	81		149	P2 = paratuberculin (0.50 mg/ml)
				+, >3 mm, d= doubtful: 2–3 mm,
				m = moved: 0–2 mm
				Fm = faecal microscopics

Results

In the faecal samples of 81 of the 230 cows (35.2%) acid and alcohol-fast germs agglomerated in nests were demonstrated. In the AGID test 23 (10%) in the CFT 21 animals (9.1%) proved positive. Out of the cows which gave positive laboratory tests (CFT, AGID, microscopic faecal test) 5 and 20 were positive in the allergic tests with bovine and avian tuberculin, respectively.

With paratuberculin 1 and 2 there were 5 and 13 positive, with the allergens 46, 44, 12 and 20 doubtful and "moved" reactions, respectively. Negative allergic tests were encountered with all 4 used allergens in 34 faecal microscopic positive, in 9 AGID positive and in 5 CFT positive animals (Table I).

The allergens together gave positive results in 58.1% of the faecal positive, in 60.9% of the AGID positive and in 76.2% of the CFT positive cows (clinical phase).

On contrary, in the animals with negative laboratory tests (in 54.4% of faecal negative, in 53.7% of AGID negative and 52.2% of CFT negative ones) positive allergic reactions were encountered (tuberculoid phase).

Discussion

In 35.2% of the cows of the herd, organisms characteristic of the paratuberculosis agent could be demonstrated in the faecal microscopic test. The positive faecal test means high probability of paratuberculosis infection [7], but only successful isolation of the agent can be taken as a proof for it. Cultural tests, however, can be definitively read only in 15 to 20 weeks which is too long for practical application. Therefore there is a need for more practical, but reliable tests to reveal infected animals.

Out of the laboratory tests, the results of the CFT were most coincident (76.2%) with those of the allergic tests.

The negative serological and faeces microscopic tests coincided in 45.6–47.8% with negative allergic tests. On this basis, the offspring of cows negative in all tests can be used to build up breeding stock free of infection when reared in isolation [2, 8].

Antigens used in the CFT and in the AGID are not standardised on an international basis. These two tests, however, combined with the microscopic investigation of faecal samples are not capable either to reveal all infected animals.

Therefore, it is advisable to use simultaneously several tests, mainly in cases in which screening with an allergic test shows many doubtful or "moved" reactions. According to Colgrove et al. [5] the blood serum ELISA is capable of revealing most infected animals.

In our investigations paratuberculin 2 gave twice as many reactions as paratuberculin 1. It should, however, be stressed that all these reactions are indicative only for a mycobacterial infection in general, but to specify it cultural tests are indispensable.

The relatively high rate of 52.2–54.4% of positive allergic tests point to a heavy infection in this herd, as usually encountered in loose holding on pasture, all over the year. In the large scale herd diseased animals were recognized only late, therefore infection was spread by scouring cows to their offspring.

Prognosis of the investigated herd has been unfavourable. Though there is a possibility to reduce losses due to the infection by culling diseased animals and to prepare for building up a healthly herd by rearing the offspring in isolation of the infected cows [7, 9]. In this case there has been no possibility to repeat the investigation because the herd has been slaughtered as a whole.

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LISTERIA ISOLATION FROM FOODS OF ANIMAL ORIGIN*

L. NITCHEVA, V. YONKOVA, V. POPOV and C. MANEV

Higher Institute of Medicine, Department of Microbiology, Varna, Institute of Hygiene, Varna, and Institute of Infectious and Parasitic Diseases, Sofia, Bulgaria

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Microbiological examination of *Listeria*, isolated from foods of animal origin was carried out during the period 1986–1987. A total of 642 samples from chicken, beef and pork raw meats, fish, eggs and from the environment were investigated. Using the cold technique, Stuart's transport medium, tryptose broth and Ralovich's medium, 76 *Listeria* strains of serogroup 1 and 2 were isolated.

There are many articles concerning the role of foodstuffs as a factor for transmission and dissemination of listeriosis [1–3] Elisherova et al. [1] isolated *Listeria* from beef, pork and chicken raw meat in 35.2%. Manev and Janakieva [4] isolated *Listeria* from frozen meat in 19.5%.

The present study is performed to clarify the ways of listeria infection in healthy carriers by way of checking possible food contamination [5].

Materials and methods

We used Stuart's transport medium (Difco) with addition of 20 mg/l of nalidixic acid, tryptoso–phosphate broth, modified trypaflavin–nalidixic–serum agar [4]. A total of 642 samples from frozen meat (fish, beef, pork, lamb), eggs, washed-off materials from kitchen equipment were collected using swabs which were then incubated in Stuart's medium for 48 h at room temperature and transferred into tryptose broth afterwards. The broth was kept in a refrigerator at +4 °C for 12 months. The cultures were monthly seeded on trypaflavin–nalidixic acid–serum agar. The isolates were identified biochemically and serologically. The samples were enriched in tryptoso–phosphate broth only, due to the lack of Stuart's medium up to 1986.

L. NITCHEVA, V. YONKOVA
Higher Institute of Medicine, Department of Microbiology, Varna, Bulgaria

V. POPOV
Institute of Hygiene, Varna, Bulgaria

C. MANEV
Institute of Infectious and Parasitic Diseases
Sofia, Bulgaria

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Results and discussion

Tables I and II represent the results of this study. The data from the investigation of frozen meat show that listeria contamination levels are high, which is contradictory to considerably lower levels according to other authors [1, 2]. A higher isolation ratio in 1987 is a result of double enrichment of samples in Stuart's medium and tryptose broth. The highest relative value is associated with strains isolated from chickens (28.9%), lamb and pork meat (17.2%). *Listeria* from frozen fish, eggs, and washed-off materials was less frequently isolated. Four strains were isolated from kitchen equipment and from kitchen stuff hands.

Table II represents the antigenic variation of isolated *Listeria* serotype 1/2a and strains with antigenic formula OV, IX, XV are prevailing. We isolated serotype 4b in two cases. Four strains had OV, XV and OV, VI, XV antigens.

Table I

Incidence of Listeria strains in the years 1986–1987

Sources	1986			1987		
	No. of samples	Listeria-positive samples		No. of samples	Listeria-positive samples	
		No	%		No	%
Chicken	72	5	7.0	152	44	28.9
Pork and beef meat	170	8	4.7	64	11	17.2
Sheep internal organs	—	—	—	14	1	7.1
Fish	—	—	—	22	2	9.1
Eggs smear	—	—	—	71	1	1.4
Kitchen environment	—	—	—	77	4	5.2
Total	242	13	5.4	400	63	15.8

Table II

Serological characteristics of the isolated Listeria strains

Origin of samples (number)	1/2a	4b	4a	4ab	OV,XV	OV,XI	OV,IX, XV	OV, VI,XV	OV, XI,XV	OV,VI, IX,XI	Not typable
Chickens (224)	28	1	1	1	1	—	14	2	1	—	—
Beef and pork meat (234)	14	1	—	—	1	1	—	—	—	1	1
Sheep internal organs (14)	1	—	—	—	—	—	—	—	—	—	—
Fish (22)	2	—	—	—	—	—	—	—	—	—	—
Eggs (71)	—	—	—	—	—	—	1	—	—	—	—
Environment (77)	2	—	—	—	—	—	2	—	—	—	—
Total (642)	47	2	1	1	2	1	17	2	1	1	1

It is interesting that in a single sample (pork and beef mince) we isolated two different virulent serotypes of *Listeria* which were haemolytic and belonged to serovars 1/2a and 4b.

Our data indicate that foodstuffs of animal origin could be one of the factors of listeria-transmission (from animal to man). The isolated *Listeria* strains in this study were antigenically identical with those isolated from many healthy carriers in the investigated region.

The results suggest a potential risk of listeria-infection for individuals who are involved in processing of meat, as well as individuals who consume raw meat or food not sufficiently heat-treated. This allows the conclusion, that frozen meat has a possible predominant role for listeria dissemination.

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PENICILLIN BINDING PROTEINS IN *LISTERIA MONOCYTOGENES**

M. F. Vicente, J. Berenguer, M. A. de Pedro, J. C. Pérez-Diaz and F. Baquero

Department of Microbiology, "Ramon y Cajal" Hospital, and Molecular Biology Centre, CSIC-UAM, Madrid, Spain

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Membranes of *Listeria* were obtained from protoplasts and treated with ^{125}I-ampicillin as probe, at different concentrations. Eight bands, corresponding to proteins labelled with the probe were detected their molecular weight ranged from 38 000 to 100 000, being the predominant ones at 95 000; 85 000; 60 000; 49 000 and 38 000. The copy number of each PBP was also estimated. By means of competitive experiments the binding pattern of ampicillin, mecillinam, piperacillin, cefalotin, cefaloridine, cefoxitin, cefotaxime, azthreonam and imipenem was studied. The most effective binding was obtained with ampicillin, piperacillin and imipenem. Cefotaxime, and particularly cefoxitin, present an extremely low binding ability. The amount of antibiotic concentration preventing an effective label by the radiactive probe to the detected penicillin binding proteins seems to correlate with the lethal concentration of the different antibiotics on *Listeria monocytogenes* and explains the natural resistance of this genus to certain beta-lactamic compounds.

Listeria monocytogenes is a Gram-positive organism presenting susceptibility to natural penicillin, aminopenicillins, carboxipenicillins, and to a lesser extent to isoxazolypenicillins. At the time of writing, no resistant strains to penicillins have been detected with the exception of strains that are apparently naturally resistant to mecillinam and a few strains presenting resistance to methicillin. On the contrary, *L. monocytogenes* seems to present a natural resistance to cephalosporins especially to those of the third generation, including compounds such as cefotaxime, ceftizoxime or ceftazidime. The same occurs with monobactam antibiotics, such as azthreonam. *L. monocytogenes* is susceptible to carbapenems, such as imipenem. This susceptibility spectrum is similar to the pattern followed by the genus *Enterococcus* where the existence of different affinity of the compounds to the penicillin-binding-proteins (PBPs) — which are involved in the last stage of peptidoglycan synthesis — has been previously explained. In contrast, there is no information about the PBPs in *Listeria*. In this study, we describe the PBPs of *L. monocytogenes*, as well as their affinities for different beta-lactam antibiotics; these affinities can lead to the elucidation of the mechanisms of beta-lactam susceptibility or resistance in this bacterial genus.

M. F. Vicente, J. Berenguer, M. A. de Pedro, J. C. Pérez-Diaz, F. Baquero
Departamento de Microbiologia, Hospital "Ramon y Cajal", Centro de Biologia Molecular, CSIC-UAM, 28034 Madrid,Spain

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Materials and methods

The strain *L. monocytogenes* LO28, serovar 1/2c, is a clinical isolate that has been described previously [1]. Minimal inhibitory concentrations (MICs) to the different beta-lactams were determined by the agar dilution method in Mueller–Hinton Agar, according to NCCLS criteria [2]. Cytoplasmic membranes were obtained from protoplasted cells and prepared as described by Spratt [3], and used in binding experiments at a concentration of 180 μg of protein per samples. Iodinated and tritiated ampicillin derivatives were prepared by the method of Schwartz et al. [4] as modified by Rojo et al. [5]. N-(2–^{3}H-propionyl) ampicillin, N-(3-)4-hydroxy-5-^{125}I iodophenyl(propionyl) ampicillin, and benzyl-^{35}S-penicillin were used as probes in binding assays. Protein electrophoresis, competition experiments and beta-lactam binding assays were performed according to the protocol of Spratt [6]; the specificity of binding was assured by treating the samples with 100 μg/ml of cold ampicillin or benzylpenicillin before labelling. For the quantitative determination of PBP-bound radioactivity, the band of the gel corresponding to the different PBPs was cut off and the ^{125}I-ampicillin radioactivity was measured in a gamma-counter.

Results

Detection and affinity of PBPs for labelled beta-lactams. The binding assays for the detection of PBPs on isolated membranes of *L. monocytogenes* LO28 were carried out using increasing concentrations of the ^{125}I-ampicillin, ^{3}H-ampicillin and ^{35}S-penicillin derivatives (Table I). Five strongly labelled proteins were identified, with apparent Mr values of 95, 80, 78, 74 and 49 Kdaltons, estimated by comparison with the PBPs of *Escherichia coli* (Fig. 1). Resolution of the bands was optimal with use of ^{35}S-penicillin F (Fig. 1/C), less clear with use of ^{3}H-ampicillin (Fig. 1/B), while use of ^{125}I-ampicillin produced no distinguishable results (Fig. 1/A). The saturation values of the *L. monocytogenes* PBPs with ^{125}I-ampicillin are shown in Fig. 2. There were significant differences among the low saturation levels of PBP1–PBP2 and the correspondence to PBP5, which never reached 100% of saturation, even with high concentrations of ^{125}I-ampicillin (75 nM). The low resolution in the gel of the pair PBP3–PBP4 made it difficult to determine the relative saturation levels.

Binding of unlabelled beta-lactams to PBPs. The pattern of PBP binding of different beta-lactams was obtained by competition experiments. The results are presented in Table II, where the differential binding abilities are clearly observed. In general, imipenem, ampicillin and penicillin G presented the best

Table I

Increasing concentrations of ^{125}I-ampicillin, ^{35}S-benzylpenicillin, and ^{3}H-ampicillin used for the binding assays

	Concentrations						
(A) ^{3}H-ampicillin (μM)	0.46	0.9	1.8	7.3	14.6	29.2	29.2
(B) ^{125}I-ampicillin (nM)	2.3	4.6	9.2	18.4	36.8	73.5	73.5
(C) ^{35}S-benzyl-penicillin (μM)	0.2	2.0	20.0	40.0	40.0	—	—

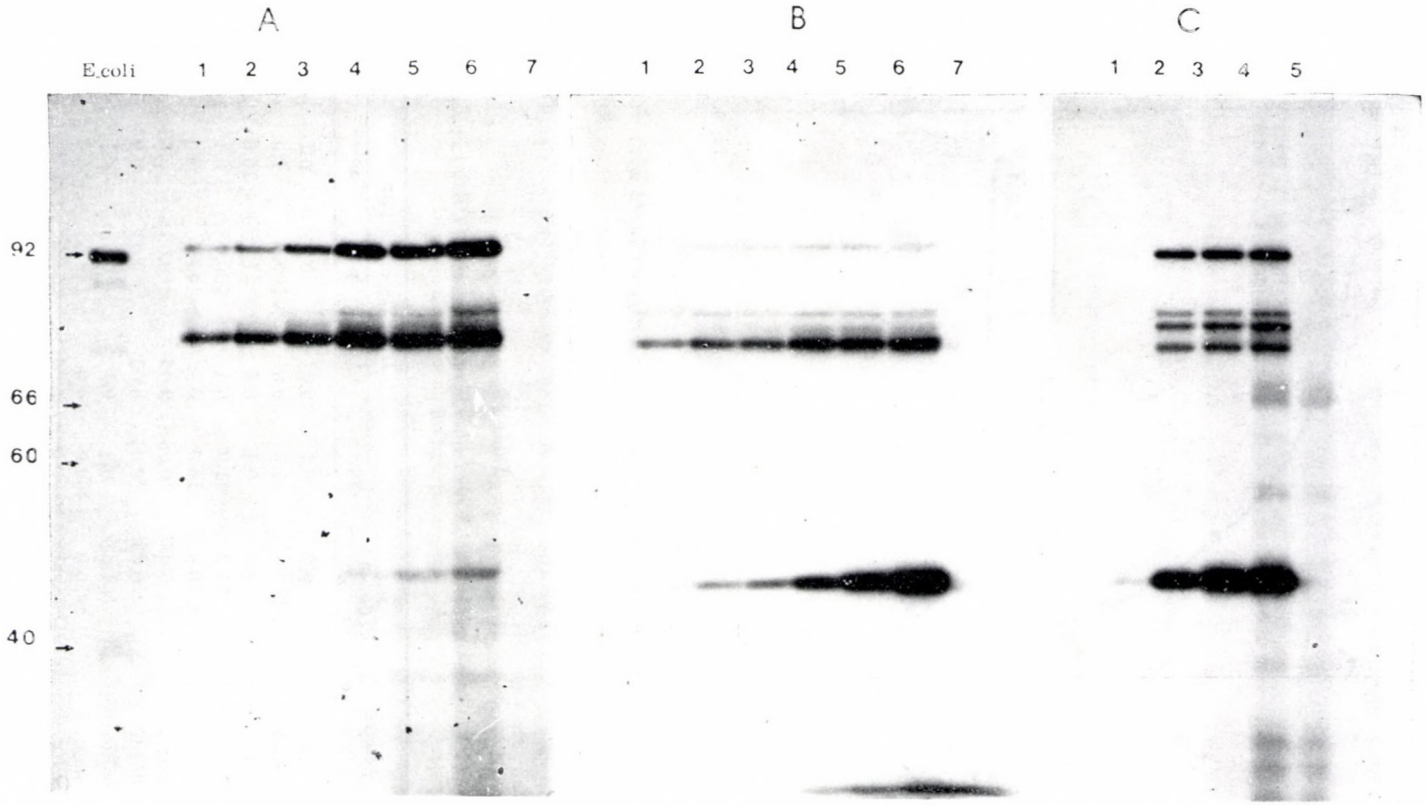

Fig. 1. Binding of different radioactive beta-lactams to PBPs of *L. monocytogenes.* (A): ^{3}H-ampicillin, (B): ^{125}I-ampicillin and (C): Benzyl-^{35}S-Penicillin. Samples A-7, B-7 and C-5 were previously treated with ampicillin (A, B) or benzyl-penicillin (C) before labelling

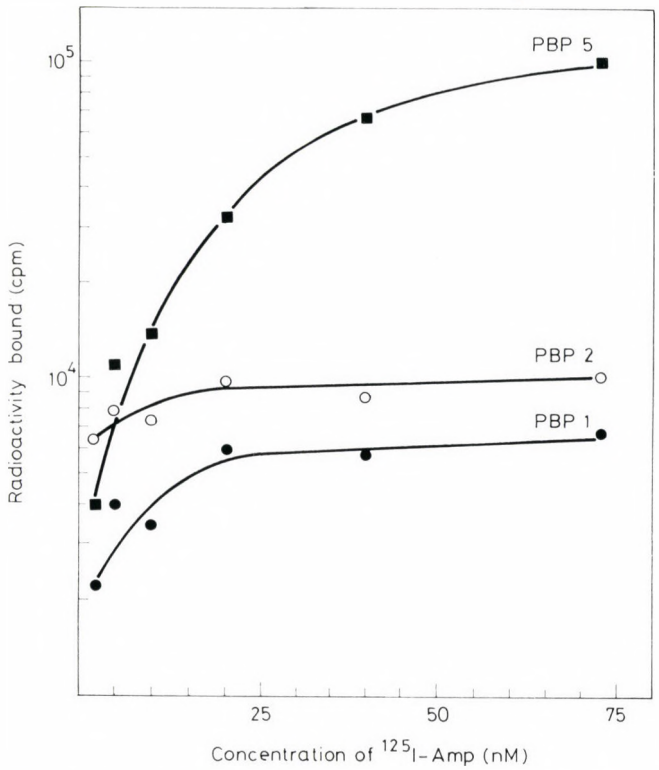

Fig. 2. Saturation values for the PBPs of *L. monocytogenes* LO28 with ^{125}I-ampicillin

Table II

Interaction of beta-lactam antibiotic with penicillin binding proteins (PBPs) in L. monocytogenes LO28

β-lactam	MIC (μg/ml)	ID90* (μg/ml) for PBP				
		1	2	3	4	5
Ampicillin	0.2	0.1	0.01	0.1	0.1	10.0
Azthreonam	>200.0	1.0	10.0	>100.0	1.0	>100.0
Cefalotin	1.0	0.1	0.1	1.0	10.0	>100.0
Cefotaxime	75.0	1.0	0.1	100.0	0.1	>100.0
Ceftazidime	>50.0	10.0	0.1	>100.0	1.0	>100.0
Imipenem	0.05	0.05	0.05	0.05	0.05	0.2
Penicillin G	0.05	0.03	0.03	0.03	0.03	10.0
Piperacillin	1.0	0.5	0.1	0.5	0.1	>10.0
Mecillinan	>50.0	>100.0	10.0	>100.0	>100.0	>100.0

* Concentration of β-lactam required to reduce ^{125}I-ampicillin of ^{35}S-benzylpenicillin binding to a given PBP by 90%

binding rate to the *L. monocytogenes* PBPs, corresponding to the lowest MIC values. Cephalosporins, and particularly third generation cephalosporins such as cefotaxime or ceftazidime, maintain a relatively good affinity for PBP2 and PBP4, and to a lesser extent for PBP1; nevertheless, PBP3 seems to be very resistant to binding. It can be suggested that the lack of inactivation of this PBP explains the increased MIC values to these antibiotics and to azthreonam.

Discussion

The PBP pattern of *L. monocytogenes* LO28 resembles the corresponding pattern of *Enterococcus*. There is obviously a similar pattern of antibiotic susceptibility in both genera, particularly concerning the natural resistance to cephalosporins and monobactams. Our results strongly suggest that the natural susceptibility pattern of *L. monocytogenes* LO28 to beta-lactam drugs reflects the susceptibility of its PBPs to these antibiotics. In particular, it can be suggested that the lack of binding, and consequently of inactivation, to PBP3 explain the increased MIC values to these antibiotics and to azthreonam. It is noteworthy that this low-affinity PBP of *L. monocytogenes* has a molecular weight [7] identical with the "low affinity PBP" of *Enterococcus faecium* or *Staphylococcus aureus* (approximately 76 Kdaltons).

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COMPARISON OF ADENOVIRAL HEXON POLYPEPTIDES (MONOMERS) AND OF NATIVE HEXONS (TRIMERS) BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

S. N. KHILKO, M. A. KIRASOVA, E. K. KISELEVA and T. I. TIKCHONENKO

Research Institute for Agricultural Biotechnology, Moscow, USSR, and D. K. Zabolotny Institute of Microbiology and Virology, Kiev, USSR

(Received September 6, 1989)

Purified hexons of 27 serotypes of human, simian, bovine and avian adenoviruses were analysed by SDS-PAGE. The apparent molecular weights of hexon polypeptides calculated by comparison with 5 non-hexon and 3 sequenced hexon polypeptide markers ranged from 98 kDa (for bovine adenovirus *Ad bos*7) to 118 kDa (for simian adenovirus *Ad sim*13; SV36). A stability of native hexon capsomers (trimers) in SDS at room temperature permitted us to resolve native (trimeric) hexon by SDS-PAGE and to distinguish them from denatured (monomeric) hexon polypeptides by electorphoretic mobilites. Hexon trimer bands with slow mobility in SDS-PAGE (unlike hexon monomer polypeptide bands) retained native hexon antigenicity as revealed by immunoblot analyses. Possible applications of simultaneous analyses of hexon trimers and monomers by SDS-PAGE are discussed.

Hexon capsomer is the main protein component of the adenovirion and its major antigen and immunogen [1]. Hexon is composed of three identical polypeptide chains and has a shape close to a pyramide with trigonal top and hexagonal base [1]. The sizes of hexon polypeptides vary from 102 to 124 kilodaltons (kDa) for different serotypes of human adenoviruses as determined by SDS-PAGE [2]. Only limited and scattered information exists about hexon polypeptide sizes for adenovirus serotypes of non-human origin (reviewed in [3]). These data cannot be readily compared because of different electrophoretic conditions used by many authors and of different proteins employed as molecular weight (Mw) markers. Recently, several protein markers in hexon-size range has become available with exact Mw values known from direct sequencing of polypeptides or respective genes (e. g. [4]) including hexon polypeptides from 3 adenovirus serotypes [5–7].

Using these marker polypeptides and a procedure of SDS-PAGE we analysed here the representative hexon polypeptides of 11 human, 10 simian, 4 bovine and 2 avian adenovirus serotypes. Results of the analysis revealed fairly wide variation of hexon polypeptide size between different adenovirus serotypes ranging from 98 kDa for BAV-7 to 118 kDa for SV36. Thus, despite

S. N. KHILKO, M. A. KIRASOVA, T. I. TIKCHONENKO
Research Institute for Agricultural Biotechnology, Moscow, USSR

E. K. KISELEVA
D. K. Zabolotny Institute of Microbiology and Virology Kiev, USSR

the numerous constraints imposed on hexon polypeptide structure [1, 8], its length may deviate for as large as 20 kDa or about 180 animo acid residues.

By means of the same procedure we analysed hexons of adenoviruses in their native form (trimers) and confirmed that hexon trimer stability in SDS-PAGE conditions at ambient temperature shown previously for SA7 [9] is the general property of adenoviral hexons for both adenovirus genera: *Mastadenovirus* and *Aviadenovirus* [10].

Materials and methods

Virus strains and their propagation. Prototype strains of human adenoviruses Ad1, Ad4, Ad7, Ad8, Ad12, Ad13 and Ad35 grown in Hep-2 cells were kindly donated for us by Dr. Gy. Berencsi. Human adenovirus strains Ad2, Ad3, Ad5 and Ad6 (from collection of Dr. R. S. Dreizin) were grown in monolayer or suspension cultures of Hep-2 cells and generously given us by Dr. T. I. Ponomareva. Simian adenovirus serotypes SV15, SV17, SV20, SV23, SV30, SV33, SV36, SV37, SV38 and SA7 (from the collection of Dr. S. S. Kalter) were grown in secondary monolayer cultures of African green monkey kidney cells and were kind gifts from Dr. T. I. Ponomareva. An antigenic variant SA7P [11] in form of purified virions was kindly provided by Dr. T. S. Denisova. Bovine adenovirus prototype strains BAV1, BAV2 and BAV3 (from collection of Dr. A. Bartha) grown in monolayer cultures of MDBK cells and a field isolate of bovine adenovirus identified previously as BAV7 [12] grown in primary monolayer culture of bovine testicular cells were kindly given by Drs R. V. Belousova and E. S. Zalmanson. The fowl adenovirus serotype 1 (CELO, Felps strain) and duck adenovirus strain EDS-76 (strain B8/78) grown in embryonated eggs were kind gifts from Drs V. I. Grabko and S. Kisary, respectively.

Purification of adenovirus hexons and virions. Hexons of mammalian adenoviruses were purified to a state of $\geq 85\%$ purity by hydrophobic and ion exchange chromatography as will be described separately [13] or by preparative SDS-PAGE [14]. For Ad1 and CELO virions purified in CsCl gradients [15] were used.

Polyacrylamide gel electrophoresis. For disc-electrophoresis of proteins in SDS-polyacrylamide slab gels we used the buffer system of Laemmli [16]. The resolving gel had the dimensions $140 \times 100 \times 0.75$ mm and contained 10 to 15% acrylamide monomer with 0.5% cross-link by N, N'-methylene bisacrylamide. The protein samples (0.1 to 0.5 μg) in 20 μl of dissociating buffer (5% SDS, 50 mM dithiothreitol, 5 M urea, 50% glycerol and 125 mM Tris-HCl buffer; pH 6.8) were either heated in a boiling water bath for 10 min or left for the same time at room temperature [9] and applied into 3.5 mm wide wells of concentrating gel (4% acrylamide cross-linked by N, N'-methylene bisacrylamide at 10% C). The electrophoretic run was carried for 1 h at 120 V and then for 2.5 h at 360 V at which time the tracking dye (Bromophenol Blue) migrated out of the gel.

Silver staining of proteins after SDS-PAGE. The resolved gels were fixed for 10 min in 50% aqueous acetone containing 0.1% formaldehyde and 1% trichloroacetic acid (the latter is essential only for native hexon trimer staining), washed twice for 5 min each in water, soaked for 20 min in 1% glutaraldehyde solution made on 50% aqueous acetone, stained for 10 min in ammoniacal silver solution prepared as in [17] and developed for 2–5 min in 0.04% aqueous formaldehyde containing 0.05% oxalic acid. The stained gels were photographed onto Micrat 300 film (TASMA, USSR).

Immunoblot staining of native hexon trimers. The proteins resolved by SDS-PAGE were transferred onto nitrocellulose membrane in semi-dry electrotransfer device essentially as described [18]. The blot obtained was blocked in 0.05% Tween 20 for 10 min and incubated (i) in rabbit antiserum against native Ad5 hexon and (ii) in a horseradish peroxidase conjugated antibody against rabbit IgG both diluted (10^{-3} and 10^{-4}, respectively) with 20 mM sodium carbonate buffer (pH 9.6) containing 0.1 M NaCl and 0.05% Tween 20. After washing the blot with 0.05% Tween 20 it was stained for peroxidase with 0.03% o-dianisidine plus 0.003% hydrogen peroxide in 20 mM 1-methyl imidazole buffer, pH 7.5.

Molecular weight estimation. All adenoviral hexon samples in gels designed for hexon polypeptide Mw determination contained also *Escherichia coli* RNA-polymerase (RP), rabbit muscle phosphorylase (PhA) and *E. coli cro*-repressor-beta-galactosidase (cro-βGal) fusion

protein as internal markers (Mw 155.2 kDa for RPβ', 150.6 kDa for RPβ, 120 kDa for cro-βGal fusion protein and 97.4 kDa for PhA). As an external marker, bovine serum albumin dimer (BSA_2) was also used (Mw 136 kDa). Calibrating line was plotted in linear-logarithmic coordinates with following modification: at abscissa axis the relative mobility of polypeptide "x" (R_X) derived by a formula: $R_x = l_{x-RP\beta'}/l_{PhA-RP\beta'}$ where $l_{x-RP\beta'}$ corresponds to distance between centres of bands "x" and RPβ', and $l_{PhA-RP\beta}$ to distance between centres of bands PhA and RPβ' in the same track. As ordinate the marker Mw on logarithmical scale was plotted. In such a system of coordinates R_x equals 1.0 for PhA and 0 for RPβ'. From this calibrating curve the Mw figures for hexon polypeptides were determined and afterwards corrected for "hexon anomaly" (see Results).

Results

Comparative analysis of adenoviral hexon polypeptides

The representative electropherogram of adenoviral hexon polypeptides covering the whole range of their Mw's is presented in Fig. 1. The hexon chains migrated in all cases between the internal markers RPβ (Mw 150.2 kDa) and PhA (Mw 97.4 kDa), thus only the central part of the gel is shown. The third internal marker, βGal had R_x of 0.58 and the external marker BSA_2 (not shown)

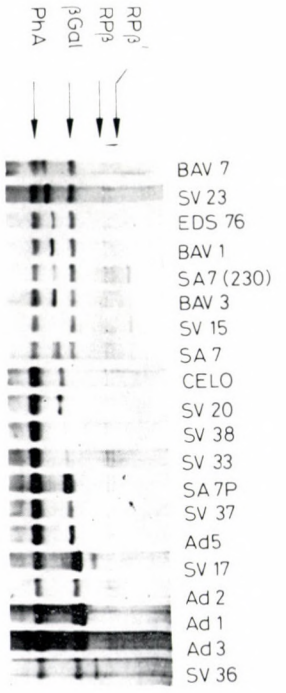

Fig. 1. SDS-PAGE (10% T) analysis of adenoviral hexon polypeptides (monomers). The central portion of 10% T polyacrylamide gel after silver staining. Adenoviral hexon polypeptides are situated between those of internal markers RPβ and PhA included in each track. The βGal marker was also included into eight leftmost tracks as well as into five rightmost tracks

migrated with R_x of 0.28. The hexon samples were applied onto the gel in approximate order of decreasing electrophoretic mobilities (i. e. of increasing Mw's). Most adenovirus serotypes analysed had revealed unique hexon polypeptide bands, but several of them contained double or triple hexon bands (e. g. Ad2, SV15, SA7) as shown previously for some human [2] and simian [19] serotypes. Of the analysed adenovirus serotypes, SV36 (Fig. 1, rightmost lane) had the largest hexon polypeptide migrating slower than BSA dimer, and BAV7 (Fig. 1, leftmost lane) had the smallest one which migrated just after PhA. The hexon polypeptides of human and simian adenoviruses not shown in Fig. 1 were well included in the Mw range defined by SV36 and BAV7 hexons [2, 19] as well as BAV2 hexon polypeptide (also not shown in Fig. 1; see Fig. 5).

To quantitate the Mw's of hexon polypeptides we constructed a calibrating curve shown in Fig. 2. Points corresponding to the five non-hexon protein Mw markers (RPβ', RPβ, BSA_2, βGal and PhA) conformed fairly enough to linearity in the coordinates chosen (upper line in Fig. 2). But the three hexon polypeptides with exact Mw values known from sequences (Ad2, Ad5 and BAV3 [5–7]) deviated significantly and systematically from the curve drawn for the non-hexon markers and gave another calibrating line shifted down (Fig. 1, lower line). The anomaly of hexon polypeptides electrophoretic mobilities could be seen also in Fig. 1 where Ad2 hexon chain (Mw 109 kDa [5]) migrated slower than *cro*-βGal marker (Mw 120 kDa). The apparent Mw's of

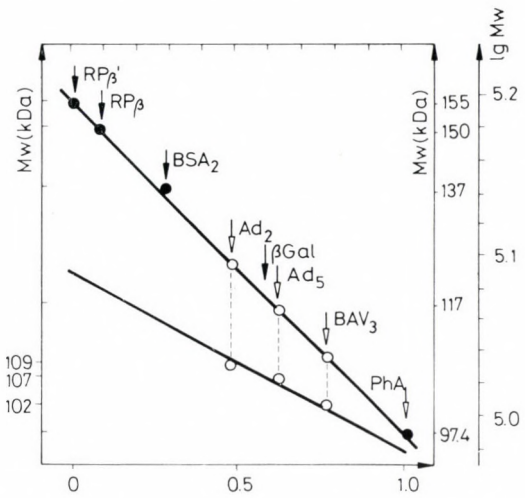

Fig. 2. Calibrating curves for hexon polypeptide molecular weight estimation. Abscissa: relative electrophoretic mobility of polypeptide "x": $R_X = l_{x-RP\beta'}/l_{PhA-RP\beta'}$, where $l_{x-RP\beta'}$ corresponds to distance between centres of bands "x" and RPβ', and $l_{PhA-RP\beta'}$ to distance between centres of bands PhA and RPβ' in the same track (see Fig. 1). Ordinate: log of Mw. Solid circles: non-hexon markers; open circles: hexon polypeptide Mw markers; upper curve: uncorrected; lower curve: corrected for "hexon anomaly" (see Results)

Ad2, Ad5 and BAV3 hexon polypeptides derived from the upper curve in Fig. 2 were higher than true values known from respective sequences (Table I). The anomaly in hexon polypeptide electrophoretic mobility reflected in proportional shift of apparent Mw could result from some inherent property of hexon amino acid composition (e. g. high proline content [5]) and/or sequence. We assumed that such a property could be common for hexon polypeptide chains from all adenovirus serotypes and constructed a second calibrating

Table I

*Apparent molecular weights for hexon polypeptides of adenoviruses**

Adenovirus				Apparent Mw derived from	
Host species	Subgenus (subgroup)	Serotype	Trivial name	non-hexon markers	hexon markers
	B	*Ad h3*	Ad3	129	112
		Ad h7	Ad7	124	109
		Ad h35	Ad35	128	111.5
		Ad h1	Ad1	128	111.5
Human	C	*Ad h2*	Ad2	126 + 124	109**
		Ad h5	Ad5	118	107**
		Ad h6	Ad6	127	111
	D	*Ad h8*	Ad8	124	109
		Ad h13	Ad13	129 + 127	112 + 111
	E	*Ad h4*	Ad4	128	111.5
	1	*Ad sim13*	Ad13	140	118
		Ad sim3	SV15	112 + 109	103 + 102
	2	*Ad sim4*	SV17	127	111
		Ad sim6	SV23	104	99
		Ad sim14	SV37	118	107
Simian monkeys		*Ad sim5*	SV20	112	103
	3	*Ad sim8*	SV30	114	104
		Ad sim11	SV33	116	105
		Ad sim15	SV38	116 + 110	105 + 102
		Ad sim16	SA7	112 + 109	103 + 102
	4	*Ad sim16*	SA7 (230)	109	102
			SA7P	116	105
		Ad bos1	BAV1	107	101
Bovine	1	*Ad bos2*	BAV2	107	101
		Ad bos3	BAV3	109	102**
	2	*Ad bos7*	BAV7	103	98
Fowl	1	*Ad gal1*	CELO	114	104
Fowl (or duck)			EDS–76	110 + 108	102.5 + 101.5

* Nomenclature suggested by ICTV Adenovirus Study Group [10]
** From sequenced genes [5–7]

line (Fig. 2) to estimate the corrected Mw values. Accordingly, all hexon polypeptide Mw figures were first determined from non-hexon markers (upper curve of Fig. 2) and then corrected for "hexon anomaly" by vertical projecting each individual point to the lower curve. The values obtained are given in Table I (two last columns, respectively).

Analysis of stable hexon trimers by SDS-PAGE.

The stability of adenoviral hexon antigenicity to a number of denaturating agents including SDS has been known for a long time [1, 20]. Because native hexon antigenicity invariantly correlates with its native trimeric structure [1, 21] it was reasonable to assume that hexon trimers are stable in SDS. Indeed, we previously demonstrated a stability of SA7 hexon trimers in conditions of SDS-PAGE performed without thermodenaturation of samples [9, 14].

Here we analysed by the same modification of SDS-PAGE the whole range of adenoviral hexons. Each hexon sample was divided to two portions, the first being denaturated by boiling in SDS-containing sample buffer ("plus" sample) and the second mildly treated by the same buffer at 20 °C ("minus" sample). Both samples were run in parallel tracks of the same SDS-polyacrylamide gel [9]. When such a procedure was applied to hexons, the pattern emerged which is illustrated by each pair of adjacent lanes marked "plus" and "minus" in Figs 3, 4 and 5. Each "plus" lane revealed denaturated adenoviral hexon polypeptide chain of mobility expected from its size (Figs 1, 2; Table I). On the other hand in "minus" lanes hexon polypeptide bands were absent and replaced by more slowly migrating bands or by a series of such slower bands (see Figs 3, 4 and 5). As shown previously for SA7 [9, 14] such bands consist of intact adenoviral hexon capsomers (trimers of polypeptides) having typical hexon shape and dimensions at electron micrographs and retaining hexon antigenicity in various immunoassays.

As illustrated in Figs 3, 4 and 5, all hexon samples in our hands displayed identical electrophoretic behaviour in the "plus–minus" test. This observation confirmed that previously reported stability of native hexon trimer in SDS-PAGE at ambient temperature [9] is the general property of hexons specified by widely differing adenovirus serotypes. Our analysis here included human adenoviruse from 5 out of the known 6 subgenera (Figs 3, 6; the Ad12 *h12* (subgenus A) hexon tracks were not analysed in experiment shown in Fig. 3 but trimers of this hexon could be seen on immunoblot in expected trimer position) [2, 10], simian adenoviruses from all 4 subgroups (Fig. 4) [3, 10, 19, 22], bovine adenovirus serotypes from both subgroups (Fig. 5) [3, 10, 23] and avian adenoviruses (Fig. 5) [3, 10] from the second genus of the *Adenoviridae* family. Thus hexon stability in SDS-PAGE may be regarded as a character

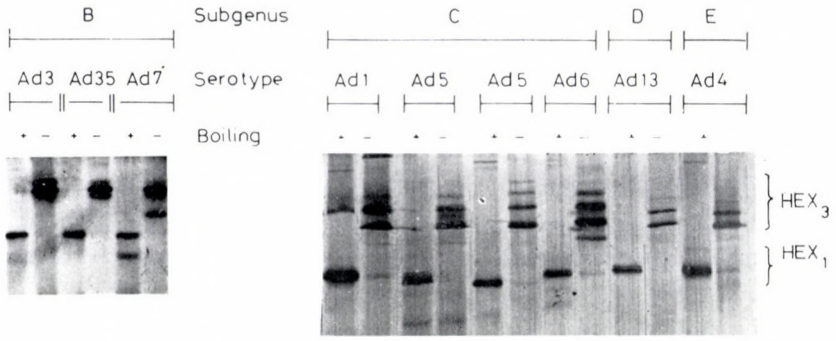

Fig. 3. SDS-PAGE (15% T) analysis of human adenovirus hexons. Each hexon preparation was separated in two parallel lanes, "plus" sample having been heated in Laemmli's [17] sample buffer for 10 min at 100 °C while "minus" sample incubated at the same buffer at 20 °C. Hex_1: positions of hexon polypeptides (monomers); Hex_3: positions of native hexon molecules (trimers)

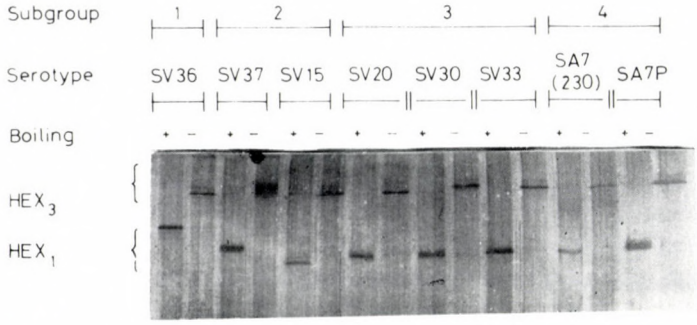

Fig. 4. SDS-PAGE analysis of simian adenovirus hexons. Conditions and designations as for Fig. 3

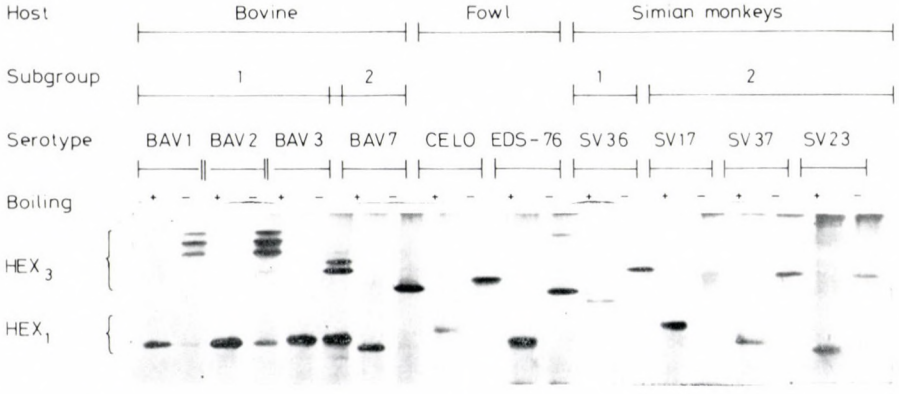

Fig. 5. SDS-PAGE analysis of hexons from bovine, avian and simian adenoviruses. Conditions and designations as for Fig. 3. The upper band in EDS-76 "minus" track is not a hexon trimer but fiber and penton base-containing oligomer (S.N.K., E.K.K., T.I.T., S.D. Osidze, N.V. Fomina, to be published)

shared by all typical adenoviruses belonging to both established genera (*Mastadenovirus* and *Aviadenovirus*) [10] including their respective prototype strains (Ad2 and CELO) as well as by serotypes deviating from these genera (BAV7 and EDS-76, respectively) in structural, antigenic and/or biological features [3, 23, 24].

It was shown previously [9, 21, 25] that antibodies raised against native adenoviral hexons do not recognize denaturated hexon polypeptides and vice versa, the native hexon antigenicity being an exclusive property of trimeric molecule [9, 25]. To prove that "slow" forms of hexon in "minus" lanes of SDS-PAGE correspond to native trimers we have probed a blot containing both "plus" and "minus" forms of adenoviral hexons with antibody against native hexon. Several human, simian and bovine adenovirus hexons were resolved in SDS-PAGE in "plus–minus" procedure. The electropherogram was subsequently transferred onto a nitrocellulose filter and reacted with an antiserum against native Ad5 hexon (Fig. 6). Such an analysis revealed immunostaining only for slow hexon bands in "minus" lanes, thus identifying them as native hexon trimers with characteristic antigenicity.

To prove the same conclusion for hexons of adenoviral serotypes not related antigenically to Ad5 (BAV7, CELO and EDS-76) we performed analogous immuno-blotting in respective systems of antigens and antibodies. The results (not shown) were in accordance with data demonstrated in Fig. 6 — i. e. only slow hexon bands in "minus" lanes were stained by respective antibodies against native hexons.

The following points in "plus–minus" analysis deserve comments. (i) The

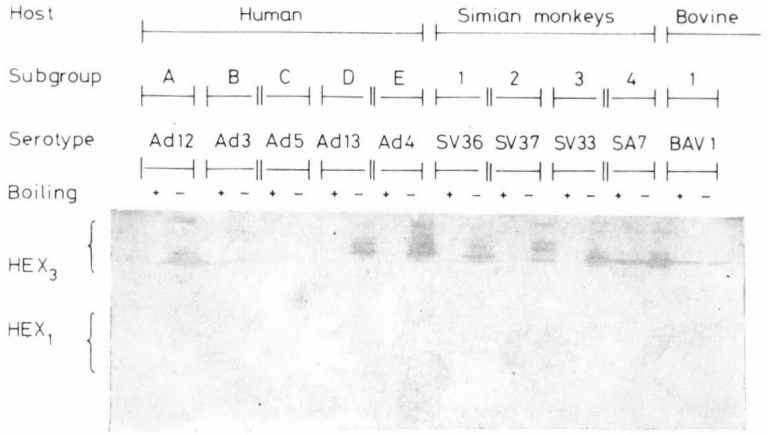

Fig. 6. Immunoblot analysis of mammalian adenovirus hexons after SDS-PAGE separation. Electrophoretic conditions as for Fig. 3. Nitrocellulose blot of the hexon electropherogram was treated with rabbit anti-(native Ad5 hexon) serum and immune complexes formed were detected by peroxidase-conjugated ovine anti-rabbit IgG antibodies. Positive reactions seen in some "plus" lanes resulted in fact from contaminating hexon trimers from nearby "minus" lanes and not from hexon monomers having higher mobilities in SDS-PAGE (see Figs 3, 4 and 5)

electrophoretic mobilities of hexon trimers did not necessarily reflect their respective molecular weights. Thus hexon trimer of SC36, the largest hexon of simian adenoviruses analysed (Table I) had the highest mobility (formally, the smallest Mw) in the same group (Fig. 4). Also BAV1 and BAV2 native hexons migrated slower than more high molecular BAV3 and CELO hexons (Fig. 5). Such an observation was not unexpected since electrophoretic mobility of compactly packed hexon trimer must not depend strictly on its mass (unlike the case of completely unfolded hexon chain complexed with SDS) but be a complex function of molecular volume, shape and charge. The last parameter for hexon trimer consists of the sum of exposed charged residues of hexon itself and of bound SDS molecules, both depending on individual hexon primary structure.

(ii) The multiple hexon trimer electrophoretic bands characteristic of some adenovirus serotypes (e. g. those of human adenoviruses, Fig. 3) could correspond either to a series of oligomers made from hexon trimer molecules or to molecules with different quantities of SDS bound. All such forms of native hexon retain antigenicity of native hexon in reaction with both polyclonal (Fig. 6) and monoclonal (data not shown) antibodies.

(iii) Not only intact hexon polypeptides changed their mobilities as a result of "plus–minus" shift but also hexon partial proteolysis products present in some samples. For example in Fig. 3 the minor degradation products of Ad6 and Ad7 hexon polypeptides could be identified in "plus" lanes while absent from respective "minus" lanes. Previously we have shown that even proteolytic fragments of hexon in the absence of intact hexon polypeptide formed stable structures resembling intact hexon trimers seen on electropherograns [25, 26]. Thus by difference between "plus" and "minus" lanes in SDS-PAGE hexon polypeptides and their degradation products could be simply differentiated from non-virion and most of adenovirion non-hexon polypeptides in complex mixtures.

Discussion

Variability of hexon polypeptide dimensions

An SDS-PAGE analysis of hexon polypeptide chains of a number of taxonomically widely different adenoviruses (Fig. 1, Table I) revealed considerable variability of their lengths. The smallest hexon polypeptide (Mw 98 kDa derived from hexon Mw markers) is characteristic of BAV7 (some additional serotypes from the second subgroup of bovine adenoviruses also contain hexon polypeptides of the same size (S. N. K., E. K. K., A. Bartha; unpublished)). Simian adenovirus SV36 contains the largest hexon polypeptide (Mw 118 kDa). The size difference between these particular hexon chains is

20 kDa or about 180 amino acid residues accounting for roughly 15% of the chain length. The latter conclusion certainly depends on the uniformity of deviation of hexon polypeptide behaviour in SDS-PAGE (formally the vertical distance between upper and lower lines on Fig. 2) but a real difference of 57 residues between Ad2 and BAV3 hexon polypeptides is established from their sequences [5, 7].

We have not observed systematic dependence of hexon polypeptide length on the taxonomical position of the respective adenovirus serotypes in our restricted sample. This result is in accordance with the conclusion that the hexon is the most variable (in size) component among adenoviral structural proteins [2, 19]. Simian subgroup 2 adenoviruses (Figs 4, 5, Table I) may be regarded as a good example of hexon size variability within a taxonomic group because their hexon polypeptides range widely in size between 99 kDa (SV23) and 110 kDa (SV17). In fact, hexon polypeptides may differ in dimensions even within a given serotype for different genome and/or antigenic variants. For example, we have shown previously that SA7 variants with different passage histories contain different combinations of hexon chains [19, 25, 27]. Moreover, individual SA7 clone 230 which may be regarded as genome type [19] differs from the prevailing SA7 genome type both in hexon polypeptide size and tryptic peptides map ([25], Fig. 1, Table I) and in hexon gene restriction site map [19, 27]. Antigenic and genome variant SA7P [12] independently isolated from African green monkey also may be distinguished from another SA7 variants by hexon size ([12, 19], Figs 1, 4; Table I). In this respect it seems interesting to search for hexon size variants among the different adenovirus Ad4, Ad7 and Ad19 genome types circulating in human population [28].

Some uniformity of hexon polypeptide dimensions in several adenovirus taxonomic groups (e. g., human subgenus C or bovine subgroup 2) may result from fairly recent phylogenetic divergence of serotypes in these taxa [28]. Even in such relatively homogenous groups individual adenovirus serotypes may deviate in hexon chain size (Figs 3, 5; Table I). On the other hand adenoviruses from different taxonomic groups often possess hexon polypeptides of apparently equal size, as those of BAV3 and SA7(230) or of Ad6 and SV37 (Table I).

In conclusion, the hexon polypeptide size may vary considerably between different adenovirus serotypes (sometimes within serotype) and such variation is not directly correlated with respective adenovirus taxonomic position.

Stability of hexon capsomers (trimers) in SDS-PAGE conditions

A simple modification of sample treatment (omitting of boiling samples in SDS-urea dissociating buffer) makes it possible to observe intact hexon trimers in SDS-PAGE ([9], this study). The bands of lower mobilities than

hexon monomers in "minus" lanes correspond to native hexon trimers, as shown by electron microscopic and immunochemical analysis for SA7 hexon [9, 14, 25]. The same kind of electrophoretic pattern in "plus–minus" analysis appears to be common for all adenoviral hexons tested (Figs 3, 4 and 5). Because this kind of hexon behaviour in "plus–minus" SDS-PAGE was consistently observed for all adenovirus serotypes analyzed here (as well as for Ad8, Ad12, BAV4, BAV5, BAV6, BAV8, BAV9, CAV1, OAV1, OAV2, OAV3 and OAV4) we believe the native hexon stability in SDS-PAGE at low temperature to be a general property of adenoviral hexon reflecting its conservative tertiary and quaternary structure.

Only slow hexon bands in "minus" lanes retain native hexon antigenicity in immunoblot analysis for typical mammalian adenoviruses (Fig. 6) as well as for bovine subgroup 2 and avian adenoviruses (results not shown) in respective antibody systems. These data strongly suggest that the slower hexon bands in "minus" lanes contain adenoviral hexon in their native (trimeric) form.

As shown previously for SA7 hexon, thermodenaturation of hexon to constituent polypeptide chains is an irreversible one-moment process without any electrophoretically discernible intermediate forms [9, 25]. The level of native hexon thermostability in Laemmli's [17] sample buffer varies for different adenovirus serotypes. We routinely observed human subgenus C and bovine serotype BAV3 hexons to be more labile while BAV7, SV23 and EDS-76 to have a higher stability than the rest of hexons analyzed. This observation may be useful in comparative studies of adenoviral hexon tertiary and quaternary structure (e. g. as in [8]) and for correlations of hexon and virion thermostability differing for various serotypes (e. g. high thermostability of avian adenoviruses).

SDS-PAGE mobilities of native adenoviral hexons do not strictly correlate with their respective polypeptide sizes. For example, SV36 hexon (calculated Mw for trimer 354 kDa) moves more rapidly than that of *Ad bosl* (Mw 300). Native hexon mobilities in SDS-PAGE are very sensitive to PAG porosity (%T, %C) unlike the mobilities of denaturated hexon chains (in fact, SV36 hexon trimer moves faster than the respective monomer in gels with $\leq 9\%$ T; 0.5% C; data not shown).

Interesting and as yet unresolved observation concerns the appearance of multiple native hexon bands in "minus" lanes (e. g. for human adenoviruses in Fig. 3). The pattern of multiple native hexon bands (their number, mobilities in given PAGE conditions and relative ratio) is reproducible for each adenovirus serotype and independent of source of hexon material (either virions, excess "soluble antigens" or purified hexons). This may indicate some form of their interconversion in SDS-PAGE conditions (e. g., reversible hexon capsomer oligomerization) but it remains to be proven by direct tests. All such isoforms of native hexon retain native hexon antigenicity (Fig. 6).

The "plus–minus" variant of SDS-PAGE permits to simply distinguish between native and denaturated hexon molecules in a given hexon preparation. For example, BAV1, BAV2 and BAV3 hexons analyzed in Fig. 3 contain substantial amounts of denaturated hexon polypeptides revealed as monomer bands in "minus" lanes. Such a variant of hexon quaternary structure analysis may be useful for analysis of hexon trimer formation in vivo and in vitro as well as for characterization of hexon state in cells infected with adenovirus *ts*-mutants defective in hexon trimerization or nuclear transport.

By a "plus–minus" SDS-PAGE analysis adenoviral hexons of all serotypes may be simply differentiated from the rest of proteins in unpurified preparations ceontaining large amounts of unrelated proteins [9] and we use this method for routine testing during hexon purification. Its sensitivity in combination with silver staining of electropherograms permits hexon detection in the presence of vast excess of unrelated proteins (the detection limit is about 0.05% of hexon in protein mixture).

We have observed a similar behaviour in "plus–minus" SDS-PAGE also for some non-hexon adenovirion oligomeric proteins: fiber [14], penton base (S. N. K., E. K. K., to be published) and have found in the literature for structural proteins of unrelated viruses [29]. In a case of phage P22 tail fiber protein [29] the situation is quite similar to one with hexon: the protein is homotrimer formed with help of virus coded scaffold protein; it is stable to proteolytic attack only in native (trimeric) state and its thermodenaturation is irreversible.

The stability of oligomeric virion proteins in SDS-PAGE may be widely spread among icosahedral and spherical viruses. From indirect literary data we suggest this property to be likely in cases of parvovirus and rotavirus outer virion proteins. If it be the case the simple and informative variant of "plus–minus" SDS-PAGE analysis will add to structural and biosynthetic studies on those virion proteins.

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MONOCLONAL ANTIBODIES IN THE TREATMENT OF ENDOTOXIN SHOCK

(A REVIEW)

J. G. SINKOVICS

Community Cancer Center, St. Joseph's Hospital, Tampa, Florida; Departments of Medicine and Medical Microbiology, University of South Florida College of Medicine; Bay Pines Veterans Administration Medical Center, St. Petersburg, Florida, USA

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I. The hybridoma principle

1. *The first "hybridoma"*

Without referring to them as "hybridomas", fusion products of lymphoma cells and specific antibody-producing spleen cells were first described in 1968–1969 [1]. A mouse lymphoma consisting of diploid malignant cells was maintained as a cell passage line in the mid-1960s. Budding retrovirus particles rendered the lymphoma cells antigenic. Mice rejecting lymphomas after small inocula lymphoma cells ($< 10^{3.7}$ cells) developed mouse leukemia virus-neutralizing antibodies and complement-dependent antibodies lytic to lymphoma cells. Inocula of $> 10^{4.7}$ lymphoma cells gave rise to large solid lymphomatous tu-

JOSEPH G. SINKOVICS
Community Cancer Center, St. Joseph's Hospital
3001 West Buffalo Avenue, Tampa, Florida 33677/4227, USA

mours leading to the death of the animals. When leukemia virus-replicating diploid lymphoma cells were mixed with spleen cells of rejector mice, the following results were observed:

1. growth of solid lymphomatous tumours and their rejection;
2. growth of solid lymphomatous tumours advancing to the death of the inoculated animals;
3. diffusely growing large cell lymphomas infiltrating all organs and growing as ascites tumours;
4. no noticeable growth of lymphoma.

Growth pattern #3 was a new observation [1, 2]. The large lymphoma cells forming this growth pattern were frequently binucleated and in the tetraploid chromosomal mode; they formed retrovirus particles that were frequently incomplete or deformed and not leukemogenic; and they produced immunoglobulins. Supernatant fluid of crude ascites or spinner bottle suspension cultures or purified immune globulins derived from these cells strongly and specifically neutralized the mouse leukemia virus [3, 4].

This unprecedented phenomenon was reported as a self-defensive act of lymphoma cells through which they gained virulence and immunoresistance. For the mechanism of the phenomenon the explanation was offered that leukemia viral antigens expressed in the membrane of the lymphoma cell attracted the immune plasma cell expressing specific antibody to the viral antigens. The two cells aligned, achieved high affinity membrane contact and fused [5, 6]. The fused cells concealed their "neoantigens", i.e. the budding virions, by coating them with self antibody thus escaping immune rejection (but remaining vulnerable to antibody-dependent cell-mediated cytotoxicity of monocytes-macrophages and thus displaying the "starry sky" histologic pattern).

It was further proposed that phenomena of cell fusion occur in the natural course of malignant lymphomas. The large tetraploid immunoresistant Burkitt's lymphoma cell arising after remission in patients with anti-EBV immunity [1], the Reed-Sternberg cell of Hodgkin's disease [6, 7] and the large Sézary cell were singled out as representatives of this phenomenon [4, 6].

This work was received with skepticism or induced no reaction at all. The National Cancer Institute's targeted cancer research program of the early 1970s gave it low priority and no funding. However, the original description of the observation as worded in 1969 stands unchanged today [1]: ". . . tetraploid immunoresistant lymphoma cells in the mouse emerge by fusion of the diploid, virus-producing lymphoma cell with a plasma cell producing virus-specific globulins. The resulting tetraploid cell will retain malignant growth potential and the genetically determined committedness of both parent cells: to produce leukemia virus as coded for by the viral genome within the neoplastic cell, and to synthesize virus specific globulins, as coded for by the genome of the plasma cell."

2. *Hybridoma technology and monoclonal antibodies*

Five years later Kohler and Milstein reported the fusion of a specific antibody-producing plasma cell with a myeloma cell thus giving rise to an expanding clone of specific antibody-producing immortal cells growing in suspension cultures or in the form of ascites tumours in mice [8]. These "hybridomas" fused not because of specific antibody-antigen reactions on their surface. The new technology allowed for the fusion of any specific antibody-producing plasma cell with a myeloma cell through the action of Sendai virus or polyethylene glycoll. Nonfused, enzyme-defective myeloma cells and nonfused mortal, normal immune spleen cells could not grow in the special medium designed for these experiments. The hybridoma technology of Kohler and Milstein provided monoclonal antibodies (McAb) of the highest specificity [9].

II. Pathophysiology of endotoxinaemia

1. *Endogenous opioids*

Endorphins are among agents that mediate hypotension in endotoxin shock [10]. Antagonists of enkephalin-preferring δ-receptors can reverse shock and morphin-preferring μ-receptor antagonists (β-funaltrexamine) can preempt this effect [11]. In patients intravenous bolus of naloxone reversed hypotension and bradycardia that was induced by endogenous opioids that were released by endotoxin [12]. While 15 min 0.3 mg/kg naloxone bolus could rise blood pressure in half of the patients with septic shock, there are entirely negative clinical trials on record. Naloxone infusion at 30 μ/kg/h for 8–16 h appears to be more effective: it significantly reduced vasopressor requirement and exerted positive inotope effect, improved stroke volume and normalized heart rate [12].

It is not only endotoxin that can derepress the gene(s) for endogenous opioids. In certain malignant tumours such as neoplasms of neuroendocrine derivation, overproduction of endogenous opioids occurs. These patients present with bradypnea, narrow pupils and hypotension and respond with dramatic improvement to naloxone drip infusion. In chemotherapy-induced remission opioid levels drop and in relapse they rise [13]. Certain viral genes can activate cellular gene(s) responsible for the encoding of endogenous opioids; for example the paramyxovirus Newcastle disease virus can induce the pro-opiomelanocortin gene [14].

2. *Bradykinin and angiotensin*

It was in the late 1960s when angiotensin II was proposed to be the natural antagonist of the nona-peptide bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) [15]. This notion was based on clinical observations in patients showing response of catecholamine-resistant endotoxin shock to angiotensin drip [15]. It is now known that bradykinin acts through its B2 receptor whose coupling to phospholipase C is mediated by a guanine nucleotide-binding (G) protein that is not acted upon by pertussis toxin: it is neither Gi nor Go [16]. However, 4 β phorbol-12 β-myristate-13α-acetate (PMA) blocks bradykinin-induced inositol monophosphate formation. Synthetic B2 receptor antagonists B4158 (DPhe-Pro-Gly-Thi-Ser-DPhe-Thi-Arg) or NPC567 (DArg [Hyp^3 DPHe^7]Bk) also block effects of bradykinin. NPC567 protected 50% of rats from endotoxin shock that was 100% fatal in untreated controls [17].

Rhinoviruses activate the bradykinin gene [18, 19]. Rhinoviruses enter cells after attachment to their receptor ICAM-1. This *c*ell *a*dhesion *m*olecule functions also as the ligand for *l*ymphocyte *f*unction *a*ssociated molecule-1 (LFA-1) and it is important for T lymphocytes executing lysis of their target cells. A McAb binding to ICAM-1 prevents rhinovirus attachment to and entry into target cells. Rhinorrhoea and sore throat during rhinovirus infection, eicosanoid formation and airway hypersensitivity in asthma appear to be bradykinin-mediated giving room to further clinical trials of bradykinin- and bradykinin receptor-antagonists in these conditions and in endotoxin shock. The oncoprotein encoded by a mutant Harvey-*ras* oncogene induces excessive expression of bradykinin receptors; for cells with overexpression of these receptors, bradykinin acts as a mitogen [20]. Endogenous opioid and bradykinin activation in certain virus infection leads to strong clinical resemblance of these infections to bacterial endotoxinaemia, but without the high mortality of true endotoxinaemia.

Natural inactivation of bradykinin is carried out by the peptidyl dipeptidase, kininase II, or angiotensin converting enzyme (ACE) on vascular endothel cells. ACE inhibitors (S-1 N^2-1-carboxyl-3-phenyl propyl) are now widely used for the treatment of hypertension. ACE is overexpressed in patients with active sarcoidosis where bradykinin levels should be low. In hypertensives treated with ACE-inhibitors bradykinin levels are elevated.

The *mas* protooncogene-oncogene was recognized in the heavy DNA of a human squamous cell carcinoma by its ability to transform NIH3T3 cells and induce tumours in athymic nude mice [21]. This oncogene encodes a GTP-binding (G) protein which responds to its cognate ligand angiotensin II. The octapeptide angiotensin II derives from the decapeptide angiotensin I under the effect of converting enzymes; angiotensin I derives from angiotensinogen α

2 globulin under the effect of the proteolytic enzyme renin released from juxtaglomerular cells of the kidney. Angiotensinogens are produced by hepatocytes and astrocytes [22]. Angiotensin II increased [^{3}H] thymidine incorporation in and is mitogenic to cells expressing its receptor. Signal transduction is through phosphoinositide hydrolysis and mobilization of Ca^{++}flux; Gi is activated and adenylate cyclase is inhibited. This receptor does not capture bradykinin, but it could be blocked by substance P, the broad-spectrum peptide antagonist ($DArg^1$, $DPro^2$, $D\text{-}Trp^{7,9}$, Leu^{11}).

3. *Tumour necrosis factor*

Of the major contributors to the pathogenesis of endotoxin shock (prostaglandins, bradykinin, opioids, etc.) tumour necrosis factor-cachectin emerges as the most important one. Tumour necrosis factor (TNF) was discovered in 1975 as a polypeptide of macrophage origin released upon exposure to bacterial-products especially to lipopolysaccharide (LPS) endotoxin [23]. LPS induces transcription of the TNF α gene and dexamethasone suppresses transcription [24, 25]. LAK and NK cells, T lymphocytes and microglial cells also produce TNF [26]. PMA is another inducer of TNF. TNFα is distinguished from lymphotoxin (LT) or TNFβ. TNFα and β share amino acid (AA) sequence homology but monoclonal antibody directed against one will not neutralize the other. Both TNFα and β induce cellular endonucleases which fragment ("dissolve") the DNA of target cells [27] thus causing programmed cell death (apoptosis) that also occurs under physiologic conditions, for example when in the developing embryo an early anlage of a primitive organ regresses [28]. LT/TNFβ is clearly different from perforins [29–31]. These latter lymphokines cause target cell death through the polymerization of proteins into microchannels of transmembrane location with resultant leakage of cytoplasmic contents from target cells. TNFα inhibits lipoprotein lipase in adipocytes (cachectin effect) [32]; induces bone resorption through osteoclast activation; exerts antiviral and antiparasitic activity; changes the phenotype from cuboid to elongated of endothel cells and causes necrosis of these cells; causes "anaemia of chronic infection" [33]; stimulates fibroblasts and increases GM–CSF and M–CSF production by fibroblasts [34]; and mediates adult respiratory distress syndrome, capillary leakage and other life threatening sequela of endotoxin shock [32–37]. r-Hu-TNFα stimulates the growth of human fibroblasts but in the presence of arachidonic acid it becomes highly cytotoxic to these cells [38]. Connective tissue cell death may occur in endotoxin shock where both TNFα and prostaglandins are overproduced.

The human TNFα gene resides in chromosome 6. It encodes a polypeptide precursor of some 231 AA of which a 155 AA chain forms the 17 000 molecular weight mature agent. Human recombinant TNFα (r-Hu-TNFα) produced in

Escherichia coli is nonglycosylated and is biologically active. It acts through binding to its high affinity receptors [39]. TNFα and β compete for the same receptor. Interaction of TNFα with its receptor results either in superoxide production ($O_2 \rightarrow H_2O_2$) leading to cell death or in the induction of the 222 AA → → 198 AA mitochondrial protein manganous superoxide dismutase (MnSOD) conveying resistance to the cell against TNFα [40]. IL-1α and β also induce MnSOD thus protect cells against TNFα [40–42].

TNFα and β induce transcription of the HIV proviral DNA. McAb to TNFα blocks HIV induction. Once activated, HIV itself can induce TNFα and its receptor. Thus the HIV proviral DNA undergoes transcription leading to the release of mature HIV virions; macrophages producing these virions do not die like CD4 T cells do [43–46]. TNFα induces RNA transcripts of the c-*abl* protooncogene in human bone marrow stromal cells. Cells with unregulated c-*abl* RNA transcripts also expressed amplified GM–CSF transcripts but procollagen I transcripts were downregulated. Thus TNFα affects haematopoietic growth factor and extracellular matrix production through protooncogene activation [47].

The most prominent physiological antagonists of TNFα are IFNβ and TGFβ. This latter agent is an inhibitor of IL-1-induced thymocyte proliferation and macrophage- or lymphocyte-mediated cytotoxicity [48–52]. McAb to TGFβ augments LAK cell and T lymphocyte proliferation. In many ways TGFβ synergizes with cyclosporin A, another antagonist of IL-2. IFNα activates macrophages and induces the release of both TNFα and TGFβ. By inducing haemoglobinization (in K-562 cell assay), TGFβ may also antagonize TNFα-induced anaemia [53, 54]. TGFβ inhibits TNFα-induced endotoxin shock in experimental animals [55]. The calcium channel blocker verapamil inhibited TNFα production by endotoxin-stimulated human monocytes and reduced mortality of endotoxin shock in rats [56]. Among immunosuppressive factors released by tumours, TGFβ is the most prominent [57]. IL-6 also inhibits LPS-induced TNFα production in human monocytes [58].

In hairy cell leukemia high levels of TNFα are produced by the leukemic cells. In this system TNFα suppresses hematopoiesis. Administration of anti-TNFα McAb reinduced hematopoiesis in an *in vitro* hematopoietic colony assay [59].

TNFα levels were elevated in patients with AIDS and AIDS-related complex [60]. While control sera seldom show higher than 28 ng/litre TNFα levels, AIDS patients sera usually contain over 50 ng/litre TNFα, as determined in a double antibody radioimmunoassay using Ernst-Boehringer's (Vienna, Austria) rabbit antiserum and ^{125}ITNFα. When expressed in pg/ml, TNFα levels were higher (50–1000 pg/ml) in patients with complicated *Plasmodium falciparum* malaria, than in patients with uncomplicated malaria (10–250 pg/ml), as determined in an immunoradiometric assay (TNFα IRMA, IRE Med-

genix, Fleurus, Belgium) [61]. When measured as cytotoxic activity of cell-free supernatants to cycloheximide-treated HeLa cells, starving healthy volunteers also had elevated TNFα blood levels. Approximately 8% or less of healthy normal individuals show occasional elevated TNFα levels [62]. Some of these sera positive in ELISA show no cytotoxicity characteristic for TNFα thus indicating that false positive ELISA results may occur [63]. Consenting volunteers injected with *E. coli* endotoxin showed elevated TNFα levels only at the onset of clinical symptoms. TNFα levels were high in meningococcaemia and directly correlated with mortality. In other septicaemias, TNFα levels were only transiently elevated early in the process and it was not possible to prove direct correlation between higher levels and increased mortality [63]. ELISA with anti-TNFα McAb was utilized in this study. The highest level (250–500 pg/ml) occurred in patients with *Enterobacter cloacae* septicaemia. Bronchial washings obtained from adult respiratory distress syndrome (ARDS), but not from patients with tuberculosis or sarcoidosis or from healthy individuals, contained highly elevated levels (> 500 U/ml $= > 12.5$ ng/ml) of TNFα [64]. Interleukin-1 (Hu-r-IL-1) also contributes to the pathogenesis of ARDS by increasing transendothelial albumin flux [41]. Interleukin-6 (IFβ2; B cell differentiation factor; hybridoma growth factor; plasmacytoma grown factor) levels were increased in septic shock and higher titres correlated with higher mortality [65]. rHu–TNFα is known to induce endogenous IL-6.

III. Early results of treatment of endotoxinaemia with monoclonal antibodies

1. *Polyclonal antisera*

Antiserum of healthy male volunteers immunized with *E. coli* J5 ameliorated endotoxin shock in patients. The lipopolysaccharide core of *E. coli* J5 lacks oligosaccharide side chains that determine antigenic diversity. The polysaccharide-lipid A complex of the core is very similar antigenically in most Gram-negative bacteria. Mortality of endotoxin shock was reduced for bacteraemic patients treated by standard means from 39% (42 of 109 patients) in the controls to 22% (23 of 103 patients) in the group treated with the addition of antiserum [66]. In profound endotoxin shock, mortality of 77% (30 of 39 patients) of patients treated with standard means was reduced to 44% (18 of 41 patients) in recipients of J5 antiserum [67]. Prophylactic administration of J5 antiserum reduced the incidence of endotoxin shock in high risk patients from 11% in control group (15 of 136 patients) to 4.7% in the treated group (6 of 126 patients); patients with abdominal surgery benefited the most: incidence of septic shock decreased from 15.6% in controls (13 of 83 patients) to 4.7% in

treated patients (2 of 71 patients) [67]. However, J5 antiserum used prophylactically in leukemic neutropenic patients failed to prevent or ameliorate Gram-negative bacteraemia [68]. One wonders how much benefit may be derived from active immunization of patients with high risk for Gram-negative bacteraemia with irradiated endotoxin (Tolerin) [69]?

2. *Murine antiendotoxin monoclonal antibodies*

Antiendotoxin murine McAb XMMEN–OE5 (XOMA) derives from Balb/c mice immunized with the J5 mutant *E. coli* strain. Splenocytes of immunized mice were fused with murine myeloma cell line B3–X63–Ag8.653. Hybridomas thus created grew as ascites tumours and released IgM antibody specifically binding the core lipid A region of endotoxin of both *Enterobacteriaceae* and *Pseudomonadaceae* sp. The purified antibody was not pyrogenic in rabbits, was free of viral contamination and contained less than 16 ng DNA. In mice septicaemic with Gram-negative bacteria, per cent survivals after treatment were 29 for saline; 45 for antibiotics and saline; 29 for high dose E5 and saline; 50 for low dose E5 and antibiotics; and 61 for high dose E5 and antibiotics [70]. Intradermal injection of 100 μg E5 serum induced no immediate hypersensitivity reaction in volunteers. In later clinical trials patients with positive skin reaction were excluded. Intravenous administration up to 60 mg/kg of the serum E5 induced no toxicity but 8 of 14 subjects produced anti-mouse antibodies. At the 15 mg/kg dose level peak serum concentration of the antibody was 188 μg/ml. Terminal elimination phase of the antibody started about 60 min after the end of intravenous infusion. Of the first 11 septicaemic patients treated, 2 died (1 with metastatic small cell carcinoma of lung; 1 with heart failure) and 9 patients survived. In a prospectively randomized double blind clinical trial 242 patients received McAb E5 and 226 patients received placebo in addition to standard therapy. Of 316 patients with blood cultures positive for Gram-negative bacteria, 179 were, and 137 were not in shock. Patients in shock and/or with disseminated intravascular coagulation were evenly distributed in the immune serum E5 and placebo groups: 56% and 56% in shock, 26% and 24% with DIC. The most common Gram-negative bacteria in both groups were *E. coli*, *Klebsiella*, *Pseudomonas* sp. and *Enterobacter* sp. Overall mortality was 12% versus 23% in immune serum-treated and control patients, respectively. Mortality and morbidity was most significantly reduced by E5 among patients with Gram-negative septicaemia who were not yet in shock [71, 72]. Allergic (anaphylactoid) reactions to E5 occurred only in 1.6% of patients but 43% of patients produced antibodies to E5.

At Centocor murine hybridomas secreting McAb to *E. coli* J5 and *Salmonella minnesota* R595 endotoxin were generated [73]. Several other groups of investigators developed murine hybridomas secreting McAb specific to lipid

A of endotoxin but no clinical trials with these McAb have as yet been reported [74—76].

3. *Human antiendotoxin monoclonal antibodies*

Very low doses (1—10 μg/mouse) of a human IgM McAb specific to *E. coli* J5 endotoxin protected mice against lethal shock [77].

Centocor and Baylor College of Medicine (Houston, Texas) have developed and are testing the human McAb HA—1A against endotoxin shock. A patient with Hodgkin's disease was immunized with endotoxin before staging laparotomy and splenectomy. Spleen cells of this patient were fused with lymphoma cells to form hybridomas secreting IgM antiendotoxin antibodies specific to lipid A. Over 36 patients with septicaemias were treated with HA—1A; in the blood of 22 patients Gram-negative bacteria were documented. There were no adverse effects and anti-Ha—1A immunoglobulin production did not take place. Serum half life of Ha—1A was 16 h. Treated patients rapidly defervesced and normalized their blood pressure [78]. Controlled and randomized clinical trials have not as yet been evaluated. Oncogen in Seattle produced human IgG1 McAb 2138 and 2B8 against type a and b flagella of *Pseudomonas aeruginosa* and showed protective effect of these immune sera in mice against lethal pseudomonas infection [79].

4. *Monoclonal antibodies to tumour necrosis factor*

TNFα/cachectin induces depletion of body fat accompanied by hypertriglyceridaemia in tumour-bearing mice. Antibodies specific to TNFα/cachectin alleviate these effects [80]. TNFα/cachectin induces IL-1 and IL-6 production; these latter cytokines amplify the effects of TNFα/cachectin and contribute to the severity of endotoxin shock [81]. Column chromatography-purified and pepsin-digested Fab_2 fragments of IgG McAb to TNFα/cachectin could neutralize in its concentration of 10 μg/ml over 50 ng/ml r-Hu–TNFα/cachectin. In baboons with *E. coli* septicaemia this antibody significantly reduced IL-1β and IL-6 production and ameliorated the outcome of endotoxin shock [81, 82].

Murine McAb CB6 developed by Celltech (Slough, England) against r-Hu–TNFα was given intravenously up to 10 mg/kg to patients with severe septic shock and hypotension not responsive to conventional treatment. Normalization of blood pressure occurred in the mean of 9h in 9 of 10 patients who survived over 72 h. Thereafter 5 patients worsened and died. At over 7 days survival rate was 5 of 10 patients [83].

IV. Discussion and summary

In the USA, nosocomial infections often resulting in septicaemia are at an extraordinarily high level due to extensive use of invasive technology for diagnosis and therapy, immunosuppressive therapeutics for collagen diseases and for organ transplant recipients, and due to extensive combination chemotherapy for malignant diseases. The annual incidence of bacteraemias is estimated to be close to 200 000 with 75 000 deaths. This high mortality occurs despite extraordinary availability of potent antibiotics. Most fatalities are consequential to endotoxin shock of Gram-negative bacterial septicaemia [84].

The mediators of endotoxin shock have antitumour effect in experimental systems. Unfortunately, when endotoxin shock occurs in patients with far advanced cancers, the molecular mediators generated seldom induce tumour regression [85, 86]. Systemic administration of bacterial products without the actual induction of endotoxin shock is somewhat more effective (as the New York City surgeon W. B. Coley observed it first at the turn of the century) [87]. Injection of live oncolytic viruses or tumour cell membranes with budding virus particles ("viral oncolysates") also induce a number of molecular mediators (IFN-α, β; IL-2; TNFα; endogenous opioids, etc.) that may cause mild clinical symptoms distantly resembling endotoxin effects. These interventions also may lead to temporary partial remissions in tumour-bearing patients or suppress the growth of subclinical tumour metastases thus delaying relapses in the course of malignant diseases [88].

If protooncogenes-oncogenes are activated or deactivated in endotoxin shock, these events are entirely unknown. Bacterial toxins have profound effects on some signal transduction pathways that protooncogene-oncogene products utilize. For example, pertussis toxin, like cholera toxin and *E. coli* endotoxin enter cells through endocytic vesicles, ribosylate ADP of GTP-binding proteins and by deregulating adenylate cyclase grossly alter intracellular cAMP concentrations. Cholera toxin enhanced the growth of human breast carcinoma cells in athymic mice; it stimulated adenylate cyclase and cAMP in colon carcinoma cells in vitro [89, 90]. cAMP suppressed the v-*ras* oncogene and cholera toxin antagonized this effect [91]. If in endotoxin shock the *mas* protooncogene is deactivated, angiotensin II receptor may undergo downregulation thus creating a state of hypotension resistant to angiotensin. If a mutant H-*ras* oncogene is amplified, overreactivity to bradykinin may follow due to excessive expression of B2 receptors.

Immune serum therapy of endotoxin shock became a most promising modality when correlation was found between recovery and high titres of circulating antibodies to *E. coli* endotoxin core in patients with pseudomonas septicaemia [92]. Abraham Braude and his associates pursued the idea of serotherapy first with polyclonal and with monoclonal antibodies [93].

Table I

Serotherapy with McAb for endotoxin-shock

McAb	Target	Comments	Major investigators and affiliations
Murine E5 IgM	Lipid A of endotoxin J5	Neutralizes endotoxin and alleviates shock in patients. Induces HAMA production	K. Gorelick; E. Ziegler; L. Young et al. XOMA Berkeley, Calif; Univ Calif, San Francisco, Calif; Univ S Florida, Bay Pines VA Hosp., Tampa and St Petersburg, Florida; St Louis Univ, St Louis, Missouri
Murine IgG Lou 4, 20, 25, 28	Lipid A of endotoxin J5 and/or *S. minnesota* R595	Neutralizes endotoxin	W. Bogard et al. Centocor, Malvern, Pennsylvania; Univ Minnesota, Minneapolis, Minn
Hamster-mouse hybridoma	8OK endotoxin receptor	Binds to endotoxin receptor	D. Morrison et al. Univ Kansas, Kansas City, Kansas
Human IgM HA-1A	Lipid A of endotoxin J5	Neutralizes endotoxin; alleviates shock in patients	C. Smith; R. P. Dellinger et al. Centocor, Malvern, Pennsylvania; Baylor College Medicine, Houston, Texas
Murine 154/6	TNFα	Binds to TNFα	G. Trinchieri, Wistar Institute, Philadelphia, Pennsylvania
Murine CB6	TNFα	Binds to TNFα Reversal of hypotension in patients	A. R. Exley; R. Riddell et al. Hammersmith Hospital, London, Celltech, Slough, England
Murine IgG Fab 2	TNFα	Neutralizes r-Hu-TNFα. Reduces IL-1β and IL-6 induction by endotoxin in baboons	K. J. Tracey et al. Cornell Medical Center, New York NY. Rockefeller University, New York NY. Chiron, Emeryville, Calif

Table I summarizes current approaches to the serotherapy of endotoxin shock [94]. The production of antimurine immune globulins when mouse McAb are used for serotherapy emerges as a setback especially when repeated infusion of the antibody are needed. The development and administration of human McAb directed to lipid A or to TNFα/cachectin hold the highest promise for success. Extra measures beyond standard therapy are essential to reduce the high mortality of endotoxinaemia.

In summary, endotoxin shock is a most complex and often fatal clinical entity. Endotoxin induces the generation of bradykinin and prostaglandins; it derepresses the gene of endogenous opioids and activates the gene of TNFα which in turn induces the overproduction of IL-1 and IL-6. Endothel cell damage leading to vascular leakage syndrome with depletion of circulating blood volume due to extravasation results in hypotension and in renal and hepatic ischemia. Consequentially metabolic acidosis sets in. Alveolar and endothelial cell damage in the lungs produces adult respiratory distress syndrome with high mortality. Consumption coagulopathy with disseminated bleeding follows. Despite prompt volume replacement, combined antibiotics, secure airways and administration of dopamine drip, endotoxin shock often progresses to death. Specific antibodies neutralizing endotoxin itself or the major mediator of the complex syndrome it causes: TNFα, are able to induce reversal of the near-fatal process. Early clinical trials for the serotherapy of endotoxin shock with monoclonal antibodies are reviewed in Table I.

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MODULATING THE MICROBIAL COLONIZATION OF THE GASTROINTESTINAL TRACT BY ORAL ADMINISTRATION OF DEFINED *ESCHERICHIA COLI* STRAINS

III. LIPOPOLYSACCHARIDE-SPECIFIC IgA IN THE INTESTINE AFTER ORAL ADMINISTRATION OF *ESCHERICHIA COLI*

R. Schröder, R. Blatz and K. Linde

Institute of Medical Microbiology, Karl Marx University, Leipzig, FRG

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The assumption that local immunologic mechanisms are responsible for findings on the specific microbial colonization of the gastrointestinal tract of mice, is confirmed by results described in the present communication. Quantitative examination of intestinal lavage fluids after oral administration of germs by ELISA showed that (i) there is a critical dose above which a significant increase of IgA synthesis is observable; (ii) low antibody levels could be detected already in the untreated control group. It may be assumed that — regardless of the biological characteristics of the administered strain, namely, substrate and receptor competition — the stimulability of local immunity provides against permanent monocolonization with displacement of the autochthonous *Enterobacteriaceae* in adult and immunocompetent hosts.

The possibility of specific microbial colonization of the gastrointestinal tract of conventional adult mice by oral administration of living *Escherichia coli* strains has been discussed in the first and second communications of this series. The rate of colonization of the gastrointestinal tract could be greatly increased by using functionally optimized cultures and "foreign" strains no occurring in this country. Notwithstanding this, predominance of the colonizing strain over the autochthonous *Enterobacteriaceae* was not detectable for more than three days. It has been assumed that local immunologic mechanisms are responsible for this finding.

Materials and methods

Experimental animals. Conventional male ICR mice obtained from VEB Versuchstierproduktion, Schönwalde, GDR, were used as experimental animals. Their as supplied body weight was between 18 and 20 g and they were used after an adaptation period of one to two weeks.

R. Schröder, R. Blatz, K. Linde
Institute of Microbiology, Karl Marx University
Liebigstr. 24, 7010 Leipzig, FRG

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Bacteria. E. coli M17 (Dr. Bondarenko, Gamaleya Institute, Academy of Medical Sciences of the USSR) a spontaneous mutant carrying rifampicin and nalidixic acid resistance as an identification marker. (For details of the method of selection see the first communication.)

Oral immunization was done using an oesophageal tube with graded doses of bacteria in 1 ml fluid.

Collection of intestinal lavage fluid. A 20 cm long piece of intestine was washed out caudally from the pylorus with 1 ml ice-cooled PBS containing 100 anti-trypsin units of Contrykal (VEB Arzeimittelwerk, Dresden, FRG). Solids were removed by centrifugation at 4 °C. The supernatant fluid was stored, before use, at −20 °C.

Immunization and sampling. Oral immunization of mice was performed on seven consecutive days with 10^7, 10^8, 10^9, 10^{10} and 10^{11} living *E. coli* M17. Samples were collected after 5, 8, 10, 15 and 20 days after the last administration of antigen.

Detection of lipopolysaccharide-specific IgA antibodies by means of an enzyme immunoassay. The coupling of alkaline phosphatase to anti-murine IgA (alpha chain-specific; Biochemical Corporation, Cleveland, USA), as well as the buffers and solutions used were described previously [1]. The enzyme immunoassay [2] was modified using the method described by Barrett et al. [3]. To assure better binding of the test antigen (LPS of *E. coli* M17), the microtitration plates (Dynatech. Alexandria, USA and VEB Polyplast Halberstadt, GDR) were precoated with 100 µl *E. coli* M17 rabbit serum per well. The plates were incubated overnight at 4 °C and subsequently rinsed five times with a washing buffer. This was followed by incubation for 2 h at 37 °C with the LPS (100 µl/well) dissolved in coating buffer. The LPS concentration was 100 µg/ml. After this, the intestinal lavage fluid (lowest dilution 1 : 2) and as a last step, the anti-murine IgA tagged with alkaline phosphatase (dilution 1 : 100) were added, and incubated for 2 and 3 h at 37 °C, respectively. After a reaction period of 1 h at 37 °C, the substrate conversion was photometrically determined at 405 nm. Only those tests were considered positive in which the so-called critical extinction was reached or exceeded. This was determined as follows. A pool was prepared from 10 intestinal lavage fluids and used to make 18 determinations in two independent series of tests. The mean value ($\bar{x}$) and the standard deviation (SD) were determined. Only measured values greater than or equal to $\bar{x} + 3$ SD were considered as reflecting positive detection of antibodies. By using these "critical extinction" results, the intestinal lavage fluids were grouped into samples with and without detectable IgA antibodies.

Statistical analysis. To determine the effects of the antigen dose on the number of reacting animals, the measured values of all experiments were compiled. Statistical comparison was made by the chi-square test ($P = 0.05$). Differences in titre were tested for significance by Finney's probit analysis ($P = 0.05$).

Results

The effect of the antigen dose upon the number of animals with detectable lipopolysaccharide-specific IgA antibodies in the intestine was detectable in less than one half of the animals already after administration of daily antigen doses as small as 10^7 living germs. It is interesting to note that antibodies were also detectable in two of the untreated controls (8%).

For doses of 10^7, 10^9, 10^{10} and 10^{11} germs/day, statistical evidence could be obtained for the difference from the control values. Dose dependence of the local IgA response could not be determined in the range of 10^7 to 10^9 germs/day. This was only observable in those cases in which larger quantities of bacteria were used. After administration of 10^{10} and 10^{11} germs/day, the number of test animals with detectable IgA antibodies increased to 52% and 76%, respectively. There was a significant difference between animals immunized with 10^9 and 10^{11} germs/day. The results obtained are summarized in Fig. 1.

The same results were obtained in those cases where samples were titrated until the critical extinction was reached. The mean titres increased significantly when antigen doses were increased from 10^9 to 10^{11} germs/day (Fig. 2).

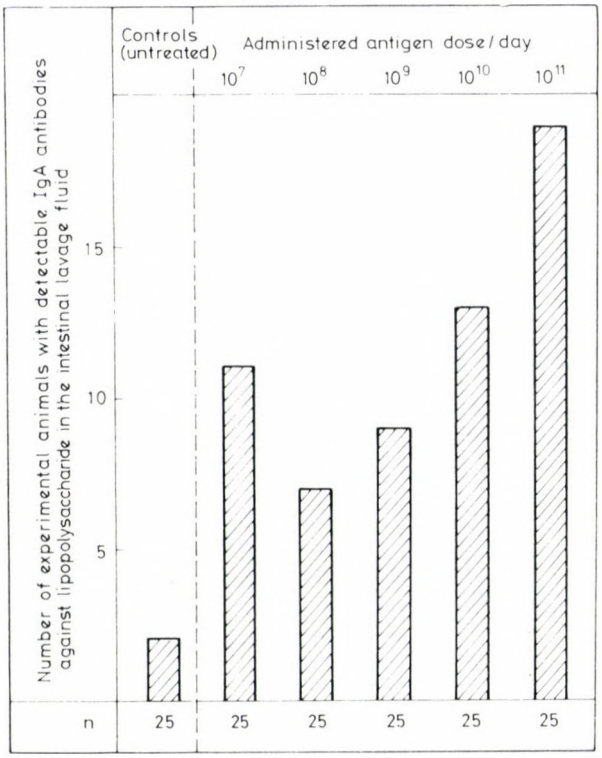

Fig. 1. Number of mice with lipopolysaccharide-specific IgA antibodies in the intestine after oral administration of different doses of living *E. coli* M17

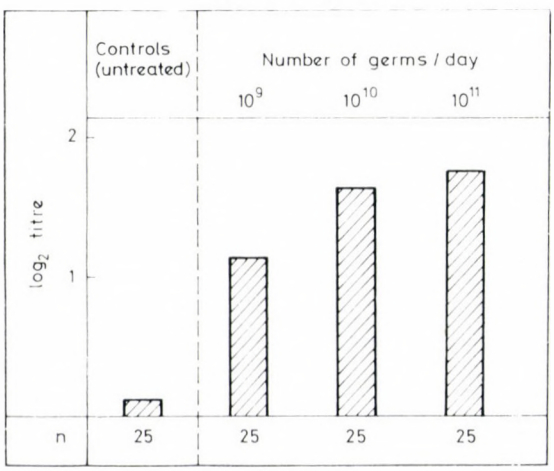

Fig. 2. Mean titre of lipopolysaccharide-specific IgA antibodies in murine intestinal lavage fluid after oral administration of different doses of living *E. coli* M17

Discussion

The present results show that oral administration of living *E. coli* in suitable multiple doses may trigger an IgA response in the murine intestine. Quite obviously, a minimum quantity of germs is necessary to induce detectable IgA antibody production.

The dose dependence of a local immune response has been described previously [4, 5]. Under the conditions used in these investigations, the majority of experimental animals may be expected to produce strong immune response to orally administered doses of 10^{10} or more germ/day. However, this also corresponds to the number of bacteria that are required to enable detectable colonization in the experimental animal model used in these studies. Accordingly, colonizing experiments are invariably accompanied by a stimulation of local immunity. Likewise, regulatory antibody synthesis may be induced in those cases in which the multiplication of a colonizable species exceeds a certain critical liminal value which for *E. coli* is believed to be in the region of 10^{10} germs. IgA is capable of preventing antigen contact with epithelial cells [6]. In addition to antibodies with a distinct specificity for fimbriae, antibodies against the O antigen of *E. coli* are also involved in the blockade of bacterial adherence [7, 8].

Accordingly, the antibodies detected in these experimental animal studies will contribute to eliminating the corresponding *E. coli* bacteria from the intestine. Obviously, the secreted antibodies are capable of affecting the rate of colonization at a very early time, for they were detectable already after five days from the last administration of the antigen. However, the immune response may be initiated at an even earlier time [9].

The immune response may also be modified by previous antigen contacts. Offering significant evidence in favour of this assumption is the fact that lipopolysaccharide-specific IgA antibodies were also detectable in two of the untreated controls. So-called natural antibodies against *Enterobacteriaceae* were detected not only in human serum but in the duodenal juice [10] or in the saliva of conventional laboratory mice [11] as well. Their principal stimulus should be the normal flora with its numerous antigenic communities [12]. The present results of experiments on conventional laboratory mice allow to make the following conclusions for the overall object of modifying existing *Enterobacteriaceae* through mucosal colonization with an apathogenetic strain. (i) Compared to nonselected strains, the selection of defined and functionally optimized strains results in a marked increase in the rate of colonization. (ii) Proper selection of strains enables a short-time colonization (with the proportion of particular strains in the autochthonous lac^+ *Enterobacteriaceae* flora being greater that 50% for one to three days) in nearly all experimental animals (see second communication). In a fully immunocompetent hosts, however, long-term

colonization is generally limited by various factors. The large oral doses required for colonization and the number of germs obtained during progressive colonization, tend to induce local immunologic defenses which, through the synthesis of s-IgA, prevent the numerical dominance over the remarking *Enterobacteriaceae* by bringing about a reduction to a tolerable number of germs or, else, eliminating the foreign germ completely. Added to these come previous antigen contacts which are due to numerous antigenic communities of *E. coli* serotypes and to relations with other *Enterobacteriaceae*. These contacts, like colonization of a corresponding strain, lead to local IgA antibody synthesis.

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PERSISTENCE OF RECEPTOR "MEMORY" INDUCED IN *TETRAHYMENA* BY INSULIN IMPRINTING

L. Kőhidai, G. Csaba and Valéria László

Department of Biology, Semmelweis University Medical School, Budapest

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Tetrahymena cells treated (imprinted) with insulin on a single occasion bound significantly more insulin than the control cells for as many as 70 days, i.e. over 664 generation changes. Although late reexposure to insulin reduced the binding of labeled hormone for 24 h, the binding value of the imprinted cells still increased significantly over the control. Maintenance under anaerobic conditions for 80 days accounted for a temporary suspension of the effect of imprinting which was, however, recovered within a week of return to aerobic conditions, in an even stronger form than observed in cultures maintained without an anaerobic episode. The experiments demonstrated that the imprinting-induced receptor "memory" lasted long, but was vulnerable to treatment with another polypeptide hormone.

The cells receive chemical or other environmental signals by means of membrane-associated or cytosol-associated highly sensitive, complex configuration, the receptors, whose structure varies with the nature of the specific signal molecule. The mechanism provides for a selective processing of the different impulses which act on the cells in biological systems, and it accounts not only for an immediate cellular response, but also for the induction of a cellular-level "memory" of the event, to judge from the consistent recurrence of the signal-evoked response pattern over a relatively long period. This ability of cells contributes to the evolvement of highly complex reaction patterns in multicellular organisms.

Biochemical memory, which operates at all levels of phylogenesis from unicellular organisms to high vertebrates, presupposes the coordinated function of all cells and all protective mechanisms of the organism. Although many details of cellular "memory" are still obscure, evidence has been accumulated that its physiological operation and changes presuppose intactness of the cell membrane and nucleus, and its modulations depend on the protein synthesis.

The foregoing considerations can also offer an explanation for the phenomenon of hormonal imprinting, which has been shown to occur in both unicellular [1] and multicellular organisms [2]. Primary interaction (imprint-

László Kőhidai, György Csaba, Valéria László
Department of Biology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

ing) of a given target cell with a hormone accounts for a changed — usually increased — response to the latter on reexposure, as if the cell "remembered" the primary event. The changed response is shown not only by the cells directly involved in primary interaction, but also by many subsequent generations. The underlying mechanism is a chain of reactions, which take place at several levels and are, for the most part, still obscure. The external membrane of the cell, the intracellular membrane systems, the interactions between intracellular compartments [3, 4], certain intranuclear changes and functional states of the cellular nucleus [5] may all be involved in the mechanism of imprinting.

The imprinting-induced modifications of cellular response can be assessed from changes in several cellular functions, such as mitotic activity [6], phagocytic activity [7], hormon binding capacity [8, 9] and membrane potential [10].

The purpose of the present study was to investigate how long the imprinting-induced increase in response to insulin was transmitted to subsequent generations of the model cell *Tetrahymena* [11]. It was also examined in this context, whether imprinting-induced receptor "memory" was influenced by later treatment with insulin itself or another polypeptide hormone (TSH), or by lasting inhibition of mitosis under anaerobic conditions of maintenance.

Materials and methods

Culture. Tetrahymena pyriformis GL cells, propagated in 0.1% yeast extract (Difco, USA) and 1% Tryptone (Oxoid, England) containing medium at 28 °C were used in the exponential phase of growth.

Treatment. Part of the mass culture was treated with 10^{-6} M insulin for 1 h, the untreated part served as control. After insulin treatment, the experimental culture was returned to plain medium for 24 h. The culture medium was subsequently changed every 48 h.

Determination of hormone binding. Samples taken from the cultures at predetermined time intervals (every seventh day between generations 9–323, and every fourteenth day between generations 323–664) were fixed in 4% formalin solution (in PBS), washed in PBS, exposed to fluorescein-isothiocyanate (FITC, BHD, England) labeled insulin for 1 h, and examined for binding of the label with a Zeiss Fluoval cytofluorimeter.

Statistical evaluation. The analogous signals of the cytofluorimeter were transformed to digital signals for assessment of mean values, standard deviation and significance of intergroup differences by means of a HP-41CX calculator connected with the cytofluorimeter. Six replica assays were done, and 20 cells were assayed in each group for intensity of fluorescence.

Second hormone treatment. Cells cultured as above were treated with 10^{-6} M insulin or thyrotropin for 1 h, were returned to plain medium for 24 h, and were finally reexposed to FITC-insulin for 1 h. Hormone binding was then assayed by cytofluorimetry, as above.

Anaerobic culturing conditions. Part of the cultures were, after propagation for 3 months, reduced to anaerobic conditions by spreading a 1 mm thick layer of paraffin oil onto the surface the nutrient medium. Eighty days later the cells were returned to aerobic conditions for one week, during which they were assayed for insulin binding on days 4 and 7. Anaerobic conditions were then provided again for 6 months, after which the cells were returned to aerobic conditions and assayed for insulin binding on day 7.

The cells were treated with 10^{-6} M insulin or TSH on day 7 after the first anaerobic episode of 80 days.

Results

In the experimental series we followed up the changes induced by primary interaction with a hormone (hormonal imprinting) in the hormone binding capacity of the interacting cells and their offspring generations (Fig. 1). The generation time of *Tetrahymena* being about 150 min, the weekly examination detected FITC-insulin binding in about every 60th generation. The hormone binding capacity of the insulin-pretreated cells differed significantly from the control throughout. Binding increase over the control was on average 110% in the initial period (generations 9–323) and 115% in the 664th generation.

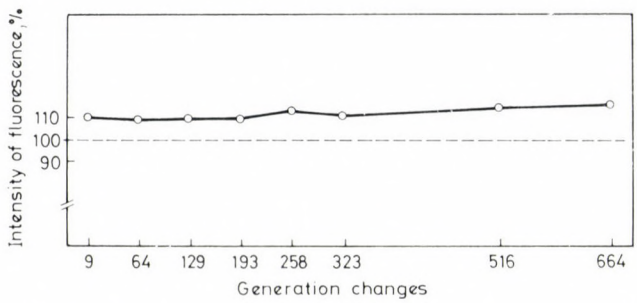

Fig. 1. FITC-insulin binding in the offspring generations of *Tetrahymena* cells treated with hormone on a single occasion (control = 100%)

In the second experimental series we studied the late effects of hormonal imprinting and the response of the cells to a second treatment with the same or another polypeptide hormone (Fig. 2). This series covered the following groups: control/control (C/C); control/insulin (C/I); control/TSH (C/T); insulin/control (I/C); insulin/insulin (I/I); insulin/TSH (I/T).

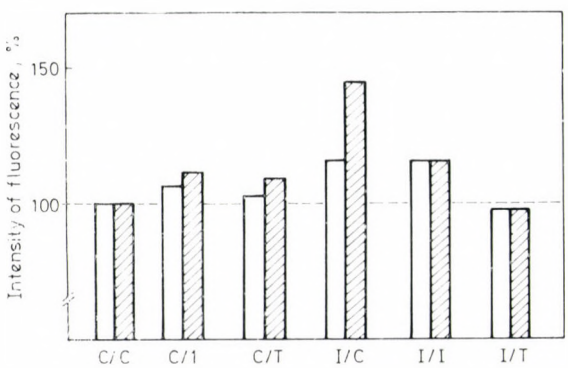

Fig. 2. Late effects of insulin-imprinting in *Tetrahymena* cells reexposed to insulin or TSH at different time intervals in the course of 664 generation changes (open columns), and 7 days after maintenance under anaerobic conditions for 80 days (shaded columns); (C = control; I = insulin; T = TSH)

The binding of FITC-insulin was low (105%) in the cells not preexposed to the hormone (C/I), and that of TSH was still lower (102%) under the same conditions of treatment (C/T). The cells preexposed, but not reexposed to insulin (I/C) and those both preexposed and reexposed to it (I/I) equally showed a significant (115%) binding increase over the control, whereas those preexposed to insulin and reexposed to thyrotropin (I/T) did not appreciably differ from the control in respect of insulin binding (98%).

The imprinted cells reduced to anaerobic conditions for 80 days recovered their insulin binding capacity gradually, to judge from a relative decrease (91%) on day 4 of return to aerobic conditions, and a significant relative increase (140%) on day 7 (Fig. 3).

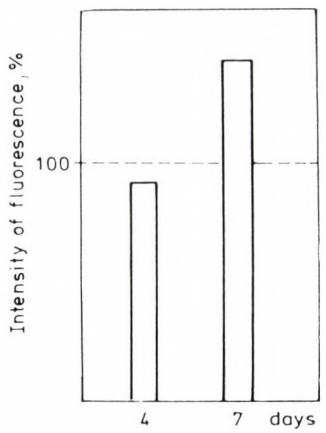

Fig. 3. FITC-insulin binding in *Tetrahymena* cells at different times after return from anaerobic to aerobic conditions (control = 100%)

Membrane-level changes shown in response to exposure to insulin or TSH after anaerobic conditions of culturing were also assessed in cultures treated on the schemes described above. Although the absolute values of FITC-insulin binding were slightly increased on day 7 after return to aerobic conditions, the bindings profile itself did not appreciably differ from the pre-anaerobic one. Although the binding values of the groups C/I and C/T increased appreciably (112, respectively 108%) over the control, they were still considerably lower than those shown by the I/C (142%) or I/I cells (116%). Mixed hormone treatment (I/T) had no influence on insulin binding (98%) in this experimental series. The cultures returned to anaerobic conditions for further 6 months showed the same patterns of response as after 80 days.

Discussion

Hormonal imprinting takes place at primary interaction between target cell and hormone. In unicellular organisms, non-specific membrane patterns transform to persistent receptor structures [12] in presence of hormone, whereas in higher organisms the genetically encoded receptor is amplified by hormonal influence [1]. Hormonal imprinting alters — usually increases — the hormone binding capacity and the specific response of the cell at later interactions [13, 14].

In earlier studies along this line, diiodothyrosine imprinting still operated in *Tetrahymena*, i.e. a "memory" of it was still demonstrable by elevation of the mitotic index, after as many as 500 generation changes [6]. Such persistent and transmissible receptor "memory" could arise either by a structural alteration of the receptor, or by a regular or hormone-induced recycling to the external membrane of receptors associated with the cytoplasmic membrane pool. A third alternative explanation is that the memory persists at post-receptorial rather than receptor level, and the changes induced by it in the post-receptorial mechanisms (e.g. cAMP elevation) account for an increased cellular response on reexposures to the hormone and usually for an increased hormone binding capacity as well.

The purpose of the present experiments was exactly to identify, whether the persistence over many generations of the functional changes associated with hormonal imprinting [6] was due to receptor-level or post-receptorial changes.

A single interaction with insulin was in fact sufficient to induce a "memory" of imprinting, and, consequently, an increased binding capacity for insulin in as many as 664 subsequent generations of *Tetrahymena*. The binding capacity was fairly uniform at the different sampling times, and tended to increase rather than to decrease towards the end of the experimental period. Although this does not exclude a periodic recurrence of negative binding results, the binding values unequivocally portrayed the persistent impact of imprinting. The present observations cannot disclose, whether imprinting had changed the structure or the number of the membrane receptors in *Tetrahymena*, but the fact remains that the insulin binding capacity was durably increased in the progeny generations of the imprinted cells.

Part of the cells were reexposed to insulin or TSH after 70 days of culturing, i. e. after 664 generation changes, to clarify whether or not the imprinting elevated binding capacity was further increased by the second treatment, and whether the response was specific. It is known that hormones or hormone-like materials may increase the binding capacity for another hormone also by nonspecific influence [15–18]. TSH was used to exclude this alternative.

The second hormone treatment gave rise to down-regulations, probably because the assay was done 24 h after insulin reexposure, when increase over the

control was appreciable, but not significant in the C/I cells, and significant relative to C/C but markedly decreased relative to I/C, in the I/I cells, owing in all probability to the down-regulating effect of the second insulin treatment. TSH was less active in the C/T group than insulin in the C/I group, and it abolished altogether the effect of insulin imprinting in the I/T series. This suggests that late reexposure to a polypeptide hormone destroys the imprinting effect of primary interaction with another polypeptide hormone. Special emphasis is laid in this context on the length of the time interval between the primary and secondary treatments, because no such effect was observed if one polypeptide hormone was employed shortly after, or simultaneously with, the other [19]. It follows that, although the effect of imprinting is strong, it can be extinguished by certain adverse conditions.

Part of the cells were, after 70 days (664 generation changes), reduced to anaerobic conditions for 80 days, and again for 6 months, to inhibit division and thereby the transmission of imprinting-induced memory to daughter cells. Since no exchange of medium was made during anaerobic culturing, the cells had to use up a considerable part of their membranes for nutrition. They indeed recovered their potentials slowly after return to aerobic conditions in fresh medium. There was no indication of the effect of imprinting after 4 days, but a significant binding increase over the original level took place by day 7. Response to reexposure to insulin or TSH on day 7 was similar to, or even greater, compared to that of the cells maintained in aerobic conditions throughout. It follows that the imprinted cells were not only able to transmit the "memory" of imprinting to daughter cells, but, as demonstrated already earlier [20] also to store it, and to use it again after return to physiological conditions. Thus, further to earlier functional evidence of receptor memory, additional evidence of it emerged from the present binding studies.

The long persistence of imprinting in both the functional and binding activities of *Tetrahymena* supports the hypothesis of a nuclear-level transmission of imprinting-induced "memory" from one generation to the other.

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CELL-TO-CELL TRANSMISSION OF HORMONAL IMPRINTING PERSISTS LONG IN *TETRAHYMENA*

G. Csaba, P. Kovács and Valéria László

Department of Biology, Semmelweis University Medical School, Budapest

(Received September 20, 1988)

Replicating and replication-inhibited (maintained under anaerobic conditions) *Tetrahymena* cells were still able to transmit to "virgin" cells insulin-induced hormonal imprinting after four weeks, i.e. after about 220 to 280 generation changes. The effect of imprinting was not uniformly demonstrable during that period, and the cells maintained under anaerobic conditions even showed a decrease in insulin binding capacity after two weeks. It appears that in the majority of the cases, the intensity of transmitted imprinting greatly depends on that of the genuine imprinting.

The reception of environmental signals, and the recognition and "memory" of their advantageous or disadvantageous nature is a life condition for unicellular organisms [1–3]. Such "memory" can be induced by molecules which are able to act (and/or to bind) at membrane level, as are above all the molecules functioning as hormones in higher organisms [2–4]. Primary interaction of target cells with such molecules gives rise to hormonal imprinting. The dynamic membrane of unicellulars always presents receptor structures, for example nutrient (food) receptors [5], which "question" the environment for information-carrying signal molecules. Imprinting accounts either for the amplification of such membrane structures, or for the formation of new receptors, on which follows the transmission of "memory" to the daughter cells by an as yet not fully understood mechanism. Thus, as a result of hormonal imprinting, scores of the offspring generations will "remember" the primary interaction with a hormone [7], and will, consequently, show a changed — as a rule increased — response to it.

Several theoretical explanations have been offered for the mode of transmission of cellular "memory" to daughter cells. It probably involves certain membrane changes which give rise to continuous receptor formation via self-assembly, and the details of membrane transmitted to the daughter cells preserve this property. It has also been suggested that hormonal imprinting

György Csaba, Péter Kovács, Valéria László
Department of Biology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

could take place intracellularly at RNA or DNA level, and transmission of "memory" could be associated with the information storing function of these molecules.

Recently evidence has been accumulated that in mammalian cell cultures not only those cells show post-receptorial changes after primary interaction with a hormone which had bound it, but also those which had no contant with the hormone itself, only with the interacting cells of the population [8–11]. This indicates a cell-to-cell transmission of the information mediated by the hormone. Transmission of hormonal information from interacting to not interacting (virgin) cells can presumably take place in the course of hormonal imprinting, not only in cultures of mammalian cells [12], but also in those of unicellular organisms [13, 14].

We investigated in the present study how long the imprinted *Tetrahymena* cells were able to transmit the information associated with imprinting, with special regard to the impact of changed life functions, such as respiration and division.

Materials and methods

Culturing conditions and treatment. Tetrahymena pyriformis GL cells, maintained in 0.1% yeast extract and 1% Tryptone (Oxoid, England) containing medium at 28 °C were used in the logarithmic phase of growth, in which they were treated with 10^{-6} M insulin (Semilente, MC, Novo, Copenhagen). After treatment the cultures were either returned to plain medium or maintained under oil overlay (maintenance medium in 10 ml distilled water: 1 cm of small intestine of starved rat + 0.5 ml paraffin oil autoclaved at 121 °C). Both types of mass cultures were transferred to plain medium one, two or four weeks later. The control cultures were transferred on the same schedule as the experimental ones. Two days after transfer the cell counts were determined in a Fuchs-Rosenthal chamber, and transfer was made again in a manner that in addition to insulin-treated and control cultures, mixed cultures, were prepared from equal volumes of experimental and control cell suspensions.

Assay for insulin binding. One day after the last transfer all cultures were fixed in a 4% neutral formalin solution (in pH 7.2, 0.05 M PBS), washed in two changes of PBS, and incubated in presence of FITC-labeled insulin for 1 h at room temperature. The protein concentration of the conjugate was 0.2 mg/ml and the FITC/protein ratio was 0.41. After incubation the cells were washed in three changes of PBS, spread on slides, dried, and examined for intensity of fluorescence with a Zeiss Fluoval cytofluorimeter.

Statistical evaluation. The analogous signals emitted by the cytofluorimeter were transformed to digital signals for recording and mathematical-statistical evaluation with a Hewlett–Packard HP 41 CX calculator, programmed for determination of the mean values and for analysis of inter-group differences for significance (by Student's *t*-test and analysis of variance).

In every case, at least six replica assays were done. Since 20 cells were assayed in each group, the values shown in Fig. 1 represent means for at least 120 cells.

Results

Under the conditions of maintenance described in Materials and methods, *T. pyriformis* cells divide at intervals of about 150 min, giving thus rise to 8–10 generations within one day. Thus about 220–280 new generations arose

during the four-week experimeental period. However, as demonstrated earlier [15], division was reduced to a minimum under the anaerobic conditions provided by the oil overlay. Return to aerobic conditions in plain medium was therefore necessary for a few days, in which the "memory" of hormonal imprinting was fully revived.

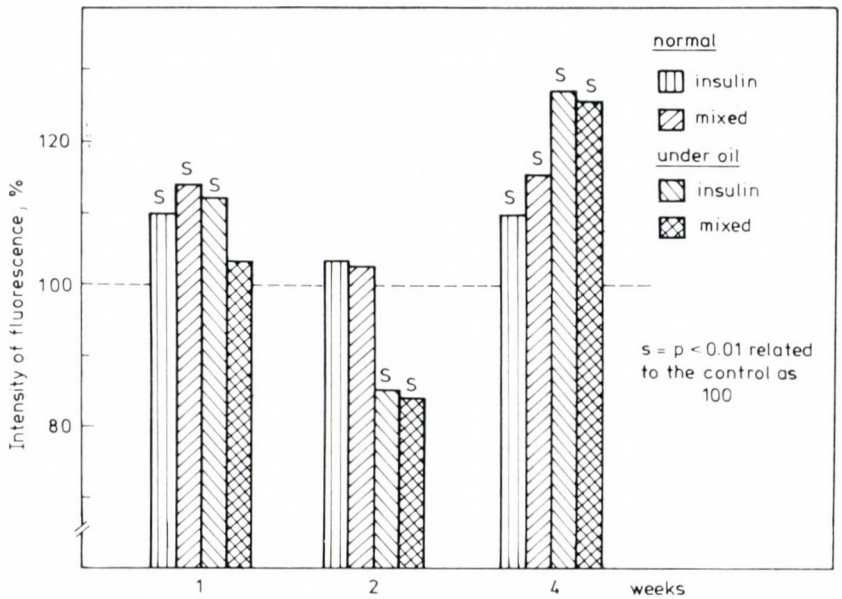

Fig. 1. FITC-insulin binding of insulin-pretreated (imprinted) and not pretreated *Tetrahymena* temporarily inhibited and not inhibited in division after primary hormone exposure

The cells which could divide freely showed after one week the characteristic effect of imprinting, i.e. a significant binding increase over the control, which was also shown by the virgin cells. Two weeks later the binding capacity and the intensity of transmission equally tended to decrease, then attained a peak again by four weeks. The cells maintained under anaerobic conditions exhibited after one week the same insulin binding capacity as those not inhibited in division, but the transmission of imprinting appeared to be disturbed. The disturbance was still more obvious at two weeks. While the insulin binding capacity of the freely dividing cells increased appreciably, although not significantly, over the control in both the pure and mixed cultures, that of the cells temporarily maintained anaerobically showed a significant relative decrease in both culture types. However, by four weeks binding peaks as high as in the freely dividing populations were measured in all cultures previously reduced to anaerobic conditions.

Discussion

The present experimental observations have unequivocally affirmed that the ability to transmit hormonal imprinting persists as long as the impact of imprinting itself. This does not, however, exclude certain fluctuations in the intensity of imprinting, such as observed at two weeks of the four-week period covered in the study. Nevertheless, it appears that the intensity of the effect transmitted to the virgin cells is equivalent to that received by the transmitter cells directly involved in imprinting.

Thus the hypothesis lies close at hand that insulin imprinting and the transmissibility of the information so acquired equally persist long in *Tetrahymena*. This suggests that the still obscure factor carrying the information associated with imprinting is durably present in the unicellular, and can therefore influence the virgin cells not involved in primary interaction with the hormone. This mechanism seems to be of great importance of the unicellular in its natural environment, for it assures the transfer of information not only to daughter cells (cells of the same clone), but also to other cells of the population.

The experimental fact that the information transfer capacity did not differ on a long term between continuously dividing and temporarily not dividing cells indicates that the information transfer factor was well preserved, and as such was not affected either by population increase via division, or by changes in life conditions in an anaerobic environment.

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DURATION OF THE MICROSOMAL ENZYME (CYTOCHROME b_{560ms}) INDUCER ACTION AND IMPRINTING POTENTIAL OF A STEROID IN *TETRAHYMENA*

VALÉRIA LÁSZLÓ, SZ. SZEBERÉNYI and G. CSABA

Department of Biology, Semmelweis University Medical School, and National Institute of Occupational Health, Budapest

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Tetrahymena subjected to a three-day exposure to the steroid triamcinolone exhibited a decreased microsomal enzyme activity, three days after treatment, followed by a slight and a considerable activity increase over the control at one and two weeks after treatment, respectively. Reexposure to triamicinolone after return to plain medium for three days accounted for a marked increase in the inducer action which, however, failed to persist for an appreciable time.

The unicellular *Tetrahymena* proved to be an excellent model for experimental studies into certain functions of the eukaryotic cells of higher organisms [1]. Being a ciliated unicellular, *Tetrahymena* represents a highly differentiated organism at its own phylogenetic level and contains as such many active molecules and/or mechanisms, whose presence is characteristic of, and/or indispensable for, the functioning of certain specialized cell populations of higher organisms. Moreover, being an aquatic organism *Tetrahymena* necessarily maintains a highly dynamic interaction with its environment, which presupposes the operation of a highly developed recognition system [2]. Owing to these properties, *Tetrahymena* can be used with success for investigations into receptor development (evolution). In presence of vertebrate hormones *Tetrahymena* is able to transform certain non-specific membrane patterns to receptor structures, which persist for a long time in many subsequent generations [3–5]. *Tetrahymena* contains itself several hormones or hormone-like molecules, which are characteristic of higher organisms [6–10]. The precise role of these active molecules at the unicellular level is obscure for the time being, unless they represent by-products of protein synthesis or of evolutionary changes. Recently presence of a microsomal enzyme, too, was demonstrated in *Tetra-*

VALÉRIA LÁSZLÓ, GYÖRGY CSABA
Department of Biology, Semmelweis University Medical School,
H-1445 Budapest, P.O.B. 370, Hungary

SZABOLCS SZEBERÉNYI
National Institute of Occupational Health
Nagyvárad tér 2, H-1096, Budapest, Hungary

hymena [11], and two of us [12] succeeded in inducing by steroid treatment production of that enzyme by the unicellular.

The vertebrate hormone exposure outlined above had such a marked effect on *Tetrahymena* that their consequences reappeared in many offspring generations. For example, the effect of primary interaction with a hormone (hormonal imprinting) was still demonstrable after 500 generation changes [13], and the steroid-induced increase in microsomal enzyme (cytochrome b_{560ms}) activity persisted over several subsequent generations of daughter cells [12].

The long duration of the effect(s) of hormonal imprinting on *Tetrahymena* prompted us to investigate the persistence of the impact of steroid treatment on the microsomal enzyme activity of the unicellular, with special regard to the influence of one or two steroid exposures on enzyme induction.

Materials and methods

Model cells and their maintenance. Tetrahymena pyriformis GL cells, cultures in 0.1% yeast extract and 1.0% Tryptone (Oxoid, England) containing medium for 72 h at 28 °C, were used in the logarithmic phase of growth. The steroid used for treatment (triamcinolone acetonide, Richter, Budapest) was dissolved in absolute ethanol and diluted in nutrient medium for use at 10^{-6} M concentration. Appropriate concentrations of ethanol were also added to the control cultures.

The cultures were treated with triamcinolone for 72 h, washed, returned to plain medium for three, seven or 14 days, and were threafter either reexposed to triamcinolone for 4 h, or were centrifuged and homogenized by sonication in 30% glycerol containing Losina-Losinsky solution. Reexposure for 4 h was followed by washing, return to plain medium for 24 or 72 h, and finally by centrifugation and homogenization. Apart from the strains returned to plain medium for 24 h after reexposure, the cultures were always homogenized after culturing for 72 h.

Determination of protein content. All homogenizates were examined for protein content by the method of Lowry et al. [14], and subsequantly adjusted to 25 mg/ml protein content.

Enzyme assay. The *Tetrahymena* cultures were examined for cytochrome b_{560ms} enzyme content by spectrophotometry. As proposed by Fukushima et al. [11], the differential spectrum of the $Na_2S_2O_4$-reduced minus oxidized enzyme content was determined in a Zeiss UV-Vis-Specord spectrophotometer between 425 and 410 nm, using an extinction coefficient of 216 $\mu m^{-1}cm^{-1}$. The results were expressed in terms of specific protein content (nmol cytochrome/mg protein).

Mathematical-statistical evaluation. The significance of inter-group differences was analyzed by Student's *t*-test.

Results and discussion

We demonstrated earlier [12] that 24-h treatment of *Tetrahymena* with steroid (triamcinolone) accounted for a gradual decrease in the microsomal enzyme content with progressing time. In the time interval between one and four days, inhibition was at peak on days 3 and 4. However, treatment with the steroid for 48 h gave rise to a marked increase in microsomal enzyme activity within 24 h, whereas treatment with it for 72 h produced a relatively lesser

activity increase. The first enzyme assays were performed in the present study after return to plain medium for 72 h of the *Tetrahymena* cells preexposed to triamcinolone for 72 h, and portrayed therefore the cytochrome b_{560ms} levels appearing in response to primary exposure. It appears that the inducer effect of the primary three-day steroid exposure had expired by that time to such an extent that microsomal enzyme activity dropped below the control level. Remarkably, however, the microsomal cytochrome concentration tended to approximate the control level one week after triamcinolone treatment, and increased over it considerably by two weeks. Taking into consideration that the *Tetrahymena pyriformis* strain used in the present study had underwent five to six generation changes (divisions) daily, there is reason to conclude that although the inducer effect had initially fluctuated to certain extent — since it had dropped below the control level from an elevated value within three days — it proved to be durable in the long run, because it was still present after 90–100 generation changes (Fig. 1).

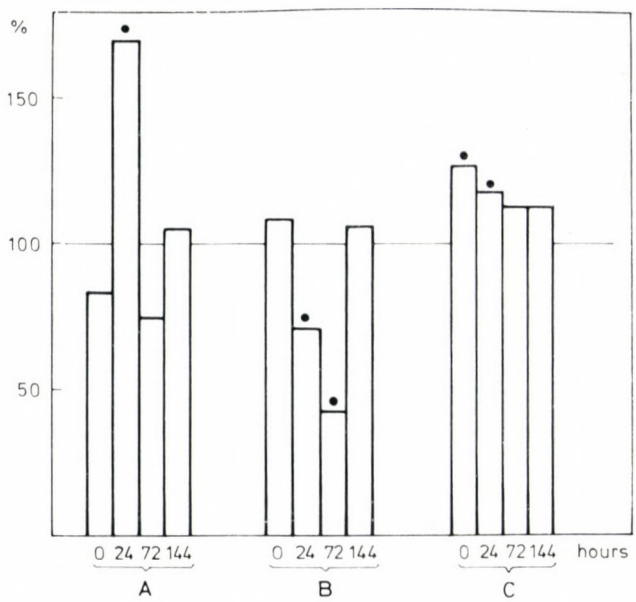

Fig. 1. Microsomal enzyme levels in *Tetrahymena* three days (A), one week (B), and two weeks (C) after treatment with triamcinolone for 72 h (O), and 24,72 and 144 h after reexposure to triamcinolone for 4 h. . = $p < 0.05$

Reexposure to triamcinolone for 4 h of the cells which had been returned to plain medium for 72 h following preexposure to the steroid also for 72 h, accounted for a considerable increase in microsomal enzyme activity. It follows that imprinting with the steroid had in fact taken place at primary exposure. This suggests that, in principle, imprinting for microsomal enzyme pro-

duction could occur in *Tetrahynema* [15, 16] by analogy of perinatal imprinting in mammals [17, 18]. However, this analogy does not hold, because induction of microsomal enzyme synthesis failed to persist at the unicellular level, to judge from a marked activity decrease after 72 h, and a relative decrease in the reexposed cultures compared to the control. It appears that, although the "memory" of lasting triamcinolone treatment had persisted in *Tetrahymena* for the next three days, it did fade away, i.e. failed to reappear on induction in the late offspring generations. It is, nevertheless, remarkable that the activity changes were not unequivocal at three and seven days after 72-h treatment with triamcinolone, and a stabilization characterized by the mentioned activity increase, which had not been influenced by reexposure, occurred only two weeks after primary steroid treatment.

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DOSE-DEPENDENT IMPACT OF PRETREATMENT (IMPRINTING) WITH HISTAMINE AND SEROTONIN ON THE PHAGOCYTIC ACTIVITY OF *TETRAHYMENA*

ZSUZSA DARVAS and G. CSABA

Department of Biology, Semmelweis University Medical School, Budapest

(Received May 3, 1989)

Serotonin and histamine stimulate the phagocytic activity of *Tetrahymena* at primary interaction. The effect of histamine is dose-dependent. While serotonin elicits an imprinting-like phenomenon at a high concentration, histamine induces no imprinting, probably for evolutionary reasons.

The hormones and hormone receptors are products of evolution. The phylogenetically low unicellular organism *Tetrahymena pyriformis*, which we have used as model system in hormone receptor studies [1], possesses non-specific, dynamic membrane patters, which are able to receive the environmental signals and transform to specific receptors under the influence of adequate signal molecules [2, 3].

We demonstrated earlier [4] that certain vertebrate hormones can bind to *Tetrahymena* and exert on it a biological influence. Of these hormones histamine and serotonin, which act on phagocytosis in higher organisms, stimulated the phagocytic activity of *Tetrahymena*, too [5, 6].

In unicellular organisms the primary interaction with a vertebrate hormone (hormonal imprinting) stimulates receptor formation, and also accounts for transmission of the receptors formed to the daughter cells over several hundreds of generations [7]. Imprinting can be induced by a wide range of hormones, including amino acid like hormones, and the basic amino acid components of the latter can also act as imprinters [8]. Earlier experiments, which had demonstrated the specificity of the phagocytosis stimulant action of certain amino acid hormones, including histamine and serotonin, on *Tetrahymena*, prompted us to investigate the sensitivity of the imprinting-induced receptors for these hormones.

ZSUZSA DARVAS, GYÖRGY CSABA
Department of Biology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

Materials and methods

Culturing and treatment. Tetrahymena pyriformis GL cells, cultured for two days in 0.1% yeast extract and 1% Tryptone (Oxoid, England) containing medium under shaking at 28 °C, were used in the early stationary phase. Before testing for phagocytic activity, the cells were starved for 4 h in Losina-Losinsky solution. The hormone treatments were performed with histamine dihydrochloride (Reanal, Budapest), or serotonin-creatinine sulphate (Reanal, Budapest).

Grain counting and statistical evaluation. Phagocytic activity was assessed from the numbers of phagocyted grains of Chinese ink, suspended in Losina–Losinsky solution for feeding to the unicellulars.

The Chinese ink containing vacuoles were counted in 50 cells in each group, and the mean value was regarded as the phagocytic index (PI). The PI values of the experimental groups were related to the PI value of the control as 100. The percentual values gave the PC (phagocytic coefficient). Five replica experiments were set up in each series, and the differences between the mean values were analyzed for significance with Student's *t*-test.

Experimental groups. Group C/C: the cells of this group did not receive hormone, only Chinese ink in Losina–Losinsky solution for 5 min, after starvation for 4 h. Groups C/H_5, C/H_9, C/S_5 and C/S_9: these cells were not pretreated and were exposed to 10^{-5} M histamine, 10^{-9} M histamine, 10^{-5} M serotonin and 10^{-9} M serotonin, respectively, simultaneously with the feeding of Chinese ink after starvation for 4 h. Groups H_5/H_5 or H_9, S_5/S_5 or S_9: the cells were, after pretreatment with 10^{-5} M hormone for 24 h, returned to plain medium for 24 h, starved for 4 h, and reexposed to 10^{-5} or 10^{-9} M hormone simultaneously with the feeding of Chinese ink for 5 min. Groups H_9H_5 or H_9, S_9/S_5 or $S/_9$: the cells were treated on the same schedule as above, except for pretreatment with 10^{-9} M histamine or serotonin.

Results and discussion

In higher organisms, histamine acts as a phagocytosis stimulating hormone, inasmuch as it enhances the phagocytic activity of the macrophages and of other potential phagocytes of the reticuloendothelial system [9–11]. Serotonin, too, is known to stimulate phagocytosis [12]. We demonstrated earlier that both hormones developed a phagocytosis stimulant action in *Tetrahymena*, too [5, 6]. The dissimilar impacts of histamine analogues and antagonists, serotonin analogues and precursors on the unicellular indicated selectivity of its response. The present experiments substantiated the phagocytosis stimulant action of histamine and serotonin on *Tetrahymena* at both tested concentrations.

The action of histamine was dose-dependent at primary exposure, whereas that of serotonin was not. Histamine was considerably more active than serotonin at 10^{-5} M concentration, but this activity difference vanished at 10^{-9} M.

On reexposure, cellular response was greater to serotonin at 10^{-9} M, and to histamine at 10^{-5} M, but in neither case did it increase over the level of primary response. It follows that — as assessed in terms of phagocytosis — no imprinting had taken place in either case.

Response to histamine did not appreciably differ from the control at either dose level of reexposure. Response to serotonin reexposure was low relative to the control in both the S_9/S_5 and S_5/S_9 system of treatment.

Since histamine and serotonin equally belong to the class of the most primitive hormones, there is reason to postulate that they could occur in the natural environment of *Tetrahymena*. The phylogenetically low organisms possess food receptors for amino acids [13, 14], which can also act as receptosr

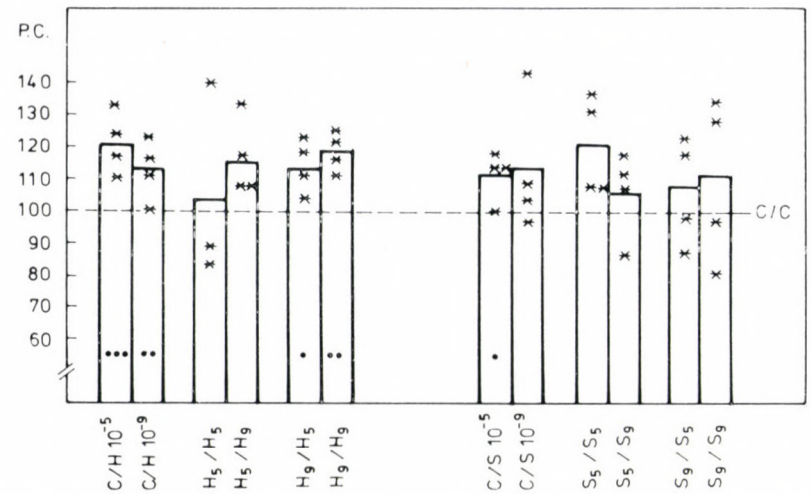

Fig. 1. Influence of single and repeated treatments with low or high concentrations of histamine and serotonin on the phagocytic activity of *Tetrahymena*. P. C. = phagocytic coefficient (per cent activity related to the control as 100). Significance: · $p < 0.05$; ·· $p < 0.01$; ··· $p < 0.001$

for amino acid hormones [8]. This can probably explain the intensive and selective response of *Tetrahymena* to such hormones already at primary interaction, and the lack of an increased response (imprinting) to reexposure as well, since imprinting (with basic amino acids or hormones) may have had occurred earlier in life and given rise to more developed receptors than those available in the membrane of the unicellular for other — e.g. polypeptide — hormones. The receptors for the two amino acid hormones tested behaved similarly under the given conditions, except in the cross-imprinting experiments.

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BACTERIAL TRANSLOCATION AFTER COLD STRESS IN YOUNG AND OLD MICE

PIROSKA ANDERLIK, ILONA SZERI, ZSUZSANNA BÁNOS and ZSUZSANNA BARNA

Institute of Microbiology, Semmelweis University Medical School, Budapest

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Spontaneous translocation of the normal intestinal flora was observed in higher rate in old mice being in a state of thymus involution than in young ones. The proportion of bacterial translocation 24 and 48 h after cold stress increased in both young and old mice, the increase of translocation as compared to controls was larger in case of young mice than in old ones. The distribution of isolated bacterial strains according to Gram stain also differed in young and old groups.

Under conventional circumstances, bacteria of the normal intestinal flora may in a small degree permeate the intestinal wall and can be translocated into organs generally regarded as sterile [1].

In certain pathological conditions, e.g. enhanced permeability of the intestinal mucosa or in excess of some bacterial species in the gut, members of the intestinal flora may permeate the intestinal wall in a larger proportion and they are translocated into mesenteric lymph nodes, spleen or liver. An increase of the degree of translocation was observed when the T-dependent immune system was lacking, for example in nude mice and in mice thymectomized in newborn age [2]. Our previous experiments have also shown the increase of bacterial translocation in mice treated with a lymphotropic cytostatic agent. The maximum of the translocation coincided with the most serious spleen atrophy caused by the agent [3]. In humans, serious traumas, burns and states of shock enhance the transfer of bacteria of the normal intestinal flora through the intestinal wall and their appearance in generally sterile organs [4].

Since Selye's description, it is known that the organism reacts to stress effects with general adaptation processes. Part of this reaction is lymphopenia in the peripheral blood some hours after stress and a transient loss of weight of the lymphatic organs detectable in 12–18 h [5, 6].

In old mice with physiological thymus involution, the number of circulating lymphocytes is low, after stress they have a decreased lymphopenic reaction and display increased sensitivity to cold [7]. In old mice, besides impaired general adaptation, the specific adaptation (specific immune response ability) is also decreased [8].

PIROSKA ANDERLIK, ILONA SZERI, ZSUZSANNA BÁNOS, ZSUZSANNA BARNA
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

In our present experiment, it has been examined whether there is increased bacterial translocation in conventional old mice in state of thymus involution, and also if cold stress influences the rate of bacterial translocation in conventional young and old mice.

Materials and methods

Experimental animals. Eight-week-old (young) and twenty-four-month-old (old) CFLP (LATI) conventional mice of both sexes were used.

Cold stress. Mice were placed in wire net cages providing individual placement into a +4 °C refrigerator for 4 h.

Examination of translocation. (a) *Cell suspension.* Two mesenteric lymph nodes, the spleen and the liver were aseptically removed from each sacrificed mouse. Cell suspensions were prepared from the lymph nodes and the spleen as well as from a 100 mg fragment of the liver; each sample was diluted in 0.5 ml PBS. (b) *Isolation of bacteria.* The entire amount of each cell suspension was poured into serum broth medium, from which subcultures were made on blood agar medium at 24 h intervals. Cultures were incubated aerobically. Identification of bacteria was carried out on the basis of macroscopical, microscopical and biochemical examination of colonies in the subcultures. The result was considered negative if no bacterial growth had appeared on any of the five subcultures by the 48th h of incubation.

Lymphocyte (ly) count was determined in caudal vein samples under standardized conditions.

Determination of relative thymus weight and translocation index. After sacrificing, the relative thymus weights of young and old mice were determined and their mean value was calculated.

$$\text{Mean relative thymus weight} = \frac{\text{mean thymus weight (mg)}}{\text{mean body weight (g)}}$$

The percentage of translocation was calculated from the number of mice or organs with positive bacterial cultures as related to the number of mice or organs with negative bacterial cultures.

$$\text{Translocation index} = \frac{\text{per cent of translocation in stressed group}}{\text{per cent of translocation in control group}}$$

Experiments and results

Seventy young and 43 old mice were exposed to cold stress. During the stress, 5 old mice died due to their increased cold sensitivity. There were no deaths among young mice.

Six hours after the stress, the ly count in the peripheral blood of 15 young and 10 old stressed and of 10 young and 10 old unstressed control mice was determined. The ly count was lower in unstressed old mice than in young ones. There was a 50% decrease in the ly count in stressed young mice as compared to the unstressed ones. The ly count in stressed old mice did not change as compared to the unstressed ones. Thus, in accordance with the known data [5–7], as opposed to young mice, there was no lymphopenic reaction in old mice.

The 45 mice used for determining the ly count were observed for one week and there were no deaths during this time. After sacrifice, the relative

thymus weights were determined, its mean value was 3.6 in case of young mice and 0.4 in case of old mice both in stressed and unstressed groups. Thus the examined old mice were in a state of physiological thymus involution.

Isolation of bacteria was carried out on the other 55 young and 28 old mice exposed to stress. After 24 h, we examined the organs of 26 young and 12 old mice, after 48 h the organs of 29 young and 16 old mice. In both experiments, 21 and 21 young and 18 and 17 old mice not exposed to stress were used as controls. In these control groups, the same results were reached in both experiments, thus they were added together. The results of isolation experiments are shown in Table I and Fig. 1.

Table I

Bacterial translocation in mice and in their organs after stress

Mouse groups			Translocation in mice		Translocation in organs		Translocation index	
			frequency*	%	frequency**	%		
Young	control		3/42	7	9/126	7	1.0	1.0
	stressed	24 h	8/26	33	12/78	15	4.7	2.1
		48 h	12/29	41	15/87	17	5.9	2.4
Old	control		10/35	28	14/105	13	1.0	1.0
	stressed	24 h	6/12	50	8/36	22	1.8	1.6
		48 h	11/16	68	12/48	25	2.4	1.9

* Number of positive mice/number of examined mice
** Number of positive organs/number of examined organs

Table I shows the frequency of bacterial translocation as referred to the number of examined mice and their organs. In case of the control young and old mice, the rate of bacterial translocations were different, there were positive results in 7% of young mice, in 28% of old mice, and, in 7% of the organs of young mice and in 13% of the organs of old ones. Twenty-four and 48 h after stress, the proportion of translocation was gradually increasing as can be seen from the translocation indices 48 h after stress. The frequency of translocation was more prominent in old mice (mice, 68%; organs, 25%) than in young animals (mice, 41%; organs, 17%). The increase of translocation, however, as compared to the controls, was more expressed in young mice than in old ones (translocation indices in mice, 5.9 and 2.4; in organs, 2.4 and 1.9).

The rate of positive cultures from the individual organs as referred to the number of organs examined is shown in Fig. 1. In the young stressed mouse group the rate of positive cultures 24 and 48 h after stress was larger in case of lymph nodes and the liver than those among the controls. The rate of po-

sitive spleen cultures after 24 h was significantly smaller than in controls, and after 48 h, however, the proportion of bacterial translocation increased in the spleens and surpassed that of the controls.

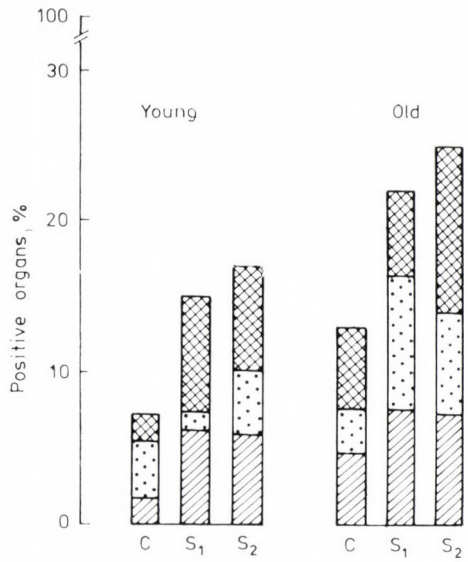

Fig. 1. Proportion of positive cultures in different organs as referred to the number of examined organs. C, control, not exposed to stress; S_1, stressed, 24 h after stress; S_2, stressed, 48 h after stress. Shaded columns, lymph node; spotted columns, spleen; hatched columns, liver

In the old stressed mouse group, the proportion of positive cultures was larger than in the controls in both periods and in all three organs. The high rate of translocation observed in the liver 48 h after stress was striking.

Comparing the effect of cold stress on translocation, in old and young mice, the most outstanding difference in the degree and the dynamism of translocation was observed in case of spleens. The rate of translocation 24 h after the cold stress significantly decreased in case of young mice, while in case of the old mice, it increased significantly. The degree of translocation 48 h after stress increased in the spleen of young mice, while it decreased in the spleen of old ones, but it was still larger than that of the controls.

Distribution of isolated bacteria according to Gram stain can be seen in Table II. From some samples, more than one bacterium was isolated, i.e. the number of isolated bacteria is larger than that of the organs. There were no significant differences in isolation results received 24 and 48 h after stress, thus these results are added together in Table II.

Table II

Number of isolated bacteria in different mouse groups and their distribution in organs according to Gram stain

Mouse groups		Isolated bacteria								
			Gram-positive*				Gram-negative**			
		Number	lymph node	spleen	liver	total	lymph node	spleen	liver	total
Young	control	9	2	5	2	9	—	—	—	—
	stressed	27	7	4	11	22	1	1	3	5
Old	control	16	—	1	4	5	4	3	4	11
	stressed	23	5	3	5	13	5	3	2	10

* *Staphylococcus, Streptococcus*
** *Escherichia, Proteus, Klebsiella*

In young control mice, only Gram-positive cocci were found, while in old controls there were more Gram-negative bacteria (11 isolates out of 16). After stress, the number of Gram-positive bacteria increased in young mice and Gram-negatives appeared in all three organs of them. In old mice the number of Gram-positive isolates increased.

Discussion

Spontaneous bacterial translocation was observed in 7% of young conventional mice and in 7% of their organs. This corresponds to other data [1]. Translocation is more rare in experimental animals kept in SPF circumstances, though even then it is 2–5%. These data show that under certain conditions the equilibrium between commensal and potentially pathogenic bacteria of the normal intestinal flora may be altered and the specific and aspecific reactions hindering microbial admission into the organs can be shifted in favour of bacteria. The reason for this can be that even in healthy animals, the intestinal wall is not completely intact [1]. In the wall of the small intestine of conventional mice, the observed slight inflammatory infiltration can be a sign of the penetration of bacteria into the intestinal wall [9].

All impacts that decrease the specific and aspecific defence mechanism of the organism can increase the degree of bacterial translocation [2, 9]. In our experiment, spontaneous bacterial translocation was observed in higher rate in old mice than in young ones. The shift of the equilibrium in favour of bacteria is due to the decreased immune responsiveness of old mice being in a state of physiological thymus involution.

In our experiment, 24 and 48 h after stress, the proportion of bacterial translocation increased in both young and old mice as compared to controls.

These changes can be in connection with the higher cortison level after stress. Cortison decreases inflammation and is of immunosuppressive effect; while there is a higher cortison level, the defensive function of the organism are decreased that can enhance the translocation of bacteria. In case of old mice, the smaller increase of translocation after stress can be in connection with the decreased function of the adrenal gland characteristic of old age. In our experiment, the decreased function of the adrenal gland was indicated by the failure of lymphopenic reaction and the increased sensitivity to cold in stressed old mice.

Neither the differences in distribution of translocated bacterial strains according to Gram stain, nor the differences in the proportion of spleens found positive after stress in young and old mice can be explained on the basis of our experiments or literary data. Functional differences of the immune- and endocrinological systems of young and old mice, the contents of normal intestinal flora, permeability of the intestinal wall are all circumstances that can actually influence the emergence of bacteria out of the intestines and the tempo and degree of their elimination.

Our results are in accordance with clinical experiences showing that clinical manifestation of endogenic infections are more frequent in organisms exposed to stress than in organisms free of stress and this is more valid in old age.

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EFFECT OF MANNOZYM TREATMENT ON PLASMA FIBRONECTIN CONCENTRATION IN GERMFREE AND CONVENTIONAL MICE

PIROSKA ANDERLIK, ILONA SZERI, ZSUZSANNA BÁNOS, ZSUZSANNA BARNA, L. KALABAY and L. JAKAB

Institute of Microbiology, and Third Department of Medicine, Semmelweis University Medical School, Budapest

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As a result of a single intraperitoneal treatment with 1 mg/ml of Mannozym (M) the plasma fibronectin (FN) level was significantly increased both in germfree (Gf) and conventional (Cv) mice, and though it started from a lower value in Gf mice, it rose to a similar level to that of Cv animals. The maximum of FN level was observed on the first day after treatment both in Gf and Cv mice. It had returned to normal by the 7th day in Cv mice, but it was higher in Gf mice as compared to untreated controls even on the 14th day. Thus the increase of FN level was of higher degree and longer duration following M treatment in Gf mice than in Cv animals. In treated Gf mice the plasma FN concentration was in the same range as in untreated Cv mice even at the termination of experiment. Both in Gf and Cv mice, there was a relative spleen weight increase, the degree of which was similar, but the duration was longer in Gf mice than in Cv ones.

Mannozym (M) is a zymosan containing cell wall derivative of *Saccharomyces cerevisiae*. In human therapy this material is used for enhancement of nonspecific immunity and to prevent unwanted side-effects of X-ray treatment [1–3]. It has been demonstrated that in animal experiments it enhances both the humoral and cellular immune responses [4]. Its mode of action is not exactly known yet, but it has been demonstrated that zymosan, influencing through different mechanisms the function of the mononuclear phagocytic system (MPS), can increase the phagocytosis [5–12].

One of the most important biological effects of plasma fibronectin (FN) is its opsonic acitivity. Further, it also mediates the motility and soluble mediator production of phagocytic cells. The effective operation of the MPS depends on the concentration of plasma FN. The phagocytic activity of MPS is impaired when plasma FN concentration is reduced and is improved if its level increases [13–15].

PIROSKA ANDERLIK, ILONA SZERI, ZSUZSANNA BÁNOS, ZSUZSANNA BARNA
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

LÁSZLÓ KALABAY, LAJOS JAKAB
Third Department of Medicine,
Semmelweis University Medical School
Eötvös út 12 ,H-1121 Budapest, Hungary

The lymphoid system of germfree (Gf) mice is underdeveloped and their phagocytic activity is decreased as compared to that of conventional (Cv) mice [16, 17]. In our earlier experiments it was proved that the plasma FN level of Gf mice is significantly lower than that of Cv animals [18]. In the present experiment it was examined whether M treatment had an influence on the plasma FN concentration of Gf and Cv mice.

Materials and methods

Experimental animals. Gf and Cv 3-month-old C3H mice of both sexes, about 25 g each were used (LATI, Gödöllő). Germfree state and its control were provided according to references [19].

Mannozym. (Institute for Serobacteriological Production and Research, Human, Budapest). The preparation contains 1 mg/ml water-insoluble glucomannan polysaccharide purified from *S. cerevisiae.* It is suspended in isotonic saline without any preservative [1].

Determination of mouse plasma FN level. Blood was taken from the caudal vein using sodium citrate as anticoagulant. FN concentration was determined from individual plasma by electroimmunodiffusion [18].

Examinations of the lymphoid system. Lymphocyte count was determined from blood taken from the caudal vein under standardized conditions. For determining relative lymphoid organ weight, body weight and after sacrifice spleen and thymus weights were determined and relative organ weight was calculated.

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

Statistical evaluation. Student's t test was applied. The accepted significance level was $p = 0.05$.

Experiments and results

Thirty Gf and 30 Cv mice were treated intraperitoneally on one occasion with 1 ml of Mannozym (groups M). This dose is equal to 40 mg/kg zymosan in case of 25 g body weight. Thirty Gf and 30 Cv mice not treated with M served as controls.

Six treated and 6 control mice were bled to death on days 1, 4, 7, 10 and 14 following M treatment, and plasma FN level was determined and the lymphoid system examined.

Plasma FN concentrations are shown in Fig. 1. FN level values of untreated Gf mice were significantly lower than that of untreated Cv mice. Already on the 1st day, the FN level following M treatment was significantly higher both in Gf and Cv mice, and though it started from a lower value in Gf mice, it rose to the same level as that of Cv mice. FN level in Cv mice returned to normal on the 7th day, but it was higher in Gf mice as compared to the values of untreated ones even on the 14th day. Plasma FN concentration of treated Gf mice was in the range of untreated Cv mice after the 4th day of the experiment.

Relative spleen weights of mice are shown in Fig. 2. In untreated Gf mice these were smaller than in untreated Cv mice. On the first day after M

treatment, the mean relative spleen weight of both Gf and Cv mice was significantly higher as compared to the untreated ones. Though it started from a lower value in case of Gf mice, it rose to the same level as that of Cv mice.

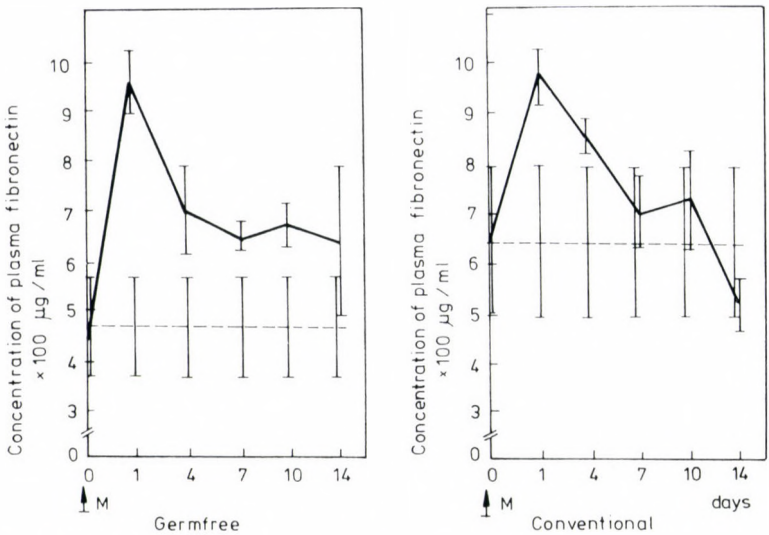

Fig. 1. Effect of Mannozym on the plasma FN level in Gf and Cv mice. ———— groups M; - - - - - controls

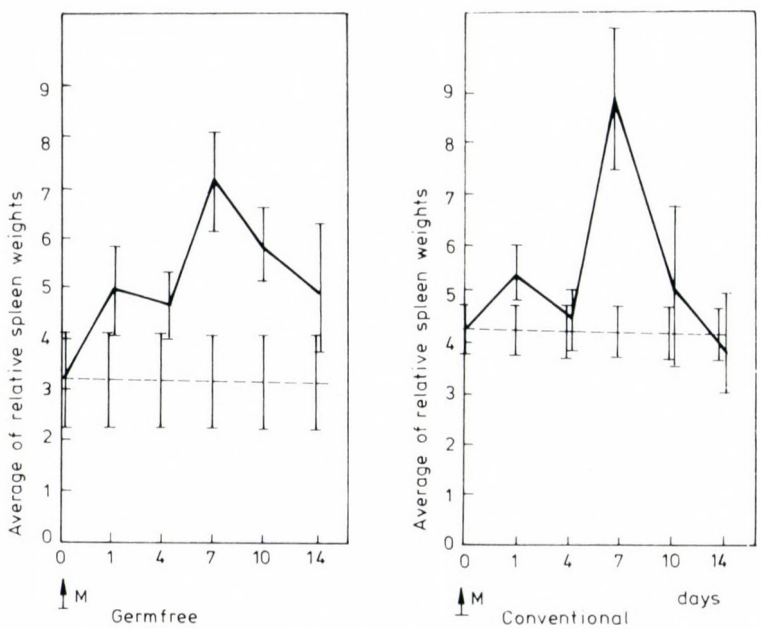

Fig. 2. Relative spleen weight of Gf and Cv mice treated with Mannozym. ———— groups M; - - - - - controls

Following a slight decrease observed on the 4th day, the maximum increase was observed both in Cv and Gf mice on the 7th day. On this day, the degree of increase as compared to controls was similar both in Gf and Cv mice. The increased relative spleen weight indicating spleen hypertrophy in Gf mice existed even on the 14th day, while this value returned to normal on the 10th day in Cv mice. Relative spleen weight of treated Gf mice was in the range of untreated Cv mice at the termination of the experiment.

Peripheral blood lymphocyte counts and relative thymus weigths of untreated Gf mice were lower than those of untreated Cv mice. In M treated Gf and Cv mice these values did not differ significantly from values of untreated controls in any of the examined days.

Discussion

We have found significantly lower plasma FN levels in 3-month-old Gf mice compared to the values of Cv mice of same age, similarly to our earlier results in case of 6-week-old and 12-month-old mice [18]. According to our earlier results, treatment with an attenuated NDV vaccine of immunomodulatory effect increased the plasma FN level of Gf mice [18]. In our present experiment, Mannozym increased the plasma FN level both in Gf and Cv mice. The maximum of increase both in Gf and Cv mice was observed on the first day after M treatment, but the increase was of a higher degree in Gf mice and did not return to normal even on the 14th day, while the values of Cv mice became normal by the 7th day. Thus in Gf mice the effect of M treatment was manifest in a FN level increase of larger degree and longer period than in Cv mice. The plasma FN concentration of Gf mice was in the range of untreated Cv mice even on the 14th day after treatment. These results support the assumptions that the low level of plasma FN may be due to lack of normal microbial flora.

In accordance with the known data [16, 17] relative lymphoid organ weights and blood lymphocyte counts of untreated Gf mice were lower than those of untreated Cv mice. Spleen hypertrophy was caused by M treatment both in Gf and Cv mice. This result is in accordance with our earlier results attained with treatments with known microbial immunomodulants as *Bordetella pertussis* vaccine and radiodetoxified *Escherichia coli* lipopolysaccharide endotoxin [20, 21].

Changes in relative spleen weight and in plasma FN level following M treatment showed a similar tendency both in Gf and Cv mice. On the first day following M treatment, mean relative spleen weight was increased in both Gf and Cv mice but the FN concentration reached the maximum on the first day, while the spleen hypertrophy was the highest on the 7th day after treatment.

Similarly to changes in plasma FN levels, relative spleen weight returned to normal only in case of Cv mice until the 14th day, while in Gf mice, it was higher even at this date as compared to untreated controls, and it was in the range of untreated Cv mice. The degree of increase of relative spleen weights after M treatment was about the same in case of Gf and Cv mice, but its duration was longer in Gf mice.

In M's effect of stimulating non-specific immunity, its effect of enhancing the function of MPS may play an important role. Our results are in accordance with this possibility. Mannozym, besides its other known effects [5–12, 22], may enhance the function of the MPS by increasing the plasma FN level.

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SEPARATION AND SOME PROPERTIES OF AN ENDO-1,4-ß-D-XYLANASE FROM *ASPERGILLUS FLAVIPES*

A. A. Sherief

Botany Department, Faculty of Science, Mansoura University, Mansoura, Egypt

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A fungal strain isolated from stored wheat straw and identified as *Aspergillus flavipes* was found to produce an extracellular endo-1,4.β-D-xylanase when grown in shake-culture of a mineral salt medium containing 0.1% wheat strawxylan as a sole source of carbon for 3 days at 28 °C. The enzyme was separated by precipitation with ammonium sulphate and desalted by Sephadex G-25 column. Fractionation and purification of the enzyme were carried out by chromatography on Bio-Gel P-100. The purified enzyme (mol wt 45 000) was found to release xylooligomers only from β-1,4-xylan. It showed maximum activity at pH 5,0 and temperature 55 °C.

The enzymatic hydrolysis of lignocellulosics, in particular, has been receiving much attention. This is due to the importance of soluble sugars in the secondary fermentation processes of food, fuel and chemical production as pointed out by Ryu and Mandelt [1] and Eriksson and Wood [2]

Lately, considerable research efforts have been focussed on ways of producing these enzymes at reasonable costs. Notable in this regard is the isolation of fungi which overproduce cellulases and hemicellulases, e.g. Moloney et al. [3], Wood et al. [4], Hoffman and Wood [5] and Brown et al. [6]. Various fungal xylanases are secreted extracellularly and a number of fungi have been used for xylanase production, e.g., *Aspergillus niger* by Conrad [7], *Aspergillus fumigatus* by Stewart et al. [8], *Trichoderma reesi* by Dekker [9] and *Aspergillus awamori* by Linko et al. [10], Stewart et al. [8], Linko et al. [11] and Poutanen et al. [12].

Many such fungal xylanases have been secreted and purified to obtain a highly effective enzyme for the digestion of wheat-straw xylan. In the present work, a strain of *Aspergillus flavipes*, isolated from Egyptian wheat straw was found to produce extracellular xylanase, the purification and properties of which are hereby described.

Abdel Dayem Aboulfetouh Sherief
Botany Department, Faculty of Science, Mansoura University
Mansoura, Egypt

Materials and methods

Isolation of fungus. Modified medium of Baker et al. [13] was used for isolation of the xylanase-producing fungus. It is composed of NH_4NO_3, 1.0 g; KH_2PO_4, 0.4 g; $MgSO_4$. $7H_2O$, 0.2 g; KCl, 0.2 g; yeast extract, 1 g; trace salt solution, 1 ml; agar, 20 g per litre of distilled water. The medium was supplemented with 2% larchwood as a sole source of carbon. A few drops of aqueous extract of rotten wheat straw were placed on Petri plates of the medium and incubated at 27 °C for 5 days. Each of the resulting colonies, outlined by a halo-clear zone, was transferred to a separate plate. The fungus was identified as *Aspergillus flavipes*, using the *Aspergillus* key of Raper and Fennell [14].

Substrates and chemicals. Ball milled wheat and barley straws (treated and untreated) besides corn cob were obtained locally and were used together with larchwood xylan of Sigma Chemicals as carbon sources for fungus growth. p-Nitrophenyl-β-D-sylopyranoside and p--nitrophenyl-β-D-glucopyranoside were obtained from Sigma. Carboxymethyl-cellulose was obtained from Fluka Ch. and Biogel P-100 from Bio-Rad Ltd.

Enzyme preparation. Baker et al. [13] medium for xylanase production consisted of wheat straw-xylan, 1 g; NH_4NO_3, 1 g; KH_2PO_4, 650 mg; $MgSO_4$, 181 mg; KCl, 149 mg; yeast extract, 1 g; trace salt solution, 1 ml per litre of distilled water. Crude xylanase was produced by growing *A. flavipes* in shake culture (80 r.p.m.) for 3 days at 28 °C.

Enzyme assays. Reaction mixtures (0.8 ml of 0.5% xylan or 1% CMC dissolved in 0.1 M acetate buffer, pH 5.0 and 0.2 ml of enzyme solution) were incubated for 30 min at 40 °C. The reducing sugars formed were determined by the Nelson—Somogyi method [15, 16] using D-xylose or D-glucose as standard.

One unit of enzyme activity (U) was defined as that amount of enzyme which produces 1 μ mole of the product in one min, under the assay conditions. β-Xylosidase and β-glucosidase activities were determined by incubation of 0.2 ml of the enzyme solution with 0.8 ml 0.0135 M of the corresponding p-nitrophenyl sugar derivatives in acetate buffer, pH 4.5 at 40 °C for 1 h. The reaction was stopped by adding 10% sodium carbonate solution. The activities were determined by measuring the released p-nitrophenol spectrophotometrically at 410 nm.

Separation of β-1,4-D-xylanase. The enzymes present in the culturefiltrate were precipitated by saturation with ammonium sulphate up to 95%. The precipitated protein was redissolved in acetate buffer, pH 5.0. The enzyme preparation was desalted on Sephadex G-25 column, using 0.05 M acetate buffer for elution. The eluant was concentrated by using an ultrafiltration cell (Model 52 Amicon Corp. Ltd.) at 4 °C, then dialyzed against distilled water for 24 h, then against acetate buffer, pH 5.0 for 24 h, with several changes of the buffer solution.

For fractionation, separation and purification of the concentrated dialyzed enzyime solution, Biogel P-100 (85 × 2 cm) equilibrated with acetate buffer, pH 5.0 was used. Elution was carried out at 6 °C at flow rate (12 ml/h) and fractions of 2 ml were collected. The action fractions of each enzyme were combined and lyophilized.

Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard. The transmittance was measured at 280 nm.

Optimization of pH and temperature. Optimization of pH was performed in 0.05 M acetate buffer adjusted to initial pH values 3.5—7.5. The reaction mixtures, containing larchwood xylan (0.5% w/v) and an appropriate volume of purified xylanase, were incubated at 40 °C for 30 min.

Temperature optimization study was similarly undertaken but in acetate buffer, pH 5.0 and assaying for reducing sugars, released after incubation for 30 min, was made at various temperatures.

Molecular weight determination. Slab gel electrophoresis (12.5% polyacrylamide in the presence of SDS) was carried out by the method of Weber and Osborn [18]. The gels were stained for protein with Comassie Blue; the protein standards for molecular weight determination were urease (Jack Bean) 272 000, albumin bovine (dimer 132 000 and monomer 66 000), egg albumin=45 000, carbonic anhydrase 29 000 and α-lactalbumin 14 200.

The molecular weight of the enzyme was also determined on the same column used for desalting and under the same conditions of separation by the Biogel. The calibration was made against the following protein standards: apoferritin (433 000), α-amylase (200 000), alcohol dehydrogenase (150 000), bovine serum albumin (57 000), carbonic anhydrase (29 000) and cytochrome C (12 400) from Sigma Chemicals. The elution volume of the standards was plotted against the $\log_{10}$ of the molecular weight to give a straight line plot from which the molecular weight of the active fractions was read off.

Results and discussion

The growth of *A. flavipes* on various natural substrates and larchwood xylan was screened. Three lignocellulosic substrates, viz., wheat straw, barley straw and corn cob were tested. Since the growth of the fungus on them was good, they were used for further work to assess the effect of the various carbon sources on xylanase production. The results (Table I) indicate that *A. flavipes* produces the highest xylanase activity in the medium containing wheat straw (untreated) as substrate. The delignified substrates, i.e., the treated wheat and barley straw supported lower production of xylanase activity, whereas the lowest activity was obtained using corn cob as substrate for growth of *A. flavipes*. Similar observations were made by Stewart et al. [8] and Ghosh and Deb [19], and indications were given that bleaching of lignocellulosics affected the hemicelluloses in the substrates.

Table I

Production of xylanase by A. flavipes on various substrates

Substrate	Xylanase activity (U/ml)
Wheat straw (untreated)	18.2
Barley straw (untreated)	12.5
Xylan (larchwood)	11.6
Wheat straw (treated)	8.8
Barley straw (treated)	8.0
Corn cob (untreated)	7.9

The fractionation of the desalted enzyme solution produced by *A. flavipes* growing on wheat straw-xylan provided a typical elution profile (Fig. 1). Three enzymes emerged from the Biogel-100 column besides xylanase. They are β-xylosidase, β-glucosidase and endoglucanase. Xylanase was pooled from the last fraction after separation of the other heavier associated enzymes.

Three components, 60 ml each, clearly recognized in Fig. 1 were designated I, II and III with fraction numbers 45–75, 70–100 and 95–125, respectively. They include three peaks of protein activity at the respective fraction numbers 55, 85 and 115.

The first component covers the activities of both β-xylosidase and β-glucosidase which starts its activity slightly later (at fraction No. 48), ends also before the former (at fraction No. 68) and reaches its peak at fraction No. 60 with a corresponding activity value of 0.75 U/ml. This is considerably lower than the 2.75 U/ml peak achieved by β-xylosidase at fraction No. 55.

The second component is mainly covered by the activity of endoglucanase with overlapping activities, in its earlier fractions (from fraction No. 70

to 75) by the concluding activity of β-xylosidase and in the latter fractions by the early activity of xylanase. This latter interference was avoided in order to obtain as pure xylanase as possible. The endoglucanase shows an activity peak of 0.2 U/ml at a corresponding fraction No. of 85.

The third component is mainly covered by xylanase with an activity peak of 10.5 U/ml at fraction No. 110.

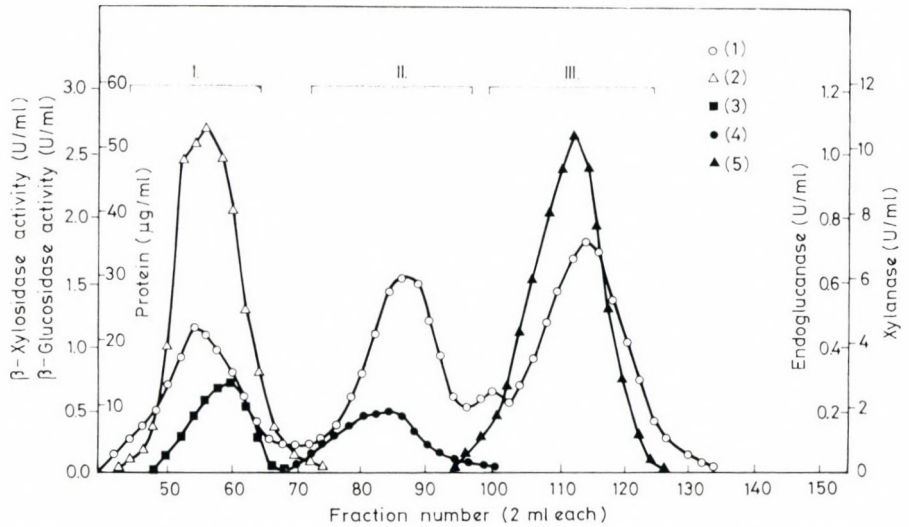

Fig. 1. Elution profile of desalted enzyme solution by *A. flavipes* showing the separation of protein peaks and enzyme activities recovered from the biogel P-100 column. o — — — — o protein; △ — — — — △ β-xylosidase; ■ — — — — ■ β-glucosidase; ● — — — — ● endoglucanase; ▲ — — — — ▲ xylanase

The fractionation data mentioned above are quite consistent with earlier studies, e.g., John et al. [20] and Stewart et al. [8]. The enzyme activities measured with xylan, p-nitrophenol derivatives and glycans are distinct, on the basis of both their physical separation and the fact that *Aspergillus* strains produce a copious amount of both xylanase and p-nitrophenyl enzymes (β-xylosidase and β-glucosidase) but only small or negligible amounts of glycanases.

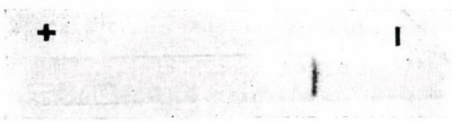

Fig. 2. Electrophoresis of the xylanase as treated with mercaptoethanol on SDS polyacrylamide gel, pH 8.6. Electrophoresis was carried out for 2.5 h at 8 mA per tube with 40 μg of enzyme protein

The analysis of xylanase after gel filtration using SDS-PAGE showed that it was homogeneous and gave a single band (Fig. 2) with a molecular weight of 45 000.

The end products of xylan hydrolysis by the purified xylanase were solely xylooligomers (X_2, X_3, X_4 and X_5). Such a hydrolysis pattern is typical of an endoxylanase. This property is well documented among other organisms by Nakajima et al. [21], Nakanishi et al. [22] and Lee et al. [23].

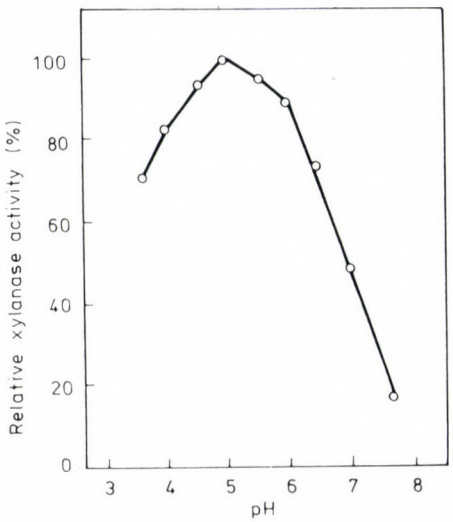

Fig. 3. pH optimum of xylanase activity assayed in acetate buffer at 55 °C for 30 min. The activity obtained at pH 5.0 was taken as the 100% reference level for xylanase activity

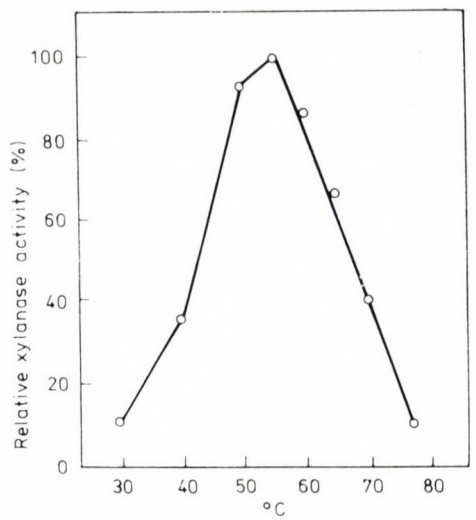

Fig. 4. Temperature optimum of xylanase activity assayed in acetate buffer (pH 5.0) for 30 min. The activity obtained at 55 °C was taken as the 100% level for xylanase activity

The data for the optimization of pH and temperature for xylanase activity are presented respectively in Figures 3 and 4. The purified enzyme showed maximum activity at pH 5.0 and temperature 55 °C.

In conclusion, the presently reported enzyme preparation can efficiently be used to enhance the degradation of arabinoxylan of wheat straw besides 1,4-β-D-xylan and could act as an endoenzyme.

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CHARACTERIZATION OF A SERIES OF MONOCLONAL ANTIBODIES SPECIFIC FOR BOVINE ADENOVIRUS SEROTYPE BAV3 HEXON ANTIGEN AND THEIR USE IN AN ELISA FOR DETECTION OF ADENOVIRAL HEXONS

V. R. Isakova, E. K. Kiseleva, M. A. Kirasova, M. L. Zelivyanskaya, V. G. Grigoryev, S. N. Khilko and T. I. Tikchonenko

Research Institute for Agricultural Biotechnology, Moscow, and Zabolotny Institute of Microbiology and Virology, Kiev, USSR

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After immunization of mice with purified hexon (the main capsid antigen) of bovine adenovirus serotype BAV3 we have obtained a set of 16 individual hybridoma clones producing MAb's against BAV3 hexon. All MAb's were shown to belong to immunoglobulin G class. Specificity of the most avid MAb marked B3Hx-1 was tested on a panel of representative hexon antigens from 16 adenovirus serotypes of human and animal origin using several immunoassays. In Western blot analysis the MAb B3Hx-1 reacted only with native (trimeric) form of hexon protein and not with denaturated hexon polypeptide chains. The epitope defined by B3Hx-1 appeared stable against SDS at ambient temperature and against chloramine-promoted iodination. The specificity of the epitope was characterized as almost genus-crossreactive: it was absent from hexons of avian and of bovine subgroup 2 adenovirus serotypes and present in most hexons of bovine, canine, simian and human adenoviruses tested. Within the latter group its expression was weak or absent only for human subgenus C serotypes. Several variants of sandwich-type ELISA were developed using MAb B3Hx-1 and different polyclonal antibodies against hexons of mammalian adenoviruses. The level of hexon detection for different adenovirus serotypes varied in range 10^{-9} to 10^{-8} g per ml.

The main capsid protein of adenoviruses called hexon has very complex antigenic structure. The two classically determined specificities in hexon (serotype-specific or ε and genus-crossreactive or α [1]) now seem to be both composed of multiple individual epitopes mostly of intermediate specificities between α and ε [2, 3]. Besides, new kinds of antigenic determinants which fall out of the range ε–α were recently detected in hexons by sensitive immunoassays: a subserotype- or clone-specific one [3, 4] and an intergenus-crossreactive determinant [2–6].

V. R. Isakova, M. A. Kirasova, M. L. Zelivyanskaya, V. G. Grigoryev, S. N. Khilko, T. I. Tikchonenko
Research Institute for Agricultural Biotechnology
Moscow, USSR

E. K. Kiseleva
Zabolotny Institute of Microbiology and Virology
Kiev, USSR

Recently several sets of monoclonal antibodies (MAb's) specific for hexons (mostly of human adenoviruses) were obtained and their taxonomic specificities characterized [7–11]. MAb's obtained had reactivities ranging from subserotype- to genus-specific for mammalian adenoviruses, many of them representing intermediate specificity. Only in one case the anti-hexon MAb's were characterized in respect of native (trimer) and denaturated (monomer) hexon forms [7]. Indeed, indirect evidence suggested that hexon reactivity with MAb could be restored from monomers after specific treatment of monomers in Western blot analysis [8, M. Rusvai, personal communication]. The latter apparently contradicted with irreversible nature of hexon dissociation to monomer chains [12, 13] and required further analysis.

In the present communication we report on the preparation and initial characterization of a series of MAb's reacting with pure hexon of bovine adenovirus serotype BAV3.

Materials and methods

Adenovirus hexons. Electrophoretically pure preparations of native hexon proteins of BAV3 and of other adenovirus serotypes of bovine, canine, simian, human and avian origin were obtained by a combination of hydrophobic and ion exchange chromatography as outlined in our invention [14] and will be described elsewhere. Sources of initial adenovirus preparations used for hexon purification are listed in our previous publications [6, 13].

Monoclonal antibodies. A group of 10 Balb/c mice was immunized by a purified BAV3 hexon (50 μg per intraperitoneal injection) four times during a 3 month period. Three days after the last immunization the splenocytes were fused with Sp2/0 myeloma cells using polyethylene glycol 4000. Hybridoma cultures obtained were screened for production of anti-hexon antibodies by indirect ELISA as described [15]. Positive hybridomas were cloned in 0.25% agar, rescreened and grown for ascites production in pristane-primed mice.

Enzyme immunoassays. Indirect ELISA on polystyrene plates was performed as described previously [15] with the following modifications: in most cases hexon was fixed in wells activated by glutaraldehyde, and ABTS was used as an alternative substrate for peroxidase reaction.

Dot-blot immunoassay on nitrocellulose filters was performed usually as described by Tarassishin [16].

Western blot immunoassay was done as in our previous publication [13].

Sandwich-type ELISA with hexon antigens in the inner (second) layer of the sandwich was performed generally by the procedure of Ádám et al. [9] except that in either lower (capturing) or upper (indicator) antibody layer polyclonal bovine, rabbit or chicken anti-hexon antibodies were used and the final layer in each case contained peroxidase-conjugated second antibodies of respective specificity (either anti-mouse, rabbit or chicken IgG). For four types of EIA we have used home-made affinity-purified anti-IgG antibodies labelled with peroxidase [15].

Radioimmunoassays. Radioimmunoprecipitation assay with electrophoretic analysis (RIPA) as well as direct and competitive radioimmunoassays (RIA) using BAV3 and SV37 hexons labeled with iodine-125 by chloramine technique were carried out using *Staphylococcus aureus* strain Cowan fixed cells for precipitation of immune complexes formed as in previous publication [6].

Results and discussion

After three fusion-cloning cycles 28 individual hybridoma clones were obtained which produced MAb's reacting with BAV3 hexon used as immunogen. The hybridoma cells from each clone were further multiplied in ascite form in pristan-pretreated mice. Sixteen ascites obtained were tested for the specific activities of MAb's by ELISA and direct RIA (Table I).

The MAb's differed widely in ELISA titres for the homologous hexon, B3Hx-1 being the most avid in this reaction (as well is RIA). Non-reactivity of some MAb's with [^{125}I]hexon of BAV3 in RIA (Table I) could result from either lability of respective epitopes in conditions of oxidative iodination of the hexon or lack for the reaction of their Fc with protein A of *S. aureus* as known for some subclasses of mouse IgG.

Table I

Reactivities of MAb's in ascite fluids with BAV3 hexon

MAb Designation*	ELISA titre**	RIA reactivity***
B3Hx-1	14	+
B3Hx-2	9	+
B3Hx-3	8	+
B3Hx-4	5	+
B3Hx-5	8	+
B3Hx-6	7	−
B3Hx-7	10	−
B3Hx-8	8	ND****
B3Hx-9	7	ND
B3Hx-10	5	ND
B3Hx-11	5	ND
B3Hx-12	5	ND
B3Hx-13	7	ND
B3Hx-14	5	ND
B3Hx-15	5	ND
B3Hx-16	8	ND

* The MAb's were designated as suggested in [7] by including abbreviated serotype (B3), hexon antigen (Hx) and a number

** Log_2 of the ascites dilution giving half-maximal response in ELISA

*** Precipitability of [^{125}I]BAV3 hexon with *S. aureus* strain Cowan fixed cells

ND = Not determined

The specificity of selected MAb's (B3Hx-1 to 6, 8, 10 and 12) in reaction with pure hexons of mammalian and avian adenoviruses was determined by dot and blot EIA on nitrocellulose filters (Table II). We have not obtained MAb's of narrow (serotype or ε) specificity in our collection. All of the MAb's tested have exhibited specificities close but nor identical to genus-crossreactive (α). Neither of them could recognize all of the hexons of typical mammalian adenoviruses tested or exhibit more wide specificities e.g. inter-genus

(such MAb's should react with hexons of avian and bovine subgroup 2 adenoviruses).

For more detailed characterization we have chosen the most avid anti-BAV3 hexon monoclonal in our collection, i.e. B3Hx-1. By indirect ELISA with class-specific reagents its IgG subclass was estimated as γ2b. The range of its reactivity with hexons included all bovine subgroup 1 serotypes tested, the only canine serotype in our collection as well as representatives of the four simian adenovirus subgroup. The MAb could apparently distinguish between subgenera of human adenoviruses, being slightly or non-reactive with subgenus C members.

Table II

Specificities of monoclonal antibodies in dot-and-blot EIA reactions with adenoviral hexons

Adenovirus			Reactivity with monoclonal antibody								
Host species	Subgroup	Serotype	B3Hx-1	B3Hx-2	B3Hx-3	B3Hx-4	B3Hx-5	B3Hx-6	B3Hx-8	B3Hx-10	B3Hx-12
Bovine		BAV3	+	+	+	+	+	+	+	+	+
	1	BAV1	+	+	+	+	+	+	+	+	+
		BAV2	+	ND	ND	ND	ND	ND	ND	ND	ND
	2	BAV7	−	−	−	−	−	−	−	−	−
Human	A	Ad12	+	+	−	−	ND	ND	−	±	±
		Ad3	+	ND	ND	ND	ND	ND	ND	ND	ND
	B	Ad7	+	ND	ND	ND	ND	ND	ND	ND	ND
		Ad35	+	+	+	+	+	+	+	+	+
	C	Ad1	±	±	+	+	+	ND	+	+	+
		Ad2	−	−	+	−	−	ND	−	−	±
		Ad5	±	±	±	±	±	D	+	N	+
	D	Ad8	+	+	±	+	+	ND	+	±	+
		Ad13	+	ND	ND	ND	ND	ND	ND	ND	ND
	E	Ad4	+	ND	ND	ND	ND	ND	ND	ND	ND
Simian	1	SV36	+	+	+	±	ND	ND	−	±	±
	2	SV37	+	+	+	+	+	+	−	+	+
	3	SV33	+	+	+	−	−	−	−	±	±
	4	SA7	+	+	+	±	±	−	−	±	±
Canine		CAV1	+	+	+	−	−	ND	−	±	±
Avian		CELO	−	−	−	−	−	−	−	−	−
		EDS76	−	−	−	−	−	−	−	−	−

ND = Not determined

As B3Hx-1 was shown to react in direct RIA (Table I) it was analyzed further in RIPA (Fig. 1). In previous report [13] we have demonstrated stability of native form of adenoviral hexon (homotrimer) against denaturation in

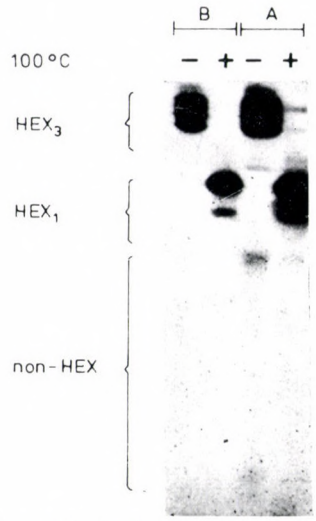

Fig. 1. Radioimmunoprecipitation analysis of simian adenovirus SV37 hexon with the monoclonal antibody B3Hx-1. Radiofluorogram of SDS-PAGE (12% T; 0.5% C). A: SV37 hexon labeled with ^{125}I was separated without precipitation in two parallel lanes, "plus" sample having been heated in sample buffer for 10 min at 100 °C while "minus" sample incubated at the same buffer at 20 °C [13]. B: The [^{125}I]hexon of SV37 was allowed to react with B3Hx-1 monoclonal antibody and the immune complexes formed were precipitated with *S. aureus* reagent and analyzed under the same conditions. HEX_1, positions of hexon polypeptides (monomers); HEX_3, positions of native hexon molecules (trimers); non-HEX, positions of non-hexon impurities present in the iodinated hexon preparation but not in the precipitate obtained

SDS-containing buffer in conditions of electrophoresis being performed without boiling of samples. Such a phenomenon permitted us to simply distinguish between native (trimer) and denaturated (monomer) forms of hexons by SDS-PAGE in "plus-minus" variant [13]. Application of thus modified electrophoretic analysis to iodinated preparation of SV37 hexon (Fig. 1) has revealed native [^{125}I]hexon trimer in "minus" lane and denaturated hexon polypeptide chains in "plus" lane. Some impurities were also present in the preparation having non-hexon nature (as they did not change mobilities as result of "plus-minus" shift). After precipitation with B3Hx-1 only hexon bands could be observed (Fig. 1) confirming the specificity of the MAb and identifying native trimer in the immune complex.

Taxonomic specificity of B3Hx-1 reaction with hexons was further tested by competitive RIA (Fig. 2). Iodinated SV37 hexon was tried to be replaced from the complex with the MAb by excess of unlabeled adenoviral hexons. Preparations of homologous BAV3 hexon as well as of heterologous hexons of bovine (BAV1), human (Ad3) and simian (SA7) origin effectively competed with the tracer giving parallel lines of replacement while human subgenus C (Ad5) and bovine subgroup 2 (BAV7) hexons were without effect. Such a specificity conformed the range of B3Hx-1 reactivity demonstrated by EIA techniques (Table II).

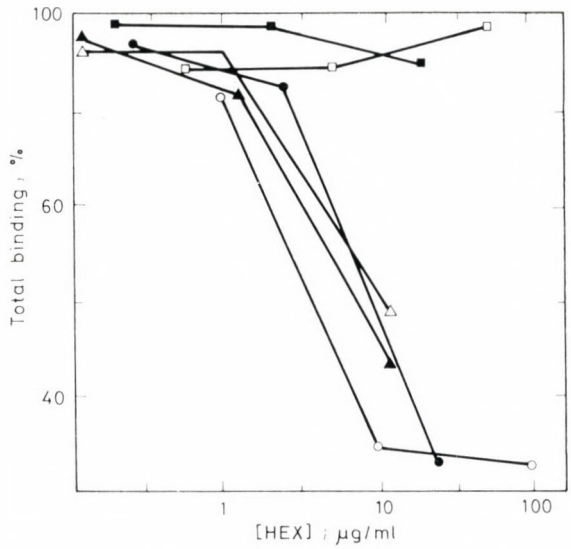

Fig. 2. Competitive radioimmunoassay for binding of hexons of mammalian adenoviruses to monoclonal antibody B3Hx-1. [^{125}I]hexon of SV37 reacted with half-precipitating dilution of B3Hx-1 monoclonal antibody in the presence of competing unlabeled adenoviral hexons. Ordinate, percent of total binding (without competition); abscissa, concentrations of the competitors in logarithmic scale. ●——●——● BAV3; o——o——o BAV1; □——□——□ BAV7; ■——■——■ Ad5; △——△——△ Ad3; ▲——▲——▲ SA7

Only native hexon trimers were shown previously to react with polyclonal anti-hexon antibodies (13 and references therein) reflecting the conformational nature of bulk antigenic epitopes in hexon structure. Such observation do not preclude in principle the existence of rare epitopes in hexon having linear nature and being present both in native trimers and in denaturated hexon monomers. As the level of presentation of such epitopes must not be high the best way to detect them seems to be the use of MAb's. Some evidence for such a possibility is presented by recent results of M. Rusvai and his colleagues [8, and M. Rusvai, personal communication]. The authors can detect reactivity of BAV2 monomers after conventional (e.g. "plus") SDS-PAGE with anti-BAV2 hexon antibodies. We have analyzed monomer or trimer specificity of our representative anti-hexon MAb (B3Hx-1) by blot EIA after "plus-minus" SDS-PAGE (Fig. 3).

Each hexon preparation in this experiment gave monomer band in "plus" (boiled sample) lane and trimer band or series of bands in respective "minus" lane (Fig. 3A). Among hexon electrophoretical bands only those of native trimers have reacted with the MAb (Fig. 3B). The reaction was weak in case of Ad5 hexon and absent for BAV7 hexon confirming the specificity of B3Hx-1 (Table II). Thus the epitope in hexon recognized by B3Hx-1 has conformational nature.

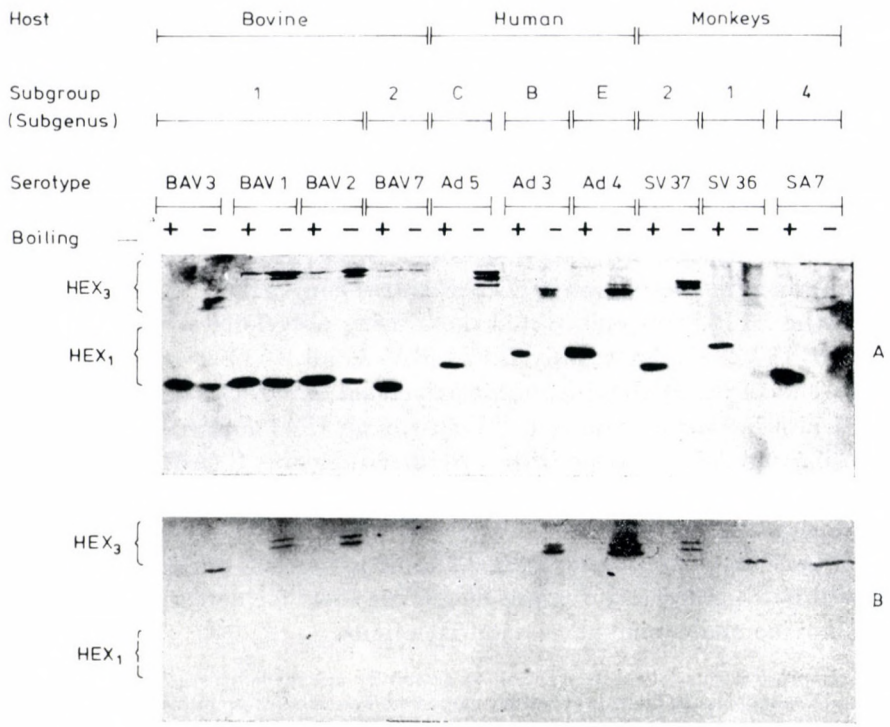

Fig. 3. Immunoblot analysis of the monoclonal antibody B3Hx-1 specificity against hexons of mammalian adenoviruses. Electrophoretic conditions and designations as for Fig. 1. A: silver stained gel electropherogram; B: nitrocellulose blot of the hexon electropherogram identical to that shown in A was treated with B3Hx-1 10^{-4}) and immune complexes formed were detected by peroxidase-conjugated rabbit anti-mouse IgG antibodies

To test the possible presence of linear epitopes in hexon that may be reversibly destroyed by SDS treatment and renaturated by mild replacement of SDS (the author's explanation of Rusvai's results) we analyzed by dot and blot EIA reactivity of adenoviral hexons boiled with or without SDS before electrophoresis (or before direct application onto nitrocellulose) with MAb's. To renaturate hexon antigenicity from monomers we tested several formulations of detergents, urea and alcohols including that used by Rusvai et al. Neither of MAb's from our collection or selected MAb's against Ad1, Ad35 (from E. Ádám) and BAV2 (from M. Rusvai) were able to react with nitrocellulose-fixed, heat-denaturated hexon monomer chains while all of them readily reacted with native hexon trimers of respective adenoviral serotypes (results not shown). We had no reasonable explanation for the divergence of our data with those of Rusvai et al. and could reach a tentative conclusion that all epitopes in native hexon antigenic structure represent conformational entities expressed only in trimeric hexon molecule.

Using anti-BAV3 hexon monoclonal antibody B3Hx-1 and several high-titered polyclonal antibody preparations we have constructed four-layer sandwich ELISA systems for detection of adenoviral hexons. The MAb was tested either in the lower layer of the sandwich for the capture of hexon antigens or in the third layer for detecting the antigens. The other antibody component of the system (detecting or capturing layer, respectively) was chosen between rabbit, bovine or chicken anti-hexon immunoglobulin preparations obtained by hyperimmunization with purified BAV3, BAV1 or Ad5 hexons. The upper layer contained respective anti-IgG peroxidase conjugate.

Of the ELISA systems tested those using polyclonal antibodies against BAV3 or BAV1 could detect only BAV1, BAV2 and BAV3 hexons. In contrast, combinations of the MAb with rabbit polyclonal anti-Ad5 hexon serum (#51) detected also hexons of canine (CAV-1), simian (SA7 and SV37) and human (Ad7, Ad12 and Ad13) adenoviruses. Neither subgenus C (Ad1, Ad5) nor subgenus E (Ad4) human adenovirus hexons were detected by any ELISA system. For both combinations of B3Hx-1 and #51 antiserum the detection levels for hexons were estimated ranging from 0.2 to 60 ng per ml. Such ELISA systems could well be regarded as potential diagnostic tools for detection of hexons in at least bovine and canine adenoviral infections.

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CHARACTERIZATION OF AN INTERMEDIATE ADENOVIRUS STRAIN

Gy. Fejér, A. Kiss, Anna Lengyel, Zs. Ruzsics, Gy. Berencsi and I. Nász

Institute of Microbiology, Semmelweis University Medical School, Budapest

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An intermediate strain of human adenovirus of subgenus D was investigated by type specific serological reactions and restriction endonuclease analysis. The latter method showed the strain identical to the prototype strain of human adenovirus type 9 as well as did serum neutralization tests. In contrast with the previous methods haemagglutination inhibition tests showed the strain related to both the prototypes strains of human adenovirus 9 and 13.

Since the discovery of adenoviruses in 1953, 47 different human serotypes have been identified [1]. Type specific identification of adenovirus strains is carried out by serum neutralization (SN) and haemagglutination inhibition (HI) tests which are based on the immunological properties of the virus. Beside these methods restriction endonuclease analysis is also being used in the identification of both human and animal adenoviruses [2–4]. The latter method revealed that isolates belonging to given serotypes sometimes possess different restriction patters [5–8]. This phenomenon reflects the fact that the genes which are responsible for the serological specificity of adenoviruses comprise less than 0.1 part of the whole genome [9]. Other strains were found to be antigenically related not to only one but to two or more prototypes [10].

In our present work we studied the characteristics of such an intermediate strain related to the prototype strains of adenovirus 9 and 13.

Materials and methods

Viruses and tissue cultures. The intermediate adenovirus strains AV 13/9 was obtained from the FRG in 1967; at that time it was believed to be the prototype strain of AV 13. Adenoviruses 9 and 13 were prototype strains from H. G. Pereira.

Viruses were propagated on the human cell line "293" transformed by the DNA of human adenovirus type 5 [11]. Cells were grown in Ca^{++} free Eagle's MEM supplemented with 0.1 part of fetal calf serum, penicillin and streptomycin.

Purification of viral DNA. Virus infected cells were harvested when the CPE was almost complete by low speed centrifugation. The cell pellet was extracted with 5 mg/ml Triton X100, 0.4 M NaCl and 10 mM Tris-HCl pH 7.5 as described previously [12]. This extract, containing mainly virion and viral DNA, was then further purified by SDS/pronase treatment followed by phenol and chloroform: isoamylalcohol extraction according to standard protocols [13–15].

György Fejér, Alexis Kiss, Anna Lengyel, Zsolt Ruzsics, György Berencsi, István Nász
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

Restriction endonuclease analysis. Restriction endonucleases *BamH* I, *EcoR* I, *Bgl* II, *Sal* I and *Hind* III were purchased from Reanal (Budapest) and were used according to the instructions of the manufacturer. The resulting DNA fragments were separated on 10 mg/ml agarose slab gels. The gels were run at 1 V/cm and subsequently stained with ethidium bromide.

Antisera. Antisera were prepared against the same virus strains used in our present experiments. They were obtained after several inoculations of unpurified virus into rabbits. In addition to these sera a WHO reference horse antiserum to AV 13 was also used.

Serological tests. Neutralization tests were performed in "293" cell tubes according to standard protocols [16] with an initial serum dilution of 1 : 2. The cells were extracted with the Triton X100-NaCl solution and neutralization was evaluated whether newly synthetized unit length DNA could or could not have been seen after SDS/pronase treatment and agarose gel electrophoresis. HI tests were performed with human red blood cells in round bottom microtiter plates with an initial serum dilution of 1 : 40.

Results

The results of SN and HI tests are summarized in Tables I and II. In HI tests the AV 13/9 strain reacted with antisera to both AV 9 and AV 13. Antiserum to AV 9 showed the same HI titre in heterologous reactions. Interestingly only the WHO reference antiserum to AV 13 cross-reacted with the AV 13/9 strain, but in this case the cross-reacting HI titre was even higher than in the homologous reaction. Similarly antiserum to AV 13/9 cross-reacted with the prototype AV 9 and in lower extent with the prototype AV 13. Antisera against the prototype strains did not cross-react with heterologous antigens.

Table I

Haemagglutination inhibition

Virus strain	Sera			
	AV 13		AV 9	AV 13/9
	1	2*		
AV 13	1280	320	40	80
AV 9	40	40	1280	320
AV 13/9	40	1280**	1280	640

* WHO reference serum
** Partial inhibition

Table II

Serum neutralization

Virus strain	Sera		
	AV 13	AV 9	AV 13/9
AV 13	256	2	2
AV 9	2	256	256
AV 13/9	2	64	32

In SN tests AV 13/9 was equally neutralized by antisera to AV 13/9 and AV 9. On the other hand AV 9 was also neutralized by both the previous antisera. Interestingly antiserum to AV 13/9 showed a significantly higher titre to AV 9 than to AV 13/9. Other cross-reactions were not detected in SN tests.

The DNA of AV 13/9 strain was cut by five restriction enzymes and the fragment patterns were compared with those of the prototype strains of AV 13 and AV 9. The restriction fragment patterns of AV 13/9 generated by *Sal* I, *BamH* I, *EcoR* I (Fig. 1) and *Bgl* II (data not shown) were identical with the respective patterns of AV 9. Even slight differences could not have been found between AV 9 and AV 13/9. They both characteristically differed from the restriction fragment patterns of AV 13. The restriction fragment patterns of the prototype strains corresponded to the ones found in the literature [17].

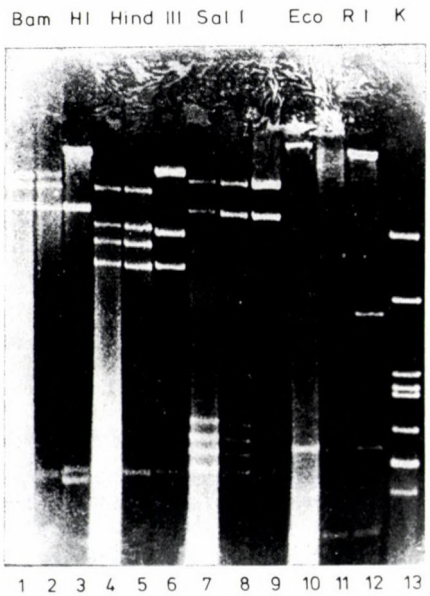

Fig. 1. DNA restriction analysis with *BamH*I, *Hind*III, *Sal*I, *Eco*RI. Lanes 1, 4, 7, 10 AV9; lanes 2, 5, 8, 11 AV13/9; lanes 3, 6, 9, 12 AV13; lane 13, AV1 DNA cut by *Hind*III, as molecular weight control

Discussion

In our present study we analysed an intermediate serotype of subgenus D. In HI tests the strain was related to both AV 9 and AV 13 while in SN tests it was related only to AV 9. In previous works the genome types of intermediate strains of subgenus D were found to be similar to that prototype strain which was related to them in HI, however, slight differences had been detected [18]. Our results show another variation when the genome type of the intermediate strain corresponds to the genome of the prototype strain related in SN.

In a previous study there were also differences between AV 13/9 (believed at that time to be the prototype strain of human adenovirus type 13) and AV 9 [20].

A possible explanation of our results is that a slight alteration exists in the fiber gene of the intermediate strain comparing to the prototype strain of AV 9. This alteration can not be recognized by the restiction enzymes used but changed the haemagglutination properties of the virus while the neutralizing epitopes remained the same.

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COMPLEX TYPING OF *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED IN AN INTENSIVE CARE UNIT*

[A NOTE]

I. Barcs, L. Jánosi, Anna Szentmihályi and K. Balázs

National Institute of Hygiene, and László Central Hospital for Infectious Diseases, Budapest

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A total of 2637 Gram-negative facultatively pathogenic bacteria were isolated in the László Central Hospital for Infectious Diseases during a 5-year-period from clinical samples of patients of the Respiratory Intensive Care Unit; 28 further strains were cultured from hospital personnel and fomites. *Pseudomonas aeruginosa*, *Klebsiella* and *Acinetobacter* spp. were most frequently isolated. Complex typing (determination of O serogroup, phage pattern, pyocin type, antibiogram and plasmid pattern) of *P. aeruginosa* strains showed the predominance of serogroup O11 (62%), but the isolates differed from each other by other characteristics. Conjugation experiments showed no common resistance plasmids in the tested population.

Gram-negative facultatively pathogenic (GNFP) bacteria (first of all *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, but frequently also *Aeromonas*, *Enterobacter*, *Proteus*, *Serratia* spp., etc.) commonly cause nosocomial infections and cause large outbreaks which are often difficult to control [1–6]. The patients in long-stay hospital units, especially in intensive wards are highly defenseless. They acquire soon one or more agents of nosocomial infections not being in association with their underlying diseases. These microorganisms persist permanently in the patients' flora and in the inanimated environment. The main problems of hospital epidemiology are the multispecies outbreaks, the patient-to-patient spread of these bacteria, and their increased resistance to antimicrobials. In particular, *P. aaeruginosa* colonization of patients was considered to be a contributing factor in higher mortality [3]. The results obtained in a hospital outbreak caused by GNFP bacteria are presented in this paper.

* This lecture was presented at the joint scientific meeting of the Bacteriology Section of the Hungarian Society of Microbiology, and the National Institute of Hygiene, Budapest, 1988.

István Barcs, László Jánosi, Anna Szentmihályi
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Károly Balázs
László Central Hospital for Infectious Diseases
H-1450 Budapest, P.O.B. 29, Hungary

Materials and methods

Patients. Of the patients treated in the Respiratory Intensive Care Unit of the László Central Hospital for Infectious Diseases in the years 1982—1986, 30% were children under 14 years of age, and 70% were adults. All the patients had serious underlying diseases (e.g. meningitis, tetanus, Guillain-Barré syndrome, etc.). Most of them were intubated and received a long-lasting combined antibiotic therapy [7].

Bacterial strains. A total of 2637 GNFP strains were isolated mainly from respiratory tract samples. From the environment (air, bed-clothes, taps, sinks, toilet seat, drain of respiratory device and suction apparatus, and hands of staff, respectively) 28 GNFP strains were isolated by a single sampling in the last quarter of 1986 [7].

The antibiotic susceptibility was tested with agar diffusion method [8] on modified Mueller-Hinton medium (Human, Budapest) using Resistest disks (Human, Budapest).

The determination of O serogroups was carried out according to Lányi and Bergan [9].

The phage typing and pyocin typing of P. aeruginosa strains was performed by Lindberg and Latta's [10] and by Gillies and Govan's [11] method.

Plasmids were detected simultaneously by methods of Bennett et al. [12] and Kieser [13] in 0.7% agarose (Seakem, Miami) gel according to Meyers and co-workers [14].

Genetic exchange was performed by using the method of Dean et al. [15]. The liquid media were supplemented with 0.02% agarose to increase the mating frequency [16]. The rifampicin-resistant mutants of *Escherichia coli* J5–3 [17] and *P. aeruginosa* PAO674 [15] were used as recipients, and selection was for both the donor and recipients markers.

Results and discussion

In clinical specimens *P. aeruginosa* occurred the most frequently while in the environment *Acinetobacter lwoffii* predominated (Fig. 1). The staff harboured no GNFP strains [7].

Both patient and environmental isolates proved to be highly resistant to commonly used antimicrobials. Table I presents the incidence of resistant isolates among the most frequently occurring species. Comparison of susceptibility data of 5 years showed that the occurrence of strains resistant to certain

Table I

Antibiotic susceptibility of Gram-negative facultatively pathogenic bacteria isolated

Species	Percentage of strains resistant to							
	AM	CB	AZL	MZ	MA	CXM	CTX	CRO
P. aeruginosa	100	62	39	42	98	98	98	36
Klebsiella	65	64	38	32	35	27	6	0
A. calcoaceticus	87	78	51	54	97	68	68	15
A. lwoffii	67	52	44	44	62	61	61	33
Other	65	75	44	38	70	43	41	33

agents — especially to aminoglycosides and new penicillins — is increasing. For example the incidence of *P. aeruginosa* strains resistant to gentamicin and tobramycin rose two-fold.

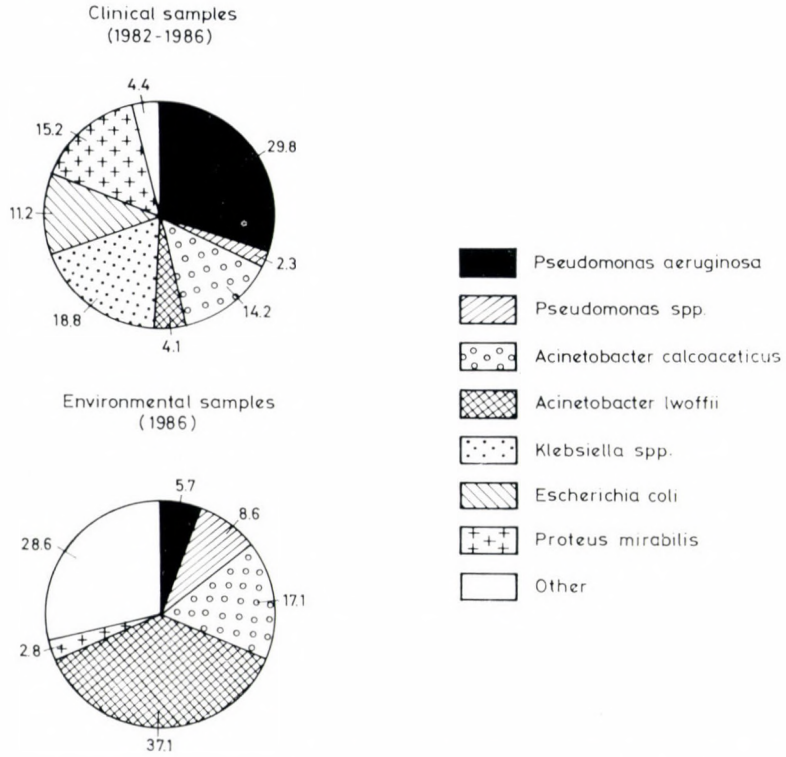

Fig. 1. Incidence of Gram-negative facultatively pathogenic bacteria in the Respiratory Intensive Care Unit of the László Central Hospital for Infectious Diseases (percentages)

from patients of the Respiratory Intensive Care Unit of László Hospital in 1986

Species	Percentage of strains resistant to							
	CAZ	GM	TM	AN	NET	TE	C	SXT
P. aeruginosa	14	69	65	13	1	96	95	98
Klebsiella	0	46	42	16	0	59	56	17
A. calcoaceticus	9	93	87	79	9	93	100	75
A. lwoffii	0	77	78	42	21	65	57	32
Other	0	47	44	5	0	67	44	19

Abbreviations: AM ampicillin, CB carbenicillin, AZL azlocillin, MZ mezlocillin, MA cephamandole, CXM cefuroxime, CTX cefotaxime, CRO cephtriaxone, CAZ ceftazidime, GM gentamicin, TM tobramycin, AN amikacin, NET netilmicin, TE tetracyclin, C chloramphenicol, SXT co-trimoxazole

Since the isolates belonging to different species of different origin exhibited similar resistance patterns, and the incidence of resistant and multiply resistant strains increased year by year [7], two possibilities were studied: whether resistant clones are spread or a continuous gene transfer occurs among the different species in the environment, and resistant strains are selected within the patients during therapy.

Our knowledge of the epidemiology and control of outbreaks developes on the availability of accurate typing procedures. Subspecification or identification of distinct types within a species is the aim of typing. Numerous schemes have been used to differentiate several species, viz. serological typing, bacteriocin production, bacteriophage typing, use of antibiograms, and plasmid patters analysis. Since no single typing method has gained widespread acceptance, two or more methods are often used for comparative typing of isolates.

Using these methods on 114 randomly selected strains revealed no predominance of any well-definable clones: every GNFP strain differed from each other at least in one characteristic [18]. A total of 36 *P. aeruginosa* isolates were examined in more detains. In respect of the results of serotyping only, the predominance of serogroup O11 was striking (62%). This serogroup has been generally considered to cause a great majority of nosocomial infections associated with intensive therapy [19–21].

Further typing experiments resulted in more exact grouping of the strains. The 36 *P. aeruginosa* strains examined belonged into 15 different pyocin types and fell into 21 different phage patterns. When these two features were considered, the 36 strains could be classified in 28 different groups. It is interesting that phage pattern 44/C21 occurred only among O11 strains, and also the incidence of pyocin type NC 1 was higher in the serogroup (48%) than in other ones (15%).

Sixty-five percent of these isolates harboured one or more plasmids with 1.4–130 Md in size. This ratio is significantly higher as indicated by literary data data [15, 22, 23]. There was no correlation among the resistance phenotype and plasmid-harbouring.

Since the resistance pattern of bacteria belonging to different species was similar, and the different species harboured plasmids with identical molecular mass [18], it seemed to be interesting to examine whether common resistance plamids were present in all of the epidemic population. To decide the presence of genetical exchange among the clinical strains we performed mating experiments.

Resistance transfer was observed in 5 experiments from *P. aeruginosa* donors, only to the same species. Intergeneric transfer occurred from *Enterobacter aerogenes* donor to *P. aeruginosa* recipient in one case, from one. *A. lwoffii*, one *E. aerogenes*, one *E. cloacae*, one *Klebsiella oxytoca*, and two *K.*

pneumoniae donors to *E. coli* recipient, respectively. The frequency of recombination occurred in range of 7×10^{-8} to 6.1×10^{-5}. One of the plasmids of the donors were detected only in three transconjugants obtained from *A. lwoffii*, *K. oxytoca* and *K. pneumoniae* donors (27 MC, 43 Md, and 83 Md, respectively). This observation suggests the appearance of transposon-mediated resistance in the epidemic population.

From our typing results it may be assumed that the ancestor of our isolates was a multiply resistant O11, phage pattern 44/C21, pyocin type NC 1 *P. aeruginosa* clone, that has been divided into subclones by acquiring some different resistance determinants sourced from the co-existing flora during a continuous antibiotic pressure.

Acknowledgement. The authors are indebted to Dr. MARIANNE KONKOLY THEGE and Dr. I. GADÓ (National Institute of Hygiene, Budapest) for the recipient strains and to Mrs JULIA PAP and Mrs MAGDA HETZMAN for skilled technical assistance.

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BOOK RECEIVED

H.-J. Rehm and G. Reed (eds): Volume 6B of Biotechnology "A Comprehensive Treatise in 8 Volumes". VHC Verlagsgesellschaft, Weinheim–Basel–Cambridge–New York 1989. pp. 810. Price DM 495; US $ 198

"Special microbial processes" are summarized in 25 chapters completed by 294 figures and 136 tables. The volume editor Hans-Jürgen Rehm wrote in the Preface .... "other new fields — models for biotechnological photosynthesis, accumulation and leaching of metals, future perspectives of biotechnology in space and more earthbound matters such as the microbial processing of tobacco, flax, and leather, ... have been treated .... because of their potential future developments". It is also mentioned in the preface that VCH Verlagsgesellschaft is already planning the Second Edition of the series with certain modificotions.

Chapters 1 to 6 are of practical interest. Microbial production of butanol, acetone, 2,3--butanediol, glycerol and related compounds, hydrocarbons, hydrogen, and poly-beta-hydroxybutyric acid is described in detail. The topic covered by Chapter 7 is of specific character; microbial production of labeled compounds are summarized completed with the enzymatic procedures, too. In chapter 8 a biomimetic model of photosynthesis is presented.

Chapters 9 and 10 are most valuable for virologists. Methodology of the mass production of eukaryotic cells both of plant and mammalian origin have been compiled by P. F. Heinstein, A. Emery, and M. Butler so as to correspond to the highest professional standard. Biosensors and "bioelectronics" in chapter 11 belong to the same subject interesting for medical and bioengineers, too.

The next group of chapters 12–16) are dealing with natural resources. They describe leaching, concentration, and elimination of metals, nitrogen, phosphorus, sulfur and coal. Two chapters (13 and 17) are of essential interest for those, who are working on the elimination of contaminations from the surrounding. The role, and characteristics of aerosols is useful for laboratory safety measurements and health safety regulation.

Four systematic works of theoretical importance was also included by the editor (chapters 18–21). Protein crystals might be used later on by the industry, and space biotechnology might be interesting for big countries, nevertheless, the chapters on gliding bacteria, and archebacteria are informative for all microbiologists.

Perspectives of biotechnology (chapters 22–25) in agriculture was outlined by G. Wenzel in addition to the three industrial topics mentioned in the citation from the editor H.-J. Rehm above, who has done an outstanding work.

György Berencsi

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CONTENTS

IM MEMORIAM ISTVÁN HORVÁTH

The unexpected death of Professor István Horváth, Director of the Second Institute for Chemistry and Biochemistry of the Semmelweis University Medical School, Budapest on the 22nd February 1990 is a griveous loss for the Hungarian Society for Microbiology.

He was born in 1920 and after studying chemistry, physics and mineralogy at the Pázmány Péter University, Budapest obtained his doctor's degree in 1944. From 1943 to 1947 he also studied the theoretical disciplines of the Medical Faculty. He began his scientific career in the Institute of Pharmacology of the same University working under the leadership of Professor Béla Issekutz. From 1948 to 1950 he was head of the Biological Department of the Chinoin Pharmaceutical Company, then from 1950 to 1972 he was head of the Microbiological Department of the Pharmaceutical Research Institute, Budapest. In the meantime from 1967 to 1972 he became also deputy director and supervised the work of the Fermentation Department. He became Professor and Director of the Second Institute for Chemistry and Biochemistry

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of the Semmelweis University Medical School, Budapest in 1973 and was working in this capacity until his unexpected death. He defended his "candidate" thesis in 1965 and his doctor thesis in 1971.

His scientific activity covers two main fields. As a researcher in the pharmaceutical industry he had a leading role in elaboration of practically all the antibiotics produced in Hungary, in addition he made important contributions in steroid-transforming and enzyme-isolation researches, too. In his theoretical research work he investigated the regulation of protein synthesis in microorganisms and the mode of action of different antibiotics. From 1973 regulation of metabolic processes of eukaryotic cells stood in the centre of his researches. He was a really successful researcher proven by about 100 scientific articles published mostly in international journals and by more than 40 patents, most of which were realized in the pharmaceutical industry.

As a Professor, István Horváth proved to be an excellent teacher. During his whole life he was deeply involved in the scientific public life. He was member of the governing body of the International Union of Pure and Applied Chemistry and of the Applied Chemistry Division Fermentation Section. He was leading member of the Hungarian Society for Biochemistry and the Hungarian Society for Microbiology, being also the secretary of the latter for a turn. He was on the Editorial Board of Journal of Antibiotics and the European Journal of Applied Microbiology and for a period of the Acta Microbiologica Hungarica.

István Horváth was a warm-hearted colleague and I am happy that I could count myself among his best friends. I think that the Hungarian microbiologists will always remember him with honour and devotion. István Horváth is survived by his wife, two sons and a daughter.

István Földes

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IMMUNOTOXICITY TESTING OF MYCOTOXINS T-2 AND PATULIN ON BALB/C MICE

J.-C. Paucod, S. Krivobok and D. Vidal

Microbiology Unit, Army Medical Corps Research Centre, La Tronche, and Botany, Criptogamy, Cellular Biology and Genetic Laboratory, Factury of Pharmacy, Meylan, France

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The effects of patulin and T-2 toxin were investigated on immunological responses of Balb/c mice. In vitro patulin had a stimulatory effect on splenocytes at lower concentration (1 nM to 10 nM) and strongly inhibited lymphocyte proliferation at higher concentrations (ID_{50} from 0.02 to 0.24 μM depending on mitogens). In the same experiments T-2 toxin was 100-fold more potent (ID_{50} from 0.7 to 2 nM). In vivo studies on immunity were performed in mice receiving *Bordetella pertussis* antigens and keyhole limpet haemocyanin. Patulin significantly reduced delayed type hypersensitivity to *B. pertussis* antigen and did not reduce anti-KLH antibody production. T-2 toxin had no effect on delayed type hypersensitivity and reduced anti-KLH antibody production. Splenocytes were harvested in mice with or without antigen stimulation to assess mitogenic responses. Patulin generally increased splenocyte proliferation, therefore T-2 toxin effect depended on the immunological status of mice and on the dose injected. At the lower doses (0.8 mg/kg), T-2 toxin enhanced responses to mitogen, but at the greater dose (1.6 mg/kg) T-2 toxin enhanced responses to mitogen of antigen stimulated mice and decreased reponses of unstimulated mice.

Mycotoxins are secondary fungal metabolites associated with illness in man and animals ingesting mouldy foodstuff. T-2 toxin, a trichothecene mycotoxin produced by different species of *Fusarium*, *Myrothecium*, and *Stachybotrys* [1] is a potent inhibitor of protein and DNA synthesis. This toxin contaminates cereals harvested and stored under damp and cold conditions [2]. It is responsible for numerous animal intoxications [3]. In natural cases as well as in experimental animals, intoxication with T-2 toxin causes a loss of body weight, emesis, inflammation, diarrhoea, haemorrhage, haematological changes and destruction of bone marrow [2, 3]. The immune system is particularly sensitive to trichothecene mycotoxins [4–8] and their effects may be explained by the ability of these toxins to inhibit protein synthesis as demonstrated in vivo [9] and in vitro [10–12].

J.-C. Paucod, D. Vidal*
Unité de Microbiologie, Centre de Recherches du Service de Santé des Armées
24 Avenue des Maquis du Grésivaudan, 38702 La Tronche, France

S. Kribovok
Laboratoire de Botanique, Cryptogamie, Biologie Cellulaire et Génétique, Faculté de Pharmacie
BP 138, 38243 Meylan, France

* Corresponding author

Patulin is a fungal toxin produced by several species of *Penicillium* and *Aspergillus* [13], and is a common contaminant of fruit, especially apples, berries and products made of these raw materials [14, 15]. Patulin is toxic for animals [16] and has broad spectrum antibiotic properties [17]. It has also been shown to possess carcinogenic [18], mutagenic [19] and teratogenic properties [20].

The purpose of this study was to compare the effects on immunity of T-2 toxin, known to be potent immunotoxic, and of patulin which has just begun to be examined for immunotoxicity [21]. We studied the effects of these mycotoxins on the in vitro mitogen-induced blastogenesis of splenocytes, and chose an experimental design to assess the in vivo effects of mycotoxins on immunity [22].

Materials and methods

Mycotoxins. T-2 toxin and patulin (Sigma Chemical Co., St. Louis, Mo.) were dissolved in ethanol at 10 mg of toxin/ml and stored at 4 °C until needed. This stock solution was diluted in phosphate buffered saline (PBS) before use.

Mitogens. Lipopolysaccharide (LPS) from *Salmonella typhi-murium* (Difco Laboratories, Detroit, MI), pokeweed mitogen (PWM, Sigma Chemical Co., St. Louis, Mo.), purified phytohaemagglutinin (PHA, Sigma Chemical Co., St. Louis, Mo.), and concanavalin A type IV (Con A, Sigma Chemical Co., St. Louis, Mo.) were diluted in PBS and stored at −70 °C until needed. They were diluted in RPMI 1640 medium (Eurobio, Paris, France) supplemented with 10% heat-inactivated and filtered fetal calf serum (batch No. 368913, Eurobio, Paris, France), 2 mM glutamine (Eurobio, Paris, France), 50 units penicillin G/ml and 50 μg streptomycin/ml (Boehringer Gmbh, Mannheim, FRG), at their optimal concentration of 10 μg/ml for PHA and Con A, 5 μg/ml for PWM, and 30 μg/ml for LPS.

In vitro assay. Spleens from 8-week-old female Balb/c mice (IFFA-CREDO, L'Arbresle, France) were aseptically excised. Cell suspensions from five animals were pooled, adjusted at 5×10^5 cells/100 μl and placed in 96 wells tissue culture plates (Costar, Cambridge, Ma.); 50 μl of mitogen (or 50 μl RPMI medium for controls without mitogen) and 50 μl of the diluted mycotoxin (or 50 μl RPMI medium for controls without toxin) were added.

In vivo treatment. Six-week-old female Balb/c mice weighing 20 g were purchased from IFFA-CREDO (L'Arbresle, France). The LD_{50} in mice of a single intraperitoneal dose of T-2 toxin and patulin were respectively 3.2 and 8 mg/kg. Doses used were 1/2 and 1/4 LD_{50}. In vivo effects of mycotoxins were assessed using a multiple assay modified from Exon et al. [22].

Mice were given intraperitoneal injection of a single dose of mycotoxin on day 0, 7 and 14 (or PBS for control mice).

In order to induce either a humoral immune response or a delayed-type hypersensibility reaction, the mice were given on days 1 and 8 a single subcutaneous dose (50 μg/10 g body weight) of keyhole limpet haemocyanin (KLH, Calbiochem, La Jolla, Ca), and on day 1 a single subcutaneous dose of *B. pertussis* antigen (2.4 mg/kg) plus on day 8, an intrafootpad dose of 48 μg/20 μl *B. pertussis* antigen (batch No. 226, kind gift of Institut Mérieux, Lyon, France). The mice were sacrificed on day 15 by cervical dislocation.

Delayed-type hypersensibility assay. Delayed-type hypersensibility (DTH) reactions were measured by footpad swelling in mice, using a manual micrometer (Oditest. Kroeplin, FRG). Briefly, on day 7, 20 μl heat aggregated(70 °C, 30 min) *B. pertussis* antigen (2.4 mg/ml) was injected in the right hind footpad of control and toxin lots. On day 8, the mean thickness of footpad of control animals was compared to that of toxin-treated animals.

Enzyme-linked immunosorbent assay of anti-KLH. Mice were given subcutaneous injections of 0.1 mg KLH/20 g body weight, on days 1 and 8. Blood samples were collected from the retroorbital sinus on day 15. The anti-KLH antibody titer of the serum was evaluated by ELISA. Briefly, 96-well microtiter plates (Costar, Cambridge, Ma.) were coated with a solution of 1.0 mg KLH/ml for 2 h at 37 °C and stored at 4 °C overnight. Plates were rinsed 5 times with PBS-Tween 20 solution and blocked with 250 μl of PBS-creamed milk (Gloria, Paris,

France): 10% solution/well for 1 h at 37 °C, then rinsed 5 times with PBS-Tween 20. Serum samples were diluted (from 1 : 100 or 1 : 200 to 1 : 102 400) in PBS-creamed milk solution and added at 100 μl amounts to the plates. The serum-containing plates were incubated at 37 °C 2 h and rinsed 5 times in PBS-Tween 20. Goat anti-mouse IgG conjugated to peroxidase (GAM/IgG/H + L) (PO, Nordic Immunological Laboratories, Tilburg, Netherlands, diluted 1 : 800) was added to each well (100 μl), incubated for 2 h at 37 °C, and then rinsed 5 times with PBS-Tween 20. The substrate for peroxidase, ABTS (2,2'-azino-di-/3-ethylbenzthiazoline sulphonate [23], Boehringer, Mannheim, FRG), was added to each well (100 μl). The colour reaction was allowed to develop for 30 min in the dark at room temperature and quantitated on a photometer (Titertek Multiskan MCC, Flow, Mc Lean, Va.) at 405 nm. The results are expressed as mean $\log_2$ (1/titre).

Ex vivo assays. Spleens were aseptically excised on day 15 and were immediately immersed into Hanks medium. Spleens from 3 animals were squeezed and the splenocytes were pooled for each experiment, then filtered through a stainless-steel sieve. After first centrifugation (250 g, 10 min, at 4 °C), cells were washed with 8.3% NH_4Cl solution during 5 min and suspended in Hanks medium. Erythrocytes and dead cells were removed from splenic cell preparations by centrifugation. After separation, cells were washed twice in RPMI 1640 medium and resuspended to a final concentration of 5.0×10^6 living cells/ml. Cell viability was assessed using the trypan blue exclusion technique [24]. Aliquots of 100 μl containing 5×10^5 spleen cells were placed in 96 well tissue culture plates (Costar, Cambridge, Ma. and 100 μl of mitogen (or 100 μl RPMI medium for each assay without mitogen) were added.

Cultures were incubated at 37 °C for 48 h in a 5% CO_2 atmosphere. Then 37 KBq of [^{3}H] thymidine (CEA, Gif-sur-Yvette, France; specific activity 1.66 TBq/mmol) was added to each well. Incubation was continued for 18 additional hours under the same conditions. The cells were collected with a multiple automated sample harvester (Flow, Mc Lean, Va) on glass-fiber filters discs (Whatman, Maidstone, UK). After dessication, the discs were placed in 3 ml of toluene scintillator (Packard, Downers Grove, Ill.) and [^{3}H] activity was determined via liquid scintillation counting (Model 2000 Tri-Carb, Packard Instrument Co., Downers Grove, Ill. The results are expressed as dpm of triplicate samples.

Statistical analysis. Means comparison tests for small samples were performed to analyze the results of in vitro and in vivo experiments.

Results

In vitro mitogen assay. Patulin at low concentration (> 0.01 μM), had a stimulatory effect, particularly with Con A (Table I). At higher concentration, patulin strongly inhibited lymphocyte proliferation, in a dose dependent manner (ID_{50} from 0.02 to 0.24 μM depending on mitogen used). In same conditions the ID_{50} of T-2 toxin ranged from 0.7×10^{-3} to 2×10^{-3} μM (Table II). T-2 toxin had a slight stimulatory effect to Con A at about 0.75×10^{-3} μM.

In vivo hypersensitivity and antibody responses. Antibody response and delayed-type hypersensitivity response are shown in Table III. T-2 toxin and patulin reduce similarly anti-KLH antibody production, but these results are significant only with 1/4 of LD_{50} of T-2 toxin ($p < 0.02$). DTH as tested by footpad thickness measurement on day 8 (24 h after stimulation by *B. pertussis*), showed that patulin significantly reduced DTH response ($p < 0.05$ for 1/4 LD_{50} and $p < 0.001$ for 1/2 LD_{50}). In contrast, T-2-toxin did not show a significant change.

Ex vivo mitogen assay. The effect toxin injection on mitogen response of splenocytes depended on the treatment of different lots.

Table I

Mitogenic response of murine (Balb/c) splenic cells treated in vitro with various concentrations of patulin

Mitogens	None	Con A	PHA	PWM	LPS
Control	1470.41[a] ±120.38	38061.00 ±6373.78	23962.60 ±2185.80	18470.20 ±610.22	6970.61 ±777.45
Patulin 0.01 μM	1945.30 ±90.42	43916.30 ±20275.00	39209.60 ±27400.00	16246.20 ±1249.19	4191.57 ±514.83
	132%[b]	115%	164%	88%	60%*
0.05 μM	1069.60 ±99.34	57482.50 ±5582.50	15390.80 ±1617.19	11419.20 ±282.93	2527.27 ±479.67
	73%*	49%*	64%**	62%***	36%*
0.1 μM	858.05 ±162.66	33788.70 ±5746.79	19925.60 ±938.71	10229.20 ±68.14	2620.45 ±771.87
	58%**	89%	83%	55%***	38%**
0.5 μM	400.23 ±31.69	727.82 ±173.98	403.12 ±30.40	459.85 ±40.21	429.75 ±31.91
	27%***	2%***	2%**	3%***	6%***
1.0 μM	469.11 ±38.60	473.74 ±61.66	364.64 ±3.79	489.94 ±8.43	461.29 ±35.39
	32%***	1%***	1%**	3%***	6%***

[a] Values are means of dpm ± SD
[b] Variation in percent of control value
Significantly different from control group at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

In animals without antigen stimulation (KLH and *B. pertussis*) (Table IV), T-2 toxin at 1/2 LD_{50} decreased slightly mitogen responses (significantly). On the other hand, patulin, at 1/4 LD_{50}, also showed no significant effect, but at 1/2 LD_{50}, patulin induced an important increase ($0.001 < p < 0.05$) in [^{3}H]TdR uptake specially in B cells.

In animals with KLH-*B. pertussis* stimulation (Table V), patulin and T-2 toxin, behave as adjuvants, increasing the [^{3}H]TdR uptake for all mitogens.

Discussion

Patulin was shown to be cytotoxic for rat alveolar macrophages in vitro, affecting mostly protein synthesis at 2 μM after 1 h [25]. Our results show that splenocytes are sensitive in vitro to patulin. Without mitogen, 0.01 μM patulin increased significantly [^{3}H]thymidine incorporation then decreased it in a dose dependent manner (ID_{50} 0.2 μM). Con A-stimulated lymphocytes were

Table II

Mitogenic response of murine (Balb/c) splenic cells treated in vitro with various concentrations of T2-toxin

Mitogens	None	Con A	PHA	PWM	LPS
Control	1470.41[a] ±120.38	38061.00 ±6373.78	23962.60 ±2185.80	18470.20 ±610.22	6970.61 ±777.45
T2-toxin 0.25 nM	1684.85 ±116.00	41811.20 ±16411.15	21139.00 ±1692.00	17758.90 ±676.37	5804.94 ±599.85
	115%[b]	110%	88%	96%	83%
0.50 nM	1583.85 ±148.86	54754.30 ±4273.70	24332.80 ±3559.84	18360.60 ±1146.34	5896.67 ±76.79
	108%	144%*	98%	99%	85%
0.75 nM	1471.27 ±66.61	47823.40 ±8750.32	16848.20 ±876.85	14685.80 ±903.66	3410.21 ±356.82
	100%	126%	70%**	80%**	49%**
1.0 nM	1108.09 ±177.98	61166.40 ±17927.97	19597.40 ±2336.45	11760.10 ±2225.50	2923.45 ±634.50
	75%	161%	82%	64%*	42%**
2.5 nM	315.44 ±37.49	605.12 ±176.81	1789.61 ±491.14	490.81 ±52.24	410.65 ±80.56
	21%***	2%**	8%***	3%***	6%***

[a] Values are means of dpm ± SD
[b] Variation in percent of control value
Significantly different from control group at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table III

The in vivo effects of T-2 toxin and patulin on the antibody production and delayed type hypersensitivity

Treatment	Antibody production (Mean $\log_2$ [1/titre] ± SD)[a]	Delayed hypersensitivity (mean mm swelling ± SD)[b]
Control	7.00±0.87	2.06±0.13
T2-Toxin (1/4 LD_{50})	5.33±0.87*	2.18±0.08
Patulin (1/4 LD_{50})	5.78±1.64	1.86±0.10*
Control	6.33±0.44	2.25±0.10
T2-Toxin (1/2 LD_{50})	5.27±1.01	2.16±0.08
Patulin (1/2 LD_{50})	5.83±1.34	2.04±0.06***

[a] By an indirect ELISA to KLH (titre = 2 × absorbance 405 nm of background)
[b] By indirect measurement of right footpad swelling between day 7 and day 8
Significantly different from control group at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table IV

Mitogenic response of murine (Balb/c) splenic cells of mycotoxins treated (1/4 and 1/2 LD_{50}) and control batches without KLH-Bp treatment

Mitogens	None	Con A	PHA	PWM	LPS
Control	479.00[c] ±89.10	76148.50 ±46709.30	22631.00 ±1907.80	58858.00 ±10367.60	34294.00 ±11839.80
T2-Toxin[a] 1/4 LD_{50}	5397.50 ±1043.00 1121%[d]*	139696.50 ±20485.69 184%	24045.00 ±2671.40 106%	60216.00 ±534.70 102%	20956.50 ±1315.90 61%
Patulin[b] 1/4 LD_{50}	3426.00 ±772.20 715%*	58375.00 ±11207.60 77%	34854.50 ±3913.80 46%	53269.50 ±262.30 90%	12050.50 ±2933.80 35%
Control	2345.78 ±586.75	52162.30 ±18888.32	56035.00 ±4830.35	26348.50 ±1383.24	11658.70 ±999.27
T2-Toxin[a] 1/2 LD_{50}	594.28 ±202.67 25%***	47821.95 ±14888.34 92%	28572.70 ±3154.36 51%***	8041.89 ±5023.79 30%***	5314.19 ±3014.22 46%*
Patulin[b] 1/2 LD_{50}	7644.71 ±2019.19 326%**	86054.72 ±15235.73 165%*	53091.33 ±7017.47 95%	46751.18 ±2421.58 177%***	53069.55 ±9150.87 455%***

[a] T2 Toxin: 1/4 LD_{50} = 0.8 mg/kg; 1/2 LD_{50} = 1.6 mg/kg
[b] Patulin: 1/4 LD_{50} = 2 mg/kg; 1/2 LD_{50} = 4 mg/kg
[c] Values are means of dpm ± SD
[d] Variation in percent of control value
Significantly different from control at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

stimulated at lower concentrations of patulin (10^{-4} μM) then strongly inhibited at higher concentrations. With PHA, a similar effect was observed but was not significant for stimulation at the lower concentration. Splenocyte proliferation was inhibited in a dose dependent manner either in the presence of PWM and LPS, (ID_{50} at 0.12 and 0.02 μM, respectively). The data suggest that very low concentrations of patulin enhanced Con A indiced stimulation of T-lymphocytes (227% at 10^{-4} μM). In the opposite, at the same concentrations of patulin, PWM and LPS stimulated B-lymphocytes and PHA stimulated T-lymphocytes were not altered. At higher concentrations, T and B-lymphocytes proliferation were strongly depressed, LPS stimulated B-lymphocytes being the more sensitive (10-fold). So, at 0.05 μM, patulin stimulated mitogen response to Con A whereas it depressed response to other mitogens. These results were in agreement to those obtained by Escoula et al. [20] or in vitro effect of patulin on mitogenic response of rabbit lymphocytes. DNA synthesis [25] was blocked at the same order of magnitude (0.5 μM) as protein synthesis

Table V

Mitogenic response of murine (Balb/c) splenic cells of mycotoxins treated (1/4 and 1/2 LD_{50}) and control batches with KLH-Bp treatment

Mitogens	None	Con A	PHA	PWM	LPS
Control	3622.70[c]	99255.30	13434.00	41900.70	42380.00
KLH-Bp	±2080.20	±32760.70	±4469.30	±9453.00	±7546.90
	3836.70	132954.30	25836.00	78206.30	47491.30
T2-Toxin[a]	±1518.60	±19326.50	±6515.30	±24895.40	±15731.90
1/4 LD_{50} KLH-Bp	106%[d]	134%	192%	187%	112%
	5853.00	107564.00	19885.70	67912.71	63051.30
Patulin[b]	±3000.90	±23339.00	±1402.00	±5092.60	±15899.40
1/4 LD_{50} KLH-Bp	162%	108%	148%	162%*	149%
Control	4579.43	89770.71	38589.90	36545.55	29190.07
KLH-Bp	±1043.97	±13140.26	±7348.90	±6170.82	±5723.87
	4122.13	168102.80	105335.90	57731.72	36847.63
T2-Toxin[a]	±2463.70	±31137.07	±23600.54	±32056.99	±9379.75
1/2 LD_{50} KLH-Bp	90%	187%**	273%***	158%	126%
	8046.31	143533.80	53078.53	51446.72	59188.95
Patulin[b]	±1592.34	±28317.68	±9038.44	±5021.71	±3694.02
1/2 LD_{50} KLH-Bp	176%**	160%**	138%*	141%**	203%***

[a] T2 Toxin: 1/4 LD_{50} = 0.8 mg/kg; 1/2 LD_{50} = 1.6 mg/kg
[b] Patulin: 1/4 LD_{50} = 2 mg/kg; 1/2 LD_{50} = 4 mg/kg
[c] Values are means of dpm ± SD
[d] Variation in percent of control value
Significantly different from control group at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

observed on macrophages (1 μM). T-2 toxin effects on mitogenic responses have been investigated using human lymphocytes [26–28] and murine lymphocytes [4, 17, 29]. Our results show that T-2 toxin at 2.5 nM blocks [^{3}H]TdR uptake ($0.7 < ID_{50} < 2$ nM) either in the absence or in the presence of mitogen (Con A, PHA, PWM, LPS) according to other investigators [4, 17, 28, 29]. However, at lower concentrations of T-2 toxin (0.25 to 1 nM) we have observed a more important stimulation of Con A-stimulated lymphocytes. Patulin is a less potent immunotoxic agent than T-2 toxin since ID_{50} is 100 fold higher.

In vivo, results showed that patulin reduced significantly DTH response, and altered slightly the anti-KLH antibody titre. T-2 toxin had no effect on DTH and decreased slightly the anti-KLH antibody production. Masuko et al. [30] observed an enhancement of DTH response and anti-SRBC antibody titre at higher doses of T-2 toxin (3 mg/kg) with toxin injections at days 0, 2 or 3 after SRBC stimulation. Otokawa et al. [31] proved the recovery of normal

DTH response and antibody production against SRBC in unresponsive mice treated by T-2 toxin two days after SRBC injection.

Splenocytes of mycotoxin treated animals were harvested on day 15 to assess mitogen responses. Effects of patulin and T-2 toxin were studied either with control or stimulated animals with KLH and *B. pertussis* antigens. Patulin generally increased splenocyte proliferation. It induced a mitogenic response as compared with control without mitogen, particularly in the animals which were not stimulated with KLH and *B. pertussis* antigens. Splenocytes of patulin treated animals (4 mg/kg) showed a higher proliferation response when stimulated with mitogens, except with PHA. Thymo-independent splenocytes (i.e. LPS stimulated cells) were the more sensitive. The effect of T-2 toxin depends on the immunological status of the treated mice and on the injected dose. At the greater dose (1.6 mg/kg) a significant decrease in [^{3}H]TdR incorporation was observed in non-stimulated mice, whereas T-2 toxin enhanced responses to mitogen in KLH/*B. pertussis* stimulated mice.

Previous studies have demonstrated the inhibitory properties of patulin and T-2 toxin towards incorporation of labeled precursors into DNA, RNA and protein synthesis in vitro [25, 32]. T-2 toxin is a more potent inhibitor than patulin. There is not real correspondence between in vitro and in vivo studies, since in our experiments both mycotoxins enhanced the splenocytes proliferation of antigen stimulated mice, but did not alter significantly delayed type hypersensitivity and anti-KLH antibody production. Immunomodulatory effects of patulin and T-2 toxin depend on immunological status of mice.

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NEW O ANTIGENS OF *MORGANELLA MORGANII* AND THE RELATIONSHIPS BETWEEN HAEMOLYSIN PRODUCTION. O ANTIGENS AND MORGANOCIN TYPES OF STRAINS

S. Vörös and B. W. Senior

Institute of Microbiology, University Medical School, Pécs, Hungary, and Department of Medical Microbiology, Dundee Medical School, Ninewells Hospital, Dundee, Scotland, UK

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A collection of 142 strains of *Morganella morganii*, principally from unrelated patients' faeces was examined to determine the relationship, if any, between haemolysin production, O antigen and morganocin production/sensitivity type. Only 55 (38.7%) were agglutinable with the existing 44 O antisera. However, when O antisera were raised to some of the non-typable strains 11 new O antigens were found and 126 (88.7%) of the strains were typable. The number of O antigenic groups in *M. morganii* is now 55. It was confirmed that the O antigenic characteristics of strains were independent from morganocin producer types. An epidemiological retrospective survey showed that finer strain recognition in *M. morganii* can be achieved by using both methods than either method alone. Approximately 30% of strains were haemolytic. The ability to produce haemolysin was more common in strains of certain O serogroups and morganocin producer types than in others.

Morganella morganii strains are occasionally isolated from clinical specimens (blood, sputum, pus, wound exudate [1] and infections of the urinary tract [2, 3]). They are less frequently the cause of urinary tract infections than *Proteus mirabilis* [4] because of their slower growth rate in urine and their inability to rapidly make it alkaline [5]. Some strains of *M. morganii* may be a cause of diarrhoea in man [6–10]. *M. morganii* strains have also been associated with nosocomial outbreaks of infection [11] with serious morbidity and mortality [12].

It has been shown that certain haemolytic strains of *M. morganii* are highly virulent for mice causing haemolytic/haemorrhagic lung oedema and death, usually within 4 h of intranasal infection [13]. It has been proved that the haemolysin of *M. morganii* is calcium dependent [14] and closely related at the molecular level to the known virulence factor of *Escherichia coli* alpha-haemolysin [15].

Sándor Vörös
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

B. W. Senior
Department of Medical Microbiology,
Dundee Medical School, Ninewells Hospital
Dundee DD1 9SY, Scotland, UK

Fine strain recognition in *M. morganii* is best achieved either by serotyping or bacteriocin typing. The antigenic schema of Rauss et al. [16, 17] at present recognizes 77 serotypes comprising 44 O antigens, 4 K and 38 H antigens, although the existence of more serotypes has been predicted. *M. morganii* strains isolated in Scotland have been differentiated into 50 morganocin producer types [18] and recently a further 15 new morganocin producer types have been found among non-British isolates of *M. morganii* [19].

The purpose of this study was to determine whether or not haemolysin production in *M. morganii* is associated with a particular O antigen or morganocin type and whether or not O serotyping is independent from morganocin typing as has been suggested in our previous study involving fewer strains [19].

Materials and methods

Bacterial strains. These were 142 of the 160 *M. morganii* strains isolated in Scotland and used for the determination of morganocin p/s type [18]. The strains were from 128 patients, most from their faeces and were maintained in pure culture on both Nutrient Agar (Na, Oxoid CM3) and Dorset's egg slopes in screw-capped bottles.

Biochemical tests. The identity of the strains was examined by conventional biochemical tests as described by Cowan [20].

Serological tests. The O antigens of the strains were determined by our standard methods [16, 17, 21, 22] by slide-agglutination tests in pooled O antisera on colonies from NA plates incubated overnight at 37 °C, and subsequently by slide and tube-agglutination tests in the respective monovalent O antisera. Strains that agglutinated in saline alone were described as "rough". Strains which did not agglutinate in any of the O antisera pools were said to be "non-typable". Some of these strains were selected at random and O antigen suspensions prepared and O antisera raised to them as previously described [16, 17, 19, 21, 22].

Haemolysin production. This was examined for both on solid and in liquid media and against different red blood cell types. Strains were plated out on NA plates supplemented with defibrinated sheep red blood cells 5% v/v. The plates were incubated overnight at 37 °C and examined for zones of haemolysis surrounding well-isolated colonies. The plates were reexamined 24 h later after incubation at room temperature. The strains were also examined for haemolysin production in nutrient broth (NB, Oxoid CM67) to horse red blood cells as previously described [14].

Bacteriocin typing. Morganocin production (p) per morganocin sensitivity (s), p/s typing, was performed as previously described [18].

Results

Biochemical tests confirmed that all strains were *M. morganii*. All produced urease, phenylalanine deaminase and indole, and fermented glucose, galactose and mannose but not lactose, sucrose, adonitol or inositol. All were negative in H_2S, citrate and Voges-Proskauer tests but positive in the methyl red test. All decarboxylated ornithine but not lysine.

Serological analysis. Using the existing 44 O antisera, only 55 (38.7%) of the 142 *M. morganii* strains were agglutinable and these were of 20 different

O antigenic groups (Table I the non-starred serogroups). Four strains (2.9%) were rough and 83 (54.4%) were non-typable. From these strains 11 were selected at random and O antisera raised to them. Cross-agglutination tests were performed on slides with O antisera diluted 1:20 and in dilutions in tubes. No significant cross-reactions were detected among the new O antigens.

Table I

The serogroups, haemolytic reactions, morganocin types and morganocin indicators of the M. morganii strains examined

Sero-group	No. of strains	No. of haemolytic strains and type (p) of morganocins produced		No. of non-haemolytic strains and type (p) of morganocins produced		Morganocin indicator strain No.
O1	9	4	p0, p1	5	p1, p44	Mg22, Mg39, Mg115
O2	1	0		1	p0	Mg121
O7	1	0		1	p37	
O13	1	0		1	p27	
O15	1	0		1	p34	
O17	1	0		1	p30	
O21	1	0		1	p0	
O22	1	0		1	p0	
O23	1	0		1	p0	
O24	1	0		1	p33	
O25	12	5	p2, p7, p8, p10, p40	7	p8, p25, p42, p43, p49	Mg8
O26	2	1	p21	1	p39	
O27	2	0		2	p0, p40	
O29	4	2	p23, p48	2	p0, p20	Mg27, Mg84
O30	1	1	p38	0		
O31	1	1	p21	0		Mg143
O32	2	0		2	p40, p41	Mg53
O37	4	4	p4, p10, p40	0		
O40	6	3	p1, p11, p26	3	p1, p46	Mg64
O44	3	0		3	p0	
O45*	3	0		3	p0, p2, p30	Mg49
O46*	16	1	p19	15	p0, p1	Mg77
O47*	23	10	p0, p1, p5, p29, p36	13	p0, p1, p3, p6, p9, p14, p21	Mg11, Mg23
O48*	16	6	p1, p23, p38, p40	10	p0, p1, p17, p18, p22	Mg3, Mg27, Mg100, Mg126
O49*	3	1	p32	2	p25	Mg1
O50*	1	1	p40	0		
O51*	2	0		2	p24, p40	
O52*	2	1	p40	1	p0	
O53*	2	0		2	p28, p43	Mg26
O54*	2	0		2	p31, p33	
O55*	1	0		1	p12	
Rough	4	0		4	p0, p1, p40	
Non-Typable	12	2	p1, p13	10	p0, p1, p25, p35, p38	
Total	142	43 (30.2%)		99 (69.8%)		

* New O serogroup

The hitherto untyped strains were then tested in slide- and tube-agglutination tests with the 11 new O antisera and 71 (85.5%) of them were now agglutinable in these new O antisera (Table I the starred O antigens). The new O antigenic type strains are Mg110 (O45), Mg77 (O46), Mg123 (O47), Mg3 (O48), Mg1 (O49), Mg51 (O50), Mg102 (O51), Mg151 (O52), Mg26 (O53), Mg112 (O54), Mg139 (O55). We believed the remaining 12 non-typable strains belong to one or more new O serogroups yet to be defined (manuscript in preparation).

Thus, 126 (88.7%) of the 142 strains were now typable into 31 different O antigenic groups, 12 (8.4%) strains remained non-typable. It was noted that 71 (50%) of the Scottish strains belonged to the newly defined O serogroups and 90 (71.4%) of these belonged to only eight serogroups. These were, in order of frequency, O47 (23 strains), O46 (16 strains), O48 (16 strains), O25 (12 strains), O1 (9 strains), O40 (6 strains), O29 and O37 (4 strains each). The remaining 36 typed strains were distributed among the other 23 different O groups (Table I).

Haemolysin production. Of the 142 *M. morganii* strains examined 43 (30.2%) were haemolytic on solid and liquid media.

Haemolytic activity and O antigenic group. Of the 126 serogrouped strains there were 41 (32.5%) that were haemolytic and 85 non-haemolytic which were distributed among 27 different O antigenic groups (Table I). Because of the small number of strains in some O groups it was not possible to state with certainty for such strains if there was a correlation between O antigen and the ability to produce haemolysin. However, it was noted that 32 (78%) of the 41 typed haemolytic strains belonged to only six of the 31 O groups, namely O1 (4 strains), O25 (5 strains), O37 (4 strains), O40 (3 strains), O47 (10 strains), and O48 (6 strains), and the remaining nine haemolytic strains were distributed among eight other O serogroups (Table I). On the other hand, strains of some O serogroups were rarely found to be haemolytic. Thus none of the three O45 strains and only one of the 16 O46 strains was haemolytic. It appeared therefore that haemolytic activity was more frequently associated with certain O antigens than others (Table I).

Morganocin type and O antigenic group. The possible association of morganocin type with O antigen was assessed. Of the original 50 different morganocin type-producers [18], 46 strains were available for O antigen analysis. Of these, 44 were serogrouped into 22 O groups and two strains were non-typable. The distribution of morganocin type-producers in the O serogroups is presented in Table I. It was noted that many different morganocin type-producers could be of the same O serogroup. For example, in the O25 serogroup were strains producing nine different morganocin types. In the O47 serogroup were strains producing 10 different morganocins and the O48 serogroup had strains producing eight different morganocins. It was also observed that strains producing common morganocins could be of many different O serogroups.

For example, morganocin type 40 producers were of eight different O serogroups, and morganocin type 1 producers were of five different O serogroups. Thus the ability to produce a particular morganocin type appeared to be independent from the O antigen of the strain and vice versa.

Of the 23 morganocin indicator strains [18] 20 were available for O antigen determination and these were of 13 different O groups. The distribution of the morganocin indicator strains among the O serogroups is also presented in Table I. It was noted that serogroup O48 contained four indicator strains, the serogroup O1 had three indicator strains and each of the serogroup O29 and O47 contained two indicator strains. The remaining nine indicator strains were equally distributed among nine other O serogroups (Table I).

Haemolytic activity and morganocin type. An analysis of the haemolytic activity of strains of the common morganocin p-types is presented in Table II. Of the 142 strains examined, 92 (64.8%) belonged to five morganocin p-types: p-type O (25 strains), p-type 1 (46 strains), p-type 40 (11 strains), p-type 35 (6 strains) and p-type 38 (4 strains). The remaining 50 strains belonged to 39 other morganocin p-types.

The 25 morganocin p-type O, that is, strains unable to produce morganocins active against any of the standard indicator strains, were of 13 different O groups, four strains were non-typable and one was rough, yet only three (12%) strains were haemolytic. These were of O groups commonly found to be haemolytic. Most of the non-haemolytic strains of this morganocin p-type were of O groups not commonly found to be haemolytic. There were six morganocin p-type 35 strains which were of two different O serogroups,

Table II

The serogroups and haemolytic activities of morganii strains of common morganocin p type

Morganocin type (No. of strains)	No. of haemolytic strains	Serogroup (and No.) of haemolytic strains	No. of non-haemolytic strains	Serogroup (and No.) of non-haemolytic strains
p0 (25)	3	O1 (1), O47 (2)	22	O2, (1), O21 (1), O22 (1), O23 (1), O27 (1), O29 (1), O44 (3), O45 (1), O46 (3), O47 (2), O48 (1), O52 (1). Non-typable 4, rough 1
p1 (46)	12	O1 (3), O40 (1), O47 (5), O48 (2). Non-typable 1	34	O1 (4), O40 (2), O46 (12), O47 (6), O48 (6). Non-typable 3, rough 1
p40 (11)	6	O25 (1), O37 (2), O48 (1), O50 (1), O52 (1)	5	O27 (1), O32 (1), O51 (1). Rough 2
p35 (6)	0		6	O25 (3), O49 (2). Non-typable 1
p38 (4)	3	O30 (1), O48 (2)	1	Non-typable 1

one strain being non-typable, yet no one was haemolytic although 50% of the non-haemolytic strains were of serogroup O25, a serogroup commonly found to contain haemolytic strains. On the other hand, 26% (12 strains) of the 46 morganocin p-type 1 strains of five different O groups were haemolytic. Of the 11 morganocin p-type 40 strains of eight different O groups, 54% (6 strains) were haemolytic, and 75% (3 strains) of four morganocin p-type 38 strains of two different O serogroups were haemolytic (Table II). Thus it appeared that haemolysin production was more commonly associated with certain morganocin p-types (p-type 1, 38 and 40) than others, like morganocin p-type 23. This association could not be explained on the basis of the O antigen of the strain.

Application of morganocin p/s typing and O serotyping to an epidemiological survey. Both morganocin p/s typing and O serotyping were done on a number of strains isolated from several patients submitting more than one specimen over limited period (Table III).

Case 1. The three isolates from three specimens taken on the same day were of the same morganocin p/s type (1M) and also the same serogroup (O1). This indicates the reproducibility of both typing methods.

Case 2. This probably illustrates a similar point to that made in Case 1 for strains isolated over 2 days.

Case 3. Four strains were isolated over 4 days and all were of the same p/s type (ON) and 3 of them were of the same serogroup (O44) but 1 was of a different serogroup (O45). This fact may indicate the carriage by the patient of another strain with different O antigen. It would have been undetected if serotyping had not been performed. It also demonstrates the independence of the two typing system.

Case 4. Three isolates of the same p/s type (25R) were obtained from a baby (Case 4a) over a period of about two weeks. Two of the isolates were also of the same serogroup (O25) but the one isolated last belonged to a different serogroup (O49). The baby's mother (Case 4b) carried both strains. This case may represent a proven cross-infection with two distinct clones. One month later the baby's father (Case 4c) was found to carry the same strain (p/s type 25R, serogroup O25) as that originally carried by the baby.

Case 5. This possibly indicates that a particular strain (morganocin p/s type OA serogroup O46) can persist in the gut for nine days.

Case 6. This indicates a point similar to that in Case 3.

Case 7. This possibly demonstrates the carriage of a particular strain for one month.

Case 8. This represents two brothers who carried at different times two strains which were seemingly related but distinct by p/s typing but clearly quite different on the basis O antigens. It illustrates the benefit of using both typing methods together.

Table III

Epidemiological survey of the types of M. morganii strains isolated from patients submitting several specimens

Case No.	Sex	Age	Date of sampling	Strain No. of isolate	Morganocin p/s type of isolate	Serogroup of isolate
1	M	1 year	10 Sep	Mg 20	1M	O1
			10 Sep	Mg 21	1M	O1
			10 Sep	Mg 22	1M	O1
2	F	29 years	2 Nov	Mg 72	40Q	rough
			3 Nov	Mg 73	40Q	rough
3	M	3 months	12 Sep	Mg 24	0N	O44
			14 Sep	Mg 25	0N	O45
			15 Sep	Mg 28	0N	O44
			15 Sep	Mg 29	0N	O44
4a	M	6 months	22 Oct	Mg 57	25R	O25
			22 Oct	Mg 60	25R	O25
			8 Nov	Mg 76	25R	O49
4b	F	26 years	22 Oct	Mg 59	25R	O49
			2 Nov	Mg 69	0Q	Non-typable
			2 Nov	Mg 70	0Q	Non-typable
4c	M	—	22 Nov	Mg 93	25R	O25
5	M	75 years	10 Nov	Mg 78	0A	O46
			19 Nov	Mg 90	0A	O46
6	F	—	26 Nov	Mg 98	1M	O48
			26 Nov	Mg 99	1M	O46
7	F	8 months	5 May	Mg 5	1A	O46
			5 Jun	Mg 14	1A	O46
8	M	—	18 Jun	Mg 12	11B	O40
	M	—	30 Apr	Mg 13	10H	O25
9	M	55 years	29 Sep	Mg 41	18R	O48
			6 Oct	Mg 45	17R	O48

Case 9. This demonstrates that isolates which appeared to be the same by O antigen analysis were distinguishable by p/s typing. Again it shows the benefit of using both typing methods for fine strain recognition in epidemiological studies.

Discussion

In this study, the relationships of three characteristics which are important in fine strain recognition and possibly virulence in *M. morganii*, namely, O antigen, morganocin type and haemolytic activity have been examined. The finding that only 38.7% of isolates were agglutinated with some of the existing 44 O antisera indicated that many of these Scottish strains of *M. morganii* were different from those isolated mainly in Hungary and to which the typing

sera had been raised. This was confirmed when it was found that 50% of the strains belonged to 11 newly defined O serogroups thus extending the number of serogroups in *M. morganii* to 55. O antisera are presently being raised to the remaining 12 non-typable strains and it is anticipated that additional new O antigens will be found. The strains are also being examined to determine whether or not they carry new K and H antigens.

Our previous study [19] suggested that morganocin p/s type characteristics were independent from those of the O antigen and that finer strain recognition in *M. morganii* could be achieved by the combination of both methods than either method alone. The results of this study confirm these previous data. A given O serogroup often contained several different morganocin p-type strains and several indicator strains. Furthermore, among the strains producing a given morganocin type were representatives of several different O serogroups. The clearest evidence for the independence of the two typing schemes was found in the epidemiological study where it was shown that some strains of a given morganocin p/s type could be of more than one O serogroup and that some strains of a given O serogroup could be of more than one morganocin p/s type.

The ratio of the strains (principally of faecal origin) found to be haemolytic on both solid media to sheep erythrocytes and in broth to horse erythrocytes was 30.2%. This ratio is similar to that of 33% found by Rauss [10]. The proportion of haemolytic strains in *M. morganii* isolates from non-faecal samples (purulent exudates, blood, CSF, etc.) may be higher as is the case in *E. coli*. Because of the small number of isolates in some O groups, it was not possible to state with certainty for these strains if there was a correlation between O antigen and haemolysin production. It was found, however, that 78% of the haemolytic strains determined belonged to only six of the 31 O serogroups (O37, O40, O1, O47, O25 and O48) and that strains of certain serogroups (O44, O45 and O46) were rarely haemolytic.

The relationship, if any, between morganocin p/s type and haemolysin production was difficult to assess. Concentrating on the more common morganocin types with respect to the distribution of these types among the different O serogroups, some of which are more likely to contain haemolytic strains than others, it appeared that isolates of morganocin p-types O and 35 were less likely to be haemolytic than those of morganocin p-types 1, 38 and 40.

We have discovered 11 new O antigens among Scottish isolates of *M. morganii*, confirmed the independence of the morganocin typing and serotyping schemes and shown some relationships between haemolysin production and the presence of certain O antigens and possibly relationships between haemolysin production and the production of certain types of morganocin. Our task in the future will be to examine more isolates of *M. morganii*, preferably of non-faecal origin and from other parts of the world before it can be stated

with certainty that an association exists between haemolysin production as a virulence marker and the O antigenic and morganocin type characteristics of *M. morganii*.

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ASSOCIATION BETWEEN CHEMILUMINESCENCE STIMULATING AND IL-2 INDUCING ACTIVITIES OF *STAPHYLOCOCCUS AUREUS* STRAINS IN HUMAN AND MOUSE MONONUCLEAR CELLS

YOSEF ASSEFA MENGESHA, KLÁRA MEGYERI and I. ROSZTÓCZY

Institute of Microbiology, Albert Szent-Györgyi University Medical School, Szeged

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The plastic adherent fraction of human mononuclear cells (MNC) responded with maximal chemiluminescence (CL) upon stimulation with $\geq$1000 bacteria per cell of heat killed preparations of *Staphylococcus aureus* strains. Different strains had different CL stimulating activities and their sequences were similar on MNC from different blood donors. IL-2 inducing and CL stimulating activities seem to be parallel features of *S. aureus* strains, since their sequence set ups established on the basis of these two properties were almost identical. The same phenomenon could also be observed in a mouse system, in which activated peritoneal cells (PC) were the most active CL exhibiting population. The IL-2 inducing activity of staphylococci in mouse spleen cells and their CL stimulating activity in activated PC followed a similar pattern too. The sequences of CL inducing activities of different staphylococcal strains were in good agreement in human and mouse cells. Representative strains with high, moderate and low CL inducing activities followed the same sequence of virulence for mice.

In a recent study we observed that the IL-2 inducing activities of staphylococci can be characteristic for different strains [1]. In the course of elucidation of responding cells we found that this activity was confined to the nonadherent E-rosette positive human MNC population. Since invading bacteria in vivo comprise the target of several cell types participating in the nonspecific and specific host defences we decided to analyse the reactivity of another cell population, the plastic adherent fraction of peripheral blood MNC. We determined their luminol mediated CL response to the staphylococci, which is considered to be a good indicator of the level of stimulation of these cells [2]. With the species specificity of the phenomenon in mind, we extended our studies to mouse PC and investigated their CL response in both their residual and activated forms.

YOSEF ASSEFA MENGESHA, KLÁRA MEGYERI, ISTVÁN ROSZTÓCZY*
Institute of Microbiology, Albert Szent-Györgyi University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

* Corresponding author

Materials and methods

The staphylococcal strains, their numbering, the preparation of heat killed suspensions of bacteria, the IL-2 induction and the assay were the same as described previously [1].

Human mononuclear cells (MNC) were isolated on Ficoll-Uromiro from the peripheral blood of individual healthy blood donors [3]. Separation of the plastic adherent cell population was performed on fetal calf serum treated plastic Petri dishes [4]. This plastic adherent cell population contained 80–90% monocytes, as determined on the basis of their morphology in May–Grünwald–Giemsa stained smears and nonspecific esterase positivity [5].

Resident mouse PC were collected from animals killed by cervical dislocation, by rinsing the peritoneal cavity with 10 ml of RPMI medium. Activated PC were obtained in the same way from mice that had been injected intraperitoneally with 10 ml of brain heart infusion broth (BHIB) (Difco) 24 h before the collection of cells.

Mouse strains (Balb/c, CBA and CFLP) were purchased from The Biological Research Center of the Hungarian National Academy of Sciences (Szeged) and from the LATI Laboratory Animal Breeding Farm (Gödöllő, Hungary), respectively.

Induction and measurement of CL. The luminol mediated CL was determined [6]. Heat killed or live staphylococci were added in 1 : 10 volume to the suspensions of responding cells at a ratio of 100 bacteria/cell. Following incubation at 37 °C for 5 min in 5% CO_2 in air atmosphere, 20 volumes of 1.1 μM luminol solution were added to the bacterium–MNC mixtures. CL was measured in a Packard Tricarb model 4530 scintillation spectrometer for 12 s at one-minute intervals in the in-coincidence summation mode of operation [6]. The cpm values were plotted on co-ordinate paper and the highest activity detected was used to characterise the individual strains. The individual variation in the CL response of the cells from various blood donors was eliminated by the introduction of the relative CL stimulating activity. This was calculated by comparing the CL responses of cells treated with PBS or staphylococcus. The CL inducing relative activity therefore indicates how many times higher the peak cpm value in the cell culture induced by the particular staphylococcal strain was in comparison with that observed in the PBS treated control.

The mouse virulence of the staphylococci was determined by calculation of the LD_{50} values in c.f.u./ml with the Reed–Muench method [7]. The serial dilutions of bacteria grown in BHIB were each inoculated intravenously into groups of 10 mice and calculations were performed on the basis of the cumulated lethality 7 days after infection.

The correlation between sequences of CL and IL-2 inducing activities was evaluated statistically with determination of the Spearman rank correlation coefficient when two ranks of order were compared. The Kendall coefficient of concordance was applied when more than two ranks of order were analysed.

Results

To investigate the dose-response relationship of the CL response we prepared different bacterium : MNC ratios. The results indicated that 50–100 bacteria per cell induced a considerable CL activity, but the maximal response was achieved at a ratio of 1000 : 1 (Fig. 1). Analysis of the responding cell population indicated that the plastic adherent cells, representing 15–20% of the MNC fraction, exhibited the same level of CL as did a proportionally higher number of unseparated MNC (Table I).

In the course of the present study, we found an up to three-fold individual variation in the intensity of CL of the MNC of various blood donors. Therefore, it was a question of interest for us whether the sequence of inducer activity of various staphylococcal strains was reproducible in the cells of donors with different activities of CL response. In order to answer this question,

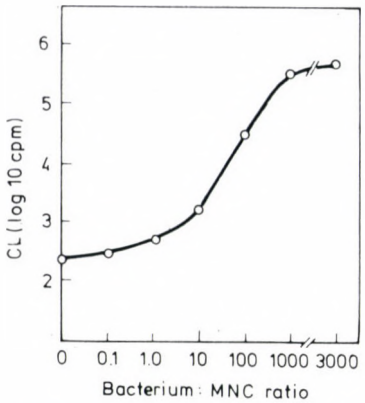

Fig. 1. Dose-response relationship of the chemiluminescence stimulating activity of heat-killed *S. aureus*

Table I

Chemiluminescence of different fractions of human MNC stimulated with heat-killed S. aureus

MCN fractions	Cell/ml	Chemiluminescence (log 10 cpm)	
		S. aureus	control
Unseparated	5×10^6	5.6	1.4
Plastic adherent	8×10^5	5.5	1.6
Nonadherent	4.1×10^6	2.7	1.3

we compared the sequences of the inducer activities of the investigated staphylococci on the MNC of three individual donors.

Table II lists the strains according to the order of magnitude of the elicited CL response. Identical positions for different blood donors are connected by horizontal bars. We found an especially good agreement for strains that exceeded or that remained below the average activity. As concerns the overall correlation, the Kendall coefficient of concordance indicated a significant agreement among the three sequences.

We also compared the sequence of the CL stimulating activity of the different strains with their recently published sequence of IL-2 inducing activity [1]. As can be seen in Table III, five strains (SA-1, 4, 10, 7 and 9) occupied the same position in both activity sequences, while another five differed only in one position, but for one strain each there was a difference of two or of three positions between the CL and IL-2 inducing activities. The Spearman rank correlation coefficient indicated a significant correlation between these two sequences.

Table II

*Sequence of chemiluminescence stimulating activity of S. aureus strains**

Blood donor		
I	II	III
sequence of SA strains		
2	1	1
1	2	2
3	3	3
10	4	4
5	10	10
4	5	6
6	7	5
7	6	8
11	8	9
9	9	7
8	12	11
12	11	12

Kendall coefficient of concordance = 0.8493 (p = 0.0032)
* Determinations on the MNCs of three individual blood donors

For optimal determination of the CL inducing activity of staphylococci in mouse cells we compared the responsiveness of residual and activated PC and that of spleen cells. The activated PC was found to exhibit the most intensive CL response. However, we observed the same sequence of the representative inducers in the activated PC and in the marginally responsive

Table III

Sequence of chemiluminescence stimulating and IL-2 inducing activities of S. aureus strains

Chemiluminescence		IL-2
sequence of SA strains	relative activity	sequence of SA strains*
1	126	1
2	110.5	3
3	87.6	2
4	60	4
10	56.7	10
5	40.5	8
6	30	5
7	25.7	7
8	23.7	6
9	21	9
11	17.2	12
12	12.6	11

* From reference 1
Spearman rank correlation coefficient = 0.93706 ($p < 0.0001$)

Table IV

Chemiluminescence inducing activity of staphylococci in mouse spleen cells, in residual and activated mouse peritoneal cells

Strain	Chemiluminescence (cpm)*		
	spleen cells	peritoneal cells	
		residual	activated
SA-1	69	340	20336
SA-2	77	371	25180
SA-9	51	138	7760
SE-1	48	70	270
—	25	30	143

* Average of three independent experiments

residual PC. The spleen cells did not react with a reproducible CL response under the present experimental conditions (Table IV).

We compared the IL-2 and CL inducing activities of 4 staphylococcal strains on the cells of 3 different mouse strains. The relative inducer activities are listed in Table V. The sequences of the strains were identical in the two tests and they behaved similarly in the cells of the 3 different strains of mice, too. We next compared the CL inducing activities of staphylococcal strains in mouse cells with those in human cells. A good agreement was found, since 4 out of 12 occupied an identical position for human and mouse cells, while for a further 6 the difference was only one place. Two strains differed in two places at most. The Spearman rank correlation coefficient was significant in this case too (Table VI). Comparison of the time frame and the magnitude of the CL response of MNC and activated mouse PC indicated very similar patterns. The magnitude of the CL response of mouse cells was somewhat superior to that of human cells. However, the sequence of activity of the investigated strains was the same in both systems (Fig. 2). The observed correlation between the IL-2 and CL inducing activities in human and mouse cells led us to

Table V

Chemiluminescence and IL-2 inducing relative activities of staphylococci in cells of CFLP, CBA, and Balb/c mice

Strain	IL-2*			Chemiluminescence**		
	Balb/c	CBA	CFLP	Balb/c	CBA	CFLP
SA-1	9.9	10.5	13.2	132	120	143
SA-2	10.1	11.8	15.9	126.8	134	175
SA-9	3.1	3.3	3.4	42	27.3	49
SA-1	1.6	1.8	1.7	3.4	2.1	4.6

* in spleen cells
** in activated PC

Table VI

Sequence of chemiluminescence inducing activities of staphylococcal strains in human and mouse cells

Human MNC		Mouse PC	
sequence of SA strains	CL (cpm)	sequence of SA strains	CL (cpm)
1	21450	2	27388
2	18777	1	24225
3	14891	3	18445
4	10200	4	14310
10	9644	5	12558
5	6879	10	9940
6	5115	8	7822
7	4374	7	7687
8	4024	6	6510
9	3573	9	6482
11	2930	12	4565
12	2146	11	4291

Spearman rank correlation coefficient = 0.78322 ($p = 0.0026$)

hypothesise that the intensity of host reactions represented here by IL-2 and CL responses might correlate to the virulence of bacteria. In order to answer this question, we decided to compare the virulence for mice of representative strains of staphylococci with high, moderate or low CL stimulating activity. Before the determination of the LD_{50} values of the selected strains, we tested the CL inducing activity of their viable forms. The comparison of the activities of heat killed and live preparations did not reveal any difference between them (Table VII). Besides the virulence test, the IL-2 inducing and CL-stimulating activities of the selected strains were also determined simultaneously. We detected the lowest LD_{50} values for SA-2 and SA-1, which were the most active inducers of IL-2 production and CL response. They were followed by SA-9. For SE-1, we could not determine the LD_{50} dose, since inoculation of undiluted BHIB cultures of this bacterium did not kill the animals (Table VIII).

Discussion

The *Staphylococcus aureus* species possesses multiplex factors of virulence [8–10]. One can assume that strains expressing a quantitatively or qualitatively more efficient set of these factors might represent a stronger stimulus for host defences. For the staphylococci investigated in the present study, the IL-2 inducing activity in T lymphocytes and the stimulation of CL in monocytes seemed to be quasi-characteristic and parallel attributes of the individual

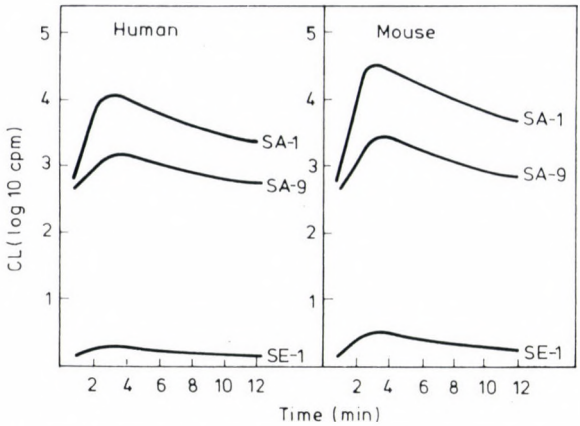

Fig. 2. Kinetics of the chemiluminescence response in human and mouse cells stimulated with heat-killed staphylococci

Table VII

Chemiluminescence inducing activity in activated mouse peritoneal cells of heat-killed and live staphylococci

Strains	Chemiluminescence (cpm)*	
	heat-killed	live
SA-1	21400	20907
SA-2	24590	26662
SA-9	7193	6873
SE-1	257	305

* Average of three independent determinations

Table VIII

Virulence for mice and chemiluminescence and IL-2 inducing activities of staphylococci

Strains	LD_{50} (log 10 c.f.u./g)	IL-2*		Chemiluminescence**	
		cpm	relative activity	cpm	relative activity
SA-1	4.18	7856	(10.6)	27450	(160)
SA-2	4.02	8811	(11.9)	27710	(170)
SA-9	5.74	2612	(3.5)	8112	(50)
SE-1	>7.78	1256	(1.7)	310	(1.9)

* In spleen cells
** In activated PC

strains. Preliminary experiments with representative strains with different inducer activities indicated that these properties correlated with the virulence of the bacteria. The observed correlation permits the assumption that the same or closely related factors are responsible for these activities. Our previous results [1] seem to argue against the participation of some already known factors of pathogenicity in the IL-2 inducing activity of staphylococci; therefore, the role of a so far unidentified constituent cannot be excluded. The isolation and chemical analysis of the IL-2 and CL inducing principle would greatly promote the elucidation of this enigma. In the continuation of the present work, our current experiments are targeted on this topic.

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POTENTIAL *SHIGELLA FLEXNERI* 2A AND *SHIGELLA SONNEI* I LIVE VACCINAL STRAINS. CHARACTERIZATION OF IMMUNOGENICITY IN ANIMAL MODELS

V. Denchev, Tatiana Sumerska, Snejina Marinova, Tch. Vassilev, Iskra Panova and K. Linde

Institute of Infectious and Parasitic Diseases, Sofia, Bulgaria, and Institute of Medical Microbiology and Epidemiology, Karl Marx University, Leipzig, FRG

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Rats and rabbits were immunized intraintestinally with different doses of virulent and nonvirulent live *Shigella flexneri* 2a and *Shigella sonnei* I strains. The nonvirulent strains had one or two attenuating markers. The period of excretion with the faeces of the bacteria and of their polysaccharide antigens, the proliferation of antigen-binding and antibody-producing cells in the spleens and gut-associated lymphoid tissues and the levels of antibodies in sera and faeces were studied. Attenuated strains *S. flexneri* 2a 77 and *S. sonnei* I 3359 induced the most potent and long lasting local immune response.

A promising approach for immunoprophylaxis of bacterial dysentery is the use of live attenuated *Shigella* strains for oral vaccination. A number of strains have been constructed by us and the stability of attenuation as well as the ability to invade gut epithelial cells have been studied [1]. The next step in the evaluation of these strains is the characterization of their immunogenicity in animal models. It is known that the only animals in which shigellae cause a disease analogous to that in humans are some primates. As such animals are not available to us another animal model for studying the development of the local immune response in short-term and long-lasting experiments following a single or multiple immunization is needed [2–4]. As protective immunity to shigellae is mainly due to the presence of secretory antibodies in the gut, the animal model should permit the study of the proliferation of immunocompetent cells in the gut-associated lymphoid tissues in response to immunization as well as the kinetics of the systemic and the local antibody responses [5]. Studies on the animal models used permit the comparative evaluation of the different attenuated strains available and to select those

V. Denchev, Tatiana Sumerska, Snejina Marinova*, Tch. Vassilev, Iskra Panova
Institute of Infectious and Parasitic Diseases
Vladimir Zaimov 26, 1504 Sofia, Bulgaria

K. Linde
Institute of Medical Microbiology and Epidemiology, Karl Marx University
Leipzig, FRG

* Corresponding author

with the best parameters for the potential use as live oral vaccines. Results from experiments on the rat and rabbit models characterizing some of the *Shigella* strains studied in our laboratory are presented in this communication.

Materials and methods

Bacterial strains. Shigella flexneri 2a: (i) strain 9108 — wild type, highly virulent, isolated from a patient in Bulgaria; (ii) strain 75 Pur — with one attenuated marker and residual virulence (purine auxotrophic); (iii) strains 77, 78 and 83 Pur^-/Rif^r — with two attenuating markers — purine auxotrophic and a virulence-reducing mutation with a metabolic drift.

Shigella sonnei I: (i) strain 1180 — wild type, highly virulent isolated in Bulgaria; (ii) strain 2923 Pur^- 5/6 with one attenuating marker — purine auxotrophy and with residual virulence; (iii) strains 3357, 3359 and 3361 — Pur^-/Rif^r — with two attenuating markers — purine auxotrophic and with a virulence reducing mutation. All strains (wild and attenuated) carried the high-molecular virulence plasmids [1].

The methods for construction, selection and primary characterization have been described before [1, 6]. Those strains to be used for enterial immunization were grown on nutrient agar overnight and suspended in saline. The number of bacteria was determined by using the Vth International optical standard of the World Health Organization for enteric bacteria. In all cases the number of live bacteria — colony forming units (c.f.u.) was determined by plating on agar serial dilutions ($1 : 10^2$, $1 : 10^3$ and $1 : 10^4$) of the suspensions.

Experimental animals. Guinea pigs weighing 180–200 g were used for the evaluation of the virulence of the strains by the keratoconjunctivitis test [7].

Inbred Wistar and Long Evans rats weighing 180–200 g were used in short-term (7–15 days) experiments for studying the response of the immunocompetent cells in the spleen and the gut-associated system to the immunization, as well as the antibody response in the serum and the gut.

Belgian giant and Chinchilla rabbits weighing 3–3.5 kg were immunized and studied in short-term and in long-lasting (6–18 months) experiments. The proliferation of antigen-specific and antibody-producing cells in the spleen, mesentery lymph nodes, Peyers' patches and the appendix were followed for two weeks after immunization. In chronic experiments the kinetics of serum antibodies and of coproantibody excretion were studied.

In every experimental group 15–20 rats and 20–30 rabbits were used. The immunogenicity of the strains was tested on rats first and if the results proved promising, additional studies on rabbits were carried out. The experiment with each strain was repeated at least three times.

Individual sera and faecal extracts from every rat and rabbit were individually tested by the passive haemagglutination method before the immunization for antibodies to *S. flexneri* 2a, *S. sonnei* I and to sheep erythrocytes. Animals with antibody titres of 1 : 20 or higher were not used.

Enteral immunization of rats and rabbits. Twenty hours before the immunization the animals were left with free access to water only. Under Hexobarbital (Artzneimittelwerk, Dresden, GDR) anaesthesia a small laparatomy was performed and the bacterial suspension was injected into the proximal jejunum. After the operation the animals had free access to food and recovered quickly [8]. The excretion of the vaccinal strains with the faeces was investigated daily by plating faecal samples from each immunized animal on MacConkey and on a selective shigella agar.

The proliferation and differentiation of immunocompetent cells in the lymphoid organs was investigated by studying the kinetics of the antigen-binding and antibody producing cells during the immune response. In each experiment 3 rats or rabbits were killed daily and the number of antigen-binding (rosette-forming) lymphocytes was determined by the method of McConnel [9] and that of the antibody-producing cells by the method of Dresser and Greaves [10]. In both cases sheep erythrocytes coated with lipopolysaccharide antigens prepared by the method of Yamada as described in [11, 12] from virulent or attenuated *S. flexneri* 2a and *S. sonnei* I strains were used. Parallel control experiments with uncoated erythrocytes were always performed.

Preparation of the faecal extracts. One gram faeces was homogenized with 9 ml of cold phosphate-buffered saline pH 7.2 containing 100 U/ml of the proteinase inhibitor Kontrical

(Artzneimittelwerk, Dresden). The suspension was cleared by centrifugation (3000 *g* for 30 min at 4 °C) and heated for 30 min at 56 °C. This treatment diminished the non-specific agglutination of the sheep red blood cells.

Passive haemagglutination was used to measure the level of antibodies in sera, in the content of the gut and in the faeces. The preparation of erythrocytes was described above. During the course of the short-term experiments the antibodies in the serum and different segments of the intestine were determined in each killed animal. In the long-lasting experiments blood was collected and tested once weekly and the faeces daily during the entire period of observation [13, 14].

Passive haemagglutination inhibition method served to study the period of excretion of the lipopolysaccharide somatic antigens of the vaccinal strains. The test was performed as described and the amount of detected free antigen was expressed as titre [15].

Results and discussion

The first problem to be solved in the continuous efforts to characterize the immunogenicity of one-, two- and three-marker attenuated strains [11, 12, 16–18] was to find the optimal immunizing doses for rats and rabbits. These doses should induce a potent and long-lasting secretory immune response in the gut and at the same time cause toxic or other side effects.

Data on the survival of rabbits treated enterally with different doses virulent *S. flexneri* 2a and *S. sonnei* I strains are presented in Table I. The *S. flexneri* 2a 9108 strain caused the death of some of the animals treated with doses higher than 5×10^{10} c.f.u. and the *S. sonnei* I 1180 strain — already in the lowest dose studied — 2.5×10^{10} c.f.u. The pathological examination of the dead animals found haemorrhagic enteritis, exudative pericarditis and toxic degeneration of the liver, described earlier by us as an experimental model of shigella intoxication [19]. In the surviving animals a marked proliferation and differentiation of immunocompetent cells was observed as well as a systemic

Table I

Toxic effect of some virulent and attenuated S. flexneri 2a and S. sonnei I strains applied into the jejunum of rabbits (50 animals per strain)

Number of bacterial cells	Virulent strains				Attenuated strains*			
	% lethality		% of survivors excreting mucus		% lethality		% of animals excreting mucus	
	S. flexneri 2a 9108	S. sonnei 1180	S. flexneri 2a 9108	S. sonnei 1180	S. flexneri 2a 77	S. sonnei 3359	S. flexneri 2a 77	S. sonnei 3359
2.5×10^{10}	0	15	0	15	0	0	0	0
5×10^{10}	10	30	10	30	0	0	0	0
7.5×10^{10}	27	55	15	30	0	0	0	0
10×10^{10}	50	70	30	20	0	0	0	0
15×10^{10}	83	90	10	10	0	0	0	0
Heat-killed bacteria 15×10^{10}	0	0	0	0	0	0	0	0

* All attenuated strains tested did not cause keratoconjunctivitis in guinea pigs

and local secretory antibody response. The higher the dose of the enterally applied strain the more potent and long-lasting the immune response was. Analogous experiments were also performed with the attenuated strains. In no case local or systemic reactions were observed. In the rabbits immunized enterally with doses of 5×10^{10} and 7.5×10^{10} c.f.u. of the attenuated strains a local and serum immune response was observed. These doses were used in subsequent experiments for the comparative characterization of the immunogenicity of the attenuated strains.

Rats were immunized by applying 0.5×10^9, 10^9, 2.5×10^9, 5×10^9 and 10×10^9 c.f.u. into the proximal jejunum. The doses 2.5×10^9 and 5×10^9 were later used for immunization during the primary screening of the *S. flexneri* 2a and *S. sonnei* I strains.

The background numbers of antigen-binding and antibody-producing cells and of the antibody levels in the sera and faeces of non-immunized rats and rabbits to the somatic antigens of *S. flexneri* 2a and *S. sonnei* I were determined (Table II). Antibodies in the faeces were not observed, but 50% of the rabbits and 10% of the rats had detectable levels of antibodies in the sera in titres 1 : 10 or higher to one of these antigens measured by passive haemagglutination. The presence of these antibodies could be due to the response of the animals to cross-reactive bacterial antigens.

Table II

Proliferation of immunocompetent cells in the spleens of rabbits and rats immunized enterally with live Shigella strains

Strain No.	Rabbits immunized with 7.5×10^{10} c.f.u. S. flexneri 2a strains		Rats immunized with 5×10^9 c.f.u. S. flexneri 2a strains	
	antigen-binding cells[1]	antibody-producing cells[2]	antigen-binding cells	antibody-producing cells
—	21±10	0.1±0.07	89±45	0.07±0.03
9108	238±61	10±4	425±54	14±12
75	300±104	11±4	126±37	8±2
77	119±26	10±2	328±80	27±9
78	76±19	2±1	143±83	11±10
83	112±30	3±2	149±100	16±3
	Rabbits immunized with 7.5×10^{10} c.f.u. S. sonnei I strains		Rats immunized with 5×10^9 c.f.u. S. sonnei I strains	
—	18±9	0.1±0.07	95±80	0.1±0.05
1180	156±34	9±3	789±138	10±9
2923	142±28	8±3	1310±280	17±13
3357	124±18	6±1	1094±955	17±7
3359	138±34	8±3	784±350	21±11
3361	58±19	3±3	346±170	4±2

[1] $\times 10^3$ per 10^8 lymphoid cells, peak value on day 3–5 (aritmetic mean ± SD)
[2] $\times 10^3$ per 10^8 lymphoid cells secreting IgM antibody, peak value on day 3–5 (aritmetic mean ± SD)

Some of the properties of the virulent and attenuated strains which are relevant to their ability to induce an immune response in the intestines are presented in Table III. Both virulent and the attenuated strains *S. flexneri* 2a 77 and *S. sonnei* I 3359 remain stable in their S-form, are being excreted with the faeces for longer periods and their somatic antigens persist in the gut 4–5 days post immunization. These two-marker attenuated strains retain the large plasmids responsible for cell-invasiveness the 120 Md plasmid in *S. sonnei* and the 140 Md one in *S. flexneri* 2a [1]. Enteroinvasiveness of shigellae is an important factor for the induction of a local immune response in the gut [20–22].

The data on the proliferation and differentiation of antigen-binding and antibody-producing cells in the spleens of rats and rabbits induced by the enteral application of virulent and attenuated *Shigella* strains confirm that the double-marker attenuated strains *S. flexneri* 2a 77 and *S. sonnei* I 3359 possess good immunogenicity — second only to the virulent and one-marker attenuated strains (Table II).

It should be noted that no local antibodies were detected during the short-term experiments in the gut of rats immunized enterally with the *S. flexneri* 2a strains, while all *S. sonnei* I strains tested induced the secretion of local antibodies. Experiments on rabbits confirmed the finding that the enteral administration of *Shigella* strains induced the development of a local secretory immune response in the gut detected on the 3–7th postimmunization day. The information obtained in tests of short duration was confirmed in

Table III

Biological characterization of the studied strains

Strain No.	Markers	% colonies in S-form	c.f.u.*	Period of excretion with faeces, days			
				live bacteria		bacterial antigen	
				in rabbits	in rats	in rabbits	in rats
			S. flexneri 2a				
9108	Wild type	100	51±13	5–6	4–6	6–7	4–6
75	Pur^{-2}	96	58±14	4–6	2–5	5–7	3–5
77	Pur^-/Rif^r	96	49±25	3–5	3–5	2–5	3–5
78	Pur^-/Rif^r	75	38±6	3–4	3–5	2–3	2–4
83	Pur^-/Rif^r	75	48±16	3–4	3–5	2–3	3–4
			S. sonnei I				
1180	Wild type	96	56±10	6–7	4–6	6–7	5–7
2923	Pur^- 5/6	90	37±25	3–5	3–4	3–5	3–4
3357	Pur^-/Rif^r 48	75	36±15	3–4	3–4	3–5	3–4
3359	Pur^-/Rif^r 57	96	38±13	3–5	3–4	3–5	3–4
3361	Pur^-/Rif^r 60	75	33±15	2–4	2–4	3–5	2–3

* Live bacteria (mean values ± SD) in the suspension from agar cultures presented as % of the number of bacteria in the same suspension determined by using an optical standard

long-term experiments on rabbits. Data presented in Tables IV and V point out that the double-marker attenuated strains *S. flexneri* 2a 77 and *S. sonnei* I 3359 cause a rise in serum antibody levels closest to that in response to the virulent strains. Several conclusions could be made regarding the local immune response in the gut: (i) coproantibody excretion starts 5 days after the primary and 4 days after the secondary immunization; (ii) the duration of the response to the attenuated strains *S. flexneri* 2a 77 and *S. sonnei* I 3359 was close to that caused by the corresponding virulent strains and (iii) the same is true concerning the levels of excreted antibodies. A finding of considerable importance was the observation of a secondary-type immune response — regarding both serum and locally secreted antibodies in the gut. Each subsequent boosting dose from all the strains tested caused a doubling of the antibody titres (Tables IV and V).

The experimental models developed by us permit the study of different parameters of the immune response to enterally applied live *Shigella* strains: the proliferation and differentiation of antigen-specific cells in the gut-associated lymphoid tissues and the spleen and the kinetics of serum and local secretory antibody production. The models could be used for the study of the immuno-

Table IV

*Immune response of rabbits to three doses of S. flexneri 2a strains applied enterally 90–110 days apart**

Strain No.	Antibodies in serum**		Antibodies in faecal extracts**			
	On day					
	15	90	responding animals, %	appear on day	are detected until day	mean titre ± SD on day 7
			Response to the priming dose			
9108	2634±2260	234±160	75	5	62	144±117
75	980±360	110±40	60	5	32	80±40
77	976±620	180±80	60	5	41	76±44
78	180±80	30±20	50	5	20	100±24
83	538±320	80±40	10	5	8	260±92
			Response to the boosting dose			
9108	1760±1280	280±30	100	4	76	178±144
75	1670±1220	240±80	90	4	46	172±126
77	4180±3120	320±160	100	4	75	185±140
78	1100±660	180±80	67	4	28	67±30
83	1472±1052	200±80	100	4	29	195±140
			Response to the second boosting dose			
9108	11360±10060	620±160	100	4	71	428±360
75	6640±4260	320±80	100	4	46	224±82
77	8180±7620	360±160	100	4	101	365±302
78	3200±2840	200±80	100	4	29	160±100
83	4600±3440	240±120	100	4	28	180±102

* At least 30 rabbits/strain were studied
** Reciprocal mean values ± SD

Table V

*Immune response of rabbits to three doses of S. sonnei I strains applied enterally 90–110 days apart**

Strain No.	Antibodies in serum** On day 15	Antibodies in serum** On day 90	Antibodies in faecal extracts** responding animals, %	appear on day	are detected until day	mean titre ± SD on day 7
		Response to the priming dose				
1180	13290±12000	370±252	20	6	51	150±110
3357	356±256	48±20	10	6	7	20
3359	2660±2550	80±40	10	5	6	60±20
3361	130±50	0	10	6	7	40
		Response to the first boosting dose				
1180	16640±15120	510±440	100	4	54	314±260
3357	550±400	120±40	100	5	35	72±40
3359	4840±4090	60±40	67	4	47	132±100
3361	366±300	30±10	100	5	20	68±40
		Response to the second boosting dose				
1180	9040±8600	360±200	100	4	107	628±103
3357	2496±2020	80±40	100	4	71	740±600
3359	1080±800	64±22	100	5	45	860±700
3361	280±200	20±10	75	4	22	36±16

* At least 30 rabbits/strain were studied
** Reciprocal mean values ± SD

genicity of other clinically relevant bacteria causing enteral infections as well as of vaccines to be used for their prevention.

From all strains tested two have been chosen as candidate-vaccinal strains — *S. flexneri* 2a 77 and *S. sonnei* I 3359. Their immunogenicity and the stable lack of virulence give ground to plan their application in humans.

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PLASMID ANALYSIS OF CLINICAL ISOLATES OF *BACTEROIDES FRAGILIS* GROUP STRAINS

ELIZABETH NAGY, ÉVA DÁNYI and J. FÖLDES

Department of Clinical Microbiology,
Albert Szent-Györgyi University Medical School, Szeged

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Cryptic plasmids were isolated from 26 of 52 *Bacteroides fragilis* group strains derived from severe infections. Four strains harboured two plasmids, one three plasmids and one five plasmids with different molecular weights. The same molecular weight plasmid (3.7 Md) was isolated from 17 of the 26 plasmid-containing *B. fragilis* group strains. No correlation was found between plasmid-harbouring and resistance against ten antibiotics and different heavy metal ions. No curing of the strains from the plasmids was achieved with ethidium bromide and acridine orange.

Bacteroides fragilis group strains are the most frequently isolated anaerobic bacteria of clinical importance. They are very often involved in purulent infections of man, alone or together with other anaerobic or facultative bacteria. The specific pathogenicity factors and resistance patterns of these species are of great interest. The pathogenicity factors of other clinically important bacteria are often encoded on plasmids, but functions connected with pathogenicity have not been detected for *Bacteroides* plasmids. Like most other bacteria *Bacteroides* strains possess plasmids which have been associated with drug resistance. Plasmid-mediated, transferable antibiotic resistance for tetracyclin, erythromycin, lincomycin and clindamycin has been described for different *Bacteroides* species by numerous authors [1–3]. Recently, surveys [4–7] have been performed on the plasmid content of different species of *Bacteroides* isolated from clinical materials or from the intestine, with the aim of identifying the plasmid-coded pathogenicity factors of these species. In these investigations, mostly small, cryptic plasmids with different molecular weights have been found in about 40% of all strains.

The purpose of the present study was to characterize a series of *B. fragilis* group isolates derived from severe infections, to determine the plasmid content of each isolate, and to attempt to find a correlation between the plasmids and the specific genetic features.

ELIZABETH NAGY, ÉVA DÁNYI, JÓZSEF FÖLDES
Department of Clinical Microbiology, Albert Szent-Györgyi University Medical School
H-6701 Szeged, P.O.B. 482, Hungary

Materials and methods

Bacterial strains. Altogether 52 clinical isolates of the *B. fragilis* group were involved in this study. All of the strains were derived from severe purulent infections that occurred after abdominal or pelvic surgery. Two strains were isolated from blood cultures. The biochemical identification of the strains was carried out by a micro method [8] and by gas–liquid chromatography.Control strains with different molecular weight plasmids were used: *B. fragilis* V479 and V503, which contain a 27 Md and a 3.7 Md plasmid, respectively. *Escherichia coli* with the plasmid PBR 322 and *E. coli* V517 containing eight different molecular weight plasmids (1.4, 1.8, 2.0, 3.4, 3.7, 4.8 and 35.8 Md) were also used. All clinical isolates and the anaerobic reference strains were cultured on brain-heart infusion (BHI) agar supplemented with vitamin K_1 (10 μg/ml), haemin (5 μg/ml) and 5% cow blood, in an anaerobic chamber (National, USA) at 37 °C in an atmosphere of 5% CO_2, 10% H_2 and 85% N_2. *E. coli* reference strains were cultured on BHI agar. All strains were maintained in chopped meat bouillon at room temperature.

Determination of resistance against antibiotics and heavy metal ions. The minimum inhibitory concentrations (MIC) of 10 antibiotics were determined by the standardized agar dilution method [9] using a Steers replicator. Antibiotics included penicillin G, ampicillin, cefoxitin, mezlocillin, piperacillin, chloramphenicol, clindamycin, erythromycin, tetracyclin and metronidazole. The dilution of penicillin G ranged from 0.125 to 128 U/ml and that of the other antibiotics from 0.125 to 128 μg/ml. As control strains for the MIC determinations *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29471 were used.

The heavy metal ion resistance of the clinical isolates was determined by means of the agar dilution method. Heavy metal salt solutions, such as sodium arsenate, sodium arsenite, cadmium nitrate, lead nitrate, mercuric nitrate and zinc nitrate, were used in a final concentration of 0.1 M to 0.0001 M in the supplemented BHI agar plates. A Steers replicator was used to inoculate the plates. The inoculum was prepared by diluting an overnight BHI broth culture to achieve a final concentration of 10^4 bacteria per spot. The MIC of the metal ions were determined as the lowest concentration preventing growth.

Determination of beta-lactamase activity. Beta-lactamase activity was revealed by the Nitrocefin stick test (Difco), as a screening method immediately after isolation of the strains. The substrate profiles of the beta-lactamases were determined semiquantitatively by microcolorimetry [10] after ultrasonic treatment of the cells. As substrates, penicillin G, ampicillin, cephalothin, cefoperazone and cefoxitin were applied.

Plasmid isolation. Plasmid DNA from the clinical isolates of the *B. fragilis* group and from the strains harbouring reference plasmids was prepared according to the method of Meyers et al. [11]. The strains were grown overnight in 30 ml of BHI broth. After centrifugation the pellet was resuspended in 2 ml of 25% sucrose in 0.05 M Tris (pH 8.0), and lysozyme (0.2 ml of a 10 mg/ml solution in 0.25 M Tris, pH 8.0) was incubated on ice for 20 min, and 0.8 ml of 0.25 M EDTA solution was added. Incubation was continued for an additional 10 min after which 0.2 ml of 20% SDS solution was added. Lysis was allowed to continue for 1 h at +4 °C. NaCl (3 M) was added to a final concentration of 1 M. After an additional 4 h storage at +4 °C, the lysates were centrifuged at 23 600 g for 30 min at +4 °C to remove chromosomal DNA and cell debris. After phenol extraction, 3 M sodium acetate was added to a final concentration of 0.3 M. The plasmid DNA was precipitated by adding two volumes of cold 95% ethanol and by holding at −20 °C overnight. The precipitated DNA was pelleted by centrifugation at 12 000 g for 20 min and resuspended in 0.2 ml TES buffer. For selected plasmid-harbouring *Bacteroides* strains, the plasmid isolation method of Birnboim and Doly [12] was also used. Plasmid DNA from each species was analysed by horizontal agarose gel electrophoresis. The electrophoresis buffer consisted of 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA; 1% agarose gel was used. The electrophoresis was carried out at 24 mA and 60 V.

Plasmid curing. Plasmid elimination was attempted by culturing selected plasmid-harbouring *Bacteroides* strains in BHI broth containing a subinhibitory concentration of acridine orange (20 μg/ml) or ethidium bromide (10 μg/ml). Strains cultured in the presence of the curing agents for 48 h were subcultured on supplemented BHI agar plates. Fifty colonies were tested for loss of any antibiotic or heavy metal ion resistance, and ten colonies were tested by plasmid isolation.

Results

Forty-five of the 52 clinical isolates from severe infection processes proved to be *B. fragilis*. Three strains were identified as *B. distasonis*, two as *B. thetaiotaomicron* and two as *B. vulgatus*. None of the strains produced any haemolysin on BHI agar plates containing cow blood or sheep blood, but *B. fragilis* strain 49 exhibited a very extensive beta-haemolysis on a BHI agar plate containing horse blood. After a 48 h incubation, the haemolytic zone was about 13 mm in diameter around a single colony. All strains were tested to detect beta-lactamase production immediately after their isolation. Thirty-two of the 52 strains exhibited beta-lactamase production in the Nitrocefin stick test. All of them were characterized as *B. fragilis*. All beta-lactamase-producing strains displayed very similar substrate profiles, hydrolysing cephalothin to a similar extents. None or only a very weak hydrolysis was observed in the cases of penicillin and ampicillin. Only 10 of the beta-lactamase-positive strains hydrolysed cefoperazone, but none of them were active against cefoxitin.

The MIC values of the 10 antibiotics against the 52 *B. fragilis* group strains can be seen in Table I. The beta-lactam antibiotics exhibited variable activity. Piperacillin and cefoxitin were the most active, with 94% and 84% susceptibility at the breakpoint. As expected for this group of microorganisms, there was a high rate (89%) of resistance to penicillin. Erythromycin and tetracyclin displayed variable activity, with a high percentage of resistant strains (61% and 65%, respectively). Among the isolates studied, 15% were resistant to clindamycin at 4 μg/ml. Metronidazole and chloramphenicol were consistently active against all strains. No marked differences in resistance

Table I

Susceptibility of the 52 B. fragilis group strains against ten antibiotics

Antibiotics	MIC_{50} (μg/ml)	MIC range (μg/ml)	Percentage of susceptible strains	
			breakpoint (μg/ml)	%
Penicillin	16	0.25–>128	2	11
Ampicillin	16	0.25–>128	8	80
Cefoxitin	8	0.5–128	16	84
Mezlocillin	4	2–>128	64	82
Piperacillin	4	1–>128	64	94
Chloramphenicol	4	0.25–8	8	100
Clindamycin	1	<0.125–32	4	84
Erythromycin	2	0.25–64	4	61
Tetracyclin	1	0.25–128	4	65
Metronidazole	0.5	0.125–2	8	100

Table II

Susceptibility of 52 B. fragilis group strains to various heavy metal salts

Heavy metal salts	Cumulative percentage of strains susceptible at			
	0.0001 M	0.001 M	0.01 M	0.1 M
Sodium arsenate	0	35	87	100
Sodium arsenite	0	18	82	100
Cadmium nitrate	0	82	100	100
Lead nitrate	0	0	10	100
Mercuric nitrate	0	100	100	100
Zinc nitrate	0	0	42	100

against the given antibiotics were observed between the *B. fragilis* and the other tested species of the *B. fragilis* group.

Results of the susceptibility testing of the 52 *B. fragilis* group strains against heavy metal ions can be seen in Table II. Of the strains 87% and 82% proved to be susceptible against 0.01 M sodium arsenate and sodium arsenite, respectively. However, all of the strains were inhibited by the same concentration of cadmium nitrate and mercuric nitrate. Against the same concentration of lead nitrate and zinc nitrate 90% and 58% of the strains proved to be resistant.

Plasmid analysis by the method of Meyers et al. [11] revealed a 50% carriage rate of one or more plasmids among the tested 52 *B. fragilis* group strains (Table III). The sizes of the plasmids were calculated by comparing them with known molecular weight plasmids isolated from reference strains of *E. coli* and *B. fragilis* strains. Four of the *Bacteroides* strains were found to harbour two plasmids with different molecular weights, and one strain was found to have three plasmids, whereas *B. fragilis* strain 56 was found to carry five different molecular weight plasmids. Twenty-two of the plasmid-containing strains belonged to *B. fragilis*, two were characterized as *B. vulgatus*, and one each as *B. distasonis* and *B. thetaiotaomicron*. The same molecular weight plasmid (3.7 Md) was isolated alone (13 strain) or together with other small or large plasmids (4 strains) from 17 of the 26 plasmid-bearing strains. Only in three *B. fragilis* isolates (strains 23, 56 and 62) was it possible to detect large molecular weight plasmids (35, 27 and 30 Md, respectively). Figure 1 shows the electrophoretic bands of the plasmids obtained from the cleared lysates of the *B. fragilis* strains. Plasmid isolation was also performed by the method of Birnboim and Doly [12] for selected strains (strains 8, 18, 21, 23, 49, 55, 56, 62, 71 and 89), to see whether plasmids with the same molecular weights can be detected by this method as well. Electrophoretic analysis of the

Table III

Plasmid-containing B. fragilis group strains

Species	No.	No. of plasmids	Molecular weight of plasmids (Md)
B. fragilis	8	2	2.8, 4.9
	9	1	3.9
	18	1	3.7
	21	1	3.7
	23	1	3.5
	25	1	4.0
	28	1	3.7
	29	1	3.7
	31	1	3.7
	32	1	3.7
	34	1	3.7
	39	1	3.7
B. distasonis	40	1	4.0
B. fragilis	41	1	3.9
	43	1	3.7
	49	1	3.7
	55	1	3.7
	56	5	2.8, 3.4, 3.7, 4.9, 27
	58	3	3.0, 3.7, 4.2
	62	1	3.7, 30
B. thetaiotaomicron	64	2	3.7
B. fragilis	65	1	3.4
	66	1	4.8
	71	2	3.7, 3.9
B. vulgatus	84	1	3.7
	89	2	2.6, 4.8

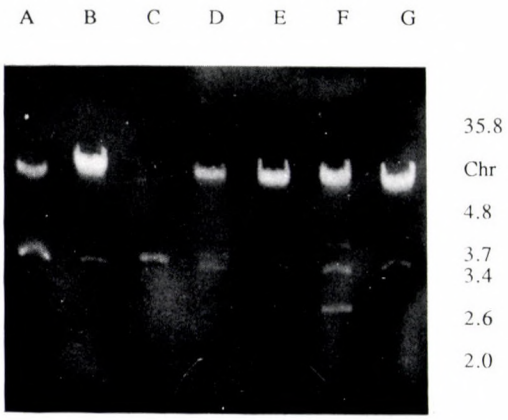

Fig. 1. Agarose gel electrophoresis of ethanol-precipitated cleared lysates DNA from *B. fragilis* group strains. Channels A: strain 25; B: strain 9; C: strain 41; D: strain 71; E: strain 28; F: strain 58; G: strain 32

plasmids isolated by the two different methods demonstrated the same patterns.

Plasmid curing was carried out for selected plasmid-bearing *Bacteroides* strains (strains 8, 18, 29, 58 and 71). The applied 25 μg/ml acridine orange and 10 μg/ml ethidium bromide treatment did not result in any loss of antibiotic or heavy metal ion resistance. Plasmid isolation from the colonies grown after treatment with acridine orange or ethidium bromide have shown the same plasmid pattern as for the untreated strains. No loss of plasmids was observed after storage of the strains for 6 months in chopped meat bouillon, with regular monthly subculturing.

Discussion

The isolation of cryptic plasmids from *Bacteroides* strains of different origins has been reported recently [4, 5, 7]. Using lysozyme treatment, Wallace et al. [7] found small plasmids in 8 of 32 *B. fragilis* group strains, with molecular weights of 2.0, 3.0 and 5.0 Md. Presuming the lysozyme resistance of the *B. fragilis* cells, Beul et al. [4] used the hot alkaline SDS treatment, which produced plasmids in 9 of the 24 tested *B. fragilis* strains, mostly with low molecular weights. We encountered no difficulties in the use of lysozyme treatment in combination with SDS for the isolation of plasmids of *B. fragilis* group strains. Twenty-six of the 52 strains (50%) proved to be plasmid-harbouring. Most of the plasmids exhibited low molecular weights. However, we could detect plasmids of high molecular weight only in three strains, in accordance with the observations [4, 6, 7] that large plasmids are seldom detectable in *B. fragilis* group strains. Even if we accept the observation of Lind et al. [13] that there can be a variation of +1 Md among plasmids with sizes between 2 Md and 20 Md, by using known molecular weight plasmids to determine the size of an unknown plasmid, we have found a great variability of the plasmids among *B. fragilis* group strains, with a molecular weight ranging from 2.6 Md to 35 Md. In this study, the most striking observation was the very frequent appearance of the plasmid with a molecular weight of 3.7 Md in *B. fragilis*, but also in *B. vulgatus* and *B. thetaiotaomicron* strains isolated from different kinds of specimens and at different times. These plasmids are very similar in size to the 3.6 Md plasmids isolated by Beul et al. [4], which accounted for about 50% of all of the plasmids they characterized.

We could not find a correlation between the plasmids harboured by the *B. fragilis* group strains and the resistance pattern of the strains against antibiotics. In contrast with the observation of Wallace et al. [7], we did not detect any association between the resistance to cadmium, mercury or zinc ions and the specific plasmids in the *Bacteroides* strains either. No evidence

was found that there is any correlation between the extreme beta-haemolysis of horse blood observed for strain 49 and the 3.7 Md plasmid harboured by it.

The transfer of large plasmids of *B. fragilis* strains harbouring lincosamide-macrolide resistance was reported by Welch et al. [14] and Tally et al. [3, 15], but no cell to cell transfer of the small plasmids was achieved by Wallace et al. [7] using resistance against cadmium, mercuric or zinc ions as selective markers. In our case no selective markers were found suitable to carry out plasmid transfer experiments.

To summarize the results of this work and of the previous investigations, it appears well established that a rather high number of different, compatible small plasmids exist in *B. fragilis* group strains. The carriage rate might be higher (in the present study up to 50%) than was presumed earlier. One question that arises is the biological functions that these plasmids encode, especially if we consider that *Bacteroides* strains isolated from the intestine also often carry plasmids with the same molecular weights. The seeming impossibility of curing the strains from the plasmids shown in this study and in those of Beul et al. [4] and Wallace et al. [7] may indicate that these plasmids are of biological importance for *Bacteroides* strains of different origins. It is possible that the apparent similarity in size of the plasmids isolated from unrelated strains of *Bacteroides* is accidental and that each plasmid may encode a totally different function. This may be supported by the fact that the occurrence of more than one plasmid in one strain is observed rather frequently. Elucidation of the biological role of small plasmids in *B. fragilis* group strains will certainly help towards an understanding of the biology and presumably the pathogenicity of this clinically important microorganism.

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SIZE-DEPENDENT REGENERATION OF *GIBBERELLA* PROTOPLASTS

Cs. Vágvölgyi, Bettina Brückner and A. Frankó

Department of Microbiology, Attila József University, Szeged, Hungary, and Department of General Microbiology, Friedrich Schiller University, Jena, FRG

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Regeneration ratios of protoplasts formed from the plant pathogenic *Gibberella fujikuroi* with a mixture of lytic enzymes were studied. The heterogeneous population of protoplasts was separated into groups differing in regeneration ratio. The frequency of regeneration was higher for large protoplasts containing an increased number of nuclei.

The regeneration ratios of different fungal species display a great diversity [1]. However, even the reversion ratios of mutants from the same strain can differ [2]. These ratios always relate to the reversion of morphologically heterogeneous protoplast population. Within this population, there are differences in the metabolic abilities of the individual protoplasts [3]. Isaac et al. [4, 5] described a connection between the regeneration ability and the site of hyphal origin of *Aspergillus nidulans* protoplasts; those formed from a distal hyphal region regenerate better. Shawcross et al. [6] found that medium-sized *A. nidulans* protoplasts selected by filtration regenerate better than smaller or larger ones.

Nycodenz, 5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N*,*N*'-bis-(2,3-dihydroxypropyl) isophthalamide, is a nonionic density gradient medium that is very versatile in biological separations [7]. Because of its ability to form isoosmotic gradients, it has been successfully used for separations of osmotically susceptible particles such as organelles [8] or yeast protoplasts [9].

The aim of this study was to clarify the relationship between protoplast size, the number of nuclei and the regeneration capability (frequency) of *Gibberella fujikuroi* protoplasts.

Csaba Vágvölgyi, András Frankó
Department of Microbiology, Attila József University
H-6071, Szeged, P.O.B. 428, Hungary

Bettina Brückner
Department of General Microbiology, Friedrich Schiller University
Neugasse 24, Jena, D-O-6900, FRG

Materials and methods

Strain and culture conditions. A colour mutant of *G. fujikuroi (Fusarium moniliforme)* m567 was maintained on YEG (0.5% yeast extract, 1% glucose, 2% agar) supplemented with 1% malt extract.

Protoplast formation. Protoplasts were formed from colonies grown on cellophane sheets on the surface of supplemented YEG [10]. After growth at 30 °C overnight, the mycelia were transferred into lytic solution (0.7 M KCl, 1.5% Protoplast-forming enzyme (Boehringer), 2% lytic enzyme produced by *Trichoderma harzianum*). The lytic enzyme of *T. harzianum* was produced in MM broth (0.1% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 1% glucose) supplemented with 0.2% chitin and 2% cell wall of *G. fujikuroi* (fresh weight) as carbon source. The colonies were incubated in the lytic solution for 2 h at 25 °C with gentle shaking. The protoplasts were separated from the undigested mycelia by G-2 filtration.

Separation of protoplast population. The protoplasts were collected by centrifugation (2500 *g* for 5 min) and suspended in 0.7 M KCl containing 5% Ficoll (5×10^8 protoplasts/ml); 100 μl was layered onto a continuous Nycodenz (Nycomed Pharma) density gradient. To form this, 4 ml aliquots of 5% and 10% Nycodenz dissolved in 0.7 *M* KCl containing 5% Ficoll were layered in sterile plastic tubes and allowed to diffuse in the vertical position [11] for 24 h at 22 °C. The gradients were spun at 1500 *g* for 5 min using a swing-out rotor. After centrifugation, 7 fractions (1.15 ml each) were taken from the top to the bottom and subjected to further analyses.

Analyses of protoplast groups. To detect the density-dependent distribution profile of the protoplasts, their numbers were determined in each fraction with a haemocytometer, and expressed as percentages of the total number of protoplasts.

To determine the protoplast size, series of samples from each fraction were photographed microscopically and the diameters were measured in the developed films by means of an episcope.

The numbers of nuclei per cell were determined by fluorescence microscopy. The protoplasts were fixed with increasing concentrations of glutaraldehyde (1.25, 2.5 and 5%) and stained overnight with 0.2 mg/ml DAPI (4',6'-diamidino-2-phenylindole) at 4 °C [12].

For regeneration protoplasts were embedded in stabilized MM medium (MM, 0.6 M KCl, 1% agar) and poured as an overlay onto stabilized YEG (YEG, 0.6 M KCl).

Results and discussion

The distribution of *G. fujikuroi* protoplasts after a rate zonal centrifugation on Nycodenz gradient is shown in Fig. 1A. This shows that the separation successfully yielded groups differing in protoplast diameter. These fractions are not totally homogeneous, but the average diameters exhibit clear differences. The lower average diameter and the higher protoplast number in the last fraction are basically due to the clumping of some of the protoplasts, which cannot be eliminated completely.

Protoplasts in the unseparated population were found to contain zero to six nuclei per cell, with an average of 1.18. The presence of numerous anucleate protoplasts in the upper part of the gradient explains the poor regeneration from these fractions (Fig. 1B). The explanation is less evident for differences in reversion measured in the lower part of the tube. In protoplasts found in fractions 4–7, on average at least 1.62 nuclei are present. Since the differences in regeneration rates are much higher than can be explained merely by the presence of more or less anucleate protoplasts in these fractions, the influence

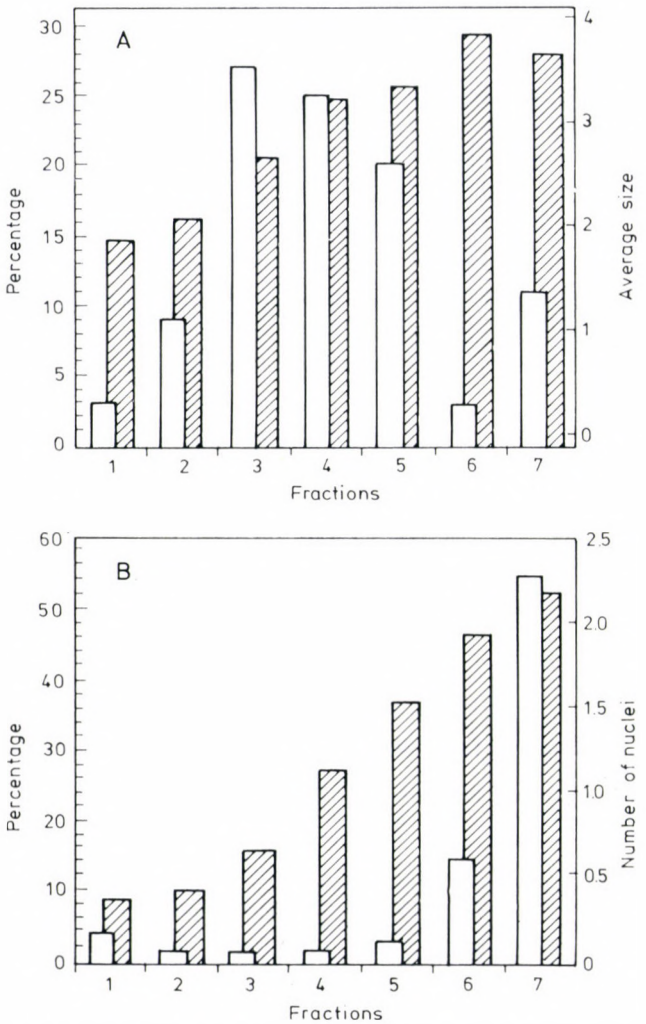

Fig. 1. Resolution of *G. fujikuroi* protoplasts population into groups on a Nycodenz density gradient. Fractions were analysed for (A) protoplast distribution (open columns), average diameter of protoplasts (shaded columns); (B) regeneration ratio (open columns), and average number of nuclei in protoplasts (shaded columns). The separation was performed as described in Materials and methods

of some other factor(s) must be suspected. The similar trends in nuclear number and in cell size (diameter) as in regeneration suggest the dependence of the regeneration on these factors. The role of the larger cell size from this respect could be the possessing of higher metabolic activity and ability to harbour more nuclei.

Mycelia of filamentous fungi always yield a heterogeneous population of protoplasts, without any very strict connection between size and nuclear

number. Under defined conditions, the diversity of the resulting protoplast population could be characteristic of a strain. The basis of this diversity being the individual susceptibility of the cell wall to the digesting enzyme. This distinct heterogeneity is a possible reason for the different regeneration ratios of mutants of the same origin. Our results suggest that with an appropriate separation technique it is possible to select groups of protoplasts with better or worse reversion rates than the average (the reversion rate of the overall population). Such a separation may be of importance in experiments (e.g. electrofusion) where protoplasts with adequately defined morphological features are required. In *G. fujikuroi*, the positive effect of larger cell size and higher nuclear number on the regeneration has been demonstrated.

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BACTERIOLOGICAL PROFICIENCY TESTING IN HUNGARIAN CLINICAL MICROBIOLOGY LABORATORIES

B. Lányi and Éva Czirók

National Institute of Hygiene, Budapest

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In course of a proficiency testing programme carried out in 1989, a total of 47 clinical microbiology laboratories of public health stations and of hospitals received freeze-dried cultures for isolation, identification and antimicrobial susceptibility testing. The specimens contained bacteria that occur in everyday work, including those that require improved methods of cultivation and identification. Nine public health laboratories and one hospital laboratory achieved excellent results. Good results were attained by 11 public health and 6 hospital laboratories. Four public health and 10 hospital laboratories were on the medium level and 4 hospital laboratories did not reach even this degree. The main failures were due to an insufficient anaerobic cultivation, unreliable identification and negligation of controls for drug susceptibility tests.

On the basis of a Government Regulation, Hungarian medical microbiology laboratories are supervised by a Committee, which includes representatives from public health and hospital laboratories. The Department of Bacteriology in the National Institute of Hygiene acts as an operating centre of the national medical bacteriology control programme.

This programme includes issuing handbooks and specific guidelines for improved diagnostic methods and in-house quality control procedures, education of personnel, day-to-day advising, and computerized analysis of laboratory data. A further tool to control diagnostic activities is the inspection of laboratories by experts who, in addition to checking the quality of routine performance and the records on the work cards, evaluate the testing of culture and identification media done with stock cultures received from our Department.

Proficiency testing, i.e. sending at intervals to the laboratories a given set of samples for processing, has long been carried out in Hungary. In 1964 we demonstrated a high variability of bacterial agglutination titres reported by different laboratories examining the same serum specimens by the use of the same bacterial suspensions [1]. Further experiments with bacterial cultures have shown that these kinds of test can be used not only to check regulatory

Béla Lányi, Éva Czirók
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

requirements, but also to keep the established methods uniform and to serve as a valuable tool for education [2].

The purpose of the present paper is to give details of the clinical bacteriology proficiency testing programme carried out in 1989. In preparing the test materials it was regarded of importance that the specimens should contain bacteria that occur in everyday work including those that require improved methods of cultivation and identification. We sought answers to the following questions: (i) what is the working level in the laboratories; (ii) how they keep recommendations of our standard methods both in view of diagnostic and formal instructions; (iii) how are they prepared for the isolation of fastidious strains?

Materials and methods

Participating laboratories. A total of 47 clinical bacteriology laboratories were requested to participate in this programme. Out of them 24, including the clinical bacteriology unit of our Department, belonged to the Public Health Laboratory Network, whereas 23 were hospital laboratories qualified to perform all kinds of bacteriology examination except that of faecal specimens.

The test preparations contained reference strains maintained in the Hungarian National Collection of Medical Bacteria (HNCMB) and also strains chosen from the routine material of our clinical bacteriology unit. The cultures were coded and suspended by one of us (B.L.) so that no one else knew their identity. After centrifugation, the bacteria were resuspended in cysteine–inositol–rabbit serum mixture [3] and freeze-dried. The range of bacterial counts varied with the purpose of the test from 10^6 to 10^3 colony forming units in 1 ml volume.

Alphabetic codes for each series of samples were written on the ampoules with a glass marker pen. After freeze-drying, self-adherent labels bearing different code numbers were attached on randomly selected ampoules and the alphabetic code was removed. The ampoules were sent to the laboratories by post. Sham request forms were enclosed, stating the type and source of the sample, clinical diagnosis and antibiotic therapy in progress.

Processing of the specimens in the laboratories was done as prescribed in our standard methods [4]. Each laboratory was expected to culture the specimens promptly after receiving them. After rehydration in 1 ml broth, the specimens had to be handled so as if they were real clinical materials, except that they contained no human cells (i.e. blood or epithelial cells.)

Evaluation of reports. The laboratories had to report as accurately and as quickly as possible the presence of a clinically significant bacterium with a guide to antibiotic therapy. Presumptive recognition of bacteria (e.g. in CSF or in case of anaerobic organisms) could be reported not only in writing but also by phone or telex.

Considering that solution of certain tests was more difficult than of others, each sample was scored individually, e.g. isolation and identification of an anaerobic bacterium was quantitated as 40 units, whereas those of an *Enterobacteriaceae* strain as 20 units. A maximum score was reached when the laboratory reported the presence of all significant bacteria included in the sample, identified them exactly and read their antibiotic susceptibility pattern correctly. Important presumptive results given in time were also considered. The scores were reduced when the laboratory could not identify correctly the bacteria, made a formal mistake in reporting the result or gave susceptibility patterns erroneously. As scores attainable varied from test to test, the optimal performance was expressed as 100% for each specimen.

Results

Altogether 32 different samples were prepared and each of them was freeze-dried in 10 ampoules. Five or six randomly selected ampoules were sent to the laboratories, the remaining ones were used for checking the viability of bacteria before and after conducting the proficiency testings. The experiments were carried out in May, 1989 (samples A_1-R_1), and in September, 1989 (samples A_2-R_2).

1. *Isolation and identification of bacteria*

Sample A_1

Pus, retroperitoneal abscess; *Escherichia coli*, *Streptococcus faecium* var. *durans*, *Bacteroides fragilis*.

E. coli and *Streptococcus* were found by all but one laboratories. Two laboratories reported *Streptococcus* group D and beta-haemolytic *Streptococcus*. Two laboratories could not cultivate the *B. fragilis* and one reported it erroneously as *Propionibacterium* (which is Gram-positive).

Sample B_1

Drainage, peritonitis post appendectomy; *Escherichia coli* H_2S-positive, *Fusobacterium nucleatum*.

Five out of six laboratories identified *E. coli* correctly, but none of them mentioned its hydrogen sulphide positivity. They may have failed to examine this reaction in a standard H_2S agar or may have not regarded this feature as worthwhile reporting. *F. nucleatum* was not isolated by two laboratories, and a third noticed that fusiform rods were seen at direct microscopy but cultivation was unsuccessful.

Sample C_1

Aspirated material, bartholinitis; *Streptococcus agalactiae*, *Peptostreptococcus prevotii*.

Streptococcus was isolated by all six laboratories that received sample C_1. Five of them identified it correctly as *S. agalactiae*, whereas one laboratory reported *S. pyogenes*. Four laboratories were unable to culture the *P. prevotii*.

Sample D_1

Pus, perianal abscess; *Streptococcus pyogenes*, *Salmonella enteritidis*, *Bacteroides thetaiotaomicron*.

S. pyogenes was isolated by all but one laboratory. All the laboratories found the *S. enteritidis* too, but one hospital laboratory failed to send it for confirmation and serotyping to the regional public health laboratory. The

anaerobic bacterium was not shown by two laboratories, and another two laboratories incorrectly identified the species *(B. fragilis* or *B. vulgatus)*.

Sample E_1

Pus, retropharyngeal abscess; *Neisseria lactamica, Peptostreptococcus prevotii*.

None of the five laboratories obtained a correct result. The *Neisseria* was reported only by one laboratory but this failed to name the species. One laboratory identified this agent as *Actinobacillus actinomycetemcomitans* obviously disregarding lactose, oxidase and nitrate positivity, which is absent in *A. actinomycetemcomitans*. The remaining laboratories misidentified *Neisseria* as alpha-haemolytic *Streptococcus, S. faecalis* and *Haemophilus influenzae*. The *P. prevotii* was shown only by one laboratory; this may be due to the fact that on the anaerobically incubated blood agar plate it produced colonies similar in morphology to *N. lactamica*. The oxidase test could be of help in distinguishing colonies of the two organisms.

Sample F_1

Drainage, suppuration after cholecystectomy; *Peptostreptococcus prevotii, Bacteroides fragilis*.

Results of three laboratories were correct according to the old nomenclature, as they reported *Peptococcus* sp. *Propionibacterium acnes* which is a rod or *Streptococcus morbillorum* which forms chains, were less satisfactory results. *B. fragilis* was correctly identified by all but one laboratories. One laboratory found, in addition *S. enteritidis* which was not incorporated in the test. Reexamining test ampoule F_1 we could not show the *Salmonella*; yet, it could not be excluded that *S. enteritidis* got in some of the ampoules F_1 by technical mistake.

Sample G_1

Aspirated pus, submandibular abscess; *Streptococcus mitis, Fusobacterium nucleatum*.

Identification of *S. mitis* as *S. sanguis* or *S. intermedius* (two laboratories) was not considered an error. Three laboratories reported alpha-haemolytic *Streptococcus* without naming the species. *S. agalactiae* (one laboratory) was a less satisfactory result. *Fusobacterium* was not reported by three laboratories, one of them identified it incorrectly as *Propionibacterium granulosum*, which is Gram-positive. One laboratory isolated *Salmonella enteritidis* from Sample G_1. As confirmed by a later testing of ampoules G_1 in our laboratory, *S. enteritidis* was, indeed present in very small numbers, probably because of a technical error during preparation of the bacterial suspension.

Sample H_1

Aspirated material, pleuritis, pulmonary abscess; *Streptococcus mitis*, *Cardiobacterium hominis*, *Peptostreptococcus prevotii*.

There was a surprising result — *S. pneumoniae* instead of *S. mitis*. Later on this laboratory changed this report to an equally unsatisfactory one (beta-haemolytic *Streptococcus* of group L). Not less than three laboratories failed to cultivate *C. hominis*. In one laboratory they misidentified it as *Capnocytophaga* sp. *H. influenzae* was considered as an entirely wrong result. Two laboratories did not report *P. prevotii*.

Sample I_1

Discharge from burn wound; *Staphylococcus aureus*, *Aeromonas hydrophila*, *Pseudomonas stutzeri*.

As an error characteristic of conservative bacteriologists, two laboratories were satisfied with *S. aureus* and failed to indicate the presence of the facultatively pathogenic Gram-negatives. Four laboratories reported *A. hydrophila* but were unable to find *P. stutzeri*. Only one laboratory isolated all the three agents.

Sample K_1

Urine, cystitis; *Citrobacter amalonaticus* 10^5/ml, *Proteus vulgaris* 10^5/ml.

Preparation K_1 contained two bacteria with uncommon biochemical properties. *C. amalonaticus* was citrate-negative and indol-positive, growth in KCN was the only feature differentiating it from *E. coli*. This could have been the reason why three laboratories identified it as *E. coli*. One laboratory was correct in giving the result: *Enterobacteriaceae* — identification in progress. Later it reported a correct result. The second pathogenic agent was an urease-negative *P. vulgaris*. Two laboratories failed to recognize that this organism belonged to *Proteus*.

Sample L_1

Ear discharge, otitis externa; *Corynebacterium diphtheriae* toxigenic, *Flavobacterium multivorum*.

In preparation L_1 a toxigenic *C. diphtheriae* of the gravis variety was included, with the aim to refresh the memory of our bacteriologists that this organism may still occur in clinical specimens. Recently in Hungary it has been isolated from some foreigners. The other organism was *Flavobacterium multivorum*. It was a serious mistake that three laboratories failed to identify *C. diphtheriae*. One laboratory recognized this species but did not perform the toxicity test. It was also unacceptable that some laboratories did not report the presence of *F. multivorum*, or identified it erroneously.

Sample M_1

Pus, obtained by mastoidotomy; *Haemophilus influenzae*, *Staphylococcus hominis*.

One out of the six laboratories failed to isolate *H. influenzae*, probably because of the unsatisfactory quality of its chocolate agar. *S. hominis* was misidentified in one laboratory as *Micrococcus* in another one as *S. epidermidis*. The report of two laboratories referred to the doubtful pathogenicity of the *Staphylococcus*, whereas one laboratory regarded it as a contaminant. The antibiotic susceptibility of this *Staphylococcus* was reported only by one laboratory — which indicates that this laboratory considered it as pathogenic or potentially pathogenic.

Sample N_1

Urine, chronic cystopyelitis; *Escherichia vulneris* 10^4/ml, *Klebsiella* sp. 10^4/ml.

It was a pleasure for us that *E. vulneris*, this recently discovered and infrequently isolated organism, was correctly identified by three laboratories. The *E. coli* result reported by three laboratories was considered only a slight mistake. The *Klebsiella* fit hardly into any of the *Klebsiella* species, it was the most closely related to *K. rhinoscleromatis*. The significance of bacteriuria (number of colony forming units) was not reported by three laboratories.

Sample O_1

Cerebrospinal fluid, acute meningitis; *Bacillus* sp.

In sample O_1 we incorporated boiled *E. coli*, imitating an antibiotic-treated meningitis, when direct microscopy indicates an infection by Gram-negative rods, but culturing is negative. During preparation the specimen was contaminated due to a technical mistake by low numbers of an aerobic spore-forming bacillus. Checking of the preparation revealed this contamination, but we considered the specimen suitable for investigation as cerebrospinal fluid can also be contaminated accidentally in practice. Thus those laboratories were right that gave a negative result either as "No pathogenic bacterium" or noticed the contamination. It was interesting that four laboratories apparently did not realize that a test preparation might contain a contaminant as a single organism, and tried to identify it — with highly unsatisfactory results (*Pasteurella* in two laboratories, *Aeromonas hydrophila* and *Pseudomonas maltophilia* in the remaining two laboratories). This variety of findings may be explained by the fact that the *Bacillus* strain was Gram-labile, weakly oxidase-positive, weakly fermentative and formed spores only after days of incubation.

Sample P_1

Cerebrospinal fluid, acute meningitis; *Neisseria lactamica.*

N. lactamica was correctly reported by two laboratories, whereas another two laboratories identified it as *N. meningitidis.* The latter results indicate that the carbohydrate decomposition spectrum was incorrecily determined. The remaining two laboratories misidentified *N. lactamica* as *Listeria monocytogenes* and alpha-haemolytic *Streptococcus.*

Sample R_1

Blood, postoperative sepsis; *Cardiobacterium hominis.*

Two out of six laboratories identified *C. hominis,* although one of them named the agent *Kingella indologenes.* This nomenclature has been accepted, since at present there is no property which would distinguish clearly between these bacteria. *Pasteurella* and *Haemophilus* represented a less correct, and *Propionibacterium* an unacceptable diagnosis.

Sample A_2

Tissue from traumatically acquired wound, femoral myonecrosis; *Aeromonas hydrophila, Pseudomonas putrefaciens, Clostridium perfringens.*

Aeromonas was determined in one laboratory. Another laboratory made a major mistake identifying it as *Enterobacter agglomerans.* Every laboratory identified *P. putrefaciens* correctly but one of them reported it as *Alteromonas.* The latter differs from the former only in sucrose and maltose positivity. Serious mistakes were that one laboratory failed to show *C. perfringens* and another identified it as *Fusobacterium* sp. Two laboratories received extra scores for carrying out the animal pathogenicity test correctly.

Sample B_2

Drainage after surgery, subacute peritonitis; *Serratia marcescens, Fusobacterium nucleatum.*

S. marcescens was isolated in every laboratory, although one of them reported it as *Enterobacter.* Four of the six laboratories isolated *F. nucleatum,* one of them identified it as *F. necrophorum.* The remaining two laboratories failed to detect this organism.

Sample C_2

Aspirated pus, thoracic empyema; *Klebsiella oxytoca, Fusobacterium nucleatum.*

Three out of six laboratories identified these bacteria correctly. *K. oxytoca* was reported as *K. pneumoniae* by one, and as *Klebsiella* by another laboratory.

One laboratory failed to isolate the *Fusobacterium* and one referred to it as anaerobic Gram-negative rods.

Sample D_2

Anaerobic blood culture after cardiac surgery, febrile condition; *Enterobacter agglomerans*, *Propionibacterium acnes*.

Four laboratories identified *E. agglomerans* correctly, one reported it according to the old nomenclature as *Erwinia herbicola* and another one as *Escherichia vulneris*, probably because of the yellow pigment production. The anaerobic *P. acnes* was found and correctly identified by all six laboratories.

Sample E_1

Aspirated material, bartholinitis; *Fusobacterium nucleatum*, *Peptostreptococcus prevotii*.

F. nucleatum was correctly identified by five laboratories, while the remaining one reported it as *Fusobacterium*. The *P. prevotii* was reported correctly by four laboratories; one laboratory reported *Peptococcus* and another misidentified it as *Veillonella*.

Sample F_2

Pus obtained after surgery, crural osteomyelitis; *Bacteroides thetaiotaomicron*.

Three laboratories identified the agent correctly. Because of using the relatively insensitive Kovács method for the detection of indole production, two laboratories reported *B. fragilis* and *B. vulgatus*. The sixth laboratory identified the organism as *B. ovatus*, which, similarly to *B. thetaiotaomicron* is indole-positive, but differs from it in the splitting of mannitol and salicin.

Sample G_2

Intravenous cannula, pyrexia after splenectomy; *Corynebacterium* JK, *Staphylococcus epidermidis*.

The following erroneous results were given: out of the five laboratories receiving this sample, two could not find *Corynebacterium* JK and another identified it as *Cardiobacterium*. In identifying *S. epidermidis* one laboratory made a slight mistake determining it as *S. haemolyticus*.

Sample H_2

Surgically removed lymph node, mesenterial lymphadenitis; *Klebsiella pneumoniae*.

Four laboratories reported *K. pneumoniae*, one used an old nomenclature *(K. aerogenes)* and one only the generic name.

Sample I_2

Blood culture after cardiac surgery, febrile condition; *Nocardia asteroides.*

Preparation I_2 contained *N. asteroides*, a pathogenic agent infrequently occurring in Hungary. It has been involved in nosocomial pyogenic septic infections. There was a minor mistake in one laboratory which identified it as *Corynebacterium* JK. However, *C. pseudodiphtheriticum* and *Actinobacillus* were major discrepancies. The agent was recognized as *Nocardia* by three laboratories.

Sample K_2

Vaginal discharge, vaginitis; *Gardnerella vaginalis, Staphylococcus epidermidis, Streptococcus faecium.*

Only one out of the six laboratories detected *G. vaginalis*. The failure of the other laboratories might have been due to a rapid decrease of viable cells in the sample: after finishing the proficiency testing, the organism could not be recovered from ampoules stored in our laboratory. *S. epidermidis* and *S. faecalis* supposed to be contaminants, were shown in all laboratories.

Sample L_2

Pus obtained by tympanocentesis, otitis media; *Streptococcus pneumoniae, Klebsiella pneumoniae, Corynebacterium pseudodiphtheriticum, Staphylococcus epidermidis.*

Two out of six laboratories reported *Streptococcus* instead of *S. pneumoniae*. The presence of *K. pneumoniae* was not reported by one laboratory, and two laboratories identified it as *Citrobacter* sp. and *Enterobacter agglomerans*. *C. pseudodiphtheriticum* and *S. epidermidis* were regarded as possible contaminating agents; the former was incorrectly identified in one laboratory as *C. pyogenes.*

Sample M_2

Cerebrospinal fluid, acute meningitis; *Flavobacterium meningosepticum.*

F. meningosepticum was correctly identified by five laboratories. The remaining one laboratory reported that the agent could be *Flavobacterium* IIb or *Pseudomonas paucimobilis*. This slight mistake may be explained by an erroneous determination of acid production from mannitol (*F. meningosepticum* is positive, *Flavobacterium* IIb — according to the new nomenclature, *F. indologenes* — is usually negative). The test strain produced indole, whereas *P. paucimobilis* is indole-negative.

Sample N_2

Blood culture, subacute endocarditis; *Aerococcus viridans, Staphylococcus epidermidis.*

A. viridans was recognized by all five laboratories that received sample N_2. Probably due to a misprint one laboratory reported "*Aerobacter viridans*". One laboratory interpreted this organism as a contaminant, since it frequently occurs in hospital environment and foods. On the other hand, according to literary data, 1% of systematic streptococcal infections is caused by this agent. *S. epidermidis* was present in small numbers, actually only two laboratories detected it. Accordingly, the clinician should have been advised to repeat the examination by collecting further blood specimens.

Sample O_2

Urine, cystitis; *Staphylococcus saprophyticus* 10^5/ml, *Candida albicans* 10^3/ml.

Recognition of *Candida* was difficult as its colony morphology on blood agar was very similar to that of *S. saprophyticus*. Probably this was the reason why four out of six laboratories could not show it. It was also a mistake that four laboratories failed to report the number of *S. saprophyticus*.

Sample P_2

Cerebrospinal fluid, acute meningitis; *Moraxella atlantae*.

Preparation P_2 contained a rare pathogenic organism *M. atlantae*. There were major errors, for example, *N. meningitidis* was a serious mistake both from clinical and epidemiological point of view. Another laboratory identified *Moraxella* as an aerobic spore-forming bacterium. In one laboratory *S. aureus* was isolated; this was absent after re-checking the ampoule. *M. atlantae* was correctly identified only in one laboratory.

Sample R_2

Bronchial discharge, pneumonia associated with tumour; *Streptococcus pneumoniae*, *Branhamella catarrhalis*, *Streptococcus salivarius*.

As an error not unfrequently occurring, the bile solubility test was misinterpreted and thus three laboratories misidentified *S. pneumoniae*. It was a serious mistake that four out of the six laboratories did not report *B. catarrhalis* — probably because in lack of the knowledge of up-to-date literature, they took it for a *Neisseria* common in the upper respiratory organs.

2. *Antimicrobial susceptibility testing*

In the course of antimicrobial susceptibility testing many unacceptable results were met with. There were several formal mistakes. Some laboratories failed to make susceptibility testing of presumably pathogenic agents, others reported only the susceptible results and not the resistant or moderately resistant ones, thus leaving the clinician in doubt as to which antibiotics had

been actually tested. One laboratory did not enclose a list of abbreviations used on its computer results. Beyond, there were professional mistakes, too. In many laboratories antibiotics more recently introduced in Hungary were not tested (for example amikacin or third generation cephalosporins). These laboratories accounted for the reason that their clinicians had not been using these new antimicrobials yet. Furthermore, there were laboratories where even the classical antibiotics were not tested, for example, nitrofurantoin and nalidixic acid sensitivity was not determined for urinary strains. Others omitted chloramphenicol sensitivity testing for *S. enteritidis*. In a few laboratories penicillin sensitivity was not determined for non-haemolytic streptococci. It was also erroneous when co-trimoxazole susceptible Gram-negative bacteria were reported as resistant.

3. *Presumptive information*

In several laboratories we encountered a delay of clinically useful presumptive informations. Other laboratories supplied excellent rapid results. E.g. that in stained smears there were Gram-positive cocci and Gram-negative rods which failed to grow aerobically. This preparation contained, indeed, only *Peptococcus* and *Bacteroides* and on the basis of the rapid diagnosis clindamycin treatment could be started. Another good example is a preliminary report, given immediately after receiving a CSF preparation, that the smears contained neisseria-like bacteria and, accordingly, penicillin therapy, which has already been started, can be continued. Laboratories that correctly diagnosed *C. diphtheriae* in their tests, sent preliminary informations.

Table I

Ranking of laboratories on the basis of proficiency tests

Per cent*	Public health laboratories	Hospital laboratories
91–100	7	1
81–90	2	–
71–80	3	3
61–70	8	3
51–60	3	5
41–50	1	5
31–40	–	3
0–30	–	1
Not evaluable	–	2
Total	24	23

* Summarized results in percentage; optimum performance, 100%

4. *Ranking of laboratories*

Table I shows the ranking of laboratories on the basis of the presented proficiency tests. Nine public health laboratories and one hospital laboratory achieved excellent results ($\geq$81%). Good results (61–80%) were attained by 11 public health and 6 hospital laboratories. Four public health and 10 hospital laboratories were on the medium level (41–60%), and 4 hospital laboratories did not reach even this degree ($\leq$40%).

Discussion

In view of the wide variation in culture media ingredients, reagents and in the competence and experience of laboratory personnel, in-house quality control and nation-wide proficiency testing programmes are regarded of great importance. Methods used for this purpose have been summarized by Bartlett [5] and by Keitges [6].

An effective means of assessing performance of personnel may be a periodical use of internal blind control [7]. This method has not so far been used in Hungarian laboratories. However, in-house quality control is regularly performed by the use of reference cultures provided by our Department.

Laboratory inspections in Hungary have proved that up-to-date performance of clinical bacteriology has improved in recent years. Results of the proficiency test in 1989 were not surprising as the best educated laboratories reached the best results. As it can be seen from Table I, public health laboratories had a better competence in performing the tests as compared to hospital laboratories. Beside their qualified personnel, equipments and facilities appropriate for up-to-date diagnostics, this can be attributed to the close connection that has developed for 30 years among these laboratories and with the Department of Bacteriology of the National Institute of Hygiene. Some hospital laboratories also achieved very good results. These laboratories have had a special interest in bacteriology and maintained a long-lasting connection with the clinical bacteriology units of the local public health laboratories.

One of the main failures in this series of proficiency tests can be attributed to the fact that many laboratories have not reached a sufficient standard of anaerobic cultivation. In lack of a proper inoculation of primary anaerobic culture plates and/or adequate anaerobic incubation, these laboratories could not recover the strict anaerobes among facultatively anaerobic bacteria. Capnophilic bacteria were sometimes not detected because of a failure to cultivate the samples in an atmosphere with increased CO_2 content. It will also be necessary to gain more experience in biochemical reactions for fastidious and non-fermentative bacteria. As acid production from carbohydrates by these bacteria cannot be reliably and rapidly examined by the classical

peptone-water and by the OF methods, some years ago we introduced the BSS ("buffered single substrate") test carried out in plastic Microtiter plates. Most of our laboratories introduced this test, but it produces accurate results only in experienced hands. Isolation technique should also be improved: part of the laboratories could not isolate bacteria incorporated in small numbers in the preparation. Furthermore, it is also advisable that laboratories advance both of their professional and their methodical knowledge concerning newly recognized or less known bacteria.

Failures in interlaboratory reproducibility of antimicrobial susceptibility results were partly due to the use of inadequate culture media (e.g. those containing sulphonamide and trimethoprim antagonists) and partly to an erroneous interpretation of inhibition zones. The latter may be associated with a failure to compare the zone borders of the examined culture with the zone borders of the standard quality control strains. The control strains, distributed by our Department to all medical microbiology laboratories, have been chosen on the basis MIC breakpoints related to the levels of drug in blood or in urine expected with the usual dose schedules. Another reason for the discrepant results may be that the inoculum was considerably heavier or lighter than that used for the control strain. As to the selection of antimicrobial agents to be tested, we are of the opinion that the laboratory should start to examine susceptibility to newer drugs without waiting for encouragement from the clinician.

Finally, it should be pointed out that personnel of the laboratories requested to take part in the proficiency testing programme, readily and enthusiastically contributed in carrying out the examinations. The best indication that the programme was successful, was that most graduates and many technologists of the Laboratory Network attended a meeting held on this topic in November, 1989, and discussed the results. We, too, believe that this proficiency examination has proved how important it is to develop clinical bacteriology on a unified and collaborative basis.

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