ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS XXV FASCICULUS 1

AKADÉMIAI KIADÓ, BUDAPEST

1978

AMAHA 5 25(1) 1–86 1978

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1054 BUDAPEST, ALKOTMÁNY UTCA 21.

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24, Bankszámla 215-11448), a külföld számára pedig a „Kultura" Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, bankszámla: 218-10990) vagy annak külföldi képviseleteinél és bizományosainál.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P.O.B. 64, Hungary

Correspondence with the editors should be sent to the same address.

The rate of subscription is $ 36.00 a volume.

Orders may be placed with "Kultura" Foreign Trade Company (H-1389 Budapest 62, P.O.B. 149, Account No. 218-10990) or with representatives abroad.

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS XXV

AKADÉMIAI KIADÓ, BUDAPEST

1978

INDEX

TOMUS XXV

Fasciculus 1

Fasciculus 2

Fasciculus 3

Fasciculus 4

Acta microbiol. Acad. Sci. hung. 25, 1—9, 1978

STABILITY OF THE PERTUSSIS COMPONENT OF DIPHTHERIA–TETANUS–PERTUSSIS (DTP) VACCINES

Z. CSIZÉR, J. ZSIDAI and I. JOÓ

Institute for Serobacteriological Production and Research "Human", Budapest

(Received June 27, 1976)

According to data of potency tests, the potency of the pertussis component of diphtheria–tetanus–pertussis (DTP) vaccines decreased below the limit of 8 IU/30 IOU in the sixth year after production. The average annual loss in potency during a 6-year interval was 0.7 IU/30 IOU. The periodicity of potency decrease was shown by analysis of variance and periodicity tests. The sources of periodicity are unknown.

According to the Requirements for Pertussis Vaccine of the World Health Organization, the single human immunizing dose of pertussis vaccines and the pertussis component of combined vaccines has to contain at least 4 IU of pertussis antigen [1]. The vaccine will meet the requirement if the manufacturers possess correct informations on the characteristics of potency decrease. Estimation of the potency decrease is one of the most important criteria for the expiry time of the vaccines in the field. According to the data of potency tests of polyvalent *Bordetella pertussis* bulk suspensions, the potency of suspensions decreased below the limit of 8 IU/30 IOU during storage for 5 to 9.8 years [2]. The stability of pertussis vaccines can be estimated in two ways. The initial potency values after production can be compared with the potency values after storage for different intervals, or the stability is estimated by the potency values of vaccines of different shelf life.

In the present paper the stability of the pertussis component of the diphtheria–tetanus–pertussis (DTP) vaccines produced in the Institute for Serobacteriological Production and Research "Human" has been investigated.

Materials and methods

Bacterial strains. Corynebacterium diphtheriae PW8 (Massachusetts), *Clostridium tetani* Harvard (Copenhagen), *Bordetella pertussis* 41405, 59, 324E, 358E, CN2894, CN2896 and CN2897 were used. For challenge, *B. pertussis* 18–323 was used.

Media and cultivation. For the cultivation of *C. diphtheriae* Linggood medium, of *Cl. tetani* beef-heart broth digested with pepsin and trypsin [3], of *B. pertussis* modified Cohen–Wheeler medium [4] were used. In the case of *C. diphtheriae* and *Cl. tetani* stationary [5], of *B. pertussis* shaken or submerged cultures [6] were applied.

Preparation of DTP vaccines. Diphtheria and tetanus toxoids purified by trichloroacetic acid, and acid-precipitated and heat-inactivated *B. pertussis* suspensions were used. The combined vaccine contains 30 Lf/ml diphtheria toxoid, 10 Lb/ml tetanus toxoid, and 30 IOU/ml *B. pertussis* bacteria adsorbed onto 6 mg/ml aluminium phosphate. Sodium ethylmercurithiosalicylate (0.01%) was used as preservative.

Mice. CFLP random bred mice of both sexes weighing 14 to 16 g were used.

Potency assays were carried out according to the Requirements for Pertussis Vaccine of the WHO [1]. Hungarian Standard Pertussis vaccine (HSPV Lot No. 4) was used as the reference preparation; it contains 51 IU of pertussis antigen per ampoule in comparison with the International Standard Pertussis vaccine [7]. The homogeneity of HSPV Lot No. 4 was shown earlier [8].

Statistical analysis. ImD_{50} values were calculated on the basis of the method of WORCESTER and WILSON [9]. Analysis of regression and that of variance performed by FINNEY's method [10], and periodicity tests (nonlinear regression analysis) were carried out using a type 709 Wang computer.

Results

The potency of the pertussis component of 21 batches of DTP vaccines produced between 1968 and 1973 was determined in 5 parallel active mouse protection tests in 1974. The year of production of the DTP vaccines and the

Table I

Mean of potency values of the pertussis component of 21 DTP vaccines in the year of production and in 1974

Batch. No.	Year of production	No. of potency tests and mean potency			
		in the year of production		in 1974	
		n	IU/30 IOU	n	IU/30 IOU
81	1968	4	18.3	5	7.5
82	1968	2	19.6	5	11.1
84	1969	2	23.1	5	9.8
85	1969	2	21.3	5	14.9
86	1969	2	23.0	5	7.4
87	1970	2	26.0	5	7.2
88	1970	1	10.2	5	15.4
89	1970	3	15.1	5	12.4
90	1970	4	20.7	5	7.8
91	1970	3	28.0	5	26.1
92	1970	1	10.2	5	19.1
93	1971	2	4.6	5	7.8
94	1971	3	6.4	5	11.2
95	1971	3	16.5	5	9.3
96	1972	6	14.2	5	16.2
97	1972	5	5.1	5	5.9
98	1972	3	5.5	5	14.0
99	1972	2	36.7	5	25.9
100	1973	2	13.8	5	16.2
101	1973	1	17.5	5	27.2
102	1973	2	24.1	5	15.1

potency values of their pertussis component tested in the year of production and in 1974 are shown in Table. I.

If the annual mean potency values determined immediately after production are regarded as 100%, the loss of potency during storage can be expressed in per cents of the potency values obtained in 1974. In the first year after the vaccine preparation the potency increased, then decreased and fell to 50% after 5 years (Fig. 1).

The annual average loss of potency can be evaluated on the basis of potency values after production and those determined in 1974. The annual

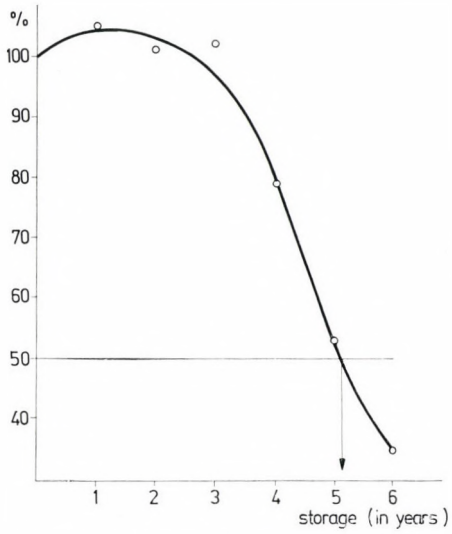


Fig. 1. Potency decrease of the pertussis component of 21 DTP vaccines in percentage of potency at the time of production

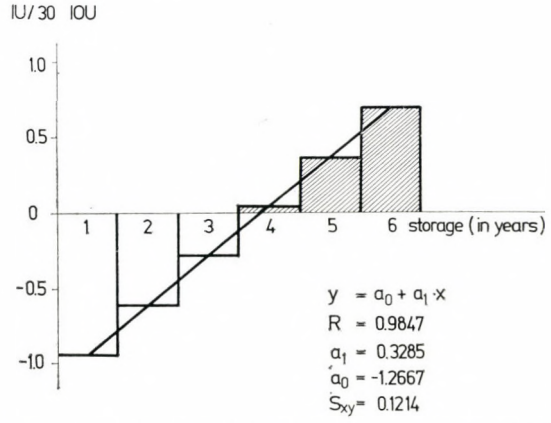


Fig. 2. Annual mean potency decrease of the pertussis component of 21 DTP vaccines

average potency decrease depends on the duration of the interval investigated [2]. In Fig. 2 the annual average potency decrease of the pertussis component of DTP vaccines is presented in IU/30 IOU. These values, plotted against time, could be approached by a straight line, and the correlation coefficient was high (R = 0.98). In the first year after production, the potency values increased and in the next 2 years there was no decrease in potency. In the fourth year the potency values decreased slightly, in the sixth year the annual average potency decrease was only 0.7 IU/30 IOU. This means that the estimated loss of potency during 6 years storage was 0.7 IU/30 IOU $\times 6 = 4.2$ IU/30 IOU.

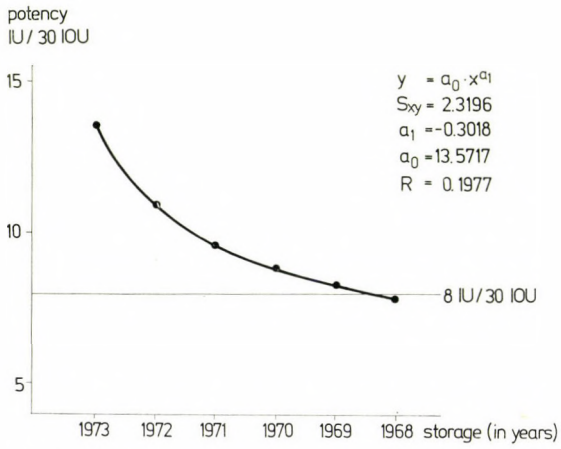


Fig. 3. Regression analysis of 105 potency values of the pertussis component of 21 batches of DTP vaccines

The potency tests of the pertussis component of 21 batches of DTP vaccines in 5 replicates resulted in 105 potency values. Regression analysis of these data plotted against the year of production was carried out by 4 different functions. The least residual variance (S_{xy}) and the highest correlation coefficient (R) were obtained by the exponential function. The exponential function of the potency loss intersects the limit of 8 IU/30 IOU in the sixth year after the production (Fig. 3).

Figure 3 shows that the correlation coefficient was low but significant ($R = 0.19$, $p < 0.05$). The cause of the low correlation may have been the wide variation of the experimental data or the lack of a correlation between the potency values and the time of production. Furthermore, in cases of nonlinear regressions there is sometimes no close correlation, but it can be significant. It is therefore suggested to evaluate the correlation coefficient before performing the nonlinear regression analysis. To find the cause of the low correlation, data of wider standard deviation than the limits of WORCESTER

and WILSON's method (1 SD less than 64 to 156%) were excluded. In this way, 54 potency values were obtained and analysed by 4 different functions. The least S_{xy} and highest R were obtained by the exponential function (Fig. 4).

Data of Fig. 4 show that S_{xy} is higher than in Fig. 3 and R is not significant. If the regression analysis was carried out only with those potency values which fall within the limits of 1 SD of 64 to 156%, no decrease in potency

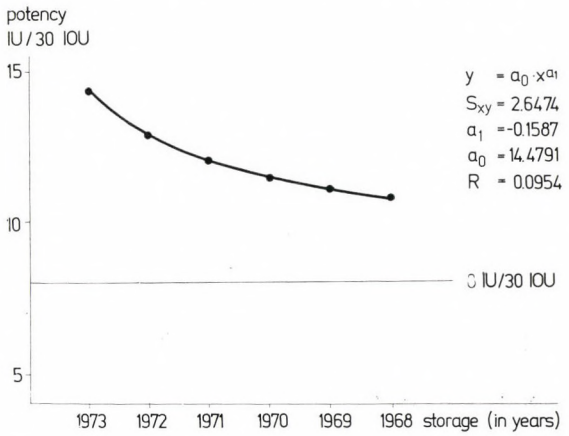


Fig. 4. Regression analysis of 54 potency values (1 SD less than 64–156%) of the pertussis component of 21 batches of DTP vaccines

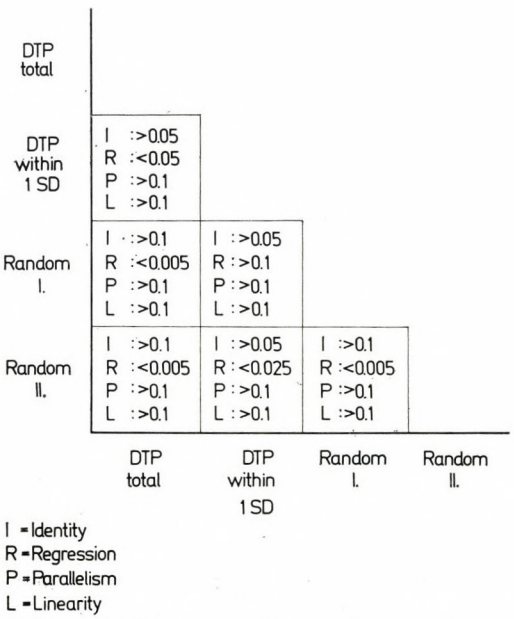


Fig. 5. Homogeneity analysis of 105 potency values of the pertussis component of 21 batches of DTP vaccines

could be demonstrated during the 6-year interval. Consequently, the results of the regression analysis of all the potency values, namely that the potency of pertussis component of the DTP vaccines decreased to the limit of 8 IU/30 IOU in 6 years, seem to be a better estimate of the stability.

For the periodicity test, the 105 potency values were grouped. The first set of data consisted of all the 105 values, the second set of the 54 data which

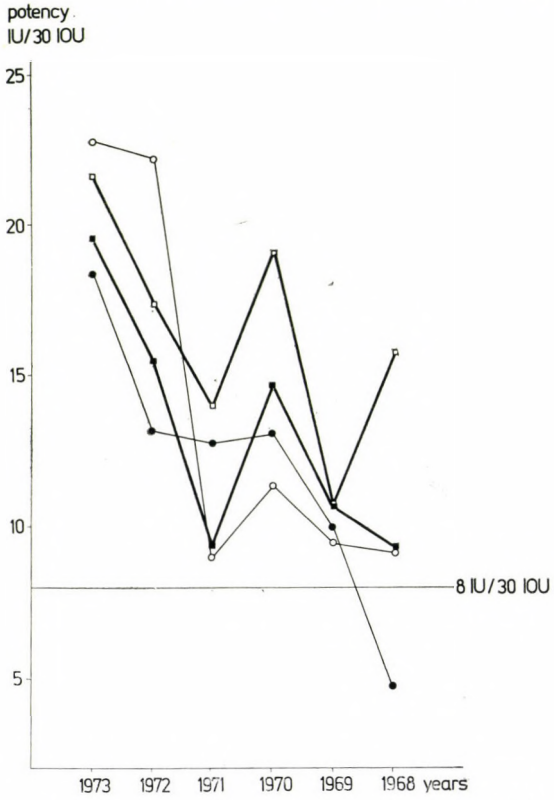


Fig. 6. Annual average potency values of DTP vaccines. ■——■ 105 potency values; □——□ data within the limits of 1 SD; ●——● potency values of random sample I; ○——○ potency values of random sample II

fall within the limits of 1 SD. The third and fourth groups were prepared by use of the random numbers (56 and 57 data, respectively). The identity of the 4 samples was tested by analysis of variance [10]. The homogeneity of samples was not dependent on the number of data in the four groups, since the random samples were identical with each other and with the sample of 105 potency values. The sample consisting of the data which fell within the limits of 1 SD was also identical with the other samples, even if their identity was close to the limits of statistical significance. Results of the analysis of variance are shown in Fig. 5.

On the basis of the analysis of variance the samples were regarded as identical, therefore if the potency decrease of the pertussis component of DTP vaccines was periodical, the periodicity had to be proven for each sample. Fig. 6 shows the annual average potency values of the samples.

The annual average potency values of the four samples were approached by nonlinear regression lines, by periodic (sinus, cosinus) functions. Mathe-

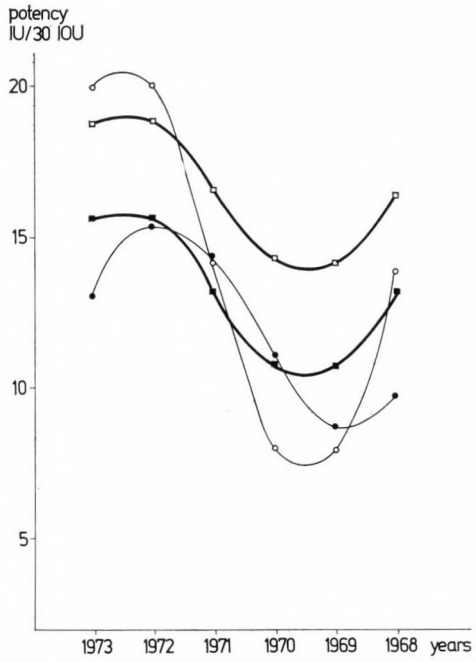


Fig. 7. Periodicity testing of annual mean potency values of DTP vaccines. ■———■ 105 potency values; □———□ data within the limits of 1 SD; ●———● potency values of random sample I; ○———○ potency values of random sample II

matically, the periodicity of a process is proved if the variance of the periodic functions approaching the data is less than their variance ($S_f < S_y$). The periodicity of potency loss was proven in each of the cases; in other words the potency loss was a periodic process (Fig. 7). The periodicity was not due to chance, because in this case the random samples would not have been periodic.

Discussion

The stability of the pertussis component of DTP vaccines was determined by KINDT *et al.* [11] in 15 batches observed for 60 to 65 months. The potency of pertussis vaccines, with an average initial potency of approximately 17

IU/ml, fell beyond the limit of 8 IU/ml after 45 months. As a pertussis component of DTP vaccines, acid-precipitated pertussis suspensions were used. The aluminium hydroxyde adsorbent content of the vaccines might have been responsible for the continuous decrease and shorter half life of the potency than in our stability tests. Owing to the strong adsorption of bacteria onto aluminium hydroxyde, the potency of pertussis vaccines decreases in the active mouse protection test. Adsorption of *B. pertussis* onto aluminium phosphate is less complete and, as a probable consequence, the vaccines prepared with this adsorbent seem to be more stable. According to PITTMAN [12] the potency of the majority of pertussis vaccines was relatively stable; in spite of this it was suggested to evaluate the stability of each manufacturer's product.

The estimates of potency of the pertussis vaccines obtained by the active mouse protection test are subject to large sampling errors. The estimate of ImD_{50} derived from a single assay, in which three groups of 15 mice each are used, has the 95% confidence limits of ranges from approximately 25% to 400%. The mean potency values for standard vaccines may show even wider variations in the course of several years [7]. The reliability of the potency estimates can be increased by the use of more than 16 mice in the treatment groups. According to recent data, the 95% confidence limits of the active mouse protection test range from approximately 33% to 300% when there are 16 mice in each treatment group and are reduced only to approximately 50% to 200% when the groups contain 32 mice [13]. If this is taken into account, it is obvious that a considerable number of assays carried out over a period of several years can only yield a good estimate of the stability of the pertussis vaccines. Because of the high expenses, the stability of each batch of pertussis vaccines cannot be tested routinely. Since for more than 10 years HSPV Lot No. 4 has been used for potency testing, the stability of vaccines could be characterized by the difference between the initial potency and the potency obtained after storage for different periods.

One of the significant variables of the active mouse protection test is the mouse strain used. When comparing different vaccines the immunogenicity of the antigens involved in protectivity may be affected to different degrees by a change of the mouse strain [7]. During long lasting stability tests it is difficult to ensure the uniformity of the mouse colony. This source of error can be eliminated by determining the potency of vaccines of different shelf life at the same date, and the stability is estimated retrospectively from the data obtained. Using two different methods for the estimation of stability, the potency of the pertussis component of DTP vaccines fell to the limit of 8 IU/30 IOU in the sixth year after production. Therefore, on the basis of the initial potency, the potency decrease can be estimated statistically for each batch of vaccine.

The periodicity of potency decrease of pertussis vaccines has been shown [2], but the cause of the periodicity is not known except for the first periodic increase of potency. The latter is explained by the rapid decrease of the vaccine's toxicity. The sources of periodicity will have to be investigated in model experiments.

REFERENCES

1. WHO Technical Report Series **274**, 25 (1964).
2. Csizér, Z., Zsidai, J., Joó, I.: Acta microbiol. Acad. Sci. hung. **22**, 83 (1975).
3. Maróczi, J., Ráskai, M.: Ann. Immunol. hung. **10**, 95 (1967).
4. Pusztai, Zs., Joó, I., Kiss, I.: Path. et Microbiol. **23**, 192 (1960).
5. Surján, M.: Schweiz. Z. Path. Bakt. **19**, 311 (1956).
6. Pusztai, Zs., Joó, I., Juhász, V. P.: Z. Immun.-Forsch. **122**, 278 (1962).
7. Csizér, Z., Joó, I., Zsidai, J., Hegedüs, L.: J. Biol. Standard **1**, 347 (1973).
8. Csizér, Z., Zsidai, J.: Ann. Immunol. hung. **18**, 11 (1974).
9. Worcester, J., Wilson, E. B.: Proc. nat. Acad. Sci. (Wash.) **29**, 207 (1943).
10. Finney, D. J.: Statistical Methods in Biologicla Assay. Charles Griffin and Co. Ltd. London 1952. p. 99.
11. Kindt, H., Milcke, L., Engelhardt, H., Reber, G., Pranter, W., Blackkolb, F.: J. Biol. Standard **2**, 183 (1974).
12. Pittman, M.: Rev. Immun. (Paris) **22**, 308 (1958).
13. Finney, D. J., Holt, L. B., Sheffield, F.: J. Biol. Standard **3**, 1 (1975).

Note. After this paper had been submitted for publication, The International Symposium on Stability and Effectiveness of Measles, Poliomyelitis and Pertussis Vaccines (Zagreb, Yugoslavia, 28–29 September, 1976) emphasized the importance of further studies aimed at improvement of the stability of pertussis component of DTP vaccines.

Address of the authors:

Zoltán Csizér, József Zsidai, István Joó
Institute for Serobacteriological Production and Research "Human"
Szállás u. 5, H-1475 Budapest, P.O.B. 4, Hungary

Acta microbiol. Acad. Sci. hung. 25, 11—15, 1978

WIRKUNGEN VON VIRAZOL (RIBAVIRIN) IN VIRUS/ PROKARYONTEN-SYSTEMEN

GISELA MENZEL und E. STENZ

Sektion Biowissenschaften, Forschungsbereich Pflanzenphysiologie und Mikrobiologie, Karl-Marx-Universität, Leipzig, DDR

(Eingegangen 25. Oktober 1976)

G. MENZEL and E. STENZ *(Section of Biosciences, Karl Marx University, Leipzig, G.D.R.)*: **Effect of Virazole (Ribavirin) in Virus/Prokaryote Systems.** Virazole (ribavirin), a compound with antiviral activity, was tested in some virus/prokaryote systems using the agar diffusion test. Virazole inhibited the formation of plaques by the cyanophage LPP-1 and had no effect on the multiplication of the bacteriophages Φ 105, λ, f 2, M 12, and Qβ. With the exception of *Bacillus subtilis* the hosts of the viruses were inhibited and partly stimulated. The plaque-inhibiting effect of virazole is due to inactivation of the free LPP 1 phages and in all probability to inhibition of the host's metabolism. Adsorption of the LPP 1 phages to *Plectonema boryanum* is not influenced.

Die antiviral wirkende Verbindung Virazol (Ribavirin) wurde auf ihre Wirkung in mehreren Virus/Prokaryonten-Systemen im Agardiffusionstest geprüft. Virazol hemmte die Plaquebildung des Cyanophagen LPP-1, ließ aber die Vermehrung der Bakteriophagen Φ 105, λ, f 2, M 12 und Qβ unbeeinflußt. Die Viruswirte wurden mit Ausnahme von *Bacillus subtilis* gehemmt und zum Teil gefördert. Die plaquehemmende Wirkung des Virazols ist auf eine Inaktivierung der freien LPP-1-Phagen und wahrscheinlich auf die Beeinträchtigung des Wirtsstoffwechsels zurückzuführen. Die Adsorption der LPP-1-Phagen an *Plectonema boryanum* wurde nicht beeinflußt.

Als erste antivirale Verbindung mit breitem Wirkungsspektrum beschrieben SIDWELL *et al.* [1] das 1-β-D-Ribofuranosyl-1,2,4-triazol-3-carboxamid, das als Virazol oder Ribavirin bezeichnet wird. Es beeinflußt zahlreiche zoo- und humanpathogene DNA- wie auch RNA-Viren [u.a. 2, 3]. Prüfungen mit pflanzenpathogenen Viren haben ergeben, daß Größe und Anzahl virusbedingter Lokalläsionen nach Virazolbehandlung geringere Ausmaße erreichten [4] und der Virusgehalt in infizierten Pflanzen niedriger blieb [5, 6].

Im Rahmen unserer Untersuchungen zur Wirkung von Synthetica auf Prokaryontenviren prüften wir, ob Virazol auch diese Viren beeinflussen kann. Es wurden sowohl DNA-Phagen (temperent und virulent) als auch RNA-Phagen einbezogen.

Material und Methode

Folgende Virus/Wirt-Kombinationen wurden für die Untersuchungen verwendet: DNA-Phagen: Φ 105/*Bacillus subtilis* 168; λ/*Escherichia coli* C 600; LPP-1/*Plectonema boryanum* (Cyanophyc.). RNA-Phagen: f 2/*E.coli* K 1046; M 12/*E.coli* W 1665 F$^+$; Qβ/*E.coli* AB 301 (zur Herkunft der Stämme vgl. MENZEL *et al.* [7]).

Die Wirkung von Virazol auf die genannten Viren und ihre Wirte wurde im Agardiffusionstest (Lochtest) unter Verwendung von Doppelschichtplatten geprüft. Methodische

Einzelheiten hierzu sind bei MENZEL *et al.* [7] angegeben: für das System λ/*E. coli* C 600 kam der von uns durch Bakterienplatten ohne Phagenzusatz erweiterte BIP-Test [8] zur Anwendung. Der sich beim Lochtest ausbildende Diffusionsgradient gestattete, Virazol als 0,1 molare Lösung (Lösungsmittel Wasser) einzusetzen.

An der Veränderung der Plaquebildung im Vergleich zu unbehandelten Kontrollplatten wurde die Virazolwirkung auf die Phagen beurteilt. Ein Einfluß der Verbindung auf die Bakterien- und Blaualgenvermehrung konnte an Hemm- bzw. Förderzonen abgelesen werden, die sich in konzentrischen Ringen um die Stanzlöcher im Agar ausbildeten.

Zur näheren Charakterisierung der Virazolwirkung auf den Cyanophagen wurden die minimale Hemmkonzentration (MIC) bestimmt und die Beeinflussung freier Phagen sowie der Phagenadsorption geprüft.

Die Bestimmung der minimalen Hemmkonzentration erfolgte in Doppelschichtplatten, in deren Deckschichtagar *Pl. boryanum* (5-Tage-Kultur), Virazol in abgestuften Konzentrationen und LPP-1-Phagen gegeben wurden. Nach 6-tägiger Kultivierung bei 25 °C und 1400 lx wurden die Plaques ausgezählt.

Für die Untersuchung der Virazolwirkung auf freie Phagen wurde ein LPP-1-Lysat mit wäßriger Virazollösung vermischt (Endkonzentration 0,01%) und nach einstündiger Kontaktzeit bei 22 °C bis 10^{-6} verdünnt. Danach erfolgte die Bestimmung des Phagentiters. Zum Vergleich diente ein LPP-1-Lysat, das mit aqua dest. versetzt und in sonst gleicher Weise behandelt wurde.

Die Wirkung des Virazols auf die Phagenadsorption wurde mit 5 Tage alten Suspensionskulturen von *Pl. boryanum* geprüft, denen 0,01% Virazol und LPP-1-Phagen zugesetzt wurden. Die Kontrollsuspension erhielt anstelle der Virazollösung eine entsprechende Menge aqua dest. Um das Adsorbieren der Cyanophagen zu ermöglichen, wurden die Ansätze für eine Stunde bei 25 °C im Dunkeln gehalten. Anschließend wurden die Blaualgen bei 5000 Umdrehungen/min. abzentrifugiert, zweimal in Nährlösung [9] gewaschen und die Anzahl der Plaque-forming units (PFU) ermittelt.

Ergebnisse

Unsere Untersuchungen mit dem Lochtest ergaben, daß Virazol die Plaquebildung in den verwendeten Phagen/Wirt-Systemen nur in einem Fall beeinflußte. Wie aus Tabelle I ersichtlich ist, bildete der Cyanophage LPP-1 in einer 25 mm breiten Diffusionszone des Virazols keine Plaques aus. Andererseits zeigte sich — mit Ausnahme von *B. subtilis* — eine starke Wirkung des Virazols auf die Vermehrung der als Viruswirte verwendeten Prokaryonten. Sowohl bei den vier *E. coli*-Stämmen als auch bei der Blaualge *Pl. boryanum* traten in höheren Konzentrationsbereichen der Verbindung deutliche Hemmwirkungen auf, ohne die Vermehrung der Prokaryonten völlig zu unterdrücken. In niedrigeren Konzentrationen förderte Virazol die Vermehrung der *E. coli*-Stämme.

Während in den Bakterienhemmzonen die Plaques in gleichem Maße wie im unbeeinflußten Bakterienrasen erschienen, fehlten sie in der Hemmzone der Blaualge. Für diesen Plaquehemmeffekt wurde die minimale Hemmkonzentration in Doppelschichtplatten bestimmt; 1×10^{-4} g/ml Virazol verhinderte die Plaquebildung vollständig. Bei niedrigeren Virazolkonzentrationen war die Plaqueanzahl im Vergleich zur Kontrolle reduziert (Abb. 1).

Da Virazol im plaquehemmenden Konzentrationsbereich auch die Vermehrung des Wirtsorganismus verringerte (s. Tab. I), war die Durchführung von Einschrittvermehrungsversuchen nicht sinnvoll.

Tabelle I

Vermehrung von Prokaryontenviren und ihren Wirten unter dem Einfluß von Virazol im Lochtest

Virus	Plaquebildung		Prokaryont	Vermehrung	
	Hemmung*	Förderung*		Hemmung*	Förderung*
Φ 105	0	0	*B. subtilis* 168	0	0
λ	0	0	*E. coli* C 600	12	5
LPP-1	25	0	*Pl. boryanum*	25	0
f 2	0	0	*E. coli* K 1046	12	7
M 12	0	0	*E. coli* W 1665 F^+	12	6
Qβ	0	0	*E. coli* AB 301	13	6

* radiale Ausdehnung des Wirkbereiches in mm

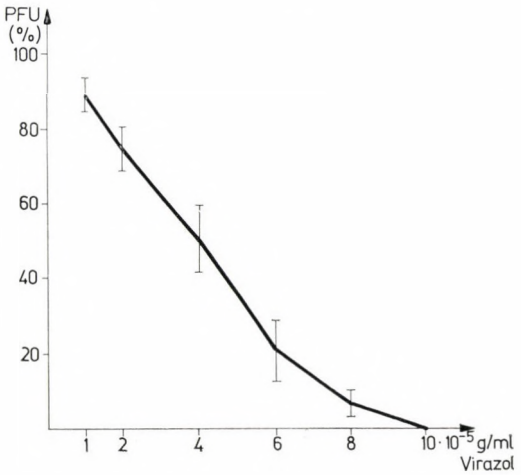


Abb. 1. Einfluß abgestufter Virazolkonzentrationen auf die Plaquebildung im System LPP-1/*Pl. boryanum*. Kontrolle = 100%

Freie LPP-1-Phagen wurden nach Einwirkung von 1×10^{-4} g/ml Virazol zu 32% inaktiviert (vgl. Tab. II). Demgegenüber hatte Virazol auf die Adsorption der LPP-1-Phagen keinen Einfluß.

Tabelle II

Wirkung von Virazol auf freie LPP-1-Phagen und auf die Adsorption von LPP-1-Phagen an Pl. boryanum

	Freie Phagen (PFU/ml)	Adsorbierte Phagen (PFU/ml)
Virazol 10^{-4} g/ml	$1{,}83 \times 10^8 \pm 1{,}1 \times 10^7 = 68\%$	$7{,}00 \times 10^4 \pm 2{,}6 \times 10^3 = 99\%$
Kontrolle	$2{,}68 \times 10^8 \pm 2{,}2 \times 10^7 = 100\%$	$7{,}04 \times 10^4 \pm 1{,}5 \times 10^3 = 100\%$

Diskussion

Im Gegensatz zu der in der Literatur beschriebenen breiten Wirkung des Virazols gegen zahlreiche DNA- und RNA-Viren in Eukaryonten fanden wir in mehreren Virus/Prokaryonten-Systemen bei Anwendung des Lochtests keine Beeinflussung der Virusvermehrung. Auch eine Störung der Lysogenisierung bzw. eine Prophageninduktion konnte bei den temperenten Phagen λ und Φ 105 nicht festgestellt werden. Es ist auszuschließen, daß mangelhafte Permeation des Virazols in die Mikroorganismenzelle die Ursache dafür ist, da die Vermehrung der meisten Wirtsstämme durch Virazol verändert wurde.

Die für LPP-1 festgestellte Plaquehemmung ist, wie die Versuche mit freien Phagen zeigen, teils auf deren Inaktivierung zurückzuführen. Der Adsorptionsprozeß der Cyanophagen an die Wirtszellen hingegen wird durch Virazol nicht beeinflußt. Da im Lochtest die LPP-1-Hemmung stets mit einer Wirtsbeeinträchtigung korreliert ist, kann man schließen, daß neben der Inaktivierung freier Phagen auch eine wirtsbedingte Blockierung der Virusreplikation für die Plaquehemmung verantwortlich ist.

In diesem Zusammenhang ist zu betonen, daß im Unterschied zu *E. coli* die Blaualge gegenüber Virazol so wie auch gegenüber vielen anderen Verbindungen [7, 10] besonders sensitiv ist. Inwieweit der photoautotrophe Stoffwechsel von Plectonema für ihre höhere Empfindlichkeit und die Beeinflussung der Virusvermehrung durch Synthetica von Bedeutung ist, kann aus den bisherigen Untersuchungen nicht abgeleitet werden. Beziehungen zwischen Photosynthese und Virusvermehrung in Blaualgen sind beschrieben worden [11–13].

Der Beeinflussung der Prokaryontenzelle durch Virazol entsprechend, kommt es bei Eukaryonten nach Einwirkung der Verbindung ebenfalls zu Zellschäden [1]. An Pflanzen wurden Chlorosen und Meristemschäden beobachtet [14]. Eine Hemmung der zellulären DNA- und RNA-Synthese in tierischen Zellen nach Virazolbehandlung stellten STREETER *et al.* [15] sowie DE CLERCQ *et al.* [3] fest. Diese Autoren sehen im Einstellen der Nukleinsäuresynthese zugleich auch die Ursache für die antivirale Aktivität des Virazols. Als Wirkungsmechanismus wird die Blockierung der Guanidinmonophosphat-Bildung angegeben, wobei der strukturellen Ähnlichkeit des Virazols mit dem Inosin bzw. Guanosin Bedeutung beigemessen wird [16, 17].

Nach der dargestellten Wirkungsweise wäre zu erwarten, daß Virazol auch die Vermehrung der Bakteriophagen beeinflußt. Das Ausbleiben einer derartigen Wirkung in unseren Versuchen könnte auf eine Inaktivierung des Virazols in der Bakterienzelle zurückzuführen sein. Dabei wäre an eine Spaltung des Moleküls in seine Triazol- und Zuckerkomponente zu denken [vgl. 18], wenngleich auch verschiedene nicht an Zucker gebundene Triazole antiphagale Eigenschaften entwickeln [19]. Außerdem könnte der Substituent in Stellung

3 am Triazol verändert werden, dessen Bedeutung für eine antivirale Wirkung WITKOWSKI *et al.* [20] beschrieben haben.

Bezüglich der Virazolwirkung auf die Prokaryonten ist zu berücksichtigen, daß selbst nach einer eventuellen Spaltung des Moleküls die Triazolkomponente allein wirksam sein kann. Die Beeinflussung der Vermehrung von Bakterien und Blaualgen durch Triazole ist mehrfach festgestellt worden [7, 21—23]. Die bei geringen Virazolkonzentrationen aufgetretene Förderung von *E. coli* ist im Vergleich zum Einfluß anderer Triazole stärker ausgeprägt, so daß möglicherweise der Riboseanteil des Virazolmoleküls hierfür verantwortlich ist.

Für die Überlassung des Phagen f 2 und des *E. coli*-Stammes K 1046 danken wir Dr. Regős, Basel. Wir danken Dr. R. W. SIDWELL, Irvine, California, der freundlicherweise dem Leiter unseres Forschungsbereiches, Prof. Dr. G. SCHUSTER, eine Probe von Virazol zur Verfügung stellte. Für technische Assistenz danken wir Frau A. GENTSCHEV und Frau H. THOMAS.

LITERATUR

1. SIDWELL, R. W., HUFFMAN, J. H., KHARE, G. P., ALLEN, L. B., WITKOWSKI, I. T., ROBINS R. K.: Science **177,** 705 (1972).
2. HUFFMAN, J. H., SIDWELL, R. W., KHARE, G. P., WITKOWSKI, J. T., ALLEN, L. B., ROBINS, R. K.: Antimicrob. Agents Chemother. **3,** 235 (1973).
3. DE CLERCQ, E., LUCZAK, M., REEPMEYER, J. C., KIRK, K. L., COHEN, L. A.: Life Sci. **17,** 187 (1976).
4. KHURANA, S. M. P., DHINGRA, M. K., NAGAICH, B. B.: 3rd Int. Congr. Virology, Madrid 1975. Abstracts C 123.
5. LERCH, B.: Jahresbericht Biol. Bundesanst. Land- u. Forstwirtschaft Berlin u. Braunschweig, S. 64 (1975).
6. SCHUSTER, G.: Ber. Inst. Tabakforsch. Dresden **23,** 21 (1976).
7. MENZEL, G., STENZ, E., TOURÉ, I. M., GEBLER, B., SCHUSTER, G.: Z. allg. Mikrobiol. **15,** 259 (1975).
8. FLECK, W.: Z. allg. Mikrobiol. **8,** 139 (1968).
9. PADAN, E., SHILO, M., KISLEV, N.: Virology **32,** 234 (1967).
10. STENZ, E., MENZEL, G.: Z. allg. Mikrobiol. Im Druck.
11. PADAN, E., GINZBURG, D., SHILO, M.: Virology **40,** 514 (1970).
12. SHERMAN, L. A., HASELKORN, R.: Virology **45,** 739 (1971).
13. MACKENZIE, J. J., HASELKORN, R.: Virology **49,** 517 (1972).
14. KLUGE, S.: Persönliche Mitteilung.
15. STREETER, D. G., KHARE, G. P., SIDWELL, R. W., SIMON, L. N.: Fed. Proc. **31,** 576 (1972).
16. PRUSINER, P., SUNDARALINGAM, M.: Nature new Biol. **244,** 116 (1973).
17. STREETER, D. G., WITKOWSKI, J. T., KHARE, G. P., SIDWELL, R. W., BAUER, R. J., ROBINS, R. K., SIMON, L. N.: Proc. nat. Acad. Sci. (Wash.) **70,** 1174 (1973).
18. VOTRUBA, I., HOLY, A., WIGHTMAN, R. H.: Biochim. biophys. Acta (Amst.) **324,** 14 (1973).
19. STENZ, E., MENZEL, G.: in Vorbereitung.
20. WITKOWSKI, J. T., ROBINS, R. K., KHARE, G. P., SIDWELL, R. W.: J. med. Chem. **16,** 936 (1973).
21. FREDRICK, J. F., GENTILE, A. C.: Arch. Biochem. Biophys. **86,** 30 (1960).
22. KRETSCHMAR, G.: Z. allg. Mikrobiol. **10,** 253 (1970).
23. KECSKÉS, M.: Symp. Biol. Hung. **11,** 405 (1972).

Anschrift der Verfasser:

GISELA MENZEL, ECKART STENZ
Sektion Biowissenschaften, Forschungsbereich Pflanzenphysiologie und Mikrobiologie, Karl-Marx-Universität Leipzig
Talstr. 33, DDR-701 Leipzig,
Deutsche Demokratische Republik

Acta microbiol. Acad. Sci. hung. 25, 17—21, 1978

ADJUVANT ACTIVITY OF ENDOTOXIN PREPARATIONS IN NORMAL AND IRRADIATED RATS*

ERZSÉBET ELEKES, L. BERTÓK and KATALIN MERÉTEY

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene Budapest and Institute of Rheumatology and Physiotherapy, Budapest

(Received February 9, 1977)

After γ-irradiation *in vitro* the adjuvanticity of detoxified endotoxin was largely retained while it diminished after treatment with potassium methylate. The adjuvant effect of the preparations was more pronounced in irradiated rats.

Endotoxins (LPS) are well-known to exert a marked adjuvant activity on the humoral immune response of mammals [1, 2]. In spite of this, data are scarce and controversial concerning the effectivity of detoxified endotoxin preparations on the immune response.

The adjuvanticity of detoxified preparations seems to depend closely on the detoxification method. According to NOWOTNY [3], treatment of endotoxin with boron trifluoride induces an expressed decrease of adjuvant activity parallel with the diminishing antigenicity. On the other hand, detoxification by potassium methylate does not influence the adjuvant capacity of endotoxin [3, 4]. An adjuvant action has been observed in lipid A after acidic hydrolysis [5] and a pronounced decrease of adjuvant activity in lipid-deprived preparations [6]. Succinated preparations also affect the adjuvant capacity.

In the present experiments the adjuvant activity of preparations detoxified by potassium methylate or by γ-irradiation and of untreated endotoxin was compared in rats. A group of normal and irradiated rats both immunized with sheep red blood cells (SRBC) served for control.

Materials and methods

Endotoxin (LPS) preparation. The LPS was isolated by the warm phenol-water method [7] from a fermentor-grown culture of *Escherichia coli* O89 and purified by repeated ultracentrifugation in Beckman L2 65B at 100 000 *g*.

Detoxification of the endotoxin preparation. Chemical detoxification of endotoxin was carried out by potassium methylate according to NOWOTNY [3]. For radiodetoxification the LPS was dissolved in distilled water and irradiated (5 Mrad) at a concentration of 10 mg/ml in a ^{60}Co-source (Noratom 3500) by Previte's method [8] as modified by us [9–11].

* Supported in part by the Scientific Research Council, Hungarian Ministry of Health (5—13—1001—O/B). Presented at the "Bacterial Endotoxins" Workshop of the European Immunology Meeting, Amsterdam, 1975.

Determination of LD_{50} of detoxified endotoxin preparation. For the determination of LD_{50} values of various LPS preparations female Wistar-R/A×Long Evans (hooded) F_1 hybrid rats (Laboratory Animal Institute, Gödöllő, Hungary) were injected intravenously. The LD_{50} values were calculated by the method of REED and MUENCH [12].

Animals. Male Wistar R/A×LE (hooded) F_1 hybrid rats of 160–200 g body weight were used.

Irradiation. The rats were irradiated with 730R by a ^{60}Co-γ-source.

Immunization. Normal rats and rats irradiated 21 days before were immunized with a single 4×10^8 dose of SRBC. One hundred μg of various endotoxin preparations were administered intravenously simultaneously with the SRBC immunization.

Determination of p.f.c. The animals were killed 5 days after the antigen injection. Their spleen was removed and the number of direct and indirect plaque forming cells (p.f.c.) were determined [13, 14]. The results were calculated on the basis of p.f.c. counts per 10^6 nucleated spleen cells and of the nucleated cell count in the whole spleen.

Serum antibody determination. Haemolysin titres of sera were determined by a micromethod both before and after 2-ME treatment [15].

Statistical analysis. Mean values and standard errors were determined; their significance was estimated by Student's *t*-test.

Results

Effect on endotoxin toxicity of ^{60}Co-gamma irradiation and chemical detoxification. E. coli O89 LPS was irradiated with 5 Mrad and the LD_{50} of the original and treated preparations was determined in rats. Irradiation increased the LD_{50} to 50 mg/kg from the initial 20 mg/kg. The decrease of toxicity was almost the same after chemical detoxification.

For native and radiodetoxified endotoxins a similar increase of the splenic p.f.c. count was obtained (Fig. 1).

Chemically detoxified endotoxin caused a slight, non-significant change. The developed 7S p. f. c. showed a change similar to that of the 19S ones.

Similar results were obtained when counting the relative number p. f. c. (Fig. 2).

The difference observed in the nucleated spleen cell count was due to the different endotoxin preparations. The increase was less with the radiodetoxified than with the untreated preparation, and the chemically detoxified one was hardly effective.

A similar order of effects was obtained when measuring the haemolysin titre (Fig. 3).

The adjuvant effect was more marked in the irradiated than in the non-irradiated animals (Fig. 4).

A significant inrease in the p.f.c. was induced by the untreated and radiodetoxified preparations ($p < 0.05$) but even the chemically detoxified preparation elicited a doubling of the splenic p.f.c. (After treatment with SRBC the p.f.c. count was 4619 ± 1660; after treatment with SRBC + chemically detoxified endotoxin it was 9180 ± 930.) The serum haemolysin titre displayed similar changes. As in the non-irradiated rats, in the irradiated animals the splenic cell count was influenced only by the untreated endotoxin.

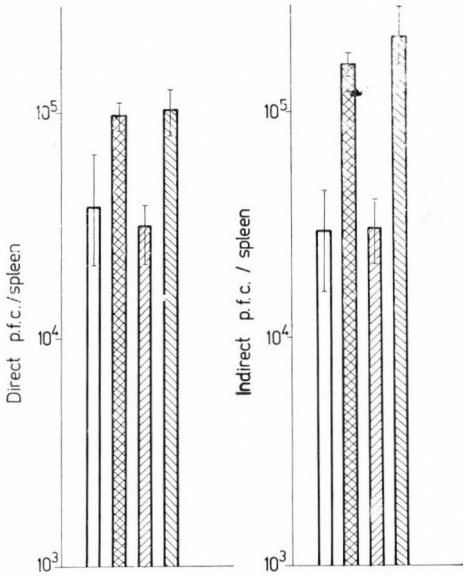


Fig 1. Effect of different endotoxin peparations on the direct and indirect p.f.c. counts; spleen in SRBC immunized rats. Open column: SRBC-treated group; hatched column: SRBC + untreated endotoxin; shaded column, left: SRBC + chemically detoxified endotoxin; shaded column, right: SRBC + *in vitro* irradiated endotoxin

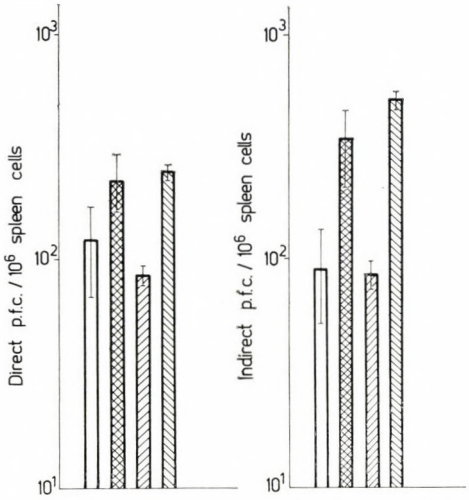


Fig. 2. Effect of different endotoxin preparations on the direct and indirect p.f.c. count/10^6 spleen cells in SRBC immunized rats. Open column: SRBC-treated group; hatched column: SRBC + untreated endotoxin; shaded column, left: SRBC + chemically detoxified endotoxin; shaded column, right: SRBC + *in vitro* irradiated endotoxin

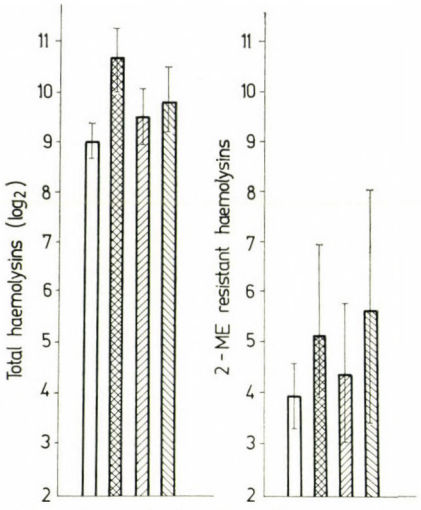


Fig. 3. Total and 2-ME-resistant haemolysin titre after treatment with different endotoxin preparations. Open column: SRBC-treated group; hatched column: SRBC + untreated endotoxin; shaded column, left: SRBC + chemically detoxified endotoxin; shaded column, right: SRBC + *in vitro* irradiated endotoxin

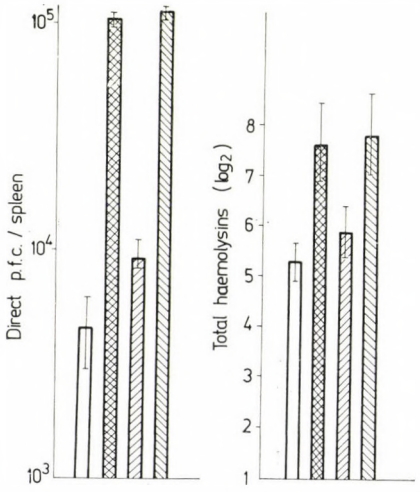


Fig. 4. Adjuvant effect of various endotoxin preparations on antibody production in rats 26 days after irradiation. Open column: SRBC-treated group; hatched column:SRBC+untreated endotoxin; shaded column, left: SRBC + chemically detoxified endotoxin; shaded column, right: SRBC + *in vitro* irradiated endotoxin

Discussion

The aim of the present investigation was to study how the different detoxyfing treatments alter the adjuvant activity of endotoxin. The results showed that irradiation *in vitro* affected the adjuvanticity of the endotoxin

molecule less than did potassium methylate treatment. In earlier experiments [16] it was shown that the untreated endotoxin preparation as adjuvant was more effective in irradiated rats. The present study proved that the adjuvanticity of detoxified preparations is also potentiated by irradiation performed 21 days before immunization. The effectivity of the endotoxin preparations investigated was not affected by the immuno-suppressive procedure. Thus, the untreated and the radiodetoxified preparations displayed a similar adjuvant effect and only the potassium methylate-treated one was less effective.

The adjuvanticity induced by different endotoxin preparations and the splenic hypertrophy caused by them thus seem to be independent phenomena. An evidence for this possibility is the discrepancy between adjuvanticity and the failure of the nucleated cell count to increase under the effect of radiodetoxified endotoxin.

REFERENCES

1. Johnson, A. G.: Bacterial Endotoxins. Rutgers University Press, New Brunswick 1964.
2. Merrit, K., Johnson, A. G.: J. Immunol. **94,** 416 (1965).
3. Nowotny, A.: Nature (Lond.) **179,** 721 (1963).
4. Johson, A. G., Nowotny, A.: J. Bact. **87,** 809 (1964).
5. Farthing, J. R.: Brit. J. exp. Med. Path. **42,** 614 (1961).
6. Schenk, J. R., Hargie, M. P., Brown, M. S., Ebert, D. S., Yoo, A. L., McIntire, F. C.: J. Immunol. **102,** 1411 (1969).
7. Westphal, O., Lüderitz, O., Bister, F.: Z. Naturforsch. **7b,** 148 (1952).
8. Previte, J. J., Chang, Y., El Bisi, H.: J. Bact. **93,** 1607 (1967).
9. Bertók, L., Kocsár, L.: Proc. 8th Annual Meeting of European Society for Radiobiology, Basko Polje, Yugoslavia 1971.
10. Bertók, L., Kocsár, L.: Izotóptechnika **11,** 543 (1972).
11. Füst, Gy., Bertók, L., Juhász-Nagy, S.: Infect. Immun. **16,** 26 (1977).
12. Reed, L. J., Muench, H.: Amer. J. Hyg. **27,** 493 (1938).
13. Jerne, N. K., Nordin, A. A., Henry, C.: In Amos, B., Koprowsky, H. (eds): Cell Bound Antibodies. Wistar Institute Press, Philadelphia 1963, p. 109.
14. Dresser, D. W., Wortis, H. H.: Nature (Lond.) **208,** 859 (1965).
15. Hege, J. S., Cole, L. J.: J. Immunol. **96,** 559 (1965).
16. Elekes, E., Merétey, K., Várterész, V.: Path. et Microbiol. **37,** 302 (1971).

Address of the authors:

Erzsébet Elekes, Loránd Bertók
"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene,
H-1775 Budapest, P.O.B. 101, Hungary

Katalin Merétey
Institute of Rheumatology and Physiotherapy
H-1719 Budapest, P.O.B. 54, Hungary

Acta microbiol. Acad. Sci. hung. 25, 23—36, 1978

COMPARISON OF ESCHERICHIA COLI ENTEROTOXIN TESTS

I. Kétyi, Éva Czirók, Adele VerténYi, Ilona Málovics and S. Pácsa

Institute of Microbiology, University Medical School, Pécs and Pest County Public Health Station, Budapest

(Received February 22, 1977)

Seventeen guinea pig eye test negative *Escherichia coli* strains and one *Enterobacter cloacae* strain isolated from clinically and epidemiologically defined cases were examined for enterotoxin production. Purified cholera toxin, *E. coli* strains of known enterotoxigenicity and a *Shigella dysenteriae* 1 strain served as controls. The rabbit skin permeability model (blueing reaction) was the most sensitive and the best for screening. The cell elongation test using the "Chinese Hamster Ovary Cell Line" and especially the rabbit ileal loop assay, were less sensitive. The results were reproducible with the suckling mouse oral test. Mitomycin C induction increased the blueing activity of filtrates and sonicated extracts.

The pioneer studies of De *et al.* [1] and Smith and Halls [2] on ligated rabbit intestine have led to the conclusion that enterotoxin-producing *Escherichia coli* strains form a new nosological unit [3—5]. Several methods have been elaborated for the detection of enterotoxin [6–12]. It has also been shown that enterotoxigenicity may vary from strain to strain [13] and that enterotoxin producing *E. coli* may be present in great numbers in the intestine of healthy individuals [14].

In the present experiments, guinea pig eye test negative cultures from well-defined sources were examined by different enterotoxin tests.

Materials and methods

Bacterial strains. Standard enterotoxigenic strains are shown in Table I. Clinical, epidemiological and immunological data for our own isolates are listed in Table II. All strains were maintained freeze-dried.

Table I

Standard strains used in the experiments

	Designation and antigenic structure of strains	Source
B2C	*E. coli* O6:H16	S.B. Formal
339T5	*E. coli* O15:H11	S.L. Gorbach
410G	*E. coli* O78:K80:H12	S.L. Gorbach
B7A	*E. coli* O148:K87:H21	S.B. Formal
P105	*E. coli* O138:K81	H.W. Smith
P99	*E. coli* O141:K85,K88	H.W. Smith
16	*S. dysenteriae* 1	Culture collection, Institute of Microbiology, Pécs

Table II

Clinical, immunological and epidemiological

	Designation and antigenic structure of strain	Source and other data
M56899	*E. coli* O114:K90a,b	Faeces of 1-month-old infant with 1 : 128 haemagglutination titre (IgM) to the homologous strain. Hospital outbreak of enteritis among infants and premature babies caused by *E. coli* O114:K90a,b uniform in antibiotic sensitivity, colicin production and lysogenicity [15].
8013	*E. coli* O148	Faeces of 2-month-old infant with mild enteritis.
23473	*E. coli* O78:K80:NM	Faeces of 6-day-old infant with meningitis. Hospital outbreak mainly with extraintestinal symptoms (sepsis, meningitis, etc.) and partly with enteritis caused by colicin positive *E. coli* O78:K80:NM uniform in antibiotic sensitivity and phage pattern [16].
Lsz 14c	*E. coli* O4	Faeces of child aged 11/2 years with dysenteriform enteritis. Homologous haemagglutination titre, 1:1024.
67643/3	*E. coli* O75	Faeces of 6-month-old infant. Hospital outbreak of enteritis among newborn infants caused by *E. coli* O75 uniform in antibiotics sensitivity, colicinogenicity and phage pattern.
74971	*E. coli* O119:K69:NM	Faeces of 4-month-old infant died with dysenteriform enteritis.
62268	*E. coli* O111:K58:H2	Faeces of 4-month-old infant. Hospital outbreak of choleriform enteritis.
17968	*E. coli* O18a,c	CSF of 1-year-old infant. Hospital outbreak of enteritis and meningitis, Wernigerode, G.D.R.

Strains listed in this table were isolated by É. CZIRÓK, except strains 3 and 4 originating from the material of the Institute of Microbiology, Pécs
NM = non-motile

Cholera toxin (Lot No. 0972) obtained from Dr. I. Joó (Institute for Serobacteriological Production and Research "Human", Budapest) was prepared by Dr. R. A. FINKELSTEIN (University of Texas, Southeastern Medical School, Dallas, Texas) [17]. The freeze-dried toxin was dissolved in twice distilled water and diluted with phosphate buffer (NaCl, 0.12 M; Na_2HPO_4, 0.016 M; NaH_2PO_4, 0.044 M; merthiolate, 0.01%; bovine serum albumin, 0.1%) so as to give 1800 Lb/ml. The stock solution was stored at 4 °C.

Cholera antitoxin was supplied by Dr. I. Joó (Institute for Serobacteriological Production and Research "Human", Budapest). The 2 ml freeze-dried sample (Swiss Serum, EC 3/A–2/67–B) was dissolved as recommended in 25 ml volume. This stock solution designated as "f", contained 357 antitoxin units. The stock solution was stored at 4 °C; the dilutions were prepared freshly before use in borate buffer saline.

E. coli anti-enterotoxin sera were prepared in rabbits with 18 hr shaken cultures grown in media for enteroxoin production. Graded doses of 0.2, 0.5, 1.0 and 2.0 ml $3–6 \times 10^8$ cells/ml) were given intravenously at 4 day intervals; the animals were bled 5 days after the last injection. The rabbits were bled 5 days after the last injection. The sera were passed through Seitz filter and preserved with 0.01% merthiolate.

data of strains tested for enterotoxigenicity

Designation and antigenic structure of strain		Source and other data
27272	*E. coli* O83:NM	Lung of 2-day-old infant died with meningitis and bronchopneumonia
40116	*E. coli* O111:K58:H2	Faeces of infant. Rapidly spreading hospital outbreak of severe enteritis.
39017	*E. cloacae*	Faeces of adult. Food poisoning of motor road-building workers.
Lsz 463b/2	*E. coli* O78:K80	Faeces of 4-month-old infant with mild enteritis and predominant respiratory symptoms. Homologous haemagglutination titre, 1 : 128.
Lsz 233a	*E. coli* O117:H27	Faeces of 8-month-old infant with moderately severe enteritis lasting 2 weeks. Homologous haemagglutination titre, 1 : 256.
Lsz 326e	*E. coli* O75	Faeces of 6-month-old infant with prolonged, moderately severe enteritis. Faecal culture in initial stage, *Salmonella infantis:* in later stages, *E. coli* O75. Haemagglutination titres, 1 : 32 for *S. infantis*, 1 : 1024 for *E. coli* O75.
T47683	*E. coli* O18a,c:NM	Faeces of 5-day-old infant. Rapidly spreading, mild hospital outbreak of enteritis among newborn infants.
T59426	*E. coli* O18a,c:NM	Faeces of newborn infant. Mild outbreak of enteritis among newborn babies.
3 and 4	*E. coli* O26:K60:H11	Faeces of infants. Hospital outbreak of enteritis.

Enterotoxin filtrates. The medium of SAKAZAKI *et al.* [18] was modified by substituting Levinthal broth for Myosat (Levinthal broth contained mannitol, 0.1% NaCl, 0.35%; K_2HPO_4, 0.368%; KH_2PO_4, 0.132%; pH 7.5). The medium was distributed at 5 ml portions into tubes, inoculated from blood agar culture and incubated at 37 °C overnight. The precultures were transferred to Erlenmeyer flasks containing a thin layer of the same medium and shaken at 37 °C for 18 hr in a New Brunswick Psychrotherm apparatus. The supernatant was separated in a refrigerated centrifuge, filtered through Göttingen Membrane-filter No. 1121 and stored at −20 °C.

Sonicated extracts. Shaken cultures prepared in the above manner were subjected to sonication at 0 °C (MSE 500 W, full energy setting, 30 min). The preparations were checked by counting the surviving bacteria: a 4–5-fold decrease in the number of colony formers was regarded as an effective exposure. The extract was filtered and stored as described above.

Mitomycin C induction. Filtrates and sonicated lysates were usually prepared from Mitomycin C induced cultures [19]. Before culturing, Mitomycin C, 0.5μ/ml (Kyowa H.K.Co. Ltd., Tokyo, Japan) was added to the medium.

S. dysenteriae 1 filtrate was prepared as described above and assayed by the HeLa cell attachment inhibition micromethod [20]. The crude filtrate gave a positive reaction up to dilution 1 : 32.

Rabbit ileal loop assay. The method of DE *et al.* [1] in New Zealand white rabbits weighing 1.8–2.6 kg was used. The test was read 20 hr after injection and the pathological change was expressed as the ratio of accumulated fluid weight/intestinal weight. Tests with an index of 1 or above were regarded as positive.

Suckling mouse test. The method of DEAN *et al.* [21] as modified for oral administration by JACKS and WU [22] was employed. One- to three-day-old mice were separated from their mothers before testing, grouped into 4–5 animals at random and given 0.1 ml volumes of filtrates orally with an S-shaped metal catheter sheathed with polyethylene. The mice were kept in an incubator at 30 °C for 4 hr, then sacrificed, weighed and dissected. Marked reaction was indicated by a visibly dilated intestine. The intestine was removed and weighed; the degree of reaction was expressed as the intestinal weight/body weight ratio: < 0.07 was considered negative, 0.07–0.09 doubtful or weak positive, and > 0.09, positive.

Intradermal (blueing) reaction. The method of EVANS *et al.* [8] was used. White rabbits of the "Pearl of Mecsek" breed were shaven on the back then 0.1 ml aliquots of the extracts were injected intradermally 2–2.5 cm apart. The lowest dilution was 1 : 2 in phosphate buffer saline containing bovine serum albumin (0.02%). Eighteen hours after the injections the dye was given intravenously. In preliminary experiments Evans blue (40 mg/kg), later 5% Pontamin Sky Blue 6XB (Serva, Heidelberg) solution was used as recommended by CRAIG [23]. Readings were performed one hour after injection of the dye.

This method was used for neutralization experiments with cholera antitoxin or *E. coli* anti-enterotoxic sera. The toxin preparation was mixed with the corresponding antiserum at equal amounts and after 30 min incubation at 37 °C the mixture was injected intradermally at 0.1 ml aliquots.

CHO tissue culture experiments were carried out as described in reference [24]. The "Chinese Hamster Ovary Cell Line" culture was supplied by Dr. B. ROWE, London. The cells were maintained on 90% MEM + 10% calf serum medium. Transfers were made at weekly intervals by trypsin-versene detachment. To obtain a tissue culture suitable for enterotoxin assay, about 10^6 cells + 15 ml medium were incubated in 250 ml flasks for 2–3 days.

Enterotoxin was detected by a micromethod using flow tissue culture microslides. By trypsinization, from each Roux flask a homogenized suspension containing $5–10 \times 10^3$ cells/0.2 ml was obtained. To the concave depression of each slide 0.2 ml cell suspensions was added and left to stand for 24 hr to allow cell attachment. Then the medium was removed by suction and 0.2 ml amounts of enterotoxin preparation diluted with MEM medium were added. After 24 hr incubation the cells were examined for elongation. Using appropriate positive and negative controls, only marked elongation was regarded as a positive reaction. The ratio of intact and elongated cells was not estimated; in positive tests at least 30% of the cells showed elongation.

The guinea pig eye test was carried out as described by SERÉNY [25].

Results

Rabbit ileal loop test. Results are shown in Table III. On the basis of a rather rigorous criterion of enterotoxigenicity (fluid weight/intestinal weight index = 1 or above), only 10 out of the 18 isolates were positive. Sonicated extracts gave better results; less positive reactions were obtained with filtrates and especially with broth cultures.

Suckling mouse test. It is generally accepted that this test detects heat stable enterotoxin (ST). After having acquired the necessary technical skill, the results were reproducible. As shown in Table IV, with some strains the intestinal weight/body weight index fell between wide ranges; this finding indicates a variation of enterotoxin production in different batches and not the standard error of the method. The negative control preparations (culture medium filtrate, *S. dysenteriae* 1 filtrate) uniformly failed to give a positive reaction. Filtrates of strain P105 and B7A used as positive controls, were strongly positive (0.09– $>$ 0.09 at 1 : 20 and even at 1 : 40 dilution).

Strain 23473 (O78 : K80 : NM), as with other enterotoxin reactions, was negative. Non-enterotoxigenic strains (with indices lower than 0.06)

Table III

Rabbit ileal loop assay of enterotoxigenicity

Designation and antigenic structure of strains		Fluid weight/intestinal weight						Result
		filtrate		sonicated extract		broth culture		
		range	$\bar{x}$	range	$\bar{x}$	range	$\bar{x}$	
23473	O78:K80:NM	.		0.1–0.2	0.15	.		negative
M56899	O114:K90a,b	.		0.1–0.2	0.15	.		negative
8013	O148							positive*
Lsz.14c	O4	0.1–0.2	0.15	0.1–0.7	0.30	0.8	0.8	negative
67643/3	O75	0.1–5.0	2.50	0.1–1.0	0.60	0.4	0.4	positive
74971	O119:K69:NM	0.1–1.2	0.70	0.1–0.5	0.30	.		positive
62268	O111:K58:H2	0.1–0.5	0.30	0.1–5.8	2.10	0.4	0.4	positive
17968	O18a,c	0.1	.	0.1–1.5	0.70	0.6	0.6	positive
27272	O83:NM	0.1–2.2	0.90	0.6–2.3	1.60	0.4	0.4	positive
40116	O111:K58:H2	0.1–4.4	1.50	0.1–1.3	0.50	0.2	0.2	positive
Lsz.463b/2	O78:K80	0.1–0.2	0.15	0.1–2.0	0.50	1.3	1.3	positive
Lsz.233a	O117:H27	0.1–0.2	0.15	0.1–0.8	0.40	0.2	0.2	negative
Lsz.326e	O75	0.1–0.2	0.15	0.1–0.5	0.30	0.1	0.1	negative
T47683	O18a,c:NM	0.1–0.2	0.15	0.1–0.4	0.20	0.3	0.3	megative
T59426	O18a,c:NM	0.1	.	0.1–0.8	0.60	0.2	0.2	negative
39017	*E. cloacae*	0.6–9.2	0.10	0.2–0.7	0.30	0.2	0.2	negative
3 and 4	O26:K60:H11	.		.		0.8–4.0	2.1	positive
16	*S. dysenteriae* 1	.		.		3.5	3.5	positive
P99	O141:K85,K88	.		4.7–9.1	7.10	0.7–6.3	3.4	positive

* Earlier experiment

sharply differed from the seven weakly positive cultures (with indices up to 0.084). The latter were regarded as enterotoxigenic organisms, since some of them (e.g. 74 971 and 62 268) were shown to vary from doubtful to strong positivity according to different batches of filtrates (Table IV).

The filtrate of the positive control strain (P105) remained positive after exposure to 100 °C for 30 min.

Intradermal test in rabbits. The dorsal region of the rabbit allows the injection of 30–40 samples and the reaction offers a simple means of screening. In a number of preliminary experiments we attempted to establish the optimum technical requirements and to produce suitable filtrates. We carefully checked the specificity of the reaction, since at the time of our first experiments the favourable results of EVANS *et al.* [8] had not yet been published and literary data had indicated that *E. coli* LT, unlike choleragen, failed to give the reaction.

Table IV

Assay of heat stable (ST) enterotoxin by the suckling mouse test using bacterial filtrates

Designation and antigenic structure of strains		No. of experiments	No. of animals	Intestinal weight/body weight range	Result
23473	O78:K80:NM	3	13	0.04–0.06	negative
M56899	O114:K90a,b	2	8	0.06–0.085	weakly positive
8013	O148	2	8	0.06–0.085	weakly positive
Lsz.14c	O4	1	3	0.078	weakly positive
67643/3	O75	1	4	0.094	positive
74971	O119:K69:NM	2	8	0.075–0.110	positive
62268	O111:K58:H2	3	12	0.075–0.109	positive
17968	O18a,c	1	4	0.093	positive
27272	O83:NM	1	4	0.094	positive
40116	O111:K58:H2	1	4	0.090	positive
Lsz.463b/2	O78:K80	1	4	0.093	positive
Lsz.233a	O117:H27	2	8	0.076–0.085	weakly positive
Lsz.326e	O75	1	4	0.098	positive
T47683	O18a,c:NM	1	4	0.093	positive
T59426	O18a,c:NM	3	12	0.076–0.084	weakly positive
3 and 4	O26:K60:H11	1–1	5–5	0.070–0.085	weakly positive
39017	*E. cloacae*	1	5	0.090	positive
Culture medium filtrate		5	20	0.030–0.050	negative
16	*Sh. dysenteriae* 1	2	9	0.050–0.060	negative
P105	O138:K81	4	25	0.098–0.110*	positive
B7A	O148:K87:H28	5	23	0.100–0.140**	positive

* At dilution 1 : 20
** 0.09 at dilution 1 : 40

We found that careful shaving was satisfactory instead of chemical epilation. In the course of the experiments we substituted the Pontamine sky blue for Evans blue which, perhaps being more readily soluble, gave a more visible coloration.

A considerable number of rabbits were unsuitable for the blueing test. These animals developed irregular red spots after shaving, showed a decreased turgor of the skin and secondary growth of hair. Most of these signs were encountered in winter. It was assumed that these animals might have suffered from allergy associated with mycosis. The only disadvantage of the blueing test is the occurrence of such kinds of rabbits; however, they can easily be recognized and false results can thus be avoided.

With undiluted preparations rich in enterotoxin the reaction is characterized not only by an infiltration of the blue dye but also by local oedema.

White spots surrounded by a blue ring were frequently observed; FINKELSTEIN *et al.* [26] attributed this phenomenon to a "blanching factor". The white spots fail to appear with choleragen which sometimes causes a definite positive reaction in rabbits unsuitable for the detection of *E. coli* LT.

Sonicated extracts were more active than filtrates. We extracted some cultures by the polymyxin-release method [26] and by alkali treatment as recommended for *S. dysenteriae* 1 [28], but failed to confirm any advantage of the procedures. In contrast, with the majority of strains Mitomycin C induction increased 2 to 20-fold the activity of filtrates and especially of sonicated extracts. As Mitomycin C induction requires no surplus labour, the method has been applied for all strains and in our experience it can be used routinely.

Boivin extract or purified LPS failed to give the reaction. After exposure to 65–100 °C for 30 min the filtrate lost its activity. As a further check of specificity, neutralization experiments were performed by adding antitoxin to the filtrates and inoculating the mixture intradermally. Cholera antitoxin at 3.57—0.71 units neutralized the blueing effect of different *E. coli* strains. Immune sera prepared from antigenically homologous and heterologous *E. coli* strains (P105, M56899, 339T5, B7A, 8013, 410G) neutralized at 1:5–1:10 dilution the activity of *E. coli* B2C (O6 : H16) filtrate. Normal rabbit serum had no neutralizing effect.

Table V shows a cross-neutralization experiment with choleragen and B2C filtrate vs. cholera antitoxin and B7A antiserum. The results show that 0.9 Lb cholera toxin (the minimum amount needed to elicit blueing reaction) was neutralized by 1.12 units of cholera antitoxin or by B7A (O148) antiserum at 1 : 8 dilution. Filtrate B2C at the highest blueing dilution was neutralized by 1.12 units of cholera antitoxin and by 1 : 40 dilution of antiserum B7A.

The data have confirmed the known serological homology of choleragen and LT of different serogroups of *E. coli* and indicated the specificity of the blueing test.

Enterotoxigenicity of different strains as assayed by the blueing reaction is presented in Table VI. Strain 23 473, which was negative in all tests, was used as negative control. The rest of the strains gave a positive reaction. Filtrates, prepared as a rule by mitomycin induction, were positive at 1 : 2–1 : 80 dilution ($\bar{x} = 19.1$), whereas mitomycin-induced sonicated lysates at 1 : 10 – 1 : 160 dilution ($\bar{x} = 75.0$). *S. dysenteriae* 1 filtrate, used also as negative control, was inactive. The sterile culture medium filtrate and BSA-PBS caused no reaction. Choleragen, used as positive control, gave the minimum degree of positive blueing effect (5 mm in diameter) at 0.09 Lb.

Chinese Hamster Ovary Cell Culture. In preliminary experiments, using *S. dystenteriae* 1 filtrate as positive control, we examined the cytotoxic effect of *E. coli* filtrates on HeLa, HEp-2 and mouse fibroblast cell cultures and

Table V

Cross-neutralization experiment with choleragen and E. coli filtrate vs. cholera antitoxin and heterologous E. coli antiserum containing antitoxin

Antitoxins		Toxins and size of blueing reaction, mm	
		choleragen 0.9 Lb	B2C filtrate, 1 : 40
Cholera antitoxin	4.46 A.U.	—	—
	2.23 A.U.	—	—
	1.12 A.U.	—	—
	0.56 A.U.	5	5
	0.28 A.U.	8	5
	0.14 A.U.	10	5
	—	10	5
		choleragen 0.09 Lb	B2C filtrate, 1 : 40
B7A (0148) serum	1 : 2	—	—
	1 : 4	—	—
	1 : 8	2	—
	1 : 16	5	—
	1 : 40	5	3
	1 : 80	5	5
	—	5	5

the inhibition of cell attachment in HeLa and HEp-2 cultures. As expected, the filtrates and purified choleragen were ineffective. On CHO cells the enterotoxin produced marked elongation, which, using negative and positive controls, was definite enough to characterize positive reactions without estimating the percentage of elongated cells. Results are shown in Table VII.

It is evident that CHO cells are at least as sensitive to choleragen as the rabbit skin in the blueing test. However, the *E. coli* filtrates caused morphological changes in CHO cells at dilutions lower (1 : 2–1 : 8) than those needed to elicit the blueing reaction. Sonicated extracts showed no appreciably higher activity. In addition to strain 23473 (O78 :K80 : NM) which was negative in every test, strain 17968 (O18a, c) and 27272 (O83 : NM) were inactive in the cell culture.

Table VI

Result of blueing test with filtrates and sonicated extracts

Designation and antigenic structure of strains		Reciprocals of dilutions showing positive reaction	
		filtrate	sonicated extract
23473	O78:K80:NM	<2	<2
M56899	O114:K90a,b	10	10
8013	O148	10	20
3	O26:K60:H11	5*	10*
4	O26:K60:H11	2	.
Lsz. 14c	O4	10*	40*
67643/3	O75	80*	80*
74971	O119:K69:NM	10*	40*
62268	O111:K58:H2	10*	160*
17968	O18a,c	10*	10*
27272	O83:NM	10*	80*
40116	O111:K58:H2	40*	80*
Lsz. 463b/2	O78:K80	40*	40*
Lsz. 233a	O117:H27	2*	160*
Lsz. 326e	O75	10*	160*
T47683	O18a,c:NM	10*	40*
T59426	O18a,c:NM	10*	80*
39017	*E. cloacae*	80*	160*
B2C	O6:H16	5*	80*
B7A	O148:K87:H21	10*	100*
Mean titre giving positive reaction ($\bar{x}$)		19.1	75.0
16	*S. dysenteriae* 1	<2	
Sterile culture medium filtrate and PBS dilutent		<2*	
Choleragen (Lot No. 0972)		0.09 Lb	

* The culture medium contained 0.5 μg/ml mitomycin

Guinea pig eye test. All examined strains were negative with SERÉNY test and after a few days the organisms could not be recovered from the conjunctival sac. These findings indicate that the cultures exerted no epithelial affinity or invasiveness.

Comparison of the results of enterotoxin tests. In view of the small number of strains tested, only limited conclusions can be drawn. Table VIII, summarizing the data, shows that the blueing test is the most sensitive and the suckling mouse test is next in order. The CHO cell reaction was negative for two strains giving the ileal loop test. The latter was negative for 7 strains yielding positive results with the other tests. The data indicate that all strains produced heat-labile (LT) and heat-stable (ST) enterotoxin in varying amounts.

Table VII

Result of Chinese Hamster Ovary cell elongation test with filtrates

Designation and antigenic structure of strains		Reciprocals of dilutions causing definite elongation
23473	O78:K80:NM	<2
M56899	O114:K90a,b	4
8013	O148	4
Lsz. 14c	O4	2
67643/3	O75	2
74971	O119:K69:NM	2
62268	O111:K58:H2	4
17968	O18a,c	<2
27272	O83:NM	<2
40116	O111:K58:H2	4
Lsz. 463b/2	O78:K80	4
Lsz. 233a	O117:H27	4
Lsz. 326e	O75	4
T47683	O18a,c:NM	2
T59426	O18a,c:NM	8
39017	*E. cloacae*	2
B7A	O148:K87:H21	8
Mean titre giving positive reaction ($\bar{x}$)		3.8
Sterile culture medium filtrate containing 0.5 μg/ml mitomycin C		<2
Choleragen (Lot No. 0972)		0.056 Lb

Table VIII

Comparison of enterotoxin tests

Rabbit loop test	Suckling mouse test	Rabbit test	CHO test	No. of strains*
+	+	+	+	6
+	+	+	−	2
−	+	+	+	7

* Total number of strains, 15. All strains produced heat-stable + heat-labile enterotoxin

Discussion

In the present study the rabbit ileal loop, the suckling mouse, the rabbit intradermal and the Chinese Hamster Ovary Cell tests were compared for sensitivity to enterotoxin. The results were supplemented with the SERÉNY test indicative of the epithelial invasiveness of the organism. Similar comparisons of different enterotoxin tests have been described in the literature partly for single cultures from well-analysed outbreaks or sporadic cases

[29], partly for series of strains with less defined origin [30, 31]. The data presented have the advantage of analysing several strains isolated from clinically and epidemiologically defined sources.

The rabbit ileal loop assay presents technical difficulties and allows a small number of examinations. Moreover, in sensitivity it is inferior to other tests and is of limited specificity: it is sensitive to LT and ST [32] but also to shigellae and salmonellae [33, 34]. On the other hand, cultures isolated from swine and found enterotoxigenic in the pig intestinal loop, were inactive in the rabbit gut assay [7, 35].

The suckling mouse test is regarded as sensitive, within the 4 hr incubation period, only to ST. We have also shown the satisfactory reproducibility and sensitivity of this test. Accordingly, using an adequate mouse strain, the reaction can be advantageous as an additional screening method. We have adopted the criteria of JACK and WU [22] and recorded tests with 0.07—0.09 intestinal weight/body weight indices as weakly positive. As such tests differed sharply from the negative ones (in many animals there was gross intestinal dilatation and fresh filtrates of the same strain frequently gave marked positive reactions), it may be concluded that the 0.09 index should not be regarded as a sharp border or as a criterion solely LT production. Some authors [14, 31] described results with positive indices lower than 0.09.

The blueing test seems to be the best method for screening. Our findings agree with those of FINKELSTEIN *et al.* [26] in that reading of the reaction elicited by *E. coli* LT is more difficult than of the choleragen reaction. Apart from this, the only disadvantage of the test was that many rabbits were unsuitable, but this may have been associated with the breed we were using.

The blueing test was the most sensitive for showing enterotoxin. Its sensitivity was improved by mitomycin induction, a simple procedure requiring no excess work. Sonication of the preparation is advantageous but may be omitted in screening. The results of heating and neutralization experiments have confirmed the specificity of the test.

The low sensitivity of the CHO cell test was an unexpected finding. Although most strains gave a positive reaction, *E. coli* LT exerted a weak activity in comparison to choleragen. The minimum amount of choleragen eliciting the reaction was about the same for the blueing test (0.09 Lb) and for the CHO test (0.056 Lb), whereas the minimum doses of *E. coli* filtrates considerably differed in this respect (1 : 19.1 for blueing and 1 : 3.8 for CHO test). Our data for the CHO test cannot directly be compared with the technique estimating the percentage of elongated cells [24]. GUERRANT *et al.* [31] regarded the CHO cell reaction as positive when at dilution 1 : 256 the percentage of elongated cells was 9.5–9.7 and when undiluted culture supernatants caused no more than 27% elongation. We recorded the result as positive if a minimum of 25% elongation occurred. In our opinion, estimation of the percentage ratio

of elongated cells would lessen the value of screening, the greatest advantage of the test.

Our results indicate that the test of choice for screening is the blueing reaction. In view of the occurrence of ST-producing strains [36], the suckling mouse test may also be recommended. The CHO cell reaction requires a concentrated enterotoxin preparation and is, therefore, more labourious. It may be added that the lower activity in the CHO cell test of *E. coli* LT as compared to choleragen can be validly stated only if the former is employed in a purified preparation.

In addition to enterotoxin, the adhesion factor(s) may play an important role in the pathogenicity of *E. coli*. The latter were studied more extensively in animal [37, 38] than in human [39] pathology. The association between enterotoxigenicity, presence of adhesion factors and clinical manifestation of the disease has not yet been elucidated. The role of enterotoxin production in different nosological units of *E. coli* is an open problem. In our material five strains were examined which belonged to serogroups associated with epidemic infantile enteritis. Two of them (serotype O26 : K60 : H11) were shown in our earlier pathomorphological study [40] to exert a limited invasiveness (characteristic of strains causing infantile enteritis) and an alteration of the brush border of the intestinal epithelium (known to take place as a result of enterotoxin action). These experiments were carried out on the rabbit intestine; under natural conditions in infants the enterotoxin effect may not manifest. We showed enterotoxin production by two guinea pig eye test positive *E. coli* strains (O28 and O143), but the association of this property with the clinical picture was questionable.

It may be assumed that the enterotoxin-type pathogenicity of a given strain should not be based only on a high level enterotoxin production demonstrable *in vitro*. To confirm the pathogenicity of the isolate, laboratory findings should be supported by clinical and epidemiological data even in the case of shigellae and salmonellae. In other words, isolation of a strain highly enterotoxic under test conditions, does not *per se* prove its aetiological role in the disease.

The present results obtained with small number of strains are insufficient to decide on the debate as to the association of serotypes with enterotoxigenicity [41, 42]. The enterotoxigenicity of our *E. cloacae* strain does not contradict the findings on the serological distribution of enterotoxin producing strains.

Acknowledgements. We wish to thank Dr. S. B. Formal (Walter Reed Army Institute, Washington D.C., U.S.A.), Dr. S. L. Gorbach (University of California, Los Angeles, California, U.S.A.) and Dr. H. W. Smith (A.R.C. Poultry Research Station, Houghton, Huntingdon, England) for standard enterotoxigenic strains. We are indebted to Dr. I. Joó (Institute

for Serobacteriological Production and Research "Human", Budapest, Hungary) for purified choleragen and cholera antitoxin and to Dr. B. ROWE (Salmonella and Shigella Reference Laboratory, Central Public Health Laboratory, London, England) for the CHO cell culture.

REFERENCES

1. DE, S. N., BATTACHARYA, K., SARKAR, J.: J. Path. Bact. **71,** 201 (1956).
2. SMITH, H. W., HALLS, S.: J. Path. Bact. **93,** 499 (1967).
3. SACK, R. B., GORBACH, S. L., BANWELL, J. G., JACOBS, B., CHATTERJEE, B. D., MITRA R. C.: J. infect. Dis. **23,** 378 (1971).
4. GORBACH, S. L., BANWELL, J. G., CHATTERJEE, B. D., JACOBS, B., SACK, R. B.: J. clin. Invest. **50,** 881 (1972).
5. EVANS, D. J. JR., CHEN, I. C., CURLIN, G. T., EVANS, D. G.: Nature (Lond.) **236,** 137 (1972).
6. CARPENTER, C. J.: J. infect. Dis. **126,** 551 (1972).
7. GORBACH, S. L., KHURANA, C. N.: New Engl. J. Med. **287,** 791 (1972).
8. EVANS, D. G., EVANS, D. J. JR., GORBACH, S. L.: Infect. Immun. **8,** 725 (1973).
9. STANISLAVSKY, J. S.: Personal communication.
10. CRAIG, J. P.: Symp. Soc. gen. Microbiol. **22,** 129 (1972).
11. GUERRANT, R. L., CHEN, L. C., SHARP, G. W. G.: J. infect. Dis. **125,** 377 (1972).
12. DONTA, S. T., KING, M., HOPER, K.: Nature new Biol. **234,** 246 (1973).
13. LARIVIÉRE, S., GYLES, C. L., BARNUM, D. A.: Canad. J. comp. Med. **36,** 319 (1972).
14. RUDOY, R. C., NELSON, J. D.: Amer. J. Dis. Child. **129,** 668 (1975).
15. CZIRÓK, É., MILCH, H., STVERTECZKY, ZS., HÉRMÁN, G., LOSONCZY, G.: Acta microbiol. Acad. Sci. hung. **22,** 299 (1975).
16. KENDE, É., SERE, G., CZIRÓK, É., BÉKÉSY, ZS., JÁKÓ, Z., KORMOS, E., KUBINYI, M., MAXIMOVA, G., MIHÁLYFI, I., NYOMÁRKAI, I., NÉMEDI, L., SALFAI, G., ADAMIS, É.: Egészségtudomány **19,** 79 (1975).
17. FINKELSTEIN, R. A., LOSPALLUTO, J. J.: J. infect. Dis. (Suppl.) **121,** 63 (1970).
18. SAKAZAKI, R., TAMURA, K., NAKAMURA, A., KUWATA, T., GHODA, A., TAKEUCHI, S.: Jap. J. med. Sci .Biol. **27,** 19 (1974).
19. ISAACSON, R. E., MOON, H. W.: Infect. Immun. **12,** 1271 (1975).
20. PÁCSA, S., HANKOVSKY, O., HIDEG, K.: Europ. J. Cancer **10,** 193 (1974).
21. DEAN, A. G., CHING, YI-CHUAN, WILLIAMS, R. G., HARDEN, L. B.: J. infect. Dis. **125,** 407 (1970).
22. JACKS, T. M., WU, B. J.: Infect. Immun. **9,** 342 (1974).
23. CRAIG, J. P.: Nature (Lond.) **207,** 614 (1965).
24. GUERRANT, R. L., BRENTON, L. L., SCHNAITMAN, T. C., REBHUN, L. I., GILMAN, A. G.: Infect. Immun. **10,** 320 (1974).
25. SERÉNY, B.: Acta microbiol. Acad. Sci. hung. **4,** 367 (1957).
26. FINKELSTEIN, R. A., LARUE, M. K., JOHNSTON, D. W., VASIL, M. L., CHO, G. J., JONES, J. R.: J. infect. Dis. **133** (Suppl.) S120 (1976).
27. EVANS, D. JR., EVANS, D. G., GORBACH, S. L.: Infect. Immun. **10,** 1010 (1974).
28. VAN HEYNINGEN, W. E., GLADSTONE, G. P.: Brit. J. exp. Path. **34,** 202 (1953).
29. FINKELSTEIN, R. A., VASIL, M. L., JONES, J. R., ANDERSON, R. A., BARNARD, T.: J. clin. Microbiol. **3,** 382 (1976).
30. MORRIS, G. K., MERSON, M. H., SACK, D. A., WELLS, J. G., MARTIN, W. T., DEWITT, D. E., FEELEY, J. C., SACK, R. B., BESSUDO, D. M.: J. clin. Microbiol. **3,** 486 (1976).
31. GUERRANT, R. L., MOORE, R. A., KIRSCHENFELD, P. M., SANDE, M. A.: New Engl. J. Med. **293,** 567 (1975).
32. EVANS, D. G., EVANS, D. J. JR., PIERCE, N. F.: Infect. Immun. **7,** 873 (1973).
33. YAHAGI, H., GHODA, A., SASAKI, S.: Keio J. Med. **16,** 119 (1967).
34. SAKAZAKI, R., TAMURA, K., NAKAMURA, A., KUWATA, T.: Jap. J. med. Sci. Biol. **27,** 45 (1974).
35. MOON, H. W., WHIP, S. C.: J. infect. Dis. **122,** 220 (1970).
36. SMITH, H. W., HALLS, S.: J. Path. Bact. **93,** 531 (1967).
37. SOJKA, W. J.: *E. coli* in Domestic Animals and Poultry. Commonwealth Bureau of Animal Health, Weybridge 1965.
38. SMITH, H. W., LINGGOOD, M. A.: Med. Microbiol. **5,** 243 (1972).
39. EVANS, D. G., SILVER, R. P., EVANS, D. J. JR., CHASE, D. G., GORBACH, S. L.: Infect, Immun. **12,** 656 (1975).

40. POLOTSKY, YU. E., DRAGUNSKAYA, E. M., SELIVERSTOVA, V. G., AVDEEVA, T. A., CHAKHUTINSKAYA, M. G., KÉTYI, I., VERTÉNYI, A., RALOVICH, B., EMŐDY, L., MÁLOVICS, I., SAFONOVA, N. V., SNIGIREVSKAYA, E. S., KARYAGINA, E. I.: Acta microbiol. Acad. Sci. hung. **24,** 221 (1977).
41. GOLDSCHMIDT, M. C., DUPONT, H. L.: J. infect. Dis. **133,** 153 (1976).
42. ØRSKOV, F., ØRSKOV, I., EVANS, D. J. JR., SACK, R. B., SACK, D. A., WADSTRÖM, T.: Med. Microbiol. Immunol. **162,** 73 (1976).
43. KLIPSTEIN, F. A., HOLDEMAN, L. V., CORCINO, J. J., MOORE, W. E. C.: Ann. intern. Med. **79,** 632 (1973).

Address of the authors:

IVÁN KÉTYI, ADELE VERTÉNYI, ILONA MÁLOVICS, SÁNDOR PÁCSA
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

ÉVA CZIRÓK
Pest County Public Health Station
H-1428 Budapest, P.O.B. 55, Hungary

Acta microbiol. Acad. Sci. hung. 25, 37—41, 1978

SOME PHYSICAL AND CHEMICAL PROPERTIES OF MYCOBACTERIOPHAGE DNA

P. A. SOMOGYI, G. V. PETROVSKY and I. FÖLDES

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest and Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, Moscow

(Received March 12, 1977)

DNA was isolated from two mycobacteriophages (Butyricum phage and Phlei phage) and some physical and chemical properties were investigated. The respective parameters obtained were: (i) T_m points in 0.1 SSC, 77.6 °C and 78.3 °C; corresponding percentual GC contents, 57.8 and 59.5; (ii) buoyant densities in CsCl, 1.7175 g/ml and 1.7179 g/ml; corresponding percentual GC contents, 58.6 and 59.2; (iii) percentual GC contents calculated from chromatography of DNA bases, 60.3 and 61.6; (iv) sedimentation coefficient ($S^0_{20,w}$), 47.8 S and 43.1 S; corresponding calculated mol. wt, 61.8×10^6 and 49.1×10^6.

Purification and partial characterization of DNA-containing bacteriophages to *Mycobacterium butyricum* and *Mycobacterium phlei* (abbreviated to Butyricum and Phlei phages, respectively) have been described previously [1]. According to electron microscopic studies and sedimentation analysis the two phages proved to be very similar and could be classified as large DNA bacteriophages. In this study we have examined some physical parameters and the base composition of the DNA's of these mycobacteriophages.

Materials and methods

The methods used for culturing host bacteria, propagation, concentration and purification of the phages were described previously [1, 2].

Phage DNA extraction. Two methods were employed for phage DNA extraction. (i) The phenol method as described by MANDELL and HERSHEY [3]. Determination of thermal denaturation profile, equilibrium centrifugations in CsCl and hydrolysis followed by chromatography to analyse DNA bases were performed using DNA obtained by this method. (ii) Mild heating detergent method: 100 μg of phage suspension were heated in the presence of 0.05% SDS at 60 °C for 10 min [4]. This DNA was used for determination of the sedimentation constant.

Host DNA extraction. For host DNA extractions the method of SAITO and MIURA [5] was used except that the cells were disintegrated in a different manner outlined as follows.

The packed cells (2 g) were suspended in a Potter homogenizer containing 4 ml Saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and 8 mg lysozime (Calbiochem) and kept at 37 °C for 60 min. The mixture was then frozen and thawed three times. After heating in a water bath at 60 °C for 10 min it was centrifuged at 2000 rpm for 20 min. The supernatant was preserved at 4 °C, the pellet was frozen again and ground in a mortar with aluminium oxide. Two parts of Tris-SDS buffer (0.1 M Tris, 1% sodium dodecyl sulphate, 0.1 M NaCl, pH 9.0) were added. After removing the aluminium oxide by slow centrifugation, the supernatants were assembled and mixed with an equal volume of phenol previously saturated with Tris-SDS buffer.

Thermal denaturation. Thermal denaturation profiles of Butyricum and Phlei phage DNA were obtained by heating samples in 3 ml teflon-stoppered quartz cuvettes containing 0.1×SSC (0.015 M NaCl, 0.0015 M Na citrate, pH 7.0) and placed in a thermostated cuvette chamber of a Unicam SP 8000 recording spectrophotometer. The absorbance measurements at 260 nm were corrected for dilutions caused by solvent expansion at higher temperatures [6].

Analytical ultracentrifugation. CsCl density gradient equilibrium centrifugations were carried out in a MOM 3170 analytical ultracentrifuge. CsCl (Calbiochem) was added to solutions containing 2 μg/ml of mycobacteriophage DNA and 1 μg/ml of T2 phage DNA as a reference to produce a density of about 1.71 g/ml in 0.01 M Tris buffer, pH 8.0. The resulting solutions were centrifuged at 40 000 rpm at 25 °C for 24 hr.

Phage DNA sedimentation was performed at 20 410 rpm in SSC at 20 °C using a Spinco Model E analytical ultracentrifuge with UV absorption optics.

Chromatographic procedure. In order to analyse nucleic acid bases chromatographically, phage and host DNAs were hydrolyzed in 70% perchlorid acid ($HClO_4$) at 100°C for 60 min [7]. After removing the bulk of $HClO_4$ with KOH, the DNA hydrolysate was fractionated by thin layer chromatography on a cellulose MN300 layer (Macherey and Nagel Co., Düren) using isopropanol –2N HCl solvent system. The spots detected by an analytical UV lamp were scraped off the plate and eluted in a test tube containing 0.1 N HCl [8].

Results

Determining the thermal denaturation profiles of both phage DNAs, an increase of more than 35% in the absorbance at 260 nm occurring in a small temperature range was observed. The midpoint of this is termed T_m. As shown in Fig. 1, the T_m points determined were 77.6 °C and 78.3 °C for Butyricum and Phlei phage DNA, respectively. The guanine and cytosine contents were calculated according to the equation of MARMUR and DOTY [9] corrected to a lower ionic strength [6, 10].

The buoyant density of DNA in CsCl increases linearly with its guanine and cytosine content. The base composition of Butyricum and Phlei phage DNA was estimated as described by SCHILDKRAUT *et al.* [11].

Base composition data for Butyricum and Phlei phage DNA obtained by thermal denaturation, by CsCl density gradient centrifugation and by chromatographic analysis are compared in Table I.

The values as calculated on the basis of both thermal denaturation and buoyant density agreed within experimental error, but were slightly higher

Table I

Biophysical properties of Butyricum and Phlei phage DNA

	T_m °C	per cent GC from T_m	Buoyant dens. in CsCl g/cm$_3$	per cent GC from buoyant density	per cent GC by chromatography	$S^0_{20,w}$	Mol. wt.×10^6 calculated from $S^0_{20,w}$
Butyricum phage	77.6±0.3	57.8	1.7175	58.6	60.3	47.8±2.0	61.8
Phlei phage	78.3±0.3	59.5	1.7179	59.2	61.6	43.1±1.0	49.1
M. butyricum	—	—	—	—	67.8	—	—
M. phlei	—	—	—	—	69.5	—	—

by chromatographic analysis. The base composition of phage DNA significantly differed from that of their host DNA (Table I).

DNA extracted from Butyricum and Phlei phages by the mild heating detergent method showed a single well-defined component in the analytical ultracentrifuge. Figure 2 shows the "least squares" plot of the reciprocals of the sedimentation coefficients plotted as a function of concentration. The $S_{20,w}$ values extrapolated to zero concentration ($S^0_{20,w}$) are listed in Table I.

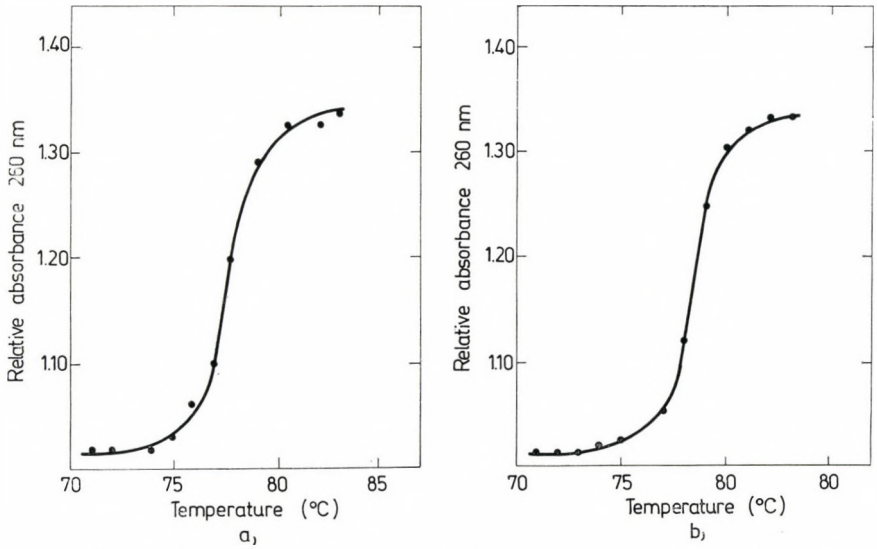


Fig. 1. Thermal denaturation of Butyricum (a) and Phlei (b) phage DNA in 0.1×SSC

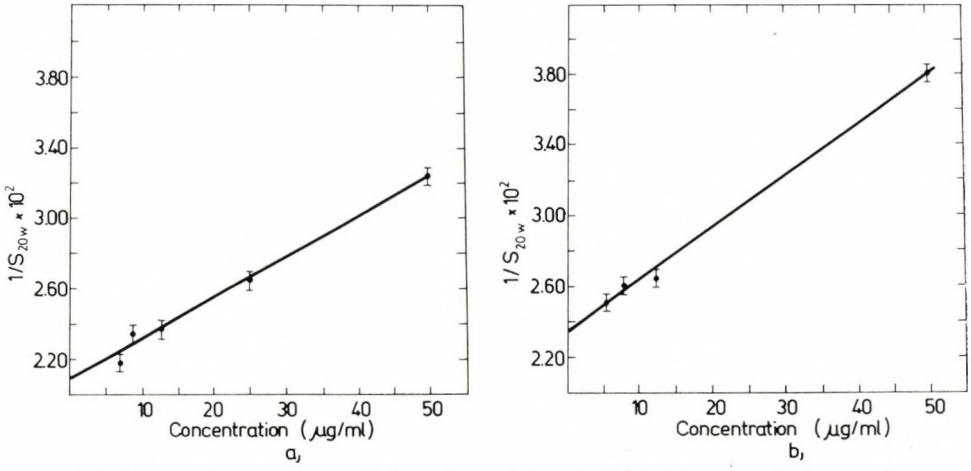


Fig. 2. Sedimentation constants of Butyricum (a) and Phlei (b) phage DNA as a function of the concentration

Discussion

According to MANDEL and MARMUR [6], if a larger quantity of glucosylated or unusual bases is present in DNA, the guanine and cytosine content calculated from the buoyant density in CsCl significantly differs from the results obtained by T_m point determination or base chromatography. In our experiments three independent methods gave the same results, so it is probable that Butyricum and Phlei phage DNA does not contain larger amounts of constituents other than the four usual DNA bases.

Thermal denaturation of phage DNA resulted in a sharp increase of absorbance (Fig. 1), which is characteristic of a double-stranded DNA [6]. The measure of this hyperchromic effect depends on the ionic strength of the DNA solution [12].

The molecular weight (M) of phage DNA can be determined from the sedimentation coefficient ($S^0_{20,w}$). FREIFELDER [13] suggested for the S – M relation the empirical equation,

$$S^0_{20,w} = 2.8 + 0.00834 \ M^{0.479}$$

This relationship is valid in the range of $20–100 \times 10^6$. Using this equation the molecular weight of Butyricum and Phlei phage DNA proved to be 61.8×10^6 and 49.1×10^6, respectively.

Knowing the DNA content of the phage (per cent of total phage weight) [1] and the molecular weight of the DNA, one can calculate the molecular weight of the phages, which proved to be 147×10^6 and 144×10^6 for Butyricum and Phlei phages, respectively. On the other hand, electron microscopic estimation of the phage molecular weight was 123×10^6 for Butyricum and 116×10^6 for Phlei phages [1]. This difference can be attributed partly to difficulties in measuring the exact DNA content of the phages, and partly to difficulties in measuring the absolute diameters (L) of phage particles by electron microscopy. Since the molecular weight is proportinal to L^3, small uncertainties in L determination result in marked changes of the calculated molecular weight.

Acknowledgements. The authors wish to thank Professors V. M. ZHDANOV and T. I. TICHONENKO, and Dr. E. VANDRA for helpful support and suggestions and Mrs. SUSAN FARAGÓ for technical assistance.

REFERENCES

1. SOMOGYI, P. A., PETROVSKY, G. V., GRIGOREV, V. B.: J. gen. Virol. **29,** 235 (1975).
2. VAJDA, B. P., HÁBER, K. J.: Amer. Rev. resp. Dis. **114,** 245 (1976).
3. MANDELL, J. D., HERSHEY, A. D.: Anal. Biochem. **1,** 66 (1960).
4. TICHONENKO, T. I., ZAK, V. I.: Biochimica **31,** 33 (1966).
5. SAITO, H., MIURA, K.: Biochim. biophys. Acta (Amst.) **72,** 619 (1963).

6. MANDEL, M., MARMUR, J.: In GROSSMAN, L., MOLDAVE, K. (eds).: Methods in Enzymology, **12B**, Academic Press, New York 1968, p. 195.
7. BENDICH, A.: In COLOWICK, S. P., KAPLAN, N. O. (eds): Methods in Enzymology. Vol. 3., Academic Press, New York 1957, p. 715.
8. RANDERATH, K., RANDERATH, E.: In GROSSMAN, L., MOLDAVE, K. (eds): Methods in Enzymology, **12A**, Academic Press, New York 1967. p. 323.
9. MARMUR, J., DOTY, P.: J. molec. Biol. **5**, 109 (1962).
10. SCHILDKRAUT, C., LIFSON, S.: Biopolymers **3**, 195 (1965).
11. SCHILDKRAUT, C., MARMUR, J., DOTY, P.: J. molec. Biol. **4**, 430 (1962).
12. FRONTALI, C., HILL, L. R., SILVESTRI, L. G.: J. gen. Microbiol. **38**, 243 (1965).
13. FREIFELDER, D.: J. molec. Biol. **54**, 567 (1970).

Address of the authors:

PÁL A. SOMOGYI, ISTVÁN FÖLDES
Microbiological Research Group of the Hungarian Academy of Sciences
Pihenő út 1, H-1529 Budapest, Hungary

G. V. PETROVSKY
Ivanovsky Institute of Virology U.S.S.R. Academy of Medical Sciences
Moscow, U.S.S.R.

Acta microbiol. Acad. Sci. hung. 25, 43—49, 1978

ADJUVANT EFFECT OF VARIOUS BORDETELLA PERTUSSIS STRAINS

ERZSÉBET ELEKES, KATALIN MERÉTEY and Z. CSIZÉR

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene and Institute for Serobacteriological Production and Research "Human", Budapest

(Received March 31, 1977)

The adjuvant effect on the antibody-producing capacity of rats of an acid precipitated precipitation (A), the bottom (C) and the supernatant (S) of a centrifuged preparation from 7 different *Bordetella pertussis* strains has been investigated. All bacterial suspensions studied were able to increase the immune response to sheep red blood cells. The adjuvant effect on the direct and indirect plaque forming spleen cells of the centrifuged cell suspensions was more expressed than of the acid-precipitated suspensions. The supernatants of the centrifuged bacteria suppressed the splenic immune response in almost every case. The serum haemolysin titres were in all cases higher than in the sheep erythrocyte treated control group.

Killed cells of *Bordetella pertussis* induce physiological, haematological and immunological changes in man and in rodents. Different factors are responsible for these effects and the concentration of these factors is different in the strains. IIDA and TAJIMA [1] showed that various *B. pertussis* strains induce a different increase in resistance. ACKERS and DOLBY [2] compared the adjuvant activity of *B. pertussis* strains in mice and APRILE [3] investigated the mouse protective activity.

The aim of our experiments was to compare the adjuvant activity of seven phase I *B. pertussis strains.*

Materials and methods

Preparation of B. pertussis adjuvants. The *B. pertussis* strains used were: 41405 (serotype 1, 2, 3), 59 (1, 3), 324E (1, 2), 2-atox (1, 2), CN 2894 (1, 2), CN 2896 (1, 2), 358E (1,2).

The bacteria were cultured in modified Cohen–Wheeler medium [4] for 18 hr.

The cells were harvested by two methods. (a) Acid precipitation (regularly used for vaccine production) was performed by the addition of hydrochloric acid at pH 4.0. After a sedimentation period of 24–28 hr, the cells were resuspended in isotonic buffered saline (pH 7.2) and heated at 56°C for 60 min in the presence of 0.02% merthiolate (preparation A). (b) After centrifugation of the cultures, the resuspended sediment (preparation C) and the supernatant (preparation S) were heated separately in the presence of 0.02% merthiolate as described for method (a).

Preparations A, C and S were tested for adjuvant activity.

Induction of primary immune response. F_1 female hybrid rats (R/A × LE/Hooded) of 230–280 g body weight were inoculated intravenously simultaneously with 4×10^8 sheep red blood cells (SRBC) and bacterial adjuvant. As shown by several authors [5, 6, 7] doses of 4×10^8 to 10^9 SRBC are able to induce a strong immune response, thus the dose of 4×10^8 SRBC used in our experiments seems to be suitable for comparing different adjuvant preparations. Vaccines A and C were administered in a dose of 3×10^9 cells and vaccine S in a

volume corresponding to the same number. Each group of 8 animals was immunized with SRBC or SRBC+A or SRBC+C and 4 rats with SRBC+S.

As it is shown in Fig. 1, the mercaptoethanol sensitive and resistant antibody titre reached the highest value on the 5th day, the mercaptoethanol resistant antibody showed a peak on the 7th day. On the basis of this figure the immune response was checked on the 5th day only. The animals were exsanguinated and the number of spleen cells was determined in a haemocytometer after staining with Türk solution. Spleen weight was determined gravimetrically. The spleen index is defined as wet spleen weight/body weight, mg/g. For the quantitative determination of plaque-forming spleen cells (PFCs) the direct [8] and indirect [9]

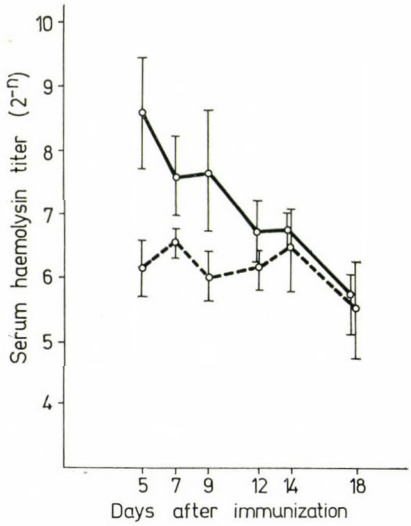


Fig.1. Serum haemolysin titre in different intervals after immunization with 4×10^8 SRBC; —— mercaptoethanol sensitive and resistant antibody titre, ------ mercaptoethanol resistant antibody titre

antibody plaque technique was used. The anti-rat immunoglobulin serum was prepared in rabbits. The antiserum inhibited the direct plaque count to more than 90%. It is generally accepted that the majority of direct PFCs represents 19S producing cells [9] whereas most of the indirect PFCs are considered 7S haemolysin producers [9]. Both the relative (PFC/10^6 spleen cells) and the absolute number (PFC/total spleen) of haemolysin producing cells were calculated. In the sera the antibody titre (MES+MER) was measured by microhaemolysis [10].

Results

Spleen indices after B. pertussis treatment. Both the vaccines A and C prepared from all *B. pertussis* strains induced an increase in relative spleen weight. The increase was significant with the exception of vaccines 59-A, 358E-C and 2-atox-C (Fig. 2). Some decrease of relative spleen weight could be found using vaccine S preparates, except CN 2896.

Influence of different B. pertussis strains on the relative antibody forming spleen cell count. As it is shown in Table I, the whole-cell vaccines A and C increased the number of PFCs. The preparations differed in the degree of their adjuvant effect. In general, the number of direct 19S PFCs increased

Table I

Adjuvant effect of various B. pertussis strains. Change in the relative direct and indirect PFCs on the 5th day after primary immunization

B. pertussis vaccine tested	Direct PFC/10^6 splenic cells Control: 164±47+			Indirect PFC/10^6 splenic cells Control: 170±66+		
	A	C	S	A	C	S
41405	318±213	638±229*	31±7*	97±26	435±162	38±4*
59	495±219	1077±538*	41±23*	414±35	253±157	58±22*
324E	461±305	506±160*	51±20*	112±12	404±185	72±26
358E	360±162	318±171	40±16*	114±31	212± 66	31± 1*
2-atox	444±223	554±162	59±10*	89±19	760±269*	48±24*
CN 2894	543±306	760±293*	160±26	274±177	1129±439*	73±37
CN 2896	414±168	1172±397*	204±180	234±91	1169±415*	70±53

+ mean value ± standard error
* the values are significant as compared to the control group ($p<0.05$)
Number of animals per group is given in the legend to Fig. 2

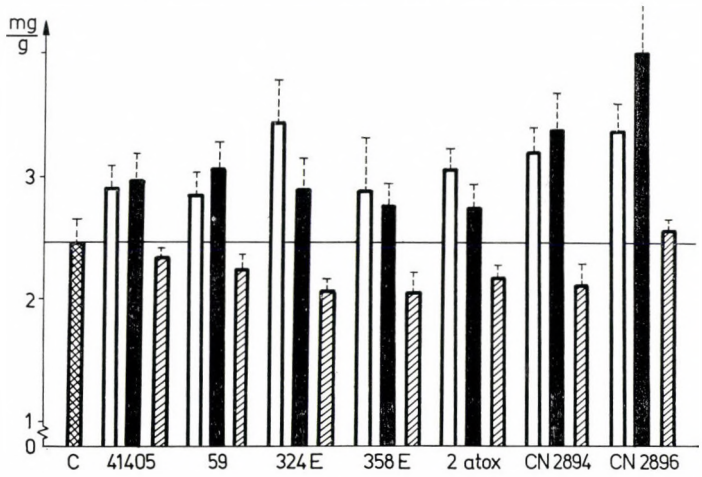


Fig. 2. Effect of treatment with different *B. pertussis* vaccines on relative spleen weight (C = control group of 8 rats immunized with SRBC; open columns = group of 8 rats treated with SRBC and A; closed columns = group of 8 rats treated with SRBC and C; hatched columns = group of 4 rats treated with SRBC and S)

mainly on using preparations C. The increase was significant in all cases except with the strains 358E and 2-atox.

The increase in indirect PFCs was not so definite. Using vaccines C, the difference proved significant in three cases only.

Comparing the effectiveness of different strains and preparations, the suspension of strain CN 2896 obtained by centrifugation had the highest

adjuvant activity. The adjuvant index (AI), i.e. the ratio of the response to SRBC in the presence of adjuvant compared with the response of that without adjuvant, was 7.1 and 6.9 on comparing the direct and indirect PFCs, respectively. Generally, preparations C had a stronger adjuvant activity than their A counterparts. The supernatants (S) had an immunosuppressive effect. The decrease in direct and indirect PFCs was significant with the majority of strains examined, except the indirect PFCs in strain 324E and direct and indirect PFCs with strains CN 2896 and CN 2894.

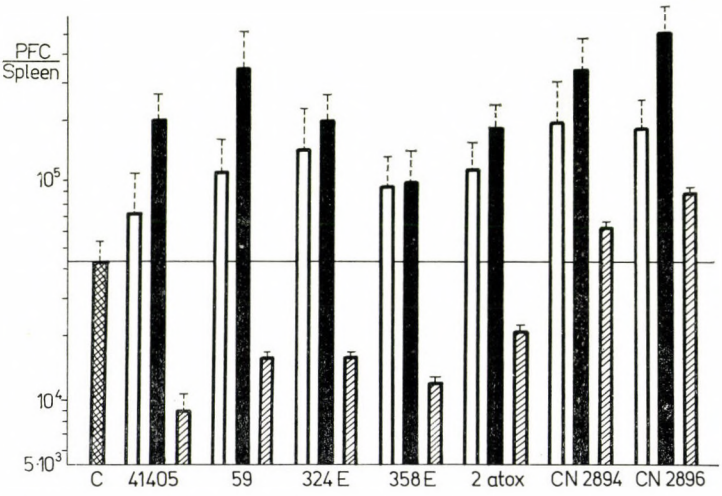


Fig. 3. Absolute direct PFC count of the spleen after treatment with different *B. pertussis* vaccines (for details, see Fig. 2)

Change of PFCs in the whole spleen. There was a close positive correlation between the absolute and relative (see above) PFC changes.

All the vaccines C (except strain of 358E) showed a significant effect on the direct PFCs of the whole spleen (Fig. 3). The preparations A had also a marked adjuvant activity, but the increase was significant only with strains CN 2896 and 2-atox.

Vaccines S were immunosuppressive also concerning this parameter. No decrease of direct PFCs was found on injecting vaccines S of CN 2896 and CN 2894 strains.

The difference in the efficiency of preparations and strains was most expressed in the absolute indirect PFC count of the spleen (Fig. 4). Vaccines C had a marked adjuvant action, significant in 5 cases (41405, 324E, 2-atox, CN 2894, CN 2896). Preparations A had no significant adjuvant activity.

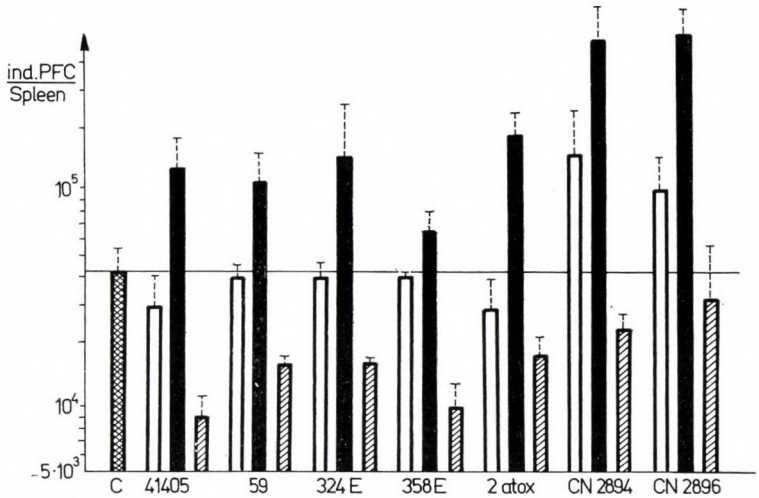


Fig. 4. Indirect PFC count in spleen under the adjuvant effect of various *B. pertussis* vaccines (for details, see Fig. 2)

Table II

Serum haemolysin titre in B. pertussis adjuvated, SRBC immunized rats

Vaccine	Total haemolysin titre Control = 8.8 ± 0.3+		
	A	C	S
41405	9.8 ± 0.4*	10.6 ± 0.1*	10.1 ± 0.6*
59	10.7 ± 0.4*	11.8 ± 0.5*	10.7 ± 0.3*
324E	9.8 ± 0.5	11.2 ± 0.5*	9.4 ± 0.8
358E	10.6 ± 0.6*	10.2 ± 0.3*	9.5 ± 0.3
2-atox	10.8 ± 0.4*	10.3 ± 0.6*	9.6 ± 0.1
CN 2894	10.7 ± 0.5*	9.1 ± 0.4	8.6 ± 1.1
CN 2895	11.3 ± 0.6*	9.8 ± 0.5	9.4 ± 1.1

+ antibody titers (mean $\pm$ SE) are expressed as base 2 logarithms
* mathematically significant changes compared to the control group ($p < 0.05$)
Number of rats tested is shown in Fig. 2

Five supernatants (S), except CN 2894 and CN 2896, had a significant immunosuppressive effect in this respect.

Effect of B. pertussis treatment on the serum haemolysin titre. The majority of A and C preparations increased the serum haemolysin titres (Table II). The acid-precipitated vaccines seemed to be more effective. All vaccines A, except 324E, induced a significant increase in haemolysin content of the sera. Among the preparations C there were two exceptions, CN 2894 and CN 2896. In the supernatant there was an increase only in cases 41405 and 59.

Discussion

B. pertussis vaccines have a definite adjuvant effect on the humoral immunity of mammals. A detailed study of this adjuvant action was done in mice. The most characteristic findings were a shortening of the latent period, higher and prolonged 19S and 7S haemolysin production, and independence of the antigen dose [11, 12].

The immune response to pertussis vaccines of rats was studied less extensively. In this species an increase of homocytotropic antibody production was characteristic [13], but the components playing a role in that effect have not been fully elucidated. In the present experiments the anti SRBC immune response has been studied in rats with and without *B. pertussis* preparations as adjuvants. First we tested the kinetics of the immune response in serum samples. Antibody production was tested on the fifth day after the injection of antigen and adjuvant, by counting the PFCs in the spleen and estimation of the serum haemolysin titre. On the 5th day after 4×10^8 SRBC injection the immune response of rats was mainly of the IgM type, in contrast with the findings in mice.

As far as the activity of different *B. pertussis* strains is concerned, only the data of ACKERS and DOLBY [2] showed that all the phase I strains investigated had a similar adjuvant activity on the serum haemolysin titre. Our results showed that all strains investigated have some adjuvant capacity on the antibody forming cell count in the spleen. There were, however, some quantitative differences among the strains in their effect on the splenic PFC count; the adjuvant effect was sometimes very slight. In present experiments the active components of *B. pertussis* were also studied. Adjuvants prepared by three methods were compared. The most expressed effect was observed from centrifuged cells, but acid precipitated suspensions were also effective. The supernatant of the centrifuged bacteria too, seemed to have an adjuvant effect, as judged from the serum haemolysin titre, though the splenic immune response was weaker than in the controls. For this inhibitory action we cannot offer an explanation.

The biological activity of *B. pertussis* fractions has been extensively studied. According to MORSE and BRAY [7] the cells contain the protective antigen, while the supernatant plays a role mainly in the histamine sensitizing activity and in the induction of lymphocytosis. It seems that a lymphocyte mobilizing factor in the supernatant fraction increases the flow of splenic lymphocytes and so it caused a discrepancy in the adjuvant action in spleen and the serum.

Our data proving the adjuvant activity of cell fractions are in good agreement with the results of ACKERS and DOLBY [2] who showed that the adjuvant activity was related to the endotoxin content. Precipitation by

acid seemed to destroy some activities as the bacteria treated in this way were less active than the centrifuged ones.

Proteolytic treatment was shown to destroy the leucocytosis promoting activity of *B. pertussis* without altering their adjuvant action. Thus, it is suggested that the leucocytosis promoting and adjuvant factors may be different but both of them increase the response. The centrifuged fraction, the supernatant and the acid precipitated fractions contain them in different ratios. The quantitative difference of various factors of the adjuvant action in the investigated 7 strains may be responsible for their different behaviour in serum and spleen.

REFERENCES

1. Iida, T., Tajima, M.: Immunology **21,** 313 (1971).
2. Ackers, J. P., Dolby, J. M.: J. gen. Microbiol. **70,** 371 (1972).
3. Aprile, M. A.: Canad. J. Microbiol. **18,** 1793 (1972).
4. Pusztai, Zs., Joó, I., Kiss, I.: Path. et Microbiol. **23,** 192 (1960).
5. Rowley, D. A., Fitch, F. W.: J. exp. Med. **121,** 683 (1965).
6. Allerdyce, R. A., James, K.: Clin. exp. Immunol. **7,** 249 (1970).
7. Morse, S. I., Bray, K. K.: J. exp. Med. **129,** 523 (1969).
8. Jerne, N. K., Nordin, A. A., Henry, C.: In Amos, B., Koprowsky, H., (eds): Wistar Institute Press, Philadelphia 1963. p. 109.
9. Wortis, H. H., Taylor, R. B., Dresser, D. W.: Immunology **11,** 603 (1966).
10. Hege, J. S., Cole, L. J.: J. Immunol. **95,** 559 (1965).
11. Finger, H., Emmerling, P.: Experientia (Basel) **24,** 832 (1968).
12. Finger, H., Emmerling, P., Schmidt, H.: Experientia (Basel) **23,** 1 (1967).
13. Mota, I.: Life Sci. **1,** 465 (1963).
14. Lehrer, S. S., Vaughan, J. H., Tan, E. M.: J. Immunol. **114,** 34 (1975).

Address of the authors:

Erzsébet Elekes
"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene
Pentz K. u. 5, H-1221 Budapest, Hungary

Katalin Merétey
National Institute for Rheumatism and Physiotherapy
H-1719 Budapest, P.O.B. 54, Hungary

Zoltán Csizér
Institute for Serobacteriological Production and Research "Human"
Szállás u. 5–7, H-1107 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 51—54, 1978

SIXTEEN COLOUR GROUPS OF THE SUBSTRATE MYCELIUM PIGMENTS OF STREPTOMYCETES

I. M. SZABÓ

Department of Microbiology, Eötvös Loránd University, Budapest

(Received March 31, 1977)

A new and improved system is recommended for the separation of strains and species of *Streptomyces* and *Streptoverticillium* according to the colour of their substrate mycelium on culture media and its change on the addition of acid (ac) and alkali (al). The 16 colour groups of this system are as follows: 1. Yellow–brown (Y–b). 2. Y–b + red. 3. Y–b + blue. 4. Y–b + green. 5. Y–b + red + green. 6. Y–b + red + blue. 7. Y–b + blue + green. 8. $Y–b_{ac} \leftrightarrow red_{al}$. 9. $Y–b_{al} \leftrightarrow red_{ac}$. 10. $Y–b \leftrightarrow green_{al}$. 11. $Y–b + blue_{ac} \leftrightarrow red_{al}$. 12. $Y–b + blue_{al} \leftrightarrow red_{ac}$. 13. $Y–b + blue \leftrightarrow green_{al}$. 14. $Y–b + red_{al} \leftrightarrow green_{ac}$. 15. $Y–b + red_{ac} \leftrightarrow green_{al}$. 16. $Y–b + green + blue_{al} \leftrightarrow red_{ac}$.

The colour of substrate mycelium has extensively been utilized by many workers in the identification and characterization of *Streptomyces* species [1]. A system of groups for the separation of strains according to their substrate mycelium colour proposed by SZABÓ and MARTON [2] has been adopted and recommended for characterization of streptomycetes by the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature [3]. In 1975 SZABÓ *et al.* [4] elaborated a new system of groups for the separation of strains and species according to their substrate colour, and they published a diagnostic key for the identification of "species" of *Streptomyces* and *Streptoverticillium* included in the International Streptomyces Project (ISP).

The present paper describes a new, more detailed system of grouping, including 16 colour groups, available for a more precise description of the pigmentation of the substrate mycelium.

Materials and methods

The media and methods used for determining the pigmentation of the substrate mycelium were identical with those outlined and officially adopted by the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature [3].

Streptomyces and Streptoverticillium strains. Type, neotype and reference strains of the ISP obtained from the Culture Collection of the Pedological Institute of the Hungarian Academy of Sciences (Budapest) were used for comparative studies.

Results and discussion

The proposed 16 colour groups, based partly on the results of our comparative investigations and partly on the data of a reevaluation of ISP descriptions [5–8], are as follows.

1. *Yellow-brown (Y-b).* No distinctive pigment in the substrate mycelium. The colour of the mycelium exhibits different hues of yellow and brown in all diagnostic culture media and in all periods of growth; these yellow–brown colours may be shaded by grey in all degrees from almost colourless light grey through dirty white to black. These pigments are not pH sensitive or they change from one hue to another of Y–b colourization on the addition of 0.05 N NaOH or 0.05 N HCl. "Strains are characterized as lacking distinctive substrate pigment if the reverse side of the colony is colourless, greenish yellow, light or pale yellow, yellowish grey, olive, light olive brown, orange yellow, yellowish brown or olive brown" [5]. Within this range, differences in the hue are neither clearly identified nor reliably constant with species, and they are not useful for species differentiation. Strains of the overwhelming majority of species of *Streptomyces (S.)* and *Streptoverticillium (Sv.)* do not produce distinctive substrate colours. Characteristic representatives of this "Y–b colour group" are type or reference strains *Actinomyces (A). griseorubens* ISP 5160, *Sv. griseocarneum* ISP 5004, *S. albus* ISP 5313, *S. hachijoensis* ISP 5114, etc.

2. *Y-b + red.* On at least one diagnostic culture medium and in a certain period of growth, the mycelium produces red or pink endopigments to such an extent that both the yellow–brown ground colour and grey shade are entirely obscured, and the mycelium becomes red or pink. The red or pink endopigments are not pH sensitive. *S. fulvissimus* ISP 5593, *A. glaucescens* ISP 5155, *A. griseoincarnatus* ISP 5274, *Sv. kashmirense* ISP 5336, *S. glomeroaurantiacus* ISP 5429, etc., represent this type of pigmentation.

3. *Y-b + blue.* Similar to the above Y–b + red (No. 2), except that the reverse mycelium pigment is blue. This pigment is not a pH indicator. *S. ambofaciens* ISP 5053 and *S. ipomoea* ISP 5383 belong to this colour group.

4. *Y-b + green.* Similar to the above Y–b + red (No. 2), except that the reverse mycelium pigment is green. This green endopigment is not a pH indicator. *A. finlayi* ISP 5218, *S. flavorirens* ISP 5062, *S. olivoviridis* ISP 5211, *S. recifensis* ISP 5115, *S. violaceoniger* ISP 5563, etc., represent this colour group.

5. *Y-b + red + green.* Two different distinctive pigments, a red and a green one can cause colourization in the substrate mycelium depending on the composition of the diagnostic medium employed. Neither of them is a pH indicator. *A. coeruleofuscus* ISP 5144 and *A. coeruleorubidus* ISP 5145 are examples for this combination of endopigments.

6. *Y–b + red + blue.* Similar to the above Y–b + red + green (No. 5), except that the yellow–brown ground colour of the substrate mycelium may be modified to red and/or to blue on the diagnostic media. These pigments are not pH sensitive. *S. bellus* ISP 5185 belongs to this colour group.

7. *Y–b + blue + green.* Similar to Y–b + red + green (No. 5), except that the colour of the substrate mycelium may be modified to blue and/or to green on the diagnostic media. These endopigments are not pH sensitive. *S. vastus* ISP 5309 represents this combination.

8. $Y\text{–}b_{ac} \leftrightarrow red_{al}$. The reverse mycelium pigment is pH sensitive changing from the yellow–brown ground colour to red or pink on the addition of 0.05 N NaOH. The red substrate mycelium pigment changes to colourless or yellow–brown by 0.05 N HCl. *S. coralus* ISP 5256, *S. erythraeus* ISP 5517, *S. erythrogriseus* ISP 5116, *S. fimicarius* ISP 5322, *S. fragilis* ISP 5044, *S. galilaeus* ISP 5481, *S. platensis* ISP 5041, *A. rubiginosohelvolus* ISP 5176, *S. tubercidicus* ISP 5261, etc., all belong to this group.

9. $Y\text{–}b_{al} \leftrightarrow red_{ac}$. Similar to $Y\text{–}b_{ac} \leftrightarrow red_{al}$ (No. 8), except that the pH sensitive mycelium pigment is changing from Y–b to red, pink or violet red on the addition of 0.05 N HCl. The red substrate mycelium pigment is changed to Y–b by 0.05 N NaOH. Type or reference strains *A. aureoverticillatus* ISP 5080, *Sv.fervens* ISP 5086, *S. griseoaurantiacus* ISP 5430, *S. longispororuber* ISP 5599, etc., represent this type.

10. $Y\text{–}b \leftrightarrow green_{al}$. Strains belonging to this type of pigmentation produce Y–b substrate mycelium on all diagnostic media. The Y–b ground colour changes, however, to green on the addition of 0.05 N NaOH. This colour group is represented by *S. coelicolor* ISP 5233 and *A. fluorescens* ISP 5203.

11. $Y\text{–}b + blue_{ac} \leftrightarrow red_{al}$. The reverse colour is generally red on the diagnostic media. This pigment is pH sensitive, changing from red to orange-red on the addition of 0.05 N NaOH and from red to violet-red or blue the addition of 0.05 N HCl. *A. biverticillatus* ISP 5272 is a member of this group.

12. $Y\text{–}b + blue_{al} \leftrightarrow red_{ac}$. The yellow to yellow–brown ground colour may persist or be modified to red or blue on diagnostic media. The substrate mycelium pigment is a pH indicator changing from red to blue on the addition of 0.05 N NaOH or from blue to red on the addition of 0.05 N HCl. Characteristic representatives of this colour group are *S. caesius* ISP 5419, *A. cyanogenus* ISP 5426, *S. violaceus* ISP 5082, *A. violarus* ISP 5205, etc.

13. $Y\text{–}b + blue \leftrightarrow green_{al}$. The colour of the substrate mycelium of strain ISP 5103 of *S.caeruleus* is dark blue, blue violet to black on the diagnostic media. This blue pigment is a pH indicator, changing from dark violet or blue to deep green on the addition of 0.05 N NaOH.

14. $Y\text{–}b + red_{al} \leftrightarrow green_{ac}$. Strain ISP 5573 of *S. thermodiastaticus* produces a reddish brown to red substrate mycelium pigment which is found also in yeast–malt agar, oatmeal agar, salts–starch agar, and glycerol–

asparagine agar. The red pigment is pH sensitive, changing from reddish-brown or red to olive-green on the addition of 0.05 N HCl, and from reddish-brown to red on the addition of 0.05 N NaOH.

15. *Y–b* + $red_{ac} \leftrightarrow green_{al}$. The substrate mycelium pigment of strain ISP 5148 of *A. cyaneofuscatus* is pH sensitive, changing from yellowish-green to pink or red on the addition of 0.05 N HCl and from pale yellow to green with 0.05 N NaOH.

16. *Y–b* + *green* + $blue_{al} \leftrightarrow red_{ac}$. Two different pigments a green and a blue ↔ red one can cause colourization in the substrate mycelium depending on the composition of the diagnostic medium. The green endopigment is not a pH indicator. The distinctive blue or purple pigment is pH sensitive, changing from violet to blue on the addition of 0.05 N NaOH and from violet to red or pink on the addition of HCl. *S. regalis* ISP 5532 is an example for this type of colourization of the substrate mycelium.

Streptomycetes are able to produce a great variety of pigments, some among them comprising two or more constituent pigments. The pH of the medium affects the nature of both water-soluble and water-insoluble pigments. The formation of these pigments is highly characteristic of the species. The colour group designations are simple and convenient in streptomyces taxonomy.

REFERENCES

1. WAKSMAN, S. A.: The Actinomycetes. Vol. II. Classification, Identification and Descriptions of Genera and Species. Williams and Wilkins Co., Baltimore 1961. p. 361.
2. SZABÓ, I., MARTON, M.: Int. Bull. Bact. Nom. Tax. **14,** 17 (1964).
3. SHIRLING, E. B., GOTTLIEB, D.: Int. J. Syst. Bact. **16,** 313 (1966).
4. SZABÓ, I. M., MARTON, M., BUTI, I., FERNANDEZ, C.: Acta botan. Acad. Sci. hung. **21,** 387 (1975).
5. SHIRLING, E. B., GOTTLIEB, D.: Int. J. Syst. Bact. **18,** 69 (1968).
6. SHIRLING, E. B., GOTTLIEB, D.: Int. J. Syst. Bact. **18,** 279 (1968).
7. SHIRLING, E. B., GOTTLIEB, D.: Int. J. Syst. Bact. **19,** 391 (1969).
8. SHIRLING, E. B., GOTTLIEB, D.: Int. J. Syst. Bact. **22,** 265 (1972).

Address of the author:

ISTVÁN M. SZABÓ
Department of Microbiology, Eötvös Loránd University
Múzeum krt. 4/a, H-1088 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 55—60, 1978

CONIDIUM ONTOGENY OF DERMATOPHYTES

J. GALGÓCZY

National Institute of Hygiene, Budapest

(Received May 3, 1977)

The special detachment of the lateral macroconidium of *Epidermophyton floccosum* and the catenulate macroconidia of dermatophytes are described. The detaching-cell of the macroconidium of several *Microsporum* and *Trichophyton* species, not studied in this respect so far, and the three possible types of detachment of microconidia are presented.

Studying the conidium ontogeny of dermatophytes, we have made new observations. In the light of these, some poorly understood phenomena described by us previously [1] have obtained clearer interpretation.

Materials and methods

Fungal species. Epidermophyton floccosum, Microsporum felineum, M. ferrugineum, M. gypseum, M. nanum, M. persicolor, Trichophyton fluviomuniense, T. megninii, T. quinckeanum, T. vanbreuseghemii.

Media. Thiamine-enriched Sabouraud–glucose agar containing antibiotics; Sabouraud–glucose agar containing 3% NaCl [2, 3].

For microscopic studies, a thin layer of medium poured on slides was inoculated with minute pieces of culture [2].

Results

Results are presented on three Plates and in two Figures.

On Plate I, two *Microsporum* and one *Trichophyton* species are shown at the stage in which a second macroconidium (mc) is developing from the apex of a mc. The former may develop on a minute stalk playing the role of the detaching cell.

Plate II shows detachment of the macroconidia of several species not having been examined in this respect so far. Pictures in order: 1. *T. rubrum*, 2. *T. fluviomuniense*, 3. *T. vanbreuseghemii*, 4. *T. megninii*, 5. *T. quinckeanum*, 6–7. *M. felineum*, 8. *M. nanum*, 9–11. *M. persicolor*, 12–13. *M. ferrugineum*. Detachment of a mc of *T. longifusum* is shown in Picture 1 of Plate III.

Pictures 2–6 on Plate III show single microconidia, and those in groups, in a stage in which the autolysis of the detaching-cell(s) has already begun or even been completed.

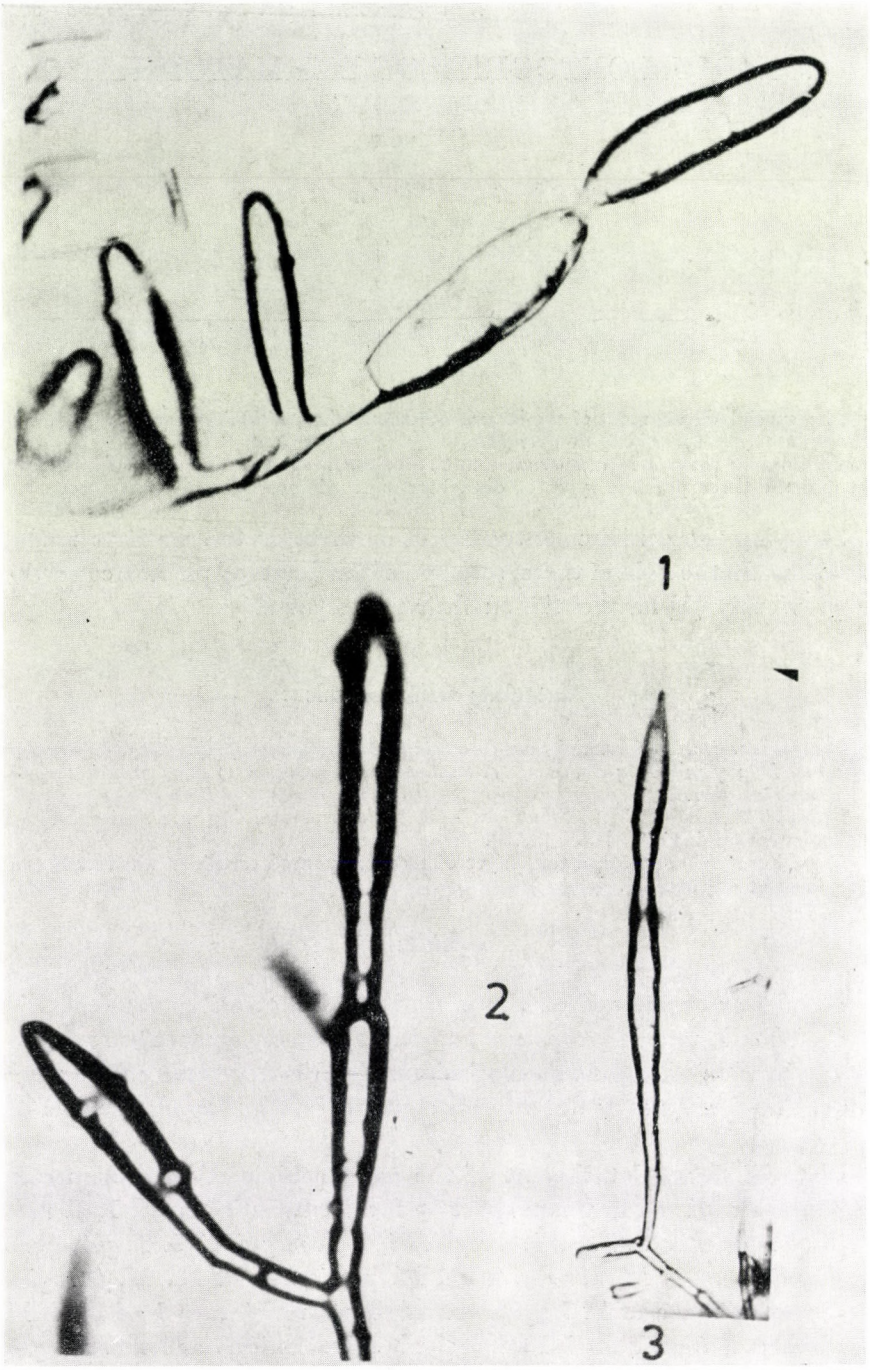


Plate I. Picture 1. A catenulate macroconidium of *M. gypseum in situ.* Original magnification, ×250. *Picture 2.* A catenulate macroconidum of *M. fulvum in situ.* Original magnification, ×160. *Picture 3.* Catenulate macroconidium of *T. longifusum in situ.* Original magnification, ×160

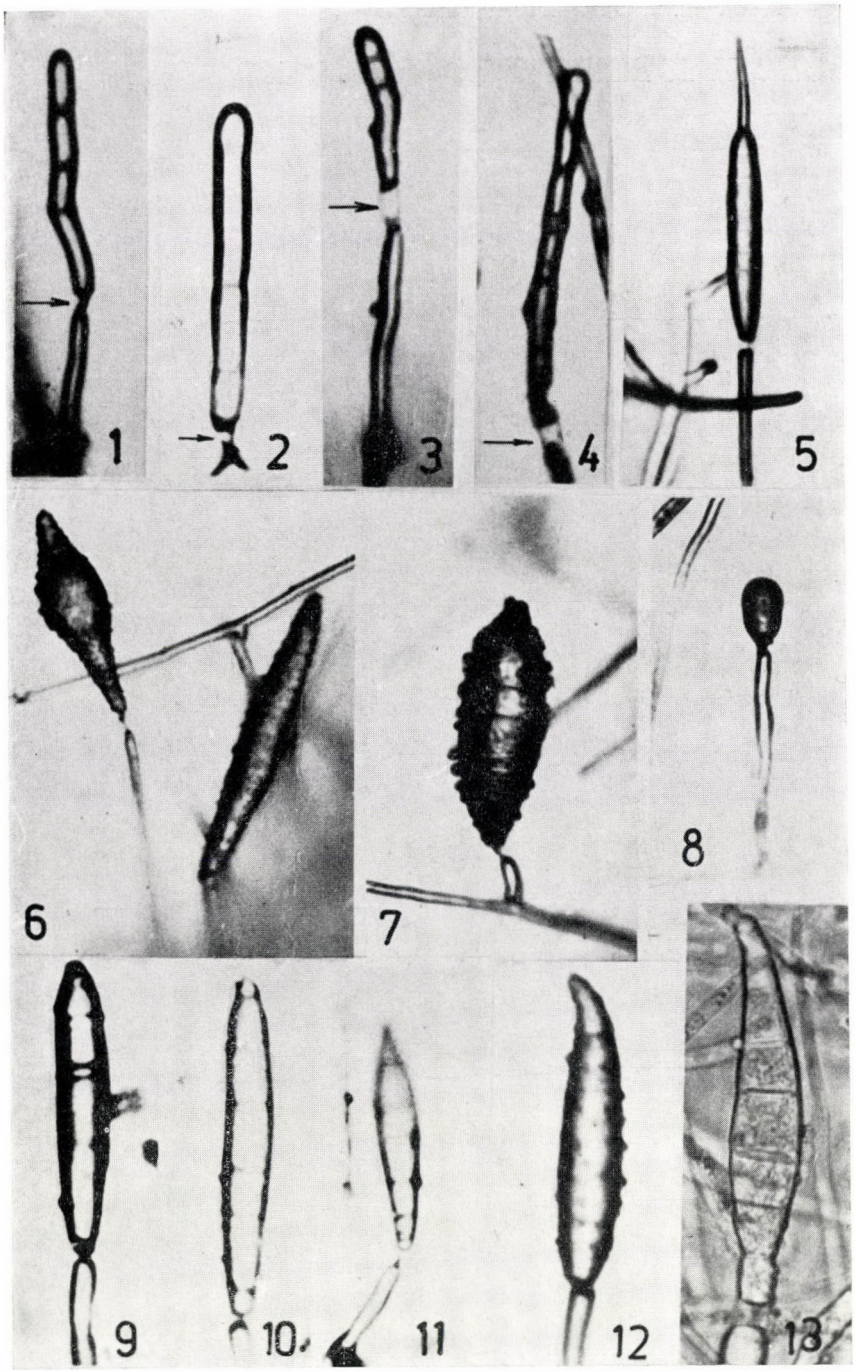


Plate II. Detachment of the macroconidia of various dermatophytes *in situ* (see text). *Picture 13* shows an aqueous preparation. Original magnification, ×160

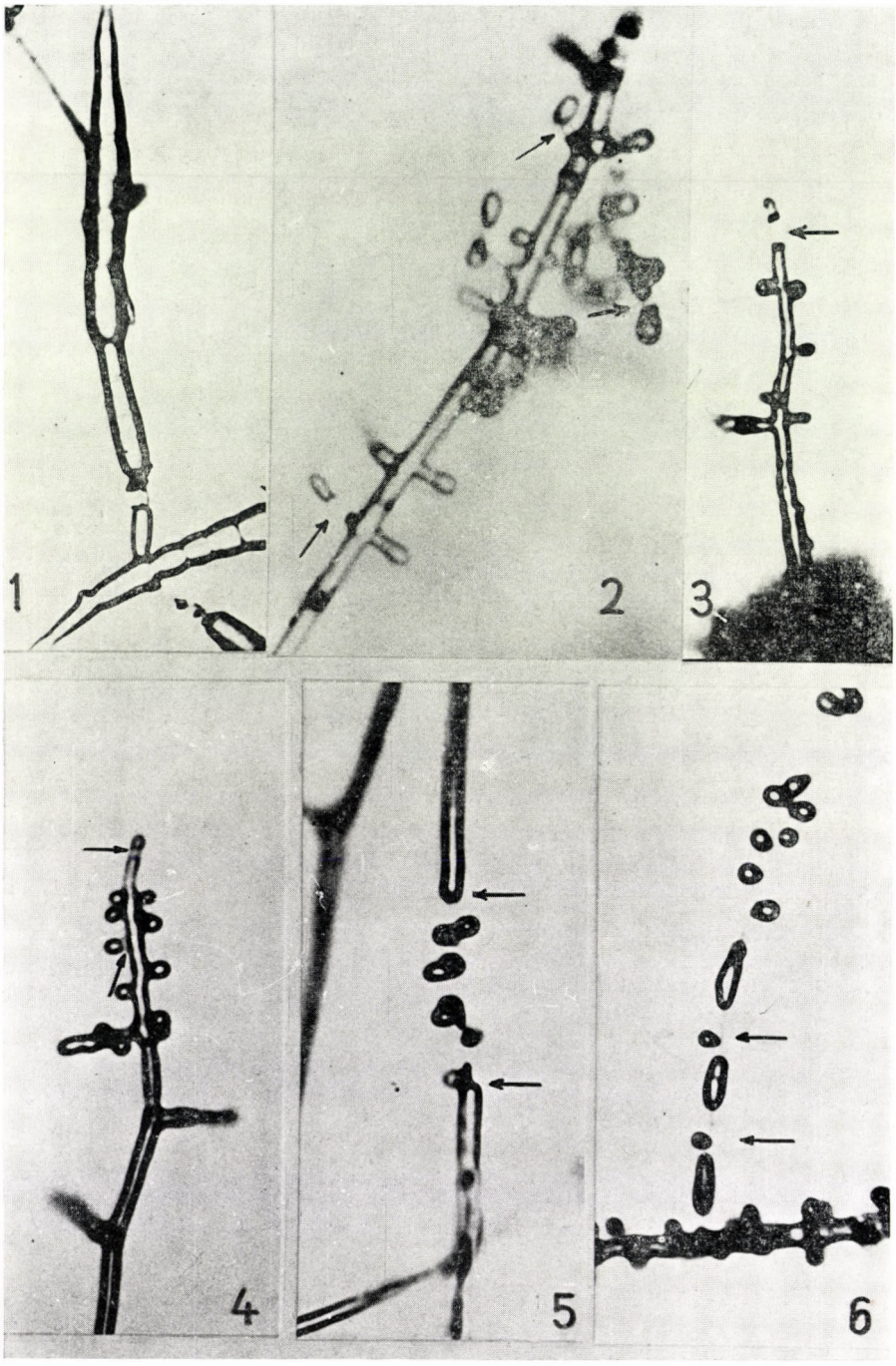


Plate III. Detachment of the macroconidium of *T. longifusum* and of microconidia of dermatophytes *in situ*. Original magnification, ×160

Figure 1 is a schematic illustration of the three possible types of dermatophyte microcondium detachment.

Figure 2 shows the ontogeny of the lateral mc of *E. floccosum*. The two cells on the right and left side of the basal part of the mc are autolysing.

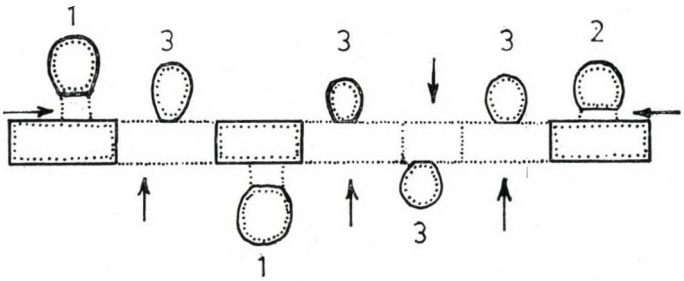


Fig. 1. Detachment of dermatophyte microconidia

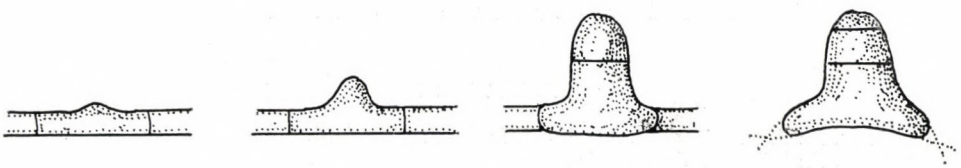


Fig. 2. Development and bilateral detachment of the lateral macroconicium of *E. floccosum*

Discussion

The formations on Plate I correspond to the catenulate type of mc. They occur rarely in the genus *Epidermophyton*, too.

The macroconidia with detaching cell shown on Plate II had been developed by holoblastic ontogeny. Those in the upper row (Pictures 1–5) present *Trichophyton* macroconidia on which protoplasm release, a phenomenon described by us [1], cannot be seen. Recently we have demonstrated that protoplasm release was an artifact arising in the course of long-lasting microscopic examination. Detachment under natural circumstances is even in the genus *Trichophyton* a more regular process than believed previously.

The detachment of microconidia, another well-organized process, does not require the conidiophore to be completely destroyed (Plate III, Fig. 1). Some of the conidia are sitting on a well-visible stalk (conidium 1 in Picture 1), others have a short stalk (conidium 2 in Picture 1), still others are sitting on the conidiophore without any stalk (conidium 3 Picture 1). The cell (or cell part) immediately below the conidium (arrows in Fig. 1) undertakes the role of detachment in each case. The picture is unequivocal if detachment is due to small stalks and the culture is not old (Pictures 2–4 on Plate III). Beginning

exsiccation of the whole culture may, however, be imitated if detaching is performed by the cells of the supporting structure. It is still more confusing when several neighbouring cells are autolysing simultaneously; nevertheless, even in the latter case, characteristic fields can be found in which the intact hypha is apparent on both sides of the autolysed segment (Picture 5 on Plate III). In Picture 6 of Plate III, autolysed detaching-cells of two stalkless lateral microconidia can be seen between three short hyphal sections. Orientation is still more difficult in old cultures in which general autolysis and protoplasm reservoirs are developing in the supporting structure.

Fig. 2 presents the ontogeny of the lateral mc of *E. floccosum*. This type of ontogeny is different from the holoblastic ontogeny of the dermatopytes. Although blowing-out starts from a part of the cell, the mature conidium still involves the whole cell, it is not demarcated by a septum from any other part. From itself it does not form a detaching cell. Detachment is due to autolysis of two neighbouring cells in every case. It seems likely that this type of ontogeny is intermediary between the holoblastic and holothallic types.

REFERENCES

1. GALGÓCZY, J.: Acta microbiol. Acad. Sci. hung. **22,** 105 (1975).
2. GALGÓCZY, J.: Bőrgombák, gombás bőrbetegségek. Medicina, Budapest 1975.
3. GALGÓCZY, J., POLYÁK, K.: Kísérl. Orvostud. **27,** 106 (1975).

Address of the author:

JÓZSEF GALGÓCZY
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Acta microbiol. Acad. Sci. hung. 25, 61–65, 1978

ISOLATION OF PURE DNA FROM FILAMENTOUS FUNGI BY CHROMATOGRAPHY ON HYDROXYAPATITE

Á. Szécsi

Research Institute for Plant Protection, Budapest

(Received May 23, 1977)

A simple technique is described for isolation of pure non degraded total DNA from fungal mycelium. The procedure involves preparation of acetone-dried powder from mycelium, lysis of the mycelium powder in 8 M urea – 0.24 M sodium phosphate buffer – 0.01 M EDTA –1% SDS – 1 M $NaClO_4$, deproteinization of the lysate with phenol–cresol–chloroform–isoamylalcohol and separation of the DNA from proteins, RNAs, carbohydrates and various low-molecular-weight substances by chromatography on hydroxyapatite. The modified technique is simple, fast and gives a high yield of pure DNA.

Double-stranded DNA (deoxyribonucleic acid) has a much higher affinity to hydroxyapatite (HA) than RNAs, proteins, carbohydrates and other cell components. Moreover, different nucleic acids can be adsorbed and eluted at different molarities of phosphate buffer [1]. Based on this principle, Britten *et al.* [2] have developed a new technique for the isolation of pure DNA from both prokaryotic and eukaryotic cells. Our method based on the passage of a crude lysate through hydroxyapatite at room temperature in the presence of 8 M urea and 0.24 M sodium phosphate buffer (SPB) permits only DNA to be adsorbed while most of the other cell constituents pass through. DNA may be recovered in a high degree of purity simply by elution from the hydroxyapatite with 0.48 M SPB. Several modifications have been proposed to improve the method [3–5].

This paper describes a modified batch technique for carrying out DNA purification from filamentous fungi (*Fusarium graminearum* Schwabe) on hydroxyapatite.

Materials and methods

Organism and cultivation. Fusarium graminearum Schwabe strain F-75 originated from corn stalk and was maintained in oil culture in the refrigerator. The isolate was grown in 500 ml Erlenmayer flasks containing 100 ml standard medium composed of peptone (Difco), 10 g; yeast extract (Oxoid), 3 g; D-glucose (Reanal), 20 g in 1 litre of water at pH 6.5. Cultures were incubated on a rotary shaker at 30 °C for 3 days. After 3 days incubation, fungal material was recovered by filtration, washed five times with tap water and once with distilled water. The material was than frozen and stored at —15 °C until use.

Preparation of acetone-dried powder from fungal material. Fungal material (120 g wet wt) was cut into small pieces and washed twice with 500 ml acetone and once with 500 ml ether

and finally dried at 50 °C overnight. The dried material was powdered using a mortar and pestle. The powder was stored in a glass stoppered flask at 4 °C. One gram of the powder corresponded to about 6 g of wet fungal material.

Preparation of granulated hydroxyapatite (GHA). GHA was prepared according to MAZIN *et al.* [6]. Silica gel powder 0.5 g (Reanal) was suspended in 25 ml of distilled water and washed twice with distilled water. The washed silica gel precipitate was added to a glass flask containing 100 ml distilled water. Simultaneously 500 ml of 0.5 M $CaCl_2$ (analytical grade) and 500 ml of 0.5 M Na_2HPO_4 (analytical grade) were added to the same vessel at the rate of 15 ml/hr by means of a ReCyChrom peristaltic pump (LKB). The content of the flask was continuously stirred. The precipitate formed was washed four times with distilled water suspended in 1 litre of distilled water and 25 ml of 40% NaOH (w/v) was added. The mixture was heated in a boiling water bath to 96–98 °C and incubated at this temperature for 1 hr; from time to time the suspension was carefully mixed by hand. The supernatant was poured off while still hot and the precipitate was washed with five portions of distilled water (1 litre). The washed precipitate was suspended in 1 litre 0.012 M SPB (equimolar solutions of NaH_2PO_4 and Na_2HPO_4) pH 6.8. The suspension was heated in a boiling water bath to 96–98 °C. The supernatant was decanted, a fresh 1 litre portion of SPB of the same molarity was added to the precipitate and the mixture was kept at 96–98 °C for 5 min. Then the buffer was changed again and the suspension was heated similarly for another 15 min. The resulting product (180 ml bed volume) was stored in 0.012 M SPB with a small amount of toluene at 4 °C. Under such conditions its properties remained unchanged for at least a year.

Determination of the rate of DNA adsorption on GHA. Deoxyribonucleic acid from chicken blood highly polymerized (Reanal, Hungary) was dissolved in 0.05 M SPB (1 mg/ml). Ten ml (bed volume) of GHA were equilibrated with 0.05 M SPB. The DNA solution was poured into 10 ml of GHA and the mixture was stirred gently and allowed to pass slowly in order to give maximum time for the double-stranded DNA to adsorb on the GHA. GHA was washed with 0.05 M SPB two times (50–50 ml) under vacuum. DNA was eluted with 0.48 M SPB. The DNA content of the eluate (10 ml) was determined spectrophotometrically.

Determination of the purity of DNA samples. The purity of DNA samples was determined by the ratio of A_{260}/A_{280} and A_{260}/A_{230}. These ratios indicate the protein contamination of the DNA. Protein content of DNA samples was determined also on the basis of the equation suggested by MAYR-HARTING *et al.* [7]: $1.45\ A_{280}–0.74\ A_{260}$. RNA content was determined by the procedure of SAVITSKY and STAND [8]. Criteria of purity of DNA were as follows: $A_{260}/A_{280} = 2 \pm 0.1$, hyperchromicity of at least 26%, no rise in optical density (OD) at 260 nm below 78 °C during denaturation [9]. The values for per cent hyperchromicity were obtained from samples during melting or thermal denaturation. The increase in absorbance at 260 nm of the DNA sample is termed "hyperchromic shift" and can be estimated from the relationship,

$$\text{per cent hyperchromicity} = \frac{\text{final absorbance} - \text{initial absorbance}}{\text{final absorbance}} \times 100 \qquad [10]$$

Thermal denaturation and melting temperature (T_m). Thermal transition profile of the isolated DNA was obtained by using Unicam SP 800 spectrophotometer (Pye Unicam Ltd., England) equipped with an automatic recording device. A DNA sample containing 50 μg DNA (1 OD) in 0.09×SSC (1×SSC, 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) was placed in a quartz cuvette (3.0 ml, 1 cm light path). A reference blank contained 0.09×SSC. The DNA sample was continuously analysed for optical density or absorbance and temperature measurements against the stationary reference blank. The sample and the reference solution were heated from 25 °C to 100 °C by using a ultrathermostate and a Scalapm Thermocouple Galvanometer (Pye and Co. Ltd., England) to control the temperature in the Constant Temperature Cell Holder (Pye Unicam Ltd., England) containing the sample and the blank. The ultrathermostate temperature was increased by 1 °C every 10 min. No corrections were made for water expansion, since evaporation was negligible in the Teflon-stoppered, siliconized cuvettes. T_m (midpoint temperature of the melting curve) value was determined by plotting absorbancy values from thermal melting data on normal probability paper [11]. The G + C content (percentage molar fraction of guanine plus cytosine) of DNA was calculated according to the equation of SCHILDKRAUT and LIFSON: $GC = 2.44\ (T_m - 81.5 - 16.6 \log M)$; M = ionic strength expressed as molarity [12].

DNA concentration. The DNA content of the samples was determined spectrophotometrically. Yield in microgram DNA (from 1 g acetone-dried mycelium powder) calculated from the OD at 260 nm by using the conversion factor 1 OD = 50 μg of DNA [13].

Isolation and purification of fungal DNA on granulated hydroxyapatite. Isolation and purification of fungal DNA was carried out according to the modified procedure of IVANOV

et al. [5]. One g acetone-dried mycelium powder was suspended in 40 ml of lysing medium (0.24 M SPB, 8 M urea, 1 M sodium perchlorate, 0.01 M EDTA; sodium salt; 1% sodium dodecyl sulphate). The lysate was stirred with an equal volume of water saturated, redistilled phenol (plus 10% m-cresol and 0.1% 8-hydroxyquinoline)–chloroform–isoamylalcohol (25 : 24 : 1) for 1 hr at room temperature and centrifuged at 5000 rpm for 10 min. The supernatant was treated once more in the same way (15 min) and then extracted two times with chloroform–isoamylalcohol (24 : 1) to remove the phenol. The DNA-containing solution was passed through a syringe with a thin needle (No. 19). Twenty ml (bed volume) of granulated hydroxyapatite (GHA) were equilibrated with 50 ml MUP (8 M urea — 0.24 M SPB, pH 6.8). The MUP was drawn of under vacuum. The DNA-containing solution was poured into a thick slurry of GHA in MUP, and the mixture gently agitated intermittently and allowed to stand at room temperature for about 1 hr. The slurry was poured into G3 sintered glass funnel (Jena) and the fluid was drawn off under vacuum. The GHA was washed free of RNAs, proteins and carbohydrates with MUP until no further material was eluted as judged by A_{260} and A_{280} measurements of the effluent (approximately 300 ml MUP/20 ml GHA). Urea was removed by washing with 0.012 M SPB (approximately 500 ml required 20 ml GHA). The removal of urea was monitored by refractive index measurements of the effluent. The GHA gel was suspended in 20 ml of 0.48 M SPB and stirred at room temperature for 20 min. The slurry was poured into G3 sintered-glass funnel and the DNA-containing buffer was drawn off under vacuum. The elution was continued to $A_{260} = 0.1$. The DNA-containing eluate (40 ml) was diluted with 80 ml distilled water to a concentration of 0.18 M SPB and filtrated through a second batch of 20 ml GHA equilibrated with 0.18 M SPB, into a G3 sintered glass funnel. Adsorption of DNA to GHA was judged by A_{260} measurement of the filtrate. The GHA was washed with 0.18 M SPB to A_{260} and $A_{280} = 0.0$. DNA was then eluted with 0.48 M SPB according to the above method. The DNA-containing eluate (40 ml) was dialysed overnight against 100 volumes of distilled water at 4 °C, brought to 0.2 M NaCl by adding solid salt, mixed with 2 vol of 96% ethanol and kept at −15 °C for 3–4 hr. The precipitate was collected by centrifugation at 6000 rpm for 20 min, dried in air at room temperature, and dissolved in 9 ml 0.1×SSC. One ml of acetate-EDTA (3.0 M sodium acetate plus 0.001 M EDTA, pH 7.0) was added. The DNA was then precipitated with 0.54 volume of isopropanol [14], and the precipitate was dissolved in 0.1×SSC and refrigerated over chloroform.

Results and discussion

MAZIN *et al.* [6] reported a modification of the original method of TISELIUS *et al.* [15] for the preparation of hydroxyapatite (HA). Their main idea was that HA is synthetized in the presence of silica gel particles. According to MAZIN *et al.* [6] the chromatographic properties of new HA are somewhat different from those of the original. The granulated HA (GHA) has a high specific capacity (600 μg native DNA/g adsorbent), it does not become denser, allows the elution to be performed at a high rate and can stand as much as 50 chromatographic cycles.

According to our studies, 10 ml GHA could bind 10 mg native DNA, but we could eluate only 6 mg DNA by 10 ml 0.48 M SPB after four filtrations through the gel. This was mainly due to the fact that large fragment size DNA cannot be eluted efficiently. Therefore, when a maximum yield of DNA is desirable, the DNA fragment size should be reduced and GHA chromatography should be repeated twice.

The characteristics of the DNA preparation isolated by the modified urea—hydroxyapatite method, are given in Table I. The A_{260}/A_{280} and A_{260}/A_{230} ratios of our DNA preparation compared with the same data from the literature are shown in Table II.

Table I

Characteristics of DNA preparation of F. graminearum

Yield mg DNA/g mycelium powder	A_{260}/A_{280}	A_{260}/A_{230}	RNA (%)	Protein (%)	T_m	%GC	Hyperchromicity (%)
1.2	2.02	2.21	1.4	1	71 °C	50.15	33.3

Table II

Comparison of the A_{260}/A_{280} and A_{260}/A_{230} ratios of DNA preparations obtained from various sources by various methods

Isolation procedure	Organism	A_{260}/A_{280}	A_{260}/A_{230}	Reference
Urea-HA	*Saccharomyces*	2 20	2.27	[5]
Urea-HA	*Allomyces*	1.90	2.00	[16]
MARMUR's + HA	*Phycomyces*	1.80	2.42	[17]
MARMUR's + HA	*Saccharomyces*	1.88	—	[18]
MARMUR's	*Blastocladiella*	1.95	1.97	[19]
CsCl centrifugation	*Dictyostelium*	1.90	2 35	[20]
Modified urea-HA	*Fusarium*	2.02	2.21	present paper

Most of the current methods [14] for the isolation of purified DNA required treatment with ribonuclease and pronase, combined with repeated deproteinization, precipitation and redissolution. Such methods take several days and involve a considerable loss of DNA. The present data showed that the modified urea-hydroxyapatite method was very satisfactory for the isolation of pure DNA from filamentous fungi (Tables I and II.)

According to our studies, GHA chromatography alone does not suffice to separate completely the DNA from protein contaminants. Treatment of the lysate with an equal volume of phenol–cresol–chloroform–isoamylalcohol mixture gave the best results. The DNA-containing solution eluted from the first batch contained a considerable amount of RNA, up to 20%. If this crude preparation is rechromatographed on a second GHA batch and precipitated with isopropanol, removal of the RNA is practically complete (RNA content about 1%).

The batch method described is simpler, faster and cheaper than the column procedure and was easily carried out in one day. The technique permits processing of small amounts of material and gives a high yield of pure DNA.

REFERENCES

1. BERNARDI, G.: In COLOWICK, S. P., KAPLAN, N. O. (eds): Methods in Enzymology. Vol. 21. Academic Press, New York 1971. p. 95.
2. BRITTEN, R. J., PAVICH, M., SMITH, J.: Carnegie Institution Year Book **68,** 400 (1969).
3. HELL, A., BIRNIE, G. D., SLIMMING, T. K., PAUL, J.: Anal. Biochem. **48,** 369 (1972).

4. MARKOV, G. G., IVANOV, I. G.: Anal. Biochem. **59,** 444 (1974).
5. IVANOV, I. G., VENKOV, P. V., MARKOV, G. G.: Prep. Biochem. **5,** 219 (1975).
6. MAZIN, A. L., SULIMOVA, G. E., VANYUSHIN, B. F.: Anal. Biochem. **61,** 62 (1974).
7. MAYR-HARTING, A., HEDGES, A. J., BERKELEY, R. C.: In NORRIS, J. R., RIBBONS, D. W. (eds): Methods in Microbiology. Vol. 7A. Academic Press, New York 1974. p. 315
8. SAVITSKY, J. P., STAND, F.: Nature (Lond.) **207,** 758 (1965).
9. CHATTOPADHYAY, S. K., DUTTA, S. K.: Neurospora Newsl. **15,** 11 (1969).
10. HARRIS, L. M., ABOKO-COLE, G. F., LEE, C. M., BOONE, L.: Trans. Amer. microsc. Soc. **95,** 599 (1976).
11. KNITTEL, M. D., BLACK, C. H., SANDINE, W. E., FRASER, D. K.: Canad. J. Microbiol. **14,** 239 (1968).
12. SCHILDKRAUT, C., LIFSON, S.: Biopolymers **3,** 195 (1965).
13. DUTTA, S. K., OJHA, M.: Molec. gen. Genetics **114,** 232 (1972).
14. MARMUR, J.: J. molec. Biol. **3,** 208 (1961).
15. TISELIUS, A., HJERTEN, S., LEVIN, Ö.: Arch. Biochem. Biophys. **65,** 132 (1956).
16. OJHA, M., TURIAN, G.: Molec. gen. Genetics **112,** 49 (1971).
17. DUSENBERY, R. L.: Biochim. biophys. Acta (Amst.) **378,** 363 (1975).
18. HARBITZ, J., OYEN, T. B.: Biochim. biophys. Acta (Amst.) **366,** 374 (1974).
19. MYERS, R. B., CANTINO, E. C.: Arch. Microbiol. **78,** 252 (1971).
20. FIRTEL, R. A., BONNER, J.: J. molec. Biol. **66,** 339 (1972).

Address of the author:

ÁRPÁD SZÉCSI
Research Institute for Plant Protection
H-1525 Budapest, P.O.B. 102, Hungary

Acta microbiol. Acad. Sci. hung. 25, 67—76, 1978

ISOLIERUNG UND REINIGUNG EINES SCHWER EXTRAHIERBAREN PROTEUS MIRABILIS ANTIGENS

R. REISSBRODT, G. KEMMER und G. SELTMANN

Institut für Experimentelle Epidemiologie, Wernigerode, DDR

(Eingegangen 23. Mai 1977)

R. REISSBRODT, G. KEMMER and G. SELTMANN *(Institute of Experimental Epidemiology, Wernigerode, G.D.R.)*: **Isolation and Some Properties of Proteus mirabilis Antigen Difficult to Extract.** Cell walls of *Proteus mirabilis* contain an antigen substance (SEPA) which cannot be extracted by anionic and neutral detergents, but becomes soluble by sonification of native or preextracted bacteria. It is assumed that SEPA is typical of *P. mirabilis*. The antigen isolated from preextracted bacteria was purified by DEAE–cellulose chromatography. On Sephadex G-200 it could be separated into 4 fractions (fraction I, $5 \cdot 10^5$–10^6 Dalton; fraction II, 1.1–$4 \cdot 10^5$ Dalton; fraction III, $\sim 10^5$ Dalton; fraction IV, $\sim 10^4$ Dalton). Rechromatography of fraction III under identical conditions resulted in the repeated formation of these fractions. Fractions I–III but not fraction IV precipitated with anti-SEPA sera. SEPA is composed mainly of amino acids, with small quantities of carbohydrates ($<2\%$) and lipids ($<1\%$). Differences in the qualitative and (as far as estimated) quantitative composition could not be found between the Sephadex G-200 fractions. In SDS–polyacrylamide gel electrophoresis, one main band ($\sim$60 000 Dalton) could be detected beside some weaker bands in the region between 10 000 and 60 000 Dalton. It is assumed that SEPA consists of two subunits (10 000 and 60 000 Dalton) which are able to aggregate as well mutually as with themselves.

Zellwände von *Proteus mirabilis* enthalten eine antigene Substanz (SEPA), die mit anionischen und neutralen Detergentien nicht extrahiert werden kann, aber nach Ultraschallbehandlung der nativen oder der vorextrahierten Bakterien löslich wird. Es wird vermutet, daß SEPA typisch für *P. mirabilis* ist. Das aus den vorextrahierten Bakterien gewonnene Antigen wurde durch DEAE–Cellulosechromatographie gereinigt. An Sephadex G-200 konnte es in 4 Fraktionen getrennt werden (Fraktion I, $5 \cdot 10^5$–10^6 Dalton, Fraktion II, $1{,}1$–$4 \cdot 10^5$ Dalton, Fraktion III, $\sim 10^5$ Dalton, Fraktion IV, $\sim 10^4$ Dalton). Rechromatographie von Fraktion III unter identischen Bedingungen ergab erneute Bildung von diesen Fraktionen. Die Fraktionen I–III, nicht aber Fraktion IV, präzipitierten mit Anti-SEPA-Seren. SEPA ist hauptsächlich aus Aminosäuren zusammengesetzt, es enthält daneben kleine Mengen Kohlenhydrate ($<2\%$) und Lipide ($<1\%$). Unterschiede in der qualitativen und (so weit bestimmt) quantitativen Zusammensetzung konnten zwischen den Sephadex G-200-Fraktionen nicht festgestellt werden. In der SDS–Polyacrylamidgelelektrophorese konnte eine Hauptbande ($\sim$60 000 Dalton) neben einigen schwächeren Banden in der Region zwischen 10 000 und 60 000 Dalton festgestellt werden. Es wird vermutet, daß SEPA aus 2 Untereinheiten (10 000 und 60 000 Dalton) besteht, die sowohl untereinander als mit sich selbst aggregieren können.

Intensive Untersuchungen zu Fragen der Proteinzusammensetzung der äußeren Membran [1], der Struktur der Lipopolysaccharide [2] und der Flagellae [3, 4] sowie der Toxizität [5, 6] sind bei *P. mirabilis* in jüngerer Zeit vermehrt unternommen worden. Über die Proteinantigene der *P. mirabilis*-Zellwand ist aus der Literatur wenig bekannt.

In der vorliegenden Arbeit werden die Isolierung und einige Eigenschaften eines mit anionischen und neutralen Detergentien wie Natriumdodecylsulfat, Brij 58, EDTA, Rodapon N 50, Tween 80 und Natriumdesoxycholat schwer extrahierbaren *P. mirabilis*-Antigens (SEPA) beschrieben.

Material und Methoden

Bakterienstämme. Die untersuchten Bakterienstämme sind in Tabelle I aufgeführt. Für die Isolierung des SEPA wurde der *P. mirabilis*-Rauhstamm R 51 verwendet, dessen Lipopolysaccharid Glucose, L-Glycero-D-mannoheptose, D-Glycero-D-mannoheptose und 2-Keto-3-desoxy-D-mannooctonsäure enthielt. Der Stamm wurde von Professor Dr. KOTELKO, Institut für Mikrobiologie, Universität Lodz, Polen, erhalten.

Nährmedien und Anzüchtung. Die Bakterien wurden auf einer Nähragar I-Platte 18 Std. bei 37 °C angezüchtet, davon eine 4 Std. Schüttelkultur in Nährbouillon I hergestellt und diese auf große Nähragar I-Platten ($\varnothing = 15$ cm) ausgespatelt (Nähragar I und Nährbouillon I von SIFIN Berlin-Weißensee). Von jeder verarbeiteten Bakteriensuspension wurde ein Ausstrich zur Reinheitskontrolle auf Galle–Chrysoidin–Glycerin–Platte [7] angelegt. Nach 16 Std. Bebrütung bei 37°C wurde der Bakterienrasen geerntet und die Bakterien wurden sofort lyophilisiert.

Extraktion. 1,6 g lyophilisierte Bakterien wurden in 300 ml 0,85%iger NaCl-Lösung mit 0.125% Rodapon N 50 16 Std. bei +4°C stehengelassen (Rodapon N 50 ist ein Natriumalkylbenzolsulfonat des VEB Deutsches Hydrierwerk, Rodleben). Anschließend wurde bei 5000 min^{-1} zentrifugiert, der Extraktionsrückstand (Sediment) 5× mit 0,85%iger NaCl-Lösung gewaschen und dann mit Ultraschall (Ultraschallgerät der Fa. Schoeller und Co., Frankfurt/Main) aufgeschlossen. Der Ultraschallextraktionsrückstand wurde bei 5000 min^{-1} abzentrifugiert und der Überstand über Nacht gegen fließendes Leitungswasser dialysiert. Die Ausbeute an aufgeschlossenem Extraktionsrückstand betrug durchschnittlich 400 mg mit 45–50% Proteingehalt (Biuret).

DEAE-Cellulosechromatographie und Sephadex G-200-Chromatographie. An einer DEAE-Celulosesäule (DEAE-Cellulose von Reanal, Budapest, nach Aktivierung in Phosphatform) mit einem $\varnothing = 8$ cm, h = 8 cm, wurde der aus 3 g lyophilisierten *P. mirabilis* R 51-Bakterien erhaltene Ultraschall-Extraktionsrückstand chromatographiert. Phosphatgepufferte isotonische NaCl-Lösung (PBS) mit steigenden Ionenstärken (1/10 PBS; PBS; PBS + 1 M NaCl) wurde verwendet. Die mit PBS bzw. PBS + 1 M NaCl erhaltenen Eluate wurden mit Ammoniumsulfat bis zu 95%iger Sättigung versetzt, das ausgefallene Material abfiltriert, der Filtrationsrückstand in aqua dest. wieder aufgelöst. Nach erneuter Dialyse gegen Leitungswasser wurde im Diaflo-Ultrafiltrationsgerät (Membran UM 2 der Fa. Amicon, Ousterhoot, Holland) auf 8–15 mg Protein/ml (Biuret) konzentriert.

1–2 ml dieser Lösung wurden sodann an einer Sephadex G-200-Säule (Fa. Pharmacia, Uppsala, Schweden) mit einem $V_t \approx 230$ ml, $V_0 \approx 70$ ml bei einer Geschwindigkeit von 25 ml/h chromatographiert (Puffer: 0,1% Ammoniumacetat). Die erhaltenen Fraktionen wurden zuerst im Rotationsverdampfer, anschließend im Diaflo-Ultrafiltrationsgerät (Membran UM 2) konzentriert. Nach Ausführung der Immunreaktionen (Immunelektrophorese bzw. Immundiffusion) wurden die Fraktionen im Exsiccator über feuchtem Natriumhydroxid/$CaCl_2$ getrocknet.

Analytische Verfahren. Immundiffusion und Immunelektrophorese, dünnschichtchromatographische Aminosäureanalyse, SDS-Polyacrylamidgel-Elektrophorese sowie gaschromatographische Kohlenhydrat- und Fettsäureanalyse wurden bereits beschrieben [8].

Die Untersuchung der Kohlenhydrate erfolgte auch durch elektrophoretische Trennung nach [9] (600 V, 20 Min., Dünnschichtelektrophoresekammer der Fa. Desaga, Heidelberg, Pyridin-Essigsäure-Puffer pH 7.0 und anschließende Dünnschichtchromatographie nach [10] im System n-Butanol-Pyridin-Wasser 6 : 4 : 3. Zusätzlich wurde nach [11] die Absorptionskurve nach Cystein-Schwefelsäure-Färbung (Gerät: Perkin-Elmer Model 124) aufgenommen.

Thermostabilität. Die mit PBS + 1 M NaCl eluierte Fraktion der DEAE-Cellulosechromatographie wurde je 1 Std. bei 60 °C und bei 100 °C gehalten, anschließend in der Immundiffusion untersucht.

Enzymatischer Abbau. 5 mg lyophilisiertes Material der mit PBS + 1 M NaCl eluierten Fraktion der DEAE-Cellulosechromatographie wurden in wäßriger Lösung bei pH 7.0–7.2

mit je 0,5 mg Pronase (Serva, Heidelberg), Trypsin (Difco, Detroit), RNAse (Ferak, Berlin), DNAse (Roth OHG, Karlsruhe) oder Lysozym (Reanal, Budapest) versetzt. Nach 1 Std. bei 37 °C wurden die Lösungen immunelektrophoretisch untersucht.

Hydrolyse der gewonnenen Fraktionen. Für Aminosäuren 16 Std. mit 6 N HCl, für Kohlenhydrate 4 Std. mit 1 N HCl oder 12 Std. mit 0,5 N H_2SO_4, Fettsäuren 5 Std. mit 4 N NaOH.

Ergebnisse

Vorkommen des Antigens. Extraktion des Stammes *P. mirabilis* 1095/67 mit anionischen und neutralen Detergentien wie Natriumdodecylsulfat, BRIJ 58, EDTA, Rodapon N 50, Tween 80 und Natriumdesoxycholat entfernte den größten Teil der Bestandteile der äußeren Membran. Zurück blieb eine Substanz, die in der Immunelektrophorese schwach anodisch wanderte und auch in allen anderen geprüften *P. mirabilis*-Stämmen einschließlich verschiedener Rauhformen bis hin zu R_e-Form nachgewiesen werden konnte (Tabelle I). Dabei war in den untersuchten *P. mirabilis*-Stämmen kein Unterschied in der Stärke der Präzipitationslinie festzustellen, lediglich in einem tet$^-$-Stamm war die Präzipitationslinie des Antigens schwächer ausgebildet.

Tabelle I

Bakterienstämme

Spezies	Bezeichnung	Marker	Herkunft	SEPA-Präzipitationslinie
Proteus mirabilis	1095/67	nic$^-$ thi$^-$ tet$^+$ fin$^+$	IEE	+
	2098	nic$^-$ thi$^-$ tet$^-$	IEE	(+)
	273	nic$^-$ thr$^-$ tet$^+$ fin$^+$	IEE	+
	1959/S	GlcUA; GalN; GalUA, GlcN; Glc; L-Hep; D-Hep; KDO	IML	+
	1959/R13	GalUA; GlcN; Glc; L-Hep; D-Hep; KDO	IML	+
	1959/R51	Glc; L-Hep; KDO	IML	+
	1959/R45	KDO	IML	+
Proteus rettgeri	6382		IEE	−
Proteus morganii	6732		IEE	−
Proteus vulgaris	6394		IEE	−
Shigella sonnei	2882/67	nic$^-$ thi$^-$ KK$^+$	IEE	−
Escherichia coli	K 12	met$^-$ pro$^-$ (λ)	IEE	−
Providencia	4619	tet$^+$	IEE	−

Abkürzungen

nic$^-$, thi$^-$, thr$^-$, met$^-$, pro$^-$ = auxotroph für Nicotinsäureamid, Thiamin, Threonin, Methionin tet$^+$($^-$) = Resistenz (Empfindlichkeit) gegen Tetracyclin, fin$^+$ = Fertilitätsinhibition. Glc = Glucose, GlcN = Glucosamin, GalN = Galaktosamin, GlcUA = Glucuronsäure, GalUA = Galakturonsäure, L-Hep = L-Glycero-D-mannoheptose, D-Hep = D-Glycero-D-manno-heptose, KDO = 2-Keto-3-desoxy-D-mannooctonsäure, KK$^+$ = positive Keratokonjunktivitis-reaktion (Meerschweinchenauge), IEE = Stammsammlung des Instituts für Experimentelle Epidemiologie, Wernigerode, IML = Institut für Mikrobiologie, Universität Lodz.

Das schwer extrahierbare *P. mirabilis-Antigen* (SEPA) konnte auch in Ultraschallaufschlüssen vorher nicht extrahierter *P. mirabilis*-Stämme nachgewiesen werden. Im Gegensatz dazu wurde unter gleichen Extraktionsbedingungen bei anderen *Proteae (Proteus morganii, Proteus rettgeri, Proteus vulgaris, Providencia)* sowie in *Shigella sonnei* und *Escherichia coli* eine solche Substanz in der Immunelektrophorese gegen *P. mirabilis*-Serum nicht gefunden (Tabelle I).

Abb. 1. Immunelektrophorese-Muster des schwer extrahierbaren *P. mirabilis*-Antigens (SEPA). Elektrophoretische Trennung 60 Min. Loch: Antigen erhalten nach Extraktion und Ultraschallaufschluß. Rinne: *Proteus mirabilis* 1095/67-Serum

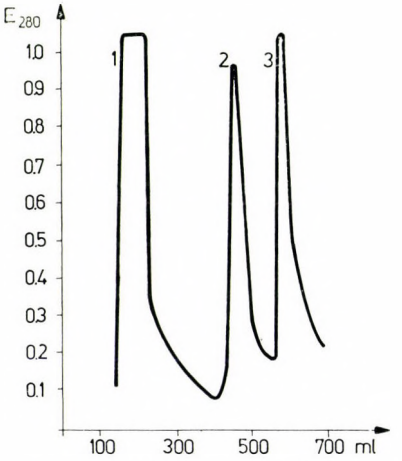


Abb. 2. DEAE-Cellulose-Chromatographie des Antigens erhalten nach Extraktion und Ultraschallaufschluß von *P. mirabilis* R 51. Fraktion 1 = PBS 1 : 10 verdünnt; Fraktion 2 = PBS; Fraktion 3 = PBS + 1 M NaCl

Extrahierbarkeit des Antigens. Der nach Vorextraktion mit 5%iger Rodapon N 50-Lösung und nachfolgendem Ultraschallaufschluß des Stammes *P. mirabilis* 1095/67 erhaltene Extrakt zeigte in der Immunelektrophorese die Präzipitationslinie des SEPA als Hauptlinie (Abb. 1), ähnlich verhielt sich der für die Isolierung verwendete Rauhstamm *P. mirabilis* R 51.

Bei Erhöhung der zur Vorextration eingesetzten Rodapon N 50-Konzentration auf das Doppelte ließ sich das SEPA in der Immunelektrophorese oder Immundiffusion nicht mehr nachweisen. Bei Einsatz der halben Rodapon N 50-Menge wurden weitere Präzipitationslinien nachgewiesen.

Reinigung des Antigens und molekulare Heterogenität. Bei der DEAE-Cellulosechromatographie (Abb. 2) wurde in der mit 1 : 10 verdünnter PBS

eluierten Substanz (Fraktion 1) kein SEPA gefunden. In der mit PBS (Fraktion 2) und der mit PBS + 1 M NaCl eluierten Fraktion (Fraktion 3) wurde das SEPA nachgewiesen, in Fraktion 3 stellte es sich als einzige Präzipitationslinie dar (Abb. 3). Bei Erhöhung der NaCl-Konzentration konnte kein SEPA mehr eluiert werden. Eine Schärfung des SEPA-Peaks in einem Elutionsschritt durch Verwendung eines Phosphatgradienten an DEAE-Cellulose gelang nicht. Das SEPA erschien auch hier diffus bei mehreren (0.3 → 0.6 M) Phosphatkonzentrationen.

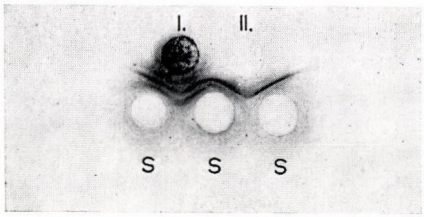


Abb. 3. Immundiffusionsmuster der DEAE-Cellulose-Chromatographie. I.: Fraktion PBS (Fraktion 2); II: Fraktion PBS + 1 M NaCl (Fraktion 3); S: *P. mirabilis* 1095/67-Serum

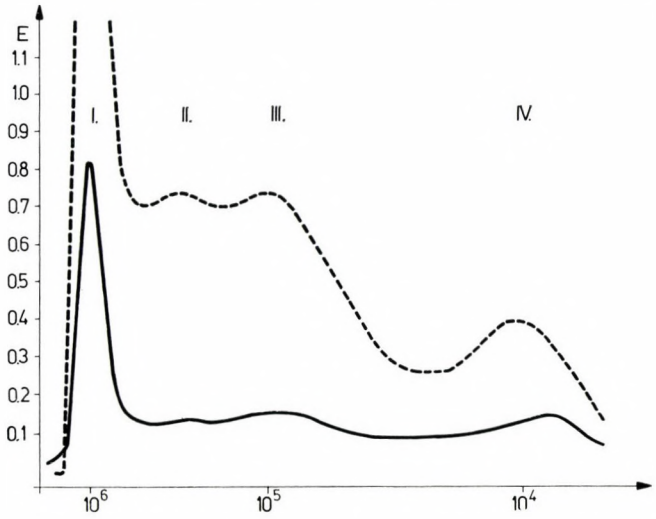


Abb. 4. Sephadex G-200-Chromatographie von Fraktion 3 der DEAE-Cellulose-Chromatographie. I–IV = erhaltene Fraktionen; —— E_{280} nm; ----- E_{206} nm

Chromatographie obiger Fraktion 3 an Sephadex G-200 führte immer zu dem in Abb. 4 dargestellten Elutionsprofil. Grundsätzlich wurden jeweils 4 Fraktionen erhalten (Fraktionen I bis IV), von denen die Fraktionen I–III in der Immunelektrophorese (s. z. B. Abb. 5) bzw. Immundiffusion (Abb. 6) die Präzipitationslinie des SEPA zeigten. Fraktion IV in einem Molekulargewichtsbereich bei $\sim 10^4$ gab keine Präzipitationslinie mit dem Anti-*P. mirabilis*-Serum.

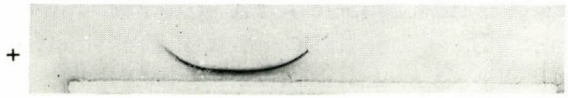


Abb. 5. Immunelektrophorese-Muster von Fraktion II (Sephadex G-200-Chromatographie) gegen *P. mirabilis* 1095/67-Serum. Elektrophoretische Trennung 90 min

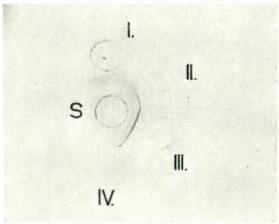


Abb. 6. Immundiffusionsmuster der Sephadex G-200-Fraktionen (I–IV) gegen *P. mirabilis* 1095/67-Serum

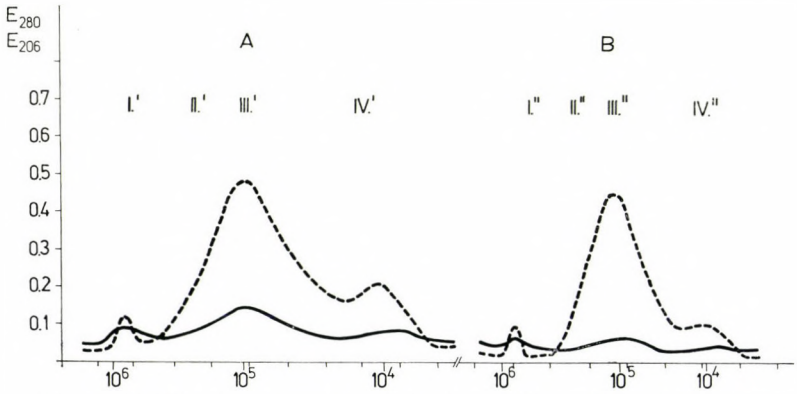


Abb. 7. Sephadex G-200-Rechromatographie der Fraktion III von der Sephadex G-200-Chromatographie (Abb. 4) (A) und wiederholte Rechromatographie von Hauptpeak III' (B). —— E_{280} nm; ----- E_{206} nm

Rechromatographie der Fraktion III an Sephadex G-200 führte zur Aufsplitterung in die 4 Fraktionen I'–IV', die Ausgangsfraktion III was als III' wieder die Hauptfraktion (Abb. 7A). Erneute Rechromatographie dieser Hauptfraktion III' an Sephadex G-200 führte zur gleichen Aufsplitterung (Abb. 7B), allerdings war der Peak IV'' gegenüber IV' deutlich verkleinert. Die hier erhaltenen Fraktionen entsprachen in ihrer serologischen Aktivität und im Elutionsverhalten den bei der ersten Sephadex G-200-Chromatographie erhaltenen Werten. Aus dem Elutionsverhalten wurden für die Fraktionen I bis IV folgende ungefähre Molekulargewichte berechnet:

Fraktion I $5 \cdot 10^5$ bis 10^6 ($\sim V_0$)
Fraktion II $1{,}1 \cdot 10^5$ bis $4 \cdot 10^5$

Fraktion III $1 \cdot 10^5$ (konstant bei 9 Chromatographieen)
Fraktion IV $8 \cdot 10^3$ bis $1{,}1 \cdot 10^4$

Zusammensetzung des Antigens. Die Antigenität des schwer extrahierbaren *P. mirabilis*-Antigens wurde durch Pronase und Trypsin zerstört. DNAse, RNAse und Lysozym blieben ohne Einfluß. Am Aufbau des SEPA müssen demzufolge Proteine entscheidend beteiligt sein. In der Immunelektro-

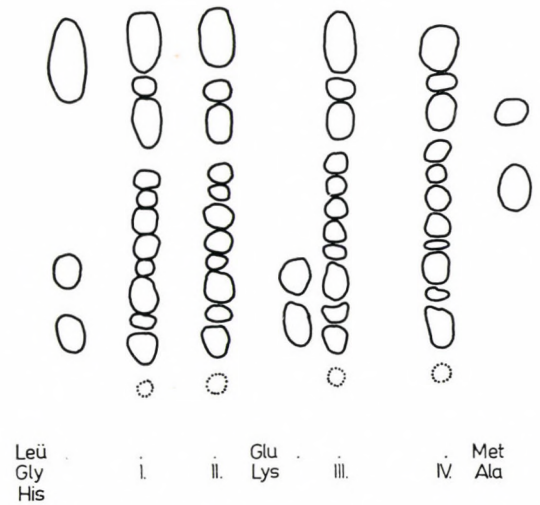


Abb. 8. Dünnschichtchromatographie der Aminosäuren nach Hydrolyse der Fraktionen I–IV (Sephadex G-200-Chromatographie) 6 N HCl, 12 Std. (Abb. 4). Anfärbung Ninhydrin; System: Butanol-Essigsäure–Wasser 12 : 3 : 5 angefärbt mit Ninhydrin und Kollidin (13.8% Kollidin in Aethanol)

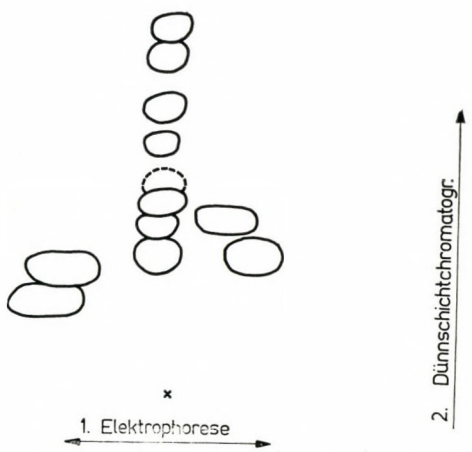


Abb. 9. Fingerprint-Technik zur Aminosäurezusammensetzung von Fraktion II (Sephadex G-200-Chromatographie). 1. Lauf: Elektrophorese; 2. Lauf: Dünnschichtchromatographie

phorese war die Präzipitationslinie des SEPA nach Erhitzen auf 100 °C (60 Min.) nicht mehr vorhanden, bei 60 °C trat sie geschwächt auf.

Bei der dünnschichtchromatographischen Aminosäureanalyse wurde gefunden, daß alle 4 Fraktionen der Sephadex G-200-Chromatographie (I–IV) eine qualitativ und, nach Schätzung der Intensität der Flecken, auch quantitativ gleiche Aminosäurezusammensetzung haben (Abb. 8). Auch nach vorheriger elektrophoretischer Auftrennung (fingerprint, Abb. 9) war kein Unterschied in der Aminosäurezusammensetzung festzustellen. Elf Aminosäure-

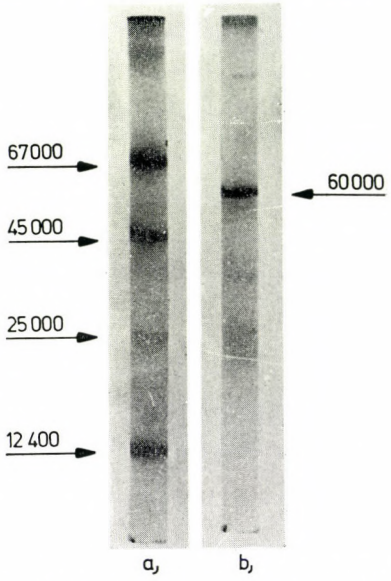


Abb. 10. SDS-Polyacrylamidgel-Elektrophorese von Fraktion II der Sephadex G-200-Chromatographie; (a) Molekulargewicht-Standards (Rinderserumalbumin, Ovalbumin, Chymotrypsinogen A, Cytochrom C); b) SEPA

flecke konnten unterschieden werden, darunter waren Leucin, Glycin, Lysin, Glutaminsäure, Asparaginsäure, Alanin und Methionin. Der Kohlenhydratgehalt war nach der gaschromatographischen und dünnschichtchromatographischen Bestimmung gering ($< 2\%$), Glucose stellte die Hauptkomponente dar. Auch hier gab es keinen Unterschied zwischen den einzelnen Fraktionen der Sephadex G-200-Chromatographie. Pentosen wurden durch die Aufnahme der Adsorptionskurve nach Reaktion mit Cystein/Schwefelsäure ausgeschlossen.

Nach der gaschromatographischen Fettsäurebestimmung enthalten alle 4 Fraktionen der Sephadex G-200-Trennung die gleichen Fettsäuren C–14, C-16 und C-18, allerdings in geringen Mengen ($\sim 1\%$).

Die SDS-Polyacrylamidgel-Elektrophorese in Gegenwart von 1% SDS, 8 M Harnstoff und 0.1% ß-Mercaptoäthanol ergab bei den Fraktionen

I bis III stets eine Hauptbande mit einem Molekulargewicht um 60 000 (s. Abb. 10), es wurden aber auch niedermolekulare Banden bis zum Molekulargewicht um 10 000 nachgewiesen. Die Bande mit einem Molekulargewicht um 60 000 ließ sich auch durch Erhöhung des Anteils an β-Mercaptoäthanol auf 5% sowie durch Veränderung des SDS-Anteils bei der SDS-Elektrophorese nicht weiter aufspalten.

Diskussion

Extrahiert man *P. mirabilis*-Stämme mit neutralen oder anionischen Detergentien, so geht in Abhängigkeit von den Versuchsbedingungen ein mehr oder weniger großer Teil der Komponenten der äußeren Membran in Lösung; es kann also allein durch Variation der Extraktionsbedingungen eine gewisse Fraktionierung erfolgen. Verwendet man 0,1% Rodapon N 50 bei +4 °C, so bleibt im wesentlichen ein Antigen in der Zellwand zurück und kann anschließend unter drastischen Bedingungen (Ultraschall) freigesetzt werden. Dieses von uns als schwer extrahierbares *P. mirabilis*-Antigen (SEPA) bezeichnete Antigen wurde in allen geprüften *P. mirabilis*-Stämmen (auch Rauhformen) gefunden, nicht dagegen in anderen *Proteae* sowie *S. sonnei* und *E. coli*.

Unterschiede in der Extrahierbarkeit der Zellwandbestandteile, insbesondere bei Proteinen der äußeren Membran von *P. mirabilis*, durch verschiedene Detergentien (SDS, Triton X-100, Natriumdesoxycholat) sind kürzlich beschrieben worden [12].

Das schwer extrahierbare *P. mirabilis*-Antigen gehört vermutlich zu den basalen Zellwand-Protein-Antigenen, da es auch unter minimalen Wachsstumsbedingungen bei fast jeder Nährmedien-Variante immunelektrophoretisch nachgewiesen werden konnte [13]. SEPA besitzt einen hohen Proteingehalt neben wenig Kohlenhydrat- und Fettsäureanteil. Der geringe Lipidgehalt des SEPA schließt eine Verwechslung mit dem S-Neurotoxin ($\sim$20%) oder L-Neurotoxin ($\sim$8%) von *P. mirabilis* aus [3]. Ebenso spricht die Thermostabilität des L-Neurotoxins (100 °C werden ertragen, ohne daß die Antigenität verändert wird) gegen eine Identität.

Vorläufige Toxizitätsbestimmungen an Mäusen zeigten, daß SEPA nur mäßig toxisch ist (LD_{50} SEPA $\sim$ 0,15 mg N; S-Neurotoxin $\sim$ 0,01 mg N; L-Neurotoxin $\sim$0,1 mg N [14], Reissbrodt, unveröffentlicht).

Aus dem Elutionsverhalten des SEPA sowohl an DEAE-Cellulose als auch an Sephadex G-200 geht hervor, daß es in wäßriger Lösung leicht in niedermolekulare Aggregate dissoziieren und in höhermolekulare Einheiten aggregieren kann (s. z. B. Abb. 7). Damit zeigte es ein ähnliches Verhalten wie das anodisch wandernde thermolabile Antigen (ATA) von Gram-negativen Bakterien [8].

Im Unterschied zu dem ATA traten aber in der Gelchromatographie und in der SDS-Gelelektrophorese 2 stabile "Untereinheiten" auf: eine mit einem Molekulargewicht von $\sim 10^4$ Dalton und eine mit einem Molekulargewicht von $\sim 6 \cdot 10^4$ Dalton. Das Gesamtmolekül und die höhermolekularen Untereinheiten geben mit Anti- *P. mirabilis*-Antiseren in der Immunelektrophorese praktisch identische Präzipitationslinien, die niedermolekulare Untereinheit dagegen nicht. Die Differenzen zwischen den mit der Gelchromatographie einerseits und der SDS-Elektrophorese andererseits bestimmten Molekulargewichte beruhen wahrscheinlich auf die Tatsache, daß die Molekulargewicht/K_{av}-Beziehung für Sephadex G-200 mit globulären Proteinen vorgenommen wurde (die Raumstruktur des SEPA ist unbestimmt). Proteine, die aus 2 Untereinheiten zusammengesetzt sind, die *in vitro* sowohl mit sich selbst als auch mit der anderen Untereinheit zu aggregieren vermögen und so in der SDS-Gelelektrophorese ein etwas verwirrendes Bild geben, sind nicht ungewöhnlich (s. z. B. [15]).

Das schwer extrahierbare *P. mirabilis*-Antigen ist offenbar für *P. mirabilis* charakteristisch. Es wurde lediglich ein Stamm gefunden, der die Präzipitationslinie des SEPA schwächer ausbildete und der gleichzeitig empfindlich gegenüber Tetracyclin war.

Der MTLA Frl. M. GRASSMAY danken wir für sorgfältige technische Assistenz bei der Durchführung der Versuche.

LITERATUR

1. HASIN, M., ROTTEM, S., RAZIN, S.: Biochem. biophys. Acta (Amst.) **375,** 381 (1975).
2. KOTELKO, K., GROMSKA, W., PAPIERZ, M., SZER, K., KRAJEWSKA, D., SIDORCZYK, Z.: J. Hyg. Epidem. (Praha) **18,** 405 (1974).
3. IZDEBSKA-SZYMONA, K.: Acta microbiol. pol. Ser. A, **6,** 163 (1974).
4. BODE, W.: Angew. Chem. **85,** 731 (1973).
5. MESROBEANU, L., MESROBEANU, I., MITRICA, N.: Arch. roum. Path. exp. **20,** 399 (1961).
6. MESROBEANU, L., MESROBEANU, I., MITRICA, N.: Ann. N. Y. Acad. Sci. **133,** 685 (1966).
7. KNORR, M., EHRENHARD, D.: Z. ges. Hyg. **19,** 370 (1973).
8. REISSBRODT, R., KEMMER, G., SELTMANN, G.: Z. allg. Mikrobiol. **16,** 385 (1976).
9. HANNIG, K., PASHER, G.: In STAHL, E. (ed.): Dünnschichtchromatographie. 2. Aufl. Springer-Verlag, Berlin—Heidelberg—New York 1967. S. 153.
10. JEANES, A., WISE, C. A., DIMLER, R. J.: Analyt. Chem. **23,** 415 (1951).
11. ASHWELL, G.: In COLOWICK, S. P., KAPLAN, N. O. (eds): Methods in Enzymology. Academic Press, New York 1957. Vol. III, P. 81.
12. RAZIN, S., MARKOWITZ, O., HASIN, M., ROTTEM, S.: Biochem. biophys. Acta (Amst.) **433,** 240 (1976).
13. REISSBRODT, R., KEMMER, G., SELTMANN, G.: Z. allg. Mikrobiol. **15,** 357 (1975).
14. IZDEBSKA-SZYMONA, K.: In MONTIES, T. C., KADIS, S., ALL, S. J. (eds): Microbiological Toxins. Vol. IIa, Academic Press, New York 1971. p. 337.
15. KUROSKY, A., MARKEL, D. E., TOUCHSTONE, B., PETERSON, J.: J. infect. Dis. **133,** 14 (1976).

Adresse der Autoren:

R. REISSBRODT, G. KEMMER, G. SELTMANN
Institut für Experimentelle Epidemiologie
DDR-37 Wernigerode, Burgstrasse 37, Deutsche Demokratische Republik

Acta microbiol. Acad. Sci. hung. 25, 77—86, 1978

STABILITY OF ESCHERICHIA COLI ADHESIVE FACTORS K88 AND K99 IN MICE

I. KÉTYI, B. KUCH and ADELE VERTÉNYI

Institute of Microbiology, University Medical School, Pécs

(Received June 23, 1977)

Strains of *Escherichia coli* carrying K88 and K99 plasmids acquired either naturally or artificially show a curing-like plasmid loss in the bowel of adult mice. Though the ratio of plasmid loss may differ significantly in various inbred mouse strains, the dynamics of the loss are identical. Under conditions *in vitro* with long incubation period, high pH and low redox values, the ratio of spontaneous plasmid loss is low. Suckling mice 3 or 10 days old infected orally with derivatives of $K88^+$ or $K99^+$ show a selective advantage for adhesive factors compared to other *E. coli* strains. In the alimentary tract of suckling mice an enrichment of adhesive factor carrying clones can be demonstrated. In this respect, there is no difference between K88 and K99. In 14-day-old mice the appearance of plasmid curing effect, together with the disappearance of their selective advantage, was observed.

In animals, the adhesive factors K88 [1, 2] and K99 [3, 4] play an important role in the pathogenicity of enterotoxigenic *Escherichia coli* strains. The present study aimed at developing a mouse model for human enterotoxigenic strains, by the aid of K88 or K99 plasmids transfered artificially. Since in these experiments a high ratio of plasmid loss was observed, a detailed analysis of this phenomenon seemed to be justified.

Materials and methods

Strains. The strains and their derivatives are shown in Table I. From strains used for animal experiments, streptomycin resistant mutants were selected.

Media. Generally, a meat broth of pH 7.2, containing 1% peptone (Bacto, Difco) and 0.5% NaCl was used, either liquid or solidified with 1.6% agar. Isolation of *E. coli* from faeces and bowel surface was performed on Endo agar. For transferring plasmids mostly 20 ml of Luria broth or sometimes liquid MINCA in Erlenmeyer flasks of 200 ml was used. Detection of spontaneous plasmid loss during a long incubation period was done in meat broth. When pH values from pH 7.2 to pH 8.5 were obtained, 2% peptone water media were used and the low redox potential was adjusted by 0.05% sodium thioglycocollic acid in the same medium. When the carried K antigen (type A) caused inagglutinability of the antigens K88 and K99, serological test for pilus surface antigen was carried out in MINCA [5] medium.

Experimental animals. Both adult and suckling mice belonged to the BALB/c strain In some of the experiments a few other inbred strains (C3H, DBA, CBA) were also used.

Pretreatment, infection and maintenance of animals. For diagnostic purposes a monoflora of the infecting agent has been established. For 2 days before infection 50 mg of streptomycin were administered orally to the animals maintained individually in glass jars. They were fed with sterilized food. Groups of jars were placed in a closed box. In case of 14- and 21-day-old mice, the litter was maintained togther; they were not separated from the mother and streptomycin was given to the latter, too. The doses were 10–15 mg in a volme. of 0.2–0.25 ml. The elimination of coliform bacteria was checked before infection.

Oral infection was carried out similarly as the streptomycin treatment, with 10^7 to 10^8 germs in a volume of 0.2, 0.25 or 0.5 ml by the aid of an S-form metal cannula. For suckling mice the cannula was covered by a soft polyethylene tube for protecting the soft tissues from injuries. Suckling mice 3 to 10 days old were infected without streptomycin pretreatment using the above technique giving 0.1 ml volumes orally.

Isolation and germ count from the bowel of adult mice. From the killed and autopsied animals, long segments of the small bowel and colon were taken and washed with saline about 20–30 ml for one mouse. Segments 9–12 mm in length were put in 5 ml of saline, shaken for one minute in a Vortex-type shaker (CSAV 5001/H), and the dilutions were plated on Endo agar.

Plasmid transfer. Twenty ml of Luria broth or liquid MINCA were inoculated with minimum quantities of donor and recipient strains and incubated at 37°C for 24 hr. Sometimes fresh media were inoculated with this mixed culture and incubated further. The optimum ratio of donor to recipient was determined for the actual pairs of strains. The recipient was selected on the basis of specific fermentation or antibiotic resistance character, and 100 or more colonies were tested by slide agglutination for the appearance of K88 or K99 antigens.

Working with *E. coli* strains, sera used for slide agglutination test were prepared against K88$^+$ (UP4460) and K99$^+$ (UP4462) derivatives of a *S. flexneri* 4b strain. These sera were unabsorbed for the *E. coli* strains and quick positive reactions were achieved without O, or K (not pilus) cross reaction.

Table I

Strains

Strains and derivatives; designation and antigenic structure	Important markers	Donor of plasmid	Origin
P99 *E. coli* O141:K85,K88a,c	Ent$^+$Str^R	.	H. W. SMITH
D432 *E. coli* O8:K87,K88a,c:H19	.	.	
D1432 *E. coli* O101:(A$^-$),K99	Str^R	.	I. and F. ØRSKOV
Lsz.14c *E. coli* O4:(K)	Ent$^+$Str^R	.	É. CZIRÓK
O4:(K),K88	—K88$^+$	D432	
B7A *E. coli* O148:K87:H21	Ent$^+$Str^R	.	S. B. FORMAL
O148:(K),K88	— K88$^+$	D432	
O148:(K),K99	— K99$^+$	UP4462	
UP40097 *S. flexneri* 4b (6:—)	IVc$^-$Nia$^-$		
UP4460 *S. flexneri* 4b 6:K88	— K88$^+$	D432	
UP4462 *S. flexneri* 4b 6:K99	— K99$^+$	D1432	

Results

1. *Transfer experiments with plasmids of K88 and K99.* Transfer of plasmid-coded adhesive factors K88 and K99 was unsuccessful into numerous human enterotoxigenic *E. coli* strains, *e. g.* serogroups O75, O78, O18a,c, O83 and others. Transfer of K88 plasmid into *E. coli* strain Lsz. 14c was successful in a single experiment. Testing 500 colonies, the transfer using *E. coli* D432 as donor occurred in 0.2%. All transfer experiments using different donor strains, various ratios between donor and recipient, or other methods, failed to introduce K99. Transfer of K88 into strain B7A (serogroup O148)

could be achieved after numerous attempts but only at a rate of 0.4% by using fluid MINCA medium. Transfer of K99 into this strain succeeded only with a *Shigella* donor (UP4462) strain.

In contrast, our *S. flexneri* 4b strain had a low restriction capacity [6–8], being a good recipient for different plasmids. The derivative of UP4460 ($K88^+$) has been produced in 1968 [7]. Using the K99 donor strain D1432, the transfer succeeded in more than 3%.

After successful transfer experiments, plasmid stability *in vitro* was checked and only a slight (1–3%) spontaneous loss was observed.

2. *Stability of adhesive factors K88 and K99 in adult mice.* In experiments performed with either the $K88^+$ derivative of Lsz. 14c (O4), or the $K88^+$ and $K99^+$ derivatives of B7A (O148), a marked loss of adhesive factors was observed. Representative data summarized in Table II show that as early as the 3rd day after oral inoculation, the ratio of colonies positive for adhesive factors was between 13 and 24%, and the decreasing tendency of adhesive factors continued on the subsequent days.

Table II

Stability of adhesive factors K88 and K99 in orally infected adult mice — analysis of the faeces

Strains	Ratio in per cent of adhesive factor positive colonies on day			
	3	7	11	15
O4:(K),K88	16.1	10.9	9.8	1.2
O148:(K),K88	24.2	11.4	12.1	3.2
O148:(K),K99	13.3	6.6	4.0	1.8

BALB/c mice pretreated with streptomycin. Data obtained with slide agglutination test in 10 mice each (100–150 colonies). In the case of derivatives of strain O148, the serological tests were performed with MINCA medium

In the next series of short experiments performed with the $K88^+$ derivative of the Lsz. 14c (O4) strain, 3 mice each were killed at the intervals indicated, and the $K88^+/K88^-$ ratio was determined in samples taken from the surface of the washed ileum. Parallel with these *in vivo* studies, the culture used for oral infection was incubated further at 37 °C, and the spontaneous loss of plasmid K88 was determined. These data are summarized in Table III. The loss of K88 began two hours after infection and continued so subsequently. In this respect, there was no difference between excreted bacteria and germs isolated from the surface of the ileum, assumed to be adhering to it. On the other hand, the same culture incubated *in vitro* showed only the usual spontaneous loss of 2–6% (Table III).

Table III

Short experiments on the stability of plasmid K88 carried out with adult BALB/c mice, investigating faeces and the surface of washed ileum, oral infection with strain O4 : (K), K88

Hours after oral infection	Percentual ratio of K88+ colonies — from		
	faeces	ileum	*in vitro*
0 (inoculum)	.	.	98
2	84.0	80.2	.
4	71.0	71.7	.
6	63.0	63.6	.
24	56.0	58.5	94
48	26.0	31.0	94
72	15.0	28.0	98
120	9.0	11.0	92

In the case of faeces, 10 animals each; in the case of ileum, 3 mice each were used, and about 100 colonies were tested in each group of animals. *In vitro* experiments were performed with the culture used for oral infection, incubated further at 37 °C and at intervals 100 colonies each were investigated for the presence of antigen K88.

As shown in Fig. 1, there was a logarithmic correlation between time and the ratio of loss.

However, the curing-like loss of plasmid observed in the bowel of mice points to environmental factor(s). Nevertheless the possible role of host receptors cannot be ruled out. Therefore, a comparative experiment was carried out in the inbred mouse strains C3H, DBA, CBA, and BALB/c. The data in Table IV, show that there were significant differences in the K88 plasmid loss among these inbred strains: the value of inhomogeneity in the lst day

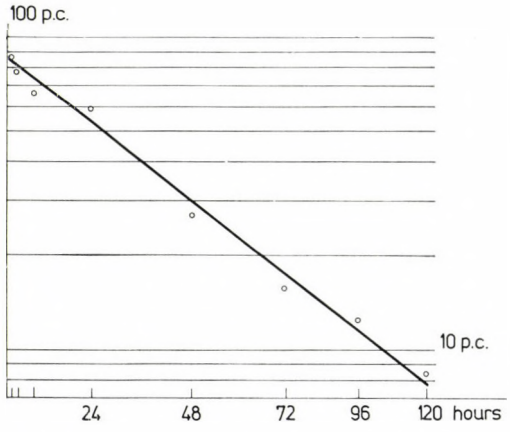


Fig. 1. The dynamics of the loss of the adhesive factor K88

was $\chi^2 = 18.780 - P_{(3)} < 0.001$ and on the 5th day $\chi^2 = 25.996 - P_{(3)} < < 0.001$. The loss and its dynamics were, however, identical in all these inbred mouse strains (Table IV).

Table IV

Stability of adhesive factor K88 in adult mice belonging to different inbred strains, on the basis of excreted bacteria: strain O4 : (K), K88$^+$

Days after oral infection	Percentual ratio of K88$^+$ colonies in mouse strains			
	C3H	DBA	CBA	BALB/c
1	87.5	67.7	54.0	61.0
5	36.0	25.0	12.2	7.7
9	12.5	2.0	7.0	2.0
13	11.0	3.3	1.0	1.2

Groups of 7–10 mice and 70–100 colonies from each group

The phenomenon might be limited to adhesive factors transferred artificially. Similar experiments were therefore performed with naturally occurring K88 and K99 carrying strains. The data summarized in Table V show that strains P99 (O141 : K85, K88a,c) and D1432 (O101 : (A$^-$),K99) lose their adhesive factors in the mouse alimentary tract, too (Table V).

Table V

Stability of adhesive factors K88 and K99 in adult BALB/c mice in the case of naturally occurring K88 and K99 carrying strains

Days after oral infection	Percentual ratio of adhesive factor positive colonies in strains	
	P99; O141:K85,K88	D1432; O101:(A$^-$), K99
1	51.2	52.5
3	41.2	25.0
5	32.5	20.0
9	5.0	3.7

Groups of 10 mice and about 100 colonies from each group

3. *Stability of the adhesive factors K88 and K99 in suckling mice.* In the first series of experiments 3-day-old suckling mice were infected orally with strains O4 : (K),K88, O148 : (K),K88 and O148 : (K),K99. Taking into consideration the technical difficulties of oral streptomycin pretreatment, and the disturbances of the litter, in these experiments no streptomycin was given and no germ-free environment was ensured, so that the suckling mice

carried *E. coli* strains. After oral infection with the above strains an increase in the number of colonies positive for adhesive factor was observed. The ratio of colonies with adhesive factor was determined on the basis of all *E. coli* colonies isolated from the bowel. The process of enrichment was especially remarkable with strain O148 : (K), K99, where the $K99^+$ ratio was only 40% in the inoculum (Table VI). These data suggest not only a lack of the curing-like effect on these plasmids, but also selective advantage for the adhesive factors in suckling mice. In this respect, there was no difference between the K88 and K99 factors (Table VI).

Table VI

Stability of adhesive factors K88 and K99 in 3-day-old BALB/c suckling mice after oral infection

Hours after oral infection	Percentual ratio of adhesive factor positive colonies in mice infected with		
	O4:(K),K88	O148:(K),K88	O148 (K),K99
0 (inoculum)	93	83	40
24	79	78.5	58
48	93	79.8	76.5
72	94	86.7	88.0
120	98	84.2	98

The animals were not pretreated with streptomycin and no germ free environment was secured. At intervals cultures were made from the small bowel and colon of 3 mice each. The percentual values were based on 70–150 colonies of *E. coli* tested by slide agglutination. In the case of derivatives of strain O148 the tested colonies were isolated on MINCA medium

The marked differences between suckling and adult mice concerning the fate of the adhesive factors raise the question at what age this switch occurs. Therefore, 10 and 14 (the average term before solid food), and 21 (the average term of weaning) day-old mice were infected with strain O4 : (K), K88. The 10-day-old litter was infected without streptomycin pretreatment, and the 14- and 21-day-old litters were pretreated like adults. According to the data summarized in Table VII, the 10-day-old animal showed the same pattern of $K88^+$ enrichment as the younger suckling mice did. The adult-like loss of K88 plasmid and the missing selective advantage appeared on the 14th day of life.

4. *In vitro stability of K88 and K99 factors under different environmental conditions.* As mentioned earlier, the spontaneous loss of plasmids K88 and K99 was usually characterized by a low ration, even during serial transfer or prolonged incubation. HIROTA [9] noted a strict correlation between higher pH and curing effect of acridine dyes on plasmids. Because of the alkalinity

Table VII

Stability of plasmid K88 in the alimentary tract of 10, 14 and 21-day-old mice infected orally with strain O4 : (K), K88

Hours after oral infection	Percentual ratio of $K88^+$ colonies in mice aged		
	10 days	14 days	21 days
0 (inoculum)	99	98	87
24	0.8 (21)	72.7	58.7
48	13.7 (70)	41.1	47.5
72	26.3 (95)	21.0	33.7
96	32	18.3	30.0
120	58	6.6	16.2

* The 10-day-old mouse litter was not pretreated with streptomycin, the $K88^+$ ratio means the ratio of all investigated *E. coli* colonies. In some cases the $K88^+$ /04:$K88^-$ ratio was also determined (values in parentheses). Litters of 14 and 21 day were pretreated with streptomycin.

of the small intestine, we investigated the plasmid stability in peptone water media of pH 7.2–7.6–8.0–8.5 with and without low redox potential values produced by 0.05% sodium thioglycolate. An enhanced loss of the plasmids could never be observed.

Discussion

The mouse model envisaged for studying human enterotoxigenic *E. coli* strains by the aid of transferring the adhesive factor K88 or K99, has not fulfilled expectations. K88 plasmid was successfully transferred into two selected human enterotoxigenic (serogroups O4 and O148) *E. coli* strains, and the adhesive factor K99 into the latter one. The low effectivity of transfer in these successful cases and numerous unsuccessful transfer experiments with other strains was remarkable. Though the background of the failure has not been studied, factors like a host specific restriction, entry exclusion by a resident plasmid, or other factors may have played a role. The data concerning the limited number of serogroups of animal and human pathogenic *E. coli* strains [10–12] were in agreement with our experience, in contrast to the principle of plasmid promiscuity.

In the alimentary tract of adult mice the rapid, curing-like loss of factors K88 and K99 of the infecting agents was clearly demonstrable. That the loss was due neither to a selection in the bowel of adhesive factor positive clones nor to an excretion of the negative ones, was proved by examination of bac-

teria originating from the surface of the washed ileum (Tables II and III). The process of loss began as early as 2 hr after the oral infection (Table III). The loss of plasmids correlated with time logarithmically (Fig. 1), which meant a constant effect on growing bacteria.

SELLWOOD *et al.* [13] studied the adhesive properties (receptors) of the jejunal brush border to factor K88 in piglets and found that brush borders had positive and negative adhesive characters inherited by Mendelian law. This observation led us to investigate the role of inherited factor(s) in the observed phenomenon. According to the data obtained with different inbred mouse strains (Table IV) they displayed significant differences in the percentual loss of plasmid K88, but the dynamics were identical.

The next experiment showed that it was not the *in vitro* transfer which was responsible for the plasmid loss; the same process takes place with naturally occurring strains carrying K88 and K99 (Table V).

The logical step was to investigate next the phenomenon in suckling mice. In these animals an opposite pattern was found: the ratio of colonies positive for adhesive factor gradually increased and 3–5 days after infection it reached nearly 100%, even when the inoculum contained only 40% $K99^+$. These suckling mice were not pretreated with streptomycin so they had not even a stabilized *E. coli* flora: they were excreting a great number of *E. coli* germs, with the recruiting possibility from their mother and environment. Adhesive factor carrying *E. coli* strains are shown to have a selective advantage when administered orally. In this respect no difference between the K88 and K99 factors was observed. The experimental results of DAVIDSON and HIRSH [14] already showed the adhesive property of K88 in suckling mice, it protected them againts infection with Ent^+ $K88^+$ bacteria. The receptor blocking effect of K99 was not investigated in that study.

The timing of the switch from baby to adult pattern in connection with the K88 adhesive factor was investigated in mice 10, 14 and 21 days of age. In the 10-day-old animal, the suckling mice pattern was observed. In this litter not only the proportion of the $K88^+$ colonies was determined at intervals but it was also compared to the proportion of the infecting O4 colonies. The latter showed rapid enrichment (Table VII). At the age 14 days, the usual time to start eating solid food, appears the adult pattern, with the curing effect of K88 plasmid.

Enterotoxigenic *E. coli* strains are usually studied in newborn animals. According to MOON and WHIPP [15] there are pig pathogenic *E. coli* strains which can only be tested in newborns, while others in 3-week-old ones. Calves according to SMITH and HALLS [1] are sensitive against infection only on the first day of life; on the 3rd day they are already resistant. Such observations are based on clinical symptoms, in which both effective colonization and enterotoxin sensitivity play a role. According to MOON and WHIPP's suggestion

[16], newborn animals are most susceptible to the particular *E. coli* strain most pathogenic to their own species. The suitability of the model is partly connected with the colonization.

The specificity of adhesive factor K88 for pigs or piglets and that of K99 for ruminants seems to be proved experimentally [3]. The ruminants does not seem to mean that without adhesive factor an *E. coli* strain cannot be pathogenic to newborn animals [17, 18], but the colonization of bacteria in the upper part of the small bowel seems to be connected solely with the adhesive factor [18, 19].

It is only natural that experiments concerning the function of adhesive factors were carried out on natural hosts and there are only few data in the literature about mouse experiments. SMITH and LINGGOOD [2] performed mouse experiments with K88$^+$ *Salmonella typhimurium* and *S. cholerae-suis* strains. These derivatives had no higher virulence than their K$^-$ forms. Their period of survival was even shorter in mouse tissues and in the alimentary tract. Without detailed data the occasional loss of K88 cannot be judged, but according to the results obtained by infecting piglets, an occasional loss of factor K88 was observed in isolates from organs.

The curing-like loss of plasmids K88 and K99 in the alimentary tract of adult mice and the absence of that phenomenon is suckling mice till to the age of 14 days point to the effect of physiological factor(s) present in the alimentary tract. The alkaline environment with a low redox potential is not, or nor alone, responsible. This fact calls for a study of other factors and other plasmid elements. The overall uniformity of a curing effect and its absence in suckling mice fed secretory antibodies speaks against an immunological effect. Furthermore, our data concern clearly the loss of plasmids and not the lack of adhesiveness. Similarly, the occasional curing effect of streptomycin can be excluded. In some experiments carried out with mice without streptomycin pretreatment the plasmid loss was enhanced (i) and, as streptomycin had been applied only before infection, its direct effect may appear only at the very beginning of the experiment (ii). There are no data about a curing effect of streptomycin, which is used extensively for selection or contraselection (iii). Taking the observed selective advantage of both the K88 and the K99 adhesive factors into consideration, further experiments are needed in connection with brush border receptors.

Acknowledgements. We are indebted to Drs I. and F. ØRSKOV (Copenhagen, Denmark), to Dr. S. B. FORMAL (Washington, D. C., U.S.A.), to Dr. H. W. SMITH (Houghton, Huntingdon, England) and to Dr. É. CZIRÓK (Budapest, Hungary) for kindly providing strains.

REFERENCES

1. Smith, H. W., Halls, S.: J. Path. Bact. **93,** 499 (1967).
2. Smith, H. W., Linggood, M. A.: J. med. Microbiol. **4,** 467 (1971).
3. Smith, H. W., Linggood, M. A.: J. Med. Microbiol. **5,** 243 (1972).
4. Ørskov, I., Ørskov, F., Smith, H. W., Sojka, W. J.: Acta path. microbiol. scand. **B83,** 31 (1975).
5. Guinée, P. A. M., Jansen, W. H., Agterberg, C. M.: Infect. Immun. **13,** 1369 (1976).
6. Kétyi, I.: Acta path. microbiol. scand. **75,** 104 (1969).
7. Kétyi, I., Ørskov, I.: Acta path. microbiol. scand. **77,** 299 (1969).
8. Kétyi, I., Ørskov, F.: Acta path. microbiol. scand. **B78,** 51 (1970).
9. Hirota, Y.: Proc. nat. Acad. Sci. (Wash.) **46,** 57 (1960).
10. Sojka, W. J., Lloyd, M. K., Sweeney, E. J.: Res. vet. Sci. **1,** 17 (1960).
11. Myers, L. L., Guinée, P. A. M.: Infect. Immun. **13,** 1117 (1976).
12. Ørskov, F., Ørskov, I., Evans, D. J. Jr., Sack, R. B., Sack, D. A., Wadström, T.: Med. Microbiol. Immunol. **162,** 73 (1976).
13. Sellwood, R., Gibbons, R. A., Jones, G. W., Rutter, J. M.: J. med. Microbiol. **8,** 405 (1975).
14. Davidson, J. N., Hirsh, D. C.: Infect. Immun. **12,** 134 (1975).
15. Moon, W. H., Whipp, S. C.: J. infect. Dis. **122,** 220 (1970).
16. Moon, W. H., Whipp, S. C.: Ann. N. Y. Acad. Sci. **176,** 197 (1970).
17. Jones, G. W., Rutter, J. M.: Infect. Immun. **6,** 918 (1972).
18. Hohmann, A., Wilson, M. R.: Infect. Immun. **12,** 866 (1975).
19. Nagy, B., Moon, H. W., Isaacson, R. E.: Infect. Immun. **13,** 1214 (1976).

Address of the authors:

Iván Kétyi, Béla Kuch, Adele VerténYi
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

NOTE ADDED IN PROOF

A plasmid curing-like effect was observed in adult mice for plasmids R100-1 (strain D368) and ColIb (strain Cl 232), but not for F-lac (strain W3287). The latter showed no spontaneous loss *in vitro*.

INDEX

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1977. IX. 17. — Terjedelem: 7,7 (A/5) ív, 34 ábra

78.4980 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:

Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P. O. B. 64, Ungarn

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $ 36.00.

Bestellbar bei dem Außenhandels-Unternehmen »*Kultura*« (H-1389 Budapest 62, P.O.B. 149, Bankkonto Nr. 218-10990) oder bei seinen Auslandsvertretungen und Kommissionären.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiène Publique, H-1966 Budapest, P. O. B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement est de $ 36.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur «*Kultura*» (H-1389 Budapest 62, P. O. B. 149, Compte courant No. 218-10990) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P. O. B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — $ 36.00 за том.

Заказы принимает предприятие по внешней торговле «*Kultura*» (H-1389 Budapest 62, P. O. B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

AUSTRALIA
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE, Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 135 Ackland Street, *St. Kilda (Melbourne), Aictoria 3182*

AUSTRIA
GLOBUS, Höchstädtplatz 4, *1200 Wien XX*

BELGIUM
OFFICE INTERNATIONAL DE LIBRAIRIE, 40 Avenue Marnix, *1050 Bruxelles*
LIBRAIRE DU MONDE ENTIER, 162 Rue du Midi, *1000 Bruxelles*

BULGARIA
HEMUS, Bulvar Ruszki 6, *Sofia*

CANADA
PANNONIA BOOKS, P.O. Box 1017, Postal Station "B", *Toronto, Ontario M5T 2T8*

CHINA
CNPICOR, Periodical Department, P.O. Box 50, *Peking*

CZECHOSLOVAKIA
MAD'ARSKÁ KULTURA, Národní třida 22, *115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 36, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

DENMARK
EJNAR MUNKSGAARD, Norregade 6, *1135 Copenhagen*

FINLAND
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128, *SF-00101 Helsinki 10*

FRANCE
EUROPERIODIQUES S. A., 41 Avenue de Versailles, *78170 La Celle St. Cloud*
LIBRAIRIE LAVOISIER, 11 rue Lavoisier, *75008 Paris*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac, *75240 Paris Cedex 05*

GERMAN DEMOCRATIC REPUBLIC
HAUS DER UNGARISCHEN KULTUR, Karl Liebknecht-Strasse 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT Strasse der Pariser Kommüne 4—3, *DDR 104 Berlin*

GERMAN FEDERAL REPUBLIC
KUNST UND WISSEN ERICH BIEBER Postfach 46, *7000 Stuttgart 1*

GREAT BRITAIN
BLACKWELL'S PERIODICALS DIVISION, Hythe Bridge Stteet, *Oxford OX1 2ET*
BUMPUS, HALDANE AND MAXWELL LTD., Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate, *Wellingborough, Northants NN8 2QT*
WM. DAWSON AND SONS LTD., Cannon House, *Folkestone, Kent CT19 5EE*
H. K. LEWIS AND CO., 146 Gower Street, *London WC1E 6BS*

GREECE
KOSTARAKIS BROTHERS, International Booksellers, 2 Hippokratous Street, *Athens-143*

HOLLAND
MEULENHOFF-BRUNA B.V., Beulingstraat 2, *Amsterdam*
MARTINUS NIJHOFF B.V., Lange Voorhout 9—11, *Den Haag*
SWETS SUBSCRIPTION SERVICE, 347b Heereweg, *Lisse*

INDIA
ALLIED PUBLISHING PRIVATE LTD., 13/14 Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD., Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTS., Books Import Division, Chandralok, 46 Janpath, *New Delhi 110001*

ITALY
EUGENIO CARLUCCI, P.O. Box 252, *70100 Bari*
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 35, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58, *20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

JAPAN
KINOKUNIYA BOOK-STORE CO. LTD., 17-7 Shinjuku-ku 3 chome, Shinjuku-ku, *Tokyo 130-91*
MARUZEN COMPANY LTD., Book Department, P.O. Box 5056 Tokyo International, *Tokyo 100-31*
NAUKA LTD., IMPORT DEPARTMENT, 2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

KOREA
CHULPANMUL, *Phenjan*

NORWAY
TANUM-CAMMERMEYER, Karl Johansgatan 31—34, *1000 Oslo*

POLAND
WĘGIERSKI INSTYTUT KULTURY, Marszałkowska 80, *Warszawa*
CKP I W ul. Towarowa 28 *00-958 Warsaw*

ROUMANIA
D E. P., *Bucureşti*
ROMLIBRI, Str. Biserica Amzei 7, *Bucureşti*

SOVIET UNION
SOJUZPETCHATJ — IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

SPAIN
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

SWEDEN
ALMQVIST AND WIKSELL, Gamla Brogatan 26, *101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB, Box 436, *401 25 Göteborg 1*

SWITZERLAND
KARGER LIBRI AG, Petersgraben 41, *4011 Basel*

USA
EBSCO SUBSCRIPTION SERVICES, P.O. Box 1934, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC., 15 Southwest Park, *Westwood, Mass, 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC., 140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC., 7250 Westfield Avenue, *Pennsauken* N.J. 08110

VIETNAM
XUNHASABA, 32, Hai Ba Trung, *Hanoi*

YUGOSLAVIA
JUGOSLAVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišiča 1, *21000 Novi Sad*

18. II. 1978 HU ISSN 0001—6187 **Index: 26.016**

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS XXV FASCICULUS 2

AKADÉMIAI KIADÓ, BUDAPEST

1978

AMAHA 5 25(2) 87–157 1978

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1054 BUDAPEST, ALKOTMÁNY UTCA 21.

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24, Bankszámla: 215-11448), a külföld számára pedig a „Kultura" Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, Bankszámla: 218-10990) vagy annak külföldi képviseleteinél.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P.O.B. 64, Hungary

Correspondence with the editors should be sent to the same address.

Subscription: $36.00 a volume.

Orders may be placed with "Kultura" Foreign Trading Company (H-1389 Budapest 62, P.O.B. 149, Account No. 218-10990) or its representatives abroad.

Acta microbiol. Acad. Sci. hung. 25, 87—96, 1978

NEW DATA ON THE ACTIVITY OF CP-20.961 (PFIZER), A NON-TOXIC INTERFERON INDUCER

E. T. GLÁZ and MARGARITA TÁLAS

Department of Pharmacology, Semmelweis University Medical School, Budapest and Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

(Received July 8, 1977)

Although CP-20.961 is a non-toxic interferon inducer, the majority of the parenterally injected drug stays locally long-lasting and induces local tissue reactions, when applied in the form of a suspension. The size of the drug particles in the suspension was found to be an important factor in the effect. It was not possible to find any other drug formulation devoid of undissolved drug particles that would be more effective than the suspension. The drug is not absorbed from the gastrointestinal tract either in the form of suspension or in the form of other preparations. The mode of action of CP-20.961 seems to be similar to other small molecular interferon inducers as regards the prolonged antiviral action *in vivo*, the prevention of activity by cycloheximide pretreatment, and the hyporeactivity occurring on repeated administration. In addition to the acid lability, a rapid deterioration of mouse serum interferon induced by CP-20.961 was also observed at 56 °C. Attempts to find the possible interferon producing cell population were unsuccessful.

Several small molecular interferon inducers have been described in recent years; most of them, however, have a very low or moderately low therapeutic index [1–4]. A notable exception is CP-20.961 — N,N-dioctadecyl-N',N'-bis (2-hydroxyethyl)propanediamine — showing a therapeutic index of at least 100 [5]. Furthermore, this was the only small molecular inducer that stimulated topical interferon production and potentiated viral interferon induction in man upon intranasal application [6–9]. These properties deserved interest for further investigation. The aim of the present study was twofold. First, to find, if possible, a more suitable drug formulation. CP-20.961 has been applied as a microdisperse suspension prepared with polysorbate 80 and glycerol. Such a preparation may be hindered in absorption as compared for instance to a true solution, and is, in fact, not absorbed from the gastrointestinal tract [5]. Also, the polysorbate 80 content may reduce activity since this agent has been shown to inhibit interferon action *in vitro* [10] and to increase the yields of poliovirus [11]. The second aim was to study some unclarified details of the mode of action of CP-20.961, in order to find eventual similarities or dissimilarities to other small molecular interferon inducers.

Materials and methods

Animals. Outbred female CFLP mice weighing either 11–16 or 15–20 g (breeding farm "LATI", Gödöllő) were mostly used, and, in a few experiments, also 4-week-old inbred Balb/c mice from the same farm, for the vaccinia virus tail lesion (antiviral) test. Hyporeactivity of interferon production was studied in female CFLP mice weighing 23–30 g. X-ray irradiation

and serum interferon stability examinations were done in inbred male Balb/c mice weighing 18–20 g.

Vaccinia virus mouse tail lesion test. The method of BOYLE, HAFF and STEWART was used with slight modifications described previously [3]. The groups consisted of 9–10 CFLP or 6–8 Balb/c mice. The infectious inoculum was $1–3 \times 10^5$ p.f.u. for the smaller and $4–8 \times 10^5$ p.f.u. for the bigger mice to attain 10–20 lesions per animal in control groups as far as possible. Since variations occurred in the results of the experiments because of the variable reactions of mice, experiments were repeated several times and their combined data were used for statistical analysis (Student's *t* test). The mice were infected 17–18 hr after injection of CP-20.961, when the highest circulating interferon level was found [5].

Interferon assay. Serum for interferon assay was obtained either from the retro-orbital venous plexus or from the tail of 5–6 mice per group and pooled. Interferon titrations were done by the cytopathic effect reduction method in mouse L cells, as described previously [12, 13], but instead of SFV the EMC virus was used for challenge and results were read 48 hr later.

X-ray irradiation. Groups of 6 inbred male Balb/c mice weighing 18–20 g were exposed to 400 R total body X-ray irradiation at a dose rate of 44.4 R/min. Other conditions of the irradiation were as described previously [12].

Drugs. CP-20.961 was kindly donated by Dr. W. W. HOFFMAN, Pfizer Central Research, Groton, Conn., U.S.A., in the form of either originally packaged formulation of the microdisperse suspension or in the form of bulk drug. The former was kept in the refrigerator and used for a period of about one and a half year. Suspension from the bulk drug was self-prepared similarly to that described for the original formulation [5]: equal weights of CP-20.961 material, polysorbate 80 (Tween 80) and glycerol were fused in a water bath at 80–90°C, then hot saline or hot distilled water was added during constant agitation. Finally, 5–20 ml of the milky suspension were sonicated with an MSE 7100 type (cabinet model) 100 W ultrasonic integrator, using a titanium probe type 25926 and tubes 23 mm in diameter, and applying 16 μm amplitude for 2–4 min. Distilled water suspensions were then made isotonic by the addition of NaCl crystals.

Cycloheximide was a product of Serva Co., Heidelberg (Actidion pure). Zymosan (Mannozym injections) was produced by Institute for Serobacteriological Production and Research "Human", Budapest.

Results

Application of CP-20.961 suspension by different routes. The route of application in mice described earlier [5] was intraperitoneal and intranasal. For an eventual parenteral application in man it seemed necessary to study the possible effect of the drug given by other routes. This was studied by the vaccinia virus mouse tail lesion test. Control groups received either saline or saline containing polysorbate 80 + glycerol, since in preliminary experiments there was no difference in the "effect" of these two solutions. It can be seen in Table I that CP-20.961 was equally highly active whether given intraperitoneally, intramuscularly or subcutaneously. On intravenous administration, however, its activity was not so explicit: in one of the four experiments treated mice had more lesions than the control animals and after combining the data of the four experiments the effectiveness was only just significant.

On subcutaneous administration the smallest active dose was 20 mg/kg of the original drug formulation. Ten mg/kg was not, or in other experiments (Table VI), only marginally active. However, 10 mg/kg of the self-prepared suspension showed highly significant effectiveness, a rather unexpected result. Microscopical examination of the suspended particles showed these in the original formulation mostly as rods a few micrometres in length and less than

Table I

Activity of the polysorbate 80-glycerol suspension of CP-20.961 administ red by different routes in the vaccinia virus mouse tail lesion test

Drug preparations*	Dose mg/kg**	Route of administration	Number ot experiments	Number of uniform experiments***	Avarage number of skin lesions	P
Control		s.c.	6		16.2	
Original suspension	50	s.c.	6	6	4.7	< 0.001
Control		s.c.	5		11.2	
Original suspension	20	s.c.	5	4	3.5	< 0.01
Control		s.c.	4		10.2	
Original suspension	10	s.c.	4	3	9.7	> 0.70
Control		s.c.	3		16.4	
Self-prepared suspension	10	s.c.	3	3	4.1	< 0.001
Control		i.p.	3		14.2	
Original suspension	50	i.p.	3	3	3.0	< 0.001
Control		i.m.	3		15.7	
Original suspension	50	i.m.	3	3	2.9	< 0.001
Control		i.v.	4		10.7	
Original suspension	50	i.v.	4	3	6.8	< 0.05

* See Materials and methods; control mice were treated with either saline or polysorbate 80–glycerol saline

** Single dose given i.v. 21 hr, and by the other routes 17–18 hr, before infection

*** Number of individual experiments showing the same tendency of activity as the pooled data of all experiments

1 μm in width. On the other hand, the round or irregular shaped granules of the self-prepared suspension had a size mostly below 1 μm with a small proportion of 1–10 μm particles. The difference in particle size of the two types of suspensions suggested that the smaller the particles the more active the suspension.

Following subcutaneous administration, a slight induration was palpable at the site of the injection. Several mice were killed 1–17 days after the injection and a bulk of the white material could be seen *in situ*. The same was found after intramuscular injections. A number of histological examinations were also done from the subcutaneous injection site. A tissue reaction consisting of a moderate mononuclear infiltration and demarcation by partially calcified fibrous tissue could be seen. Significant amounts of the material were found in or around the widened lymph capillaries showing the probable way of slow absorption.

Trial of other drug formulations. Considering the local irritation and the persistence of the greater part of the suspended material *in situ* following its

injection, and considering the eventual disadvantage caused by the polysorbate 80 present in the suspension, efforts were made to find a more suitable drug preparation. Although CP-20.961 is soluble in several solvents, it is precipitated on dilution with water. A single solvent was found to retain the drug in true solution even after dilution with water: tetrahydrofuran (THF). A solution of SP-20.961 in 5–10% THF was therefore given either orally or subcutaneously to mice. According to the data of Table II no effect was obtained. The substance had precipitated upon contact with the tissues since at autopsy white material was found at the subcutaneous injection site. Thereafter a solution of the drug in sunflower oil was administered (Table II). Neither oral nor subcutaneous administration had any effect, while intramuscular administration seemed satisfactory. The effect by this route was highly significant computing the pooled data of 6 experiments, but in 2 individual experiments no difference was observed between control and treated group. At autopsy partly the oil solution and partly some precipitated drug material was seen among the muscles. Finally the trial of an oil in water emulsion is shown in Table II. Lipid particles or drops smaller than 1.5 μm are considered to be

Table II

Activity of different drug formulations of CP-20.961 in the vaccinia virus mouse tail lesion test

Drug formulation	Single dose, mg/kg	Hours before infection	Route of administration	Number of experiments	Number of uniform experiments*	Average number of skin lesions	P
Control[1]		42–50	i.m.	6		15.5	
Solution in oil[1]	100	42–50	i.m.	6	4	8.7	< 0.001
Control[1]		48	s.c.	2		15.8	
Solution in oil[1]	100	46	s.c.	2	2	10.4	N.S.**
Control[1]		42	p.o.	2		14.5	
Solution in oil[1]	400	41–45	p.o.	2	2	17.9	N.S.
Control[2]		18	p.o.	3		22.5	
Oil in water emulsion[2]	100	18	p.o.	3	3	19.4	N.S.
Control[3]		17	s.c.	2		12.3	
Solution in THF[4]	50–100	18–23	s.c.	2	2	15.3	N.S.
Control[5]			p.o.	2		14.5	
Solution in THF[4]	100	23	p.o.	2	2	13.8	N.S.

* See Legend to Table I
** Not significant
[1] Sunflower oil
[2] Sunflower oil – tragacanth powder – polysorbate 20 emulsion
[3] Saline
[4] 5–10% tetrahydrofuran
[5] Not treated

absorbed *in toto* by the intestinal villi. An emulsion from 1 ml of the sunflower oil solution of the drug was therefore prepared by the aid of 0.25–0.75 g tragacanth powder, 0.2 ml polysorbate 20, and 2.8 ml water. The oil drops obtained were between 1–3 μm in diameter as measured microscopically, a considerable part of them being smaller than 1.5 μm. Given orally, this preparation, too, was ineffective.

Duration of the antiviral action of CP-20.961. The next experiments are related to the mode of action of the drug. The original polysorbate 80 – glycerol suspension was used in these investigations mostly.

In the vaccinia virus mouse tail lesion test, several interferon inducers had a prolonged activity [13, 14]. This could be reproduced with CP-20.961. As it can be seen in Table III, a single dose of 50 mg/kg given subcutaneously

Table III

*Duration of activity of the polysorbate 80 – glycerol suspension in the vaccinia virus mouse tail lesion test**

Treatment**	Days before infection	Average number of skin lesions	P
Control	6	8.2	
Drug	8	5.3	> 0.10
Drug	7	4.0	$\leqslant 0.05$
Drug	6	3.2	$\leqslant 0.01$

* Single dose of 50 mg/kg given s.c.; 3 parallel exp.-s
** Original drug formulation; control as indicated in Legend to Table I

6 days before the infection was effective. Treatment applied 7 days before the infection showed marginally significant activity, one applied 8 days before was ineffective.

Effect of cycloheximide on the antiviral action of CP-20.961. Cycloheximide, a so called protein synthesis inhibitor, was given intraperitoneally 1 hr before subcutaneous administration of the drug suspension. Results are shown in Table IV. Cycloheximide pretreatment abolished the antiviral action of CP-20.961. In previous investigations [3], cycloheximide *per se* in the dose applied (60 mg/kg) did not influence the number of skin lesions.

Hyporeactivity to repeated doses. This was studied in the relation of serum interferon production. CP-20.961 was administered subcutaneously on two occasions at different time intervals and 17–18 hr after the second dose blood was collected for serum interferon titration. As shown in Table V, a total or partial hyporesponsiveness occurred when the interval between the two doses

Table IV

Effect of cycloheximide pretreatment on the activity of CP-20.961 in the vaccinia virus mouse tail lesion test

Treatment	Number of experiments	Number of uniform experiments*	Average number of skin lesions	P to control
Control*	4		15.8	
CP-20.961**	4	4	5.7	< 0.001
CP-20.961** + cycloheximide***	4	3	11.8	> 0.10

* See Legend to Table I
** Original drug formulation, single dose of 50 mg/kg given s.c. 18 hr before infection
*** Single dose of 60 mg/kg given i.p. 1 hr before CP-20.961

was 1–3 days. Five or 7 days after the first dose, a second dose of 50 mg/kg induced the same interferon level as a single dose in the control.

Attempts to find the interferon producing cell population. It has been suggested that CP-20.961 may induce interferon production in macrophages, although peritoneal macrophages (as well as peripheral lymphocytes) did not produce interferon or develop resistance to virus in response to the drug *in vitro* [5]. Therefore the *in vivo* reaction of two cell systems was studied. The first was the system of fixed macrophages which was stimulated by intravenous administration of zymosan to mice 4 days before the injection of CP-20.961. This pretreatment did not enhance the antiviral response to the drug (Table VI),

Table V

*Hyporeactivity after repeated doses of CP-20.961**

Exp.	First dose (mg/kg)	Second dose (mg/kg)	Days between doses**	Serum interferon titre***
1	30	–		64
	30	30	2	4
	100	–		64
	100	100	2	4
2	50	–		20
	50	50	1	< 5
	50	50	3	10
	50	50	5	20
	50	50	7	20

* Polysorbate 80–glycerol suspension given s.c.
** Blood collection 17–18 hr after the second dose
*** In 0.5 ml serum; reciprocal of serum dilution

Table VI

Effect of zymosan and X-ray pretreatment on antiviral activity and on interferon stimulation, respectively

Pretreatment	Treatment	Number of experiments	Average number of skin lesions	P to control	Serum interferon titre*
Nil	Saline[1]	3	11.9		
Nil	CP-20.961[2]	3	7.5	$\leqslant 0.05$	.
Zymosan[3]	CP-20.961[2]	3	7.4	$\leqslant 0.05$	.
Nil	CP-20.961[5]	2	.		20
X-ray[4]	CP-20.961[5]	2	.		20

* See Legend to Table V
[1] Given s.c., 17–18 hr before infection
[2] Original polysorbate 80–glycerol suspension, 10 mg/kg s.c., 17–18 hr before infection
[3] 5 mg/kg i.v., 4 days before inducer
[4] 400 R, 3 days before inducer
[5] Self-prepared polysorbate 80 – glycerol suspension, 50 mg/kg s.c. 18 hr before bleeding
. Not done

indicating that the cells affected by zymosan may not be responsible for interferon induction by CP-20.961. As the second, the role of lymphocytes was studied. Mice were irradiated with X-rays and after 3 days (when their periph eral white blood cell count and spleen weight had reached about 20% of the initial value [15]), they were injected with CP-20.961. Table VI shows that even in this case interferon induction was not altered.

Heat stability of mouse serum interferon. Mouse plasma interferon induced by CP-20.961 was found to be partially stable to acid [5]. This acid lability could be reproduced in the present experiments (Table VII). The hitherto unreported heat stability was then studied. Mouse serum of 1 : 20 interferon titre was incubated at 56 °C for 30 and 60 min. As shown in Table VII, interferon activity already deteriorated after 30 min incubation.

Table VII

*Acid and heat stability of CP-20.961 induced serum interferon**

Condition	Interferon units**	
	before incubation	after incubation
pH 2 at 4 °C for 48 hr	20	< 5
56°C for 30 min	20	< 5

* Serum obtained 18 hr after 50 mg/kg of the drug given s.c. in the form of polysorbate 80 – glycerol suspension
** See Legend to Table V

Discussion

Although the polysorbate 80 – glycerol suspension of CP-20.961 is equally active by the intraperitoneal, intramuscular or the subcutaneous routes in mice, this preparation does not seem to be suitable for parenteral application considering the local tissue reaction and local persistence of the drug. The weaker effect on intravenous administration is not clear. It may be speculated that on intravenous administration drug particles are ingested mostly in RES cells which differ from the cells of local infiltrations and regional lymph nodes that may participate in interferon production after parenteral administration by other routes. Antiviral activity in EMC infected mice by the subcutaneous, intramuscular and, to a lesser extent, the intravenous route, was observed recently [16].

The small particles in the drug suspension seemed to be an important factor in effectiveness since freshly prepared suspensions were more active than the original one. Especially the suspension prepared with distilled water (see Materials and methods) showed small particle size and, correspondingly, high activity. It is supposed that the original suspension had lost potency, perhaps by aggregation during shipping [16]. Unfortunately, even the self-prepared suspension caused subcutaneous induration in mice and, at autopsy, infiltration of the subcutaneous tissues with a white material was observed.

The drug-free vehicle (placebo) applied intranasally to human volunteers induced interferon in the nasal washings in the experiments of STANLEY *et al.* [9]. (The ingredients of the packaged microdisperse suspension besides the drug are: polysorbate 80, glycerol, small amounts of phenyl ethyl alcohol, sodium metabisulphite and buffer salts). Interferon induction by placebo in similar experiments was not found by DOUGLAS and BETTS [7]. In the present experiments saline containing polysorbate 80 – glycerol showed no antiviral activity.

Attempts to find a suitable preparation devoid of undissolved particles and of polysorbate 80 were unsuccessful. The only active preparation in these trials was the solution of the drug in oil (given intramuscularly). However, the effect of this preparation was not always reproducible, and did not exceed that of the microdisperse suspension; furthermore, even with this formulation, precipitation of the drug material was observed in the muscles.

The mode of action of CP-20.961 seems to be similar to other small molecular interferon inducers, considering the protracted antiviral action, the prevention of activity by cycloheximide pretreatment (indicating probably *de novo* interferon synthesis), and the hyporeactivity. This last property was rather favourable with this inducer, since a second dose 5 days after the first one was fully active. Hyporesponsiveness to a smaller dose (25 mg/kg) given intraperitoneally lasted for an even shorter period, about 2 days [16].

Our attempts at finding the interferon producer cell population after injection of CP-20.961 were far from complete. Only two cell systems were examined. Stimulation of the RES was done by intravenous injection of zymosan according to NAGANO and MAEHARA [17], who were able to enhance the endotoxin induced serum interferon level. Since this could not be achieved with CP-20.961, RES cells probably do not play an important role in interferon production by this drug. A similar conclusion has been drawn earlier in this Discussion. It must, however, be considered, that the Hungarian zymosan preparation used (Mannozym) differs to some extent from other preparations of zymosan: it contains a greater proportion of mannan in consequence of the application of a yeast-extraction method somewhat different from the original Pillemer's method [18]. The effect of such a difference is unknown. The second cell system studied was the lymphoid system which is considered to be damaged primarily by whole body X-ray irradiation [19, 20]. No reduction in interferon production on subsequent CP-20.961 injection was found. Interferon induction by tilorone, another small molecular inducer, was substantially decreased by 650 R of X-rays in mice [20, 21] and, correspondingly, tilorone was found to induce interferon in human lymphoid cells *in vitro* [22]. The lack of interferon induction by CP-20.961 in peritoneal macrophages and peripheral lymphocytes [5], together with our failure to identify the producer cell system with either the RES or the lymphoid tissue, may indicate that other tissues are stimulated for interferon production by CP-20.961. Or else, a subtle collaboration between macrophages and lymphoid cells in necessary for interferon production similarly to the case of other inducers, for instance LPS [23], the mitogens [24, 25], and BL-20803, a small molecular inducer agent [26]. No further elucidation of the cell system stimulated by CP-20.961. could be attained by the results of HOFFMAN [16], showing a slight depression of interferon production by the drug following splenectomy and no alteration following steroid pretreatment in mice. The possible role of T lymphocytes in interferon production, instead of that of the whole lymphoid system, is now under study.

The mouse serum interferon was found to be acid labile. Based on this phenomenon, the antiviral substance induced by CP-20.961 can be classified as type II or "immune" [27–29] interferon. In addition to the acid lability, the rapid deterioration of CP-20.961 induced interferon at 56°C has also been observed. This is, however, not an absolutely characteristic property of type II interferons: several of them showed heat lability [24, 29, 30] but one of them (PPD) was heat stable [28] and, on the other hand, virus-induced human fibroblast (type I) interferon lost $^2/_3$ of its activity on exposure to heat [29].

Acknowledgements. We are indebted to Dr. B. SZENDE (First Department of Pathological Anatomy, Semmelweis University Medical School, Budapest) for the histological examinations and to Dr. P. MEDVECZKY (Institute of Microbiology, Semmelweis University Medical School, Budapest) for the sonication of drug preparations. The skilled technical assistance of Mrs. J. PARÓCZAI and Mrs. A. BALÁZS is acknowledged.

REFERENCES

1. SIMINOFF, P., BERNARD, A. M., HURSKY V. S., PRICE, K. E.: Antimicr. Agents Chemother. **3,** 742 (1973).
2. SOEHNER, R. I., GAMBARDELLA, M. M., HOU, E. F.: Proc. Soc. exp. Biol. (N.Y). **145,** 1114 (1974).
3. GLÁZ, E. T., TÁLAS, M.: Arch. Virol. **48,** 375 (1975).
4. NICHOL, F. R., WEED, S. D., UNDERWOOD, G. E.: Antimicr. Agents Chemother. **9,** 433 (1976).
5. HOFFMAN, W. W., KORST, J. J., NIBLACK, J. F., CRONIN, T. H.: Antimicr. Agents Chemother. **3,** 498 (1973).
6. GATMAITAN, R. G., STANLEY, E. D., JACKSON, G. G.: J. infect. Dis. **127,** 401 (1973).
7. DOUGLAS, R. G., BETTS, R. F.: Infect. Immun. **9,** 506 (1974).
8. PANUSARN, C., STANLEY, E. D., DIRDA, V., RUBENIS, M., JACKSON, G. G.: New Engl. J. Med. **291,** 57 (1974).
9. STANLEY, E. D., JACKSON, G. G., DIRDA, V. A., RUBENIS, M.: J. infect. Dis. **133,** Suppl., A 121 (1976).
10. BLALOCK, J. E., GIFFORD, G. E.: Arch. ges. Virusforsch. **45,** 161 (1974).
11. KOCH, A., LOMNICZI, B., GYÖRGY, E.: Acta microbiol. Acad. Sci. hung. **13,** 243 (1966).
12. TÁLAS, M., SZOLGAY, E., VÁRTERÉSZ, V., KOCZKÁS, G.: Arch. ges. Virusforsch. **38,** 143 (1972).
13. GLÁZ, E. T., SZOLGAY, E., STÖGER, I., TÁLAS, M.: Antimicr. Agents Chemother. **3,** 537 (1973).
14. DECLERCQ, E., DESOMER, P.: Proc. Soc. exp. Biol. (N.Y.) **132,** 699 (1969).
15. TÁLAS, M., SZOLGAY, E., VÁRTERÉSZ, V., KOCZKÁS, G.: In WEISZFEILER, GY. J. (ed.): Proc. Microbiological Research Group, Hungarian Academy of Sciences. Vol. IV. Publishing House of the Hungarian Academy of Sciences, Budapest 1971. p. 49–52.
16. HOFFMAN, W. W.: Personal comment on the manuscript of this paper.
17. NAGANO, Y., MAEHARA, N.: In NAGANO, Y., LEVY, H. B. (eds): Interferon. Igaku-Shoin, Tokyo 1970. p. 120.
18. NAGY, I., CSÁKY, G., LUST, I., CSEH, G.: Krebsarzt **16,** 170 (1961).
19. DEMAEYER, E., DEMAEYER-GUIGNARD, J., JULLIEN, P.: Proc. Soc. exp. Biol. (N.Y.) **131,** 36 (1969).
20. GLASGOW, L. A.: Fed. Proc. **30,** 1846 (1971).
21. STRINGFELLOW, D. A., GLASGOW, L. A.: Antimicr. Agents Chemother. **2,** 73 (1972).
22. DENNIS, A. J., WILSON, H. E., BARKER, A. D., RHEINS, M. S.: Proc. Soc. exp. Biol. (N.Y.) **141,** 782 (1972).
23. HO, M., BREINING, M. C., MAEHARA, N.: J. infect. Dis. **133,** Suppl. A 30 (1976).
24. EPSTEIN, L. B., CLINE, M. J., MERIGAN, T. C.: Cell. Immunol. **2,** 602 (1971).
25. WALLEN, W. C., DEAN, J. H., LUCAS, D. O.: Cell. Immunol. **6,**, 110 (1973).
26. SIMINOFF, P.: Infect. Immun. **12,** 1051 (1975).
27. FALCOFF, R.: J. gen. Virol. **16,** 251 (1972).
28. YOUNGNER, J. S., SALVIN, S. B.: J. Immunol. **111,** 1914 (1973).
29. VALLE, M. J., JORDAN, G. W., HAARH, S., MERIGAN, T. C.: J. Immunol. **115,** 230 (1975).
30. SAITO, N., SUZUKI, F., SASAKI, K., ISHIDA, N.: Antimicr. Agents Chemother. **10,** 14 (1976).

Address of the authors:

ERVIN T. GLÁZ
Department of Pharmacology, Semmelweis University Medical School,
H-1450 Budapest, P.O.B. 91, Hungary

MARGARITA TÁLAS
Microbiological Research Group of the Hungarian Academy of Sciences
Pihenő út 1, H-1529 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 97–104, 1978

SIZE AND SEQUENCE OF THE BAM-HI FRAGMENTS OF ADENOVIRUS h1 DNA

GY. BERENCSI, P. MEDVECZKY, B. S. NARODITSKY, N. M. CHAPLYGINA, B. A. ZAVIZION, T. I. TIKCHONENKO and I. NÁSZ

Institute of Microbiology, Semmelweis University Medical School, Budapest and Ivanovsky Institute of Virology, Academy of Medical Sciences of the U.S.S.R. Moscow, U.S.S.R.

(Received August 22, 1977)

The DNA of the prototype strain of adenovirus h1 was shown to have three specific recognition sites for the *Bam–HI* restriction endonuclease. Molecular weights of the four DNA fragments *A*, *B*, *C* and *D* are 10.0 ± 1.13, 6.8 ± 0.21, 4.0 ± 0.34 and 2.2 ± 0.47 megadaltons, respectively. In contrast to the *Bam–HI* restriction patterns of adenoviruses h2 — h6 fragment *A* is located within the G : C-rich half of the the adenovirus h1 DNA. The physical order of the fragments is *A–C–B–D*, which was deduced from the analysis of partial hydrolysates. In addition to the results concerning the *Bam–HI* map of adenovirus h1 DNA trace amounts of a different restricase activity *(Bam–HII)* were found in the *Bam–HI* enzyme preparations.

The sequence-specific restriction endonuclease *Bam–HI* from *Bacillus amyloliquefaciens* [1] recognizes the hexanucleotide sequence GGATCC [2]. In order to work with purified homogeneous gene clusters of adenovirus h1 in the DNA form, the size and sequence of the fragments produced by the enzyme are to be determined. This work has been accomplished and the results are presented in the present paper.

Materials and methods

Virus and cell culture. The prototype strain of adenovirus h1 was grown on HEp-2 cells in suspension cultures [3]. Complete virions were separated from the soluble components by sedimentation through a CsCl cushion (1.2 g/ml) onto a cushion of 1.35 g/ml density in a Superspeed A50 MSE ultracentrifuge using a 3 × 23 ml rotor. Complete and incomplete virions were separated by a second centrifugation step using a preformed linear CsCl gradient (1.2–1.4 g/ml) at 26 000 rpm for 4 hr in the same rotor as above.

DNA preparation. The viral DNA was isolated by the pronase–SDS–phenol procedure as described earlier [4]. Only DNA preparations isolated from complete virions were used.

Agarose gel electrophoresis. Electrophoresis of the DNA fragments was performed in 0.6 × 9 cm siliconized glass tubes or in 0.2 × 16 × 18 cm slab gels under the conditions described by HELLING *et al.* [5]; 0.7% agarose gels (SeaKem, Marine Colloids Inc., Rockland, Maine, U.S.A.) were used in both systems. To improve separation of incomplete digestion fragments, the voltage was set below 1.0 V/cm. The fluorescence of DNA–ethidium bromide complexes were located photographically.

Molecular weight of the DNA fragments was calculated using a calibration curve based on the mobility of complete lambda and adenovirus h1 DNAs, the EcoRI fragments of lambda phage DNA [6] and the linear form III of PM2 DNA [7].

Restriction enzymes and reaction mixtures. Bacillus amyloliquefaciens strain HM was kindly supplied by Dr. J. ORTIN, Madrid, Spain, and large quantities were produced in shaken tryptose–phosphate broth at 37 °C [8]. Purification of the enzyme was performed according to the procedure of ORTIN *et al.* [9], with certain modifications. The bacterial pellet washed in ice-cold Tris–2-mercaptoethanol buffer (0.02 M each) was sonicated by 10 strokes of 30 seconds.

The DNA was pelleted at 120 000 *g* for 60 min in a Spinco L-2 ultracentrifuge with Ti-60 rotor. Crude separation of the enzyme was done by gel filtration on a 42 × 2.5 cm Sepharose 6B column (Pharmacia, Uppsala, Sweden) equilibrated with 1.0 M NaCl in tris–2-mercaptoethanol buffer pH 7.5. The active fractions of the eluate were pooled and dialysed into 0.01 M Na-phosphate buffer ph 7.5, supplemented with 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM EDTA and 0.001% Triton X-100. The dialysed pool was further fractionated on a 20 × 2 cm Whatman P-cellulose column with 0.1–1.0 M KCl gradient in the dialysis buffer. The active fractions of the eluate were pooled and dialysed into the buffer used for the reaction mixture containing 0.05 M tris–HCl, 12 mM $MgCl_2$, 5–50 mM NaCl or KCl and 6 mM 2-mercaptoethanol pH 7.5. The enzyme preparations were stored in 20% glycerol at —20 °C.

The *EcoRI* enzyme preparation was a kind gift of Drs B. SAIN and P. VENETIANER, Szeged, Hungary. It was used under the same conditions as *Bam–HI* except for the incubation temperature of the reaction mixture. The incubation temperature was 28 °C, if the reaction mixture contained *Bam–HI*, and 37 °C for *EcoRI* digestion. In the case of combined treatment with both *Bam–HI* and *Eco–RI*, the samples were incubated first at 28 °C and then at 37 °C.

Melting of DNA fragments. The enzyme reactions were stopped by the addition of Na_2EDTA (50 mM final concentration) and SDS (1.4% final concentration). In melting experiments, 50% formamide (analytical grade Merck A. G., Darmstadt, G.F.R.) was added and mixed thoroughly. The mixture was then distributed into Plastibrand reaction vials of 1.5 ml. Each vial contained 1–3 μg DNA and could be stored at +4 °C for 10–15 days without alteration. Each sample was incubated separately at the required temperature ± 0.1 °C for 15 min in a water bath. The samples were quenched immediately following incubation in an ice-bath and diluted with equal amounts of ice-cold electrophoresis buffer in order to prevent rehybrization. The total amount of the samples was applied to the agarose gels without the addition of bromophenolblue and sucrose.

Results

Purity and specific activity of the Bam–HI enzyme. Five different *Bam–HI* batches were used. All of them produced the same four final fragments if adenovirus h1 DNA was digested. Before molecular weight determination of the fragments it seemed necessary to test the non-specific endonuclease contamination of the enzyme in order to exclude an aspecific degradation of the specific fragments.

It was observed in a separate series of experiments, that the DNA of PM2 phage has no specific *Bam–HI* cleavage site (BERENCSI and MEDVECZKY, unpublished results). PM2 DNA preparations [7] containing approximately equal amounts of form I (superhelical), form II (nicked circular) and form III (linear) molecules were tested with three separate enzyme batches under standard conditions. Calculations on the basis of the disappearence of the superhelical PM2 fraction in concentration- and time-dependence experiments suggested that less then one nick had been introduced by aspecific endonucleases during 100 events of specific *Bam–HI* cleavage.

Size and sequence of specific Bam–HI fragments. The DNA of adenovirus h1 could completely be digested with *Bam–HI*. Four homogeneous fragments were present in the completely digested reaction mixtures. The molecular weight of fragments *A*, *B*, *C* and *D* was calculated to be 10.0 ± 1.13 (13 individual determinations), 6.8 ± 0.21 (7 individual determinations), 4.0 ± 0.34 (10 individual determinations), and 2.2 ± 0.47 (9 individual determinations) megadaltons, respectively.

Time and concentration-dependence was studied to identify and measure the size of incompletely cleaved fragments. Six such "incomplete" fragments could regularly be identified; they are shown as unlabelled circles in Fig. 1/c,

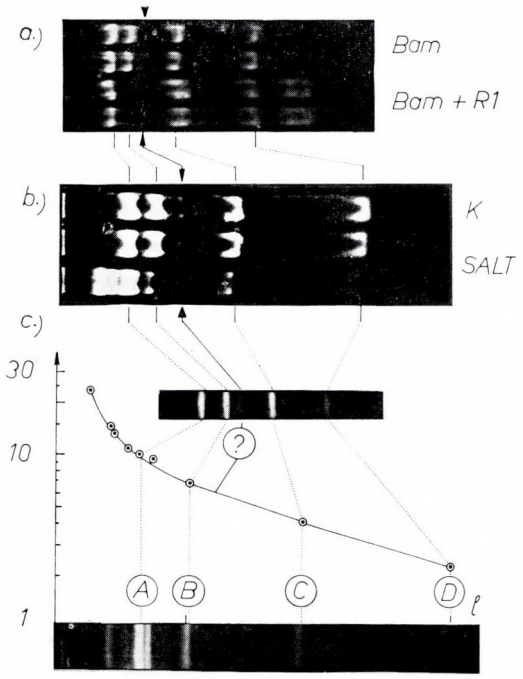


Fig. 1. Agarose electrophoresis patterns of the complete and incomplete *Bam–HI* fragments of adenovirus h1. a) 50 μl samples were cleaved by 1 μl (first and third samples) *Bam–HI* and by 2 μl (second and fourth sample) *Bam–HI* at 24 °C for 2 hr followed by incubation at 37 °C for 1 hr. Samples 3 and 4 were completed with *Eco–RI* (1 μl each). The electrophoresis was run in 0.8% agarose gel for 20 hr at 1.0 V/cm. b) 3 μg of virion DNA were digested with *Bam–HI* at 25 °C for 4 hr in the presence of 10 mM (K), 30 mM and 110 mM KCl ("salt"). c) Ordinate: molecular weight of complete and "incomplete" fragments $\times 10^6$ daltons. Abscissa: distance of migration of the fragments in 0.7% agarose. Individual data were standardized according to the migration distance of fragment *B*. The direction of electrophoresis was from left to right in each photograph. Identical positions of the final fragments are shown by broken lines. The comparable positions of the 6.1 megadalton fragment (question-mark) are shown by arrows. Other details see in the text

"Complete" fragments are labelled by capital letters, but these are not mentioned in the following paragraph.

The largest "incomplete" fragment shown at the left terminus of the curve is the undigested viral DNA of 23 megadaltons. The second "incomplete" molecule of 14.3 $\pm$ 0.27 megadaltons (second circle from left to right in Fig. 1/c) can only be the sum of the "complete" fragments $A + C$. The fragment of 12.8 $\pm$ 0.47 megadaltons may have been composed by $A + D$ or $B + C + D$ or $C + D + B$ or $C + B + D$, but the sequence $B + C + D$ could be

excluded, since the $A + C$ fragment had been identified. The presence of a fragment of 10.8 $\pm$ 0.24 megadaltons indicated that $B + C$ are neighbouring fragments. Thus, the fragment of 12.8 $\pm$ 0.47 megadaltons may be either $A + D$ or $C + B + D$. The fifth incomplete fragment of 8.87 $\pm$ 0.38 megadaltons may be composed of fragments $B + D$ only. Since the existence of fragments $B + C$ and $B + D$ is excluding the possibility of a sequence $A + D$ for the third incomplete molecule, the final order of the fragments seemed to be $A + C + B + D$ or $D + B + C + A$. Nevertheless, in a considerable number of experiments a sixth incomplete fragment of 6.1 $\pm$ 0.18 megadaltons was identified (labelled with a question-mark in Fig. 1/c). The presence of such an incomplete fragment ($C + D$) is compatible with a physical order of $A +$ $+ C + D + B$ if the fragment $B + C$ is absent. More than ten virus DNA preparations were systematically tested and both $B + C$ and $C + D$ were occasionally present in each of them. It was therefore suggested that the prototype adenovirus h1 was heterogeneous genetically.

Homogenety of the prototype adenovirus h1 DNA. Two different observations indicated that the virus strain was not heterogeneous. 1. *Sal–I, Eco–RI* and *Hind–III* restriction patterns of the same DNA preparations proved to be homogeneous ([10] and unpublished results). 2. The incomplete fragment corresponding to $C + D$ never appeared if other incomplete fragments were still visible in the gels. The upper and lower photographs are presented for comparison in Fig. 1/c.

Evidence is presented in Figs 1/a and 1/b that the fragment of 6.1 megadaltons was produced by a different enzyme activity present as contamination in the *Bam–HI*.

The two upper samples in Fig. 1/c contained the same amount of DNA, but the amount of *Bam–HI* was twice as much in the second reaction mixture than in the upper one. The digestion was complete even in the upper reaction mixture, therefore the second material must have contained excess enzyme. The "incomplete" fragment (indicated by arrows in Fig. 1/a and 1/b) occurred only in the second gel. If the production of an aberrant fragment was due to the presence of an additional DNA site for *Bam–HI*, both samples would have contained approximately the same amount of the fragment.

The third and fourth samples shown in Fig. 1/a were incubated simultaneously with *Bam–HI* and *Eco–RI*. The fourth reaction mixture contained twice as much *Bam–HI* as the third one. The 6.1 megadalton fragment also disappeared upon *Eco–RI* treatment, which is cutting only within the *Bam–HI* fragment B [10]. This would indicate that the specific site for the contaminating enzyme activity was located within that fragment.

The samples shown in Fig. 1/b were also digested with an overdose of *Bam–HI*. The upper sample contained only 10 mM KCl, and the aberrant fragment was produced. The second sample was digested in the presence of

30 mM KCl. The specific hydrolysis by *Bam–HI* was still complete, but the aberrant fragment disappeared. The third reaction mixture was prepared with 110 mM KCl, and this sufficed for a partial inhibition of specific *Bam–HI* cleavage. The 3 μg amount of the adenovirus h1 DNA was sufficient in the samples to detect a subpopulation possessing an additional site for *Bam–HI* even in the third, incompletely hydrolysed reaction mixture. It was therefore concluded that the contaminating enzyme activity is more sensitive to KCl than is the specific *Bam–HI* activity.

Polarity of the Bam–HI map of adenovirus h1 DNA. The G : C-rich half of the adenovirus maps is drawn left in order to compare the results obtained with different virus types [11]. Aliquots from the same reaction mixtures were incubated at different temperatures and electrophoresed. Part of an experiment of this kind is shown in Fig. 2. Thermal stability in 50% formamide was the weakest for fragment *D;* it was between 64.5 and 65.5 °C. The single strands of the melted fragment *D* were visible on the film, but could not be reproduced photographically. Upon heating the undenatured fragments showed heterogeneous electrophoretic bands, since small single-stranded regions were melted off irregularly due to the nicks produced by aspecific endonucleases during incubation. The thermal stability of fragment *B* was about 65.5–66.0 °C. The single strands were large enough to give sufficient fluorescence to be reproduced photographically (Fig. 2). Fragments *A* and *C* melted above 66.5°C. All this suggests that fragments *A* and *C* are neighbouring fragments located within the G : C-rich half of the molecule, in contrast to fragments *B* and *D* that are

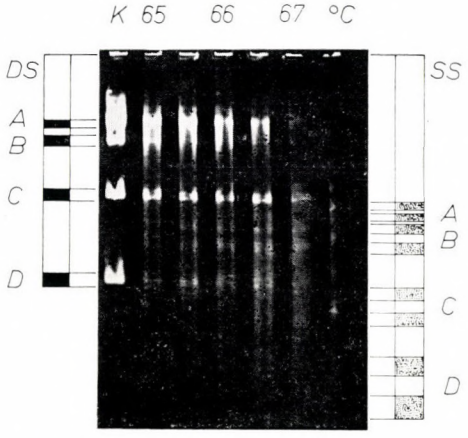


Fig. 2. Thermal stability of the *Bam–HI* fragments of adenovirus h1 in 50% formamide. The left panel shows the positions of the double stranded (DS) fragments A, B, C, and D. The right panel is indicating the migration distances of the pairs of denatured single stranded (SS) molecules, which are visible in the case of fragments *B* and *C*. The others could not be visualized photographically. Sample "K" was not heated. Intermediate temperatures are not indicated in the Figure. The electrophoresis was run in 0.7% agarose gel at 0.8 V/cm for 24 hr without cooling. Further details see in text

located within the A : T-rich DNA region. Thus, the physical order of the *Bam–HI* fragments of adenovirus h1 DNA is *A—C—B—D*, as illustrated in Fig. 3.

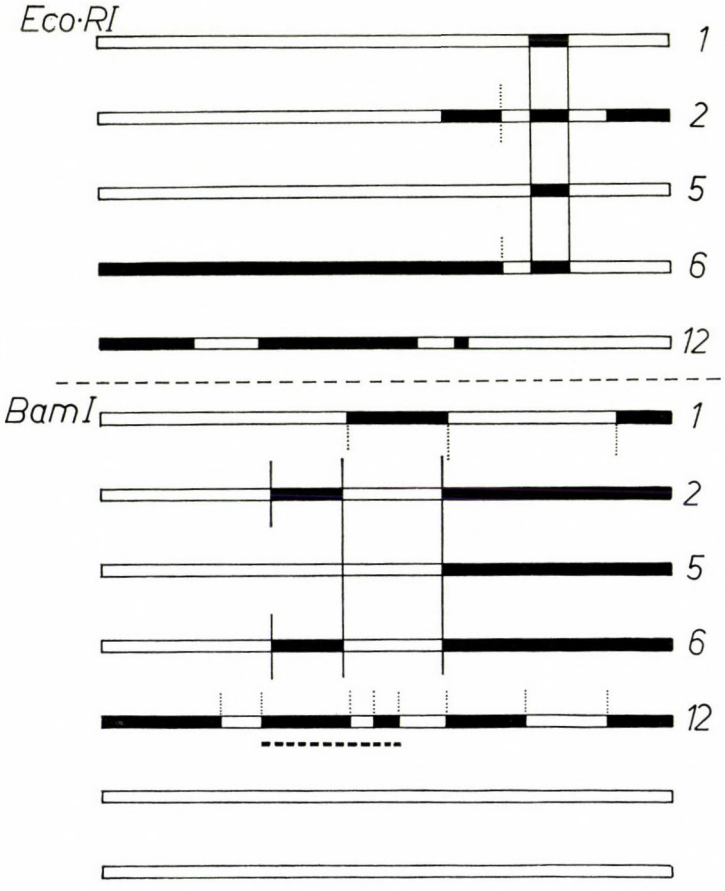


Fig. 3. Relative size and distribution of the DNA fragments produced by the restriction endonuclease *Eco–RI* and *Bam–HI* of adenovirus types 1, 2, 5, 6, and 12. See references [9—13] including the data presented in this paper. The horizontal double lines are representing the unit-length DNA. The bars, indicating the location of the individual fragments, are filled in alternatingly. The cleavage sites thought to be identical in different virus types are shown by vertical lines. Numerals are indicating the type of the viruses

Discussion

Adenoviruses are supposed to be of identical evolutionary origin. Although the attachment sites of restriction enzymes characterize only very short DNA sequences, some data suggest that the distribution of specific sites follows a similar pattern within the DNA molecules of related types [11–13].

The *Eco–RI* and *Bam–HI* maps completed with the results of our groups on adenovirus h1 and h6 ([13] and unpublished observations of the Soviet coauthors) are plotted in Fig. 3. Members of the non-oncogenic subgroups obviously possess homologous localizations. We believe that the small dislocation of the two central type 1 *Bam–HI* sites was due to an experimental error. The similar pattern of the central *Bam–HI* fragments of adenovirus h12 cannot be compared and evaluated at present, their exact sequence being unknown ([9], broken line in Fig. 3). DNA heteroduplex studies indicate [14, 15] that the two *Eco–RI* sites in adenovirus h1 which seem to be stable in all the four types examined, is located within the DNA region possessing very weak homology. The DNA region carrying the central *Bam–HI* site within adenovirus h1 is also heterogeneous, if the thermal stability of the heteroduplexes are examined [14, 15]. One may speculate whether the stable sites within heterogeneous DNA fragments may indicate the essential biological significance of the palindromes cut by the enzymes.

Isolation of microgram quantities of the *Bam–HI* fragments allow experimentation with non-infectious groups of adenovirus genes. DNA sequences of the *Bam–HI* fragment *B* is coding for the adenovirus h1 fibre and fragment *C* is carrying the sequences for the hexon protein [16].

Combined digestion of the adenovirus h1 DNA with *Eco–RI* and *Bam–HI* showed (Fig. 1/a) that the polarity of the physical map is correct since only the *B* fragment of *Bam–HI* is cleaved by *Eco–RI* (see Fig. 3 for comparison).

The additional fragment produced under special conditions is suggested to be the result of an enzyme activity *(Bam–HII)* different from *Bam–HI*. Earlier experiments failed to reveal its presence, since the enzyme preparations had not been dialysed off from the KCl [9]. Nevertheless, the higher concentrations of glycerol introduced into the reaction mixture with the enzyme, or pH changes due to the prolonged storage of the enzyme preparations, may have activated a *Bam–HI'* activity similar as the *Eco–RI'* activity [17].

Acknowledgement. The enthusiastic technical assistance of Miss K. GÖTTLINGER and Mrs. K. TURCZER-BUDAI is greatly appreciated.

REFERENCES

1. WILSON, G. A., YOUNG, F. E.: J. molec. Biol. **97,** 123 (1975).
2. WILSON, G. A., YOUNG, F. E.: cit. N. I. H. Guidelines on Recombinant DNA Research. Bethesda, Md. 1976.
3. GREEN, M., PINA, M.: Virology **20,** 199 (1963).
4. MEDVECZKY, P., CSUZI, S., NÁSZ, I., ÁDÁM, É., BERENCSI, GY., ANTONI, F.: Arch. Virol. **52,** 315 (1976).
5. HELLING, R. B., GOODMAN, H. M., BOYER, H. W.: J. Virol. **14,** 1235 (1974).
6. THOMAS, M., DAVIES, R. W.: J. molec. Biol. **91,** 315 (1975).
7. FRANKLIN, R. M.: Cur. Top. Microbiol. **68,** 107 (1974).
8. ORTIN, J., DOERFLER, W.: J. Virol. **15,** 27 (1975).

9. Ortin, J., Scheidtmann, K. H., Greenberg, R., Westphal, M., Doerfler, W.: J. Virol. **20,**, 355 (1976).
10. Завизион, В. А., Чаплыгина, Н. М., Народицки, Б. С., Тихоненко, Т. И., Беренчи, Д., Медвецки, П., Нас, И.: Вопр. Вирусол. In press.
11. Sambrook, J., Williams, J. F., Sharp, P. A., Grodzicker, T.: J. molec. Biol. **97,** 369 (1975).
12. Forsblom, S., Riegler, R., Ehrenberg, M., Pettersson, U., Philipson, L.: Nucl. Acid Res. **3,** 3255 (1976).
13. Народицки, Б. С., Карамов, Е. В., Чаплыгина, Н. М., Завизион, Б. А., Тихоненко, Т. И., Дрейзин, Р. С., Золотарская, Е. Е., Вихнович, Е. М., Пономарева, Т. И.: Вопр. Вирусол. 539 (1977).
14. Garon, C. F., Berry, K. W., Hierholzer, J. C., Rose, J. A.: Virology **54,** 414 (1973).
15. Нас. И., Беренчи, Д,: Вопр. Вирусол. 643 (1976).
16. Беренчи, Д., Нас, И.: Ж. Микробиол. (Киев) **38,** 515 (1976).
17. Roberts, R. J.: CRC Critical Rev. Biochem. 123 (1976).

Address of the authors:

György Berencsi, Péter Medveczky, István Nász
Institute of Microbiology, Semmelweis University Medical School
Hőgyes E. u. 7–9, H-1092 Budapest, Hungary

B. S. Naroditsky, N. M. Chaplygina, B. A. Zavizion, T. I. Tikchonenko
D. I. Ivanovsky Institute of Virology, Academy of Medical Sciences of the U.S.S.R.
Gamaleya Street 16, 123098 Moscow, U.S.S.R.

Acta microbiol. Acad. Sci. hung. 25, 105—114, 1978

SENSITIVITY OF SHIGELLA SONNEI PHASE I AND II IMMUNODETERMINANTS TO CHEMICAL TREATMENTS USED IN STRUCTURE ANALYSIS

T. KONTROHR and B. KOCSIS

Institute of Microbiology, University Medical School, Pécs

(Received September 14, 1977)

Purified lipopolysaccharide antigens of *Shigella sonnei* phase I and phase II bacteria were fragmented by partial acid hydrolysis in order to determine the proper hydrolytic conditions for removing lipid A without a loss of serological activity. The phase I immunodeterminant retained its full activity after hydrolysis in strong mineral acids at 100°C, while the phase II lipopolysaccharide lost its serological activity even under mild conditions of hydrolysis. In contrast, the chemical modification of the phase I immunodeterminant by hydrazinolysis or esterification-reduction of the free carboxyl groups markedly reduced its serological activity. Short-term periodate oxidation destroyed the activity of the phase II lipopolysaccharide. The activity of the phase I immunodeterminant was eliminated only partly.

The O-antigens of *Enterobacteriaceae* are known as lipopolysaccharides of the cell wall. On mild acid hydrolysis they dissociate readily to a polysaccharide and lipid A. The polysaccharide moiety consists of a core region and a side chain region, the O-side chain.

The lipid fraction, the less specific part of the lipopolysaccharides, carries some nonspecific biological properties of the molecule e.g. toxicity, pyrogenicity, etc.

The core region may be identical to various groups of enteric bacteria such as *Escherichia, Salmonella, Shigella* [1] while the O-side chain represents the im munospecific structure in the lipopolysaccharide of the smooth forms of these bacteria. In the lipopolysaccharide of rough mutants the latter part of the molecule is lacking; in this case some regions of the core determine the serological specificity.

Shigella sonnei as most of the enterobacteria, has smooth and rough mutants; phase I is serologically equivalent to the smooth (S) form, and phase II represents the first stage of rough (R) mutation. There are also R mutants with more degraded polysaccharide structure [2–4].

The *S. sonnei* lipopolysaccharides containing only the "core-specific" sugars glucose, galactose, glucosamine and heptose seem to represent one of the most simple polysaccharide structure [5]. It has, however, been shown that these sugars neither represent the immunodeterminants of *S. sonnei* mutants nor take part in building them up [6, 7].

As the chemical character of the immunodeterminants of *S. sonnei* phase I and II is not known, in the present study we have attempted to compare

some of the chemical and serological characteristics of the groups having a role in the serological specificity, in order to find a way to prepare them in pure active form suitable for chemical characterization.

Materials and methods

The bacterial strains used were *S. sonnei* phase I and II described elsewhere [2, 7]. Mass cultivation was performed in well-aerated liquid medium at pH 7.2 containing 20 g per litre of casein hydrolysate ("Lactacid", Institute for Serobacteriological Production and Research Human, Budapest), 2.5 g glucose and 5 g yeast extract ("Cellamin", Human, Budapest). The latter two substances were sterilized separately. After cultivation at 37 °C for 7 to 8 hr, phenol was added to a final concentration of 0.5% and after standing at + 4 °C overnight the bacteria were harvested by centrifugation, washed with water and dried with acetone. Yield: 100–120 g dry mass per 30 litre medium.

The lipopolysaccharides were prepared by the hot phenol–water method and purified by repeated ultracentrifugation [8].

Enzymatic methods were used for the determination of glucose with glucose oxidase (Boehringer, Mannheim, G.F.R.) and of galactose with galactose oxidase as modified by FISCHER and ZAPF [9]. Glucosamine was determined according to the method of ROSEMAN and DAFFNER [10]. Heptose and 2-keto-3-deoxy-mannooctonic acid (KDO) were determined according to OSBORN [11] and HEATH and GHALAMBOR [12], respectively. The polysaccharide fractions of the gel filtration experiments were tested with phenol–sulphuric acid according to DUBOIS *et al.* [13]. Total phosphate was determined by the method of LOWRY and LOPEZ [14]. N-acetyl was determined as the difference of total acetyl and O-acetyl assayed by the method of LUDOWIEG and DORFMAN [15] and SNYDER and STEPHENS [16], respectively.

Gas chromatography of sugars as alditol acetates according to SAWARDEKER *et al.* [17] was performed with a Varian Aerograph Model 2740 equipped with glass column ¼" × 6' containing either ECNSS-M (1% on Gas-Chrom Q) or OV-225 (1% on Gas-Chrom Q) at a column temperature of 180 °C with nitrogen as the carrier gas. As internal standard, myo-inositol was used.

Fatty acids were identified as methyl esters obtained by hydrolysis of about 2 mg lipopolysaccharide or lipid A in 1 ml M HCl in dry methanol at 100 °C for two hours. Separation was achieved on an ⅛" × 6' stainless steel column packed with 3% SE-30 on Gas-Chrom Q at a temperature program between 160 °C and 250 °C at a rate of 6 °C/min.

Paper chromatography was performed by the descending technique on Whatman No. 1 or Schleicher Schuell No.2043/b paper in the following solvent systems: (A) butanol–pyridine–water (6 : 4 : 3); (B) pyridine–ethyl acetate–acetic acid–water (5 : 5 :3 : 1). The spots were detected either with alkaline silver nitrate or with ninhydrin.

Passive haemolysis inhibition test was performed by a micromodification described elsewhere [18]. The rabbit immune sera to *S. sonnei* phase I and phase II bacteria were prepared and kindly supplied by Dr. M. ÁDÁM (National Institute of Hygiene, Budapest).

Experiments and results

Sensitivity to acid hydrolysis of the immunodeterminants. As the phase I immunodeterminant is highly resistant to acid hydrolysis [19, 20], this experiment has been carried out only with the phase II lipopolysaccharide. Ten mg phase II lipopolysaccharide were dissolved in 1.0 ml 1% acetic acid. The solution was divided in 100 μl parts, heated in sealed ampoules at 100 °C for various length of time, was chilled in ice water. Then 100 μl of 0.1M sodium hydroxyde were added to each sample. Aliquots were withdrawn for serial dilution for testing by passive haemagglutination inhibition.

The same method was used for hydrolysis in 0.1 M hydrochloric acid, but neutralization with sodium hydroxyde was done carefully with an Agla microburette.

The change in serological activity is demonstrated in Fig. 1. It is seen that the activity increased rapidly during the first 30 min of acetic acid hydrolysis and then decreased at about the same rate. The hydrochloric acid rapidly destroyed the serological activity of the phase II lipopolysaccharide.

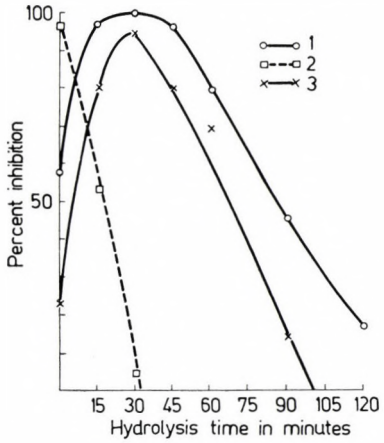


Fig. 1. Behaviour of the serological activity of phase II lipopolysaccharide on acid hydrolysis. Inhibitor: 4 μg in 0.1 M HCl (□– – –□); 1 μg (○——○); and 500 μg (×——×) in 1% acetic acid

Splitting of the lipopolysaccharide by mild acetic acid hydrolysis and separation on Sephadex G-50. Two-hundred mg lipopolysaccharide were suspended in 40 ml 1% acetic acid and heated in a boiling water bath for 1.5 hr for phase I and 1 hr for phase II. After freeze-drying, the material was redissolved in water and centrifuged at 10 000 *g* for 15 min. The lipid A pellet was washed twice with water. The supernatants which contained the polysaccharide fractions were collected, concentrated in vacuum and freeze-dried. After redissolving in about 2 ml water the polysaccharide fraction was centrifuged at 15 000 *g* to remove insoluble particles and applied on a column of Sephadex G-50 (2.5 × × 95 cm). Pyridine acetate (10 ml pyridine + 4 ml glacial acetic acid per litre) was used as eluant [21]. Five ml fractions were collected and tested with phenol-sulphuric acid in 50 μl aliquots [13]. The fractions corresponding to the peaks were collected, concentrated in vacuum and freeze-dried.

For sugar analysis, 2–3 mg polysaccharide fractions were dried over phosphorus pentoxide in vacuum and, after adding 50 μM inositol as internal standard, were hydrolysed in 500 μl 0.25 M sulphuric acid at 100° C for 18 hr. After dilution with water and neutralization with barium hydroxide the sample was centrifuged. The precipitate was extracted twice with 1 ml water

and the pooled supernatants were evaporated to dryness. The dry hydrolysate was redissolved in 500 μl 0.1 M ammonium hydrocarbonate (pH 10) and incubated with 2 μl alkaline phosphatase from *Escherichia coli* (Sigma) at 37 °C for 14 hr in the presence of toluene.

As Fig. 2 shows, the phase I degraded polysaccharide gave on the Sephadex column three peaks while phase II gave only two. The first peak was found only in phase I while the second and third ones were common in both samples. Each of the fractions obtained from the peaks were assayed in passive haemo-

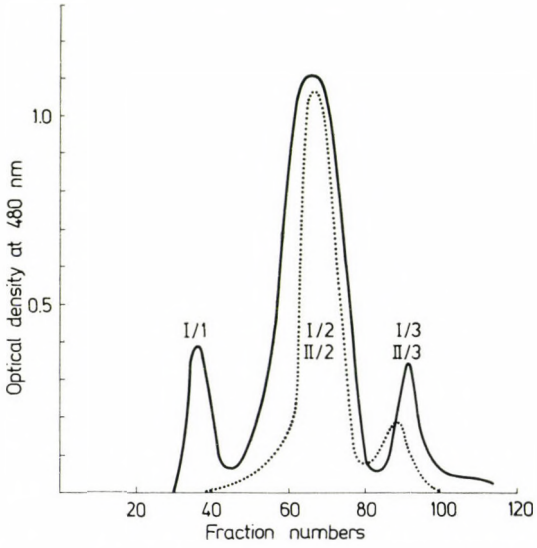


Fig. 2. Separation of degraded polysaccharides on Sephadex G-50 column. Phase I (———) and phase II (· · · ·) degraded polysaccharides

lysis inhibition test in both of the phase I and phase II systems (Fig. 3). The results corroborated the findings of ROMANOWSKA *et al.* [6] in that the first peak represents the O-side chain with core-stubs while the second one the core polysaccharide.

The fractions contained only traces of long chain fatty acids, gas chromatography showed a fairly complete splitting off of lipid A.

The analytical values for the fractions are shown in Table I. The bulk of the carbohydrate components was recovered in the I/2 and II/2 fractions in a similar ratio as in the lipopolysaccharide [7] with the exception of KDO, which had been set free during hydrolysis and was recovered in the I/3 and II/3 fractions. The latter contained only some percents of KDO, the nature of the other constituents remained unknown. Fraction I/1 contained 6.5–7.5% total carbohydrate. It is believed that the O-side chain of the phase I lipopolysaccharide does not contain common sugar constituents as galactose, glucose, etc.

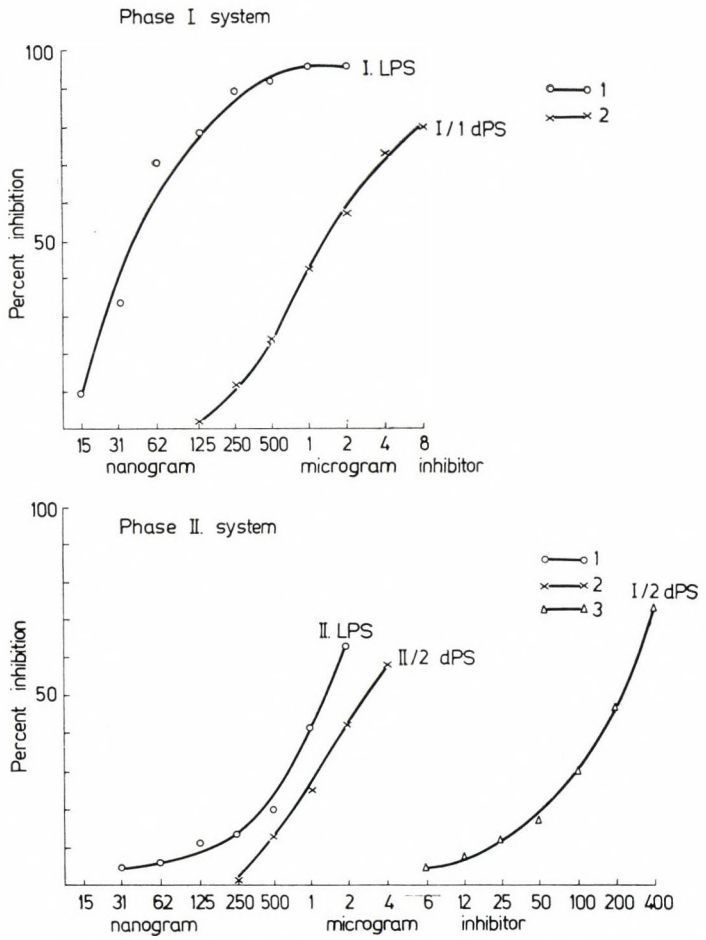


Fig. 3. Activity of degraded polysaccharide fractions in the passive haemolysis test

Table I

Chemical composition of degraded polysaccharide fractions

Lipopolysaccharide mM/100 g	Phase I		Phase II	
	fraction 1	fraction 2	fraction 2	fraction 3
Galactose	9.8	52.5	80.5	4.5
Glucose	13.1	81.4	161.8	7.0
Heptose	7.7*	41.8*	126.8	3.0
KDO	3.8	7.5	7.5	135.0

* Without dephosphorylation

The percentage found was ascribed to core fragments carrying O-chains or to a lipid A impurity with some glucosamine content.

Hydrazinolysis of phase I lipopolysaccharide [22]. A 100 mg aliquot of phase I lipopolysaccharide dried over phosphorus pentoxide was dissolved in 5 ml anhydrous hydrazine and maintained at 100 °C for 18 hr. After adding about 25 ml toluene, the hydrazine was evaporated in vacuum at 35 °C. Addition of toluene (20 ml each) and evaporation was repeated three times. The hydrazinolysed lipopolysaccharide was dried over sulphuric acid in vacuum then redissolved in water and lyophilized. The activity of the product was 25–50 times lower than that of the genuine lipopolysaccharide as tested by passive haemolysis inhibition in the phase I system (Fig. 4).

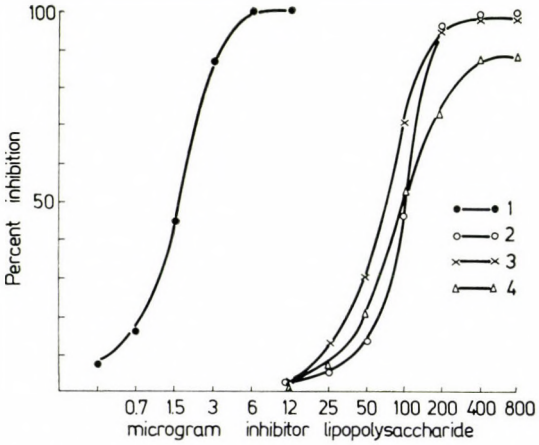


Fig. 4. Serological activity of the chemically modified phase I lipopolysaccharide. Free carboxyl form (●—●); ethylester (×—×); reduced (○—○);hydrazinolysed (△—△)

Esterification and reduction of the free carboxyl groups of phase I lipopolysaccharide. Five-hundred mg phase I lipopolysaccharide were dissolved in 50 ml distilled water, and 15 ml freshly regenerated Amberlite CG-120 cation exchanger in H^+-form was added. The suspension was shaken at 4 °C for one day. The resin was filtered off and washed with distilled water. The washings were collected and lyophilized (recovery: 318 mg). The lipopolysaccharide was redissolved in 50 ml distilled water, cooled in ice bath and 50 ml cold ethylene oxide were added. The reaction mixture was kept at room temperature for 5 days. After dialysis against distilled water and lyophilization, the esterified material was redissolved in 50 ml water and reduced with sodium borohydride in the presence of glycerol as described by HUNGERER *et al.* [23]. After dialysis and lyophilization, 298 mg carboxyl-reduced lipopolysaccharide was obtained. After esterification, the infrared spectrum showed well-defined carbonyl absorption at 1740 cm^{-1}. After reduction this absorption band could not be demonstrated.

Table II

Chemical composition of periodate oxidized phase I and phase II lipopolysaccharides

Lipopolysaccharide mM/100 g	Phase I		Phase II	
	untreated	oxidized	untreated	oxidized
Mannose	–	11.3	–	14.3
Galactose	30.3	4.6	31.0	3.7
Glucose	41.2	40.0	52.5	37.0
Heptose*	27.0	13.7	39.4	15.7

* Without dephosphorylation

In serological test the carboxyl reduced lipopolysaccharide was 25–50 times less active than the untreated sample, as calculated from the 50% inhibition values (Fig. 4).

Short-term periodate oxidation of phase I and phase II lipopolysaccharides. Two hundred mg lipopolysaccharide were dissolved in 20 ml water and 4 ml 0.1 M sodium metaperiodate were added. After 2 hr at 4 °C the oxidation was terminated by adding 2 ml ethylene glycol. Fifteen minutes later the reaction mixture was reduced by adding 80 ml 2% sodium borohydride. After 15 min the solution was acidified with glacial acetic acid to destroy the remaining borohydride and dialysed against frequently changed distilled water at 4 °C for 3 days. The product was then freeze-dried and the activity was tested in the phase I and phase II systems. The analytical values for the sugar composition after periodate oxidation are shown in Table II and the serological activities in Fig. 5.

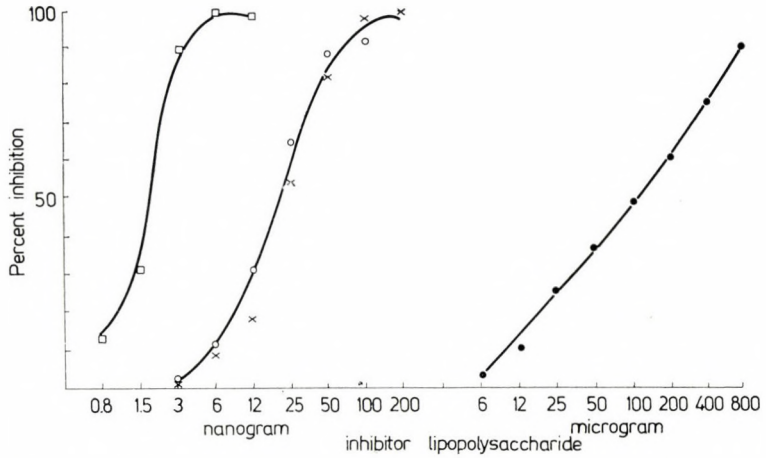


Fig. 5. Serological activity of the periodate oxidized phase I and phase II lipopolysaccharides. Phase I: untreated (□——□); oxidized (×——×); phase II: untreated (○——○); oxidized (●——●)

Discussion

In order to obtain fragments of the lipopolysaccharide molecule bearing the immunodeterminants, we used acetic acid degradation and separation of the split products on Sephadex G-50 according to FREEMAN [24] and MÜLLER-SEITZ *et al.* [21]. The phase I and phase II immunodeterminants are, however, different in their sensitivity to acid hydrolysis. The phase I immunodeterminant retains its full activity after hydrolysis in 1 to 6 N mineral acid at 100 °C for 15–20 hr, while the phase II lipopolysaccharide loses its serological activity in 0.1 N mineral acid at 100 °C for less than 30 min [cf. 25].

Our first aim was to determine the hydrolytic conditions in the Freeman degration, especially for the phase II lipopolysaccharide and to split off lipid A completely without a considerable loss of serological activity. As it was shown, 0.1 M hydrochloric acid rapidly destroyed the activity but even diluted acetic acid markedly diminished it. In the first 30 min of acetic acid hydrolysis a twofold increase in activity was demonstrated. Assuming a similar rate of deactivation as estimated on the declining part of the graph, i.e. about the same rate of activation as the inactivation, we concluded that only about half or one third of the existing phase II immunodeterminants are available in the genuine phase II lipopolysaccharide. It is not known whether the phenomenon is due to the poor solubility of the highly aggregated molecule or to the masking effect of other parts of the repeating units.

This extreme acid lability of the phase II lipopolysaccharide explains the weaker reactivity of the I/2 fraction in comparison with the II/2 fraction in the phase II inhibition system. (For the complete removal of lipid A, the phase I lipopolysaccharide needs prolonged treatment with acid and therefore, more destruction occurs).

Fractionation of the degraded polysaccharide on Sephadex and analysis of the fractions corroborated the findings of former workers [21, 6]. The first peak represents the O side-chain with core-stubs (this peak is absent in the phase II lipopolysaccharide), while the second peak contains the core polysaccharide and has the same chemical composition in both mutants. The first peak of phase I contains only minute amounts of the common sugar components. Its main constituents are the immunodeterminant compound of unknown structure [20] and the amino-uronic acid discovered by ROMANOWSKA and REINHOLD [26], the structure of which was determined in our laboratory to be 2-amino-L-altruronic acid, a component hitherto unknown in nature [27].

The amino compounds of the I/1 fraction are N-acetylated and part of their carboxyl groups is free. The role of these groups in the immunospecificity was studied by hydrazinolysis (for removing N-acetyl groups) and carboxyl reduction. The serological activity could be reduced by both treatments, espe-

cially by hydrazinolysis. Unambiguous conclusions could not be drawn, considering uncontrolled side reactions during the hydrazinolysis of amino sugar containing polymers newly reported in [28].

The phase I and phase II immunodeterminants differ susceptibility to short-term periodate oxidation. Phase II activity was practically destroyed while phase I activity only diminished. Two moles of galactose and two of glucose were oxidized. The mannose, which is absent in the native lipopolysaccharide, originated from the heptose substituted at C_3 and free in C_6 and C_7 positions. One heptose remained intact and another mole was destroyed to lower fragments.

The nature of the immunodeterminant of the *S. sonnei* phase I and phase II lipopolysaccharides is unknown. Our study showed that extreme care must be taken in modifying lipopolysaccharide antigens with unknown immunodeterminants. Some methods used routinely and considered mild can destroy the immunospecific part of the molecule, making thus impossible to identify the original structure. On the other hand, a chemical modification with simultaneous tracing of the serological activity of the antigen molecule allows to recognize the nature of unknown immunodeterminants.

REFERENCES

1. Jann, K., Westphal, O.: The Antigens. Vol. 3, Academic Press, New York 1975. p. 1–125.
2. Rauss, K., Kétyi, I., VerténYi, A., Vörös, S.: Acta microbiol. Acad. Sci. hung. **8,** 53 (1961).
3. Slopek, St., Mulczyk, M., Lachowicz, T. M., Krukowska, A.: Arch. Immunol. **8,** 593 (1961).
4. Rauss, K., Kontrohr, T., VerténYi, A., Kétyi, I.: Biochem. Z. **336,** 126 (1962).
5. Jesaitis, M. A., Goebel, W. F.,: J. exp. Med. **96,** 409 (1952).
6. Romanowska, E., Lugowski, C., Mulczyk, M.: J. Hyg. Epidem. (Praha) **18,** 391 (1974).
7. Kontrohr, T., Westphal, O.: Acta microbiol. Acad. Sci. hung. **14,** 205 (1967).
8. Westphal, O., Jann, K.: In Whistler, R. H. (ed.): Methods in Carbohydrate Chemistry. Vol. 5. Academic Press, New York 1965. p. 83.
9. Fischer, W., Zapf, J.: Hoppe-Seylers Z. physiol. Chem. **337,** 186 (1964).
10. Roseman, S., Daffner, I.: Anal. Chem. **28,** 1743 (1956).
11. Osborn, M. J.: Proc. nat. Acad. Sci. (Wash.) **50,** 499 (1963).
12. Heath, E. C., Ghalambor, M. L.: Biochim. biophys. Res. Comm. **10,** 340 (1963).
13. Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., Smith, F.: Nature (Lond.) **168,** 167 (1951).
14. Lowry, O. H., Lopez, J. A.: J. biol. Chem. **162,** 421 (1946).
15. Ludowieg, J., Dorfman, A.: Biochim. biophys. Acta (Amst.) **38,** 212 (1960).
16. Snyder, F., Stephens, N.: Biochim. biophys. Acta (Amst.) **34,** 244 (1959).
17. Sawardeker, J. S., Sloneker, J. H., Jeanes, A.: Anal. Chem. **37,** 1602 (1965).
18. Kontrohr, T., Péterffy, K. K.: J. immunol. Methods **13,** 271 (1976).
19. Romanowska, E., Mulczyk, M.: Biochim. biophys. Acta (Amst.) **136,** 312 (1967).
20. Kontrohr, T., Kocsis, B.: Abstr. 9th Meeting of European Biochemical Society 1974. p. 499.
21. Müller-Seitz, E., Jann, B., Jann, K.: FEBS Letters **1,** 311 (1968).
22. Fraenkel-Conrat, H., Chun Ming Tsung: In Colowick, S. P., Kaplan, N. O. (eds): Methods in Enzymology. Vol. 11, Academic Press, New York 1967. p. 151.
23. Hungerer, D., Jann, K., Ørskov, F., Ørskov, I.: Europ. J. Biochem. **2,** 115 (1967).

24. FREEMAN, G. G.: J. Biochem. **36,** 340 (1942).
25. ROMANOWSKA, E., MULCZYK, M.: Europ. J. Biochem. **5,** 109 (1968).
26. ROMANOWSKA, E., REINHOLD, V.: Europ. J. Biochem. **36,** 160 (1973).
27. KONTROHR, T.: Carbohydrate Res. **58,** 111 (1977).
28. ERBING, C., GRANATH, K., KENNE, L., LINDBERG, B.: Carbohydrate Res. **47,** 5 (1976).

Address of the authors:

TIVADAR KONTROHR, BÉLA KOCSIS
Institute of Microbiology, University Medical Schoo
Szigeti út 12, H-7643 Pécs, Hungary

ANNUAL MEETING OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY

GYULA, AUGUST 29–SEPTEMBER 1, 1977

ABSTRACTS OF PAPERS

Bacteriology and Bacterial Genetics

PILI (FIMBRIAE) AS COLONIZING FACTOR FOR PORCINE ENTEROPATHOGENIC ESCHERICHIA COLI

B. Nagy, H. W. Moon and R. E. Isaacson

Veterinary Institute, Szombathely and the National Animal Disease Center, Ames, Iowa, U.S.A.

Colonization of the small intestine is an important step in the pathogenesis of enterotoxic diseases caused by enteropathogenic *Escherichia coli* (EEC). Porcine EEC commonly carry the antigen K88, which is known as adhesive virulence factor for $K88^+$ ECC. There are, however, several EEC strains which do not carry K88 and yet they are enteropathogenic for newborn pigs. The experiments reported were done with these $K88^-$ EEC of serogroups O9, O20 and O101. Newborn, colostrum deprived pigs were exposed intragastrically to the $K88^-$ EEC. Sixteen hours post exposure bacterial counts, immunfluorescence microscopy, scanning and transmission electron microscopy of the ileal segments all suggested that the $K88^-$ EEC adhere to the ileal mucosa. The bacteria were found near the microvilli with surface appendages reaching towards them. Electron microscopy detected pili on all the $K88^-$ EEC. One of the $K88^-$ EEC (strain 987) was found to be richly piliated (P^{++}) when grown in the porcine small intestine *in vivo* but poorly piliated ($P^{\pm}$) when grown *in vitro*. The P^{++} variants adhered well to isolated intestinal epithelial cells *in vitro*, but the $P^{\pm}$ variants did not. Other variants, which had lost their *in vivo* pilus production (P^-) did not cause diarrhoea and did not colonize the small intestine of newborn pigs, although they retained their enterotoxin producing ability. It is concluded that the $K88^-$ EEC tested colonize the porcine small intestine by their adhesiveness, which is in close correlation with the presence of pili.

STABILITY OF ESCHERICHIA COLI K88 AND K99 ADHESION FACTORS

I. Kétyi, B. Kuch and A. VerténYi

Institute of Microbiology, University Medical School, Pécs

In the intestine of adult mice *Escherichia coli* strains carrying adhesion factors K88 and K99 show a rapid and considerable loss of plasmid. The loss is of "plasmid-deletion" character and affects both natural and plasmid-transferred $K88^+$ or $K99^+$ strains. The degree and dynamics of the loss are identical in different strains of inbred mice. The phenomenon cannot be demonstrated

in suckling mice, the intestine of which is selective for $K88^+$ and $K99^+$ strains and clones. Factors R100-1 and Col Ib losing plasmids *in vitro* showed an increased loss in adult mice whereas F-lac factor not exhibiting a spontaneous loss, failed to do so. Alkaline pH alone or in combination with low redox conditions caused no increased plasmid loss *in vitro*.

THERMOSTABLE (ST) ENTEROTOXIN PRODUCTION BY SHIGELLA FLEXNERI

I. Kétyi, I. Málovics, T. Kontrohr, S. Pácsa, A. VerténYi and B. Kuch

Institute of Microbiology, University Medical School, Pécs

The filtrate or sonicated lysate of *Shigella flexneri* 3 and 4b strains gave positive "rapid PF" and suckling mouse tests; the rabbit intestinal loop test was also positive if read after 4 hr. LT-specific tests (mouse pad oedema, CHO cell elongation and "delayed PF" reaction) were negative. The "rapid PF" test was positive for Ent^+ and Ent^- *Escherichia coli*, but the latter failed to give the suckling mouse and early rabbit intestinal reactions. *S. flexneri* extracts fractionated on Sephadex G-100 column showed a sharp peak with the "rapid PF" and suckling mouse positive fractions; the "blanching" effect was associated with the same fraction. The control Ent^- *E.coli* extract contained the "rapid PF" fractions at about the same site as shigellae, but gave no suckling mouse reaction. A shiga-like cytotoxic effect was not demonstrated either with crude or with partly purified *S. flexneri* filtrate.

NUTRITIONAL REQUIREMENTS OF LISTERIA STRAINS

B. Ralovich, M. Woodbine and M. Shahamat

Institute of Hygiene and Epidemiology, University Medical School, Pécs and Microbiology Unit, Department of Applied Biochemistry and Nutrition, Faculty of Agricultural Sciences, University of Nottingham, Bonington, Loughborough, England

On a minimal medium suitable for genetical experiments, 15 *Listeria monocytogenes* strains were examined for nutritional requirements. DL-6,8-Thioctic acid was not essential. Growth was detected on thiamine-free medium, but colonies of normal size developed only when biotin and riboflavin were also present. Low redox potential attained with thioglycollate, was essential together with Fe^{++}, Mg^{++} and Ca^{++} ions. Citrate was not convenient as sole carbon source but was necessary as a chelating agent. Ammonium salt was not sufficient as single nitrogen source, but in the presence of essential amino acids

it promoted growth. The individual strains differed somewhat in amino acid requirements; leucine, isoleucine, valine and cysteine were essential almost in every case. There was no definite association between nutritional requirements, serotype, agglutinability, haemolytic activity and virulence of the strains. One of two EMS mutants originated from a virulent *Listeria* strain became avirulent and auxotrophic for arginine, glutamine and methionine.

AMINO COMPONENTS OF PSEUDOMONAS AERUGINOSA LIPOPOLYSACCHARIDES

A. Szentmihályi

National Institute of Hygiene, Budapest

The amino components of Westphal-type phenol water extracts of 3 *Pseudomonas aeruginosa* strains containing degraded O antigens were examined using the BIO-CAL model BC 200 automatic amino acid analyser and the single column ion exchange method with three changes of buffer. In the sequence of elution, the LPS hydrolysates contained the following compounds: glucosamine phosphate, threonine, serine, unidentified amino compound (U) glutamic acid, proline, glycine, alanine, valine, galactosamine, glucosamine, U, tyrosine, phenylalanine, fucosamine, U, ammonia (decomposition product), U and arginine. Seven of the above amino acids and unidentified substances have not been proviously described in *P. aeruginosa* LPS.

SEROLOGICAL EXAMINATION OF PSEUDOMONAS AERUGINOSA O4a,b LIPOPOLYSACCHARIDE HYDROLYSATES

M. M. Ádám and T. Kontrohr

National Institute of Hygiene, Budapest and Institute of Microbiology, University Medical School, Pécs

Passive haemolysis of sheep erythrocytes sensitized with *Pseudomonas aeruginosa* O4a,b LPS was inhibited by LPS hydrolysed with N HCl at 100 °C for 10, 30, 60, 120 and 240 min and by "degraded polysaccharide" (dPS) obtained after heating with 1% acetic acid at 100 °C for 1 hr. Three ng untreated LPS and 31 ng dPS exhibited 50% inhibition; the activity of LPS hydrolysates decreased rapidly at the beginning of HCl treatment then remained at the same level (10 μg after 10 min and 20 μg after 30, 60, 120 and 240 min). It is concluded that the immunodeterminant group of *P. aeruginosa* O4a,b is acid stable and that the passive haemolysis inhibition test is suitable for the serological analysis of isolated mono and oligosaccharide fragments.

HEAT-STABLE ANTIGENS OF A NEW GROUP OF FLUORESCENT PSEUDOMONADS

B. Lányi, A. W. Hoadley and G. W. Ajello

National Institute of Hygiene, Budapest and School of Civil Engineering, Georgia Institute of Technology, Atlanta, Georgia, U.S.A.

UFP (unidentified fluorescent pseudomonas) bacteria described by Hoadley and Ajello resemble *Pseudomonas aeruginosa* not only in cultural, but also in basic serological properties (agglutination-inhibiting effect of mild heating, ethanol, saturated sodium chloride and formalin and agglutination-restoring effect of heating at 100 °C or above). In serological specificity, UFP strains differed from *P. aeruginosa*. Biotypes I and III of 115 UFP strains were classified into 17 serological groups; two subgroups were distinguished in each of groups O2, O5, O10, O12 and O16. Determination of the O antigens of UFP strains was suitable for epidemiological tracing and rapid identification of these bacteria from *P. aeruginosa*.

ROLE OF BILE ACIDS IN THE PATHOGENESIS AND PREVENTION OF ESCHERICHIA COLI DYSPEPSIA

L. Bertók, L. Kocsár, T. Bereznai and J. Mészáros

"Fréderic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest Phylaxia Veterinary Biologicals and Feedstuffs Ltd., Budapest and Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

Model experiments demonstrated the primary role of bile acid deficiency in the absorption of endotoxin from the intestines and in the pathogenesis of endotoxin shock. As bile acids have been shown to exert a protective effect, a bile acid preparation (Detertoxon) was employed for the prevention of diarrhoea of newborn pigs and calves. Detertoxon given prior to the first suck prevented the development of coli diarrhoea. The preparation supplemented bile acids lacking in the newborn animals and, owing to the detergent effect, disintegrated endotoxins of Gram-negative bacteria into atoxic fragments. Part of the bile acids, after absorption from the intestine, stimulated the liver to bile production and excretion of bile from the gallbladder to the intestine.

INCREASE OF NATURAL RESISTANCE AFTER INJECTION OF IRRADIATION-DETOXIFIED ENDOTOXIN

L. Bertók

"Fréderic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

After exposure to 5–20 Mrad ^{60}Co-gamma irradiation, the toxic, pyrogenic, complement-activating, local Shwartzman reaction-eliciting, lysosomal membrane-damaging and blood-coagulating effect of *Escherichia coli* endo-

oxin is decreased or eliminated, but its natural resistance, enhancing capacity and adjuvant effect is retained. The irradiation altered the structure of the lipopolysaccharide, first of all the lipid part of the macromolecule. Pretreatment with radio-detoxified endotoxin prevents the development of shock in the rat, mouse, golden hamster, pig and dog challenged with a lethal dose of endotoxin. In the rat it exerts protection against irradiation (70%), prevents or decreases the incidence of peritonitis and septic shock after intraperitoneal injection of colonic contents (90%), schock developing after occlusion of the superior mesenteric artery (62%), and tourniquet shock (60%); it also prevents the development of lethal *Pasteurella multiseptica* (100%), *Klebsiella pneumoniae* (100%) and Aujeszky virus (70%) infection. The radio-detoxified endotoxin inhibits endotoxin-induced abortion in pregnant rats (90%), provides endotoxin tolerance in rats immunosuppressed with antilymphocyte serum and protects against endotoxin injury of the lysosomal membrane. As an adjuvant, in rats it increases the primary immune response. In dogs it inhibits endotoxic haemodynamic changes and haemorrhagic shock (80%) and decreases liver injury after occlusion of the coronary arteries.

COAGGLUTINATION TEST FOR GROUPING BETA-HAEMOLYTIC STREPTOCOCCI

Z. Rácz, I. J. B. Szabó and J. Szita

National Institute of Hygiene, Budapest

Coagglutination and precipitation tests gave identical results in grouping 119 out of 121 beta-haemolytic streptococcal strains. One of the remaining strains belonged to group B by coagglutination and was ungroupable by precipitation, the other to group G by precipitation, but, due to the B–G relationship, could not be classified by coagglutination. Seventy strains belonged to group A, 23 to B, 5 to C and 8 to G; 13 group D strains failed to react with reagents A, B, C and G, but in lack of reagent D, could not be grouped by coagglutination. The results were in agreement with other data in the literature.

KILLED STAPHYLOCOCCI FOR COAGGLUTINATION TEST

R. Varró, I. Barna-Vetró and T. Brózik

Institute for Serobacteriological Production and Research "Human", Budapest

Heated and formalinized *Staphylococcus aureus* backteria retain their immunglobulin-binding capacity mediated by surface protein A molecules. Antiserum-treated killed staphylococcal cells bound IgG and were suitable for grouping streptococci and for detecting protein and polypeptide antigens.

INCIDENCE OF AEROBIC GRAM-NEGATIVE BACTERIA IN A PULMONOLOGY UNIT

Cs. Csukás

Institute of Pulmonology, Szolnok

A total of 7000 bacteriological specimens were obtained from the respiratory organs including 2507 sputum and 1307 other samples. Gram-negative bacteria were present in significant numbers in 1121, in pure culture in 95 samples. The following organisms were cultured: *Neisseria* sp. 346, *Haemophilus* sp. 188, unidentified bacteria 179, *Enterobacter* sp. 168, *Escherichia coli* 151, *Pseudomonas aeruginosa* 98 (including 37 strains growing in pure culture), *Pseudomonas stutzeri* 69, *Proteus mirabilis* 66, *Klebsiella* sp. 55, *Pasteurella multocida* 39, *Pseudomonas* sp. 37, *Moraxella* sp. 33, *Acinetobacter* sp. 32, *Alcaligenes* sp. 29, *Citrobacter* sp. 13, *Proteus* sp. 11, *Proteus vulgaris* 9, *Serratia* sp. 8, *Flavobacterium* sp. 8, *Proteus morganii* 6, *Aeromonas* sp. 6, *Proteus rettgeri* 4 and *Chromobacterium* sp. 2.

OXOLINIC ACID MEDIUM FOR CULTURING LISTERIA MONOCYTOGENES

A. Forray and T. Angyal

Food Hygienic Control Service and Outpatients' Unit Laboratory, Pécs

Solid medium containing oxolinic acid at 40 μg/ml concentration is excellent for selective culturing; as compared to nalidixic acid agar, *Listeria monocytogenes* produces more distinct colonies and other bacteria are more effectively inhibited. The superiority of the medium was confirmed by inoculating throat swabs from 114 slaughtered healthy swine into broth, leaving to stand the tubes in the refrigerator, preparing oxolinic acid + 5% ox serum agar subcultures at 14 and 28 day intervals and incubating them at 37 °C.

RESISTANCE TO RADIATION OF BACILLUS PUMILUS E 601 AND BACILLUS SPHAERICUS C_1A IN HUNGARIAN γ-SOURCES

L. Gazsó and S. Igali

"*Fréderic Joliot-Curie*" *National Research Institute for Radiobiology and Radiohygiene, Budapest*

As recommended by the International Atomic Energy Agency, standard spore preparations were used for the evaluation of these factors in the radiation sterilization plants. D_{10}-value of the spore preparation of *Bacillus pumilus* in Hungarian γ-sources was as follows: Noratom, 0.36; PX- γ -30, 0.30; γ-source

of the Isotope Institute of the Hungarian Academy of Sciences, 0.27; JS-6900 Radiation Sterilization Plant, Debrecen, 0.21 Mrad. C_1A and D_{10}-values of *Bacillus sphaericus* itemized as above were, 0.99; 0.70; 0.83 and 0.60 Mrad, respectively. The results support the observation that the resistance of the products exposed to the same dose of irradiation may be different depending either on sources or dose-rates.

STERILIZATION OF A DISPOSABLE SET OF MEDIUM BY IONIZING RADIATION

M. Rodler, N. László, I. Rodler, A. Urbán, J. Simon and I. Pogány

Public Health Station, Szekszárd, Ministry of Health, Budapest, Public Health Station, Zalaegerszeg and Phylaxia Veterinary Biologicals and Feedstuffs Ltd., Budapest

A disposable set of medium for the counting of bacteria was effectively sterilized by 1 Mrad ionizing radiation. Examination of about 1000 food, sewage, surface water, urine and hospital quality control samples with the new method and the standard technique gave identical results.

RADIORESISTANCE OF MICROORGANISMS ISOLATED FROM VARIOUS ENVIRONMENTS

L. Gazsó and S. Igali

"*Fréderic Joliot-Curie*" *National Research Institute for Radiobiology and Radiohygiene, Budapes*

In qualitative and quantitative examination of the natural microflora of dry catgut to be exposed to radiation sterilization, contamination was determined for eight catgut thickness types. Examination of the radioresistance of 42 strains showed that a 100 krad dose sufficed for the inactivation of 50% of the microorganisms (at 10^6 germs). As for the other 50%, the D_{10} value fluctuated between 25 and 265 krad. Calculating with the highest D_{10} value, 3.5 Mrad sufficed for the inactivation of 10^{13} cells. Supposing that every sample contains 100 microorganisms of the same resistance as above, only a single cell would survive in every 10^{11} sample after 3.5 Mrad irradiation. This indicates a very favourable safety factor for the product. The radioresistance of microflora in the gamma pool water of our 650 Ci activity γ-source was also examined using tap water as control. Thirty-two strains were insolated from gamma pool water and 31 strains from tap water. Maximum D_{10} values were 17.5 and 45 krad, respectively. The bacterial flora of tap water supplying the source-surroundings was more resistant than the population of the gamma pool water. It is concluded that the development of a radioresistant bacterial population in water circulating around a low activity γ-source is improbable.

ELECTRIC-HEATED BACTERIOLOGICAL SEEDING LOOP

A. Forray and F. Studinger

Food Hygienic Control Service, Pécs and Institute of Physiology, University Medical School, Pécs

A special, 0.2–0.3 mm thick metal wire, is sterilized by direct electric heating (6 V, 4–5 A). Use of the thin wire allows a rapid cooling and easy seeding. Air pollution by gas flame is avoided in the laboratory. At field operations, the seeding loop is heated from a car battery.

ATYPICAL MYCOBACTERIA FROM AQUARIUM FISH

B. Körmendy, S. Tuboly, R. Bánki and G. Csaba

Central Veterinary Institute, Budapest and Institute of Epidemiology, University of Veterinary Sciences, Budapest

Thirty mycobacterial strains were isolated from aquarium fish imported for biological experiments. The strains were classified on the basis of temperature, nutrient requirement and time of growth, pigment production, nitrate reduction, catalase and peroxidase activity, carbohydrate and carbonamide breakdown. The strains identified were: *Mycobacterium aquae* (2), *M. smegmatis* (3), *M. marinum* (4), *M. fortuitum* (8), *M. terrae* (2) and *M. parafortuitum* (1). Of seven strains not classifiable into species, 5 belonged to Runyon group IV, 2 to Runyon group III. The taxonomic position of 3 strains could not be determined.

THYMINE AND THYMIDINE UPTAKE BY MYCOBACTERIA BEFORE AND AFTER PHAGE INFECTION

P. Somogyi and I. Földes

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

(i) Unlike *Escherichia coli* and *Bacillus subtilis*, *Mycobacterium phlei* and *Mycobacterium smegmatis* var. *butyricum* incorporated thymine for a short interval in the absence of deoxyribose. The finding is ascribed to a larger deoxyribose pool in mycobacteria than in *Escherichia* and *Bacillus*. DNA synthesis in mycobacteria can effectively be analysed by labelled thymidine incorporation; a very high (500–1000 μg/ml) uridine concentration is necessary for the process. (ii) Phage infection causes a considerable change in thymine and thymidine uptake; the latter becomes very low compared to the former and increasing of the ribose pool fails to improve either thymine or thymidine incorporation; a high uridine concentration even inhibits thymine incorporation.

COMPARISON OF MYCOBACTERIUM AVIUM STRAINS FROM DIFFERENT SOURCES

E. T. Illés, S. Tuboly and I. Szabó

Central Veterinary Institute, Budapest, Institute of Epidemiology, University of Veterinary Sciences, Budapest and National Korányi Institute of Pulmonology, Budapest

Twenty *Mycobacterium avium* strains were isolated from pheasants and poultry. All strains decomposed nicotinamide and pyrazinamide and gave other biochemical tests with essentially the same results. The pheasant strains, with one exception, were of the Schaeffer serogroup II; 2 strains from poultry belonged to serogroup I and 4 strains to serogroup II. One strain from each group of birds proved to be composed of two serotypes. The strains were uniformly sensitive to INH and viomycin, with two exceptions to cycloserine and with one exception to streptomycin and ethymbutol. Sensitivity to 5 μg rifampicin and rigenicide varied from strain to strain.

ISOLATION OF DOUBLE LYSOGENIC MYCOBACTERIAL CELLS

K. Háber and B. Vajda

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

Phages of *Mycobacterium smegmatis* strain butyricum form plaques not only on their original host cells but also on *M. smegmatis* strain Rabinowitz cells lysogenised with bacteriophage V72 (M. sm. R./V72). The average burst size of M. sm. R./V72 cells infected with bacteriophage butyricum depends on the culture medium used. The average burst size of M. sm. R./V72 cells infected with bacteriophage butyricum was 6 in YRP medium, and 60 in broth. A considerable number of M. sm. R./V72 cells survived the infection by bacteriophage butyricum. These phenomena suggested the possibility of the formation of double lysogenic mycobacterial cells. Experiments carried out for the isolation of double lysogenic cells were successful.

SCOTOCHROMOGENIC MYCOBACTERIA, MIXED MYCOBACTERIOSES AND THEIR CLINICAL SIGNIFICANCE

F. Gimpl

Department of Pulmonology, Semmelweis University Medical School, Budapest

In 1976–1977, a total of 29 662 samples were obtained from patients showing X-ray changes. *Mycobacterium tuberculosis* occurred in 1680, other acid fast bacteria in 123 samples. Most of the 18 scotochromogenic strains

were identified as *Mycobacterium aquae* and its variants. From 5 patients *M. tuberculosis* was isolated with other mycobacteria. All scotochromogenic strains were resistant to INH, sensitive to ethambutanol and, with two exceptions, sensitive to rifampicin.

A NEW PATHOGENIC TREPONEMA SPECIES

I. Horváth, F. Kemenes and L. Molnár

National Institute of Determatology and Veneral Disease, Budapest and Institute of Epidemiology, University of Veterinary Sciences, Budapest

A pathogenic *Treponema* strain was isolated from the genital organs of hares. The microorganisms, for which the name *Treponema leporis* has been proposed, is maintained by passage in domestic rabbits. The finding is in contrast to literary data stating that the hare has no treponematosis.

PROPERTIES OF POULT UREOPLASMA STRAINS

L. Stipkovits and A. Rashwan

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

Four *Ureoplasma* strains isolated from pathological changes in chickens differed biochemically and serologically from known human and mammal ureoplasma strains. The organisms exerted a slight pathogenicity in chick and duck embryos. Artificial infections of 3 week and 2 month-old chicken and turkey resulted in pneumonia and air sac inflammation.

IDENTIFICATION OF HAEMOPHILUS

Zs. Csukás and P. Sutka

Institute of Microbiology, Semmelweis University Medical School, Budapest and Central Station for Artificial Insemination, Budapest

Using 110 human strains of *Haemophilus*, various methods of growth factor determination (Alexander; Butler; Zinnemann; Sims; Cowan and Steel; Parker; Pickett and Steward) were compared. Results obtained by Sims' technique were the most reproducible and corresponded best to biochemical properties. For species differentiation the splitting of sucrose and xylose, indole production and ONPG test were the most useful. *H.influenzae* failed to ferment sucrose, was ONPG negative and usually xylose and indole positive. All *H.parainfluenzae* and *H. parahaemolyticus* strains split surcose, were xylose and indole negative and most of them gave ONPG reaction.

PROTOPLASTS OF BACILLUS SUBTILIS MUTANTS

A. Miczák and B. Prágai

Institute of Microbiology, University Medical School, Szeged

Tetrapyrrole membrane enzymes of *Bacillus subtilis*, if present in decreased amounts, may affect the membrane structure of porphyrin mutants. Protoplasts of the mutants showed different stability in buffers. In 0.7 M sucrose buffer they usually were stable, whereas in more dilute buffers they were lysed in varying degrees. The lysis curves showed that *B. subtilis* 168 *trpC* parent strain was the most stable. B_{12} vitamin producing porphyrin mutants were lysed more readily. Mutants not producing B_{12} vitamin were highly labile. Membrane stability depended on the ingredients of the culture medium, among others.

ANTIBACTERIAL, PLASMID CURING AND MUTAGENIC EFFECT OF CHLORPROMAZINE DERIVATIVES

J. Molnár, B. Schneider and M. A. Albert

Institute of Microbiology, University Medical School, Szeged and Psychopharmacology Research Branch, National Institute of Mental Health, Rockville, Maryland, U.S.A.

Chlorpromazine derivatives were bacteriostatic and bactericidal to both Gram-negative and Gram-positive bacteria. 7-Hydroxychlorpromazine (60 μg/ml) eliminated at 2% frequency the F'lac plasmid of *Escherichia coli* K12 LE 140; while 7,8-dihydroxy and 7,8-dioxochlorpromazine (120 μg/ml) exhibited no plasmid curing effect. Chlorpromazine (60 μg/ml) was effective at 30% frequency. Ca^{++}, Mg^{++} and Mn^{++} chelates of chlorpromazine were ineffective even at 500 μg/ml. *Salmonella typhi-murium* His^- frameshift mutant and control cells showed the same degree of back-mutation after chlorpromazine treatment. From *E. coli* K12 LE 140 cultured with chlorpromazine derivatives, lon^- mutants were selected at different frequency: 1–3% for 7-hydroxy-chlorpromazine, 0–0.3% for 7,8-dihydroxychlorpromazine, and nil for 7,8-dioxochlorpromazine.

MUTAGENIC EFFECT OF STREPTOZOTOCIN ON SALMONELLA TYPHI-MURIUM TA 1530 in RAT TISSUE EXTRACTS

S. Igali and L. Gazsó

"*Fréderic Joliot-Curie*" *National Research Institute for Radiobiology and Radiohygiene, Budapest*

Streptozotocin, a cytostatic antibiotic was given intramuscularly in 0.1 mg/kg doses to RA×LE (H) F_1 male rats weighing 200 g. One half hour after injection, cell-free extracts were prepared from the blood, liver and

testicles. After incubating the extracts with *Salmonella typhi-murium* TA 1530 for one hour, the frequency of $his^- \longrightarrow his^+$ base-substitution reversion was determined on selective medium. The values for induced mutation frequency were $1.01 \times 10^{-6} \pm 0.4$ for blood; $2.78 \times 10^{-6} \pm 0.3$ for testicle; and $1.43 \times 10^{-4} \pm 0.5$ for liver. When studying and evaluating the mutagenic activity of of chemicals, the organ-specific metabolic activation of different substances should be considered.

CORRELATION BETWEEN LINKAGE AND MAP DISTANCE IN R68.45 CONJUGATION SYSTEM

Zs. Bánfalvi, É. Vincze, Gy. B. Kiss and Á. Kondorosi

Biological Research Centre, Hungarian Academy of Sciences, Szeged

Plasmid R68.45 readily promotes transfer of chromosomal markers in *Escherichia coli* K12. Linkage data for different marker pairs in *E. coli* were compared with the physical distances between the marker pairs. (i) R68.45 is able to mobilize the chromosome starting from several chromosomal sites. (ii) The order of markers is identical with that obtained with F sex factor. (iii) Using a map function, the calculated distance values between marker pairs are approximately additive and, on the other hand, proportional to actual physical distances. (iv) The length of donor fragments can also be calculated from the map function. (v) The frequency of chromosome mobilization and the average length of donor fragments vary with certain *E. coli* recipients.

CONSTRUCTION OF A GENETIC MAP OF RHIZOBIUM MELILOTI

Á. Kondorosi, Gy. B. Kiss, É. Vincze, T. Forrai and Zs. Bánfalvi

Biological Research Centre, Hungarian Academy of Sciences, Szeged

Plasmid R68.45 promotes chromosome transfer in *Rhizobium meliloti* strain 41. Recombinants for various chromosomal markers were produced at the same frequency (10^{-4}–10^{-5} per donor cell), indicating that R68.45 promotes gene transfer from a range of chromosomal sites. Two and three-factor crosses between multiple marked derivatives of *R. meliloti* were performed and the linkage (contransfer) frequencies for various marker pairs were determined. Using an empirical function, the linkage values were expressed in additive map distance values. From the linkage analysis of 20 markers a circular linkage map of the *R. meliloti* chromosome was established. On the basis of its

broad host range and chromosome donor ability, it may be assumed that R68.45 is suitable for the mapping of various Gram-negative bacteria. In principle, the function used in this study can be applied to these conjugation systems.

ELECTROPHORETIC VARIABILITY OF PROTEINS IN RHIZOBIA

K. Köves-Péchy and K. Szende

Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest

Electrophoretic variation in malate dehydrogenase (Mdh), esterase and tetrazolium oxidase loci of 86 clones of 7 *Rhizobium* species of different geographic origin was examined by the polyacrylamide gel method. The data, especially the variation in Mdh locus allele, were useful for a differentiation of the taxons. As the resolving power of electrophoresis is limited due to the identity of netto charge differences, heat and inhibitor sensitivity alterations expressing molecular structural differences were also considered for showing the so-called hidden variability. In certain cases the different structure of Mdh and esterase allozymes, exhibiting identical electrophoretic mobility, was demonstrated

GENERAL TRANSDUCTION IN RHIZOBIUM MELILOTI

T. Sik, J. Horváth, A. K. Misra and S. Chattopadhyay

Biological Research Centre, Hungarian Academy of Sciences, Szeged

By means of the temperate phage 11, in *Rhizobium meliloti*, leucine, adenine, histidine, tryptophan, methionine and cysteine auxotroph recipients were transduced and streptomycin, chloramphenicol and azide resistance was transferred. The linkage between markers of resistance and auxotrophy was determined with cotransduction and thus a partial genetic mapping of the bacterial chromosome was achieved. To elucidate the formation of transducing particles and the mechanism of transduction, the phage-host interaction was studied. The original transducing phage was temperate. Phages induced from lysogenic bacteria segregated and became gradually more virulent. Concomitantly bacteria surviving phage infection or the transductants showed alterations in immunity to superinfection. The relations of phage derivatives to bacteria with different host-immunity was determined by testing infectivity, plating efficiency, phage growth inhibition and modification. The results indicated a non-classical host-phage modification in *Rhizobium*.

ASSIMILATORY NITRATE REDUCTASE DEFICIENT MUTANTS OF RHIZOBIUM MELILOTI

GY. B. KISS and Á. KONDOROSI

Biological Research Centre, Hungarian Academy of Sciences, Szeged

Thirty-nine mutants defective in nitrate assimilation, were isolated from *Rhizobium meliloti* strain 41 and classified into 3 categories. (1) Defect in assimilatory nitrate reductase but not in respiratory activity; (2) in both activities; (3) only in nitrite reductase activity. The mutants did not significantly differ from the wild type organism in symbiotic nitrogen fixation. No benzylviologen dependent nitrate reductase activity was detected in mutants of the second category.

EFFECT OF FERMENTATION PARAMETERS ON LABORATORY SCALE PRODUCTION OF RHIZOBIUM JAPONICUM INOCULUM

P. BIACS, K. GRUIZ and G. VERECZKEY

Institute of Agricultural Chemical Technology, University of Technical Sciences, Budapest

Rhizobium japonicum strain BME 011 isolated from soybean root nodules was grown in shaken flasks then in 10 litre fermentors at 30 °C, pH 6.5–7.0. Mannitol used as carbon source, gave higher yields than glucose. The organism was sensitive to aeration: with mannitol, maximum yields were obtained at 1 litre sterile air (litre/min.), whereas with glucose the degree of aeration had no important influence on growth. Based on optical density measurement, the growth rate curves were drawn using the Kono–Asai mathematical model. Growth kinetic calculations showed that in the presence of mannitol the growth rate coefficient ($k = 0.48–0.78$) was reversely proportional to the cell count of the inoculum. With glucose the corresponding value was 0.37–0.55.

CAPSULE PRODUCTION BY METICILLIN SENSITIVE AND RESISTANT STAPHYLOCOCCUS AUREUS

F. ROZGONYI and G. SELTMANN

Institute of Microbiology, University Medical School, Debrecen and Institute of Experimental Epidemiology, Wernigerode, G.D.R.

Three pairs of meticillin-sensitive (MS) substrains and meticillin resistant (MR) stable mutants selected from 3 independent heteroresistant *Staphylococcus aureus* strains produced mucoid colonies on modified Difco „Staphylococcus aureus agar No.110" medium. MS and MR counterparts of the same

parent strain showed no difference in uronic acid content and chemical composition of the capsule polysaccharide. The uronic acid content per g dry bacteria was considerably higher in MR than in MS of the same pair. There were important differences between the pairs in capsule production, capsule uronic acid content and composition of the capsular polysaccharides according to different parent strains. Meticillin resistance and capsule production are not associated in *S. aureus*.

BIOCHEMICAL AND GENETIC ANALYSIS OF PENICILLIN AND AMPICILLIN RESISTANCE. I. RESISTANCE AND BETA-LACTAMASE PRODUCTION OF CLINICAL ISOLATES

J. Földes, K. Domokos and É. Eperjesi

Microbiology Department, Central Laboratory and Institute of Microbiology, University Medical School, Szeged

Penicillin beta-lactamase (PBL) production by fresh clinical isolates of different bacteria was examined parallel with a colorimetric micromethod, iodometry and chromatography. (i) Penicillin (P) and ampicillin (Ap) resistant strains showed MIC values of > 100 μg/ml for Ap in 90%. (ii) Varying with the species, P and Ap resistant strains produced adaptive and constitutive PBL in 35–75%. (iii) P and Ap resistance was most frequently associated with Su, Sm, Tc, Nm and Cm resistance. (iv) Multiple resistant strains producing PBL occurred in less than 50% in *E. coli;* in other bacteria the incidence of PBL-producing strains was twice that of PBL negative strains. (v) In the case of Gram-negative bacteria the curve for linked resistance with PBL negative strains showed one peak of 6 linked resistance, whereas with PBL positive strains, a double peak appeared.

AN AMINOGLYCOSIDE ANTIBIOTIC WITH REMARKABLE ANTITUBERCULOTIC ACTIVITY

I. Koczka, Cs. Rétháti and I. Sági

Research Institute for Pharmaceutical Chemistry, Budapest

In the fermentation broth of *Micromonospore purpurea* (strain K-148), in addition to members of the gentamicin C complex, 30 to 40 chemically different biologically active and inactive minor components were found. They were isolated and examined. Some of them were new antibiotics with significant activity. Gentamicin B and B_1, described earlier by Waitz *et al.* were especially activive against mycobacteria including *Mycobacterium tuberculosis* and *Mycobacterium bovis*, both *in vitro* and *in vivo*. Gentamicin X showed antifungal activity.

STANDARDIZATION OF ANTIBIOTIC DISK SENSITIVITY TESTING

M. Konkoly Thege

National Institute of Hygiene, Budapest

On modified Mueller–Hinton medium the inhibiton zone-narrowing effect of divalent cations present in the acid hydrolysate of casein decreased on the addition of phosphate at appropriate concentration. Buffering of the medium to pH 7.4 with phosphate was effective for eliminating double zones around aminoglycoside antibiotics. For a more precise checking of the medium and interpretation of zones with routine isolates, a series of 8 control strains has been selected; the cultures corresponded in minimal inhibitory concentration to serum and urine levels attainable by the usual dosage and each of them was suitable for the checking of several different disks.

Virology

REPLICATION OF THE "Ckp" STRAIN OF TGE VIRUS IN VITRO, FOLLOWED UP BY IMMUNOFLUORESCENCE

E. Mocsári

Central Veterinary Institute, Budapest

Secondary pig thyroid cell cultures grown on coverslips were infected with $10^{5.14}$ $TCID_{50}$ of the "Ckp" strain of TGE virus. Virus synthesis was demonstrated by cytoplasmic immunofluorescence as early as in the 3rd hr incubation at 37 °C. At the same time, mature virus appeared in the maintenance medium. Subsequently, the virus titre in the medium increased parallel with the quantity of the fluorescent antigen until a maximum had been reached at 12 and 15 hr, at the time when the first CP changes were observed in native cell cultures. When the maximum CP effect had been reached, (i. e. after 24–30 hr), virus synthesis slowed down and, soon thereafter, the titre of the infectious virus began to decline, supposedly due to heat inactivation at the incubation temperature.

IMMUNIZATION WITH BOVINE PARAINFLUENZA-3 VACCINE

A. Bartha, K. Bognár, L. Zsák and J. Kojnok

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest and Phylaxia Veterinary Biologicals and Feedstuffs Ltd., Budapest

A vaccine containing live attenuated parainfluenza-3 virus was prepared and 30 000 cattle were immunized with the vaccine in three neighbouring areas

during a winter epizootic. Seroconversion needed at least $10^{3.5}$ $TCID_{50}$ of virus administered either as intranasal spray or by intramuscular injection. The intranasal method proved superior, as after muscular application antibody failed to appear in the mucosal secretion. In the areas of vaccination, the animals remained free of disease, except for four fatal cases of respiratory disease. In two cattle stocks, the IBR virus was identified as aetiological agent by virus isolation.

FACTORS INFLUENCING THE SEROLOGICAL RESPONSE TO A BOVINE PARAINFLUENZA-3 VACCINE

L. Zsák and A. Bartha

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

The serological response to an experimental parainfluenza-3 vaccine was studied by the HI test in paired serum samples from 2000 vaccinated cattle. After intranasal spraying the antibody titre reached depended on the age of the animal and on the prevaccination serum antibody titre. The antibody response increased with age and it was depressed by the prevalent maternal antibodies in the same degree as by the actively produced antibodies.

INFLUENZA A (H3N2) VIRUS INFECTION IN ZOO BIRDS, WILD BIRDS AN ZOO MONKEYS

J. Romváry, J. Mészáros, J. Tanyi, Gy. Takátsy, J. Rózsa and L. Fábián

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest, Veterinary Institute Debrecen, National Institute of Hygiene, Budapest, County Veterinary Station, Szeged and Municipal Veterinary Station, Budapest

Serum antibodies to type A human influenzavirus were demonstrable during the epidemic due to variant Victoria/75 in 17.7–40% of captive and migrating wild birds (doves, mallards, black-headed gulls) in the premises of the Budapest Zoo, and in 15.6 and 28.6%, respectively, of a total of 35 species of Zoo birds, after transfer in two lots at 5-week interval from the closed aviary to the open bird lake of the Zoo. Among representative groups of doves, crows and sparrows captured or shot along the river Tisza for the purpose of serological examination, 37.7 and 18.1%, respectively, had HI antibodies not only to Victoria/75, but occasionally also to the earlier variants Port Chalmers/73 and England/72. The birds exhibiting a positive serological immune response belonged to 17 species. Rhesus monkeys kept in the Zoo showed more pronounded immune response to the variants Port Chalmers/73 and/or England/72 than

to Victoria/75. It follows that the Zoo monkeys had become infected during the preceding pandemics, and the last infection by Victoria/75 had a booster effect on the immune response(s) to the former influenzavirus A variants. Virus strains identified as Victoria/75 were isolated from the lings of a Zoo gibbon died with pneumonia, and from a sparrow captured in its habitat along the river Tisza.

ISOLATION OF AVIAN INFLUENZA VIRUSES FROM WILD BIRS IN HUNGARY

J. Romváry, J. Mészáros, J. Tanyi, J. Rózsa and L. Fábián

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest, Veterinary Institute, Debrecen, County Veterinary Station, Szeged and Municipal Veterinary Station, Budapest

Subtype *Hav*5 strains of avian influenzavirus were isolated from the airways of a migrating mallard duck, captured in the premises of the Budapest Zoo, of a dove shot along the southern course of the river Tisza, and of 3–4 weeks old captive Muscovy ducks. Specific antibodies to subtype *Hav*5 were demonstrated in the blood sera of various Zoo birds. Strains of subtype *Hav*3, and of not yet identified type(s) other than *Hav*1–*Hav*7 were isolated from both respiratory tract and cloacal mucosa of pheasants and coots shot along the river Tisza. A subtype *Hav*6 strain was detected in the sinus exudate and cloacal mucosa of a 8-month-old captive mallard duck, and type A influenzavirus other than *Hav*1–*Hav*6 was isolated from 4–5 weeks and 4 months old mallards reared in captivity in other farms. Subtype *Hav*7 influenzavirus was demonstrated in common terns and black-headed gulls captured at Lake Balaton by the end of the spring migration period. Positive isolations of subtype *Hav*5 influenzavirus from migrating wild birds were successful for the first time in Europe. Introduction of *Hav*5 and of the other Hav-subtypes by migrating wild birds thus seems possible, as the infected bird populations had invaded the main migration areas and habitats.

PURIFICATION OF INFLUENZA VIRUS VACINE STOCK SUSPENSION BY ION-EXCHANGE CHROMATOGRAPHY

G. Nagy, Zs. Takátsy and Gy. Takátsy

National Institute of Hygiene, Budapest

Influenza virus suspended in 0.05–0.1 M approximately neutral or slightly alkaline buffer binds to QAE-Sephadex A-27 gel reversibly. The gel has a high virus-binding capacity: it is saturated at 100 000 HA units of virus per ml gel.

The virus is eluted by 1.0 M NaCl solution. By the use of these procedures, 80% of the virus otherwise lost during influenza vaccine preparation was recovered. The virus concentrate obtained contained 20 000–100 000 HA units/ml, depending on the concentration in the starting material. The degree of concentration was 12–83-fold. The 88–375 HA units of virus/μg total protein showed that the product was sufficiently pure to be used as vaccine in man. According to preliminary results, the method is suitable for concentrating other viruses as well.

INFLUENCE OF BCG IMMUNIZATION ON THE COURSE OF EXPERIMENTAL HERPES SIMPLEX VIRUS INFECTION IN RABBITS

F. Lehel, M. Harsányi and I. Tóth

Public Health Station and Municipal Institute of Public Health, Debrecen

BCG-immunized and non-immunized rabbits were infected with *Herpesvirus hominis* (HSV-2) intradermally. The non-immunized animals developed a dose-dependent characteristic skin lesion 2 or 3 days after inoculation and died of encephalomyelitis on days 7–10. The BCG-immunized rabbits developed similar skin lesions with some delay and the virus could rarely be reisolated from the lesions. These animals did not develop encephalomyelitis. A similar degree of protection was induced by combined administration of leucocytes from BCG-immunized rabbits and anti-HSV immunoglobulin.

INTERACTION BETWEEN GOOSE PARVOVIRUS AND DUCK HERPESVIRUS REPLICATION IN THYMIDINE (TdR)-BLOCKED CELL CULTURE

J. Kisary

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

The replication of the B strain of goose parvovirus requires simultaneous host DNA synthesis. The latter is blocked before the S phase of the mitotic cycle by the presence of 2 mM TdR in the maintenance medium. The block is relieved and the parvovirus replicates again in a medium rich in calf serum. A similar effect was achieved in TdR-blocked parvovirus cultures by superinfecting the cultures with a herpesvirus replicating independently of host DNA synthesis. On the other hand, herpeshirus replication is considerably inhibited by simultaneous parvovirus infection.

RADIOPROTECTIVE ACTIVITY OF INTERFERON INDUCERS

E. Szolgay, M. Tálas and L. Bátkai

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest

The radioprotective activity of interferon inducers was investigated against acute X-ray (450–700 R) and prolonged ^{60}Co-gamma (0.7 rad/min) irradiation. Inbred BALB/c and DBA mice of both sexes weighing 28–30 g (12–14 weeks old) were treated with interferon inducers. The inducers used were tilorone, 200 mg/kg: Acranil, 60 mg/kg; poly I:poly C, 1 mg/kg; poly G:poly C, 8 mg/kg, *Escherichia coli* endotoxin, 1 mg/kg. The agents were administered 1–4 days before or 30 min after irradiation. The radioprotective effect was evaluated by the endogenous haemopoietic spleen colony-formation test and on the basis of survival. In irradiated mice treated with interferon inducers prophylactically, 20–40 times more spleen colonies could be detected than in the controls and mean survival increased by 20–60%. Administering the agents 30 min after irradiation, only *E. coli* endotoxin proved effective. The radioprotective effect was the most marked when irradiation was carried out at the time of the peak interferon level. It is assumed that the radioprotective effect of interferon inducers depends on the interferon induced.

RELATIONSHIP BETWEEN INTERFERON SYNTHESIS AND VIRUS-SPECIFIC MACROMOLECULAR PROCESSES IN ADENOVIRUS INFECTED CHICK CELLS

B. Taródi and W. C. Russell

Institute of Microbiology, University Medical School, Szeged and National Institute for Medical Research, Mill Hill, London, U.K.

Adenoviruses are suitable models for studying the mechanism of interferon induction by DNA viruses. In the present work, among others, two temperature-sensitive mutants (*ts* 18 and *ts* 19) of human adenovirus type 5 were used. These mutants do not induce interferon at the nonpermissive temperature. Polypeptide synthesis and, polypeptide phosphorylation and the appearance of ds RNA were studied in infected chick cells. Although the human adenovirus infection is abortive in these cells, virus-specific polypeptides and DNA as well as structural antigens are synthesized in them. In chick cells infected with *ts* mutants, on the other hand, only the early events can be observed. Interferon induction results from an early event of virus-cell interaction; the same event is necessary for the synthesis of viral DNA. Supposedly, structural antigen(s) of the virion play an important role in this interaction, and the participation of ds RNA in the induction mechanism cannot be ruled out.

REPLICATION OF OVINE ADENOVIRUSES

S. Belák, V. Pálfi and S. Tuboly

Institute of Epizootiology, University of Veterinary Sciences and Central Veterinary Institute, Budapest

The 5 prototype strains, and Hungarian isolates of type 1, 4 and 5, of ovine adenoviruses as well as viruses related to bovine adenovirus type 2 proved to replicate well in a number of primary and secondary cultures of various mammalian cells (MDBK, trachea, etc.). The cytopathic (CP) effect was type and strain-dependent, the nuclear inclusions were highly variable. The progress of CP changes was followed up in native and stained preparations and by direct and indirect immunofluorescence. The rate of replication and the virus yield varied. In cultures infected by the ORT/111 strain the fluorescence appeared in the 10th hr and CP effect and intranuclear virus appeared in the 12th hr, whereas with the homotypic strain Het/3, the morphological changes were evident consistently later and the extra and intracellular virus titres remained far below those of strain ORT/111. Virus replication was not considerably influenced by the age of the culture at inoculation (from 0 to 7 days).

SOME PHYSICAL AND CHEMICAL CHARACTERISTICS OF SOLUBLE ADENOVIRUS PROTEINS

É. Ádám, I. Nász and P. Medveczky

Institute of Microbiology, Semmelweis University Medical School, Budapest

Soluble viral proteins produced in excess in HEp-2 cell cultures infected by adenovirus type 1 were separated and purified by anion-exchange chromatography. The chromatographic charcteristics of the capsid proteins were determined on DEAE Sephadex A-50 column and their migration in electric field was investigated. The iso-electric points of the hexon (pI = 4.55), penton (pI = 4.69) and fibre proteins (pI = 7.07) agreed well with the chromatographic characteristics of the respective proteins and with the results of the experiments performed in electric field. The amino-acid composition of acid hydrolysates of the separated and purified capsid components, of the virion and of HEp-2 cells were determined. Differences were found in the basic and acid amino acids.

EXAMINATION OF THE HUMAN ADENOVIRUS TYPE 1 WITH RESTRICTION ENZYMES

Gy. Berencsi, P. Medveczky, I. Nász, B. A. Zavizion, N. M. Chaplygina and T. I. Tikchonenko

Institute of Microbiology, Semmelweis University Medical School, Budapest and Institute of Virology, Academy of Medical Sciences of the U.S.S.R., Moscow, U.S.S.R.

The DNA of the prototype strain of adenovirus h1 was shown to have three specific recognition sites for the *Bam–HI* restriction endonuclease. The physical order of the four DNA fragments was found to be *A–C–B–D*. Melting experiments on the fragments led to the conclusion that *A* and *C* are located within the *G* : *C*-rich half of the molecule. The size of the fragments *A–B–C–D* was measured as 10.0 ± 1.13, 6.8 ± 0.21, 4.0 ± 0.34 and 2.2 ± 0.47 megadaltons, respectively. Evidence was presented that the *Bam–HI* enzyme preparations were contaminated by a KCl-sensitive restrictase activity, which has no recognition site on the DNA of adenovirus h2. Recently, international cooperation has made available the physical maps of adenovirus h1 DNA with the *Eco–RI*, *Sal–I* and *Hind–III* enzymes. The sequence of the *Eco–RI* fragments is *A–C–B* of 17.3 ± 0.1, 1.67 ± 0.12 and 3.75 ± 0.16 megadaltons, respectively. The size and sequence of the *Sal–I* fragments were found to be 6.0 *(B)*; 0.2 *(E)*; 5.0 *(C)*; 8.2 *(A)* and 4.1 *(D)* megadaltons from the *G* : *C*-rich end to the *A* : *T*-rich terminus. The tentative sequence of the *Hind–III* fragments seems to be *J–F–I–B–D–E–A–(G* or *H)–C–(G* or *H)–K*. The *Hind–III* fragments were found to be 5.38 ± 0.19 *(A)*; 3.5 ± 0.07 *(B)*; 2.3 ± 0.03 *(C)*; 2.18 ± 0.02 *(D)*; 2.11 ± 0.03 *(E)*; 1.72 ± 0.03 *(F)*; 1.43 ± ± 0.04 *(G)*; 1.43 ± 0.04 *(H)*; 1.23 ± 0.03 *(I)*; 0.72 ± 0.03 *(J)* and 0.54 ± ± 0.02 *(K)* megadaltons.

BIOCHEMICAL PROPERTIES OF THE ADENOVIRION-ASSOCIATED ENDONUCLEASE

P. Medveczky, S. Csuzi, É. Ádám, I. Nász and É. Szomolányi

Institute of Microbiology and First Institute of Chemistry and Biochemistry, Semmelweis University Medical School, Budapest

DNase was separated together with the penton antigen from purified adenovirus virions on CsCl gradient. Enzyme activity was measured by a method developed by the authors using isotope-labelled, cellulose adsorbed DNA substrate. The optimum concentration of the different ions was between pH 7 and 8 in 1 mM $MgCl_2$. The enzyme is inhibited by EDTA, 0.5 M NaCl,

0.5 M Na_2SO_4 and 100 mM $CaCl_2$. It is inactivated at 70 °C within 5 min. The endonuclease cannot be separated from the penton antigen by DEAE chromatography and only partially on DNA cellulose column. As an effect of the enzyme, random nicks arise in the superhelical DNA of the PM-2 bacteriophage.

EXPERIMENTAL CARRIERSHIP OF HUMAN ADENOVIRUS IN MICE

J. Horváth, P. Dán, G. Kulcsár, I. Nász and P. Geck

Institute of Microbiology, Semmelweis University Medical School, Budapest

Mice were infected with human adenovirus type 1 in the late phase of pregnancy. The virus could not be re-isolated from the progeny, though virus particles were present in the spleen for several months after birth. After neonatal infection, viral antigen (immunofluorescence), virus particles (electron microscopy) and infectious virus were demonstrated in some of the animals. In the maternal spleen and ovary, the virus was demonstrated by immunofluorescence for two months after inoculation. It is concluded that the latent-type adenovirus is capable of passing through the placenta and perinatal adenovirus infection may lead to carriership in mice.

DEMONSTRATION OF ADENOVIRUS ANTIBODIES BY MICRO-IMMUNODIFFUSION

I. Cserba, A. Lengyel and É. Ádám

Institute of Microbiology, Semmelweis University Medical School, Budapest

Blood samples were taken in heparinized glass capillaries and pooled virus (adenovirus type 1 or 5) obtained from infected tissue cultures or adenovirus type 1 hexon antigen purified by chromatography was used as antigen in the micro-immunodiffusion test. The micro-test proved to be practicable when blood sampling from veins meets with difficulties.

ONCOGENIC TRANSFORMATION OF HAMSTER EMBRYO FIBROBLAST CELLS BY UV-IRRADIATED HUMAN CYTOMEGALOVIRUS

I. Boldogh, É. Gönczöl and L. Váczi

Institute of Microbiology, University Medical School, Debrecen

After UV-irradiation (80 ergs/s/mm^2, 22 min) of human cytomegalovirus (CMV) inoculum, the virus genom is fully reactivated (infectious virus production) in permissive human embryonic fibroblasts and partially reactivated

(cytoplasmic antigens in 1–2 of 10^6 cells) in semi-permissive hamster embryonic fibroblast cells. In the hamster embryonic fibroblast monolayer infected with the UV-irradiated virus, 1–2 piled-up foci of 1–2 $\times$ 10^6 cells were observed at 20–22 days after infection. One of these foci could be propagated and a cell line (87-TRH-5) was established. The 87-TRH-5 cells induced tumour in newborn Syrian hamsters. CMV-specific surface and intracytoplasmic antigens could be detected in the cells by immunofluorescence using CMV-specific human and rabbit antisera. Sera of the tumour-bearing hamsters contained CMV-specific antibodies. Infectious virus could not be detected in the 87-TRH-5 cells after treatment of the cells with IUdR or fusion with permissive human embryonic fibroblast cells. The anti-p30 sera of RLV, FeLV and HaLV showed no immunofluorescence with the cell line.

Gp70 ONCORNAVIRUS ANTIGEN AND ANTIBODIES IN PATIENTS WITH MYELOID LEUKAEMIA AND PRELEUKAEMIA

F. D. Tóth, L. Váczi, I. Rédai, J. Jákó and K. Rák

Institute of Microbiology and Second Department of Medicine, University Medical School, Debrecen

Leukocytes from 12 patients with acute myeloid leukaemia (AML) and 15 with preleukaemia and those from 44 control subjects were tested for the presence of gp70 antigen by indirect membrane immunofluorescence. Rauscher–MuLv and Gibbon–LV anti-gp70 sera were used. Antigen of gp70 specificity was demonstrated on the leukocyte surface of 6 AML patients in advanced stage of the disease. Of the preleukaemia patients 4 proved positive for gp70 antigen while all the control samples were negative. In an advanced stage of AML, none of the serum samples contained anti-gp70 which, however, appeared during remission. Six subjects with preleukaemia were seropositive; 4 of 9 seronegative ones developed acute leukaemia within a year. Anti-gp70 was present in 17 control subjects.

DEMONSTRATION OF ANTIBODIES OF VARIOUS IMMUNOGLOBULIN CLASSES TO THE EPSTEIN–BARR VIRUS NUCLEAR ANTIGEN

J. Czeglédy, L. Gergely, L. Váczi and A. Szalka

Institute of Microbiology, University Medical School, Debrecen and László Central Hospital for Infectious Diseases, Budapest

A previously unknown antibody, viz., anti-EBNA-IgM. was demonstrated in acute EBV infection by the very sensitive anti-complement immunofluorescence (ACIF) test. The anti-EBNA-IgM present in the convalescence phase

of infectious mononucleosis (IM) disappeared within several weeks. The antibody could not be demonstrated in serum samples from either patients with IM of cytomegalovirus origin or anti-EBV seropositive healthy subjects. Anti-EBNA-IgM appears simultaneously with the anti-EBV-IgM, but its titres are considerably lower. Since it was demonstrated in all of the fresh EBV infections tested (29 cases), the positive test may serve as a further evidence for acute EBV infection. Control tests were performed to exclude the disturbing effect of nonspecific antinuclear antibodies expectable during the acute phase of IM. In EBV seropositive subjects, the majority of the anti-EBNA antibodies belong to the IgG class; non-complement-fixing IgA antibodies occur rarely.

PREPARATIONS OF AN ANTIGEN FOR THE DEMONSTRATION OF BOVINE LEUCOSIS ANTIBODIES IN THE IMMUNODIFFUSION PRECIPITATION TEST

L. Zsák

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

A simple rapid method has been evolved for the preparation of viral antigen from established cell cultures infected by the enzootic bovine leucosis virus. The antigen, purified by the fluorocarbon method and concentrated by ultracentrifugation, is of high purity and contains the whole virus antigen. It has been successfully applied for demonstration of precipitating antibodies in the immunodiffusion test.

SEARCH FOR AVIAN LEUCOSIS VIRUSES IN A HUNGARIAN POULTRY STOCK

Cs. Drén

Veterinary Research Institute, Hungarian Academy of Sciences, Budapest

A hundred birds were selected at random for virological and serological tests from a poultry stock. As a serological assay, the focus-reduction test was carried out in tissue cultures with the A, B, C and D subgroups of Rous sarcoma virus (RSV). The seronegative birds were examined for viraemia by performing the phenotypic mixture test using their plasma with RSV (RAV-O) and C/O cells. The poultry stock proved to be extensively infected by the B subgroup and to a lesser extent by the A subgroup of the virus. In a second series. 50 birds were included. The results agreed well with those of the first series, The low level of infection with the A serotype may reflect a genetic property of the stock.

DEMONSTRATION OF THE MC-29 VIRUS BY MEASURING THE REVERSE TRANSCRIPTASE ACTIVITY AND BY RADIOIMMUNOASSAY

K. NAGY, M. TÁLAS, L. G. ZAKHAROVA, I. STÖGER, A. D. ALTSTEIN and E. G. PIKER

Microbiological Research Group of the Hungarian Academy of Sciences, Budapest and Ivanovsky Institute of Virology, Academy of Medical Sciences of the U.S.S.R., Moscow, U.S.S.R.

The optimum circumstances (incubation temperature, incubation time, bivalent ion and detergent concentration and template primer complexes) for the MC-29 virus RNA-dependent-DNA-polymerase activity were determined. The activity was much higher with poly (rA). oligo (dT) than with poly (dA). oligo (dT) as template. This suggest the viral origin of the enzyme. A radioimmunoassay was developed for the demonstration of the MC-29 virus, using the group-specific proteins of avian myeloblastosis virus. Compared to the reverse transcriptase test, the radioimmunoassay proved to be a much more sensitive procedure.

STABILITY OF THE B.K. VIRUS HAEMAGGLUTININ AND THE CIRCUMSTANCES INFLUENCING B.K. VIRUS HAEMAGGLUTINATION

M. KENDE, GY. SZÜCS and M. UJ

Public Health Station, Pécs

The haemagglutinin of the B. K. virus retained its original titre for a week at any of the temperature 4, 20, 37 and 56 °C, irrespective of the NaCl molarity of the medium (from 0.15 M to 2.5 M and it was also stable in the pH range 5.5–9.5 between 4 and 37 °C, but tended to decline at 56 °C. In 1 M $MgCl_2$ solution, its titre fell significantly within 4 hr. The optimum pH of haemagglutination was between 5.5 and 6.5 and the HSAG (HEPES, salt, albumin, gelatin) buffer of pH 6.0 was found to be the most favourable medium.

HAEMAGGLUTINATION-INHIBITING ANTIBODY TO THE B.K. VIRUS IN A HUNGARIAN POPULATION

GY. SZÜCS, M. KENDE and M. UJ

Public Health Station, Pécs

A total of 694 serum samples divided in 10 age groups were tested for haemagglutination-inhibiting (HI) antibodies to the human papovavirus B. K. Group-O human erythrocytes were used in HSAG buffer of pH 6.0 at 4 °C.

Antibody was demonstrated in 72% of the samples. In the age group under 2 years 18%, in the group from 6 to 10 years 36%, of the sera were positive. The highest frequencies, 91–93, were found between 16 and 30 years of age, and the titres were the highest in the same age groups. Of the 51 sera with titres 1 : 640, 31 belonged to these groups. Titres 1 : 160 or higher occurred in 2% over 60 years, though antibody was demonstrated in 71% of the same sera. The specificity of the HI antibodies has been confirmed by indirect immunofluorescence tests carried out in Vero cells. Of the 72 serum pairs tested, 3 showed a 4-fold rise in titre. The clinical picture corresponded to an undefined respiratory infection in these cases.

ATTEMPTS TO PROPAGATE HEPATITIS B VIRUS IN HUMAN LIVER TISSUE CULTURES

I. Hollós, Á. Pálfi, B. László, Ö. Pohl, M. Börzsönyi and T. Neumark

National Institute of Hygiene, László Central Hospital for Infectious Diseases and National Institute for Rheumatology and Physiotherapy, Budapest

Attempts were made to propagate hepatitis virus in human embryonic liver tissue cultures. Cultures of series 1 were inoculated with serum samples taken from 10 patients in the early phase of acute hepatitis B. The characteristic electron microscopic picture of the core in the cell-nucleus and the appearance of HB_sAg in the culture medium were chosen as criteria of virus multiplication. This was proved in a single case only. In series 2, cultures were inoculated with isolated lymphocytes and blood plasma samples from patients. The method yielded reproducible results. Virus multiplication was proved in 9 of 12 experiments. Attempts to propagate the virus in serial passages have failed.

SEQUELAE OF RUBELLA DURING PREGNANCY

I. Mezey, M. Tass and E. Molnár

National Institute of Hygiene, Budapest

During the 1973–1974 rubella epidemic in Hungary, serum samples from 5905 gravidae were examined by the rubella HI method. The first samples from 5441 women proved to be positive. These patients were regarded as being immune. In 128 cases, fresh rubella was proved by seroconversion or by a significant titre rise. In further 781 pregnancies, the history and the serological findings were suggestive of rubella virus infection. The outcome of these,

altogether 909, pregnancies and the condition of the live-born infants were studied by use of questionnaires. Data of 623 questionnaires returned showed that 284 of the pregnancies had been interrupted artificially, whereas 339 gravidae had undertaken the risk. Spontaneous abortion occurred in 29 cases. Of the 310 live-born children, 7 showed signs attributable to the teratogenic effect of rubella virus. Diagnosis of the rubella syndrome could, however, be confirmed in a single case only.

CMV MONONUCLEOSIS VERIFIED BY SEROLOGICAL METHODS

Zs. Kósa, M. Koller, L. Binder, Z. Pintér and A. Pálinkás

National Institute of Hygiene and László Central Hospital for Infectious Diseases, Budapest

Thirty-three cases of CMV mononucleosis verified by virus serological methods are reported. The clinical symptoms and the results of laboratory tests were analysed in 15 of these cases. The CMV complement-fixing antibody titres and the demonstrability of specific IgM antibodies were recorded systematically.

IN VITRO AGGREGATION OF ADENOVIRUS SOLUBLE HEXONS

I. Nász and É. Ádám

Institute of Microbiology, Semmelweis University Medical School, Budapest

Highly purified soluble adenovirus type 1 hexons were crystallized by dialysing against 0.5 M acetate buffer, pH 4.5, for 3 days. Crystals of tetraedral and prismatic shapes were produced after storage at 4 °C for 1 to 10 days with edge sizes ranging between 0.01 and 0.1 mm. The crystals could be washed in acetate buffer and dissolved in basic Tris-buffer. In agar gel precipitation test the solution of the crystalls gave specific reaction with adenovirus type 1 anti-hexon immune sera. In the solution many single hexons were visible by electron microscopic examination. The course of crystallization was followed closely with electron microscope. In the early stage only diffusely dispersed single hexons could be observed similar to the control hexon material and to hexons in the solution of crystals. Later two-dimensional hexon crystals developed. The hexon-sheet contained hexons located close to each other without any holes forming regular crystalline arrays. This structure is different from the two-dimensional crystal lattice of adenovirus type 5 hexons, reported by H. G. Pereira and N. G. Wrigley and by E. B. Carstens and R. C. Marusyk. It can be assumed

that the formation of such compact two-dimensional hexon crystals may play a role in the growth mechanism of three dimensional crytals. Filamentous structures made up of three or more hexon rows, and multilayered microcrystals were also detected.

Agricultural and Industrial Microbiology

EARLY DEFENCE MECHANISM INDUCED BY PATHOGENIC BACTERIA IN TOBACCO PLANT

J. Burgyán and Z. Klement

Research Institute for Plant Protection, Budapest

A new type of defence reaction of plants induced by bacteria has been demonstrated. Inhibition of bacteria developed as early ad 3–6 hr after infection. Experiments indicated that the inhibitor was produced in compatible host-pathogen combinations, too, but it was ineffective for the compatible pathogens. The results suggest that the host–bacterium interaction studied is different from the hypersensitivity reaction or premunity of plants.

EFFECT OF HERBICIDES ON THE GROWTH AND N-FIXING CAPACITY OF AZOTOBACTER CHROOCOCCUM

B. Helmeczi

Institute of Soil Science and Microbiology, University of Agricultural Sciences, Debrecen

Nine strains of *Azotobacter chroococcum* were isolated from leached chernozem, rust coloured forest soil, solonchak-solonetz meadow solonetz soil types originating from Czechoslovakia, Romania and the Soviet Union. The following herbicides and combinations were tested: Afalon, Aniten, Banvel, Buvinol, Dikonirt, Dikotex, Gramoxone, Hungaria K64, Hungazin PK, NaTa, Nitrikol, Pyramin, Ramrod, Satecid, Sys 67 ME; and Satecid 60 WP + Terbutrin 50 WP, Satecid 60 WP + Terbutrin 50 WP + Aktikon 90 WP, Trikomb, Satecid 65 WP + Aktikon 90 WP, Satecid 65 WP with urea + Aktikon 90 WP, Satecid 65 WP with urea + Terbutrin 50 WP + Aktikon 90 WP, Propachlor 40 EC + Sutan 80 EC, Propachlor 40 EC + Eptan SH 202, Satecid 65 WP with urea + Afalon 20 EC, Aktikon 90 WP + Eptan EC, Satecid AT + methyl-carbamate. Herbicides not applied in combinations affected the N-fixing capacity of *Azotobacter* differently but not significantly. According to their effect the herbicides could be grouped as stimulators (Nitrikol, Ramrod,

Buvinol, Sys 67 ME); neutrals (Hungazin PK, NaTa, Banvel) and inhibitors (Gramoxone, Hungaria K 64, Dikotex, Dikonirt, Aniten, Afalon, Satecid, Pyramin). The combinations on growth of *Azotobacter* proved to be similar in their effect on the N-fixing capacity. The greatest inhibitory effect was shown by the Satecid AT + methyl-carbamate + Propachlor 40 EC + Aktikon 90 WP combinations.

EFFECT OF SURFACTANTS APPLIED IN PESTICIDE FORMULATIONS ON THE PHYSIOLOGICAL ACTIVITY OF SOIL MICROBES

T. Cserháti and M. Kecskés

Research Institute for Plant Protection, Budapest and Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest

The effect of non-ionic (alkyl-phenyl-polyglycolether type), anionic and mixed surfactants occurring in pesticides was studied on the physiological activity of soil microorganisms by the resazurine reduction test. All of them but one inhibited the reduction capacity of the microorganisms. A regression was found between the surface tension decreasing effect and the microbial activity but the regression explained only 68% of the total effect. The Hansch constant characteristic for lipophilia of the compounds was determined by reversed phase thin layer chromatography, but this, too, failed to explain the biological activity. The constant for the CH_3 group surfactants agreed with data in the literature but the values obtained for the C_2H_4O group agents deviated significantly. It is concluded that the Hansch constants for the substituting groups given in the literature can not be applied for estimating the biological—activity of surfactants.

BIOLOGICAL STABILITY OF [$^{14}CH_3$]6-METHYL URACIL IN PLANTS AND ITS BIODEGRADATION BY SOIL MICROORGANISMS

E. Lakatos, L. Horváth and B. I. Pozsár

Institute of Isotopes of the Hungarian Academy of Sciences, Budapest

The 6-methyl uracil as a decomposition end-product of many uracil-type herbicides is active biologically (chlorophyll preservation, increase of permeability) and relatively stable in leaves of Pinto bean *(Phaseolus vulgaris* L.) variety. 6-Methyl uracil preparations labelled with radiocarbon were incorporated into leaves at identical aliquots, resolved and measured by the liquid scintillation method. The level of 6-methyl uracil decreased by 15% at the end of the first day, by 18% at the end of the first week and by 33% at the end of the second week as compared to the initial state. Decomposition is not

due to demethylation, because it was not detectable to a significant degree in the two most important methyl acceptors (methionine, choline). The biodegradation of 6-methyl uracil by soil microorganisms is primarily associated with the effect of soil algae. The methyl oxidase activity of *Chlorella* sp. could be detected in quantities almost negligible compared to the intensive methyl-oxidase activity observed with [$^{14}CH_3$]CCC.

EFFECT OF INTERACTIONS OF ZOOCIDES AND LINURON HERBICIDE ON BACTERIAL AND FUNGAL STRAINS

Cs. Dobolyi and M. Kecskés

National Institute of Hygiene, Budapest and Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest

Technical preparations of prothoate "o,o-diethyl-S-(N-isopropylcarbamoyl-methyl)" and phosphorodithioate "o,o-dimethyl-S-(N-methylcarbamoyl-methyl) phosphorodithioate" with increasing stimulatory effect were studied on the growth of 17 strains of different microbial groups by the microfermentor and biophotometer tests. Protoate (10 ppm) stimulated the growth of *Klyveromyces marxianus* 74/K_2, *Cryptococcus albidus* var. *diffluens* P_7, *Torulopsis candida* S 579, *Candida pseudotropicalis* 372, *C. utilis* 472, *Rhodotorula rubra* 1165 yeast strains, of yeast-like *Prothotheca wickerhamii*, and of *Geotrichum candidum* 76/K65, *Aspergillus tamarii* P_{31} and *Fusarium oxysporum* P_{17} filamentous microscopic fungi. It exerted a slight inhibitory effect on bacterial strains belonging to *Pseudomonas*, *Rhizobium* and *Arthrobacter* genera. Phosphamidon "2-chloro-2-diethylcarbamoyl-1-methyl vinyl dimethyl-phosphate" in doses applied in agricultural practice (1–10 ppm) did not or did hardly influence the growth of the microbial strains investigated. The inhibitory effect of linuron "N-3,4-dichlorophenyl(-N-methoxy-N-methylurea)" applied together with trichlorfon, dimethoate, prothoate and phosphamidon zoocides was unequivocal.

MICROBIOLOGICAL AND CHEMICAL DECOMPOSITION OF CHLORBROMURON IN DIFFERENT SOILS

É. János and T. Cserháti

Research Institute for Plant Protection, Budapest

Decomposition of the herbicide chlorbromuron was investigated in non-sterilized and sterilized soils: in peat brown earth (Raman type brown forest soil) and chernozem with forest residue soil types at 10, 18, 26 and 37 °C for

10 weeks. The amount of chlorbromuron was measured by gas chromatography. The amount of chlorbromuron decreased logarithmically with time; the correlation between the logarithm of chlorbromuron concentration and incubation time was linear, indicating that decomposition occurs according to a reaction of the first order. In soils containing the natural microflora the decomposition was faster in evergy case. The enery of activation calculated from the rate constants of microbial decomposition was found to be 15–16 kcal/mole, significantly smaller than the energy needed for the hydrolytic decomposition of chlorbromuron (20–22 kcal/mole). The microbial decomposition rate increased linearly between 10–26 °C; at 37 °C it decreased a little. The proportion of chemical decomposition was important at 10 and 37 °C.

MOULD INFECTION OF ROUGH FODDER

I. Fésüs

National Inspectorate for Animal Breeding and Feed Control, Budapest

In a survey for mycotoxins, 100 fibrous fodder samples were subjected to mycological investigation. The "total" mould counts for 44% of the samples investigated were above 10 000/g and the average mould number per sample was 23 032/g. In the mould flora of the samples the following genera were represented: *Acremonium, Acrostalagmus, Alternaria, Acremoniella, Botrytis, Cercospora, Chaetomium, Cladosporium, Glyocladium, Monocillium, Nigrospora, Paecilomyces, Scopulariopsis, Spicaria, Stemphylium, Stachybotrys, Penicillium, Aspergillus, Circimella, Mucor, Fusarium.*

THIOBACILLUS FERROOXIDANS IN BIOMETALLURGICAL PROCESSES

J. Görög, G. Pap, Z. Lakatos and M. Miskei

Department of Agricultural Chemical Technology of Budapest University Technical School and Research Engineering and Prime Contracting Centre of the Hungarian Aluminium Corporation, Budapest

Among the many *Thiobacillus ferrooxidans* strains collected from sulphide ores and pyrite coal fields, the strain isolated from Parádsasvár was the most active in the oxidation of ferro compounds. Using this strain, a continuous three stage oxidation process was developed, by which 90 g per litre $FeSO_4$ could be almost completely oxidized to $Fe_2(SO_4)_3$ by an once through treating. This $Fe_2(SO_4)_3$ solution is suitable for oxidative leaching of ores, spoil banks, metal-containing industrial wastes and for water cleaning.

MICROBIOLOGICAL RECULTIVATION OF OIL POLLUTED AREAS

F. Buday, G. Török, Z. Gergely, Á. Szakács, A. Koltai and I. Farkas

Department of Microbiology University of Agricultural Sciences, Gödöllő, Hungarian Mineral Oil Trade Company, Budapest, Gas and Oil Transporting Company, Siófok and Phylaxia Veterinary Biologicals and Foodstuffs Co., Budapest

Mixed cultures were isolated from samples of oil-polluted soil. *Nocardia*, *Pseudomonas*, *Flavobacterium* and *Candida* species were predominant in the mixture which yielded the most active cultures using oil as carbon source. These cultures, which could decompose in five days 80–85% of the added oil were tested in small plot experiments. As these yielded favourable results, an area of 2.5 hectares, where a broken oil pipe had polluted the site, was inoculated at 6 litres/m^2 with liquid medium (Phylaxia, Budapest), containing 3.5×10^9 cells, in autumn 1975. By spring 1976, 96% of the oil was decomposed in the "A" level of the soil, 60% in the "B" level and 30% in the "C" level.

VIRULENT RHIZOBIOPHAGES IN HUNGARIAN SOILS

E. Manninger

Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest

In plants producing rhizobium inocula as well as in agricultural practice rhizobiophages cause much damage. Their occurrence in Hungarian soils has not been studied so far. For this reason, rhizobiophages were isolated from samples of twelve sites of different soil types, using G5 bacterium filter or chloroform and 10^8/ml titre cell suspensions. For the purpose, 60 *Rhizobium* strains were isolated from root nodules of *Vicia villosa*, *Phaseolus vulgaris*, *Trifolium pratense*, *Soya max*, *Medicago sativa*, *Lupinus luteus* and *Robinia pseudoacacia*. Effectivity, morphological and biochemical characteristics and serological behaviour of the strains were determined. Both methods were suitable for isolating rhizobiophages present in the soil: phages were obtained from two-thirds of nearly 1500 samples. It is concluded that for the common failure of rhizobium inoculations the fact might be responsible that the phages are much more frequent than it was supposed so far.

Mycology

PROTOPLAST FUSION IN SACCHAROMYCES CEREVISIAE STRAINS OF IDENTICAL AND DIFFERENT MATING TYPES

A. MARÁZ, M. KISS and L. FERENCZY

Department of Microbiology, Attila József University, Szeged and Department of Dermato-Venerology, University Medical School, Szeged

Protoplasts of *Saccharomyces cerevisiae* are able to fuse in the presence of polyethylene glycol as inducer. When auxotrophic mutants are used, the fusion products can selectively be regenerated. The fusion of protoplasts is not affected essentially by mating factors, since the fusion frequency of identical (a + a or $\alpha + \alpha$) or different (a + α) mating types is nearly equal. The fusion products are diploid; they resemble in biochemical and genetical properties the diploid cells produced by sexual processes. Fusion products (a + α) can be induced to sporulate and the parental haploid types can be recovered by meiotic segregation. Sporulation cannot be induced in (a + a) and ($\alpha + \alpha$) diploids. By induced haploidization, cells of parental biochemical characters can, however, be recovered by mitotic segregation.

MITOCHONDRIAL TRANSFER BY PROTOPLAST FUSION

L. FERENCZY, A. MARÁZ and M. KISS

Department of Microbiology, Attila József University, Szeged and Department of Dermato-Venerology, University Medical School, Szeged

Attempts have been made to transfer mitochondria of *Saccharomyces cerevisiae* from respiration-sufficient ("grande") cells into respiration-deficient ("petite") cells by protoplast fusion. Characteristics of the haploid donor mutant were, α ura 1 trp 1 nuclear markers and ω^+ E_{221} mitochondrial markers; those of the haploid recipient mutant, α ad 2 nuclear markers and ϱ^0 mitochondrial marker. Polyethylene glycol (mol wt 4000) was used as fusion inducer. The mitochondrial genetic transfusion occurred at 0.6% frequency. The fusion products proved to be α ϱ^+ E^R prototrophic diploids. From the diploids α ad 2 ϱ^+ haploids and aneuploids harbouring mitochondria of the donor strain were recovered by induced haploidization. Interspecific mitochondrial DNA transfer also seemed to be possible.

GENETICAL CHARACTERIZATION OF PROTOPLAST-FUSION PRODUCTS OF RHODOSPORIDIUM AND SCHIZOSACCHAROMYCES STRAINS

M. Sipiczki

Department of Microbiology, Attila József University, Szeged

Prototrophic fusion products are obtained as a result of the fusion of different auxotrophic mutants. Fusion and complementation may occur between cells of both identical and opposite mating types. Prototrophic fusion products were isolated at about the same frequency whether (A and α) and (α and α) strains of *Rhodosporidium toruloides* or (h^- and h^-) and (h^- and h^+) strains of *Schizosaccharomyces pombe* were subjected to fusion. As a result of fusion, prototrophs of various genotypes may arise. Ploidy can be concluded at from the genetical and cytological results. Cell volume and the amount of DNA were approximately double in the fusion products. On the basis of random spore analysis and mitotic segregation data, the most probable state is diploid.

PROTOPLAST FUSION AND COMPLEMENTATION BETWEEN ASPERGILLUS NIDULANS AND ASPERGILLUS RUGULOSUS AUXOTROPHS

F. Kevei and J. F. Peberdy

Department of Microbiology, Attila József University, Szeged and Department of Botany, University of Nottingham, Nottingham, U.K.

Interspecific heterokaryons were produced between auxotrophic mutants of *Aspergillus nidulans* and *Aspergillus rugulosus* by polyethylene glycol-induced fusion of protoplasts. The heterokaryons grew at a slower rate and differed in colony form from the intraspecific heterokaryons of the parent strains. When cultivated on complete medium, the heterokaryons readily segregated into parental strains in sectors. During incubation for 7 to 10 days on minimal medium, many of the heterokaryons gave rise to vigorously growing sectors characterized by the secretion of brown pigment and by their stability when subcultured on complete medium. Protoplasts isolated from the heterokaryons yielded the same new type of colonies when regenerated on minimal medium. The new type of colony appeared besides auxotrophic parents in the course of regeneration on complete medium; their proportion increased with time. Based on the properties of the new colony type, it is suggested that an interspecific diploid has arisen.

PRODUCTION AND CHARACTERIZATION OF ANUCLEAR PROTOPLASTS OF GEOTRICHUM CANDIDUM

L. MANCZINGER and L. FERENCZY

Department of Microbiology, Attila József University, Szeged

Attempts were made to develop a method suitable for transfer of mitochondrial genetic information without the disturbing effect of the nuclear genome. For this purpose enucleated or non-nucleated protoplasts containing mitochondria seemed suitable. In the case of *Geotrichum candidum* the best results were obtained when arthrospores in the initial stage of germination were protoplasted. When protoplast formation was performed after a germination time of 120–150 min and the protoplast suspension was fractionated at 900 *g* for 5 min, the supernatant contained practically only non-nucleated protoplasts. The amount of non-nucleated protoplasts was 27% of the original. Ultracentrifugal separation of the mitochondrial DNA from nuclear DNA, and electron microscopic observations revealed mitochondria in the protoplasts of the fraction.

YEAST CONTAMINATION OF PUBLIC BATHS IN HAJDÚ-BIHAR COUNTY

K. ANDRÁSSY and I. HORVÁTH

Public Health Station, Debrecen

A total of 1713 water samples was collected from public baths. Among them, 389 proved positive for yeasts. *Candida albicans* was isolated from 9 samples. It is suggested that the role of public baths in the summer peak of mycotic skin and mucosa diseases should not be neglected. Demonstration of yeasts in bath water and elsewhere in public baths (pavement, shower etc.) is a good indicator of hygienic conditions.

EFFECT OF OCHRATOXIN A, PATULIN T-2 TOXIN AND BUTENOLID IN BROILER CHICKEN

Z. BITAY, R. GLÁVITS and G. SELLYEY

Central Veterinary Institute, Budapest

Mycotoxins were mixed into the feed of five groups of chicken of 6–8 birds each for a period of 2–8 weeks. The toxin concentrations per kg feed were as follows. Group A, 5.65 mg patulin; group TB_1, 2.45 mg T-2 toxin + 2.12 mg butenolid; group TB_2, 1.22 mg T-2 + 1.06 mg butenolid; group TB_3, 0.61 mg

T-2 + 0.53 mg butenolid; group O, 2.00 mg ochratoxin A. Two groups served as normal controls. Clinical observations, and pathological, histopathological, bacteriological, virological and analytical investigations were carried out. Lesions in group A were restricted to the liver, those in group O to the liver and the kidneys. T-2 + butenolid caused mucosal necroses in the mouth and the crop, atrophy of the bursa of Fabricius and of the thymus and an inflammation of the intestinal mucosa. The chicken were retarded in growth; individual differences in body weight *vs.* the controls ranged from 300 to 655 g and the production of 1 kg live weight required an extra feed of 430 to 1085 g.

Immunology

STIMULATION OF CELL-MEDIATED IMMUNE RESPONSE TO INFECTION WITH LCM VIRUS IN SUCKLING MICE

Zs. Bános, P. Anderlik, I. Szeri and B. Radnai

Institute of Microbiology, Semmelweis University Medical School, Budapest and István Hospital, Budapest

The neurological symptoms and the fatal lymphocytic choriomeningitis following intracerebral LCM virus infection are due to the cellular immune response of the host. Since the outcome of the infection depends on the immunological reactivity of the animals it depends also on their age. In the present study one-week-old mice were infected intracerebrally with LCM virus. Part of the animals had been treated with phytohaemagglutinin (PHA) prior to infection while the other part (litter-mates) served as controls. PHA-pretreated mice showed a higher mortality rate, a shorter incubation time and more pronounced meningeal infiltration than did the controls. The course of the infection in the PHS-pretreated one-week-old mice was comparable to that in the two-week-old ones without pretreatment with PHS. It is concluded that PHA-pretreatment contributed to the development of fatal meningitis by accelerating the development of the cellular immune reactivity.

SPECIFIC PROPHYLAXIS OF PNEUMOENTERITIS OF LAMBS CAUSED BY ADENOVIRUS INFECTION

V. Pálfi and S. Belák

Central Veterinary Institute, Budapest and Department of Epizootiology, University of Veterinary Sciences, Budapest

Pneumoenteritis due to adenovirus infection was observed in suckling and in fattening lambs. Fever and enteritis appeared after an incubation time of 7 to 8 days, followed 2 to 3 days later by acute respiratory symptoms.

During the acute phase sporadic death may occur. Most deaths are caused by bacterial superinfections in the chronic phase of the disease. Virological examination of epidemic cases revealed Gy/14 and ORT/111 viruses, related to ovine 1 and bovine 2 type adenoviruses, respectively. From the virus strains isolated a vaccine was prepared. It proved efficient in both laboratory experiments and under natural conditions in farms. It was found that suckling lambs must be protected passively, by immunization of the pregnant ewes. Seronegative ewes or those with a low virus neutralization titre can be immunized by 2 injections at a 2 week interval, the second injection being performed 3 to 4 weeks before yeaning. By the end of the 2nd month of age the lambs born of vaccinated ewes have a low titre (less than 1 : 8), therefore they must be vaccinated once at this time. Vaccination of lambs cannot be done earlier because passive immunity with a neutralization titre of 1 : 16 or more interferes with active immunization. The above vaccination method ensures protection against adenovirus diseases in both suckling and fattening lambs.

TOXOPLASMA ANTIBODIES IN PREGNANT WOMEN

I. Málovics and S. Pácsa

Institute of Microbiology, University Medical School, Pécs

Sera of pregnant women were titrated for toxoplasma antibodies with the complement fixation (CF) and the indirect immunofluorescent (IF IgG and IF IgM) methods, in order to provide further data on the number of subjects infected with *Toxoplasma* and to compare the results of the CF and of the IF methods. CF antibody could be demonstrated in 25% of the 560 serum samples. While the IF method proved more sensitive than the CF method, results of the CF and IgM/IF techniques were almost the same.

DEMONSTRATION OF HAEMOPHILUS INFLUENZAE ON THE RESPIRATORY MUCOSA BY IMMUNOFLUORESCENCE

Z. Csizér

Institute for Serobacteriological Production and Research "Human", Budapest

The immunofluorescent method proved suitable for detecting non-capsular *Haemophilus influenzae* by the colonization on, and penetration into, the tissues of the respiratory tract. Colonization of *H. influenzae* was found in the upper respiratory tract of 80% of both healthy and atopic subjects. In the material tested no *H. influenzae* could be demonstrated in the bronchi and the lung tissue.

THE AGE-DEPENDENT IMMUNOLOGICAL (ANTITOXIN-PRODUCING) POTENTIAL OF HUMANS

L. RÉTHY

Institute for Serobacteriological Production and Research "Human", Budapest

On the basis of human immunizations with adsorbed tetanus and/or diphtheria toxoids, a strict correlation was established between the age and the antitoxin-producing capacity of humans. The antibody-producing capacity increases with age in children, reaches its peak in adolescence, remains stable between 20 and 40 years of age then decreases slowly between 40 and 50 years and a rapid decline is characteristic beyond 50 years. The antitoxin-producing capacity could be increased by prolonging the immunization interval in children, adolescents and adults between 20 and 40 years. Above 40 years of age, prolongation of interval does not result in a significant increase in antitoxin production. On the administration of immunopotentiating agents i.e. endotoxins or endotoxin-like substances, together with the toxoid, the immune potential, as measured by the amount of produced antitoxin, significantly increases in all age groups. Exploiting the immunopotentiating activity of the mentioned substances, even subjects above 60 years of age can successfully be immunized, and full protection can be ensured.

SEASONAL VARIATIONS IN THE IMMUNE (ANTITOXIC) RESPONSIVENESS OF HUMANS AND ANIMALS

L. HEGEDÜS, L. RÉTHY and L. BÁCSKAI

Institute for Serobacteriological Production and Research "Human", Budapest

The role of factors contributing to the seasonal variations in antitoxic immune response of humans and experimental animals was studied. Experimental data were evaluated by factorial analysis. Deficiencies in the quality of food were found of utmost importance. The immunological reactivity of malnourished animals could be restored with additional food, thus, well-fed animals did not display seasonal variations. Another factor of importance was the type of the vaccine used. The immune response to vaccines with mineral or bacterial adjuvants was not influenced by the season. In humans, seasonal variations affected the antibody response only in the case of tetanus toxoid vaccination i.e. vaccine not containing adjuvant of bacterial origin. The importance of seasonal effects on both the primary and the secondary immune response of malnourished animals was observed, i.e. immunological memory was not affected.

COMPARISON OF PPD PREPARATIONS PREPARED FROM HUMAN AND BOVINE STRAINS OF MYCOBACTERIUM TUBERCULOSIS

J. Zsidai, Z. Csizér, Zs. Vas and I. Joó

Institute for Serobacteriological Production and Research "Human", Budapest

Groups of guinea pigs were sensitized with either human or bovine strain of *Mycobacterium tuberculosis* or both, suspended in paraffin oil. Six weeks later the potency of PPD preparations from the same strains or of their mixtures was tested against a standard, in each group. Titrations were carried out by intradermal injections of 20 and 100 TU/0.1 ml. Readings were done 24 and 48 hr later. Dose-response lines were analysed by analysis of variance. Bovine PPD was the most potent in all groups; no statistically significant differences were found between the PPD preparations tested.

COMPETITION OF ANTIGENS IN PERSONS WITH INHERITED ANTIGEN-SPECIFIC PASSIVE IMMUNITY

L. Réthy and L. Hegedüs

Institute for Serobacteriological Production and Research "Human", Budapest

In a previous study, a negative correlation was found between the amount of concurrent antigen associated with the test antigen and the intensity of humoral response, in case of T-dependent antigens. The present study deals with the competition of antigens in the presence or absence of maternal antibodies in three-month old children vaccinated with diphtheria-pertussis-tetanus vaccine. A competition of antigens was observed even when antibody specific either to the test or to the competing antigen was present as early as before vaccination. The observed antigenic competition might not be part of the negative feedback mechanism. Immunological memory was not affected by the concurrence of antigens. It is suggested that in the case of specific inherited immunity, the features of antigenic competiton of the intermolecular type are comparable to the features of those intercellular immunological processes which may have a part in intramolecular antigenic competition.

DETERMINATION OF THE MINIMUM ANTIGEN DOSE NECESSARY FOR COMPLETE PROTECTION AGAINST DIPHTHERIA IN CHILDREN

L. Réthy, L. Hegedüs and L. Bácskai

Institute for Serobacteriological Production and Research "Human", Budapest

To define the minimum antigen dose of diphtheria toxoid ensuring full protection of children, comparative immunological laboratory and field in-

vestigations were performed using DTP–AP vaccines. The children were immunized twice or three times at four week intervals. The criteria of safe protection were (i) an individual antitoxin titre: ≥ 0.03 AU/ml serum; (ii) a diphtheria antitoxin titre of ≥ 0.03 AU/ml had to be found in $\geq 95\%$ of the immunizees. (i) In trials based on the "one stimulant method USP" 53 DTP–AP samples were tested in 2700 children. The results showed that the diphtheria component induces full protection if the guinea pigs produce at least 5 AU/ml antitoxin immune response after single shot immunization. (ii) In a comparative study based on Prigge's active protection laboratory test 16 DTP–AP samples were tested in 1200 children. Full protection could be achieved with two-shot immunization by 150 PU per dose in children aged 3 months or by 100 PU in 6 months old children. With three-shot immunization, the above degree of anti-diphtheria protection could be reached by 100 PU per dose in three-month-old children. It is suggested to revise the laboratory minimum requirements.

INDEX

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója. Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1978. II. 20. — Terjedelem: 7,65 (A/5) ív, 8 ábra

78.5530 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:

Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P.O.B. 64, Ungarn

An die gleiche Anschrift ist jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $36.00.

Bestellbar bei »Kultura« Außenhandelsunternehmen (H-1389 Budapest 62, P.O.B. 149, Bankkonto Nr. 218-10990) oder seinen Auslandsvertretungen.

Les *Acta Microbiologica* paraissent en fransais, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiene Publique, H-1966 Budapest, P.O.B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement: $36.00 par volume.

On peut s'abonner à l'Entreprise du Commerce Extérieur «Kultura» (H-1389 Budapest 62, P.O.B. 149, Compte courant No. 218-10990) ou chez représentants à l'étranger.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P.O.B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — $36.00 за том.

Заказы принимает предприятие по внешнец торговле «Kultura» (H-1389 Budapest 62, P.O.B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

AUSTRALIA
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE, Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 145 Ackland Street, *St. Kilda (Melbourne), Victoria 3182*

AUSTRIA
GLOBUS, Höchstädtplatz 3, *1200 Wien XX*

BELGIUM
OFFICE INTERNATIONAL DE LIBRAIRIE, 30 Avenue Marnix, *1050 Bruxel'es*
LIBRAIRIE DU MONDE ENTIER, 162 Rue du Midi, *1000 Bruxelles*

BULGARIA
HEMUS, Bulvar Ruski 6, *Sofia*

CANADA
PANNONIA BOOKS, P.O. Box 1017, Postal Station "B", *Toronto, Ontario M5T 2T8*

CHINA
CNPICOR, Periodical Department, P.O. Box 50, *Peking*

CZECHOSLOVAKIA
MAD'ARSKÁ KULTURA, Národní třida 22, *115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 46, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

DENMARK
EJNAR MUNKSGAARD, Norregade 6, *1165 Copenhagen*

FINLAND
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128, *SF-00101 Helsinki 10*

FRANCE
EUROPERIODIQUES S. A., 31 Avenue de Versailles, *78170 La Celle St.-Cloud*
LIBRAIRIE LAVOISIER, 11 rue Lavoisier, *75008 Paris*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac, *75240 Paris Cedex 05*

GERMAN DEMOCRATIC REPUBLIC
HAUS DER UNGARISCHEN KULTUR, Karl-Liebknecht-Strasse 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT, Strasse der Pariser Kommüne 3—4, *DDR-104 Berlin*

GERMAN FEDERAL REPUBLIC
KUNST UND WISSEN ERICH BIEBER, Postfach 46, *7000 Stuttgart 1*

GREAT BRITAIN
BLACKWELL'S PERIODICALS DIVISION, Hythe Bridge Street, *Oxford OX1 2ET*
BUMPUS, HALDANE AND MAXWELL LTD., Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate, *Wellingborough, Northants NN8 2QT*
WM. DAWSON AND SONS LTD., Cannon House, *Folkestone, Kent CT19 5EE*
H. K. LEWIS AND CO., 136 Gower Street, *London WC1E 6BS*

GREECE
KOSTARAKIS BROTHERS, International Booksellers, 2 Hippokratous Street, *Athens-143*

HOLLAND
MEULENHOFF-BRUNA B.V., Beulingstraat 2, *Amsterdam*
MARTINUS NIJHOFF B.V., Lange Voorhout 9—11, *Den Haag*
SWETS SUBSCRIPTION SERVICE, 347b Heereweg, *Lisse*

INDIA
ALLIED PUBLISHING PRIVATE LTD., 13/14 Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD., Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTD., Books Import Division, Chandralok, 36 Janpath, *New Delhi 110001*

ITALY
EUGENIO CARLUCCI, P.O. Box 252, *70100 Bari*
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 45, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58, *20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

JAPAN
KINOKUNIYA BOOK-STORE CO. LTD., 17-7 Shinjuku-ku 3 chome, Shinjuku-ku, *Tokyo 160-91*
MARUZEN COMPANY LTD., Book Department, P.O. Box 5050 Tokyo International, *Tokyo 100-31*
NAUKA LTD. IMPORT DEPARTMENT, 2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

KOREA
CHULPANMUL, *Phenjan*

NORWAY
TANUM-CAMMERMEYER, Karl Johansgatan 41—43, *1000 Oslo*

POLAND
WĘGIERSKI INSTYTUT KULTURY, Marszałowska 80, *Warszawa*
CKP I W ul. Towarowa 28 *00-958 Warsawa*

ROUMANIA
D. E. P., *Bucureşti*
ROMLIBRI, Str. Biserica Amzei 7, *Bucureşti*

SOVIET UNION
SOJUZPETCHATJ — IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

SPAIN
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

SWEDEN
ALMQVIST AND WIKSELL, Gamla Brogatan 26, *S-101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB, Box 346, *401 25 Göteborg 1*

SWITZERLAND
KARGER LIBRI AG, Petersgraben 31, *4011 Basel*

USA
EBSCO SUBSCRIPTION SERVICES, P.O. Box 1943, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC., 15 Southwest Park, *Westwood, Mass, 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC., 140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC., 7250 Westfield Avenue, *Pennsauken N. J. 08110*

VIETNAM
XUNHASABA, 32, Hai Ba Trung, *Hanoi*

YUGOSLAVIA
JUGOSLAVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišića 1, *21000 Novi Sad*

12. VII. 1978 HU ISSN 0001—6187 Index: 26.016

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS XXV FASCICULUS 3

AKADÉMIAI KIADÓ, BUDAPEST

1978

AMAHA 5 25(3) 159–231 1978

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1954 BUDAPEST, ALKOTMÁNY UTCA 21.

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24, Bankszámla: 215-11448), a külföld számára pedig a „Kultura" Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, Bankszámla: 218-10990) vagy annak külföldi képviseleteinél.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P.O.B. 64, Hungary

Correspondence with the editors should be sent to the same address.

Subscription: $ 36.00 a volume.

Orders may be placed with "Kultura" Foreign Trading Company (H-1389 Budapest 62, P.O.B. 149, Account No. 218-10990) or its representatives abroad.

Acta microbiol. Acad. Sci. hung. 25, 159—164, 1978

HAEMATOLOGIC EFFECT AND SHWARTZMAN REACTIVITY OF RADIODETOXIFIED ENDOTOXIN*

T. Szilágyi, Hedvig Csernyánszky, E. Gazdy and L. Bertók

Institute of Pathophysiology, University Medical School, Debrecen and "Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

(Received June 25, 1977)

Comparative experiments were made in rabbits with *Escherichia coli* O89 endotoxin and endotoxin detoxified by ionizing radiation (^{60}Co-gamma, 5 Mrad). Radiation significantly weakened the leukopenia and thrombocytopenia provoking effect of endotoxin. Radiodetoxified endotoxin decreased the fibrinogen level only slightly and caused insignificant changes in reptilase time. The complement level was decreased less by the detoxified than by the parent endotoxin. Even the local Shwartzman phenomenon inducing capacity of radiodetoxified endotoxin decreased significantly, particularly when it was used for preparation and provocation, too.

In recent years, several methods have been used for the preparation of slightly toxic endotoxin with unchanged tolerance inducing and natural resistance increasing capacity [1–4]. Bertók *et al.* [5–7] exposed endotoxin to ^{60}Co-gamma radiation. This treatment was found to decrease toxicity to a great extent while the tolerance inducing and shock preventive effects were left unaffected [5, 6, 8]. It was even demonstrated that parallel to lethal and haemodynamic effects, the complement activating effect of the endotoxin was also decreased [9]. It was therefore studied how the haematologic effect and Shwartzman reactivity of the radiodetoxified endotoxin had changed as compared to parent endotoxin.

Materials and methods

Endotoxin was extracted by the phenol–water method [10] from the fermentor culture of *Escherichia coli* O89 and purified by repeated ultracentrifugation (Beckman L2 65B) at 100 000 *g*. For detoxication the endotoxin was suspended in distilled water (10 mg/ml) and irradiated by 5 Mrad (^{60}Co-gamma, Noratom 3500) according to the modified method of Previte *et al.* [11]. In haematologic tests, 100 μg/kg parent endotoxin and 1.5 mg/kg radiodetoxified endotoxin were injected intravenously to male New Zealand albino rabbits weighing 2300–2500 g.

Haematologic examinations. Leukocyte and erythrocyte counts were determined in a Buerker chamber. Platelet count was registered according to Feissly and Lüdin [12] by phase-contrast microscopy in Buerker chamber. The haematocrit value of heparinized blood was determined with the Janetzky TH12 centrifuge.

Fibrinogen evaluation was performed by *Grannis*'s method [13]. For the estimation of reptilase time, 0.1 ml citrated rabbit plasma and 0.2 ml reptilase (Disperga) solution were mixed in a water bath at 37 °C. Coagulation time was determined by the Clotek coagulometer (Hyland). The haemolytic complement titre (CH_{50}) was estimated according to Taliaferro and Taliaferro [14].

* Supported in part by the Hungarian Ministry of Health, Contract No. 5–13–1001–O/B.

Local Shwartzman phenomenon was induced in male New Zealand albino rabbits weighing 2500 g. Two days prior to the experiment, the back of the rabbits was depilated. Fifty μg parent endotoxin (0.2 ml) and 200 μg radiodetoxified endotoxin (0.2 ml) as well as control physiological saline were injected intracutaneously at two sites of the back and 24 hr later, 100 μg/kg of parent endotoxin or 1 mg/kg of radiodetoxified endotoxin were injected intravenously to induce Shwartzman phenomenon. Results obtained were read from the haemorrhagic and necrotic areas on the back after further 24 hr. Specimens from the back were then excised, fixed in Susa's fluid, embedded, and stained with haematoxilin–eosin.

Results

One hour after the intravenous injection of parent endotoxin, the leukocyte count decreased to one-third. Its initial value was regained only 24 hr later and an intense reactive leukocytosis developed 48 hr afterwards. Radiodetoxified endotoxin caused the leukocyte count to decrease to half; the initial value was regained after 48 hr without any reactive leukocytosis (Fig. 1).

The platelet count decreased significantly less under the effect of radiodetoxified endotoxin and its regeneration was slower than in rabbits treated with parent endotoxin (Fig. 2).

The erythrocyte count and the haematocrit value increased slightly in rabbits treated with parent endotoxin; in those treated with radiodetoxified endotoxin no haemoconcentration was observed.

One hour after the injection of parent endotoxin the fibrinogen level decreased (40%) and displayed a minor increase after 24 hr. Radiodetoxified endotoxin caused a moderate decrease of the fibrinogen level (Fig. 3). Reptilase time was prolonged about twofold one hour after the administration of parent

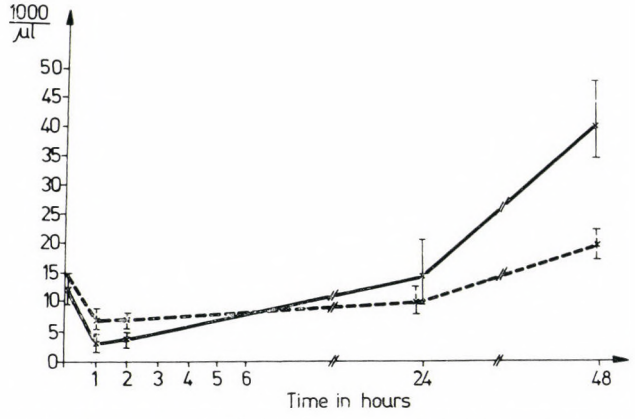


Fig. 1. Effect on leukocyte count of intravenously injected parent endotoxin (———) and radiodetoxified endotoxin (– – – – – – –). Mean of 10 experiments

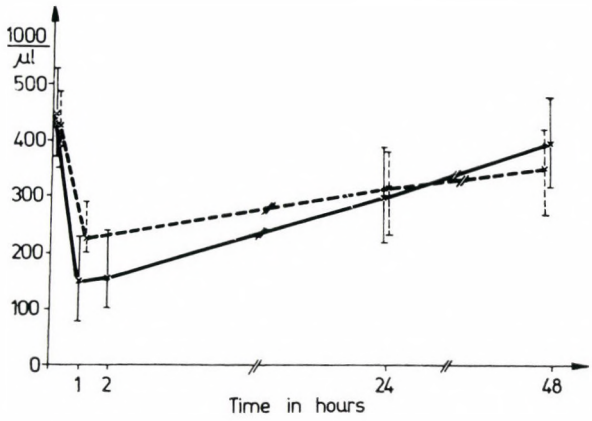


Fig. 2. Effect on platelet count of intravenously injected endotoxin (——) and radiodetoxified endotoxin (- - - -). Mean of 10 experiments

endotoxin, while radiodetoxified endotoxin did not cause any significant change (Fig. 4).

Changes in the complement level were expressed in per cents of the normal value. Parent endotoxin caused a 35% decrease while radiodetoxified endotoxin decreased the complement level only by 10% (Fig. 5).

To examine the local Shwartzman phenomenon, rabbits were subdivided to four groups. Animals of the first and third groups were prepared intracutaneously with parent endotoxin, those of the second and fourth groups with radiodetoxified endotoxin. The first and second groups (Fig. 6/I)

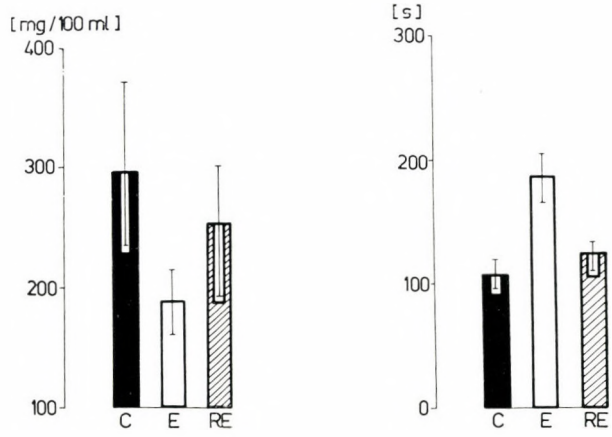


Fig. 3. Fibrinogen level one hour after the intravenous injection of physiological NaCl (C), endotoxin (E), and radiodetoxified endotoxin (RE). Means of 6 experiments each
Fig. 4. Reptilase time one hour after the intravenous injection of physiological NaCl (C), endotoxin (E), and radiodetoxified endotoxin (RE). Means of 6 experiments each

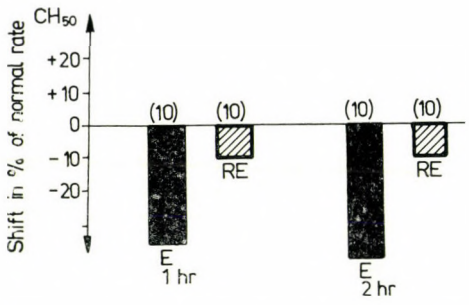


Fig. 5. Complement level in per cents of the normal value, after intravenous endotoxin (E), and radiodetoxified endotoxin (RE) injection. Means of 10 experiments each

were injected intravenously with a provoking dose of parent endotoxin, while the third and fourth groups (Fig. 6/II) with radiodetoxified endotoxin. The reaction elicited by radiodetoxified endotoxin was significantly slighter, extending to about half of the area of the classical reaction; the haemorrhagic halo surrounding the necrotic area was also small.

After preparation with parent endotoxin, a significantly slighter reaction was provoked by the radiodetoxified endotoxin. Finally, when radiodetoxified endotoxin was applied for preparation or provocation, the reaction was minimal. Histological examination, too, revealed a significant difference in the Shwartzman phenomenon induced by parent (Fig. 7) or radiodetoxified endotoxin (Fig. 8).

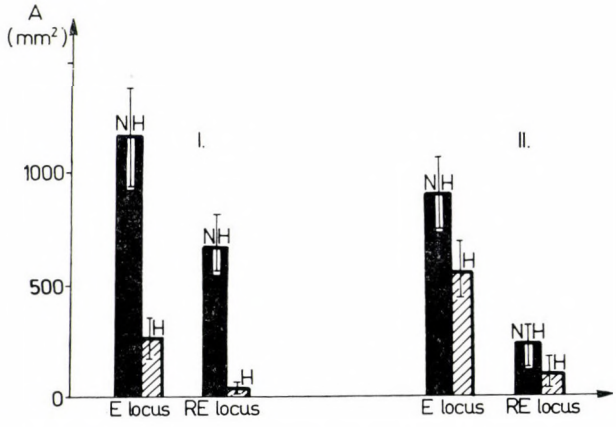


Fig. 6. Extension in mm² of local Shwartzman reaction. Means of 9 experiments; N = = necrotic skin area; H = haemorrhagic skin area; E locus = preparation with parent endotoxin; RE locus = preparation with radiodetoxified endotoxin; I = induction by parent endotoxin; II = induction by radiodetoxified endotoxin

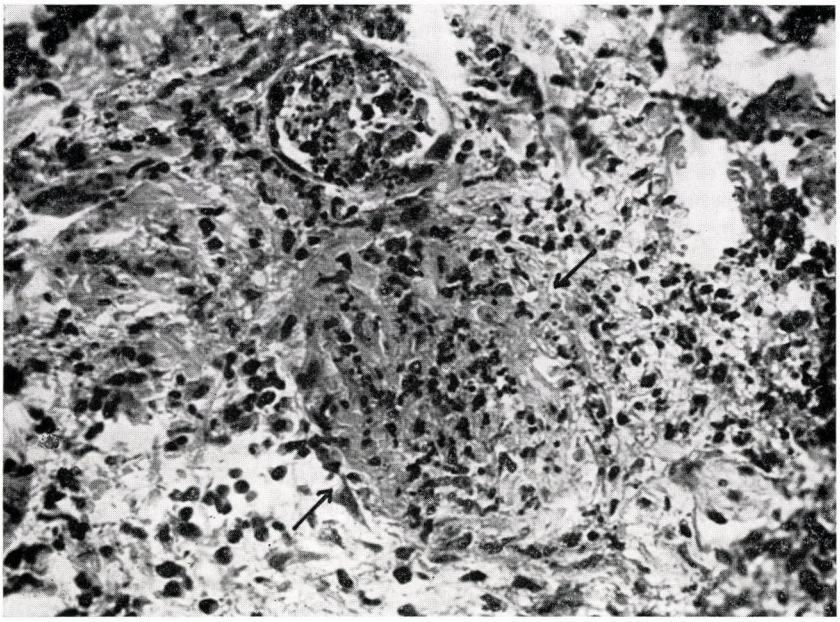


Fig. 7. Local Shwartzman phenomenon. Preparation and provocation by endotoxin. Necrotic and thrombotic vessel wall (arrows). Haematoxylin–eosin staining, × 250

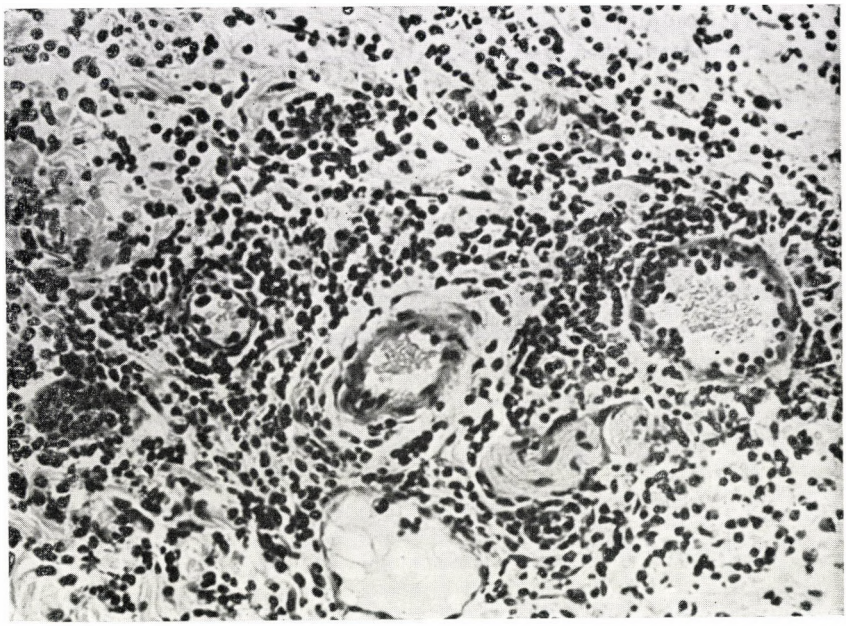


Fig. 8. Local Shwartzman phenomenon. Preparation and provocation by radiodetoxified endotoxin. Moderate leukocytic infiltration under the vascular structure. Haematoxylin–eosin staining, × 150

Discussion

Experiments aimed at comparing the haematologic parameters and local Shwartzman activity of radiodetoxified endotoxin with those of parent endotoxin showed that the effect of radiodetoxified endotoxin on blood elements was considerably weaker than that of the parent endotoxin: leukocyte and platelet counts decreased far less. The decrease of the leukocyte count caused by radiodetoxified endotoxin was not followed by secondary leukocytosis either, nor were the fibrinogen level and reptilase time affected.

Thus, gamma irradiation had changed the complement activating effect of endotoxin *in vivo*. GALANOS [15] and recently FÜST *et al.* [9] obtained similar results *in vitro*.

The local Shwartzman phenoemnon was quite slight if preparation and provocation had been performed with radiodetoxified endotoxin. The weaker effect of radiodetoxified endotoxin when applied for preparation was clearly shown by parallel experiments when the parent endotoxin had been used for provocation. This explains why the leukotactic effect of radiodetoxified endotoxin is weaker than that of the parent endotoxin.

NOWOTNY [16] showed that although chemically detoxified endotoxin failed to activate the complement, its local Shwartzman phenomenon provoking effect was much stronger than that of the parent endotoxin. In our experiments, after pretreatment with the parent endotoxin, provocation by radiodetoxified endotoxin elicited a marked Shwartzman reaction. This supports the view that the complement has no determining role in provocation of the local Shwartzman phenomenon.

REFERENCES

1. HASKINS, W. T., LANDY, M., MILNER, K. C., RIBI, E.: J. exp. Med. **114,** 665 (1961).
2. KIM, Y., WATSON, O. W.: Proc. Soc. exp. Biol. (N. Y.) **115,** 140 (1964).
3. NOLL, H., BRAUDE, A. J.: Fed. Proc. **20,** 260 (1961).
4. NOWOTNY, A.: Nature (Lond.) **197,** 721 (1963).
5. BALOGH, Á., BERTÓK, L.: Langenbecks Arch. Chir. (Suppl.) Chir. Forum **337,** 293 (1974).
6. BALOGH, Á., BERTÓK, L.: Acta chir. Acad. Sci. hung. **15,** 21 (1974).
7. BERTÓK, L., KOCSÁR, L., BEREZNAI, T., VÁRTERÉSZ, V., ANTONI, F.: Hungarian Patent No. 62 973 (1973).
8. BALOGH, Á., BERTÓK, L., NEMES, A., JUHÁSZ-NAGY, S.: Orv. Hetil. **115,** 2055 (1974).
9. FÜST, GY., BERTÓK, L., JUHÁSZ-NAGY, S.: Infect. Immun. **6,** 26 (1977).
10. WESTPHAL, O., LÜDERITZ, O., BISTER, F.: Z. Naturforsch. **7b,** 148 (1952).
11. PREVITE, J., CHANG, Y., EL BISI, H.: J. Bact. **93,** 1607 (1967).
12. FEISSLY, R., LÜDIN, H.: Rev. Haemat. **4,** 48 (1949).
13. GRANNIS, G. F.: Clin. Chem. **16,** 486 (1970).
14. TALIAFERRO, W. H., TALIAFERRO, L. G.: J. infect. Dis. **87,** 37 (1950).
15. GALANOS, CH.: Z. Immun.-Forsch. **149,** 214 (1975).
16. NOWOTNY, A.: Bact. Rev. **33,** 72 (1969).

Address of the authors:
TIBOR SZILÁGYI, HEDVIG CSERNYÁNSZKY, ENDRE GAZDY
Institute of Pathophysiology, University Medical School
H-4012 Debrecen, P.O.B. 23, Hungary

LÓRÁND BERTÓK
"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene,
H-1775 Budapest, P.O.B. 101, Hungary

Acta microbiol. Acad. Sci. hung. 25, 165—171, 1978

HEAT-STABLE ENTEROTOXIN PRODUCED BY SHIGELLA FLEXNERI*

I. Kétyi, Ilona Málovics, Adele VerténYi, T. Kontrohr, S. Pácsa and B. Kuch

Institute of Microbiology, University Medical School, Pécs

(Received August 2, 1977)

Filtrates and ultrasonic extracts of *Shigella flexneri* showed rapid permeability factor (PF) test and proved positive in suckling mice and ligated rabbit loop tests within 4 hr. Delayed PF was not detected and the rabbit loop dilatation test read after 18 to 24 hr, the mouse pad oedema reaction, the test for elongation effect of CHO cells were also negative. In the delayed PF test a strong "blanching" effect was observed. A filtrate of an Ent$^-$ *Escherichia coli* strain was positive only in the rapid PF test, while filtrate and ultrasonic extract prepared from an Ent$^+$ *E. coli* strain showed a positive reaction in all tests for enterotoxins (ST and LT) including the rapid PF test. Ultrasonic extracts of a *S. flexneri* and an Ent$^-$ *E. coli* strain concentrated by freeze-drying were fractionated on Sephadex G-100 column. *S. flexneri* fractions of 60–70 ml were positive for rapid PF, dilatation capacity in suckling mice, and the blanching effect in the delayed PF test. No positive reaction was found in the delayed PF test and in CHO cell culture. Similar fractions of Ent$^-$ *E. coli* carried substances responsible for the rapid PF and the blanching effect, but without suckling mice positivity.

It has been shown (1–3) that on injecting *Shigella flexneri* or *Salmonella* into the ligated rabbit ileal loop, fluid accumulation occurs. In the case of *Salmonella*, Koupal and Deibel [4] observed a positive reaction in suckling mice 2.5 hr after inoculating them with salmonella filtrates. With crude filtrates of *Salmonella typhi-murium*, Sandefur and Peterson [5] obtained negative results in CHO cell culture and in the blueing test (giving the dye 18 hr after the intradermal inoculation — "delayed PF test"). The latter authors, injecting Evans blue one hour after intracutaneous inoculation of the filtrate, demonstrated a so-called "rapid Permeability Factor (PF)". By fractionation of the crude material on Sephadex G-100 column, the rapid PF activity could be separated from the delayed PF capacity and elongation effect for CHO cells.

In the present paper, some data concerning the enterotoxigenic character of *S. flexneri* will be summarized.

Materials and methods

Strains. Freshly isolated strains of *S. flexneri* type 3a (No. 140 and 219) were obtained from Dr. V. László (National Institute of Hygiene, Budapest). A further *S. flexneri* type 4b strain (No. UP40097) as well as a strain of *S. dysenteriae* 1 (No. 16) originated from our strain

* Supported by the Scientific Research Council, Hungarian Ministry of Health (3—10—0305—03—1/K).

collection. The Ent$^-$ strain of *E. coli* serotype O78:K80:NM (No. 23473) was provided by Dr. É. Czirók (Pest County Public Health Station, Budapest) and the Ent$^+$ strain of *E. coli* serotype O141 : K85, K88 was kindly sent by Dr. H. W. Smith (A.R.C. Poultry Research Station, Houghton, England).

Choleragen (Lot No. O972) purified by Dr. F. A. Finkelstein was also used (obtained together with cholera antitoxin from Dr. I. Joó, Institute for Serobacteriological Production and Research "Human", Budapest).

Sera. Cholera antitoxin (Swiss Serum EC 3/A-2/67-B) and sera prepared from living strains of *S. flexneri* type 4b and different Ent$^+$ *E. coli* serotypes were used in neutralization experiments.

Media and cultures. The medium of Sakazaki et al. [6] modified by us [7] was used for the production of enterotoxin; it consisted of Levinthal broth, 1%; mannitol, 0.1%; NaCl, 0.35%; K_2HPO_4, 0.368%; KH_2PO_4, 0.132%; pH 7.6. The cultures were incubated at 37 °C for 18 hr in a shaker (Psycrotherm, New Brunswick).

Filtrates were prepared from cooled cultures after sharp centrifugation and filtration of the supernatants through a membrane filter (No. 1121, Göttingen).

Ultrasonic treatment of cultures was performed at 0 °C (MSE, 500 W, "full energy setting"). Effectivity was measured on the basis of cell destruction; lowering of the germ count by 4–5 log10 exponents usually yielded optimal extraction. Ultrasonic lysates were also filtered through membrane filter. All the filtrates were preserved at −20 °C.

Molecular filtration was carried out according to Sandefur and Peterson [5] as follows. Ultrasonic lysates were dialysed, freeze-dried, further dehydrated over P_2O_5 and the vials were filled with N_2 gas. Sephadex G-100 column measuring 1.5 × 90 cm was used for 200 mg material. This was dissolved in a buffer used also as eluent: 0.005 M Tris HCl pH 7.6, containing 0.001 M EDTA. Eluation speed was 20 ml/hr and 5 ml samples were collected. Optical density was measured at 280 nm (Uvicord II, LKB). Fractions were stored at −20 °C.

Ligated rabbit loop test was performed according to De *et al.* [8] using white rabbits weighing about 2–3 kg. Results were read after 3, 4, 6, 18 and 24 hr. The index expressing fluid accumulation was calculated on the basis of total-empty/empty weight. A rate above one was taken as positive [9].

Suckling mice test was carried out according to Dean et al. [10], with the modification of Jacks and Wu [11], by oral application, following the suggestion of Koupal and Deibel [4], reading the reaction after 2.5 instead of 4 hr. Dilatation was expressed by an index calculated on the basis of bowel/body weight. Values below 0.07 were considered negative, 0.07 to 0.09 positive, and above 0.09, strongly positive.

Permeability factors. Rapid PF was demonstrated by injecting the dye 1 hr after the intradermal introduction of filtrates, while the delayed PF [5] according to Evans *et al.* [12]. In both cases Pontamine Sky Blue (Serva, Heidelberg, GFR) was used according to Craig [13]. Rabbits (2.5–3.5 kg) were shawed 1 hr before the test. Blueing was read one hour after injection of the dye, by measuring the diameter of blue spots. The minimal requirement for a positive reaction was a diameter of 5 mm.

CHO (Chinese Hamster Ovary) cell line was kindly provided by Dr. B. Rowe (Salmonella and Shigella Reference Laboratory, Central Public Health Laboratory, London, England). The cells were maintained in 90% MEM and 10% newborn calf serum. Passages were made twice weekly by trypsin–versene treatment. Roux flasks (250 ml) were seeded with 10^6 cells suspended in 15 ml of the medium (MEM completed with 20% newborn calf serum). Cells were harvested after 2 to 3 days incubation.

Enterotoxicity (LT) was tested with the aid of a micromethod using microplates (FLOW). In a volume of 0.2 ml 5-10 × 10^3 cells were given in each well. After 24 hr incubation the medium was substituted by 0.2 ml test material diluted in MEM. After a further incubation for 24 hr the CHO cell elongation was estimated. Percentual ratio of the elongated cells, according to Guerrant *et al.* 14], was not determined. A 25 to 75% reaction was considered positive.

Mouse pad oedema test recommended by Lexomboon *et al.* [15] was used according to the description of Durikhin *et al.* [16]. The materials (0.1 ml) were injected subcutaneously into one of the hind foot pads of mice (5 in each group); the other hind leg served as control (Sakazaki medium). After 48 hr the mice were killed and the weight difference between test and control pad was measured, and the mean ±SD was calculated. Results were expressed in biological units, taking an oedematous weight of 75 mg as one unit.

Cytotoxicity of filtrates or lysates was determined on the basis of cell attachment inhibition in a microtest [17]. Cell lines of HeLa and human embryonic fibroblast (HEF) were used.

Results

1. *Experiments with crude filtrates and ultrasonic lysates.* Of freshly isolated *S. flexneri* strains, two strains of serotype 3a (No. 140 and 219) were studied in detail. An old laboratory stock culture (*S. flexneri* type 4b) was also investigated. As controls, the Ent$^-$ *E. coli* strain, serotype O78 : K80 (No. 23473), the Ent$^+$ *E. coli* strain, serotype O141 : K85, K88 (P99) and choleragen were applied.

According to the data of Table I, filtrates of *Shigella* strains up to a dilution of 1 : 10, ultrasonic lysates up to the dilutions of 1 : 20 and 1 : 40 showed the presence of rapid PF. The activity was not influenced by heat treatment (100 °C for 30 min). Investigating the delayed PF, no blueing reaction but a strong blanching effect [18] was observed. Undiluted shigella lysates were strongly positive in the suckling mice model (0.094 to 0.100) and weakly positive in rabbit loop test when the reaction was read after 4 hr; the positive reaction disappeared after 18–24 hr. Tests carried out with CHO cells, and the mouse foot oedema test were negative.

The Ent$^+$ control strain of *E. coli* was positive for all enterotoxic activities tested, including the rapid PF. In LT specific tests with choleragen, similar results were obtained except for rapid PF activity. Filtrates prepared from the Ent$^-$ strain of *E. coli* (No. 23473) — in contrast to shigella filtrates — were positive only for rapid PF and blanching, all the other tests were negative (Table I).

O'Brien *et al.* [19] showed a cytotoxic, *S. dysenteriae* 1-like toxin in the supernatant of a *S. flexneri* type 2a culture. By the method of cell attachment inhibition, cytotoxicity of extracts from *Shigella* or *E. coli* could not be demonstrated for HeLa and HEF cell lines. A simple crude filtrate prepared from a strain (No. 16) of *S. dysenteriae* 1, used as a control material, inhibited the attachment of HeLa cells in a dilution of 1 : 8, and that of HEF cells in a dilution of 1 : 16.

2. *Experiments with Sephadex G-100 fractions.* As described in Materials and methods, Sephadex G-100 fractions were collected from a freeze-dried material of *S. flexneri* type 3 (No. 140); control experiments were performed with an analogous material of Ent$^-$ *E. coli* strain (No. 23473).

Data for shigella fractions are shown in Fig. 1. The rapid PF appeared in fractions 10 to 18, and it showed higher activity in fractions 13 and 14 (60 to 70 ml). No delayed PF was demonstrable, but a strong blanching effect appeared together with the rapid PF. These fractions (Nos 13 and 14) had suckling mice activity (index: 0.088). Other, randomly chosen fractions (Nos 4, 5, 6, 25, 26, 31, 32) were negative in this test (indices between 0.049 and 0.057). Similarly as in the LT specific delayed PF test, no elongation by any of the samples was observed in CHO cell culture. The connection between

Table I

Enterotoxic activity of crude filtrates and ultrasonic extracts of S. flexneri and E. coli

Test	Materials	*S. flexneri*			*E. coli*		Choleragen
		3a	3a	4b			
		140	219	UP 40097	P99	23473	
Rapid PF	Filtrate	1 : 10	1 : 10	.	1 : 10	.	< 18
	Lysate	1 : 20	1 : 20	1 : 40	1 : 80	1 : 20	
	Lysate 100 °C	1 : 20	1 : 20	1 : 40	1 : 80	1 : 20	
Delayed PF	Lysate	—	—	—	1 : 40	—	0,09 Lb
"Blanching"	Lysate	+++	+++	+++	+++	+++	—
Suckling mice (undiluted —0.1 ml)	Lysate	0.094	0.100	0.093	0.095	0.059	.
CHO cells	Filtrate	—	—	—	1 : 8	—	0.056 Lb
	Lysate	—	—	—	1 : 8	—	
Mouse foot oedema test (undiluted — 0.1 ml)	Lysate	0.58	.	.	1.41	0.05	0.9 Lb
Ligated rabbit loop test	Lysate 4 hr	2/3	1/3		3/3	0/3	
		1.1	1.3	.	1.6	<0.5	.
	Lysate 20 hr	0/3	0/3		3/3	0/3	.
		<0.5	<0.5	.	3.9	<0.5	.

The values for rapid PF, delayed PF and CHO tests are expressed in titres, the blanching effect with crosses. In the case of the suckling mice test, the figures are the indices of bowel/body weight: values over 0.09 mean a strongly positive, below 0.07, a negative response. In the foot oedema test 75 mg of oedema is equal to 1 biological unit (0.9 Lb of choleragen was equal to 1.52 biological units). There are two data in the case of ligated rabbit loop test: the upper figures mean the ratio of positive/tested loops, the lower figures the index (total weight — empty weight/empty weight).

rapid PF, suckling mice activity and the optical density peak at 280 nm was questionable (Fig. 1).

In the case of the control Ent⁻ *E. coli* (sample No. 23473), the rapid PF activity appeared in the same fractions as in the case of *Shigella* (fractions 10, 11, 12, 13). These results are summarized in Fig. 2. Here, too, crude filtrates had no suckling mice activity. These fractions and some randomly chosen others showed indices ranging from 0.043 to 0.054; neither CHO, nor delayed PF activity was demonstrated in any of them. The blanching effect appeared together with the rapid PF. No connection has been found between rapid PF activity and optical density at 280 nm (Fig. 2).

3. *Neutralization with antisera and inhibition by the blanching factor.* Cholera antitoxin did not neutralize the rapid PF. Neither was it neutralized by immune sera prepared with living *Shigella* and *E. coli* strains in rabbits.

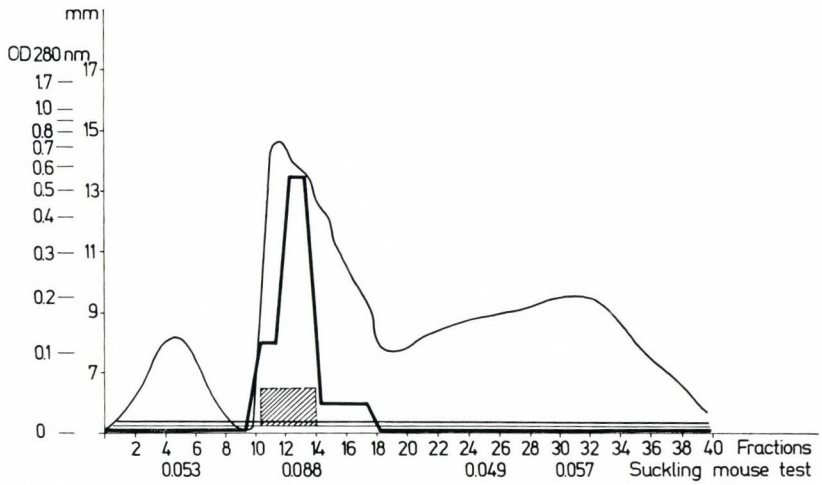


Fig. 1. Sephadex G-100 fractions of the concentrated lysate of *S. flexneri* 3 "140". From the dialysed and freeze-dried ultrasonic lysate of *S. flexneri* type 3 No. 140, 200 mg was fractionated on Sephadex G-100 column; 40 samples of 5 ml each were collected. ——— Optical density at 280 nm; ——— values in the rapid PF test (mm diameter); i.g.: 0.088 = index of bowel/body weight in suckling mice of the given fractions (values below 0.07 are negative, above 0.07 are positive); shaded area = blanching effect

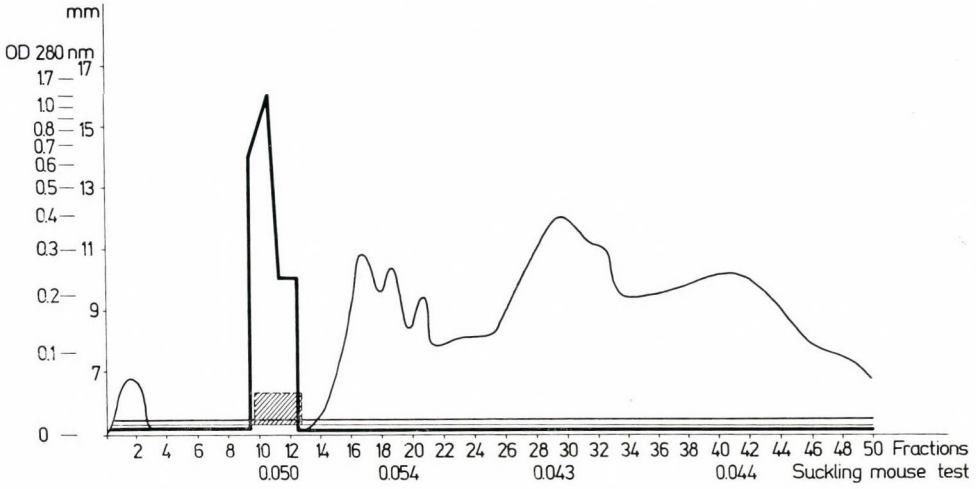


Fig. 2. Sephadex G-100 fractions of the concentrated lysate of Ent⁻ *E. coli* O78 : K80 (No. 23473). From the dialysed and freeze-dried ultrasonic lysate of Ent⁻ *E. coli* O78 : K80, 200 mg was fractionated on Sephadex G-100 column; 50 samples of 5 ml each were collected. ——— Optical density at 280 nm; ——— values in rapid PF test (mm of diameter); i.g.: 0.054 = index of bowel/body weight in suckling mice of the given fractions (values below 0.07 are negative, values above 0.07 are positive); shaded area = blanching effect

No inhibition of the delayed PF reaction by choleragen or *E. coli* LT (strain P99) could be observed when these samples were mixed before the test with concentrated shigella filtrates of high blanching activity.

Discussion

Studies of the enterotoxigenicity of *Salmonella* strains [4, 5] revealed the presence of both heat-labile (LT) and heat-stable (ST) toxins. LT seems to be inhibited in crude filtrates and is detectable only in purified materials [5]. Neither toxins were present in *S. flexneri*. The rapid PF of SANDEFUR and PETERSON [5] was present in all *S. flexneri* culture supernatants investigated. This activity was resistant to heat-treatment. All the rapid PF positive samples were positive in the suckling mice test, which is considered specific for heat-stable (ST) enterotoxin [20]. Weak positive reactions were obtained in ligated rabbit loops after 4 hr. Molecular filtration on Sephadex G-100 column showed that rapid PF, suckling mice and blanching activity were present in the same fractions. These data point to the presence of a heat-stable enterotoxin in the supernatant of *S. flexneri* culture. On the other hand, heat-labile enterotoxin could not be demonstrated either in crude filtrates, extracts, or in the collected fractions. This does not yet exclude the existence of LT producing strains.

The question arises whether the rapid PF was identical with the ST observed in suckling mice and in ligated rabbit loops. A somewhat surprising property of the Ent$^-$ strain of *E. coli* was the expressed rapid PF activity, without suckling mice or ligated rabbit loop dilatation positivity. This may mean that the rapid PF and the ST are different substances. At the same time, the ST and LT producing strain of *E. coli* (P99) also contains rapid PF.

The neutralizing capacity of cholera antitoxin supports [21, 22] the serological relationship of choleragen with LT, in agreement with the results of SANDEFUR and PETERSON [23]. The missing or weak antigenicity of ST [24] may be responsible for the lack of neutralization of sera produced by *Shigella* and Ent$^+$ *E. coli* strains.

The nature of the blanching factor remains unclear. Since it appears in the same fractions as the rapid PF (and ST) in *Shigella* as well as in Ent$^-$ *E. coli* (rapid PF only) the question of the identity of the rapid PF and the blanching factor may arise. To settle the question, further purification and serological investigations will be needed.

With the sensitive cytotoxicity test, a *S. dysenteriae* 1-like toxin could not be demonstrated. The negative finding may be due to the low quantities of the toxin. According to O'BRIEN *et al.* [19] the toxic ratio between *S. flexneri* and *S dysenteriae* 1 is 1 to 10^3. The rabbit loop positivity of *S. flexneri* found by these authors may be due to a "Shiga-toxin"-like substance or to the ST observed by us.

The pathogenic role of ST in bacillary dysentery is an open question. Watery diarrhoea may sometimes appear alone, or together with symptoms of colitis. KINSEY *et al.* [25] infected monkeys intracoecally with *S. flexneri*

and recorded only a colitis. When monkeys immunized with a dysentery vaccine containing Boivin antigens [26] were challenged with virulent *S. flexneri* bacteria, watery diarrhoea lasting a single day was observed in the protected animals. This may indicate the lack of anti-enterotoxic immunity, which may mean that in the clinical appearance of bacillary dysentery, the enterotoxicity of *Shigella* strains may have a role.

Acknowledgements. We are indebted to Dr. V. László, Dr. É. Czirók (Budapest), and to Dr. H. W. Smith (Houghton, England) for kindly providing strains, to Dr. I. Joó (Budapest) for choleragen and cholera antitoxin, and to Dr. B. Rowe (London, England) for the CHO cell line.

REFERENCES

1. Arm, H. G., Floyd, T. M., Faber, J. E., Hayes, J. R.: J. Bact. **89,** 803 (1965).
2. Yahagi, H., Ghoda, A., Sasaki, S.: Keio med. J. **16,** 119 (1967).
3. Sakazaki, R., Tamura, K., Nakamura, A., Kuwata, T.: Jap. J. med. Sci. **27,** 45 (1974).
4. Koupal, L. R., Deibel, R. H.: Infect. Immun. **11,** 14 (1975).
5. Sandefur, P. D., Peterson, J. W.: Infect. Immun. **14,** 671 (1976).
6. Sakazaki, R., Tamura, K., Nakamura, A., Kuwata, T., Ghoda, A., Takeuchi, S.: Jap. J. med. Sci. **27,** 19 (1974).
7. Kétyi, I., Czirók, É., Verténvi, A., Málovics, I., Pácsa, S.: Acta microbiol. Acad. Sci. hung. **25, 23** (1978).
8. De, S. N., Battacharya, K., Sarkar, J.: J. Pathol. Bact. **71,** 201 (1956).
9. Pesti, L., Semjén, G.: Acta vet. Acad. Sci. hung. **23,** 227 (1973).
10. Dean, A. G., Ching Yi-Chuan, Williams, R. G., Harden, L. B.: J. infect. Dis. **125,** 407 (1970).
11. Jacks, T. M., Wu, B. J.: Infect. Immun. **9,** 342 (1974).
12. Evans, D. G., Evans, D. J. Jr., Gorbach, S. L.: Infect. Immun. **8,** 725 (1973).
13. Craig, J. P.: Nature (Lond.) **207,** 614 (1965).
14. Guerrant, R. L., Brenton, L. L., Schnaitman, T. C., Rebhun, L. I., Gilman, A. G.: Infect. Immun. **10,** 320 (1974).
15. Lexomboon, U., Goth, A., Finkelstein, R. A.: Res. Comm. Path. Pharmacol. **2,** 245 (1971).
16. Дурихин, К. В., Попова, А. Е.: Ж. М. Э. И. **9,** 52 (1974).
17. Pácsa, S., Hankovsky, O., Hideg, K.: Europ. J. Cancer **10,** 193 (1974).
18. Finkelstein, R. A., La Rue, M. K., Johnston, D. W., Vasil, M. L., Cho, G. J., Jones, J. R.: J. infect. Dis. **133,** (Suppl.) S 120 (1976).
19. O'Brien, A. D., Thompson, M. R., Gemski, P., Doctor, B. P., Formal, S. B.: Infect. Immun. **15,** 796 (1977).
20. Giannella, R. A.: Infect. Immun. **14,** 95 (1976).
21. Smith, N. W., Sack, R. B.: J. infect. Dis. **127,** 164 (1973).
22. Gyles, C. L.: J. infect. Dis. **129,** 277 (1974).
23. Sandefur, P. D., Peterson, J. W.: Infect. Immun. **15,** 989 (1977).
24. Evans, D. J. Jr., Evans, D. G., Pierce, N. F.: Infect. Immun. **7,** 873 (1973).
25. Kinsey, M. D., Formal, S. B., Dammin, G. J., Giannella, R. A.: Infect. Immun. **14,** 368 (1976).
26. Dhikidze, E. K., Kavtaradze, K. N., Krilova, R. I., Rauss, K., Kétyi, I., Verténvi, A.: Acta microbiol. Acad. Sci. hung. **24,** 7 (1977).

Address of the authors:

Iván Kétyi, Ilona Málovics, Tivadar Kontrohr, Sándor Pácsa, Adele Verténvi, Béla Kuch
Institute of Microbiology, University Medical School, Szigeti út 12
H-7643 Pécs, Hungary

Acta microbiol. Acad. Sci. hung. 25, 173—178, 1978

MYCOBACTERIUM VALENTIAE SP. NOV. A NEW SPECIES OF RAPIDLY GROWING MYCOBACTERIA

J. F. G. SABATER and A. AMADOR

Servicio de Medicina Preventiva y Analisis Clinicos, Sección de Bacteriologia, Hospital Militar, Valencia, Spain

(Received August 11, 1977)

Five strains of rapidly growing scotochromogenic mycobacteria have been isolated from soil and sputum. This study comprises 101 biological, biochemical and morphological characteristics, as well as the behaviour against the common antituberculous drugs at several concentrations. The organisms are considered to belong to a new species of the genus *Mycobacterium* and are named *Mycobacterium valentiae* sp. nov. They have been deposited in the American Type Culture Collection, Rockville, Md., U.S.A. as 29356 and the Czechoslovak Collection of Microorganisms in the Czechoslovak National Collection of Type Cultures, Institute of Epidemiology and Hygiene, Prague, as My 220/77.

Within the genus *Mycobacterium* the fourth group of Runyon (rapidly growing mycobacteria) has considerably developed in the last seven years (1971–1977) when ten new species have been described: *M. duvalii* and *M. gilvum* [1], *M. rhodesiae* [2], *M. obuense* [3], *M. neoaurum* [4], *M. agri* [5], *M. chubuense*, *M. aichiense* and *M. tokaiense* [6], and *M. gadium* [7]. All these species have been designated after the study of their biological and biochemical characteristics.

In the present study we shall describe a new species of rapidly growing scotochromogenic mycobacteria, *Mycobacterium valentiae*, the latin name of Valencia (Spain), the place of its isolation.

Materials and methods

Bacterial strains. Representative strains of rapidly growing scotochromagenic mycobacteria used in this study included five strains of *M. valentiae:* Nos. 1.30 (ATCC 29356), 2.30 and 3.30 isolated from soil, and Nos. 4.30 and 5.30 from sputum of a patient with pulmonary disease (isolation associated with *M. tuberculosis); M. rhodesiae* ATCC 27024 obtained from H. BOISVERT, Institut Pasteur, Paris, France; *M. phlei* SN 101 obtained from I. TARNOK, Forschungsinstitut Borstel, 2061 Borstel, West Germany; *M. chubuense* ATCC 27278, *M. aichiense* ATCC 27280 and *M. tokaiense* ATCC 27282 received from M. TSUKAMURA, Chubu Chest Hospital, Obu, Aichi-ken, Japan 474.

Procedures of isolation. A rapidly growing scotochromogenic mycobacterium (ATCC 29356) isolated from a sample of soil coming from a cultivated land situated near the Military Hospital of Valencia (Spain) in March, 1976. The sample was decontaminated with 4% sodium hydroxide and 5% oxalic acid [8], and seeded in Ogawa egg medium [9]. Growth was observed after three days incubation at 37 °C. Yellow-orange colonies (in cultures of twenty days), originally yellow, presented some difficulty at emulsion in distilled water.

Character determination. Identification of the strains studied was based on the following biological, biochemical and morphological characteristics: acid-fastness, colony morphology

and pigment, cord formation, photochromogenicity, rapid growth, niacin production [10,] tween hydrolysis (5 days) [11], acid phosphatase [12], semiquantitative catalase [13], beta-galactosidase [14], nitrate reduction [15], aryl-sulphatase (24 hr) and three-days-aryl-sulphatase [16], urea hydrolysis [17], resistance to cycloheximide (Actidione, 0.5 μg/ml [18] obtained from ICN. K&K Laboratories, Inc. Plainview, N. Y., and Cleveland, Ohio, USA, growth on Mac Conkey agar [19], growth on Mueller–Hinton agar, growth temperature, tolerance to 0.1% (w/v) salicylate in Ogawa egg medium, salicylate degradation in Sauton agar, gelatin hydrolysis [18], amino acids as sole nitrogen source (concentration, 0.02 M in the basal medium [20]: L-arginine, L-tyrosine, L-ornithine, L-asparagine, L-valine, L-cystine, L-cysteine, L-serine, L-leucine, creatinine, L-tryptophan, L-phenylalanine, L-citrulline, L-alanine and L-proline; amides as nitrogen and carbon source (concentration, 0.02 M in the basal medium) [21]: acetamide, benzamide and nicotinamide; amides as sole nitrogen source (concentration, 0.02 M in the basal medium) [21]: acetamide, benzamide, nicotinamide, succinamide and allantoin; organic acids as sole carbon source (concentration, 0.02 M in the basal medium) [22]: succinate, malate, oxalate, propionate, malonate, pyruvate, formiate, citrate, acetate and fumarate; acid formation from carbohydrates (added to the basal medium at 0.5% final concentration; with carbohydrate-free control) [23]; glucose, mannose, galactose, arabinose, xylose, rhamnose, trehalose, mannitol, inositol and sorbitol; utilization of carbohydrates as sole carbon source (carbohydrates were added to the basal medium to obtain a 0.5% final concentration; with carbohydrate-free control) [23]: glucose, mannose, galactose, arabinose, xylose, rhamnose, trehalose, mannitol, inositol and sorbitol; enzymatic activity in the ZYM API system [24]: alkaline phosphatase (substrate, 2-naphthyl phosphate pH 8.5), esterase C_4 substrate, 2-naphthyl-butyrate pH 7.1), esterase lipase (C_8 substrate, 2-naphthyl-caprylate pH 7.1), lipase (C_{14} substrate, 2-naphthyl-miristase pH 7.1), leucine aminopeptidase (substrate, leucyl-2-naphthylamide pH 7.5), valine aminopeptidase (substrate, L-valyl-2-naphthylamide pH 7.5), cystine aminopeptidase (substrate, L-cystil-2--naphthylamide pH 7.5) trypsin (substrate, N-benzoyl-DL-arginine-naphthylamide pH 8.5), chymotrypsin (substrate, N-benzoyl-DL-phenylalanine naphthylamide pH 7.1), acid phosphatase (substrate, 2-naphthyl-phosphate pH 5.4), phosphoamidase (substrate, naphthol--AS-B1-phosphodiamine pH 5.4), α-galactosidase (substrate, 6-Br-2-naphthyl-α-D-galacto-pyranoside pH 5.4), β-galactosidase (substrate, 2-naphthyl-βD-galactopyranoside pH 5.4), β-glucoronidase (substrate, naphthol-AS-B1-βD-glucoronic acid pH 5.4), α-glucosidase substrate, 2-naphthyl-glucopyranoside pH 5.4), β-glucosidase (substrate, 6-Br-2-naphthyl-βD--glucopyranoside pH 5.4), β-glucosaminidase (substrate, 1-naphthyl-N-acetyl-βD-glucos-aminide pH 5.4), α-mannosidase (substrate, 6-Br-2-naphthyl-αD-mannopyranoside pH 5.4) and α-fucosidase (substrate, 2-naphthyl-αL-fucopyranoside pH 5.4).

Drug sensitivity. Resistance to antituberculous drugs of the isolated strains was estimated by the method of proportions according to CANETTI *et al.* [25] utilizing the following drugs: INH (0.2, 1 and 10 μg/ml), streptomycin (5 and 10 μg/ml) PAS (0.5 and 1 μg/ml), rifampicin (20 and 40 μg/ml) and ethambutol (1.5 and 2 μg/ml).

Results

All five strains showed similar biological and biochemical characteristics.

Description of the type strain of M. valentiae. Strain ATCC 29356 (originally designated CMHM 1.30), isolated from a sample of soil is the type strain of *M. valentiae.*

Morphology. Rods, approximately 3 μm by 0.5 to 0.6 μm, no compact grouping. Non-motile. Gram-positive. Strongly acid-fast.

Biological characteristics. Rough colonies, yellow-orange after three days on Ogawa egg medium and on Löwenstein–Jensen medium. Scotochromogenic. No growth in Mac Conkey agar; grows on Mueller–Hinton agar and on Ogawa egg medium with salicylate (0.1%). Grows at 28 °C and 37 °C but not at 45 °C and 52 °C.

Biochemical characteristics. Niacin test negative. β-Galactosidase and tween hydrolysis (5 assays) positive. Acid phosphatase and catalase semiquantitative test negative. Nitrates reduced. Aryl-sulphatase (24 hr) negative, three-days-aryl-sulphatase test positive. Resistance to cycloheximide (0.5 μg/ml) positive. Salicylate degradation on Sauton agar negative. Urea hydrolosys (24 hr) positive and gelatin hydrolysis (7 days) negative.

Acetamide, benzamide and nicotinamide utilized as simultaneous nitrogen and carbon source. L-arginine, L-ornithine, L-asparagine, L-valine, L-cystine, L-cysteine, L-tyrosine, creatinine, L-leucine, L-citrulline, L-alanine and L-proline utilized as the sole source of nitrogen; L-tryptophan, L-serine and L-phenylalanine not utilized.

Acetamide, nicotinamide, succinamide and allantoin utilized as the sole source of nitrogen; benzamide not utilized. Succinate, malate, propionate, pyruvate, acetate and fumarate utilized as sole source of carbon within seven days at 37 °C; oxalate, malonate, formiate and citrate not utilized. Acid from glucose within two weeks at 28 °C; acid not formed from mannose, galactose, arabinose, xylose, rhamnose, trehalose, mannitol, inositol and sorbitol; glucose, mannose, mannitol and sorbitol utilized as sole source of carbon; galactose, arabinose, xylose, rhamnose, trehalose and inositol not utilized. Strong enzymatic activity with esterase (C_4) and esterase lipase (C_8), and weak enzymatic activity with alkaline phosphatase, leucine aminopeptidase and phosphoamidase; no enzymatic activity with lipase (C_{14}), valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase and α-fucosidase.

Resistant to PAS and INH, and sensitive to streptomycin, rifampicin and ethambutol.

Discussion

Differentiation of M. valentiae (ATCC 29356) from other scotochromogenic mycobacteria. Differentiation from *M. rhodesiae* is based upon nitrate reduction, β-galactosidase, gelatin hydrolysis, L-serine (N source), acetamide and benzamide (N–C source), citrate as C source, acid from mannose, xylose, inositol and mannitol; xylose, inositol and sorbitol as C source; enzymatic activity (strong): phosphatase acid and α-glucosidase; enzymatic activity (weak): β-galactosidase and phosphoamidase.

The differences from *M. aichiense* are, nitrate reduction, β-galactosidase, catalase semiquintative, L-serine (N source), acetamide and benzamide (N–C source), malonate as C source, acid from mannose and mannitol; sorbitol as C source; enzymatic activity (strong) leucine aminopeptidase, acid phosphatase and α-glucosidase; enzymatic activity (weak): β-galactosidase.

Table I

Differentiation of M. valentiae from other rapidly growing mycobacteria

C	Characteristics	*M. valentiae*	*M. rhodesiae*	*M. aichiense*	*M. chubuense*	*M. tokaiense*	*M. phlei*
1.	Acid-fast	+	+	+	+	+	+
2.	Colony morphology	S	S	S	S	S	S
3.	Pigment production	+	+	+	+	+	+
4.	Photoinduction	−	−	−	−	−	−
5.	Rapid growth	+	+	+	+	+	+
6.	Niacin test	−	−	−	−	−	−
7.	Tween hydrolysis (5 days)	+	+	+	+	−	+
8.	Acid phosphatase	−	−	+	−	−	+
9.	Catalase (semiquantitative)	−	−	+	−	+	−
10.	Galactosidase	+	−	−	−	−	+
11.	Nitrate reduction	+	−	−	+	−	+
12.	Arylsulphatase (24 hr)	−	−	−	−	−	−
13.	Arylsulphatase (3 days)	+	+	+	−	−	−
14.	Urea hydrolysis (24 hr)	+	+	+	+	+	+
15.	Resistance to cycloheximide	+	+	+	+	−	+
16.	Growth on Mac Conkey agar	−	−	−	−	−	−
17.	Growth on Mueller–Hinton agar	+	+	+	+	+	+
18.	Growth at 28 °C	+	+	+	+	+	+
19.	Growth at 37 °C	+	+	+	+	+	+
20.	Growth at 45 °C	−	−	−	−	−	+
21.	Growth at 52 °C	−	−	−	−	−	+
22.	Salicylate tolerance	+	+	+	+	+	+
23.	Salicylate degradation	−	−	−	−	−	−
24.	Gelatin hydrolysis	−	+	−	−	−	−
25.	L-serine (N)	+	−	−	+	−	−
26.	Acetamide (N–C)	+	−	−	−	−	+
27.	Benzamide (N–C)	+	−	−	−	−	−
28.	Benzamide (N)	−	+	−	−	−	−
29.	Nicotinamide (N)	+	+	+	+	+	+
30.	Succinate (C)	+	+	+	+	+	+
31.	Malate (C)	+	+	+	+	+	+
32.	Pyruvate (C)	+	+	+	+	+	+
33.	Acetate (C)	+	+	+	+	+	+
34.	Fumarate (C)	+	+	+	+	+	+
35.	Malonate (C)	−	−	+	−	−	+
36.	Citrate (C)	−	+	−	−	−	−
37.	Glucose (acid)	+	+	+	+	+	+
38.	Mannose (acid)	−	+	+	+	+	−
39.	Galactose (acid)	−	−	−	−	−	−
40.	Arabinose (acid)	−	−	−	−	+	+
41.	Xylose (acid)	−	+	−	+	+	+
42.	Rhamnose (acid)	−	−	−	+	+	−
43.	Trehalose (acid)	−	−	−	−	−	+
44.	Mannitol (acid)	−	+	+	+	+	+
45.	Inositol (acid)	−	+	−	+	+	−
46.	Sorbitol (acid)	−	−	−	+	+	+
47.	Glucose (C)	+	+	+	+	+	+
48.	Mannose (C)	+	+	+	+	+	+
49.	Galactose (C)	−	−	−	−	−	+
50.	Arabinose (C)	−	−	−	−	+	+

Table I *cont.*

C	Characteristics	*M. valentiae*	*M. rhodesiae*	*M. aichiense*	*M. chubuense*	*M. tokaiense*	*M. phlei*
51.	Xylose (C)	—	+	—	+	+	+
52.	Rhamnose (C)	—	—	—	+	+	—
53.	Trehalose (C)	—	—	—	—	—	+
54.	Mannitol (C)	+	+	+	+	+	+
55.	Inositol (C)	—	+	—	+	+	—
56.	Sorbitol (C)	+	—	—	+	+	+
57.	Alkaline phosphatase	w	w	w	w	—	w
58.	Esterase (C_4)	S	S	S	S	S	S
59.	Esterase-lipase (C_8)	S	S	S	S	S	S
60.	Lipase (C_{14})	—	—	—	—	w	S
61.	Leucine aminopeptidase	w	w	S	S	S	S
62.	Valine aminopeptidase	—	—	—	—	w	S
63.	Cystine aminopeptidase	—	—	—	—	w	S
64.	Trypsin	—	—	—	—	—	S
65.	Chymotrypsin	—	—	—	—	—	w
66.	Acid phosphatase	—	S	S	w	—	S
67.	Phosphoamidase	w	—	—	—	—	S
68.	α-Galactosidase	—	—	—	—	—	—
69.	β-Galactosidase	—	w	—	—	—	—
70.	β-Glucoronidase	—	—	—	—	—	—
71.	α-Glucosidase	—	S	—	—	w	—
72.	β-Glucosidase	—	—	—	—	w	—
73.	β-Glucosaminidase	—	—	—	—	—	—
74.	α-Mannosidase	—	—	—	—	—	—
75.	α-Fucosidase	—	—	—	—	—	—

+ = positive w = weak

— = negative S = strong

M. chubuense differs in β-galactosidase, three-days-aryl sulphatase, acetamide and benzamide (N–C source), acid from mannose, xylose, rhamnose, inositol, mannitol and sorbitol; xylose, rhamnose and inositol as C source; enzymatic activity (strong); leucine aminopeptidase, and α-glucosidase; enzymatic activity (weak): acid phosphatase and β-galactosidase.

M. tokaiense can be differentiated by resistance to the actidione, nitrate reduction, β-galactosidase, tween hydrolysis (5 days), three-days-aryl-sulphatase, L-serine (N source), acetamide and benzamide (N–C source); acid from mannose, arabinose, xylose, rhamnose, inositol, mannitol and sorbitol; arabinose, xylose, rhamnose, and inositol as C source; enzymatic activity (strong): leucine aminopeptidase and α-glucosidase; enzymatic activity (weak): valine aminopeptidase, cystine aminopeptidase, phospho-amidase and β-glucosidase.

M. phlei will be differentiated by aryl-sulphatase (3 days), growth at 45 °C and 52 °C, malonate as C source; acid from mannitol and sorbitol;

galactose, arabinose, xylose and trehalose as C source; enzymatic activity (strong): lipase (C_{14}), valine aminopeptidase, cystine aminopeptidase, trypsin, phosphatase acid and phosphoamidase; enzymatic activity (weak): chymotrypsin. The differences are listed in Table I.

Acknowledgement. We are indebted to Dr. H. BOISVERT, Service de Tuberculose et des Mycobacterioses, Institut Pasteur, Paris, France; Dr. I. TARNOK, Forschungsinstitut Borstel, 2061 Borstel, West Germany; and Dr. M. TSUKAMURA, National Sanatorium Chubu Chest Hospital, Obu, Aichi-ken, Japan 474, for providing some strains used in the present study.

REFERENCES

1. STANFORD, J. L., GUNTHORPE, W. J.: Brit. J. exp. Path. **52,** 627 (1971).
2. TSUKAMURA, M., MIZUNO, S., GANE, N. F. F., MILLS, A., KING, L.: Jap. J. Microbiol. **15,** 407 (1971).
3. TSUKAMURA, M., MIZUNO, S.: J. gen. Microbiol. **68,** 129 (1971).
4. TSUKAMURA, M.: Med. and Biol. (Tokyo) **85,** 229 (1972).
5. TSUKAMURA, M.: Med. and Biol. (Tokyo) **85,** 153 (1972).
6. TSUKAMURA, M.: Med. and Biol. (Tokyo) **86,** 13 (1973).
7. CASAL ROMAN, M., REY CALERO, J.: Tuberc. **55,** 299 (1974).
8. BEERWERTH, W.: Perax. Pneumol. **25,** 661 (1971).
9. OGAWA, T., SANAMI, K.: Kekkaku **24,** 13 (1949).
10. MEDVECZKY, E.: Amer. Rev. resp. Dis. **81,** 757 (1960).
11. WAYNE, L. G., DOUBECK, J. R., RUSSELL, R. L.: Amer. Rev. resp. Dis. **90,** 588 (1964).
12. TSUKAMURA, M., MIZUNO, S., MURATA, H., OSHIMA, T.: Iryo (Tokyo) **28,** 275 (1974).
13. KUBICA, G. P., JONES, W. D., ABBOTT, V. D., BEAM, R. E., KILBURN, J. O., CATER, J. C.: Amer. Rev. resp. Dis. **94,** 400 (1966).
14. TACQUET, A., TISON, F., POLSPOEL, B., ROOS, PH., DEVULDER, B.: Ann. Inst. Pasteur **111,** 86 (1966).
15. BOISVERT, H.: Ann. Inst. Pasteur **100,** 352 (1961).
16. PATTYN, S. R., HERMAN BOVEROULLE, M. T.: Amer. Rev. resp. Dis. **92,** 297 (1965).
17. TACQUET, A., GERNEZ-RIEUX, CH., GAUDIER, M.: Ann. Inst. Pasteur **87,** 335 (1954).
18. SABATER, J. F. G.: In press.
19. JONES, W. D., KUBICA, G. P.: Amer. J. med. Tech. **30,** 187 (1964).
20. TSUKAMURA, M.: Rep. Cont. Taxon. Bact. (Brno) 369 (1965).
21. TSUKAMURA, M., TSUKAMURA, J.: Amer. Rev. resp. Dis. **94,** 104 (1966).
22. GORDON, R. E., SMITH, M. M.: J. Bact. **66,** 41 (1953).
23. TSUKAMURA, M.: Identification of Mycobacteria. Research Laboratory of the National Sanatorium Chubu Chest Hospital, Japan 1975. p. 76.
24. AMADOR, A. Y., SABATER, J. F. G.: Laboratorio **369,** 223 (1976).
25. CANETTI, G., RIST, N., GROSSET, J.: Rev. Tuberc. **27,** 217 (1963).

Address of the authors:

JOSE FRANCISCO G. SABATER, ALFREDO AMADOR
Military Hospital,
Valencia, Spain

Acta microbiol. Acad. Sci. hung. 25, 179–183, 1978

HAEMOPHILUS PARAPHROPHILUS ISOLATED FROM ENDOCARDITIS

Zsuzsanna CSUKÁS and Éva BÁN

Institute of Microbiology, Semmelweis University Medical School, and Municipal Hospital for Infectious Diseases, Budapest

(Received September 8, 1977)

Isolation and microbiological features of a *Haemophilus paraphrophilus* strain which caused a subacute bacterial endocarditis are described. The isolate conformed to the first description by ZINNEMANN *et al.*, except that it was sensitive to ampicillin.

A new V factor dependent *Haemophilus* species preferring increased CO_2 tension for growth, was reported by ZINNEMANN *et al.* [1] and named *Haemophilus paraphrophilus*. This proposal of a new species was noted by the Subcommittee on the Taxonomy of Haemophilus of the International Committee on Nomenclature of Bacteria in Mexico City, 1970 [2]. The sites of the lesions due to *H. paraphrophilus* were different [1]. The microbiological features of a *H. paraphrophilus* strain causing subacute bacterial endocarditis are described in this paper.

Material and methods

Report of a case. A healthy sportswoman aged 22 who had been under regular medical control and had had no cardiac symptoms was admitted to the infectious ward for intermittent fever and exhaustion for the last 10 days. On admission physical examination was negative. She had a temperature of 40 °C, the ESR (Westergreen) was 82 mm in the first hour; WBC 1380/μl with 64% neutrophils; total serum protein, 6.4 g/100 ml with normal protein fractions.

Chest X-rays and the ECG were normal, serological investigations were negative. No pathogen was isolated from urine and a throat swab. After a few days the PCG showed an atrial sound and a presystolic murmur and on the ECG slight disturbances of repolarisation appeared. These findings suggested an acute involvement of the bicuspid valve.

Treatment was started with 2 g/day of chloramphenicol. Having received the result of the antibiotic sensitivity test of the infective organism, ampicillin 4 g/day with oxytetracyclin 2 g/day and gentamicin 160 mg/day and sulphamethoxazole 1.6 g and trimethoprim 320 mg/day were prescribed. This treatment was continued until the 55th day.

Blood cultures. Three blood samples were taken during the first 48 hr, when her temperature was 40–40.5 °C. Ten ml whole blood without anticoagulant were added to 100 ml Witte peptone vitamin broth. Subcultures were performed daily on blood agar (5% ox blood) and heated blood plates. Further blood samples were taken on the 4th and 6th days.

Culture media. Nutrient agar was prepared from beef infusion with 2% agar and 1% Witte peptone pH 7.4. To prepare chocolate agar, nutrient agar at 80 °C was supplemented with 5% ox blood and the bottles were left to stand at the same temperature for 30 min. For the enrichment of chocolate agar, 10% yeast extract was employed (250 g baker's yeast

were boiled for a few minutes in 750 ml distilled water; on the next day the supernatant was passed through Seitz filter).

Growth factor requirement. Requirements of accessory growth factors were demonstrated according to Alexander's method [3], COWAN and STEEL [4] and SIMS [5] using haematin (National Biochemicals Corp., Cleveland) and NAD (Reanal, Budapest). Growth factor requirement was examined by seeding the plates with one loopful of bacteria mixed in 1 ml physiological saline.

Biochemical tests. Indole, nitrate, H_2S, methyl red, Voges-Proskauer reactions were tested in Levinthal broth. Indole production was tested with Kovács's reagent after 24 hr, the Voges-Proskauer reaction with Barrit's reagent after 3 days; the methyl red and nitrate tests were performed in 3-day cultures [4]. H_2S production was detected with lead acetate paper strips. Acid production from carbohydrates was tested with 1% of the test sugar in peptone water, containing 10 μg/ml haematin solution, 10 μg/ml NAD solution, 2 mg/ml albumin and 1% bromothymol blue (stock solution 0.2%) as indicator. The tests were incubated for 10 days. The following rapid biochemical tests were carried out. (a) Urease activity: to 2% aqueous urea (pH 7.6) 0.5% phenol red stock solution (0.02%) was added; one ml solution was seeded with a loopful of bacteria; the same medium without urea was used as control [6]. (b) ONPG was dissolved and filtered; one half ml solution was inoculated with a loopful of bacteria and the result was read after 2 and 4 hr [7]. (c) Tryptophan and phenylalanine deaminase tests: 1% amino acid was dissolved in distilled water and 0.5 ml aliquots were seeded with one loopful of culture; after 4 hr incubation 0.1 ml ferric chloride reagent was added [4].

Antibiotic sensitivity was tested by the use of susceptibility disks (Institute for Serobacteriological Production and Research, Human, Budapest) applied on chocolate agar plates swabbed densely with saline suspensions.

Results

Blood culture. Inoculation of one of the early haemocultures on the 7th day of incubation yielded 1–2 mm colonies on chocolate agar, which failed to form colonies at the first passage on blood and chocolate plates. The colonies could be recovered from the blood sample taken on the 4th day, when small colonies as observed before developed on the aerobic chocolate plate whereas colonies of 3–4 mm appeared on the chocolate plate incubated in air with 10% CO_2. The strain could be maintained in CO_2 atmosphere for 6–8 weeks.

Morphology. Gram-stained smear from growth in air with 10% CO_2 showed small Gram-negative bacilli regular in shape. The strain was non-spore-forming and non-motile in digest broth incubated at 37 °C for 48 hr.

Cultural characteristics. The organism initially isolated from the blood of the patient failed to grow on nutrient agar and blood agar not only in air but in air with 10% CO_2 and on chocolate agar in air. The colonies growing on chocolate agar in air with 10% CO_2 were 3–4 mm in diameter, smooth white and opaque. The culture showed CO_2 atmosphere requirement for 2 months (subcultured twice a week), subsequently it started to adapt itself to aerobic conditions. No haemolysis was produced on horse blood agar at 37 °C for 48 hr.

Growth factor requirement. The strain was found to have V-factor requirement with all the three methods, but regular growth was obtained

on V and XV factors containing plates only when they were incubated in air with 10% CO_2.

The strain showed weaker growth on basic media without NaCl, when V factor was supplied by a feeder organism in the form of a streak of *Staphylococcus aureus* and when cultures were incubated in air. It showed stronger growth on this medium when the plates were incubated in air with 10% CO_2.

Biochemical reactions. Biochemical properties of the organism are shown in Table I. Features characteristic of *H. paraphrophilus* were nitrate reduction; urease, catalase and ONPG tests were positive, neither indole nor H_2S were produced. Acid without gas was produced from dextrose, fructose, maltose and saccharose.

Antibiotic sensitivity. The organism was sensitive to ampicillin, carbenicillin, chloramphenicol, tetracyclin, oxytetracyclin, nitrofurantoin, nalidixic acid and showed partial sensitivity to cephalosporin, novobiocin, pristinamycin, streptomycin, kanamycin, neomycin, gentamicin, paromomycin, polymyxin B and colistin. The strain was resistant to benzyl penicillin, oxacillin, methicillin, erythromycin, oleandomycin, vancomycin, lincomycin and spiramycin.

Table I

Characteristics of H. paraphrophilus strain isolated in this study

X factor	—	Methyl red	—
V factor	+	Voges-Proskauer	—
Serum	—	Phenylalanine deaminase	—
CO_2	+	Tryptophan deaminase	—
Haemolysis	—	ONPG	+
Motility, 22 and 37 °C	—		
Capsule	—	Arabinose	—
Sporulation	—	Xylose	—
Optimal growth, °C	37	Glucose	+
Gelatin hydrolysis	—	Fructose	+
Litmus milk	—	Galactose	—
Indole	—	Lactose	—
Urea	+	Maltose	+
Nitrate to nitrate	+	Sucrose	+
H_2S	—	Trehalose	—
Oxidase	—	Mannitol	—
Catalase	+		

Discussion

H. paraphrophilus belongs to the group of haemophili with V factor requirement. Its culturing and undisturbed growth needs increased CO_2 tension [8, 9]. The pathogen occurs in the normal flora of the human mouth and pharyngeal cavity and was isolated so far in 22 cases from different lesions: vaginitis, cerebral abscesses, osteomyelitis, paronychia, appendicitis, urogenital lesions, throat and mouth [1].

KHAIRAT [10] questioned the existence of this new species. In the numerical taxonomy of *Haemophilus* and related bacteria, SNEATH and JOHNSON [11] studied and classified *H. paraphrophilus*. HOLLAENDER and MANNHEIM [12] characterized the haemophilic bacteria by their respiratory quinones and cytochromes, and acknowledged the existence of the *H. paraphrophilus* strain. FRAZER *et al.* [13] found the *H. paraphrophilus* species to be homogeneous serologically. KILIAN [14] classified *H. paraphrophilus* into the *Haemophilus* genus.

Some characteristic biochemical reactions of the *H. paraphrophilus* species including catalase and oxidase reported by ZINNEMANN *et al.* [1] and KILIAN [14] are not in complete agreement. In culturing requirements, morphological and biochemical characteristics, our isolate conformed to the first description by ZINNEMANN *et al.* [1], except that our strain was sensitive to ampicillin.

The incidence of bacterial endocarditis has increased along with the valve implantations and other heart operations, insertion of venous pacemakers and other interventions in the last 10 years [15, 16]. DE SILVA *et al.* [17] were the first to diagnose endocarditis due to *H. paraphrophilus* in a prolapsed mitral valve. Then GERACI *et al.* [18] reported 14 patients with haemophilus endocarditis representing four different species: *H. influenzae* (1), *H. aphrophilus* (5), *H. paraphrophilus* (5) and *H. parainfluenzae* (4). One patient had both *H. aphrophilus* and *H. paraphrophilus* infection.

The organism fails to be demonstrable from a considerable number of haemocultures, so that no aimed antibiotic therapy or a reliable determination of the serum antibiotic level, needed for appropriate dosage, are possible. When performing inoculation from haemocultures, it is advisable to incubate duplicate chocolate plates in CO_2 atmosphere, to support the growth of *H. paraphrophilus* and other members of this genus which require increased CO_2 tension for optimal growth [19].

REFERENCES

1. ZINNEMANN, K., ROGERS, K. B., FRAZER, J., BOYCE, J. M. H.: J. Path. Bact. **96,** 413 (1968).
2. LAPAGE, S. P., ZINNEMANN, K.: Int. J. Syst. Bact. **21,** 132 (1971).
3. DUBOS, R. J., HIRSCH, J. G.: Bacterial and Mycotic Infections of Man. J. B. Lippincott Co. Philadelphia 1965.

4. Cowan, S. T., Steel, K. J.: Manual for the Identification of Medical Bacteriology. University Press, Cambridge 1965.
5. Sims, W.: J. med. Microbiol. **3,** 615 (1970).
6. Csukás, Zs., Füzi, M., Schmidt, M.: Egészségtudomány **17,** 228 (1973).
7. Füzi, M.: Kísérl. Orvostud. In press.
8. Buchanan, R. E., Gibbons, N. E.: Bergey's Manual of Determinative Bacteriology. 8th ed. Williams and Wilkins Co., Baltimore 1975.
9. Wilson, G. S., Miles, A. A.: Topley and Wilson's Principles of Bacteriology and Immunity. 6th ed. Arnold, London 1975.
10. Khairat, O.: Brit. med. J. **1,** 728 (1971).
11. Sneath, P. H. A., Johnson, R.: Int. J. Syst. Bact. **23,** 405 (1973).
12. Hollaender, R., Mannheim, W.: Int. J. Syst. Bact. **25,** 102 (1975).
13. Frazer, J., Zinnemann, K., Boyce, J. M. H.: J. med. Microbiol. **8,** 89 (1975).
14. Kilian, M.: J. gen. Microbiol. **93,** 9 (1976).
15. Goldsweig, H. G., Matsen, J. M., Castaneda, A. R.: J. thorac. cardiovas. Surg. **63,** 408 (1972).
16. Gullott, R. F.: Chest **63,** 446 (1973).
17. De Silva, M., Rubin, S. J., Lyons, R. W.: J. clin. Path. **66,** 922 (1976).
18. Geraci, J. E., Wilkowske, C. J., Wilson, W. R.: Mayo Clin. Proc. **52,** 209 (1977).
19. Frazer, J., Zinnemann, K., Boyce, J. M. H.: J. med. Microbiol. **2,** 563 (1969).

Address of the authors:

Zsuzsanna Csukás
Institute of Microbiology, Semmelweis University Medica School
Hőgyes Endre u. 7-9, H-1092 Budapest, Hungary

Éva Bán
"László" Central Hospital for Infectious Diseases
H-1450 Budapest, P.O.B. 29, Hungary

Acta microbiol. Acad. Sci. hung. 25, 185–189, 1978

MATHEMATICS OF THE INVERSE SOLID-PHASE RADIOIMMUNOASSAY

S. BERNÁTH and L. ZALOTAI

State Institute for the Control of Veterinary Serobacteriological Products, Budapest and Institute of General and Physical Chemistry, Attila József University, Szeged

(Received September 22, 1977)

Use of a simplified, idealized model allows a mathematical treatment of the inverse solid phase radioimmunoassay. Equations are presented for calculation of the following values: bound/free antibody ratio (R) in the state of equilibrium; the equilibrium constant (K), the concentrations of the directly non-measurable values (Ab_f; Ab_fAg^*: Ab_s and Ag^*) as well as the concentration of the antibody tested $[Ab_f]_0$. The model has the same parameters as the test system, thus the equations may be used in planning of the test in the laboratory.

To perform and evaluate correctly the results of radioimmunoassays it is necessary to be familiar with the mathematical principles involved. This has been the topic of several papers in respect of both the classical radioimmunoassay (RIA) [1, 2] and the immunoradiometric assay (IRMA) [3, 4].

As a methodical advantage of RIA, reagents coupled to a solid phase system are often used [5, 6]. The coupled reagent is usually the antibody, thus allowing easy separation of bound from free antigen. The methodical principles of this procedure are similar to those of the classical RIA, thus the same mathematics apply to both.

Recently, inverse solid-phase radioimmunoassay [7–10] has been extensively applied particularly for the quantitation of antibodies against bacterial exotoxins (*Clostridium tetani*, *Clostridium botulinum*, *Clostridium perfringens* Dε). In this test the labelled component is the antigen. During incubation the solid-phase bound antibody competes with the tested one to bind the labelled antigen. When the reaction is complete, the solid and fluid phases are separated. The fluid phase contains free antibody, free antigen and antigen-antibody complex.

Because of the above reasons the mathematics valid for the other radioimmunological tests cannot be applied to the inverse solid-phase radioimmunoassay. Mathematics describing the latter are given in this paper in the form of an idealized model. Experimental standard curves were obtained by quantitative tests performed with *C. perfringens* Dε antitoxin.

Materials and methods

C. perfringens Dε prototoxin was produced in protein free medium and purified according to HABEEB [11], then labelled with ^{125}I as described earlier [9]. The specific activity of the final product was 1 mCi/mg protein.

The standard curve was obtained with the international standard serum against *C. perfringens* Dε toxin received from the WHO International Laboratory (New Haw, Weybridge, Surrey, England). The test was performed by the antibody-coated tube method described earlier [9]. The slightly modified procedure was as follows. Dilutions of the antibody studied and the labelled antigen were measured into antibody coated tubes and incubated for 4 hr. Then the fluid phase was removed, the tubes were rinsed with saline and their radioactivity was measured in an Autogamma spectrometer.

Definition of terms

$[Ab_f]_0$ – total concentration of the studied antibody in the fluid phase;
$[Ab_f]$ – free antibody concentration in the fluid phase in state of equilibrium;
$[Ab_f\ Ag^*]$ – antigen (labelled) – antibody complex concentration in the fluid phase in state of equilibrium;
$[Ab_s]_0$ – total concentration of solid phase bound antibody;
$[Ab_s]$ – free antibody concentration on the solid phase at the state of equilibrium;
$[Ab_sAg^*]$ – concentration of solid phase bound antibody and antigen (labelled) complex in state of equilibrium;
$[Ag^*]_0$ – total concentration of labelled antigen;
$[Ag^*]$ – concentration of free labelled antigen in state of equilibrium;
k_1 and k_{-1} – reaction rate constants (fluid phase);
k_2 and k_{-2} – reaction rate constants (solid phase);
K_1, K_2 and K – equilibrium constants for fluid phase, solid phase, and the total system;
R_1, R_2 and R – bound/free antibody ratios for fluid phase, solid phase and the total system.

Results

Standard curve. The curve presented in Fig 1. was obtained by the method used. Measurement of the radioactivity of the fluid phase yielded a similar curve in an inverse position.

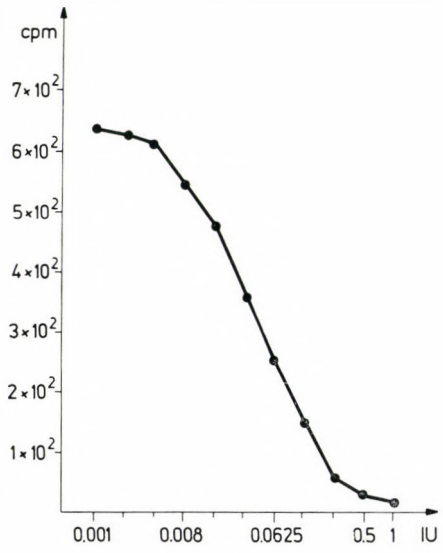


Fig. 1. Standard curve obtained by quantitative determination of *C. perfringens* Dε antitoxin in an inverse solid-phase radioimmunoassay using the antibody coated tube method

The idealized model. Certain conditions and requirements are necessary to allow mathematical treatment of the process. These are difficult or impossible to realize experimentally although they are in full accordance with the basic principles of the procedure.

(1) The labelled antigen represents a homogeneous population of a single uniform compound.

(2) The tested and the solid-phase bound antibodies are both homogeneous populations of a single uniform compound. Binding to the solid phase does not affect reactivity.

(3) Labelling does not change the antigen's original physical chemical characteristics or its reactivity.

(4) Both the antigen and the antibody are univalent, i.e. only one molecule of each is involved in the reaction.

(5) The antigen-antibody reaction exhibits first order kinetics.

(6) All reagents are present simultaneously and the reaction continues until an equilibrium has been reached.

(7) It is possible to separate quantitatively the antigens bound by the solid phase antibody and free in the fluid phase.

The amount of antibody bound to the solid phase is exactly measurable and is identical throughout. The amount of labelled antigen added to the fluid phase is also known. Thus we may make the following statements:

(i) Known concentrations:

$$[Ab_s]_0;\ [Ag^*]_0$$

(ii) Concentration measurable as radioactivity:

$[Ab_sAg^*]$ — in the solid phase
$\{[Ag^*] + [Ab_fAg^*]\}$ — in the fluid phase

(iii) Concentration calculated from (i) and (ii):

$$[Ab_s]$$

Equations

Under the conditions specified above the following equations hold

$$Ab_f + Ag^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ab_fAg^* \tag{1}$$

$$K_1 = \frac{[Ab_fAg^*]}{[Ab_f] \cdot [Ag^*]} \tag{2}$$

$$Ab_s + Ag^* \underset{k_{-1}}{\overset{k_2}{\rightleftharpoons}} Ab_sAg^* \tag{3}$$

$$K_2 = \frac{[Ab_sA_g^*]}{[Ab_s] \cdot [A_g^*]} \tag{4}$$

According to condition (2)

$$K_1 = K_2 \equiv K \tag{5}$$

Applying the mass law we have

$$[Ag^*]_0 = [Ag^*] + [Ab_fAg^*] + [Ab_sA_g^*] \tag{6}$$

where $[Ag^*] + [Ab_fAg^*]$ and $[Ab_sAg^*]$ are concentrations measured in the fluid and solid phase, respectively.

$$[Ab_f]_0 = [Ab_f] + [Ab_fAg^*] \tag{7}$$

$$[Ab_s]_0 = [Ab_s] + [Ab_sAg^*] \tag{8}$$

where $[Ab_s]_0$ is known, $[Ab_sAg^*]$ is measurable and $[Ab_s]$ can be calculated. Further, using equations (2) and (4) we have

$$R_1 = \frac{[Ab_fAg^*]}{[Ab_f]} = \frac{[Ab_fAg^*]}{[Ab_f]} \cdot \frac{[Ag^*]}{[Ag^*]} = K_1 \cdot [Ag^*] \tag{9}$$

and

$$R_2 = \frac{[Ab_sAg^*]}{[Ab_s]} = \frac{[Ab_sA_g^*]}{[Ab_s]} \cdot \frac{[Ag^*]}{[Ag^*]} = K_2 \cdot [Ag^*] \tag{10}$$

Since $[Ab_sAg^*]$ is measurable and $[Ab_s]$ can be calculated, equation (10) is solved for R_2. According to equation (5):

$R_1 = K_1 \cdot [Ag^*] = K_2 \cdot [Ag^*] = R_2$, hence $R_1 = R_2 \equiv R$.

Equation (6) may be written as:

$$[Ag^*] = [Ag^*]_0 - [Ab_sAg^*] - [Ab_fAg^*] \tag{11}$$

Hence, using equation (9) or (10), we have

$$R_1 = R_2 \equiv R = K \cdot [Ag^*] = K \cdot \{[Ag^*]_0 - [Ab_sAg^*]\} - K \cdot [Ab_fAg^*] \tag{12}$$

Plotting R against $\{[Ag^*]_0 - [Ab_sAg^*]\}$ yields a direct line with the slope $tg\ \alpha = K$ and with an intercept on the negative part of the R axis at $-K \cdot [Ab_fAg^*]$. In the knowledge of $[Ab_fAg^*]$, $[Ag^*]$ and $[Ab_f]$ can be calculated from equations (11) and (2). Hence:

$$[Ab_f] = \frac{1}{K} \cdot \frac{[Ag_fAg^*]}{[Ag^*]}$$

Thus by using equation (7) the concentration $[Ab_f]_0$ is:

$$[Ab_f]_0 = [Ab_f] + [Ab_fAg^*] = \frac{1}{K} \cdot \frac{[Ab_fAg^*]}{[Ag^*]_0 - [Ab_sAg^*] - [Ab_fAg^*]} +$$

$$+ [Ab_fAg^*] =$$

$$= \frac{[Ab_fAg^*] + K \cdot [Ag^*]_0 \cdot [Ab_fAg^*] - K \cdot [Ab_sAg^*] \cdot [Ab_fAg^*]}{K \cdot \{[Ag^*]_0 - [Ab_sAg^*] - [Ab_fAg^*]\}} -$$

$$- \frac{K \cdot [Ab_fAg^*] \cdot [Ab_fAg^*]}{K \cdot \{[Ag^*]_0 - [Ab_sAg^*] - [Ab_fAg^*]\}} = \tag{13}$$

$$= \frac{[Ab_fAg^*] \cdot \{1 + K([Ag^*]_0 - [Ab_sAg^*] - [Ab_fAg^*])\}}{K \cdot \{[Ag^*]_0 - [Ab_sAg^*] - [Ab_fAg^*]\}}$$

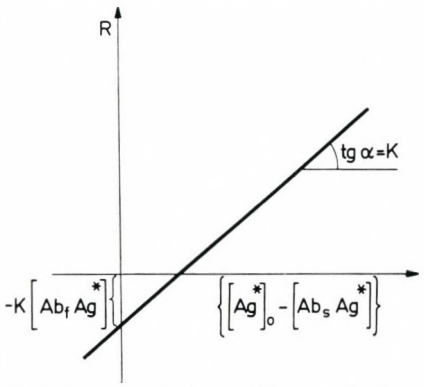


Fig. 2. Plot of equation (11) showing the bound/free antibody ratio *vs.* the radioactivity in the fluid phase in the state of equilibrium

REFERENCES

1. Feldmann, H., Rodbard, D.: In Odell, W. D., W. W. Daughaday (eds): Principles of competitive protein binding assays. Lippincott, Philadelphia 1971. pp. 158–199.
2. Yalow, R. S.: Pharmacol. Rev. **25,** 161 (1973).
3. Rodbard, D., Weiss, G. H.: Anal. Biochem. **52,** 10 (1973).
4. Kalmakoff, J., Parkinson, A. J., Crawford, A. M., Williams, B. R. G.: J. immunol. Meth. **14,** 73 (1977).
5. Catt, K., Tregear, G. W.: Science **158,** 1570 (1967).
6. Catt, K., Niall, H. D., Tregear, G. W.: Nature (Lond.) **213,** 825 (1967).
7. Habermann, E., Wiegand, H.: Naunyn-Schmiedberg's Arch. exp. Path. Pharmak. **276,** 321 (1973).
8. Bernáth, S., Habermann, E.: Med. Microbiol. Immunol. **160,** 47 (1974).
9. Bernáth, S.: Appl. Microbiol. **30,** 499 (1975).
10. Habermann, E., Bernáth, S.: Acta vet. Acad. Sci. hung. **25,** 377 (1975).
11. Habeeb, A. F. S. A.: Arch. biochem. **130,** 430 (1969).

Address of the authors:

Sándor Bernáth
State Institute for the Control of Veterinary Serobacteriological Products
Szállás u. 8., H-1107 Budapest, Hungary

Lajos Zalotai
Institute of General and Physical Chemistry, Attila József University
Renich Béla tér 1, H-6720 Szeged, Hungary

Acta microbiol. Acad. Sci. hung. 25, 191—197, 1978

EFFECT OF DIALYSABLE LEUKOCYTE EXTRACTS ON ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY AND LYMPHOCYTE Fc RECEPTORS

K. NÉKÁM, I. LÁNG, B. FEKETE, P. GERGELY and GY. PETRÁNYI

Second Department of Medicine, Semmelweis University Medical School, Budapest

(Received October 15, 1977)

Dialysable leukocyte extracts significantly inhibited the antibody-dependent cell-mediated cytotoxicity of normal human peripheral blood lymphocytes in both xenogeneic and allogeneic assays. The inhibitory effect was dose-dependent. Fractions of dialysable leukocyte extracts separated on Sephadex G-25 column differed in their capacity to suppress cytotoxicity. Dialysable leukocyte extracts did not block EA-rosetting but inhibited the resynthesis of Fc receptors. Some explanations for the observed suppressive effect are discussed.

The potential role of antibody mediated cellular cytotoxicity (ADCC) in various malignancies has widely been studied [1–3]. Though its significance *in vivo* has not been directly proved in man, cumulating data indicate that it may be important in the immunological defence of the host against tumour cells [4–6].

Immunostimulation, now widely used in the treatment of malignancies [7–11], may have a direct enhancing effect on ADCC, as it was shown by CAMPBELL and MACLENNAN [12] using BCG for active immunotherapy.

In this paper we report on the effect *in vitro* of the immunostimulant dialysable extract of leukocytes (transfer factor, TF_d) on ADCC and discuss possible explanations for the observed inhibitory effect.

Materials and methods

Preparation of TF_d. Four-hundred ml of heparizined venous blood were drawn from seven healthy blood donors and separated on Ficoll (Pharmacia, Uppsala) – Uromiro (Bracco, Milano) gradient [13]. TF_d was prepared according to the method of LAWRENCE [14]: leukocytes were ten times freeze–thawed and then the disrupted cells were treated with 100 μg/ml DNase (Calbiochem, Luzern) in 0.01 M $MgSO_4$ at 37 °C for 60 min. The leukocyte extract was dialysed against distilled water at 4 °C for 48 hr, lyophilized, reconstituted in tissue culture medium 199 (TC 199) and stored prior to use at −30 °C. Biological activity of each preparation was controlled *in vitro* by its enhancing effect on active rosettes [15] and on the production of migration inhibitory factor [15, 16]. One preparation was reconstituted in distilled water and separated on a Sephadex G-25 (Pharmacia, Uppsala) column [15] with 0.01 M ammonium bicarbonate elution buffer (Fig. 1). Biological activity present in the crude preparation was found in fraction III only (Table III). Tubes between V_0 and fraction I were not tested because of low optical density at 260 and 280 mn.

The amount of TF_d was expressed in lymphocyte equivalents, i.e. the number of lymphocytes used for preparation.

Separation of lymphocytes for ADCC. Thirty ml of heparizined venous blood were drawn from healthy blood donors. Lymphocytes were separated on Ficoll–Uromiro gradient [13]. Phagocytic cells were removed by carbonyl iron treatment. Finally, the cells were suspended in medium and adjusted to the concentration desired. Viability of cells was greater than 98% as judged by trypan blue exclusion. Monocyte contamination was less than 2%.

Media. In the chicken red blood cell (CRBC) assay, TC 199 supplemented with 10% heat inactivated (56 °C, 60 min) pooled human AB serum, and in the human RBC assay, TC 199 supplemented with 10% heat inactivated fetal calf serum (FCS), was used.

ADCC assay. Xenogeneic system. The original method of PERLMANN and PERLMANN [17] was used. ^{51}Cr labelled CRBC (10^5) were added to glass test tubes and mixed with an appropriate amount of effector cells according to the desired effector/target cell ratio. Anti-CRBC serum (final dilution 1 : 500) was added, and the volume was made up to 0.5 ml with medium. Controls with target and effector cells without antiserum and target cells and antiserum without effector cells were included in each experiment (background). After 16 hr incubation of the tubes at 37 °C, radioactivity of the pellet and the supernatant was determined. Cell damage was expressed as per cent isotope release into the supernatant. Background release never exceeded 5%. Maximum isotope release was about 90%, as determined by treatment of the target cells with 1% saponin. Cytotoxicity was expressed as

$$\frac{\text{test tube percentage} - \text{background percentage}}{\text{maximum release} - \text{background percentage}} \times 100$$

The means of triplicate samples are given.

Allogeneic system. The method of URBANIAK [18] was used with minor modifications [19]. ^{51}Cr labelled Rh(+) human erythrocytes (3×10^4) were placed into test tubes and mixed with an appropriate number of effector cells according to the desired effector/target cell ratio. Heat inactivated human anti-D serum (final dilution 1 : 500) was added and the volume was made up to 0.1 ml with medium. The tubes were incubated at 37 °C for 16 hr. Further processing was carried out as described above.

In both the xenogeneic and allogeneic systems TF_d was incubated together with the target and effector cells for 16 hr. If not indicated otherwise, the final concentration of TF_d was 1 : 1, i.e. the number of effector lymphocytes was equal to the number of lymphocytes used for the preparation of TF_d (equivalent dose).

Percentage inhibition was calculated according to the formula:

$$1 - \frac{\text{cytotoxicity with TF}}{\text{cytotoxicity without TF}} \times 100$$

Statistical analysis was carried out by the Student's *t*-test.

EA-rosettes. EA-rosettes were formed according to the method of BRAIN and MARSTON [20] with slight modifications. Rh positive human erythrocytes were incubated with a subagglutinating dilution of anti-D rabbit antiserum at 37 °C for 30 min. After washing, the cells were resuspended to a concentration of 10^8 erythrocytes/ml and 0.1 ml erythrocyte suspension was added to 0.3 ml lymphocyte suspension containing 10^6 cells. The tubes were centrifuged at 200 *g* for 10 min, and allowed to stand at 4 °C for 30 min. After gentle resuspension, 200 lymphocytes were examined for EA-rosettes.

TF_d treatment of lymphocytes. Lymphocytes (10^6 in 1 ml medium containing 40 mM HEPES, Serva, Heidelberg) were incubated at 37 °C for 2–16 hr in the presence of TF_d equivalent to 10^6 lymphocytes. After washing, EA rosettes were formed and counted as described above.

Results

The effect of crude TF_d (all preparations exerted *in vitro* biological activity in the active rosette and migration inhibition assays) on the cytotoxicity of normal human leukocytes in xenogeneic and allogeneic ADCC assays is shown in Table I. (Crude TF_d even at the highest concentration used had no influence on spontaneous chromium release). In the allogeneic

ADCC system total inhibition was observed at an effector/target cell ratio of 5 : 1, and significantly decreased cytotoxicity was found at an effector/-target cell ratio of 30 : 1. In the CRBC assay, the 4 TF_d preparations tested caused a marked impairment of ADCC activity. Percentage inhibition was about 30%.

Table II shows the effect of TF_d concentrations on ADCC activity. The decrease in cytotoxicity was still remarkable at a final dilution of 1 : 100. At a dilution of 1 : 500 impairment was slight, while no inhibition could be demonstrated at the dilution of 1 : 1000.

Table I

Effect of TF on ADCC in allogeneic and xenogeneic test systems

TF preparation[a]	Target cell	Effector/ target ratio	Cytotoxicity per cent[b]		Percentage inhibition	p
			Control	TF treated		
TF 1	HRBC	5 : 1	10.1 ± 1.2	1.2 ± 0.4	88.1	<0.001
TF 2	HRBC	5 : 1	8.9 ± 0.9	1.1 ± 0.3	87.7	<0.001
TF 1	HRBC	30 : 1	47.9 ± 1.8	35.7 ± 1.3	25.5	<0.05
TF 2	HRBC	30 : 1	42.4 ± 1.7	32.6 ± 1.8	23.1	<0.05
TF 3	CRBC	20 : 1	70.0 ± 2.3	44.7 ± 2.5	36.1	<0.01
TF 4	CRBC	20 : 1	84.9 ± 2.8	55.4 ± 2.7	34.8	<0.01
TF 5	CRBC	20 : 1	60.3 ± 2.1	42.9 ± 1.9	28.9	<0.01
TF 6	CRBC	20 : 1	69.0 ± 2.6	43.7 ± 1.8	36.7	<0.01

[a] 1 : 1 lymphocyte equivalent dose
[b] mean ± s.e.m.

Table II

Effect of TF concentrations on ADCC
(CRBC targets, effector: target cell ratio, 20 : 1)

TF dilution	Percentage inhibition*	p
1 : 1	34.8 ± 1.8	<0.01
1 : 100	21.2 ± 4.3	<0.01
1 : 500	11.2 ± 1.4	<0.05
1 : 1000	no inhibition	

* mean of four experiments ± s.e.m.

Table III

Effect of TF fractions on ADCC
(CRBC targets, effector: target cell ratio 10 : 1)

TF treatment*	Percent cytotoxicity	Percentage inhibition	*In vitro* activity of the fraction (see text)	
			Enhancing effect on "active" rosettes	Inhibitory effect on leukocyte migration
None	48.9** ± 1.9	—	—	—
Crude TF	29.2 ± 1.4	40.3	+***	+***
Frcation I	19.1 ± 1.1	60.9	—	—
Fraction II	33.0 ± 1.6	32.5	—	—
Fraction III	41.5 ± 1.8	15.1	+***	+***

* 1 : 1 ratio for lymphocyte equivalent TF *vs.* effector cell
** mean ± s.e.m.
*** all significant at 0.05 level

Next, we investigated the effect of TF_d fractions on ADCC of normal human lymphocytes on CRBC. The percentage inhibition of the crude preparation was about 40%. The strongest inhibition was exerted by fraction I. Fraction II was less potent in this respect and fraction III (having almost all biological activities in Ea-rosette and leukocyte migration assays) impaired cytotoxicity moderately (Table III).

The effect of TF_d on the EA-rosetting ability of lymphocytes is shown in Fig. 2. When incubated at 37 °C, the number of Fc positive cells declined reaching a minimum after 4 hr owing to the shedding of Fc receptors. This was followed by a receptor resynthesis resulting in an increase of EA-rosettes.

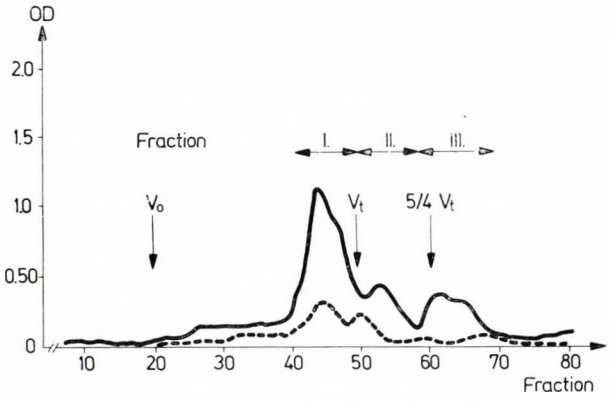


Fig. 1. Sephadex G-25 gel filtration of crude TF. (Fraction volume: 3.3 ml) V_0, void volume V_t, column volume; ——— 260 nm, – – – – – 280 nm

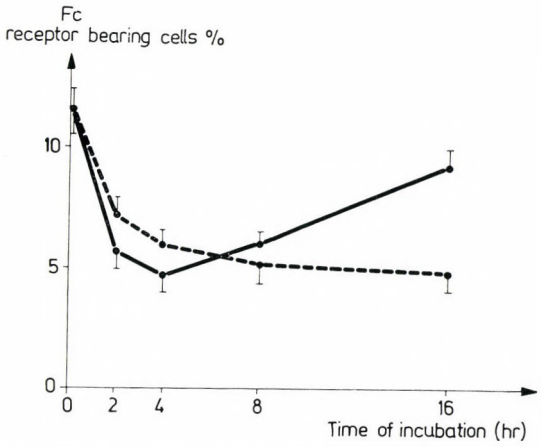


Fig. 2. Effect of TF on spontaneous changes of lymphocyte Fc receptors (incubation at 37 °C) Mean of eight experiments ± s.e.m.; ——— without TF, – – – – – with TF

After 16 hr the number of Fc positive cells was again near to the original value. The presence of TF_d in the culture medium did not affect the EA binding, since no significant differences were seen in the percentages of EA rosettes during 8 hr observation. The lower values obtained after 16 hr incubation ($p < 0.001$) can be explained by the inhibition of receptor resynthesis, since it was not due to cell death as proved by trypan blue exclusion.

Discussion

TF_d was found by many authors to stimulate cellular immune reactions *in vitro*. It stimulates lymphocyte blastogenesis [21], mediator production [16, 22) and mixed lymphocyte culture [23]. Its enhancing effect was demonstrated also in *in vivo* skin tests [14], leukocyte chemotaxis [24], graft rejection [14] and cytotoxicity against tumour cells [25]. On the other hand, inhibition of DNA synthesis [26, 27], of T lymphocyte receptors [15] and suppression of cellular immune responses following repeated injections of TF_d have also been reported [28].

The inhibitory material seems to be of higher molecular weight and can be separated from the stimulatory one by chromatography [26, 29]. Among the fractions of TF_d eluted from the Sephadex G-25 column, fraction I proved to be the most inhibitory. Fraction III possessing stimulatory activity according to tests *in vitro* impaired cytotoxicity only slightly. The more striking effect of fraction I than that of crude TF_d might be explained by the dilution of the active component. The inhibition was dose-dependent similarly as some other effects of TF_d [24, 30, 31]. The marked inhibition

at the effector cell/lymphocyte equivalent TF_d ratio of 1 : 1 was probably due to the supraoptimal concentration. Nonetheless, significant inhibition was also found at higher dilutions being often used successfully *in vitro*.

A possible explanation for the inhibitory effect may be the influence of TF_d on the cyclic nucleotide system. Besides enhancing the intracellular cGMP level in monocytes [32], TF elevates the cAMP level of peripheral lymphocytes [32, KALMÁR and NÉKÁM, unpublished]. The latter phenomenon is known to be connected with the suppression of mediator release, resulting in a depressed cytotoxic activity [33, 34]. In our experiments, however, the first fraction yielded by Sephadex chromatography was less active than fraction III in elevating the cAMP levels.

A further explanation would be that TF_d stimulates suppressor cells residing among effector cells; this would bring about an adverse effect. This explanation does not, however, seem likely since the most inhibitory fraction I is chromatographically clearly separated from the antigen-dependent TF_d activity of the dialysate (Table III) and so the inhibition of ADCC may not be connected with latter. The first fractions separated under similar conditions also inhibited the "active" T-rosetting [15].

We cannot exclude the possibility that the TF_d preparation procedure (dialysis against distilled water or medium) may influence the stimulatory–inhibitory effects of TF_d, a phenomenon still debated in literature [27, 30, 35, 36]. The inhibitory effect of low-molecular-weight dialysable iodine salts from Uromiro can be ruled out because TF_d from leukocytes separated on 6% dextran (mol wt approximately 200 000, Pharmacia, Uppsala) showed the same inhibition (unpublished observations).

The presence of Fc receptors on effector (killer) cells is a well known prerequisite of ADCC activity [37, 38]. TF_d was found to impair Fc receptor resynthesis, but did not block the Fc receptors on the cell surface. We do not think, therefore, that this effect of TF_d may offer a clue to the ADCC suppression. The higher molecular weight fraction I may be responsible for the inhibitory effects of crude TF_d preparations observed sometimes in therapy [24]; therefore, use of the purified fraction III is recommended.

The exact mechanism of the effects of TF_d on ADCC reactivity *in vitro* remains unsolved and further studies are needed to elucidate its possible significance *in vivo*.

REFERENCES

1. HERSEY, P., ISBISTER, J., EDWARDS, A., MURRAY, E., ADAMS, E., BIGGS, J., MILTON, G. W.: Lancet **1**, 825 (1976).
2. NELSON, K., POLLACK, S. B., HELLSTRÖM, K. E.: Int. J. Cancer **16**, 932 (1975).
3. POLLACK, S.: Int. J. Cancer **11**, 138 (1973).
4. HAKALA, T. R., LANGE, P. H., CASTRO, A. E., ELLIOT, A. Y., FROLEY, E. E.: New Engl. J. Med. **291**, 637 (1974).

5. Peter, H. H., Kalden, J. R., Seeland, P., Diehl, V., Eckert, G.: Clin. exp. Immunol. **20,** 193 (1975).
6. Zighelboim, J., Gale, R. P., Chin, A., Bonavida, B., Ossorio, R. C., Fahey, J. L.: Clin. Immunol. Immunopath. **3,** 193 (1974).
7. Carter, S. K.: Amer. Sci. **64,** 418 (1976).
8. Kirkpatrick, C. H., Gallin, J. I.: Oncology **29,** 46 (1974).
9. MacLennan, I. C. M.: Proc. roy. Soc. Med. **68,** 216 (1975).
10. Rosenberg, S. A.: Cancer **36,** 821 (1975).
11. Vetto, R. M., Burger, D. R., Nolte, J. E., Vandenbark, A. A., Baker, H. W.: Cancer **37,** 90 (1976).
12. Campbell, A. C., MacLennan, I. C. M.: Lancet **1,** 856 (1976).
13. Böyum, A.: Scand. J. clin. Lab. Invest. **21,** Suppl. **97** (1968).
14. Lawrence, H. S.: Advanc. Immunol. **11,** 195 (1969).
15. Nékám, K., Kalmár, L., Gergely, P., Kelemen, G., Fekete, B., Láng, I., Lévai, J., Petrányi, Gy.: Clin. exp. Immunol. **27,** 416 (1977).
16. Salaman, M. R.: Immunology **26,** 1069 (1974).
17. Perlmann, P., Perlmann, H.: Cell Immunol. **1,** 300 (1970).
18. Urbaniak, S. J.: Brit. Haemat. **33,** 409 (1976).
19. Láng, I., Fekete, B., Gergely, P., Petrányi, Gy.: Haematologia (Budapest). In press.
20. Brain, P., Marston, R. H.: Eur. J. Immunol. **3,** 6 (1973).
21. Hamblin, A. S., Maini, R. N., Dumonde, D. C.: Clin. exp. Immunol. **23**, 290 (1976).
22. Brandes, L. J., Goldenberg, G. J.: Cancer Res. **34,** 3095 (1974).
23. Dupont, B., Ballow, M., Hansen, J. A., Quick, C., Yunis, E. J., Good, R. A.: Proc. nat. Acad. Sci. (Wash.) **71,** 867 (1974).
24. Gallin, J. I., Kirkpatrick, C. H.: Proc. nat. Acad. Sci. (Wash.) **71,** 498 (1974).
25. Levin, A. S., Byers, V. S., Fudenberg, H. H., Wybran, J., Hackett, A. J., Johnston, J. O., Spitler, L. E.: J. clin. Invest. **55,** 487 (1975).
26. Erickson, A. D., Holzman, R. S., Valentine, F. T., Lawrence, H. S.: In Ascher, M. S., Gottlieb, A. A., Kirkpatrick, C. H. (eds): Transfer Factor Basic Properties and Clinical Applications. Academic Press, New York 1976. p. 23.
27. Mueller-Eckhardt, C., Ritts, R. E.: Blut **32,** 353 (1976).
28. Kirkpatrick, C. H., Gallin, J. I.: Cell Immunol. **15,** 470 (1975).
29. Neidhart, J. A., Schwartz, R. A., Hurtubise, P. E., Murphy, S. G., Metz, E. N., Balcerzak, S. P., LoBuglio, A. F.: Cell Immunol. **9,** 319 (1973).
30. Ascher, M. S., Andron, L. A.: In Ascher, M. S., Gottlieb, A. A., Kirkpatrick, C. H. (eds): Transfer Factor Basic Properties and Clinical Applications. Academic Press, New York 1976. p. 3.
31. Dunnick, W., Bach, F. H.: Proc. nat. Acad. Sci. (Wash.) **72,** 4573 (1975).
32. Kirkpatrick, C. H.: J. Allerg. clin. Immunol. **55,** 411 (1975).
33. Gale, R. P., Zighelboim, J.: J. Immunol. **114,** 1047 (1975).
34. Strom, T. B., Garavoy, M. R., Bear, R. A., Gribik, M., Carpenter, C. B.: Cell Immunol. **20,** 247 (1975).
35. Arala-Chaves, M. P., Ramos, M. T. F., Rosado, R., Branco, P.: Int. Arch. Allerg. Appl. Immunol. **46,** 612 (1974).
36. Burger, D. R., Vetto, R. M., Vandenbark, A. A.: Cell Immunol. **14,** 332 (1974).
37. Gale, R. P., Zighelboim, J., Ossorio, R. C., Fahey, J. L.: Clin. Immunol. Immunopath. **3,** 377 (1975).
38. Larsson, A., Perlmann, P.: Int. Arch. Allergy **43,** 80 (1972).

Address of the authors:

Kristóf Nékám, István Láng, Péter Gergely, Gyula Petrányi
Second Department of Medicine, Semmelweis University Medical School
H-1444 Budapest, P.O.B. 277, Hungary

Béla Fekete
"Korányi" National Institute of Tuberculosis and Pulmonology
H-1529 Budapest, P.O.B. 1, Hungary

Acta microbiol. Acad. Sci. hung. 25, 199—201, 1978

STIMULATION OF PHAGOCYTOSIS BY HUMAN LYMPHOKINES

S. Sipka, P. Szerze, M. Szabó, Cs. Balázs and T. Szilágyi

Institute of Pathophysiology and First Department of Medicine, University Medical School, Debrecen

(Received October 25, 1977)

Supernatants of concanavalin A stimulated human lymphocytes increased the phagocyting and intracellular killing activities of human macrophages and neutrophils tested with particles of *Saccharomyces cerevisiae.*

Lymphokines, the biological active products of lymphoid cells responding to antigen or mitogens (e.g. concanavalin A, phytohaemagglutinin) are considered potential mediators of cellular immunity [1]. Their role manifests itself with a delayed type hypersensitivity reaction, in protective immunity and in cell cooperation effects [2].

Beside the macrophage migration inhibitory factor there are other biologically active products of lymphocytes affecting the macrophages, such as macrophage chemotactin, a substance attracting macrophages and the macrophage activation factor which enhances macrophage motility and phagocytosis [3]. Fowler *et al.* [4] demonstrated the enhancement of macrophage bacteriostasis by the products of activated lymphocytes. Pantalone and Page [5] described the lymphokine-induced production and release of lysosomal enzymes by macrophages. According to previous data [4, 5] lymphokines can be regarded as macrophage activators.

The role of the lymphokine factor, neutrophil chemotactin, has been shown in the processes of lymphocyte–neutrophil interactions [3], but there is hardly any information whether lymphokines can increase the phagocytosis of neutrophils.

To obtain direct evidence of the effect of the lymphokine containing supernatants of concanavalin A stimulated human lymphocytes on the phagocyting and intracellular killing activities of human macrophages and neutrophils, we examined the particles of *Saccharomyces cerevisiae* phagocyted by these cells [6].

Materials and methods

Preparation of lymphokines. Normal human blood anticoagulated by citrate was used. Preparation of lymphocytes was carried out in Uromiro–Ficoll solution in Parker's 199 medium at room temperature. No serum was added. The samples were centrifuged at 800 *g* for 15 min, washed twice. They contained 90–95% mononuclear cells.

Lymphocytes (10^7 cells) were cultured at 37 °C in 5% carbon dioxide and 95% air in the presence of 300 μg concanavalin A (Con A; Sigma) completed with penicillin and streptomycin 100 IU/ml) at 37 °C for 36 hr. The cell-free supernatants were chromatographed on Sephadex G-75 column using 0.01 M phosphate buffer pH 7.4 for elution. In the control preparation, concanavalin A was added at the end of cultivation. The protein containing fractions were collected and concentrated to about 1 mg/ml.

The preparations containing the leukocyte migration inhibitory factor and skin reactive factor were termed active supernatant (AS) and the control was designated CS. Leukocyte migration inhibitory factor activity was tested on human leukocytes according to KINCSES and SZABÓ [7]. Skin reactive factor testing was carried out on guinea pig skin by measurement of the blue areas at the inner surface of the skin 4 hr after the intracardiac injection of 1% Evans blue.

Separation of human macrophages was carried out by a quantitative modification of REBUCK's skin window technique [8]. An 1 cm^2 area of both forearms of patients was abraded to the papillary layer of corium. Sterile coverslips were fixed on the injured area and the adhering cells forming a macrophage monolayer during 8 hr, were collected, covered with some drops of Parker's 199 medium completed with 15% own serum.

Separation of human neutrophils was carried out according to MOVAT *et al.* [9]. The cells were kept in Parker's 199 medium and completed with 15% own serum.

Evaluation of the phagocytic activity of macrophages. 50 μg AS and CS were preincubated with 10^5 macrophages at 37 °C for 30 min in a wet chamber. Without washing, 10^6 of the particles of opsonized *S. cerevisiae* preincubated with the patient's own serum for 60 min were added to the samples at 37 °C for 60 min in the wet chamber. After washing with culture medium containing own serum the preparations were dried and stained according to May–Grünwald–Giemsa and the particles phagocyted by 100 macrophages were counted.

Evaluation of the phagocytic activity of neutrophils. Active and control supernatants were preincubated with 10^5 cells at 37 °C min. Without washing, 10^6 opsonized saccharomyces cells were added to the sample at 37 °C for 60 min. The preparations were smeared on coverslips and stained and estimated similarly as the macrophages.

Evaluation of intracellular killing of S. cerevisiae particles by macrophages and neutrophils. After incubation with the saccharomyces particles at 37 °C for 60 min, the samples were stained with 1% methylene blue. The counts of stained phagocyted particles found intracellularly were estimated within 5–10 min.

Results

The active supernatants of concanavalin A stimulated human lymphocytes elevated the phagocytic and intracellular killing capacity of human macrophages and neutrophils (Table I).

Table I

Effect of supernatants of concanavalin A stimulated human lymphocytes on the phagocytosis and intracellular killing of human macrophages and neutrophils

Samples	Macrophages		Neutrophils	
	Phagocytosis	Killing	Phagocytosis	Killing
Control supernatants (CS)	254 ±16.86	84 ±8.92	282 ±21.33	88 ±10.42
Active supernatants (AS)	328 ±27.39 p<0.001*	115 ±17.04 p<0.001	367 ±32.61 p<0.001	179 ±31.49 p<0.001

The results are averages of three experiments
* = Student's *t* test

Discussion

In our experiments the counting of *S. cerevisiae* particles phagocyted by leukocytes was found to be a simple and reproducible method.

Our results showed the stimulating effect of supernatants of concanavalin A stimulated human lymphocytes on the phagocytosis and intracellular killing of peripheral macrophages and neutrophils. Beside the study of PANTALONE and PAGE [5] who showed that lysosomal enzyme release could be induced by lymphokines in macrophages, our data proved directly the enhanced phagocytic activity of human macrophages and leukocytes on the influence of lymphokines. The slighter killing effect of macrophages than that of neutrophils may be connected with the lower amount of lysosomal enzymes in the macrophages than in the neutrophils.

The ability of lymphokines to activate macrophages and neutrophils might be part of their biological significance.

REFERENCES

1. PICK, E., TURK, J. L.: Clin. exp. Immunol. **10,** 1 (1972).
2. DUMONDE, D. C., KELLY, R. H., PRESTON, P. M., WOLSTENCROFT, R. A.: In FURTH, R. (ed.): Mononuclear phagocytes in immunity, infection and pathology. Blackwell, Oxford 1975. pp. 675–699.
3. COHEN, S.: Hum. Path. **7,** 249 (1976).
4. FOWLER, E. R., FAJARDO, I. M., LEIBOWITCH, J. L., DAVID, J. R.: J. exp. Med. **138,** 952 (1973).
5. PANTALONE, R. M., PAGE, R. C.: Proc. nat. Acad. Sci. (Wash.): **72,** 2091 (1975).
6. WENKATARAMAN, M., MOHAPATRA, L. N., BHOYAN, U. N.: Sabouraudia **9,** 183 (1973).
7. KINCSES, É., SZABÓ, G.: Probl. Ophthal. **16,** 95 (1976).
8. MAGLIULO, E., DE FES, V., STRIPE, A., RIVA, S., SAVOLA, D.: Clin. exp. Immunol. **14,** 371 (1973).
9. MOVAT, H. Z., STEINBERG, S. G., HABAL, F. M., EANADIVE, N. S.: Agents and Actions **3,** 284 (1973).

Address of the authors:

SÁNDOR SIPKA, TIBOR SZILÁGYI
Institute of Pathophysiology, University Medical School,
H-4012 Debrecen, P.O.B. 23, Hungary

PÉTER SZERZE, MÁTÉ SZABÓ, CSABA BALÁZS
First Department of Medicine, University Medical School,
H-4012 Debrecen, P.O.B. 19, Hungary

Acta microbiol. Acad. Sci. hung. 25, 203—207, 1978

ANTIBODIES TO RUBELLAVIRUS, HERPES SIMPLEX VIRUS AND TOXOPLASMA GONDII IN SERIAL SERUM SAMPLES OF PREGNANT AND NON-PREGNANT WOMEN*

A. S. PÁCSA and B. PEJTSIK

Institute of Microbiology, University Medical School, and County Hospital, Pécs

(Received October 28, 1977)

By testing serial serum samples of 213 pregnant women for rubellavirus, of 196 for herpes simplex virus and of 134 for *Toxoplasma gondii*, it was found that during pregnancy there was a fall in the humoral antibody level. Presence and titre of antibodies were lower in sera of pregnant than of non-pregnant women. Alteration of the humoral antibody level during pregnancy may influence serological studies aimed at clarifying the role of infections in fetal malformations. Serial serum samples (4 samples from each pregnant woman involved) should be tested for obtaining reliable data regarding the frequency of infections during pregnancy.

It is estimated that approximately 14% of fetal malformations are associated with well-defined genetic factors [1]. Apart from these, a considerable part of the malformations is due to environmental hazards [2–4]. Among these, infections during pregnancy play a significant role. Viruses like rubella [5–7], herpes simplex [8–10], cytomegalovirus [11, 12], influenza [13], varicella [14, 15] and coxsackie B [16] may produce a variety of fetal damages. Other microbial agents such as *Listeria monocytogenes* [17] and *Toxoplasma gondii* [18, 19] are also important in this respect.

Though we know a number of agents which may inhibit normal fetal development, the frequency of damages and clinical symptoms due to a particular infection need further clarification. A further open question is whether the presence of maternal serum antibodies protects the fetus against reinfections [20].

Infections caused by TORCH (Toxoplasma, Others, Rubella-, Cytomegalo- and Herpesviruses) agents [21] — which possibly play the most significant role in the causation of fetal malformations — may occur subclinically. Symptomless infections may, however, be responsible for severe fetal damages [4, 13]. For introducing effective preventive measures against fetal malformations [22], collaborative studies are needed to determine the mode of action of intrauterine infections.

In 1975, we have started a project aimed to clarify the role of intrauterine infections in fetal malformations. In the first series of the study, antibody titres of pregnant and non-pregnant women were compared.

* Supported by Grant No. 5, Scientific Council, Ministry of Health, Budapest.

Materials and methods

Population. (a) Pregnant women. In town Pécs and County Baranya, Hungary, Pregnancy Care Units (PCU) are operating for monitoring women during their pregnancy. This organization provides an excellent network to obtain serial serum samples (one in each trimester) from pregnant women aged between 20 and 30 years. The County Hospital admits for delivery the majority of women covered by our project and then an additional sample is taken. By this organization, we cover now approximately 5000 pregnancies a year. It is planned to build up a serum bank containing serial samples from 15 000 pregnancies.

(b) Control group. Blood samples were collected from 98 age-matched, non-pregnant women wearing IUDs. The first sample was drawn at the time of IUD insertion and three more samples were taken at three months intervals. Registered and coded sera were stored at −20 °C.

Serology. For titration of rubella antibodies the haemagglutination inhibition (HI) test was used. The procedure described in Flow Manual [23] has been followed.

Antibodies to herpes simplex virus (HSV) and *T. gondii* were determined with a modified version [24] of the indirect immunofluorescent antibody (IFA) technique [25]. In the case of HSV, HEp-2 cells grown in Roux flasks were infected with 100 mean tissue culture infective doses of virus strain type 1 (HIL). After incubation at 35 °C overnight, cells were washed and collected into 2 ml phosphate-buffer-saline (PBS). From this cell suspension 8 drops were placed by a capillary pipette on cleaned slides. After drying, the drops were fixed in cold acetone for 5 min and kept at −20 °C till use.

For titration of *T. gondii* antibodies, cells of human amnion (AV-3) origin were infected with parasite-rich mouse ascitic fluid. After incubation at 37 °C for 48 hr, the cells were treated as in the case of HSV-infected cells.

Antibody titration. Serum dilutions were dropped on cells infected with HSV or *T. gondii* for 1 hr. After washing them with PBS, anti-human-IgG conjugated with fluorescein-isothiocyanate (FITC; conjugates were purchased from Human, Institute for Serobacteriological Production and Research, Budapest), was layered on the drops. After incubation for 1 hr the slides were washed again, counterstained with Evans blue, mounted in glycerol–PBS and examined under the fluorescence microscope. The titre of a serum sample was the last dilution still giving a characteristic fluorescence in the cytoplasm. Standard positive and negative serum samples as well as non-infected cells served as controls.

Results

Testing 4 serum samples from each non-pregnant woman (IUD group), it was found that their serum antibody level to the three agents known to play a role in fetal malformations remained practically unchanged for a period of nine months.

Of 98 women examined so far, 3 showed titre rises to rubellavirus. Fall in rubella antibody titre could not be detected. Similar titre rises in sera from 213 pregnant women could be found in six cases; of these, 37 showed an at least fourfold fall in rubella antibody titre. In the IUD group, antibodies to rubellavirus were present in 90% during the nine months period. In contrast, in sera of pregnant women, antibodies were present in 79% in the first trimester, and only in 68% at the time of delivery (Fig. 1). Between IUD and pregnant groups the difference was statistically significant; $P < 0.1$ in the first trimester and $dP < 0.05$ at delivery.

Antibodies to HSV were determined in 196 pregnant and in 98 non-pregnant women. Along with 13 fourfold titre rises, in 12 pregnant women the antibody titre decreased. Comparing the two HSV groups, comparatively higher titres were more frequent in the IUD group than in the pregnant

group. Taking 1/160 titres into account (65% in the IUD and 25% in the pregnant groups) the difference between the two groups was significant: $P < 0.05$ (Fig. 2).

Antibodies to *T. gondii* were determined in 134 pregnant women, and in the IUD group. A fall in antibody titre was found in 4 of the pregnant sera. Their mean antibody titre was also lower than that of the IUD group. Antibody titres of pregnant women in the third trimester were compared to the titres found in the third sample of the IUD group (Fig. 3). Statistical analysis based on titre 1/40 revealed a difference of $P < 0.1$ between the two groups.

The antibodies determined were of the IgG type as it was impossible to obtain serum samples frequently enough for IgM determination.

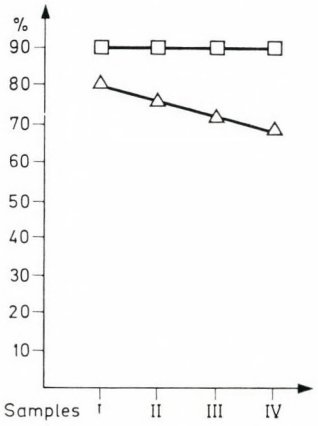


Fig. 1. Rubella HI antibodies in serial serum samples of pregnant and non-pregnant women. Comparison is based on antibody titre found in serial serum samples of 213 pregnant women (I = first trimester, II = second trimester, III = third trimester and IV = time of delivery; △ — △) and of 98 non-pregnant women wearing an intrauterine device (IUD: □ — □). In their case I = first sample taken at the time of IUD insertion, II, III and IV = three consecutive samples at three months intervals

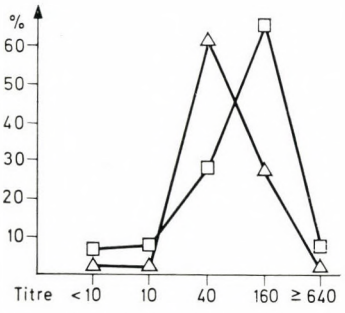


Fig. 2. HSV IF antibodies in sera of pregnant (△ — △) and non-pregnant (IUD: □ — □). women. Comparison is based on titres found in sera of 196 pregnant women and in sera of 98 non-pregnant women

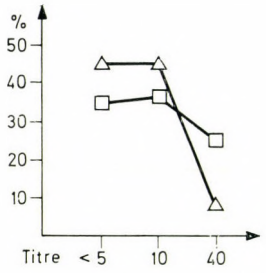


Fig. 3. Toxoplasma IF antibodies in sera of pregnant (△ — △) and non-pregnant (□ — □) women. Comparison is based on titres found in sera of 134 pregnant women in third trimester and in 98 sera of non-pregnant women

Discussion

Our previous serological study, comparing a first sample collected early in pregnancy to a second one obtained at the time of delivery, revealed a certain impairment of humoral immunity during pregnancy [26]. Then, 30 to 40 days after delivery, the titre returned to the level measured in the early phase of gestation. Reduction of humoral immunity is associated with the complex feto-maternal interactions [27–29]. Apart from feto-maternal immune reactions, alteration of immunity during pregnancy may jeopardize the value of serological assessment.

It seemed therefore justified to continue our serological study on serial serum samples to clarify the extent of alterations during pregnancy. The results indicate that the level of humoral immunity decreases with the progress of pregnancy. This fact should be taken into account when intrauterine infections are determined with the use of serological tests. Nevertheless, serial serum samples (at least one in each trimester) provide a reasonable background for obtaining reliable data in detecting infections during pregnancy.

Determining titre changes in sera of non-pregnant women was justified by the scarcity of such data [30, 31] for the normal population.

We consider that our serum bank gives a solid base for analising the relationship between intrauterine infections and fetal malformations.

Acknowledgements. We are indebted to Drs T. Angyal and Z. Nagy and their staff for cooperation in collecting blood samples; and to Miss Éva Klement, Miss Éva Nagyréti and Miss Magdalena Koronics for excellent technical assistance.

REFERENCES

1. Kleinebrecht, J., Degenhardt, K. H.: Lancet **2,** 1097 (1975).
2. Smithells, R. W.: Brit. med. Bull. **32,** 27 (1976).
3. Dudgeon, J. A.: Brit. med. Bull. **32,** 77 (1976).

4. Fucillo, J. A., Sever, J. L.: Bact. Rev. **37,** 19 (1973).
5. Gregg, N. M.: Trans. ophthal. Soc. Aust. **3,** 35 (1941).
6. Lundström, R.: Acta paediat. scand. **51,** Suppl. **133,** 9 (1962).
7. Michaels, R. H., Mellin, G. W.: Pediatrics **26,** 200 (1960).
8. South, M. A., Tompkins, W. A. F., Morris, R., Rawls, W. E.: J. Pediat. **75,** 13 (1969).
9. Nahmias, A. J., Dowdle, W. R., Josey, W. E., Naib, Z. M., Painter, L. M., Luce, C.: J. Pediat. **75,** 1194 (1969).
10. Montgomery, J. R.: Amer. J. Dis. Child. **126,** 364 (1973).
11. Hanshaw, J. B.: Pediat. Clin. N. Amer. **13,** 279 (1966).
12. Stern, H., Tucher, S. M.: Brit. med. J. **2,** 268 (1973).
13. Hakulinen, T., Hovi, L., Karkinen-Jaaskelainen, M.: Brit. med. J. **4,** 265 (1973).
14. McKendry, J. P. J., Bailey, J. D.: Canad. med. Ass. J. **108,** 66 (1973).
15. Savage, M. O., Moosa, A., Gordon, R. R.: Lancet **1,** 352 (1973).
16. Brown, G. C., Evans, T. N.: J. Amer. med. Ass. **199,** 183 (1967).
17. Gray, M. L., Seeliger, H. P. R., Potel, D.: Clin. Pediat. **2,** 614 (1963).
18. Desmonts, G., Courver, J.: New Engl. J. Med. **290,** 1110 (1974).
19. Kean, B. H., Kinball, A. C.: Amer. J. Dis. Child. **131,** 21 (1977).
20. Stagno, S., Reynolds, D. W., Huang, E. S., Thames, S. D., Smith, J. R., Alford, C. A.: New Engl. J. Med. **296,** 1254 (1977).
21. Nahmias, A. J., Roizman, B.: New Engl. J. Med. **289,** 781 (1973).
22. Medearis, D. N.: New Engl. J. Med. **296,** 1289 (1977).
23. Standardized rubella hemagglutination-inhibition test. U.S. DHEW. October, 1970, Flow Labs. 1975.
24. Pácsa, A. S., Kummerländer, L., Pejtsik, B., Pali, K.: J. nat. Cancer Inst. **55,** 775 (1975).
25. Leiniki. P.: Arch. ges. Virusforsch. **35,** 349 (1971).
26. Pácsa, A. S., Pejtsik, B.: Lancet **1,** 330 (1977).
27. Anderson, J. M.: Lancet **2,** 1077 (1971).
28. Billingham, R. E.: New Engl. J. Med. **270,** 667 (1964).
29. Gudson, J. P.: Immunology of human reproduction. Academic Press, New York 1976.
30. Sever, J. L.: Int. J. Obstet, Gynec. **8,** 763 (1970).
31. The Collaborative Perinatal Study (NIH): The women and their pregnancies. DHEW Publication No. 73–379, NIH 1972.

Address of the authors:

A. Sándor Pácsa
Institute of Microbiology, University Medical School
Szigeti út 12, H–7643 Pécs, Hungary

Béla Pejtsik
County Hospital
H-7621 Pécs, Hungary

Acta microbiol. Acad. Sci. hung. 25, 209–212, 1978

DIPLOID FORMATION OF CANDIDA TROPICALIS VIA PROTOPLAST FUSION

C. VALLIN and L. FERENCZY

Department of Microbiology, Attila József University, Szeged

(Received November 10, 1977)

Haploid auxotrophic mutants were produced from *Candida tropicalis*, and protoplast usion was induced by polyethylene glycol. The resulting nutritional complementation was due to heterokaryon formation and, at a much lower frequency, to spontaneous diploidization. During cultivation, heterokaryotic clones regularly gave rise to heterozygous diploids from which, in turn, haploids could be isolated. The technique of protoplast fusion gives an opportunity for genetic analysis of this and similarly asexual fungal species.

Protoplast fusion and nutritional complementation of yeasts has been described with *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Rhodosporidium toruloides* [1–4]. Normally, in these species the diploid formation is a consequence of mating reactions and nuclear fusion between two cells of opposite mating-types. By means of protoplast fusion techniques, diploids can be produced also within the same mating-type [1–4].

In *Candida tropicalis* no mating reactions or parasexual events resulting in diploid formation are known. The only means of temporary diploidization has been described as "autoploidization" [5].

We have now made attempts at protoplast fusion with haploid auxotrophic mutants of *C. tropicalis* to obtain nutritional complementation and heterozygous diploids as the first step in the genetic analysis of the species. The method of protoplast fusion seems to be the only possibility to start this kind of investigation when sexual processes or heterokaryon formation by anastomosis are not known. At the same time, the protoplast fusion technique is easy to carry out with good efficiency in the case of *C. tropicalis*, as it has been shown previously [6].

Materials and methods

Strains. A series of auxotrophic mutants was produced from *C. tropicalis* CBS 644 by UV treatment (Philips TUV 15 W lamp, 5% survival). The frequency of auxotrophic mutants was 0.4%, practically the same as for haploid strains of *S. cerevisiae* or *S. pombe*. Stable mutants requiring adenine *(ade)* and cysteine *(cys)* were selected for the fusion experiments. At low adenine concentrations the *ade* mutant produced red pigment with maximum yield at an adenine concentration of 6 μg/ml. The prototrophic strain and the mutants showed the same spectrum of carbohydrate assimiliation which corresponded to the standard description [7]. No reverse mutation has ever been observed from the selected auxotrophs.

Media. YL: 0.5% yeast extract (Oxoid) and 1% glucose; YA: YL supplemented with 2% agar (Difco Bacto-agar); MA: minimal medium containing 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1% glucose, 2% agar and vitamins. OMA: MA supplemented with 0.9 M mannitol as osmotic stabilizer. When required, MA or OMA was also supplemented with 6 μg/ml adenine and/or 25 μg/ml cysteine–HCl.

Protoplast formation. Cells of *ade* and *cys* mutants were grown under shaking in YL at 30 °C for 12 hr, collected by centrifugation, washed twice with distilled water, and treated with 1.5% (w/v) snail enzyme (freeze-dried gastric juice of *Helix pomatia* produced in this institute) dissolved in 0.6 M KCl containing 0.1% beta-mercaptoethanol. The cell concentration was about 5×10^7/ml. After 1 hr at 30 °C, at least 99% of the cells were converted into protoplasts, which were collected and washed by centrifugation, and then transferred into 0.9 M mannitol.

Protoplast fusion and regeneration. Protoplast suspensions of the two mutants, each containing 10^6/ml protoplast, were mixed in a 1 : 1 ratio, and 10 ml portions were sedimented by centrifugation. The supernatant was removed and 2 ml polyethylene glycol (PEG) mol wt 4000, 40% in 0.1 M $CaCl_2$ solution, was layered onto the protoplasts and the tubes were gently shaken several times. After 30 min the suspensions, containing protoplast aggregates of different sizes, were diluted in PEG solution, mixed into OMA at 43 °C and transferred as a thin layer to the surface of already solidified OMA plates with and without adenine and cysteine. About 5×10^2 colony-forming units (single protoplasts and aggregates of protoplasts) were applied to each 5 cm diam. plate. The plates were incubated at 30 °C for 5 days and colonies developing on OMA were isolated and transferred to MA plates.

The frequency of complementation was determined by comparing the number of colonies growing on OMA to that of colonies growing on OMA supplemented with both adenine and cysteine; and also by comparing the number of colonies growing on OMA to the number of protoplasts involved, counted by a haemocytometer before adding PEG to the mixed suspension of the two mutants.

The controls employed were: protoplasts from the parental mutants without PEG, both separately and in a mixture; protoplasts from the parental mutants separately with PEG; intact cells from the parental mutants, with or without PEG, and both separately and in a mixture.

All procedures were carried out under aseptic conditions.

Cytological analysis. The original prototrophic strain, the two mutants, fusion isolates and their prototrophic segregants were inoculated onto YA. After 3 days of cultivation at 30 °C, samples were taken, photographed under the microscope and the cell dimensions were measured. The number of nuclei was determined by acridine orange staining and fluorescence microscopy after fixation with ethanol–acetic acid (1 : 1), and the amount of DNA per cell was estimated using chicken erythrocyte DNA as standard, as described previously [2].

Results and discussion

Colonies were not observed when protoplasts of the two mutants were inoculated separately in OMA, or when they were mixed without PEG-treatment before inoculation. Similarly, no growth was found when intact cells of the mutants were used separately or mixed, with or without PEG-treatment. These facts indicate that mutants are stable auxotrophs; cross-feeding between the *ade* and *cys* mutants is negligible; and without protoplast formation and aggregation by PEG there is no nutritional complementation. On the other hand, pink, slow-growing, frequently sectored prototrophic colonies appeared in OMA due to protoplast fusion, somewhat similar to those obtained with *Geotrichum candidum* [8, 9]. At a frequency of about 1% of the pink colonies, fast-growing white prototrophic colonies also appeared. The frequency of nutritionally complemented colonies was 4.3×10^{-3} when the number of colonies in nutritionally non-supplemented and supplemented

media was compared, and 2.6×10^{-4} when the comparison was made concerning the number of colonies in OMA and that of protoplast pairs measured with the haemocytometer.

Acridine orange staining revealed that cells of the pink fusion colonies contained 1 to 8, most frequently 3 or 4 nuclei, while the original prototrophic strain and both mutants contained only 1 nucleus per cell. The fusion colonies could be maintained indefinitely on MA. On YA or on MA supplemented with adenine and cysteine, the fusion cells of pink colonies regularly gave rise to cells of the parental types with high frequencies. This indicates the existence on MA of unstable heterokaryons.

Pink, unstable heterokaryons regularly gave rise to stable and fast-growing white-colony prototrophs, the cells of which contained only 1 nucleus each. Spontaneous segregation into the parental mutants on nutritionally-rich media was isolate-dependent, with a low frequency of 10^{-5} or less. The dimensions of 3 randomly selected uninucleate prototrophic isolates, their DNA contents and those of the parental mutants are seen in Table I.

The fusion isolates were more ellipsoid than the parental mutants, usually larger, and contained much more DNA. Although with low frequency, both parental mutant types could be recovered from the same stable prototrophic isolates. All these data indicate that the stable prototrophic isolates were diploids.

The distribution of DNA between the nucleus and the mitochondria, and the relationships between this distribution and ploidy are unknown. A high mitochondrial DNA content and/or its little ploidy-dependency might be an explanation why the DNA contents of the cells do not exactly follow the ploidy pattern. Neither can the existence of aneuploids be excluded.

C. tropicalis differs from the other yeasts studied by protoplast fusion in that the heterokaryotic state can be maintained indefinitely, as in the

Table I

Cell size and DNA content of stable prototrophic fusion isolates and the parental mutants
Standard errors are based on 50 measurements and four DNA determinations.
Both prototrophs and parental mutants are uninucleate

Strains	Length, μm	Width, μm	DNA content (fg DNA per cell)
Prototroph S-3	8.02 ± 0.26	5.44 ± 0.16	79.9 ± 3.6
Prototroph S-4	8.13 ± 0.29	5.43 ± 0.09	76.3 ± 6.2
Prototroph S-5	9.37 ± 0.28	6.32 ± 0.19	82.1 ± 4.1
Auxotroph *ade*	6.70 ± 0.16	5.59 ± 0.15	49.7 ± 4.0
Auxotroph *cys*	6.50 ± 0.17	5.32 ± 0.11	50.0 ± 4.1

case of filamentous fungi [8–10]. This characteristic is attributed to the ability of this yeast to form filaments. Linkage group studies by induced haploidization are in progress.

Acknowledgements. This work was conducted while C. V. was in receipt of a fellowship sponsored jointly by the Hungarian Academy of Sciences and UNDP–UNESCO, which is gratefully acknowledged. The authors are indebted to Mr. L. NAGY and Mrs. ÉVA FORRAI for excellent technical assistance.

REFERENCES

1. SIPICZKI, M., FERENCZY, L.: Molec. gen. Genet. **151,** 77 (1977).
2. FERENCZY, L., MARÁZ, A.: Nature (Lond.) **268,** 524 (1977).
3. SOLINGEN, P. VAN, PLAAT, J. B. VAN DER: J. Bact. **130,** 946 (1977).
4. SIPICZKI, M., FERENCZY, L.: FEMS microbiol. Letters **2,** 203 (1977).
5. SUKROONGREUNG, S., MIRANDA, L. R. DE: Antonie van Leeuwenhoek **39,** 65 (1973).
6. FERENCZY, L., VALLIN, C., MARÁZ, A.: Proc. Fifth International Specialized Symposium on Yeasts. Keszthely 1977. Part II, p, 85.
7. LODDER, J. (ed.): The Yeasts. North-Holland Publ., Co., Amsterdam–London, 1970.
8. FERENCZY, L., ZSOLT, J., KEVEI, F.: Abstr. 3rd International Symposium on Yeast Protoplasts. Salamanca 1972. p. 74.
9. FERENCZY, L., KEVEI, F., ZSOLT, J.: Nature (Lond.) **248,** 793 (1974).
10. FERENCZY, L., KEVEI, F., SZEGEDI, M.: Experientia (Basel) **31,** 50 (1975).
11. FERENCZY, L., KEVEI, F., SZEGEDI, M.: Experientia (Basel) **31,** 1028 (1975).

Address of the authors:

CARLOS VALLIN
Instituto de Quimica y Biologica Exp. A.C.C.
Ave 26, No. 1605, Nuevo Vedado, Habana, Cuba

LAJOS FERENCZY
Department of Microbiology, Attila József University
H-6701 Szeged, P.O.B. 428, Hungary

NOTE ADDED IN PROOF

Since this manuscript was submitted for publication, a paper entitled "Recombination after protoplast fusion in the yeast *Candida tropicalis*" by P. FOURNIER, A. PROVOST, C. BOURGUIGNON and H. HESLOT has appeared (Arch. Microbiol., **115,** 143, 1977). The results reported in the two papers are in no way contradictory, and complement each other.

Acta microbiol. Acad. Sci. hung. 25, 213—217, 1978

SEROLOGIC DISTRIBUTION OF ANTIBODIES AGAINST ADENOVIRUSES IN SHEEP OF LARGE-SCALE FARMS

V. PÁLFI and S. BELÁK

Central Veterinary Institute, Budapest and Institute of Epizootiology, University of Veterinary Science, Budapest

(Received November 22, 1977)

The common soluble antigen of the first subgroup of bovine adenoviruses was used for assaying 793 sheep sera by the agar gel diffusion test. Of the 50 farms included in the study 43 were found infected. The ratio of reacting samples was 73.7% of the sera obtained from infected farms. Virus neutralization tests revealed that a considerable number of sera reacted specifically with all types of ovine adenoviruses, even with serotypes which had never been isolated in Hungary. The results yielded by the agar gel diffusion tests were compared with the results of virus neutralization tests. Of 850 cattle serum samples, agar gel diffusion tests gave positive results in 33.4%. Virus neutralization test was done only with the bovine adenovirus type 2. No differences could be detected in antibody titres when the prototype strain (No. 19) and the strain isolated from sheep (ORT/111) were compared in parallel titrations. Both ruminant species were found to be infected with bovine adenovirus type 2. Nevertheless, inapparent infection with these strains seemed to be less frequent among cattle than in sheep flocks.

Pneumoenteritis in lambs caused by adenoviruses has frequently been observed in Hungary [1]. Ovine adenovirus type 1 and adenovirus strains closely related to bovine adenovirus type 2 were isolated during the outbreaks [2–4]. Ovine adenovirus types 4 and 5 also were demonstrated in the nasal discharge of lambs showing mild respiratory symptoms [5, 6]. No reports are available on the isolation of bovine adenovirus type 2 from cattle in Hungary, although closely related or identical viruses have often been isolated from-sheep [7].

One of these isolates (Het/3 strain of bovine adenovirus type 2 of sheep origin) proved pathogenic for both lambs and calves in experimental infection [8–11].

A serological survey was performed in order to clarify the occurrence of bovine adenovirus type 2 antibodies in the two ruminant species on some large-scale farms in Hungary. In addition, the sheep sera were tested with all known types of ovine adenovirus.

Materials and methods

Sera. A total of 793 samples of sheep sera from 2–6-year-old breeders were received from 50 large-scale farms and 850 cattle sera were collected from various age groups on 30 farms.

Virus strains. Ovine adenovirus reference strains PX 515 (type 2), PX 611 (type 3), 7769 (type 4) and *SAV* (type 5) were kindly supplied by Dr. J. B. MCFERRAN, Belfast, North

Ireland. The type 1 strain used (GY/14) was a local isolate. The sheep sera were tested with an ovine isolate (ORT/111) of bovine adenovirus type 2. Cattle sera were tested with ORT/111 and with the reference strain No. 19 of bovine adenovirus type 2 (received from Dr. A. BARTHA, Budapest).

Serological techniques. Undiluted sera were screened first in double gel-diffusion test against the common soluble adenovirus antigen. Purified Bacto agar (Difco, Detroit, Michigan), 1.5% in 0.15 M NaCl with 0.03% merthiolate and methyl orange (Reanal, Budapest) was used. The antigen was prepared by concentration with 22.8% w/v ammonium sulphate (Reanal, Budapest) of calf kidney tissue cultures infected with bovine adenovirus type 3. Cultures were frozen and thawed three times.

Virus neutralization tests were done with 200 sheep sera and 200 cattle sera, positive in the gel-diffusion test (10 each from 20 farms). Serum samples were tested in 1 : 5 dilution with 100 $TCID_{50}$ of the viruses. Twenty samples reacting with the strains used in the study were selected and their final titre was determined.

Results

Gel-diffusion tests revealed that none of the sheep sera submitted from 7 farms gave a positive reaction. These were omitted from further calculations. Of 681 samples collected from infected farms 502 (73.7%) gave a positive test. Only 284 cattle sera (33.4%) were positive in the gel-diffusion test.

The frequency of neutralizing antibodies against the different adenovirus serotypes in sheep sera and of those against the bovine adenovirus type 2 in cattle sera is summarized in Table I. Sheep sera were found to contain antibodies against types 2 and 3, which had not been isolated in Hungary. The geometric mean titres were 1 : 12, 1 : 13 and 1 : 14 for the strains 7769, GY/14 and ORT/111, respectively. Results listed in Table I indicate that sheep of infected farms possess antibodies against two or three different adenovirus serotypes.

Table I

Frequency of neutralizing antibodies against adenovirus serotypes in sheep and cattle sera

	Ovine adenovirus serotypes					Bovine adenovirus type 2	
	OA 1	OA 2	OA 3	OA 4	OA 5		
Per cent of positive sera of those reacting in agar gel diffusion test	24.0	49.0	64.0	88.0	29.0	55.0	21,5*
Positive sera per cent of the total number of sera tested**	17.9	36.3	47.0	64.9	21.4	40.0	6.9*

* Cattle sera tested in 1 : 5 working dilution.

** Calculated values to show the real frequency of infection. Mathematical calculations indicate that these values are underestimated due to the low sensitivity of the agar gel diffusion test. The actual per cent of positive sera is less than the upper, but higher than the lower, number in the Table. See text for further details.

Cattle sera were tested with bovine adenovirus type 2 in the neutralization test, and 21.5% of the samples were found positive. Antibody titrations were done in parallel with the reference strain (No. 19) and with the local ovine isolate (ORT/111), but no significant difference could be detected.

Results of the virus neutralization test and of agar-gel precipitation were compared on a mathematical basis. Results are plotted in Fig. 1. The percentage of polynegative sera is shown on the ordinate. The calculation was done on basis of the virus neutralization tests. The number of randomly selected virus serotypes consecutively tested is shown on the abscissa. The expression

$$N_n = \frac{(100 - P_1) \times (100 - P_2) \times \ldots\ldots \times (100 - P_{n-1}) \times (100 - P_n)}{100^{n-1}}$$

gives in per cent the highest possible number of the sera negative against all virus types *(n)* tested, if the percentage of positive sera (P_1; P_2 etc.) was applied in increasing order (P_1 P_2 ... etc.). The reversed order (P_1, P_2 ... P_n) will give the lowest percentage of polynegative sera (N_n) calculated on the basis of the data listed in Table I. The extremes are represented by open circles in Fig. 1.

It was anticipated that the percentage of polynegative sera calculated (N_n) equalled the percentage of sera proving negative in the agar gel precipitation test (arrow in Fig. 1), provided the sensitivity of the two tests was the

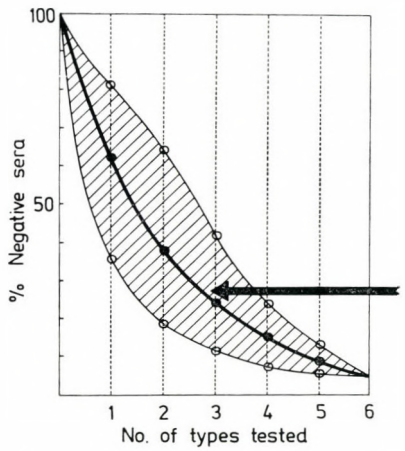


Fig. 1. Calculated per cent of polynegative sheep sera, if different numbers of adenovirus serotypes are consecutively tested in the virus neutralization test. Ordinate: per cent of negative sera. Abscissa: number of serotypes involved. Open circles: maximum and minimum values calculated on the basis of the data given in Table I. Closed circles: arithmetical mean of the percentages given in Table I were used for calculation. Shadowed area: range of variation on the basis of the data given in Table I. Arrow indicates per cent of sera unreactive in agar gel diffusion test using the common soluble antigen (i.e. 26.3% of sheep sera tested with serotypes in the virus neutralization test)

same. The results indicate that this was not the case. Sheep sera were tested with 6 different serotypes and the calculated mean value of polynegative sera (full circles in Fig. 1) was about 5.7%. In contrast, negative sheep sera amounted to 26.3% in the agar gel precipitation test. This value is shown by the arrow in Fig. 1.

Discussion

Adenovirus infections are known to play an important aetiological role in respiratory and enteric diseases of lambs. Specific pneumoenteritis was often seen causing severe losses [1, 2, 4]. Unlike in highly industrialized farms we have observed only mild clinical disease in lambs reared under traditional conditions [7].

The serological survey reported revealed that even seronegative flocks could be found when solely the agar gel precipitation test was used. Nevertheless, these results should be considered carefully. Lack of antibodies detectable by the agar gel precipitation test may result from the time dependent decrease of the antibody level against the common soluble antigen. An antibody level still detectable by the agar gel precipitation test in the sera of adults may be attributed to the booster effect of the hexon antigen due to reinfection with either serotype. The results of the virus neutralization test seem to confirm this hypothesis since it revealed that, on the average, the sera of adults contained antibodies against two or three serotypes.

The results plotted in Fig. 1 allow a comparison of the data obtained in the case of cattle sera using the two serological techniques: 66.6% of the cattle sera were negative in the agar gel precipitation test, and 21.5% of the positive ones reacted with bovine adenovirus type 2 in the virus neutralization test. This is only 6.9% of the total number of sera examined. The presence in Hungarian cattle herds of bovine adenovirus type 2 has been proven by these results. A comparison of the positivity of the agar gel precipitation test with those of sheep sera in Fig. 1 suggests the presence of other bovine adenovirus serotypes.

During the preparation of this manuscript, bovine adenovirus type 2 was isolated from calves (L. Csontos, personal communication). The sheep isolates of this serotype are pathogenic for both ruminant species [8–11]. Whether the virus neutralization titres were the same if tested with different strain(s) of sheep and/or cattle, remains to be determined.

In other countries, isolation of sheep adenoviruses was successful from lambs with respiratory symptoms, and also from apparently healthy animals [5, 6, 12]. The strains were isolated only from faeces whereas our local isolates (i.e. ovine adenovirus types 1, 4 and 5) were heavily shed with both faeces and nasal discharge during the acute phase of the disease.

Serological studies revealed that large-scale farms are infected even with ovine serotypes (i.e. types 2 and 3) which had never been isolated in Hungary. The conclusion may be drawn that similarly as the bovine adenoviruses [13], ovine adenoviruses frequently cause inapparent infections. Increased import and local transfer for breeding purposes have probably contributed to the wide distribution of various ovine adenovirus serotypes. Several strains of these types were proven to be pathogenic. Therefore, the need for new epidemiological measures and the necessity of vaccination have to be taken into consideration when highly industrialized sheep farms are operated.

REFERENCES

1. Belák, S., Pálfi, V.: Acta vet. Acad. Sci. hung. **24,** 241 (1974).
2. Belák, S., Pálfi, V., Palya, V.: Zbl. Vet.-Med. **23,** 320 (1976).
3. Belák, S., Pálfi, V., Palya, V.: Magy. Állatorv. Lap. **31,** 629 (1976).
4. Palya, V., Belák, S., Pálfi, V.: Zbl. Vet.-Med. **24,** 529 (1977).
5. Bauer, K., Müller, M., Gürtürk, S.: Zbl. Vet.-Med. **22,** 656 (1975).
6. Sharp, J. M., McFerran, J. B., Rea, A.: Res. vet. Sci. **17,** 268 (1974).
7. Belák, S., Pálfi, V.: Arch. ges. Virusforsch. **44,** 177 (1974).
8. Belák, S., Pálfi, V., Tury, E.: Acta vet. Acad. Sci. hung. **25,** 91 (1975).
9. Belák, S., Pálfi, V., Szekeres, T., Tury, E.: Zbl. Vet.-Med. **24,** 542 (1977).
10. Tury, E., Belák, S., Pálfi, V.: Acta vet. Acad. Sci. hung. **25,** 171 (1975).
11. Tury, E., Belák, S., Pálfi, V., Szekeres, T.: Zbl. Vet.-Med. In press.
12. McFerran, J. B., Nelson, R., Knox, E. R.: Arch. ges. Virusforsch. **35,** 232 (1971).
13. Rossi, C. R., Kiesel, G. K., Emrich, V. R.: Amer. J. vet. Med. **34,** 841 (1973).

Address of the authors:

Vilmos Pálfi
Central Veterinary Institute
Tábornok u. 2, H-1149 Budapest, Hungary

Sándor Belák
Department of Epizootiology, University of Veterinary Science
Hungária krt. 22, H-1581 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 219—227, 1978

UNIQUE FEATURES OF HEAT-STABLE ENTEROTOXIN OF SHIGELLA FLEXNERI*

I. KÉTYI, ADELE VERTÉNYI, ILONA MÁLOVICS, T. KONTROHR and S. PÁCSA

Institute of Microbiology, University Medical School, Pécs

(Received November 23, 1977)

Sephadex G-100 fractions of ultrasonic lysate of *Shigella flexneri* were compared to the fractions of *Escherichia coli* lysates of Ent^-, LT^+ST^+, LT^+, and ST^+ strains. The range of molecular weight of *S. flexneri* ST fractions was the same as that of *E. coli* LT fractions. Rapid PF activity was associated with the ST peak in the case of *S. flexneri*, and followed the LT activity in the *E. coli* (LT^+ST^+) fractions, and appeared in the same molecular weight range in the case of Ent^- *E. coli* lysate. Cross neutralization could be demonstrated between *S. flexneri* ST and *E. coli* LT. Antigenic relationship between shigella ST and choleragen seemed to be less expressed and rather unilateral.

In a previous paper [1] we described that in the filtrates and ultrasonic lysates of all but few *Shigella flexneri* 3a and 4b strains and also in type 2a strain M42–43 of O'BRIEN *et al.* known as a Shiga-like toxin producer, a rapid permeability factor (PF) and a heat-stable (ST) enterotoxin were present. These activities appeared in the same Sephadex G-100 fractions. LT specific activity was observed neither in crude materials nor in Sephadex fractions. The appearance of shigella ST in the early Sephadex fractions revealed an unusually high molecular weight of the toxin. This fact has made us to conduct further comparative studies on the antigenicity of the ST of *S. flexneri*.

Materials and methods

Strains used are listed in Table I.

Enterotoxin production. Bacteria were cultivated in a modified Sakazaki medium, as described previously [1]. Ultrasonic lysates were prepared from fresh cultures killed by merthiolate. After dialysis against tap water for 2 days and against distilled water for 2–3 days and membrane filtration, the lysates were freeze-dried, distributed in phials, dried over P_2O_5, filled with N_2 gas and stored at -20 °C. Choleragen (Lot No. 0972) was prepared by Dr. R. A. FINKELSTEIN.

Sephadex G-100 fractions were prepared as described previously [1].

Enterotoxic activity was assayed in the case of LT by the classical ("delayed") blueing-test in white rabbits, and in CHO and Y-1 cell cultures as described earlier [1]. ST activity was assayed in 3-day-old suckling mice inoculated orally [1, 2]. The rapid PF discovered by SANDEFUR and PETERSON [3] was investigated according to their original description in white rabbits.

Antisera against *S. flexneri* ST (strain No. 140, type 3a), *E. coli* LT and ST (strain B7A), as well as against rapid PF (strain No. 23473, Ent^-) were produced by immunizing

* Supported by the Scientific Research Council, Hungarian Ministry of Health (3–10–0305–03–1/K).

rabbits according to CALLAHAN *et al.* [4]. Sephadex G-100 fractions containing the highest activity were mixed with Freund adjuvant (Difco) (1 : 1) and rabbits weighing 2.5–3.5 kg were inoculated 4 times with 0.5 ml and thereafter with 1 ml of the mixture subcutaneously into the axillary or inguinal region at weekly intervals. One week after the last injection, the animals were bled, sera were collected, sterilized by filtration and stored at −20 °C without chemical conservation.

The cholera antitoxin used was a Swiss Serum preparation (EC 3/A-2/67-B).

Neutralization tests. Neutralization of shigella ST was performed in suckling mice. A dose of 3 mg freeze-dried concentrated crude *S. flexneri* material was used. It caused slight intestinal dilatation (bowel/body mean index, 0.081). A 0.05 ml volume of 3 mg crude enterotoxin was mixed with 0.05 ml of serum dilution incubated at 37 °C for 30 min and at 4 °C for one hr. With each mixture, four animals were inoculated. After 3 hr, body weight and bowel weight of the suckling mice were measured. For evaluation, the last negative (below index 0.070) and the first positive dilution of a given serum were used. The neutralizing dose of a serum was determined by graphical plotting, corresponding to the mean control value (0.081). It was expressed in absolute quantity.

Neutralization of LT and choleragen activity were performed in CHO cells, estimating the highest serum dilution still inhibiting the cytotonic effect (elongation of cells) of 2–4 tissue culture cytotonic doses (TCCD).

Table I

List of strains

Designation	Species and antigenic structure	Enterotoxigenicity	Origin
140	*S. flexneri* type 3a	ST^+	G. V. LÁSZLÓ
23473	*E. coli* O78:K80:NM	Ent^-	É. CZIRÓK
"B7A"	*E. coli* O148:K87:H21	LT^+ST^+	S. B. FORMAL
Barnum 2176E8	*E. coli* O138:K81	ST^+	B. NAGY
Moon No. 263	*E. coli* O8:K87 K88a, b:H19	LT^+	B. NAGY

Results

1. *Sephadex G-100 fractions of S. flexneri and E. coli.* Ultrasonic lysates were prepared from all strains. After dialysis they were concentrated by freeze-drying. The dry material was dissolved at 200 mg aliquots in 0.005 M Tris–HCl (0.001 M EDTA) buffer pH 7.6, and fractionated on the same Sephadex G-100 column. Fractions of 5 ml each were collected and stored at −20 °C. In cases of *S. flexneri* 3a No. 140, Ent^- (O78 : K80 : NM, No. 23473) *E. coli* and LT^+ST^+ (O148 : K87 : H21, No. B7A) *E. coli* the fractions were tested first for rapid PF activity, thereafter selected fractions were tested in suckling mice, and all the fractions were investigated in the classical ("delayed") PF test as well as in CHO cells. Samples prepared from LT^+ strain No. 263, and ST one No. 2176E8 were tested for LT activity only in CHO cells and selected fractions for ST in suckling mice. Results are summarized in Table II and illustrated in Fig. 1.

Table II and Fig. 1 clearly indicate that the molecular size of the studied *S. flexneri* ST was in the order of magnitude of 100 000. Its molecular weight was in the range characterizing *E. coli* LT produced either by LT^+ST^+ or by only LT^+ strains, while the ST of *E. coli* originated either from LT^+ST^+ or only ST^+ strains, both of a low molecular size.

In respect of molecular size, the position of rapid PF seems to be similar as that of the LT. On the other hand, it is associated with the ST activity of *S. flexneri* and the LT activity of *E. coli*. Its appearance in the Ent^- *E. coli* strain No. 23473 was unexpected.

The large molecular size of *S. flexneri* toxin, together with the chemical and biological properties of ST (non dialysable, resistant to 100 °C for 30 min and to pH 1.0 for 90 min, positive in the suckling mice model and negative in LT specific tests) indicated the probability of an antigenic character and the possibility of a serological relationship to the LT of *E. coli*.

2. *Antigenicity and antigenic relationship of S. flexneri ST.* Rabbits were immunized with active Sephadex fractions containing *S. flexneri* ST, *E. coli* LT, ST (B7A), and rapid PF (23473). The only system that could be

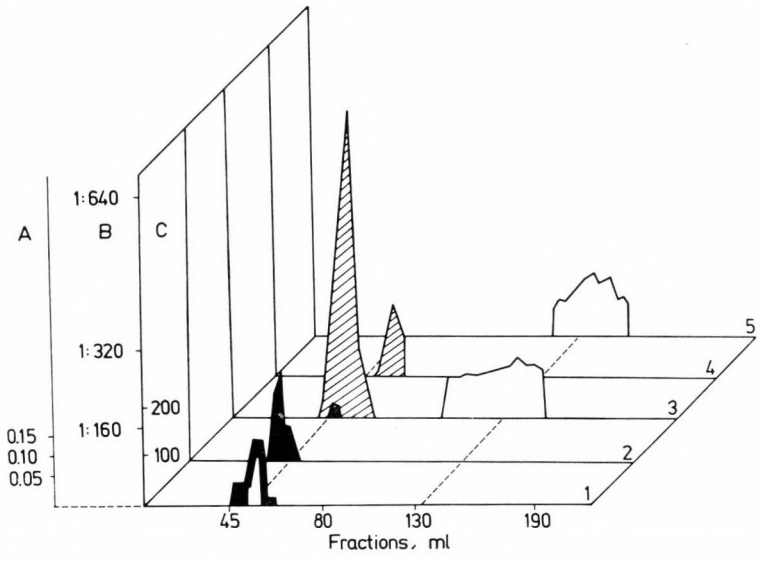


Fig. 1. Comparison of molecular weight ranges and biological activities of Sephadex G-100 fractions of *S. flexneri* 3a, Ent^-, LT^+ST^+, LT^+ and ST^+ *E. coli*. Open area = ST activity: bowel/body weight indexes measured in suckling mice; Shaded area = LT activity measured in CHO cells, titre of cytotonic (elongation) activity; Closed area = rapid PF effect in mm^2 of blueing area on rabbit skin. 1 = *S. flexneri* 3a, 2 = Ent^- *E. coli* No. 23473 (O78 : K80 : : NM), 3 = LT^+ST^+ *E. coli* "B7A" (O148 : K87 : H21), 4 = LT^+ *E. coli* No. 263 (O8 : K87, K88a, b : H19), 5 = ST^+ *E. coli* No. 2176E8 (O138 : K81). Fractions of 50, 5 ml aliquots were collected, activity ranges are designated in volume. Scale "A" = bowel/body weight indices over 0.070. Scale "B" = reciprocal values of cytotonic titres higher than 1 : 4 in CHO cells. Scale "C" = blueing areas in mm^2 above 20 mm^2

Table II

Enterotoxigenicity of Sephadex G-100 fractions prepared from the ultrasonic

Fractions (ml)	*S. flexneri* 3a 140				Ent⁻ *E. coli* 23473 (O78 : K80 : NM)			
	rapid PF (mm^2 area)	delayed PF (mm^2 area)	CHO cells (titre)	suckling mice (bowel/body)	rapid PF (mm^2 area)	delayed PF (mm^2 area)	CHO cells (titre)	suckling mice (bowel/body)
0– 45	—[1]	—[1]	—[2]	.[3]	—	—	—	.
45– 50	—	—	—	.	153	—	—	.
50– 55	50	—	—	.	201	—	—	—
55– 60	50	—	—	.	78	—	—	.
60– 65	153	—	—	0.088	78	—	—	—
65– 70	153	—	—	0.088	—	—	—	—
70– 75	28	—	—	.	—	—	—	—
75– 80	28	—	—	.	—	—	—	.
80–110	—	—	—	.	—	—	—	.
110–125	—	—	—	.	—	—	—	.
125–130	—	—	—	.	—	—	—	.
130–135	—	—	—	.	—	—	—	.
135–140	—	—	—	.	—	—	—	.
140–145	—	—	—	.	—	—	—	.
145–150	—	—	—	.	—	—	—	.
150–155	—	—	—	.	—	—	—	.
155–160	—	—	—	.	—	—	—	.
160–165	—	—	—	.	—	—	—	.
165–170	—	—	—	.	—	—	—	.
170–175	—	—	—	.	—	—	—	—
175–180	—	—	—	.	—	—	—	.
180–185	—	—	—	.	—	—	—	.
185–190	—	—	—	.	—	—	—	.
190–250	—	—	—	.	—	—	—	.

[1] blueing area under 20 mm^2, [2] under 1 : 10, [3] not tested.

used for neutralization experiments of *S. flexneri* ST, was the suckling mice model. Results of these experiments are summarized in Table III.

The data presented in Table III clearly show the neutralizing effect of homologous serum, proving the antigenicity of the shigella ST. Furthermore, a strong cross neutralization was given by the *E. coli* anti-LT serum. Sera against ST, or rapid PF of *E. coli*, were lacking neutralizing capacity. The fact that a high dose of cholera antitoxin was needed for neutralizing the effect of shigella enterotoxin, was indicative of a weak antigenic relationship.

The cross neutralizing capacity of shigella anti-ST serum was studied on CHO cells. As enterotoxins the most active fractions of B7A LT and 263 LT, as well as choleragen were tested. Before the neutralization test, the toxins were titrated and the last dilution still having a cytotonic effect (elongation of at least 60% of the cells) was determined (TCCD). Such a titration was made as a control parallel with every neutralization experiment. The results

lysates of S. flexneri and control strains of E. coli

LT+ST+ *E. coli* B7A (O148 : K87 : H21)				LT+ *E. coli* 263		ST+ *E. coli* 2178E8	
rapid PF (mm^2 area)	delayed PF (mm^2 area)	CHO cells (titre)	suckling mice (bowel/body)	CHO cells (titre)	suckling mice (bowel/body)	CHO cells (titre)	suckling mice (bowel/body)
—	—	—	—	—	.	—	.
—	—	—	—	—	.	—	.
—	50	40	—	—	.	—	.
50	50	320	—	—	.	—	.
39	200	640	—	10	—	—	.
—	28	160	—	80	—	—	—
—	—	8	—	80	—	—	—
—	—	—	—	—	.	—	.
—	—	—	—	—	.	—	.
—	—	—	.	—	.	—	.
—	—	—	.	—	—	—	—
—	—	—	0.097	—	—	—	—
—	—	—	0.100	—	—	—	—
—	—	—	0.120	—	—	—	0.072
—	—	—	0.140	—	—	—	0.086
—	—	—	0.120	—	—	—	0.085
—	—	—	0.120	—	—	—	0.100
—	—	—	0.100	—	—	—	0.125
—	—	—	—	—	—	—	0.131
—	—	—	—	—	—	—	0.112
—	—	—	—	—	—	—	0.120
—	—	—	—	—	—	—	0.093
—	—	—	—	—	—	—	0.086
—	—	—	—	—	—	—	—

of repeated neutralization experiments on CHO cells are summarized in Table IV.

The data in Table IV show a good agreement with the neutralization experiments made in suckling mice. The cytotonic effect of heat-labile *E. coli* toxins of B7A and 263 origin was effectively neutralized not only by *E. coli* anti-LT (B7A) serum, but also by *S. flexneri* anti-ST serum. The cholera antitoxin had a weak neutralizing effect on *E. coli* LT despite its strong neutralizing activity against homologous toxin. No neutralization was observed by the serum prepared against *E. coli* rapid PF. Evaluation of the neutralizing activity of anti-*E. coli*-LT, or of anti-shigella-ST sera, to choleragen was hampered by the fact that at lower dilutions the sera showed a cytotoxic effect and at higher dilutions no neutralization was observed. If these sera had any neutralization capacity, it was weak and insignificant.

The neutralization experiments proved the antigenicity of *S. flexneri* ST (type 3a, No. 140), its strong relationship to the heat-labile *E. coli* enterotoxin, as well as a weak antigenic relation to the choleragen.

Table III

Neutralization experiments in suckling mice test
S. flexneri 3 No. 140 concentrated material (ST) in one dose of 3 mg

Sera	Dilution (reciprocal)	Bowel/body weight index	Absolute serum quantity (ml) reaching control values (L+ dose)
S. flexneri 3 No. 140 anti-ST	2	0.048	*0.004*
	4	0.067	
	8	0.074	
	16	0.085	
E. coli B7A anti-LT	2	0.063	*0.002*
	4	0.065	
	8	0.064	
	16	0.068	
	32	0.091	
E. coli B7A anti-ST	2	0.090	>0.025
	4	0.085	
E. coli 23473 Anti-rapid PF	2	0.082	>0.025
	4	0.084	
Cholera antitoxin*	2	0.062	*0.013* (4.95 IU)
	4	0.087	
	8	0.090	
Control: *S. flexneri* 3 No. 140 3 mg of conc. material; mean index value		0.081	

* Swiss Serum – 375 IU/ml "f" solution.

Table IV

Neutralization experiments in Chinese Hamster Ovary (CHO) cells
(against *E. coli* LT fractions and choleragen)

Sera	Reciprocals of minimum neutralizing serum titres against					
	B7A – LT[1]		263 – LT[2]		choleragen[3]	
	4 TCCD	1 TCCD	4 TCCD	1 TCCD	8 TCCD	2 TCCD
Anti-Shigella-ST	<32	128	16	.	<16	<4
Anti-coli-LT	<32	>512	16	.	<4	<32
Anti-coli-rapid PF	.	<8	.	<8	.	<8
Cholera antitoxin	<0.55**	.	1.13	.	.	<0.034

[1] and [2] The most active Sephadex G-100 fractions of *E. coli* strains B7A and 263. [3] choleragen Lot No. 0972 prepared by Dr. R. A. FINKELSTEIN. TCCD means the dilution of enterotoxin causing cytotonic effect (Tissue Culture Cytotonic Dose) in CHO cells, values over TCD_{60}. The expression e.g. 4 TCCD means 4 times the dose read in parallel. Cholera antitoxin (Swiss Serum, dilution "f" of 375 IU/ml) values are expressed in IU.

Discussion

It is not an easy task to interpret the unique features of *S. flexneri* enterotoxin. Its high molecular weight is in contrast to its heat-stable character, while its antigenicity and close serological relation to *E. coli* heat-labile enterotoxin raise some problems.

On the other hand, enterotoxins of *E. coli* display wide variations in molecular weight, heat stability and diffusibility. The heat labile toxin may range in molecular weight from 20 000 [5], 35 000 [6] to over 100 000 [6, 7]. Similarly, the ST varies greatly, its mean molecular weight being 6000, but active fragments may be under that value [8]. The high molecular weight of ST was attributed by JACKS *et al.* [9] to complex formation with lipopolysaccharide. Further information supporting the variability of the molecular weight of ST is provided by dialysis but some data distinguish dialysable [10, 11] and non-dialysable [9, 12] heat-stable enterotoxins. Crude toxins differ also in sensitivity to heat [12, 13]. The lack of immunogenicity is a general feature of non-purified ST [14, 15]. Thus, the characteristic biological activity seems to be a dependable quality in differentiating between LT and ST.

The *S. flexneri* enterotoxin studied in our experiments showed beside its heat and acid stability all biological characters of heat-stable enterotoxins: producing dilatation after 3 hr in suckling mice, and an early (4 hr) but no delayed (18 hr) rabbit loop positivity [15]. Furthermore, it is negative in the foot oedema test, classical (delayed) blueing reaction and cytotonic effect for Y-1 and CHO cells.

A possible explanation of the observed high molecular weight may be that a complex formation occurs between enterotoxin and lipopolysaccharide [7], which may cause heat stability and large molecular size. Without further investigations using purified toxin, the possibility cannot be excluded. Nevertheless, the validity of the hypothesis is weak as in addition to heat-stability, ST exerts biological activity. Furthermore anti-serum prepared in rabbits gave a very low titre (1 : 10) in precipitation with the fractionated antigen and this contradicts a high O-antigen content.

The relation of *E. coli* LT to ST is a complex problem. There are two toxins with basically identical activity (fluid accumulation). In case of LT-ST producing strains only one plasmid is responsible for both toxins, which may involve the action of two "*tox*" genes or different chemical entities originate from a single gene information [16]. Both toxins are characterized by molecular heterogeneity. So the idea of the origin of ST did not change since 1970 when SMITH and GYLES supposed that the ST might be the active toxin and the LT was a protein linked precursor [14]. According to FINKELSTEIN *et al.* [6], a proteolytic cleavage may chiefly be responsible for the large-

scale molecular heterogeneity. As a working hypothesis, such an intermediary position of *S. flexneri* ST is acceptable

The data obtained in neutralization experiments are in agreement with the literature. The choleragen and the LT of *E. coli* show a rather unilateral antigenic relationship when tested on the ligated rabbit loop [17]. Similar results were obtained in tissue cultures of Y-1 and CHO [18, 19]. Summarizing these data, we may say that cholera antitoxin neutralizes *E. coli* LT in a somewhat lesser degree than it neutralizes the homologous choleragen, while anti-coli-LT has no or a very weak neutralizing effect on choleragen. Our results show that the suckling mice model is also suitable for neutralization experiments.

No antigenic relationship between the rapid PF of high molecular weight and enterotoxins was found. Similarly, SANDEFUR and PETERSON [20] showed the lack of neutralizing capacity of cholera antitoxin against *Salmonella* rapid PF.

Although the pathological role of *E. coli* ST has been proven [21], clarification of the role of *S. flexneri* ST in the pathogenesis of bacillary dysentery needs further studies. Watery diarrhoea is a common symptom of bacillary dysentery. Experiments carried out on monkeys showing a similar clinical pattern revealed that infection of the coecum did not result in this "jejunal reaction" [22].

A further factor in the toxic effect may be a *S. dysenteriae* 1-like toxin. A cytotoxic enterotoxin has been found by O'BRIEN *et al.* [23]. The possible pathological and immunological role of this kind of enterotoxin was shown by KEUSCH and JACEWICZ [24] who demonstrated antitoxin titres against "Shiga-toxin" in the sera of patients convalescing from *S. flexneri* or *S. sonnei* infections. As already mentioned [1], this type of toxin was not found in materials prepared from the *S. flexneri* strains used by us. On the other hand, strain M42–43 of O'BRIEN *et al.* [23] is a ST producer.

Acknowledgements. Freshly isolated *S. flexneri* strains were kindly supplied by Dr. G. V. LÁSZLÓ (National Institute of Hygiene, Budapest). The Ent^- *E. coli* No. 23473 strain originated from extraintestinal pathological processes and was provided by Dr. É. CZIRÓK (Pest County Public Health Station, Budapest). The LT^+ST^+ "B7A" strain was kindly sent by Dr. S. B. FORMAL (Walter Reed Army Institute, Washington, D. C., USA), while the ST standard (No. 2176E8) and LT standard (No. 263) strains of *E. coli* were supplied by Dr. B. NAGY (Veterinary Institute, Szombathely, Hungary). The choleragen prepared by Dr. R. A. FILKENSTEIN and cholera antitoxin were obtained from Dr. I. JOÓ (Institute for Serobacteriological Production and Research "Human", Budapest).

REFERENCES

1. KÉTYI, I., MÁLOVICS, I., VERTÉNYI, A., KONTROHR, T., PÁCSA, S., KUCH, B.: Acta microbiol. Acad. Sci. hung. **25**, 165 (1978).
2. GIANNELLA, R. A.: Infect. Immun. **14**, 95 (1976).
3. SANDEFUR, P. D., PETERSON, J. W.: Infect. Immun. **14**, 671 (1976).

4. Callahan, L. T., III., Ryder, R. C., Richardson, S. H.: Infect. Immun. **7,** 611 (1971).
5. Evans, D. J., Evans, D. G., Jr., Gorbach, S. L.: Infect. Immun. **10,** 1010 (1974).
6. Finkelstein, R. A., LaRue, M. K., Johnston, D. W., Vasil, M. L., Cho, G. J., Jones, J. R.: J. infect. Dis. **133,** (Suppl.): S120 (1976).
7. Dorner, F.: J. biol. Chem. **250,** 8712 (1975).
8. Mitchell, I. de G., Tame, M. J., Kenworthy, R.: J. med. Microbiol. **7,** 439 (1974).
9. Jacks, T. M., Wu, B. J., Braemer, A. C., Bidlack, D. E.: Infect. Immun. **7,** 178 (1973).
10. Kohler, E. M.: Amer. J. vet. Res. **29,** 2263 (1968).
11. Lariviére, S., Gyles, C. L., Barnum, D. A.: Canad. J. comp. Med. **36,** 319 (1972).
12. Smith, H. W., Halls, S.: J. Path. Bact. **93,** 531 (1967).
13. Truszczynski, M., Pilaszek, J.: Res. vet. Sci. **10,** 469 (1969).
14. Smith, H. W., Gyles, C. L.: J. med. Microbiol. **3,** 387 (1970).
15. Evans, D. G., Jr., Evans, D. J., Pierce, N. F.: Infect. Immun. **7,** 873 (1973).
16. Gyles, C. L., So, M., Falkow, S.: J. infect. Dis. **130,** 40 (1974).
17. Sack, R. B., Gorbach, S. L., Banwell, J. G., Jacobs, B., Chatterjee, B. D., Mitra, R. C.: J. infect. Dis. **123,** 378 (1971).
18. Donta, S. T.: J. infect. Dis. **129,** 284 (1974).
19. Guerrant, R. L., Brunton, L. L.: J. infect. Dis. **135,** 720 (1977).
20. Sandefur, P. D., Peterson, J. W.: Infect. Immun. **15,** 989 (1977).
21. Whipp, S. C., Moon, H. W., Lyon, N. C.: Infect. Immun. **12,** 240 (1975).
22. Kinsey, M. D., Formal, S. B., Dammin, G. J., Giannella, R. A.: Infect. Immun. **14,** 368 (1976).
23. O'Brien, A. D., Thompson, M. R., Gemski, P., Doctor, B. P., Formal, S. B.: Infect. Immun. **15,** 796 (1977).
24. Keusch, G. T., Jacewicz, M.: Nature new Biol. **241,** 31 (1973).

Address of the authors:

Iván Kétyi, Adele VerténYi, Ilona Málovics,
Tivadar Kontrohr, Sándor Pácsa
Institute of Microbiology, University Medical School,
Szigeti út 12, H-7643 Pécs, Hungary

NOTE ADDED IN PROOF

The high molecular weight nature of *S. flexneri* ST was further proved by Amicon ultrafiltration, showing the active material in 97.2% in the XM 100 A retentate.

Acta microbiol. Acad. Sci. hung. 25, 229–231, 1978

HIGH MOLECULAR WEIGHT RIBOSOMAL RIBONUCLEIC ACIDS FROM VEGETATIVE HYPHAE AND SPORES OF STREPTOMYCES GRISEUS

GABRIELLA VALU and G. SZABÓ

Institute of Biology, University Medical School, Debrecen

(Received December 7, 1977)

High molecular weight ribosomal ribonucleic acids (rRNAs) were isolated from young vegetative cells and spores of a streptomycin non-producing *Streptomyces griseus*, and their electrophoretic mobility was compared to each other and to that of rRNAs of *Escherichia coli* K-12. The electrophoretic mobility of 23 and 16S rRNAs from vegetative cells and spores of *S. griseus* was identical, but the 23S rRNAs of streptomyces ribosomes migrated more slowly on polyacrylamide gel than those of *E. coli* ribosomes. Intact, electrophoretically homogeneous rRNAs could be isolated from *S. griseus* (No. 45-H) only in the presence of diethyl pyrocarbonate (DEP), and intact rRNAs could be obtained from spores only if DEP had been added before breaking the spores. Otherwise instead of two distinct bands, three were obtained on polyacrylamide gel.

In previous studies we examined the stability [1], the protein composition [2], and the *in vitro* amino acid incorporating activity [3] of ribosomes isolated from young vegetative hyphae and spores of *Streptomyces griseus* strain 45-H. In this work we wished to supplement our and other workers' data (4–6) on streptomyces rRNAs.

Materials and methods

Organisms. A streptomycin non-producing *S. griseus* strain (No. 45-H) and *Escherichia coli* K-12 strains were used. The *S. griseus* variant had a complete life-cycle even in liquid soybean medium, producing conidia in great number. A detailed description of the strain has been published previously [7].

S. griseus was grown at 27 °C in a rotary shaker on filtered soybean medium and harvested at 16 hr of age, which corresponded to an exponentially growing culture. Isolation and purification of spores were performed as described previously [1].

Isolation of rRNAs. By the combination of different methods [8–11], total RNA was obtained and the high molecular weight rRNAs were precipitated with 3.0 M sodium acetate [10, 12]. The main steps of isolation were as follows. Vegetative cells and spores were washed twice in freshly prepared buffer containing 0.01 M Tris–acetic acid pH 7.7, 0.01 M magnesium acetate, and 5 ml DEP per 1000 ml buffer [13, 14]. Washed pellets (2–3 g of *E. coli* and 6–8 g of *Streptomyces)* were resuspended in 10 ml of the buffer, and 5 ml phenol : m-cresol : 8-hydroxychinoline mixture (phenol crystals, 500 g; m-cresol, 70 ml; bisdistilled water, 55 ml; 8-hydroxychinoline, 0.5 g) was added, then the cell or spore suspension was broken in a Braun cell homogenizer (model MSK). After centrifugation the upper phase was pipetted off and preserved. The interphase was mixed with 10 ml buffer (0.01 M Tris–acetic acid pH 6.6, 0.01 M magnesium acetate and 5 ml DEP per 1000 ml) containing 0.5% sodium dodecyl sulphate SDS) and 5 ml phenol : m-cresol : 8-hydroxychinoline mixture. After centrifugation the upper phases were combined, the SDS concentration was adjusted to 0.5%, and phenol treatment was repeated three times. One tenth of 2 M potassium acetate was added and the nucleic acids were precipitated with two volumes of cold ethanol, then kept at −20 °C overnight. The precipitate was collected by centrifugation at 12 000 *g* for 20 min, then washed three times with 70% ethanol at −20 °C. The sediment was dissolved at room temperature

in 0.15 M sodium acetate pH 6.0 containing 0.5% SDS, then precipitated again with two volumes of cold ethanol. After centrifugation the pellet was dissolved in 3 M sodium acetate buffer pH 6.0, and kept at 4 °C for 12–14 hr. During this time high molecular weight rRNAs were precipitated. The precipitate was sedimented, washed three times with 3 M sodium acetate, dissolved in 75% ethanol–0.5% SDS solution, and stored at −20 °C until used.

The absorption ratio (A_{260} per A_{280}) of RNA isolated by the method described above was found between 2.02 and 2.22.

Polyacrylamide gel electrophoresis of rRNA. High molecular weight rRNA samples were electrophoresed in 2.4% gel [15]. To 4 ml of acrylamide solution (acrylamide 15%, bis-acrylamide 0.75%) 12.45 ml bisdistilled water and 8.33 ml concentrated electrophoresis buffer (Tris, 108 g; Na_2EDTA, 9.3 g; boric acid, 55 g; to 1000 ml bisdistilled water pH 8.3 [16]) were added. After deaeration 20 μl N,N',N,N'-tetramethyl-ethylethylene diamine (TEMED) and 200 μl 10% freshly prepared ammonium persulphate were added and 7 cm long gels were prepared in plexi tubes with an inner diameter of 6 mm. RNA samples (30–50 μg) were dissolved in tenfold diluted electrophoresis buffer, then layered over the gels in volumes of 10 μl containing 10% saccharose (Serva, RNase free) and 0.005% bromophenol blue. Electrophoresis was carried out at 4 °C with constant current of 3 mA per gel column, using a tenfold dilution of electrophoresis buffer pH 8.3. The gels were then removed from the tubes and put into 1.0 M acetic acid for 10–15 min [16], then stained with 0.2% methylene blue dissolved in a buffer containing 0.4 M sodium acetate and 0.4 M acetic acid. After staining the gels were transferred into water and washed for some hours. They were scanned in a Kipp and Zonen Densitometer (DD2) at 600 nm.

All the experimental procedures were performed in a cold chamber at about 4 °C. Glasswares were sterilized to reduce ribonuclease activity.

Results and discussion

Polyacrylamide gel electrophoresis patterns of high molecular weight rRNAs from *E. coli* K-12, from young vegetative hyphae and spores of *S. griseus*, are shown in Fig. 1. Electrophoretic mobility of 23 and 16S rRNAs of young vegetative cells and that of spores was identical, but the 23S rRNAs of *S. griseus*, both from vegetative cells and spores, migrated more slowly on the polyacrylamide gels than the 23S rRNA of *E. coli*. The difference in electrophoretic mobility of 23S rRNAs from *S. griseus* and *E. coli* may be

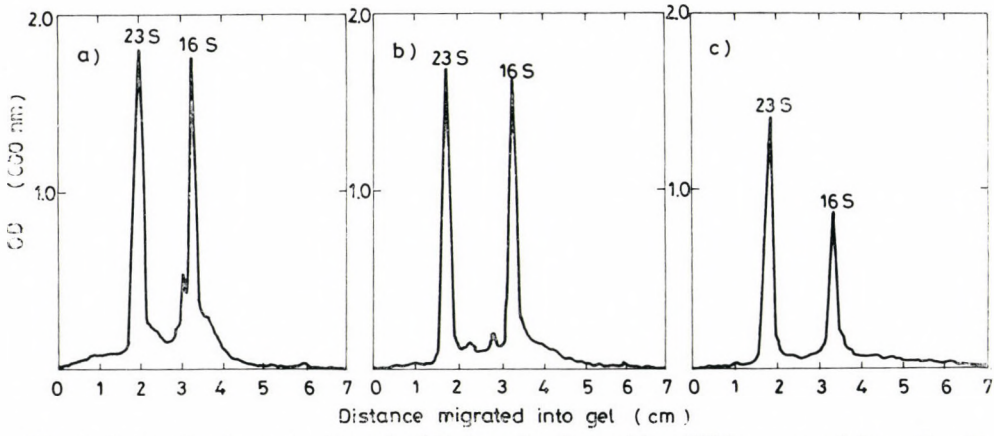


Fig. 1. Scans of polyacrylamide gel of high molecular weight rRNAs prepared from *E. coli* K-12 (a), 16 hr cells (b) and spores (c) of *S. griseus* No. 45-H.

due to the different molecular weight and/or different conformation of these rRNA molecules. Conformation independent molecular weight determination [17] of the rRNA samples was not performed.

These results are in good agreement with LOENING's data [4], who examined the molecular weight of rRNAs from different sources by polyacrylamide gel electrophoresis and found that in the prokaryotic group streptomycetes had slightly higher molecular weight 23S RNA than had *E. coli* cells ($1.11–1.13 \times 10^6$ versus 1.07×10^6).

The negligible difference observed may be considered an additional information in support of the idea that streptomycetes occupy an intermediate position between bacteria and eukaryotic organims.

As to the isolation of high molecular weight rRNAs from spores, intact, electrophoretically homogeneous rRNA could be obtained if the RNase inhibitor (DEP) had been added before breaking the spores. Otherwise instead of two distinct bands three were obtained on the polyacrylamide gels.

Acknowledgement. We are indebted to Mrs. F. RÓZSA for skilled technical assistance.

REFERENCES

1. VALU, G., SZABÓ, G.: Acta biol. Acad. Sci. hung. **24,** 171 (1973).
2. VALU, G., SZABÓ, G.: Acta biol. Acad. Sci. hung. **26,** 151 (1975).
3. VALU, G., SZABÓ, G.: Proc. International Symposium on Nocardia and Streptomyces, Warsaw 1976. G. Fischer Verlag, Stuttgart. In press.
4. LOENING, U. E.: J. molec. Biol. **38,** 355 (1968).
5. MIKULIK, K., JANDA, I., RICICOVÁ, A., VINTER, V.: In GERHARDT, P., SADOFF, H. L., COSTILOW, R. N. (eds): Spores. Vol. VI. Amer. Soc. Microbiol. Washington, D. C. 1975. p. 15.
6. TIMKO, J., ZELINKA, J.: J. Antibiot. **30,** 644 (1977).
7. SZABÓ, G., BARABÁS, GY., VÁLYI-NAGY, T.: Arch. Mikrobiol. **40,** 261 (1961).
8. GIERER, A., SCHRAMM, G.: Nature (Lond.) **177,** 702 (1956).
9. DINGMAN, W., SPORN, M. B.: Biochim. biophys. Acta (Amst.) **61,** 164 (1962).
10. KIRBY, K. S.: Biochem. J. **96,** 269 (1965).
11. LOENING, U. E.: Biochem. J. **113,** 131 (1969).
12. KERN, H.: Anal. Biochem. **67,** 147 (1975).
13. SOLYMOSY, F., FEDORCSÁK, I., GULYÁS, A., FARKAS, G. L., EHRENBERG, L.: Europ. J. Biochem. **5,** 520 (1968).
14. SOLYMOSY, F., LÁZÁR, G., BAGI, G.: Anal. Biochem. **38,** 40 (1970).
15. BISHOP, H. L., CLAYBROOK, J. R., SPIEGELMAN, S.: J. molec. Biol. **26,** 373 (1967).
16. PEACOCK, A. C., DINGMAN, C. W.: Biochemistry **6,** 1818 (1967).
17. BOEDTKER, H.: Biochim. biophys. Acta (Amst.) **240,** 448 (1971).

Address of the authors:

GABRIELLA VALU, GÁBOR SZABÓ
Institute of Biology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

INDEX

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária

A kézirat nyomdába érkezett: 1978. III. 21. — Terjedelem: 7,0 (A/5) ív, 20 ábra

78.5652 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

THE JOURNAL OF GENERAL MICROBIOLOGY

Volume 104, Part 1, January 1978

Volumes 104-109 £114.00 ($264.00 U. S. A. and Canada)
Single Parts £11.00 ($26.00 U. S. A. and Canada) each
Enquiries about advertising in the journal should be sent direct to the Press

CAMBRIDGE UNIVERSITY PRESS

Bentley House. 200 Euston Road, London NW1 2DB
32 East 57th Street. New York. N. Y. 10022

Beiträge zur Sterilisation und Aseptik

Proceedings in Sterilization and Aseptic Methods

Herausgegeben von Dipl.-Phys. Rolf Machmerth, Erfurt, und Dr. rer. nat. Annelore Günther, Erfurt
(Mikrobielle Umwelt und antimikrobielle Maßnahmen. Band 2)
1976. 424 Seiten, 72 Abbildungen, 48 Tabellen
Kunstleder 48,— M · Bestell-Nr. 793 528 4

Der Band enthält die Vorträge des V. Symposiums über Sterilisation, Desinfektion und Antiseptik, das vom 23. bis 25. Oktober 1974 mit mehr als 500 Fachleuten aus Wissenschaft und Praxis, darunter 60 ausländischen Gästen, in Erfurt stattfand. Die Beiträge zeigen die Fortschritte auf den Gebieten der experimentellen und praktischen Keimtötung der letzten Jahre und sollen sowohl neue Erkenntnisse schnell in die Praxis überführen als auch die Grundlagenforschung auf die weitere Entwicklung und offene Probleme aufmerksam machen.

Bestellungen an den Buchhandel erbeten

JOHANN AMBROSIUS BARTH LEIPZIG

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

will submit papers based exclusively on original researches from the field of microbiology (and within it from bacteriology, virology, mycology and protozoology). Papers of immunological subject will only be accepted for publication if microbiological aspects are also involved. The main object of Acta Microbiologica is to make the results of Hungarian microbiological researches known to readers abroad.

ACTA MICROBIOLOGICA is published mainly in English, but also in French, German and Russian. Four issues make up a volume of some 400 to 500 pages yearly.

Subscription rate per volume: US $36.00

AKADÉMIAI KIADÓ
Publishing House of the Hungarian Academy of Sciences
BUDAPEST

AKADÉMIAI KIADÓ
H-1363 Budapest P. O. B. 24

We should like to order:
ACTA MICROBIOLOGICA ACADEMIAE SCIENTIARUM HUNGARICAE

☐ Subscription starting with

(date) . . . . . .

Subscription rate per volume: US $36.00

☐ Specimen copy (free of charge) . . . . . .

Name (in block letters): . . . . . .

Address (in block letters): . . . . . .

MEDICAL PERIODICALS OF THE HUNGARIAN ACADEMY OF SCIENCES

ACTA CHIRURGICA

ACTA MEDICA

ACTA MICROBIOLOGICA

ACTA MORPHOLOGICA

ACTA PAEDIATRICA

ACTA PHYSIOLOGICA

HAEMATOLOGIA

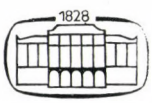
INTERNATIONAL UROLOGY AND NEPHROLOGY

These periodicals of the Hungarian Academy of Sciences publish original scientific treatises in English, German, French or Russian. The papers are written by outstanding scientists from Hungary and other countries. The editorial board of each periodica consists of professors of international reputation.

The volumes published so far have had a favourable reception in the international scientific world: the treatises are reviewed by the corresponding international reference papers.

Our periodicals are, as a rule, quarterlies: four issues make up a volume of some 400 to 500 pages.

Subscription rate per volume: US $36.00



AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

BUDAPEST

Distributor: KULTURA H-1389 Budapest, P.O.B. 149

Ask for the quarterly lists of our forthcoming books!

Address: AKADÉMIAI KIADÓ H-1361 Budapest, P.O.B. 36

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:

Acta Mirobiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P.O.B. 64, Ungarn

An die gleiche Anschrift ist jede für die Redaktion bestimmte Korrespondenz zu richten. Aonnem entspreis pro Band: $ 36.00.

Bestellbar bei »Kultura« Außenhandelsunternehmen (H-1389 Budapest 62, P.O.B. 149, Bankkonto Nr. 218-10990) oder seinen Auslandsvertretungen.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiène Publique, H-1966 Budapest, P.O.B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement: $ 36.00 par volume.

On peut s'abonner à l'Entreprise du Commerce Extérieur «Kultura» (H-1389 Budapest 62, P.O.B. 149, Compte courant No. 218-10990) ou chez représentants à l'étranger.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P.O.B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — $ 36.00 за том.

Заказы принимает предприятие по внешней торговле «Kultura» (H-1389 Budapest 62, P.O.B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

AUSTRALIA
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE, Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 145 Ackland Street, *St. Kilda (Melbourne), Victoria 3182*

AUSTRIA
GLOBUS, Höchstädtplatz 3, *1200 Wien XX*

BELGIUM
OFFICE INTERNATIONAL DE LIBRAIRIE, 30 Avenue Marnix, *1050 Bruxelles*
LIBRAIRIE DU MONDE ENTIER, 162 Rue du Midi, *1000 Bruxelles*

BULGARIA
HEMUS, Bulvar Ruski 6, *Sofia*

CANADA
PANNONIA BOOKS, P.O. Box 1017, Postal Station "B", *Toronto, Ontario M5T 2T8*

CHINA
CNPICOR, Periodical Department, P.O. Box 50, *Peking*

CZECHOSLOVAKIA
MAD'ARSKÁ KULTURA, Národní třida 22, *115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 46, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

DENMARK
EJNAR MUNKSGAARD, Norregade 6, *1165 Copenhagen*

FINLAND
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128, *SF-00101 Helsinki 10*

FRANCE
EUROPERIODIQUES S. A., 31 Avenue de Versailles, *78170 La Celle St.-Cloud*
LIBRAIRIE LAVOISIER, 11 rue Lavoisier, *75008 Paris*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac, *75240 Paris Cedex 05*

GERMAN DEMOCRATIC REPUBLIC
HAUS DER UNGARISCHEN KULTUR, Karl-Liebknecht-Strasse 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT, Strasse der Pariser Kommüne 3—4, *DDR-104 Berlin*

GERMAN FEDERAL REPUBLIC
KUNST UND WISSEN ERICH BIEBER, Postfach 46, *7000 Stuttgart 1*

GREAT BRITAIN
BLACKWELL'S PERIODICALS DIVISION, Hythe Bridge Street, *Oxford OX1 2ET*
BUMPUS, HALDANE AND MAXWELL LTD., Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate. *Wellingborough, Northants NN8 2QT*
WM. DAWSON AND SONS LTD., Cannon House, *Folkestone, Kent CT19 5EE*
H. K. LEWIS AND CO., 136 Gower Street, *London WC1E 6BS*

GREECE
KOSTARAKIS BROTHERS, International Booksellers, 2 Hippokratous Street, *Athens-143*

HOLLAND
MEULENHOFF-BRUNA B.V., Beulingstraat 2, *Amsterdam*
MARTINUS NIJHOFF B.V., Lange Voorhout 9—11, *Den Haag*
SWETS SUBSCRIPTION SERVICE, 347b Heereweg, *Lisse*

INDIA
ALLIED PUBLISHING PRIVATE LTD., 13/14 Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD., Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTD., Books Import Division, Chandralok, 36 Janpath, *New Delhi 110001*

ITALY
EUGENIO CARLUCCI, P.O. Box 252, *70100 Bari*
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 45, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58, *20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

JAPAN
KINOKUNIYA BOOK-STORE CO. LTD., 17-7 Shinjuku-ku 3 chome, Shinjuku-ku, *Tokyo 160-91*
MARUZEN COMPANY LTD., Book Department, P.O. Box 5050 Tokyo International, *Tokyo 100-31*
NAUKA LTD. IMPORT DEPARTMENT, 2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

KOREA
CHULPANMUL, *Phenjan*

NORWAY
TANUM-CAMMERMEYER, Karl Johansgatan 41—43, *1000 Oslo*

POLAND
WĘGIERSKI INSTYTUT KULTURY, Marszałkowska 80, *Warszawa*
CKP I W ul. Towarowa 28 *00-958 Warsawa*

ROUMANIA
D. E. P., *Bucureşti*
ROMLIBRI, Str. Biserica Amzei 7, *Bucureşti*

SOVIET UNION
SOJUZPETCHATJ — IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

SPAIN
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

SWEDEN
ALMQVIST AND WIKSELL, Gamla Brogatan 26, *S-101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB, Box 346, *401 25 Göteborg 1*

SWITZERLAND
KARGER LIBRI AG, Petersgraben 31, *4011 Basel*

USA
EBSCO SUBSCRIPTION SERVICES, P.O. Box 1943, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC., 15 Southwest Park, *Westwood, Mass, 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC., 140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC., 7250 Westfield Avenue, *Pennsauken N. J. 08110*

VIETNAM
XUNHASABA, 32, Hai Ba Trung, *Hanoi*

YUGOSLAVIA
JUGOSLAVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišića 1, *21000 Novi Sad*

12. VII. 1978 HU ISSN 0001—6187 Index: 26.016

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

TOMUS XXV FASCICULUS 4

AKADÉMIAI KIADÓ, BUDAPEST

1978

AMAHA 5 25(4) 233–331 1978

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: 1954 BUDAPEST, ALKOTMÁNY UTCA 21.

Az *Acta Microbiologica* német, angol, francia és orosz nyelven közöl értekezéseket a mikrobiológia tárgyköréből.

Az *Acta Microbiologica* változó terjedelmű füzetekben jelenik meg. Több füzet alkot egy kötetet.

A közlésre szánt kéziratok a következő címre küldendők:

Acta Microbiologica, Országos Közegészségügyi Intézet, 1966 Budapest, Pf. 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (1363 Budapest Pf. 24, Bankszámla: 215-11448), a külföld számára pedig a „Kultura" Külkereskedelmi Vállalatnál (1389 Budapest 62, Pf. 149, Bankszámla: 218-10990) vagy annak külföldi képviseleteinél.

The *Acta Microbiologica* publish papers on microbiological subjects in English, German, French and Russian.

The *Acta Microbiologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to

Acta Microbiologica, National Institute of Hygiene, H-1966 Budapest, P.O.B. 64, Hungary

Correspondence with the editors should be sent to the same address.

Subscription: $36.00 a volume.

Orders may be placed with "Kultura" Foreign Trading Company (H-1389 Budapest 62, P.O.B. 149, Account No. 218-10990) or its representatives abroad.

Acta microbiol. Acad. Sci. hung. 25, 233—236, 1978

OBSERVATIONS CONCERNING THE STAINING PROPERTIES OF THE MACROCONIDIA OF CERTAIN DERMATOPHYTON SPECIES

J. GALGÓCZY

National Institute of Hygiene, Budapest

(Received September 8, 1977)

Staining of the macroconidia of several *Dermatophyton* species by lactophenol–cotton blue was investigated. Young macroconidia stain variably; their cytoplasm may appear homogeneous or inhomogeneous. The possible explanation seems to be variation in the composition of the cytoplasm. In some macroconidia dark-staining filament was seen along the longitudinal axis. Mature macroconidia showed basal or basal+apical homogeneous deep staining. The intensive apical staining suggests that the apical structure differs from the structure of the side wall.

In earlier reports [1, 2] we have suggested that the macroconidium was the most important microbiological marker in the identification and differential diagnosis of the *Dermatophyton* species. We examined these features (growths) *in situ*, in aqueous and lactophenol–cotton blue preparations in routine work. The regularities found in the staining of macronidia are described in the present report.

Materials and methods

Dermatophyton species: *Microsporum gypseum* (5 strains), *M. fulvum* (1 strain), *M. felineum* (2 strains), *M. vanbreuseghemii* (1 strain), *M. persicolor* (1 strain), *M. ferrugineum* (1 strain), *M. cookei* (2 strains), *M. racemosum* (1 strain), *M. bullardii* (1 strain), *Trichophyton ajelloi* (3 strains), *T. vanbreuseghemii* (1 strain).

Preparations were stained with lactophenol–cotton blue,

Results

The upper row in Fig. 1 shows stained young macronidia of *M. felineum*. The photos were taken after 24 hr staining. The cytoplasm of some macroconidia shows only a few dark-staining particles (Picture 1), in others a great mass of dark (deep-blue) particles occur (Pictures 2 and 3) or the whole cytoplasm stains homogeneously (Pictures 4–6). In the lower row of Plate I some further young macronidia of *M. felineum* are shown. In the cytoplasm of these, a filamentous structure staining more intensely than its environment is seen along the longitudinal axis.

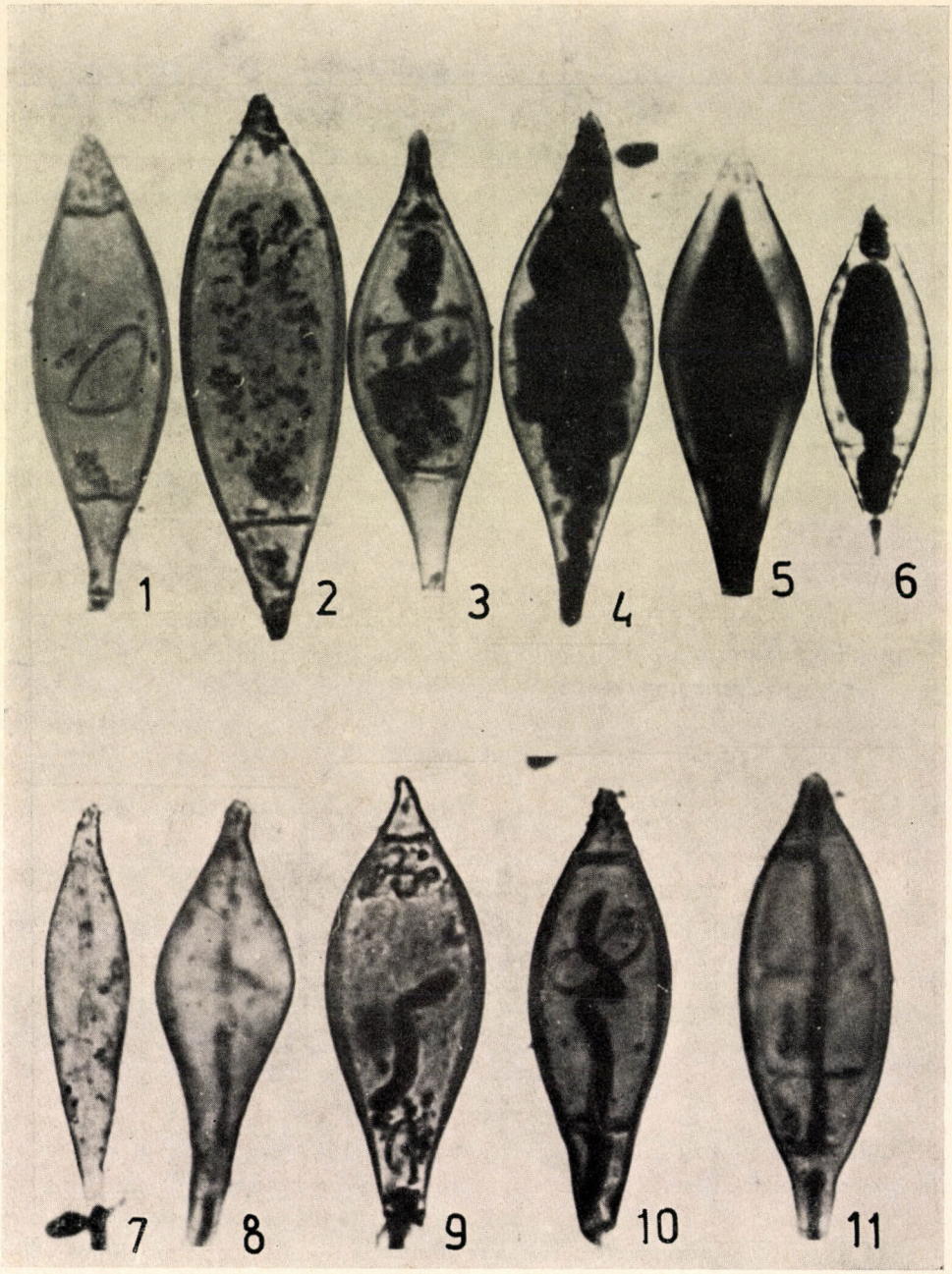


Fig. 1. M. felineum. Young macroconidia. Lactophenol–cotton blue staining. Original magnification ×250

In Fig. 2, mature macronidia of *T. ajelloi* (Picture 1), *M. felineum* (Picture 2) and *M. racemosum* (Picture 3) are shown. The photos were taken after a staining period of 2 hr. In Picture 1, inflow of dye at the basal end is seen. The macronidia in Pictures 2 and 3 show deep staining at both ends.

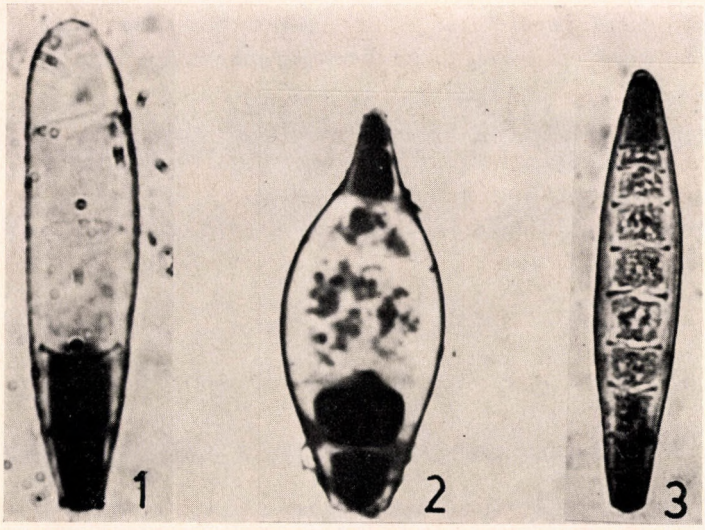


Fig. 2. Mature macroconidia of *T. ajelloi* (Picture 1), *M. felineum* (Picture 2) and *M. racemosum* (Picture 3). Lactophenol–cotton blue staining. Original magnification ×250

Discussion

The macronidia of the other species studied took the stain similarly as those shown in the Pictures. The partly granular, partly homogeneous staining of the young macronidia appears to represent a general rule. Some macroconidia stain faintly even after a staining period of several days, others take the stain intensely in a few hours. We have failed to explain this great variability which is due presumably to individual variations in the composition of the cytoplasm of macronidia or to slight differences in the degree of maturation not demonstrable micromorphologically.

The filaments in the lower row of Fig. 1 suggest that intensely staining components may be situated along the longitudinal axis of the cytoplasm. One might assume that the filaments represent a fungus parasitizing the dermatophyte. However, dematophytes living under natural conditions are rarely parasitized by fungi and the dermatophytes shown in our photos had been taken from primocultures obtained directly from pathological processes, not from collections.

Picture 1 of Fig. 2 presents the condition in which a mature macronidium is beginning to stain homogeneously at its basal end. The staining substance accumulating in the cytoplasm during one or two days accounts for the homogeneous deep blue staining of the whole cytoplasm. The macronidial wall and the septum remain much lighter. In a considerable proportion of the cases, inflow of the dye stops at one of the septa, presumably due to closing of the septal pore. In such cases, invasion of the dye stops before reaching the apex. The same picture was seen in lactophenol–cotton blue stained preparations stored for several years.

The most important phenomenon is seen in Pictures 2 and 3 of Fig. 2. In many macroconidia, the dark staining starts from the basal and apical ends simultaneously in both genera under study. It is therefore assumed that the wall at the apex differs from the side walls not only in being thinner, but also in structure.

REFERENCES

1. GALGÓCZY, J., NOVÁK, E. K.: Az Országos Közegészségügyi Intézet működése az 1961. évben. (The Activities of the National Institute of Public Health in 1961). Budapest, 1962. p. 217.
2. NOVÁK, E. K., GALGÓCZY, J.: Acta botan. Acad. Sci. hung. **15**, 123 (1969.)

Address of the author:

JÓZSEF GALGÓCZY
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

Acta Microbiol. Acad. Sci. hung. 25, 237—243, 1978

DEMONSTRATION OF EBNA (EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN) ANTIBODIES OF DIFFERENT IMMUNOGLOBULIN CLASSES

JUDIT CZEGLÉDY, L. GERGELY, L. VÁCZI and A. SZALKA

Institute of Microbiology, University Medical School, Debrecen and László Central Hospital for Infectious Diseases, Budapest

(Received September 16, 1977)

Anti-EBNA IgM, a previously unknown antibody, was detected by the antihuman globulin anticomplement immunofluorescence (ACIF) method in serum samples from acute infectious mononucleosis (IM) of Epstein–Barr virus (EBV) origin. The antibody disappears from the serum in some weeks during convalescence. It was absent in anti-EBV-positive sera of healthy donors and in serum samples taken from patients with IM caused by cytomegalovirus. The antibody appears simultaneously with anti-EBV IgM and, reaching a lower titre than the latter, its titre curve runs parallel with the anti-EBV IgM curve. Since in acute EBV infections, anti-EBNA IgM always appeared, its presence may serve as an additional evidence of the acuteness of EBV infection. In EBV-seropositive healthy subjects, the bulk of antibodies belongs to the IgG class, non-complement-fixing IgA antibodies occur only sporadically.

Among the Epstein–Barr virus (EBV) antigens described so far, the nuclear antigen (EBNA) has attracted most interest. EBNA is present in the nucleus of all EBV-carrying lymphoid cells, even in the absence of all other EBV antigens. EBNA is, therefore, a reliable indicator of the presence of EBV [1, 2]. In this respect, EBNA is similar to the T antigen of small DNA viruses, except that it binds to the DNA of chromosomes being in the metaphase (so-called DNA-binding protein) [3]. Recent findings have suggested that it might function as a repressor of virus transcription, thus contributing to the maintenance of virus latency and the transformed state of cells (G. KLEIN, personal communication).

In EBV-seropositive subjects, EBNA antibodies can always be demonstrated. However, in EBV-positive infectious mononucleosis (IM) they appear as late as 2 months or more after other EBV specificities, e.g. the greatest numbers of EBV-positive lymphoid cells are seen in the first few weeks of the illness [4, 5]. To explain the delay, it was assumed that the antihuman globulin anticomplement immunofluorescence (ACIF) method, the procedure generally used for the detection of EBNA antibodies, is not sensitive enough for detecting small amounts of the antibody. The so-called combined test of SCHMITZ *et al.* [6], based on the combination of indirect IF and ACIF, is more

Table I

Demonstration of EBV antibodies

	Indirect immunofluorescence (IF)	Anti-complement immunofluorescence (ACIF)	Anti-human-globulin (ACIF/combined ACIF)
Step 1	Antigen–antibody	Antigen–antibody	Antigen–antibody
Step 2	FITC-anti-human globulin	Complement (C)	Unlabelled anti-human globulin
Step 3	—	FITC-anti-human C	Complement
Step 4	—	—	FITC-anti-human C

Table II

Horizontal study with serum samples from

Case	PBD titres	Time interval between first and actual sampling	EBV IgM titres (IF)	EBV VCA IgG titres (IF)	EBNA antibody titres determined by the combined ACIF method			Anti-EBNA titres (ACIF)
					EBNA IgM	EBNA IgG	EBNA IgA	
32 yr. ♂	1 : 128	0 day	20	80	16	32	negative	<5
		8 days	80	320	16	64	negative	<5
		10 months	<15	40	negative	32	negative	20
		14 months	<15	<40	negative	16	negative	40
19 yr. ♀	1 : 1024	0 day	40	40	16	64	16	<5
		7 days	80	160	32	128	32	<5
		25 days	40	80	32	64	32	<5
		6 months	<15	40	negative	32	16	15
		11 months	<15	<10	negative	32	16	40
22 yr. ♀	1 : 64	0 day	20	80	16	64	16	<5
		12 days	40	80	64	64	32	<5
		17 days	80	160	64	64	32	<5
		1 month	40	160	16	64	2	<5
		8 months	<15	10	negative	64	2	40
17 yr. ♀	1 : 64	0 day	15	40	16	negative	16	<5
		3 days	20	40	64	negative	16	<5
		9 days	40	80	32	negative	16	<5
		1 month	80	160	16	negative	32	<5
		4 months	<15	80	16	negative	16	20
		8 months	<15	80	negative	negative	16	40
16 yr. ♂	1 : 128	0 day	15	40	16	16	16	<5
		11 days	40	40	32	32	16	<5
		1 month	40	10	32	16	16	<5
		12 months	<15	10	negative	16	16	20

sensitive and permits differentiation of ACIF-detectable antibodies belonging to different immunoglobulin classes.

In the present work we have made attempts to demonstrate EBNA antibodies appearing EBV-positive IM and those present in subjects who had contracted EBV infection earlier.

Materials and methods

Serum samples. (I) Forty-nine samples from 10 patients who showed the typical clinical, and haematological picture of IM. Each of the patients was positive for heterophilic antibody and anti-EBV IgM.

(ii) Twenty-one samples from 5 patients with CMV-positive IM.

(iii) Twenty-one samples from EBV-positive healthy donors (Blood Supply Service, Debrecen).

patients with IM of EBV origin

Case	PBD titres	Time interval between first and actual sampling	EBV IgM titres (IF)	EBV VCA IgG titres (IF)	EBNA antibody titres determined by the combined ACIF method			Anti-EBNA titres (ACIF)
					EBNA IgM	EBNA IgG	EBNA IgA	
24 yr. ♂	1 : 4096	0 day	15	10	16	16	16	<5
		4 days	20	40	32	16	16	<5
		1 month	40	40	32	16	16	<5
		4 months	<15	40	negative	16	16	20
19 yr. ♀	1 : 8192	0 day	20	10	32	64	16	<6
		4 days	40	10	32	128	16	<5
		10 days	80	40	32	64	16	<5
		17 days	80	40	16	64	32	<5
		1.5 months	<15	10	16	16	16	<5
24 yr. ♀	1 : 512	0 day	20	10	32	16	negative	<5
		5 days	40	40	16	16	negative	<5
		10 days	40	80	16	32	negative	<5
		3.5 months	<15	40	negative	16	negative	10
		9 months	<15	10	negative	16	negative	40
15 yr. ♀	1 : 64	0 day	20	40	16	32	negative	<5
		5 days	40	40	32	32	negative	<5
		1 month	40	40	16	128	negative	<5
		7 months	<15	40	negative	64	negative	40
20 yr. ♂	1 : 1024	0 day	40	10	16	16	32	<5
		5 days	80	40	32	32	64	<5
		10 days	80	80	64	64	64	<5
		15 days	80	160	64	128	64	<5
		1.5 months	15	80	16	64	32	<5
		9 months	<15	40	negative	64	16	20
		9 months	<15	40	negative	32	16	40

The sera were kept at 56°C for 30 min and stored at —20°C until used.

Source of complement. Native human serum negative for EBV antibodies.

Detection of anti-EBV IgM. This method was applied on acetone-fixed smears of B 95–8 cells. The original method [7] was modified. The serum samples were diluted in buffered salt solution (BSS pH 6.9. 0.8% NaCl; 0.014% $CaCl_2$; 0.04% KCl; 0.02% $MgSO_4 \cdot 7H_2O$; 0.06% KH_2PO_4; 0.06% $Na_2HPO_4 \cdot 2H_2O$) 1 : 15 and cells were incubated with the diluted serum for 20 hr. The preparations were then incubated with FITC-labelled anti-human IgM (μ-chain-specific) at 37°C for 30 min.

Detection of complement-fixing anti-EBNA by the ACIF method. The preparation and fixation of smears has been described elsewhere [1]. Anti-EBNA was titrated according to Henle's three-step ACIF method [8]. Sera were diluted 1 : 4, 1 : 10 and 1 : 40, human complement 1 : 10 and FITC-labelled anti-human complement (β_{1C}/β_{1A}) conjugates 1 : 20. BSS was used as diluent throughout.

Detection of complement-fixing and non-complement-fixing anti-EBNA by the anti-human globulin ACIF test. The reaction was performed in 4 steps on smears prepared from EBNA-positive RAJI and B 95-8 cells. The cell lines were kindly supplied by Professor G. KLEIN (Karolinska Institute, Stockholm).

1. Smears were incubated at 37°C for one hr, except those serving for the demonstration of complement-fixing IgM, which were incubated for 20 hr.

2. The smears were washed and incubated with 1 : 50-diluted goat anti-human IgG serum (γ-chain-specific), anti-human IgA serum (α-chain-specific) or anti-human IgM serum (μ-chain-specific) at 37°C for 1 hr.

3. The preparations were washed again and covered with human complement (1 : 10) at 37°C for 1 hr.

4. The preparations were washed, and stained with 1 : 20-diluted FITC-anti-human complement at 37°C for 20 min, then washed, dried, covered with buffered glycerol and examined under a Leitz Orthoplan UV microscope provided with Ploem-Opak illuminator.

The preparations were washed and the reagents were diluted with BSS throughout.

With regard to the possible presence of nonspecific anti-nuclear antibodies, we pre-incubated the acute-phase IM sera with cells obtained from chronic lymphoid leukaemia (CLL) and human embryonic fibroblasts, (HuEFb), and the reaction was carried out with CLL lymphocytes, HuEFb cells and umbilical cord lymphocytes as well.

Demonstration of heterophilic antibody. The modified method of Paul–Bunnell–Davidsohn was used [9].

Table I shows the combined ACIF method in comparison with the two basic methods.

Results

A. *EBV IgM, EBV-VCA IgG and EBNA antibodies in EBV-positive IM.* The antibody changes in 49 serum samples taken from 10 patients with IM of EBV origin are shown in Table II.

In the acute phase, small amounts of EBNA-specific antibodies appeared parallel with EBV-specific IgM and IgG antibodies; those belonging to the IgM class appeared in each case, whereas the IgG and IgA antibodies were demonstrable in 9 and 7 of the 10 cases, respectively. Like the EBV IgG and IgM antibodies, the EBNA antibodies reached their highest serum level in the first weeks of the illness. During convalescence, the titre of each antibody was declining, the anti-EBV IgM and the anti-EBNA IgM even disappeared. EBV-VCA IgG, EBNA IgG and EBNA IgA were still detectable several months after convalescence.

In the first weeks of IM, the ACIF reaction was negative in every case. The first seroconversion was demonstrated 3.5 months later in Patient No. 8.

Figure 1 shows the typical dynamics of serum antibodies in a young male patient.

B. *EBV IgM and EBNA antibodies in IM of CMV origin.* Twenty-one such serum samples were examined from 5 patients, all seropositive for EBV. EBV antibodies of the IgM type were not detectable either by the IF or the ACIF technique. In contrast, every sample contained little amounts of EBNA-specific antibodies (Table III).

Table III

Horizontal study with serum samples from patients with IM of CMV origin

Case	Time interval between the first and the actual sampling	EBV antibody titre		
		Anti-EBV IgM (IF)	Anti-EBNA IgM (combined ACIF)	Anti-EBNA (ACIF)
16 yr. ♂	0 day 14 days 6 months 9 months	<15	negative	20
24 yr. ♀	0 day 12 days 2 months 7 months 10 months	<15	negative	15
15 yr. ♂	0 day 16 days 7 months 12 months	<15	negative	5
20 yr. ♂	0 day 8 days 1 month 7 months 10 months	<15	negative	15
10 yr. ♂	0 day 8 days 10 months	<15	negative	5

C. *EBV IgM and EBNA antibodies in EBV-positive sera* of twenty-one serum samples kindly supplied by the Blood Supply Service of Debrecen, all contained little amounts of EBNA late antibodies, but none of them were found to be positive for anti-EBNA IgM.

The sensitive combined ACIF method showed IgG-type antibodies in 19 and IgA-type antibodies in 9 of the 21 cases (Table IV).

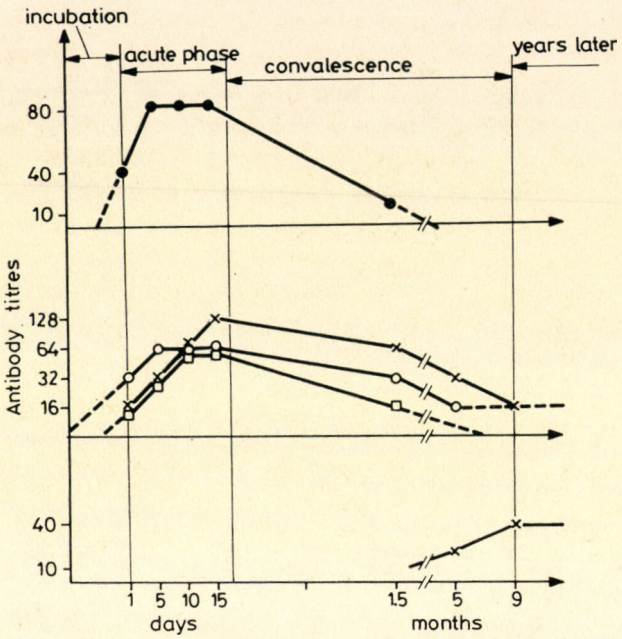


Fig. 1. Dynamics of serum antibodies in a young male patient (heterophilic antibody titre 1 : 1024). Upper graph: anti-EBV antibody titres detected by IF test. Middle graph: anti-EBNA antibody titres detected by combined ACIF test. Lower graph: anti-EBNA antibody titres detected by original ACIF test. ●——● Anti-EBV–IgM; ×——×anti-EBNA–IgG; ○——○ anti-EBNA–IgA; □——□ anti-EBNA–IgM

Table IV

Virus-specific antibodies in EBV-seropositive samples collected from 21 healthy donors

Total No. of sera	21
No. of sera positive for anti-EBV IgM (IF)	0
No. of sera positive for anti-EBNA IgM (combined ACIF)	0
No. of sera positive for anti-EBNA IgA (combined ACIF)	9
No. of sera positive for anti-EBNA IgG (combined ACIF)	19
No. of sera positive for anti-EBNA (ACIF)	21

Discussion

1. EBNA IgM antibody was consistently detected in EBV-specific IM. The same antibody could not be detected in serum samples from subjects who had contracted EBV infection at an earlier time. The appearance and early

disappearance of the antibody remind one of the EBV IgM antibody, and its detection may serve as an additional evidence of the acuteness of an EBV infection.

2. In serum samples collected from acute EBV infections and healthy EBV carriers, the antibodies detectable irregularly may represent the non-complement-fixing IgG4 subclass and/or the IgA class. The importance of their presence or absence is not known.

3. Small amounts of complement-fixing anti-EBNA IgG (IgG1 IgG2 and IgG3) are persisting in the serum of every EBV-seropositive subject.

It may be concluded that the human immune response to EBNA, the earliest viral product, is continuous. The early antibodies, *viz.*, the complement-fixing IgM, appearing consistently, and the non-complement-fixing IgG and IgA antibodies, appearing irregularly, are soon replaced by complement-fixing IgG antibodies. The antigenic stimulus may be due to lymphocytes releasing virus spontaneously, as well as to immune lysis of non-productive EBV-genome-carrying B lymphocytes. Although acute infection is followed by a sudden decrease in the number of EBNA-positive cells [5], unaffected carrier cells may multiply for a long time, until killed by the immune response [10].

REFERENCES

1. Reedman, B. M., Klein, G.: Int. J. Cancer **11,** 499 (1973).
2. Zur Hausen, H.: In Homburger, F. (ed.): Oncogenic Adenoviruses. Progress in experimental tumor research. Vol. **18,** Karger, Basel 1973, p. 240.
3. Henle, W., Guerra, A., Henle, G.: Int. J. Cancer **13,** 751 (1974).
4. Henle, G., Henle, W., Horwitz, Ch. A.: J. infect. Dis. **130,** 231 (1974).
5. Klein, G., Svedmyr, E., Jondal, M., Persson, P. O.: Int. J. Cancer **17,** 21 (1976).
6. Schmitz, H., Kampa, D., Heidenreich, W.: Int. J. Cancer **16,** 1030 (1975).
7. Schmitz, H., Scherer, M.: Arch. ges. Virusforsch. **37,** 332 (1972).
8. Ohno, S., Luka, J., Lindahl, T., Klein, G.: Proc. nat. Acad. Sci. (Wash.) **74,** 1605 (1977).
9. Lee, C. L., Zandren, F., Davidsohn, I.: Clin. Path. **21,** 631 (1968).
10. Zur Hausen, H.: Cancer Res. **36,** 678 (1976).

Address of the authors:

Judit Czeglédy, Lajos Gergely, Lajos Váczi
Institute of Microbiology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

András Szalka
László Central Hospital for Infectious Diseases
Gyáli út 5, H-1097 Budapest, Hungary

Acta Microbiol. Acad. Sci. hung. 25, 245—252, 1978

CHROMOSOME CHANGES IN SUBACUTE SCLEROSING PANENCEPHALITIS

ÉVA CSONKA, F. FORNÁDI and A. S. KOCH

Second Institute of Pathology and First Department of Neurology, Semmelweis University Medical School, Budapest

(Received September 16, 1977)

In two out of a total of 6 cases of subacute sclerosing panencephalitis with manifest chromosome alterations, chromosome studies were repeated 4 and 5 years of standing, respectively, after manifestation of the disease. In one case the incidence of chromosome alterations increased while in the other no change was found. Both cases were clinically similar in their history with remission.

The importance of measles virus in the aetiology of subacute sclerosing panencephalitis (SSPE) has repeatedly been emphasized in the last 10 years [1, 2]; an involvement of other viruses could not be excluded [3–5].

Earlier we have reported chromosome breaks and pulverization in leukocytes from SSPE patients [6]. Such changes are generally ascribed to virus infection [7, 8], so that the finding was an additional circumstantial evidence in favour of a possible viral aetiology of the disease. AMATO and PELLEGRINO [9] failed, however, to observe chromosome alterations in the leukocytes of one patient while they found them in the cultured cells from a brain biopsy specimen of an SSPE patient.

In this paper we report on chromosome studies in some additional SSPE patients. Two of those had been studied for chromosome alterations 4 and 5 years earlier, respectively. Since that time these patients remained in a clinically stagnating condition. The results of chromosome studies are presented together with neurological and laboratory findings.

Methods

Measles virus antibody titre in the sera was determined by haemagglutination inhibition (HI) test using TAKÁTSY's Microtitrator [10].

Chromosome studies were performed in 48 hr leukocyte cultures using the conventiona air drying method.

Results

The clinical history, the results of relevant laboratory tests as well as those of the chromosome studies are surveyed in Table I.

Among the clinical tests, the greatest diagnostic value is generally attributed to the high gamma globulin levels in the CSF by electrophoresis

Table I

Clinical history of patients, results of laboratory tests and chromosome studies

Patient, year of birth	Onset of disease	Dates of examinations	Phase	EEG Radermacker complex	Gamma globulin, per cent			Measles HI*		Chromosome alternations, per cent					
					CSF	Serum	Q	CSF	Serum	Mitosis	GAP	Break	Fragmentation	Dicentrism	Total
P. J.	1973	5. 1973	II	+	n.d.	n.d.		n.d.	1 : 640						
1961		5. 1973	II–III	+	37.6	25.2	1.49	1 : 80	1 : 1280	93	5.38	6.45	5.38	2.15	19.36
♂		6. 1973	II–III	+	20.4	18.5	1.10	1 : 40	1 : 640						
Sz. J.	1969	9. 1969	I	+	n.d.	n.d.		n.d.	1 : 640						
1960		3. 1971	II	+	34.3	n.d.		n.d.	1 : 160						
♂		4. 1973	I		n.d.	30.0		1 : 80	1 : 1280						
		5. 1973		neg.	48.6	16.5	2.94	n.d.	1 : 1280	160	10	6.25	1.88	0.62	18.75
H. I.	1964	1964	I–II	neg.	n.d.	n.d.		n.d.	n.d.						
1956		4. 1968	II	neg.	11.6	23.5	0.49	n.d.	1 : 1280						
♂		8. 1969	I	n.d.	n.d.	n.d.	n.d.	n.d.	1 : 160	125	6.8	3.2			10.0
		3. 1971	I	neg.	8.0	8.0	1.0	n.d.	1 : 320						
		3. 1973	I	neg.	25.0	12.3	2.0	neg.	1 : 160						
		5. 1973	I	n.d.	n.d.	n.d.		n.d.	n.d.	112	12.5	13.5	4.5	0.9	31.4
W. Gy.	1967	1967	I–II	+	63.2	n.d.		n.d.	n.d.						
1954		5. 1968	III	+	45.5	14.6	3.1	n.d.	1 : 640	240	13.1	8.2	5.4		26.7
♂		9. 1968	III	+	47.0	14.6	3.2	n.d.	1 : 1280						
		1. 1969	III	+	30.4	13.7	2.2	n.d.	n.d.						
		2. 1970	II	+	20.0	15.5	1.3	n.d.	1 : 2560						
		4. 1973	I	neg.	38.0	16.8	2.3	1 : 80	1 : 1280	218	7.3	6.0	11.4	2.3	27.0

S. I.	1957	5. 1968	II	+	n.d.	n.d.	n.d.	n.d.	1 : 320						
1946		7. 1968	II	n.d.	n.d.	n.d.	n.d.	n.d.	1 : 320						
♀		1. 1969	II	+	n.d.	n.d.	n.d.	n.d.	1 : 320	140	4.8	5.3	3.0	7.8	20.9
N. M.	1960	1960	I–II												
1945		1961	II	+	n.d.	n.d.		n.d.	n.d.						
♀		7. 1962	I	+	n.d.	54		n.d.	n.d.						
		10. 1963	I	neg.	n.d.	n.d.		n.d.	n.d.						
		4. 1966	I–II	n.d.	n.d.	n.d.		n.d.	n.d.						
		2. 1967	I–II	neg.	25.6	18.3	1.40								
		11. 1967	I–II	neg.	n.d.	18.3		n.d.	n.d.						
		4. 1968	II–III	neg.	22.6	12.8	1.76	n.d.	1 : 80						
		10. 1968	II–III	neg.	17.4	16.1	1.08	n.d.	n.d.						
		12. 1968	III	+	n.d.	18.8		n.d.	1 : 80						
		1. 1969	III	neg.	n.d.	n.d.		n.d.	1 : 80						
		7. 1969	III	n.d.	n.d.	n.d.		n.d.	1 : 160	100	12.8	16.2	3.0	—	32.0
		7. 1969	III	n.d.	n.d.	n.d.		n.d.	1 : 160						

* Haemagglutination inhibiting antibodies
n.d. = not done

as well as to the ratio of gamma globulin in CSF and serum. The presence of Radermacker complex in EEG is also of diagnostic value. The clinical status of patients was designated by roman numbers I, II and III. Phase I is characterized by behavioural disturbances and the appearance of some cortical symptoms. In phase II, extrapyramidal hyperkinesis develops. Phase III represents the stage with rigor and progressive decortication. In addition to the clinical and laboratory data the results of measles HAI tests and chromosome studies are shown.

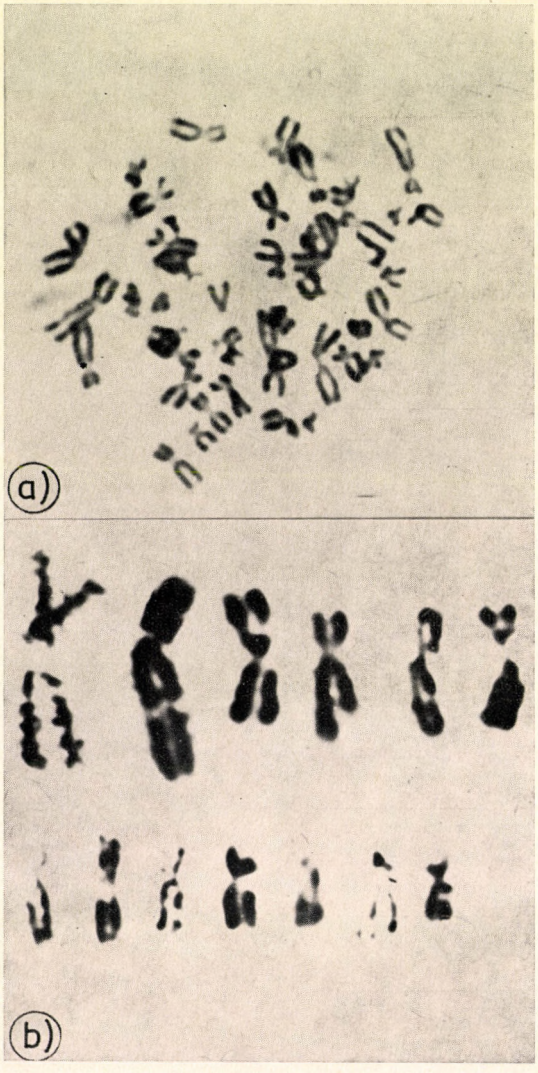


Fig. 1. a) Case No. 1. Breaks and gaps. b) Case No. 2. Breaks and gaps

Detailed results of the chromosome studies are also presented in Table I. The chromatid gaps are not any more considered to represent chromosome damage. Since, however, we had to compare the present results with earlier ones, we still take into account the chromatid gaps in a separate group; their incidence of this particular chromosome was 0.8% or less in healthy individuals.

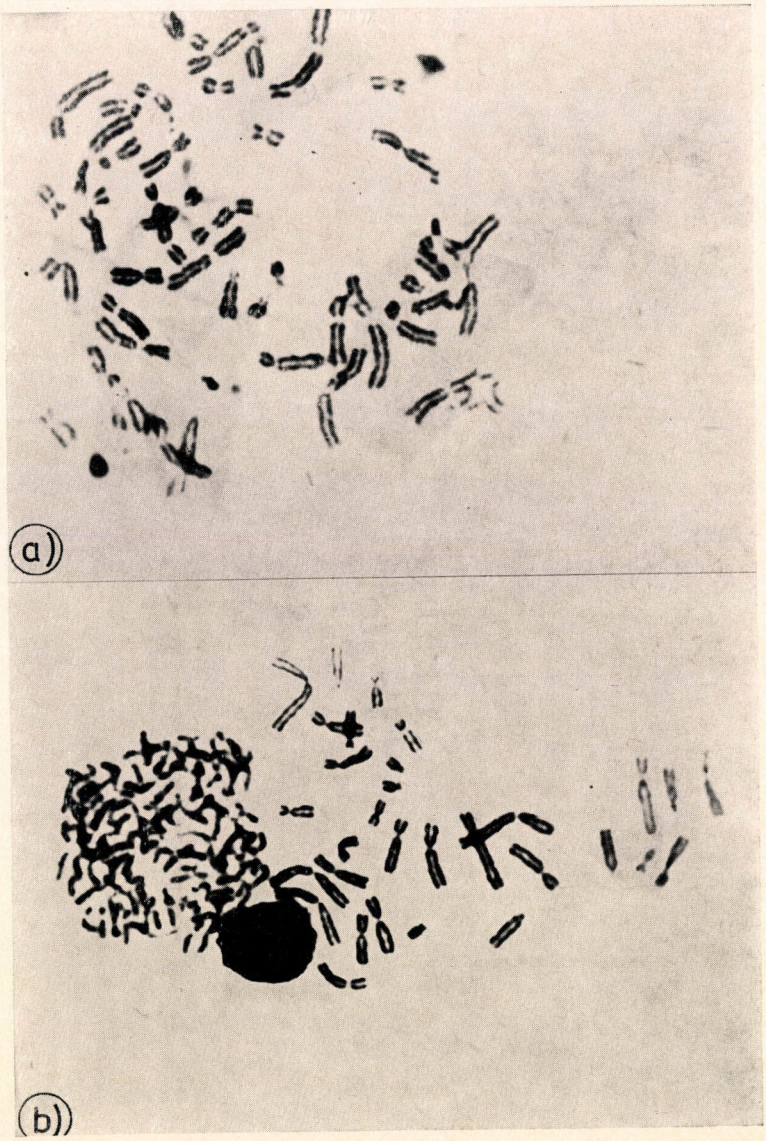


Fig. 2. a) Case No. 3. Undefinable multiple chromosomal rearrangements.
b) Case No. 4. Chromosal fragmentations

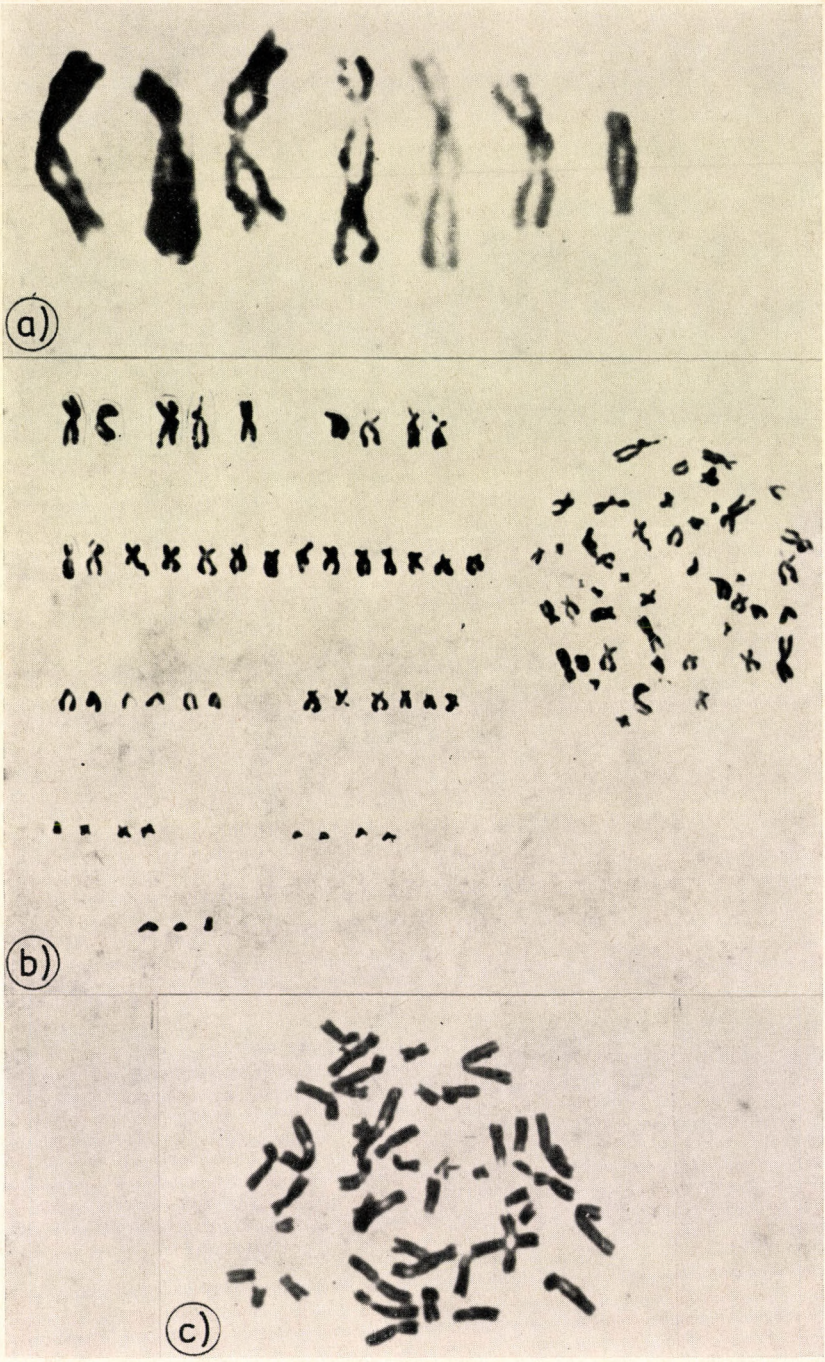


Fig. 3. a) Case No. 5. Dicentrism. b) and c) Case No. 6. Undefinable multiple major chromosomal rearrangements

With patient P. J. the history is typical of SSPE. He had high measles antibody titres in both serum and CSF. Breaks and fragmented chromosomes occurred with approximately the same frequency (Fig. 1/a).

Sz. J. underwent remission after a condition corresponding to phase II of the disease. High measles antibody titres were demonstrable. This patient exhibited the lowest incidence of chromosome alterations (Fig. 1/b).

H. J.: the disease ran an atypical course in this patient. The CSF–serum gamma globulin quotient, the elevated measles antibody level and the presence of M-component in the CSF were typical, but no Radermacker complex was seen in the EEG. Chromosome studies revealed marked alterations and their incidence increased by 21.4% in four years. No drug or treatment was given to the patient that could cause chromosome alteration (Fig. 2/a).

W. E. exhibited clinical symptoms characteristic of SSPE and the blood measles antibody titre was high. The only unusual symptom was a marked remission with disappearance of the Radermacker complex after a two-year period of phase III condition. Chromosome studies repeated in the phase of remission 5 years after the first study did not reveal any change in the incidence and type of the changes (Fig. 2/b).

S. I. was admitted in 1968 and in 1969, thus the data on the course of the disease are scarce. The patient could not be reached since her discharge. Chromosome studies revealed a high incidence of dicentric figures (Fig. 3/a).

N. M.: this patient improved considerably after a period of phase II condition. Some years later, however, the condition again became worse. A Radermacker complex was not always observed even in the severe periods. The measles antibody level was consistently low, but chromosome studies revealed marked alterations (Fig. 3/b, c).

Discussion

In the SSPE patients studied we could not demonstrate any correlation of the clinical condition with the measles antibody level or the frequency of chromosome alterations. The chromosomes of two patients were studied repeatedly after 4 and 5 years, respectively. In one case no change occurred in the frequency of chromosome alterations while in the other a significant increase was observed.

In patients 3 and 6 some similarities were evident. Their age (17 and 24) was comparatively high, both experienced a several years' long remission, in one the Radermacker complex was consistently missing and in the other it disappeared. In both cases, low measles HAI titres were found and both exhibited chromosome alterations similar in degree and frequency.

The finding do not permit to draw conclusions. The variability in the relation of chromosome alterations to the measles antibody levels and the

course of the disease are, however, suggestive of a possible heterogeneity of the aetiological agent(s) involved [4, 5].

REFERENCES

1. Horta-Barbosa, L., Fuccillo, D. A., London, W. T., Jabbour, J. T., Zeman, W., Sever, J. L.: Proc. Soc. exp. Biol. (N. Y.) **132,** 272 (1969).
2. Payne, F. E., Baublis, J. V., Itabashi, H. H.: New Engl. J. Med. **281,** 585 (1969).
3. Katz, M., Koprowski, H.: Arch. ges. Virusforsch. **41,** 390 (1973).
4. Müller, D., Meulen, V., Katz, M., Koprowski, H.: Lab. Invest. **25,** 337 (1971).
5. Sato, T. A., Yamanouchi, K., Shishido, A.: Arch. ges. Virusforsch. **42,** 36 (1973).
6. Csonka, É., Lipcsák, M., Szirtes, G.: Nature (Lond.) **222,** 292 (1969).
7. Csonka, É., Ruzicska, P., Koch, A. S.: Acta microbiol. Acad. Sci. hung. **22,** 41 (1975).
8. Nichols, W. W.: Ann. Rev. Microbiol. **24,** 479 (1970).
9. Amato, R. S., Pellegrino, R. J.: Neurology **21,** 553 (1971).
10. Takátsy, Gy.: Acta microbiol. Acad. Sci. hung. **3,** 191 (1955).

Address of the authors:

Éva Csonka, Sándor Koch
Second Institute of Pathology, Semmelweis University
Medical School
Üllői út 93, H-1091 Budapest, Hungary

Ferenc Fornádi
First Department of Neurology, Semmelweis University Medical School
Balassa u. 6, H-1083 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 253—264, 1978

REPLICATION OF OVINE ADENOVIRUSES

S. Belák, V. Pálfi and S. Tuboly

Institute of Epizootiology, University of Veterinary Sciences and Central Veterinary Institute, Budapest

(Received September 21, 1977)

The replication of ovine adenoviruses shows intertypic and intratypic variations, e.g. the extracellular and intracellular virus yield of ORT/111, a strain related to bovine adenovirus type 2, reached its peak 40 to 46 hr postinfection, in contrast to GY/14, a strain classified as ovine adenovirus type 1, which required 52 to 58 hr to reach the highest yield. The replication cycle was not appreciably influenced either by rolling of the tube cultures or the age (between 0 and 7 days) of the supporting ovine cell culture. All the strains under study replicated at 40°C more rapidly than at 34°C or 37°C. There was some intratypic variation in the replication of strains at different temperatures. Ovine adenoviruses replicated well after three consecutive passages in cultured lamb testicle or secondary heterologous cells such as calf kidney, calf testicle or pig kidney cell cultures as well as the MDBK cell line and a cell line from the calf trachea. The tissue spectra of strains showed intratypic variations. As examined by direct immunofluorescence, fluorescing adenovirus antigen appeared first at 8 hr postinfection and was present in 90% of the cells at 16 hr. Intertypic differences in the intensity and character of fluorescence are described.

Recently [1–4] we have shown the aetiological role in pneumoenteritis of lambs of several ovine adenoviruses and of strains related to bovine adenovirus type 2. In the present work, we have studied the replication of the ovine strains.

In the literature available one single paper [5] was found to deal with the replication of ovine adenovirus, of strain SAV, the prototype strain of type 5. Since in preliminary experiments we had found essential differences between strains belonging to different serotypes, it seemed justified to study the strains comparatively. Experiments were performed in various cell cultures of various ages and at various temperatures. The appearance and intracellular location of adenovirus antigen were examined by direct and indirect immunofluorescence (IF).

Materials and methods

Cell cultures. The strains were propagated in secondary fetal ovine kidney cell cultures. Their tissue spectra were investigated in lamb testicle, calf testicle, calf kidney and pig kidney secondary cell cultures and in two cell lines, viz., the MDBK calf kidney line and a calf trachea cell line.

Viruses. Of the ovine adenovirus type 1 strains the prototype strain S1 and the Hungarian isolate GY/14, and of the type 5 strains the prototype strain SAV and the Hungarian isolate PA/8, were included in the study. The replication of the bovine type 2 adenovirus strains

ORT/111 and Het/3, both isolated from sheep, was compared with that of the prototype strain No. 19 isolated from calves. The ovine prototype strains were kindly supplied by Dr. J. B McFERRAN, Belfast, Northern Ireland, the prototype No. 19 by Dr. A. BARTHA, Budapest.

Study of replication in stationary cultures. Secondary fetal ovine kidney cells cultivated in tubes were washed in PBS and inoculated with 10^7 $TCID_{50}$ virus/tube. The high multiplicity served for approaching synchronous infection of cells. The replication of ORT/111 and GY/14 was studied in detail. After an adsorption period of 2 hr at 37°C, the cultures were washed three times, each was covered with 1.5 ml Earle's medium and further incubated at 37°C. Six samples were taken at 0 hr, 5 hr and 10 hr and, subsequently, in every 6th hr until 82 hr post-inoculation. The sampled cultures were examined under the light microscope. The culture media from the six parallel cultures were pooled and centrifuged at 5000 rpm for 30 min; the cell layers were washed with PBS and the cells sedimented from the supernatant were resuspended and added (in 1.5 ml Earle's medium/tube) to the cell sediment. The cells were then frozen and thawed six times and the debris was removed by centrifugation. The virus released into the supernatant as well as the cell-fixed virus was titrated always in the same batch of cell culture, simultaneously.

Study of replication in rolling tubes. Except for incubation in rolling drum, the experiments were performed as described above.

Influence of the age of cell culture. Secondary fetal ovine kidney cell cultures were inoculated with 10^6 $TCID_{50}$/0.1 ml of the GY/14 strain on days 0, 1, 3, 5 or 7 of culturing. Samples taken daily were treated and titrated as described above.

Influence of incubation temperature. Cell cultures were inoculated with 100 $TCID_{50}$/0.1 ml of virus and incubated at 34, 37 and 40°C. Six samples were taken on days 2, 3, 5 and 7 post-inoculation. To determine infectivity, we inoculated cell cultures with serial virus dilutions and incubated them at 37°C.

Replication in various cell cultures. The strains propagated in ovine fetal kidney cell cultures were transferred to various cell cultures. Each tube was inoculated with 100 $TCID_{50}$/0.1 ml of virus. The cultures were then incubated at 37°C for 10 days while examined for CPE daily. Those showing well-defined CPE and the cultures negative on day 10 were subjected to further two consecutive passages in cultures of the same type. Samples from the last passage were frozen and thawed three times, freed from cell debris, and titrated in fetal sheep kidney cell cultures parallel with the starting material. The virus recovered was identified by virus neutralization tests.

Direct immunofluorescence. Antiserum to fluorocarbon (Arklone, P, ICI) treated strain ORT/111 was raised in initially seronegative goats. The animals were inoculated four times at weekly intervals intravenously and intramuscularly and bled on the 10th day following the last inoculation. The pooled sera gave positive gel precipitation test with an antigen prepared from serotype 1 of bovine adenovirus and neutralized ORT/111 up to serum dilution of 1 : 512. To prepare conjugate the globulins were salted out with Na_2SO_4. The globulin fraction was dialysed and its 2% solution was labelled with FITC (Fluka AG, Buchs SG). The free FITC was removed by filtration of Sephadex G-50 and the optimum dilution of the conjugate was determined. The ORT/111 strain was propagated in secondary sheep kidney cell coverslip cultures. These were inoculated as described above. Samples were taken every second hr. The different ovine adenovirus strains were comparatively studied by IF. The cultures to be examined were fixed in acetone at 4°C, incubated with the conjugate at 37°C for 30 min, washed again, and counterstained with 1 : 40 000-diluted Evans blue.

Indirect IF. Fixed preparations were incubated with a serum obtained from immunized ewes (neutralization titre, 1 : 128), washed, and incubated with FITC-labelled anti-sheep immunoglobulin prepared from the blood of rabbits immunized with an antigen prepared from pooled sheep serum by alum treatment.

In the evaluation of fluorescence Zeiss NU-2 microscope, BG 12 and BG 3 objectives and OG 1 eye-piece were used.

Results

Replication in stationary and rolling cultures. In native cultures of ORT/111 of GY/14 CPE appeared in the 16th hr. Initially, there was no appreciable difference between stationary and rolling cultures. Later, rolling cultures

showed more pronounced CPE. In ORT/111-infected cultures the damaged cells were diffusely refractive, rounded up and swollen, those in cultures infected with the GY/14 strain were also refractive and formed clusters. In cultures infected with the ORT/111 strain, nearly all cells were damaged 52 to 58 hr after inoculation and many of them were tending to detach. The same changes developed slower in GY/14-infected cultures. The virus titres are shown in Figs 1–4.

Influence of the age of cultures. Figures 5 and 6 show the dynamics of virus replication in cultures of different ages. The rate of replication seemed to be independent of the age of culture.

Influence of incubation temperature. Of the bovine type 2 adenovirus strains, ORT/111 reached the same titre irrespective of temperature, while the titre reached by Het/3 was 10^{-3} at 34°C, 10^{-4} at 37°C and 10^{-6} at 40°C (Table I).

Table I

Infective titres at different temperatures

Virus strain	Temperature, °C	log $TCID_{50}$/ml virus on days postinfection			
		2	3	5	7
Het/3	34	1	2	2	3
	37	1	2	3	4
	40	1	2.5	4	6
GY/14	34	3	4	4	5
	37	3	4	5	7
	40	4	5	6	7
ORT/111	34	3	5	6	7
	37	3.5	5	6	7
	40	3	6	6	7
Sl	34	3	3.5	4	6
	37	3	4	4.5	7
	40	4	5	6	7

All the other strains replicated at the highest rate at 40°C. On the 7th day, however, the virus yield was identical at 37°C and 40°C.

Virus multiplication in different cell cultures. All strains replicated well in mammalian cell cultures (Table II). The strains isolated from sheep and the bovine strain No. 19 replicated nearly equally in ovine, bovine and pig cells. The ORT/111 strain, related to bovine adenoviruses, reached somewhat

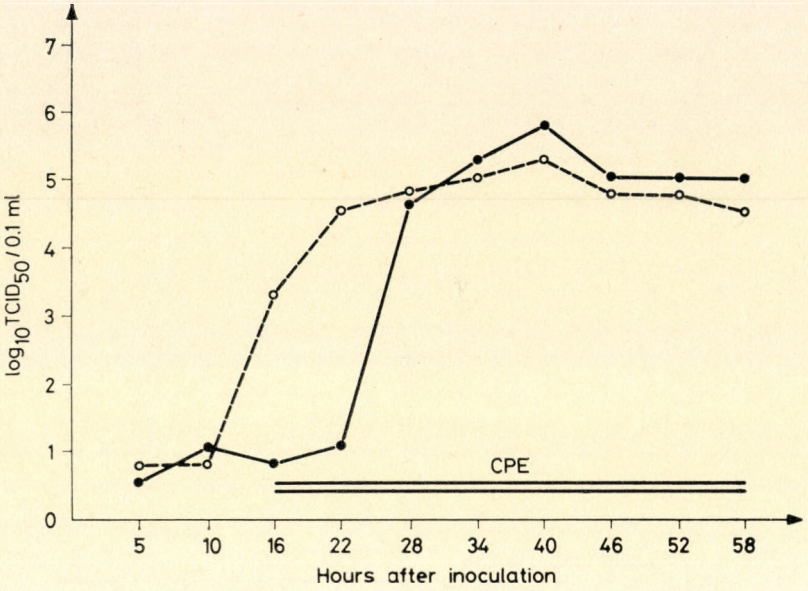


Fig. 1. Multiplication of the ORT/111 virus strain in stationary fetal sheep kidney cell cultures; ● —— ● extracellular virus, ○ ---- ○ intracellular virus

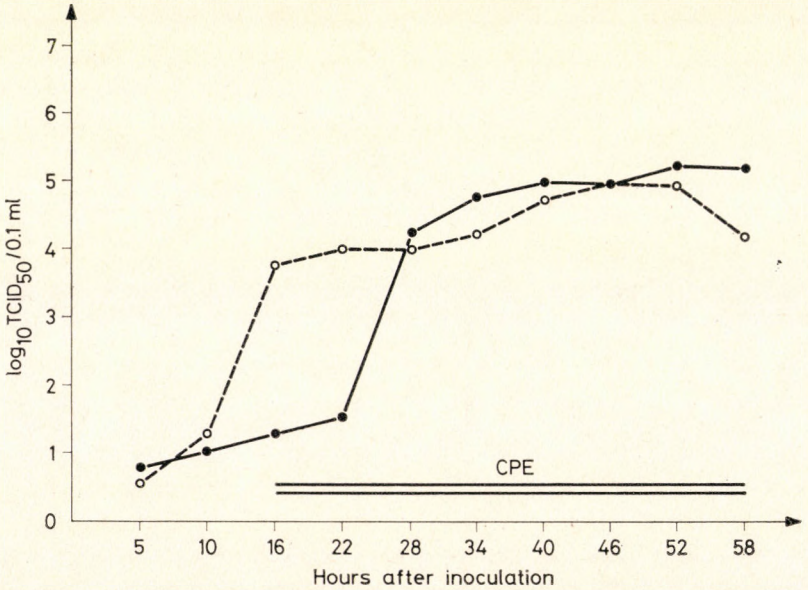


Fig. 2. Multiplication of the ORT/111 virus strain in rolled fetal sheep kidney cell cultures; ● —— ● extracellular virus, ○ ---- ○ intracellular virus

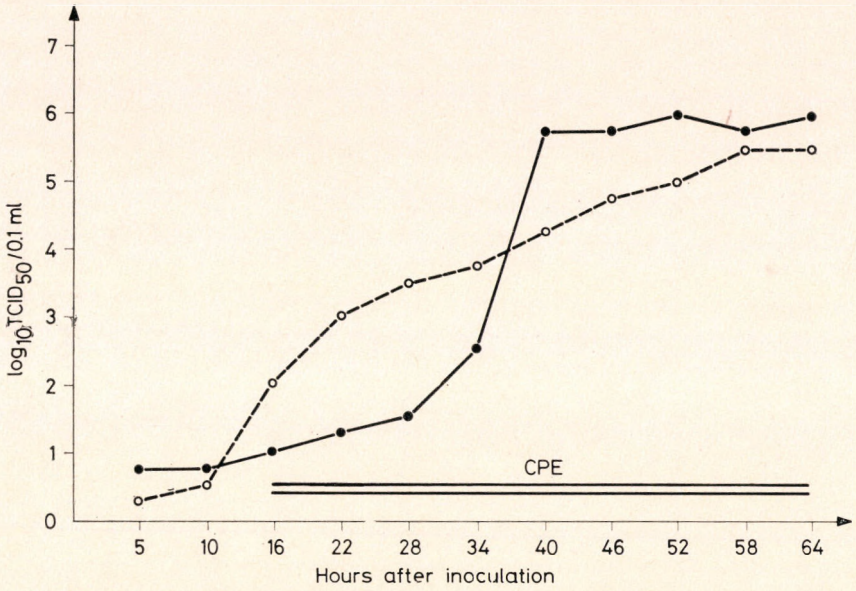


Fig. 3. Multiplication of the GY/14 virus strain in stationary fetal sheep kidney cell cultures; ● —— ● extracellular virus; ○ ---- ○ intracellular virus

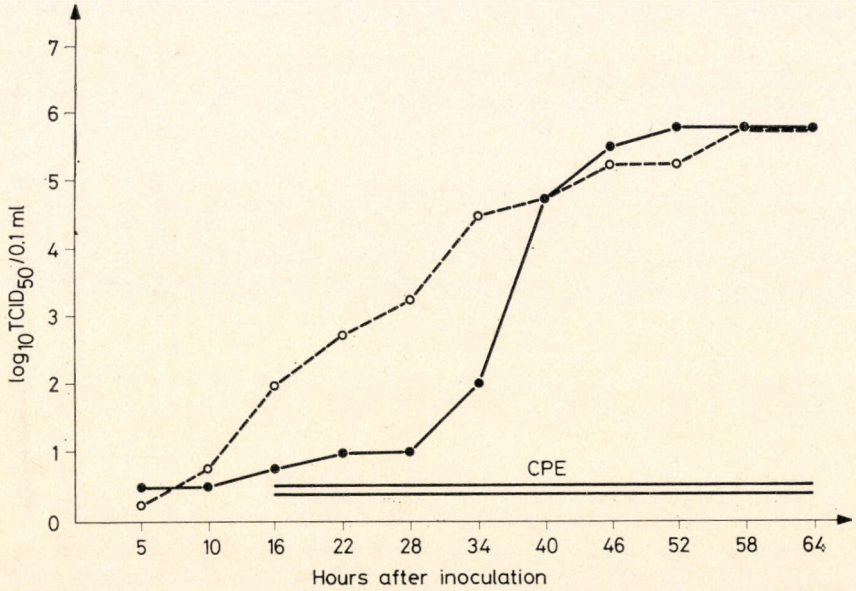


Fig. 4. Multiplication of the GY/14 virus strain in rolled fetal sheep kidney cell cultures; ● —— ● extracellular virus, ○ ---- ○ intracellular virus

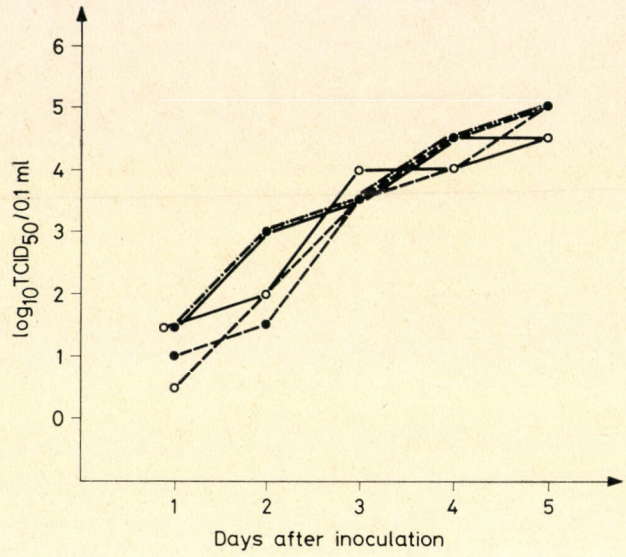


Fig. 5. Virus titres of the supernatants of fetal sheep kidney cell cultures inoculated with GY/14 virus strain at different intervals. Age of inoculum, days: ○ ---- ○ 0, ● ---- ● 1, ● ------ ● 3, ○ —— ○ 5, ● —— ● 7

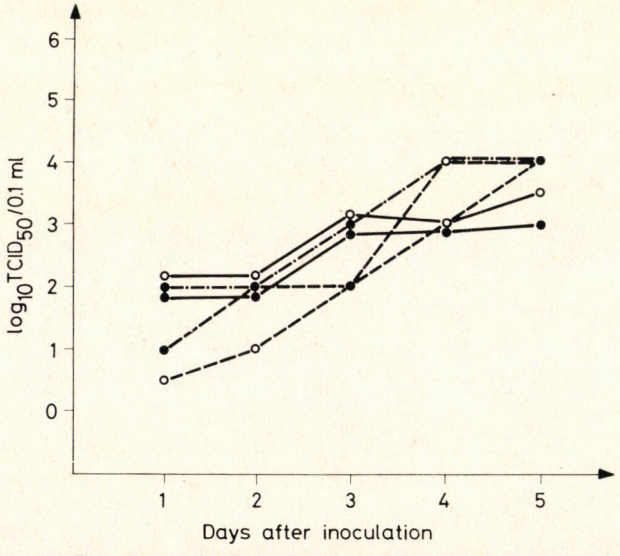


Fig. 6. Titres of intracellular virus in fetal sheep kidney cell cultures inoculated with the GY/14 strain of different days of culturing. Age of inoculum, as in Fig. 5

Table II

Multiplication of virus strains in various cell cultures

Virus strain	Initial titre in lamb kidney cells*	Titre in lamb kidney cell cultures after 3 serial passages in*					
		lamb testicle	calf kidney	calf testicle	MDBK	calf trachea	pig kidney
		cell cultures					
Sl	6.0	5.0	4.0	4.0	4.75	5.0	5.25
GY/14	6.0	5.25	4.0	3.5	4.5	5.0	5.0
SAV	6.25	5.5	5.25	6.0	5.25	5.0	5.5
PA/8	5.25	4.0	4.0	4.0	4.0	2.5	4.5
ORT/111	6.5	6.0	6.0	7.0	5.25	5.0	6.5
Het/3	5.0	4.75	5.0	5.25	4.75	3.0	5.75
No. 19	4.75	4.25	5.0	5.0	5.75	4.0	4.0

* $\log_{10}$ $TCID_{50}$/ml

higher titres in calf testicle cell cultures than in lamb kidney ones, whereas an opposite difference of two orders of magnitude was observed in the multiplication of the ovine type 1 strain GY/14.

IF studies. Zero and 2 hr after inoculation, direct IF showed the background of the infected cultures faintly glimmering while the cell nuclei appeared as dark areas. The same was observed in non-infected control cultures (Fig. 7).

In the 4th hr, the glimmering of the cytoplasm tended to become more intensive and the nuclear membrane sharper. In the 6th hr, the outline of the nuclear membrane became brighter. In the 8th hr, this pattern of fluorescence was still more pronounced and coarse fluorescing granules appeared in a few nuclei. At the same time the fluorescence of the nuclear membrane began to weaken. In the 10th hr, well-defined granular fluorescence was observed in several nuclei (Fig. 8).

In parallel cultures examined in the native state, CPE was first seen in the 12th hr. At the same time, an intense fluorescence was detected in about 10% of the nuclei. Sometimes the fluorescing structures appeared as a fine granulation, in other cases the nucleus was diffusely filled with coarse granules (Fig. 9).

Cells with fluorescing nuclei were scattered in the cultures. In the 14th hr, some 50% of the nuclei were fluorescing, many of them were enlarged and filled with bright granules (Fig. 10).

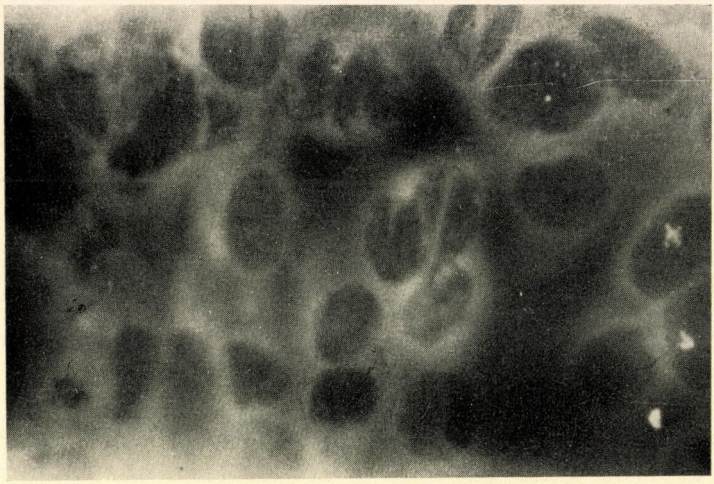


Fig. 7. Direct IF by cells supporting the replication of strain ORT/111. Secondary fetal sheep kidney cell cultures; 0 hr sample

In other nuclei, bright structures like adenovirus inclusions and round structures similar to nucleoli were seen surrounded by fluorescent substance. In the 16th hr, 90% of the cells showed this picture (Fig. 11).

At the same time, the intranuclear fluorescence tended to weaken and fluorescent granules seemed to attach themselves to the nuclear membrane, sometimes simulating a bright ring lining its inner surface. Many nuclei displayed an intense homogeneous fluorescence. The picture showed hardly any

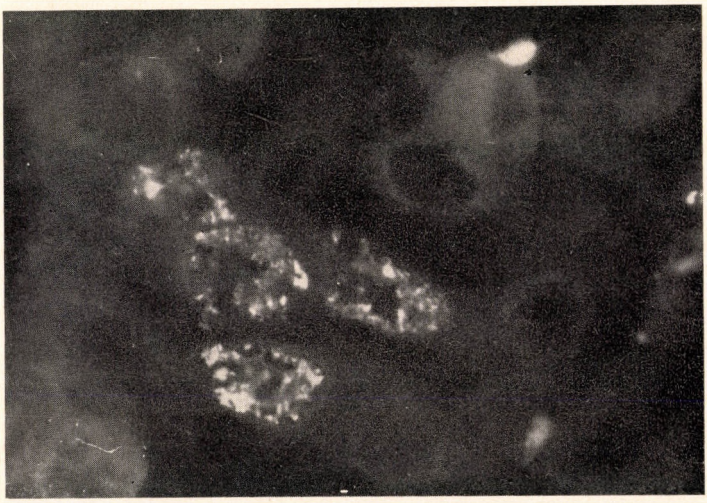


Fig. 8. Same as Fig. 7; 10 hr sample

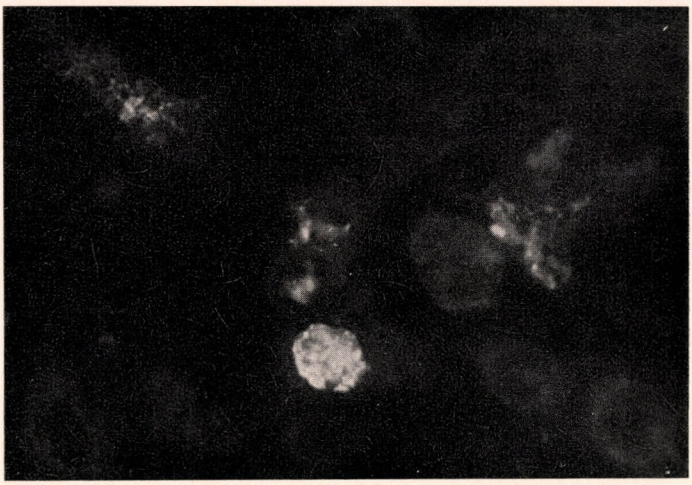


Fig. 9. Same as Fig. 7; 12 hr sample

change until the end of the observation period in the 40th hr. The control cultures remained unchanged.

With the ORT/111 conjugate, fluorescence was observed in cultures infected by any of the strains involved in the study. Both representatives of ovine type 5 induced granular fluorescence similar in intensity and appearance to that seen in nuclei of cells infected by ORT/111. The type 1 ovine strains behaved differently; in cells infected by GY/14, mainly the clustered

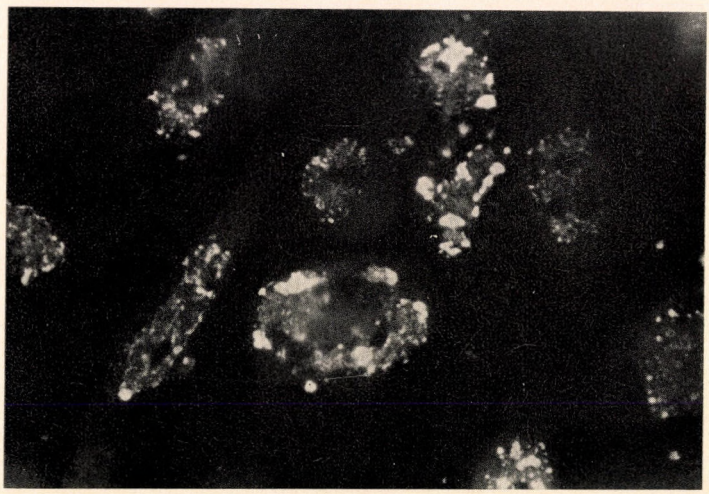


Fig. 10. Same as Fig. 7; 14 hr sample

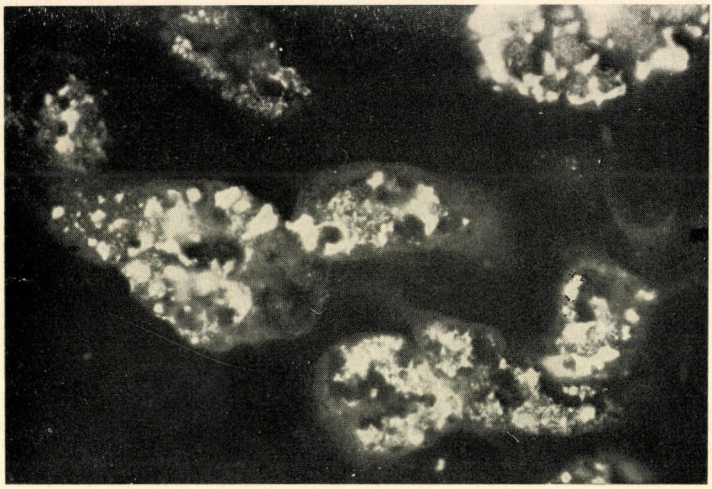


Fig. 11. Same as Fig. 7; 16 hr sample

cells were fluorescing, but less intensely than the cells infected by ORT/111. The fluorescence involved the nuclear membrane in the first place, intranuclear granular fluorescence occurred rarely. The fluorescence induced by strain S1 was more intensive, and the intranuclear granular fluorescence occurred more frequently in S1-infected cells than in those infected by GY/14. Nevertheless, the fluorescence due to the S1 strain was weaker than that observed in ORT/111-infected cells.

Indirect IF tests were performed only with the ORT/111 strain. The fluorescence was similar in type but weaker than the direct one.

Discussion

Replication of the studied virus strains seemed to be similar to the replication of adenoviruses in general [6–9]. The different appearance of the strains ORT/111 and GY/14 suggests that pronounced differences, like those observed among human adenovirus strains, may be common among ovine strains. According to our unpublished findings (BELÁK and PÁLFI), even intratyping differences may occur, e.g. the replication cycle of Het/3 seems to be prolonged as compared to that of ORT/111.

CPE was found to be more pronounced in rolling cultures than in stationary ones. Since, however, ageing of cells is faster in rolling cultures, rolling may be disadvantageous when strains of prolonged replication cycle are studied.

According to investigations performed with human adenoviruses in human amniotic cell cultures [10], old cultures are more susceptible to CPE

than young ones. In the first week of incubation replication was not influenced by the age of the culture and the area covered by the monolayer, whereas CPE was more pronounced in young cultures.

The influence of incubation temperature on the replication of ovine adenovirus has not been studied before. We found that 40 °C was the temperature at which all the strains multiplied at the highest rate. The yield of strain ORT/111 was, however, equal at 34°C and 40°C while that of Het/3 was higher by 3 orders of magnitude at 40°C than at 37°C, in spite of the fact that both strains belong to the same serotype.

The wide CPE spectrum of adenoviruses include a number of heterologous cells [6, 10], but replication was most intensive in cells of the homologous species [11].

Bovine adenoviruses replicate in ovine kidney cells with a low virus yield [12]. Replication of ovine adenoviruses has been reported in lamb kidney [13] and sheep thyroid [14] cell cultures. It was found [15] that the type 5 prototype strain, SAV, lost its cytopathogenicity and infectivity during three consecutive passages in calf kidney, calf testicle, chicken embryonic fibroblast, BHK21, PK15 or MDCK cells. In contrast, like the other ovine adenovirus strains SAV was found to replicate on a wide scale of mammalian cell cultures.

It deserves interest that, e.g., in calf testicle cultures, the ovine isolates related to bovine adenovirus type 2 attain or even surpass their original titres while the titre of ovine type 1 adenovirus strains declines.

Our IF tests have thrown light on the intracellular location of adenovirus antigens and on the proportion of cells involved in replication. CPE appeared slightly later than did the intracellular adenovirus antigen. The fluorescence detected during the replication of ORT/111 agreed in character with that of cells infected with other adenoviruses [16]. The fine and coarse granulation first lining the nuclear membrane then appearing inside the nucleus was extremely intensive. The cytoplasmic fluorescence was, however, weaker even in the 40th hr of replication than that described for other adenoviruses [16–18].

The representatives of type 1 ovine adenovirus, first of all GY/14, elicited an IF different in intensity and character from the fluorescence of ORT/111 demonstrated with the same conjugate (ORT/111) while for different type 5 strains the picture was approximately the same. The phenomenon reminds one of the variations observed among bovine adenovirus strains [18], which might be attributed to different degrees of intertypic antigenic relation. Our IF studies support the observation of McFERRAN *et al.* [13] that ovine adenoviruses have a common antigen, closely related to the common antigen of human adenoviruses. This would raise the possibility of differences in the antigenic relationship between different strains. We observed similar phenomena with the immunodiffusion test.

REFERENCES

1. BELÁK, S., PÁLFI, V., TURY, E.: Acta vet. Acad. Sci. hung. **25,** 91 (1975).
2. BELÁK, S., PÁLFI, V., PALYA, V.: Zbl. Vet. Med. B. **23,** 320 (1976).
3. PALYA, V., BELÁK, S., PÁLFI, V.: Zbl. Vet. Med. B. **24,** 529 (1977).
4. TURY, E., BELÁK, S., PÁLFI, V.: Acta vet. Acad. Sci. hung. **25,** 171 (1975).
5. MÜLLER, H.: Charakterisierung und Typisierung eines aus Schafen isolierten Adenovirus. Dissertation, Giessen 1974.
6. ANDREWES, C., PEREIRA, H. G.: Viruses of Vertebrates. Bailliere Tindall, London 1972.
7. DENNY, F. W., GINSBERG, H. S.: J. Immunol. **86,** 567 (1961).
8. FENNER, F., MCAUSLAN, B. R., MIMS, C. A., SAMBROOK, J., WHITE, D. O.: The Biology of Animal Viruses. Academic Press, New York–London 1974.
9. LIEBERMAN, M., FRIEDMAN, M.: J. Immunol. **82,** 1 (1959).
10. NÁSZ, I., BÉLÁDI, I., LENGYEL, A.: Az adenovírusok és kórokozó szerepük. Akadémiai Kiadó, Budapest 1967.
11. GINSBERG, H. S.: Virology **18,** 312 (1962).
12. MOHANTY, S. B.: Amer. J. Vet. Res. **32,** 1799 (1971).
13. MCFERRAN, J. B., NELSON, R., KNOX, E. R.: Arch. ges. Virusforsch. **35,** 232 (1971).
14. SHARP, J. M., MCFERRAN, J. B., RAE, A.: Res. Vet. Sci. **17,** 268 (1974).
15. BAUER, K., MÜLLER, H., GÜRTÜRK, S.: Zbl. Vet. Med. **22,** 656 (1975).
16. PEREIRA, H. G., ALLISON, A. C., BALFOUR, B.: Virology **7,** 300 (1959).
17. MOULTON, J. E., ZEE, Y. C.: Amer. J. vet. Res. **30,** 2051 (1969).
18. SARTMADSHIEV, K., GUENOV, I.: Zbl. Vet. Med. B. **17,** 249 (1969).

Address of the authors:

SÁNDOR BELÁK, SÁNDOR TUBOLY
Institute of Epizootiology, University of Veterinary Sciences
H-1581 Budapest, P.O.B. 22, Hungary

VILMOS PÁLFI
Central Veterinary Institute
Tábor u. 2, H-1143 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 265—268, 1978

IMMUNOFLUORESCENT STUDIES ON CANDIDA ALBICANS*

ERZSÉBET HESZLER and ÉVA SZABÓ

Department of Dermatology and Venerology, University Medical School, Szeged

(Received November 14, 1977)

The indirect immunofluorescent titres of *Candida albicans* 0656 CBS hyperimmune rabbit serum were investigated against *C. albicans* antigens prepared from strains originating from mucous membranes of healthy persons and patients with thrush. Using pathogenic strains as antigens, a definitely strong fluorescent reaction was obtained with hyperimmune rabbit serum in a dilution of 1 : 2500. In the case of apathogenic strains the fluorescent reaction was either very weak or negative in a dilution of 1 : 2000. Absorption studies also seemed to reveal a difference in reactivity between pathogenic and apathogenic strains. Hyperimmune rabbit serum absorbed with apathogenic *C. albicans* strains showed a positive fluorescent reaction only with pathogenic ones as antigens. In the opposite case a negative reaction was obtained.

Cutaneous and systemic infections by yeasts have become increasingly frequent in recent years. In most cases the pathogenic agent is *Cancida albicans*, a facultative pathogen, a normal inhabitant of the alimentary tract and the mucocutaneous region. It is often difficult for the mycologist, especially in the lack of adequate information, to decide the pathogenic role of the isolates [1, 2].

Some authors have tried to differentiate between pathogenic and apathogenic strains on the basis of differences in the antigens of *Candida* or the antibody response to them [1, 3—6]. These investigations were mainly concerned with the reactivity to the microorganism, in other words, the relation of the pathogenic agent with the host organism.

The question arises whether there are differences in the yeast itself on the basis of which pathogenic strains could be separated from apathogenic ones and whether there is some factor related to the ability of causing disease.

In order to clarify the question, the reactivity of an anticandida hyperimmune rabbit serum was studied using the immunofluorescent method on *C. albicans* cells as antigens originating from healthy and thrushy mucous membrane.

Materials and methods

Antigen. C. albicans was isolated from the mucous membrane of 10 healthy subjects and 10 patients with thrush. Culturing was done on Sabouraud agar, identification on rice agar [7]. Preparation of the antigen was carried out according to SEELIGER [8].

* Part of this work was presented at the Fifth International Specialized Symposium on Yeasts, Keszthely, 1977.

Immune serum. Production of hyperimmune anticandida rabbit serum was done using SEELIGER's method [8]. Rabbits were immunized intravenously with 10 mg/ml suspension of the above mentioned antigen prepared from *C. albicans* strain 0656 CBS (Delft). After five weeks immunization the titre of the serum with homologous antigen was 1 : 1280 in agglutination and 1 : 2500 in immunofluorescence.

Conjugate. Goat anti-rabbit serum labelled with fluorescein isothiocyanate (Code No.: 65-173-Miles Yeda) was used in 1 : 65 dilution.

Microscopy. A Leitz Orthoplan Fluorescence microscope with Xenon X BO 150 w bulb and Leitz vertical illuminator was used. Filter combination: KG 1 (2 mm), BG 38 (4 mm). Kp 500; secondary filters, TK 510/K 515. Objectives: Apo 40/63 ×. Ocular: Periplan GF 12.5 ×. Microphotographs were taken with an Orthomat camera using Anscochrome 500 daylight film.

Immunofluorescent technique. C. albicans cells layered on a slide were dried at room temperature, then incubated for 30 min in a wet chamber with different dilutions of anti-candida rabbit serum. After washing in Nairn buffer pH 7.2, they were incubated with conjugate for 30 min.

Immunoabsorption. Anticandida rabbit serum was absorbed with pathogenic and apathogenic *C. albicans* strains: 0.3 ml heat killed *C. albicans* suspension was added to 0.5 ml immune serum diluted 1 : 20 and incubated at 37°C for 2 hr., then at 4°C for 20 hr. After centrifugation the process was repeated.

Results

First the final titre of the anti-candida serum was defined. The highest dilution causing a bright green fluorescent ring on the cell walls (Fig. 1), which was easily differentiated from the yellowish-brown auto-fluorescence, was considered the titre of the serum. It depended on whether pathogenic or apathogenic *C. albicans* was used as antigen, and the difference could be measured quantitatively. Results are summarized in Table I, where it is seen that pathogenic strains reacted with anti-candida rabbit immune serum more intensely than apathogenic ones.

To obtain further information on the difference in reactivity between pathogenic and apathogenic isolates, the hyperimmune anti-candida rabbit serum was absorbed with four pathogenic and four apathogenic *C. albicans* strains. Back-titration was done with further 6 strains of both types each. Results are shown in Table II.

The serum absorbed with pathogenic strains in 1 : 20 dilution gave a negative fluorescence pattern with both pathogenic and apathogenic strains without further dilution. In the opposite case, serum absorbed with apatho-

Table I

Difference between pathogenic and apathogenic strains of C. albicans as measured with indirect immunofluorescence

Antigens (strains)	No. of strains	Degre of fluorescence			
		1 : 200 serum dilution		1 : 2500 serum dilution	
		−	+	+	++
Pathogenic	10	—	—	7	3
Apathogenic	10	4	6	—	—

genic strains in 1 : 10 dilution gave a distinct fluorescence with pathogenic strains, even on an additional 1 : 10 dilution. With the last type of absorbed serum, in a dilution of 1 : 100, a mosaic pattern [5] was observed, viz., besides negative cells about 1–2% positively stained cells were seen (Fig. 2).

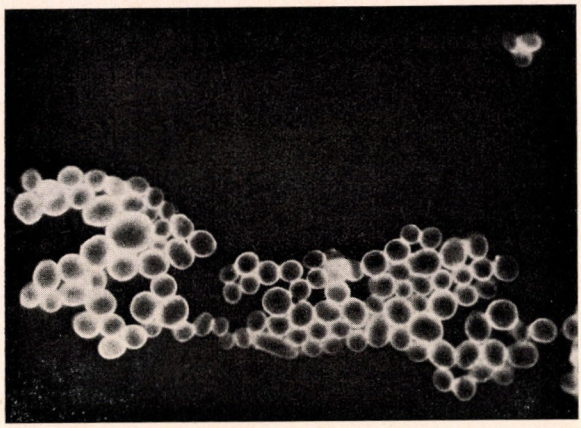


Fig. 1. Homogeneous, ring-like reaction on the cell walls of *C. albicans.* ×800

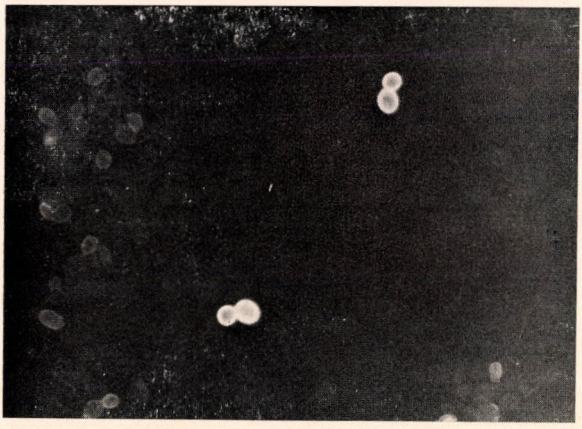


Fig. 2. Mosaic pattern. ×500

Table II

Indirect immunofluorescence of a hyperimmune anti-candida rabbit serum absorbed with apathogenic and pathogenic C. albicans strains

Antigens (strains)	Reaction of the serum absorbed with	
	apathogenic *C. albicans* strains	pathogenic *C. albicans* strains
Apathogenic	negative	negative
Pathogenic	positive (1 : 10 dilution)	negative
	mosaic pattern (1 : 100 dilution)	

Discussion

Our results seem to point to a difference in reactivity between *C. albicans* strains from healthy oral mucosa and from mucosa with thrush. With immune absorption the difference could be demonstrated qualitatively. As the serotypes A and B of *C. albicans* have not been considered, it is supposed that the difference demonstrated lies within the range of the antigenic structures represented by these two types.

The question arises whether the *C. albicans* strains different in reactivity are alternative colonizers of the diseased or healthy host or have a common origin. In the latter case the antigenic change may be correlated with the acquisition of pathogenicity and reflect the host's defence. Pathological conditions such as diabetes, malignant disease, etc., and iatrogenic effects such as antibiotic or cytostatic therapy may contribute to the process. Despite the unanswered questions, the above methods are considered useful in clinical practice.

REFERENCES

1. Bienas, L.: Mykosen **14,** 114 (1971).
2. Seeliger, H. P. R., Tomsikova, A., Török, I.: Mykosen **18,** 51 (1974).
3. Ansorg, R., Gunesch, D., Bandelow, B., Thomssen, R.: Mykosen **20,** 167 (1977).
4. Böhme, H.: Derm. Mschr. **159,** 359 (1973).
5. Gordon, M. S., Elliot, J. C., Hawkins, T. W.: Sabouraudia **5,** 323 (1967).
6. Vogel, R. A., Padula, J. F.: Proc. Soc. exp. Biol. (N. Y.) **98,** 135 (1958).
7. Ajello, L., Georg, L. K., Kaplan, W., Kaufman, L.: Laboratory manual for medical mycology. U. S. Government Printing Office, Washington 1963. p. E 21.
8. Seeliger, H. P. R.: In: Jadassohn, N.: Handbuch der Haut und Geschlechtskrankheiten. Vol. IV/4. Springer Verlag, Berlin 1963, p. 631.

Address of the authors:

Erzsébet Heszler, Éva Szabó
Department of Dermatology and Venerology, University Medical School
H-6701 Szeged, P.O.B. 480, Hungary

Acta microbiol. Acad. Sci. hung, 25, 269—275, 1978

TRANSFORMATION OF HAMSTER EMBRYONIC FIBROBLAST CELLS BY UV-IRRADIATED HUMAN CYTOMEGALOVIRUS*

I. Boldogh, Éva Gönczöl and L. Váczi

Institute of Microbiology, University Medical School, Debrecen

(Received November 17, 1977)

Infection of hamster embryonic fibroblast (HEF) cells with UV-irradiated human CMV resulted in appearance of transformed clones. The established cell line (87-TRH-5) when inoculated subcutaneously induced tumour in newborn hamsters. The 87-TRH-5 cell line and the cell lines (87-TRH-5-Tsc_1, 87-TRH-5-Tsc_2, 87-TRH-5-Tsc_3) developed from the induced tumour, proved to bear CMV-specific cytoplasmic and surface antigens. Demonstration of infectious CMV or CMV-specific nuclear antigen in 87-THR-5-cells has failed.

The cell-transforming capacity of herpesviruses of man (EBV, HSV-1, HSV-2) and lower animals (MDV, HSV, HVA, LHV etc.) has been supported by a number of authors [1–3]. The same has not proved unequivocally for human cytomegalovirus (HCMV), though Albrecht and Rapp [4] and Géder *et al.* [5] induced transformation of hamster embryonic fibroblasts (HEF) and human embryonic lung cells, respectively, with HCMV.

We have succeeded in infecting semipermissive mouse cells with HCMV [6]. In the infected semipermissive cells the virus genome persisted in a latent form for long periods of time while the virus functions were under host control [7]. Both homologous (human) and heterologous (mouse) DNA synthesis was stimulated by partially inactivated HCMV [8]. In this report, we present further data on transformation by HCMV.

Materials and methods

HEF cells were prepared from 14-day-old Syrian hamster embryos as described by Duff and Rapp [9]. The cells were propagated in plastic (Nuclon) vessels in minimum essential medium (MEM, GIBCO) supplemented with 10% fetal calf serum (FCS), 0.075% $NaHCO_3$, 100 μg/ml penicillin and 100 μg/ml streptomycin.

The C-87 strain of HCMV was propagated titrated and stored as described [10, 11]. Specific HCMV antigens were detected by indirect immunofluorescence [12, 13]. Anti-HCMV human convalescent sera, were used antisera. Fluoresceinisothiocyanate-labelled anti-human IgM conjugate (Hyland) was applied instead of IgG conjugate because of the IgG–Fc binding activity of the cells (unpublished data).

The virus suspension was UV-irradiated at 80 erg/sec/mm² from a distance of 20 cm for 16 min. Methods described earlier [7, 14] were used in 5-iodo-2'-deoxyuridine (IUdR, Sigma) treatment and fusion with human embryonic fibroblasts (HuEF) of 87-TRH-5 cells.

* This work was supported by a grant from the Hungarian Ministry of Health, Contract No. 2-11-0801/VG.

Results

HEF cells (4×10^6) were infected at a multiplicity of about 1 p.f.u./cell with partially UV-inactivated HCMV strain C-87. The virus had been titrated on HuEF cultures. After an adsorption period of 2 hr at 37°C, the unadsorbed virus was removed by washing with PBS. The cells were maintained in MEM enriched with 10% FCS in plastic Petri dishes 60 mm in diameter in an atmosphere containing 5% CO_2. The medium was changed weekly.

Nineteen to 24 days after inoculation, rapidly multiplying foci of cells of altered morphology (Fig. 2/b) appeared in the infected monolayers. In non-infected control HEF monolayers, transformed foci could not be observed.

Eleven of the foci were isolated. Figure 1 shows that of these only one clone (87-TRH-5) remained capable of multiplying. The clone caused tumour in

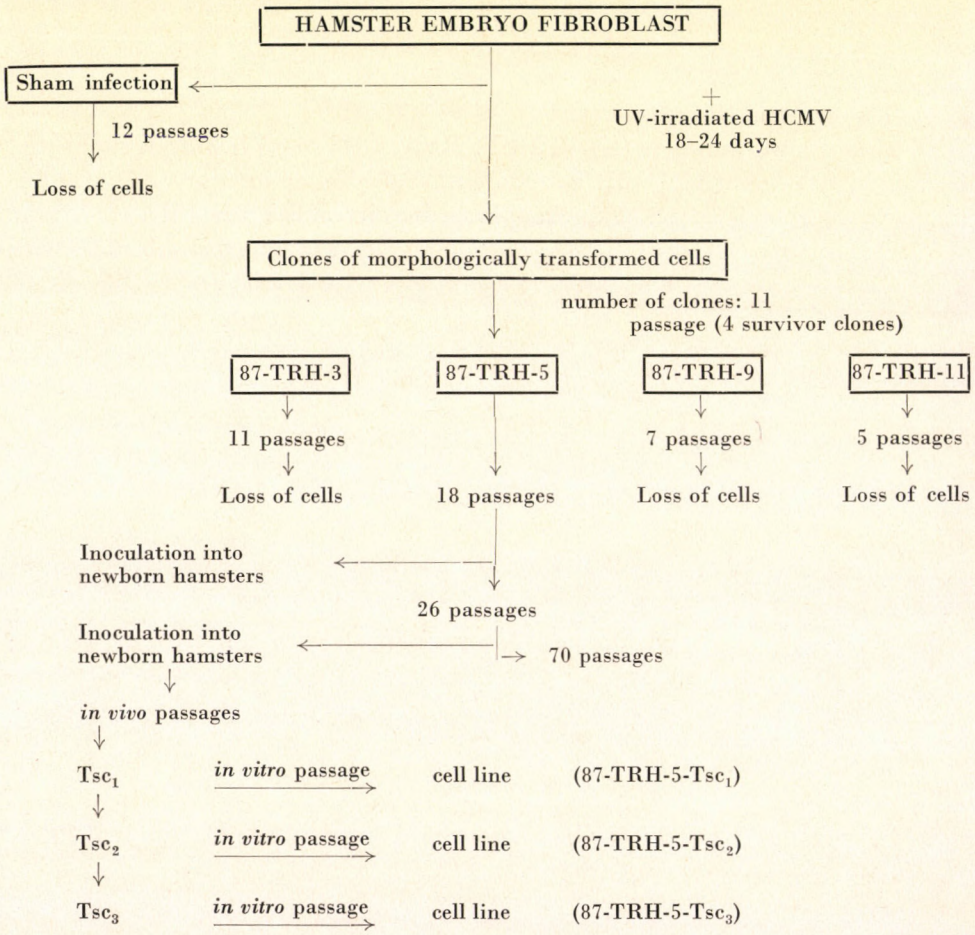


Fig. 1. History of oncogenic transformation of hamster embryo cells by UV-irradiated HCMV

serial *in vivo* passages in hamsters. A cell line was isolated from each of three consecutive passages of 87-TRH-5-tumours (lines 87-TRH-5-Tsc_1, 87-TRH-5-Tsc_2 and 87-TRH-5-Tsc_3). In the following, the properties of the four cell lines are described.

The cells of the 87-TRH-5 line are fibroblast-like, some of them multinuclear. The nuclei are larger (Fig. 2/c) than those of normal hamster cells in general (Fig. 2/a).

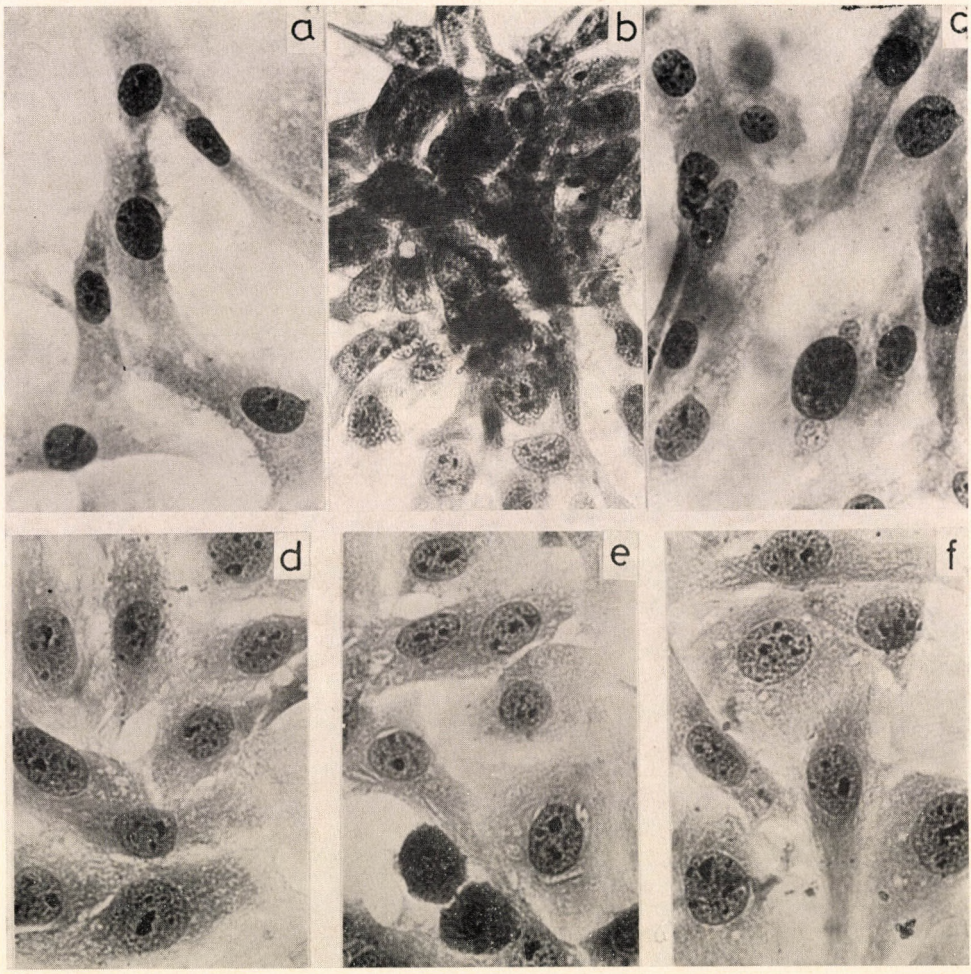


Fig. 2. Normal and HCMV-transformed hamster cell lines: a) normal hamster embryo fibroblast (HEF) culture; b) HCMV-transformed hamster embryo fibroblast, a clone of morphologically altered cells; c) culture of 87-TRH-5 cells after the 18th passage; d) culture of 87-TRH-5-Tsc_1 cells (15th passage) after isolation from tumour induced by injection of 87-TRH-5 cells into newborn hamster; e) culture of 87-TRH-5-Tsc_2 cells (12th passage) after isolation from tumour induced by injection of 87-TRH-5-Tsc_1 cells into newborn hamster; f) culture of 87-TRH-5-Tsc_2 cells (12th passage) after isolation from tumour induced by injection of 87-TRH-5-Tsc_2 cells into newborn hamster

The derivative cell lines (Figs 2/d, 2/e, 2/f) do not differ from one another morphologically, but do differ from the parental line 87-TRH-5. They show fibroepitheloid morphology. Occasionally multinuclear giant cells and giant cells of bizarre morphology occurred.

The oncogenecity of the cell lines is summarized in Table I. Cells of 87-TRH-5 were detached and inoculated into newborn hamsters (10^5, 5×10^5, 10^6 and 5×10^4 cells/animal) and weanling hamsters (10^6 and 10^7 cells/animal).

Tumour developed in one of the newborn hamsters inoculated with 10^5 cells and in 23 of the 24 inoculated with 10^6 cells. Of the 14 weanlings inoculated with 10^7 cells 2 developed tumour.

Tumour induction by cell lines of tumoural origin were examined in weanling hamsters. Tumours were removed under sterile conditions, trypsinized and transferred through 5 serial passages *in vitro*. Hamsters were inoculated with 10^4, 10^5 or 10^6 cell/animal. Table I shows that the oncogenicity of the cell

Table I

Tumour induction in Syrian hamster by HCMV-transformed cell lines

Number of animals	Number of inoculated cells/hamster	Tumour incidence: Weeks after injection									Total
		2	4	6	8	10	12	14	20	24	
Newborn hamster	HEF										
10	10^7	—	—	—	—	—	—	—	—	—	—
	87-TRH-5										
12	10^5	—	—	—	—	—	1	—	—	—	1
10	5×10^5	—	—	—	2	1	1	—	—	—	4
10	10^6	—	—	2	4	2	1	—	—	—	9
14	5×10^6	—	2	2	6	4	—	—	—	—	14
Weanling hamster (4 weeks old)	87-TRH-5										
12	10^6	—	—	—	—	—	—	—	—	—	—
14	10^7	—	—	—	—	—	—	—	1	1	2
	87-TRH-5-Tsc_1										
10	10^4	—	—	—	4	2	2	—	—	—	8
8	10^5	1	6	—	—	—	—	—	—	—	7
11	10^6	3	7	—	—	—	—	—	—	—	10
	87-TRH-5-Rsc_2										
10	10^4	—	—	4	3	2	—	—	—	—	9
7	10^5	2	5	—	—	—	—	—	—	—	7
8	10^6	4	4	—	—	—	—	—	—	—	8
	87-TRH-4-Tsc_3										
10	10^4	—	2	5	3	—	—	—	—	—	10
10	10^5	3	6	1	—	—	—	—	—	—	10
8	10^6	2	6	—	—	—	—	—	—	—	8

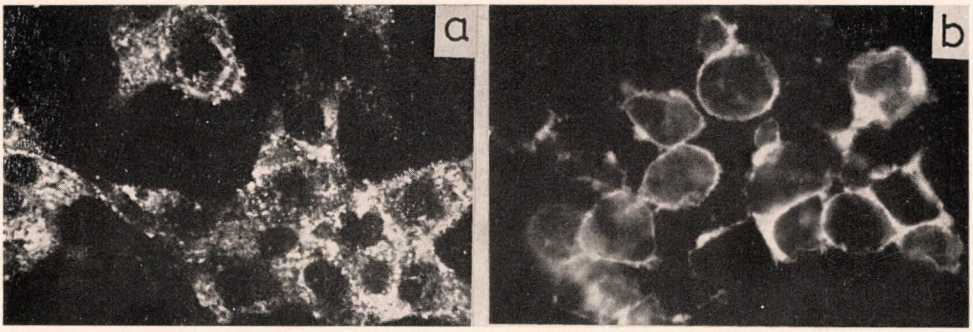


Fig. 3. Immunofluorescence of HCMV antigens localized in 87-TRH-5 transformed hamster cells. ×252
a) Antigen(s) localized in cytoplasm; b) antigen(s) on the surface of 87-TRH-5 cells

lines had increased; 10^4 cells/hamster caused tumour in 90 to 100% of the inoculated animals.

Hamsters inoculated in neonatal age with 10^7 normal hamster cells/animal developed no tumour during the 8-month observation period.

The tumour induced by any of the four cell lines showed the histological picture of fibrosarcoma.

The 87-TRH-5 cell line and the lines obtained from tumour were examined by indirect immunofluorescence for HCMV-specific antigens (Table II).

Table II

Immunofluorescence studies

Cells	Fluorescence with					
	human sera positive for IgM antibodies to HCMV		human sera positive for IgM antibodies to HSV-2		human sera negative for anti-HCMV and anti-HSV-2	
	I	S	I	S	I	S
87-TRH-5	+	+	—	—	—	—
87-TRH-5-Tsc$_1$	+	+	—	—	—	—
87-TRH-5-Tsc$_2$	+	+	—	—	—	—
87-TRH-5-Tsc$_3$	+	+	—	—	—	—
HCMV (C-87)-infected HuEF[a]	+	+	—	—	—	—
HCMV (C-87)-infected HEF[b]	+	+	—	—	—	—
Hamster embryo fibroblast	—	—	—	—	—	—
HSV-2-infected HEF	—	—	+	+	—	—
II/d SV$_{40}$ tumour cells	—	—	—	—	—	—

I = intracellular antigen(s)
S = antigen(s) on the cell surface
[a] human embryonic fibroblasts 48 hr after infection
[b] hamster embryonic fibroblasts 24 hr after infection

HCMV-specificity of both the cytoplasmic and the surface antigen was detected in the transformed cells (Figs 3/a and 3/b). No fluorescence occurred with either anti-HSV-2 or normal sera.

Attempts were made to detect infectious virus or HCMV-specific nuclear antigen(s) suggestive of the presence of the complete virus genome or its parts responsible for coding for the nuclear antigen(s).

(1) IUdR-treated (48 hr, 100 μg/ml) and untreated 87-TRH-5 cells were sonicated (1.5 A, 1 mm) and HuEF cells were inoculated with the disintegrated cells;

(2) IUdR-treated and untreated 87-TRH-5 cells were co-cultivated with HuEF cells;

(3) IUdR-treated and untreated 87-TRH-5 cells were induced to fuse with HuEF cells and the resulting hybrids were treated with IUdR.

We failed to isolate infectious virus from any of the experiments. Fluorescence was seen to appear in 87-TRH-5/HuEF nuclei, but the antigen responsible for the fluorescence could not be distinguished immunologically from the cytoplasmic antigen, suggesting that the antigen detected in the nuclei was due to an inflow of cytoplasmic antigen.

Discussion

Infection of HEF cells with partially UV-inactivated HCMV resulted in the appearance of morphologically transformed cells. Multilayered foci were isolated and cultivated further. One clone survived and developed into a continuous cell line. Inoculated into newbornor weanling hamsters, the cell line induced local tumour growth. Uninfected HEF cells kept under the same conditions did not transform.

Both the parental cell line and its derivatives showed HCMV-specific cytoplasmic and membrane fluorescence. Attempts at isolating infectious HCMV or inducing HCMV-specific nuclear antigens have, however, failed. We assume that the viral genome in the tumour cells is incomplete.

It may be concluded that infection of HEF cells with HCMV resulted in the development of malignant cells. Whether the malignization was induced by the viral genome directly or through induction of endogenous oncornavirus needs further investigations.

Acknowledgements. The authors are indebted to Mrs. MARIA ROZGONYI SZÉCSI and Miss MAGDOLNA LOVÁSZ for excellent technical assistance.

REFERENCES

1. Rapp, F., Advanc. Cancer Res. **19,** 265 (1974).
2. Braun, R., Flügel, R. M., Munk, K.: Nature (Lond.) **265,** 744 (1977).
3. zur Hausen, H.: Biochim. biophys. Acta (Amst.) **417,** 25 (1973).
4. Albrecht, T., Rapp, F.: Virology **55,** 53 (1973).
5. Géder, L., Kreider, J., Rapp, F.: J. nat. Cancer Inst. **58,** 1003 (1977).
6. Gönczöl, É., Boldogh, I., Váczi, L.: Acta microbiol. Acad. Sci. hung. **22,** 275 (1975).
7. Boldogh, I., Gönczöl, É., Gärtner, L., Váczi, G.: Arch. Virol. **53,** 101 (1977).
8. Boldogh, I., Gönczöl, É., Gärtner, L., Váczi, G.: Arch. Virol. In press.
9. Duff, R., Rapp, F.: J. Virol. **5,** 568 (1970).
10. Plummer, G., Benyesh-Melnick, M.: Proc. Soc. exp. Biol. (N. Y.) **117,** 145 (1964).
11. Andersen, H. K.: Arch. ges. Virusforsch. **36,** 143 (1971).
12. Váczi, L., Gönczöl, É.: Acta virol. (Prague) **17,** 189 (1973).
13. Andersen, H. K.: Arch. ges. Virusforsch. **35,** 133 (1972).
14. Váczi, L., Boldogh, I., Gönczöl, É.: Arch. ges. Virusforsch. **44,** 62 (1974).

Address of the authors:

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

Acta microbiol. Acad. Sci. hung. 25, 277—283, 1978

COURSE OF LYMPHOCYTIC CHORIOMENINGITIS (LCM) VIRUS INFECTION IN SUCKLING MICE TREATED WITH BORDETELLA PERTUSSIS VACCINE

ZSUZSANNA BÁNOS, PIROSKA ANDERLIK, ILONA SZERI and B. RADNAI

Institute of Microbiology, Semmelweis University Medical School, Budapest and István Hospital, Budapest

(Received December 9, 1977)

Death occurred earlier and the mortality rate was higher in one and two-week-old mice pretreated with *Bordetella pertussis* vaccine and infected intracerebrally with LCM virus, than in not pretreated animals of the same litter. Pertussis vaccine treatment contributed to the course of LCM virus infection ending in lethal meningitis in suckling mice, by accelerating the development of their cellular immune response.

Besides several other effects in mice, *Bordetella pertussis* vaccine causes transitory splenomegaly, leucocytosis [1, 2] and alteration of the immunological reactivity. The alteration may be an increases of the humoral immune response or a decrease of the cellular immune response to heterologous antigens [3, 4].

The cellular immune response is known to be the basis of lymphocytic choriomeningitis, a neurological symptom of intracerebral LCM virus infection, and T lymphocytes play a basic role in its development [5–7]. The course of virus infection depends on the immune response of the animals and so on their age. The infection ends in fatal lymphocytic choriomeningitis in adult mice with intact immune system while newborn mice with undeveloped immune system do not develop meningitis, they survive the infection and become lifelong virus carriers. In suckling mice the mortality rate and time of death depend on the age of the animal at the time of inoculation [8].

In the present work the effect of pertussis vaccine pretreatment has been studied in the course of LCM virus infection in suckling mice.

Materials and methods

Experimental animals. CFLP mice of different age and both sexes were used.

Pertussis vaccine. The vaccine contained 30×10^9/ml killed bacteria in physiological saline (Institute for Serobacteriological Production and Research Human, Budapest).

LCM virus infection. The W. E. strain used in our experiments was maintained in our Institute in serial mouse brain passages. PBS (pH 7.0) was used for brain suspensions and virus dilutions. Virus was titrated with intracerebral inoculation in young adult mice, using 100 LD_{50} in 0.03 ml of the pretitrated virus. Neurological symptoms characteristic of lymphocytic chorio-

meningitis (tremor, convulsion) were observed twice daily and the occurrence of death was registered.

Reisolation of virus. At the end of the experiments the brain suspensions prepared from surviving animals were inoculated intracerebrally in a 1 : 10 dilution. Reisolation of LCM virus was confirmed by the characteristic neurological symptoms and death in the inoculated mouse groups.

Histological examination. Sections prepared from the brain of the animals dying during the experiments were stained with haematoxylin–eosin.

Examinations of the lymphoid system. (a) Absolute lymphocyte count was determined from blood taken from the caudal vein under standardized conditions. (b) Mean relative spleen weight of animals sacrificed 4 and 10 days after pertussis vaccine treatment and the spleen index characteristic of vaccine treated groups was determined.

$$\text{Mean relative spleen weight} = \frac{\text{mean spleen weight mg}}{\text{mean body weight g}}$$

$$\text{Spleen index} = \frac{\text{mean relative spleen weight of pertussis vaccine treated group}}{\text{mean relative spleen weight of control group}}$$

Statistical evaluation of data. Evaluation of the results was carried out by calculating the mean values of the data groups ($\bar{x}$) and the deviations of the means ($\pm S\bar{x}$) and by comparing them with statistical methods. Student's two sample t test was applied for statistical evaluation. The accepted significance level was $p = 0.05$.

Results

The effect of pertussis vaccine was studied in the course of LCM virus infection in 1-, 2- and 6-week-old mouse groups. Twenty-six litters were included in the experiments. Each litter consisted of 8 animals and half of them was vaccinated intraperitoneally. The animals received on two occasions 0.3 ml vaccine (18×10^9 bacteria) on successive days. The remaining animals were given PBS solution. On the second day of treatment, 5 litters of 1-week-old animals and 8 litters of 2-week-old animals were infected with LCM virus. Simultaneously, 13 litters were inoculated with the brain suspension of non-infected animals. Treatment of 6-week-old mice and of the controls was identical to that of suckling mice. Control groups consisted, however, not of the siblings. One-week-old vaccine treated mice belonged to the P-LCM-I group, 2-week-old animals to the P-LCM-II and the 6-week-old ones to the P-LCM-III group. PBS-treated one-week-old mice formed the C-LCM-I, 2-week-old mice the C-LCM-II and the 6-week-old ones the C-LCM-III group. Animals inoculated with normal brain suspension and pretreated with vaccine belonged to the P-I, P-II and P-III, those pretreated with PBS to the C-I, C-II and C-III groups. The experiments ended on the 21st day after virus infection. Table I demonstrates the mouse groups and treatments.

The effect of vaccine treatment on the lymphoid organs of mouse groups not infected with virus was studied 4 and 10 days after treatment. Absolute lymphocyte counts were determined. Subsequently four animals were sacrificed 4 days and eight animals 10 days after the treatment in the P-I and C-I

Table I

Mouse groups and their treatments

Group	Number	Age	Intraperitoneal treatment	Cerebral inoculation
P-LCM I	20	1 week	pert.	LCM virus
C-LCM I	20		PBS	LCM virus
P-I	20		pert.	×
C-I	20		PBS	×
P-LCM II	32	2 weeks	pert.	LCM virus
C-LCM II	32		PBS	LCM virus
P-II	32		pert.	×
C-II	32		PBS	×
P-LCM III	16	6 weeks	pert.	LCM virus
C-LCM III	16		PBS	LCM virus
P-III	32		pert.	×
C-III	32		PBS	×

× = normal brain suspension
pert. = *B. pertussis* vaccine

groups and twelve animals each were sacrificed 4 and 10 days following the treatment in the P-II, C-II, P-III and C-III groups and the relative spleen weights and spleen indexes were determined. According to the results summarized in Table II, spleen hypertrophy and lymphocytosis developed in each age group of mice treated with pertussis vaccine.

Table II

Data of the lymphoid organs

Group	Lymphocyte count/μl		Relative spleen weight		Spleen index	
	4th day	10th day	4th day	10th day	4th day	10th day
P-I	13500 ± 4085	6050 ± 2407	10.7 ± 1.04	14.23 ± 4.1	1.82	1.60
C-I	2450 ± 819	2633 ± 787	5.87 ± 0.94	8.87 ± 1.36		
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$		
P-II	19800 ± 6000	2822 ± 707	9.46 ± 0.85	9.96 ± 1.8	1.68	1.60
C-II	2237 ± 972	2212 ± 895	5.91 ± 0.85	6.2 ± 1.83		
	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$		
P-III	9990 ± 6814	4083 ± 1200	7.17 ± 1.52	8.15 ± 1.66	1.37	1.76
C-III	5108 ± 3874	4587 ± 1333	5.21 ± 1.37	4.62 ± 0.61		
	$p < 0.02$	$p > 0.1$	$p < 0.001$	$p < 0.001$		

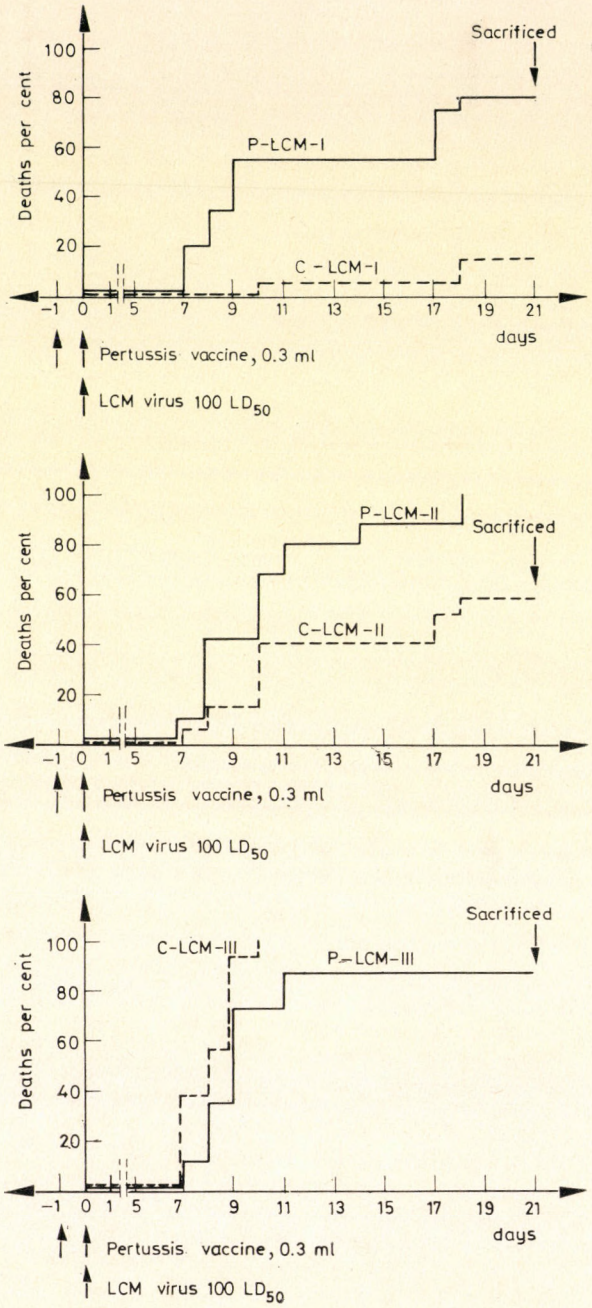


Fig. 1. Time course and rate of mortality

Rate and time course of deaths are presented in Fig. 1.

Virus infected 6-week-old mice not treated with pertussis vaccine (C-LCM-III group) all succumbed with characteristic neurological symptoms, 7–10 days after infection. Two-week-old mice (C-LCM-II group) showed 58% and the one-week-old animals (C-LCM-I group) 15% mortality rate. Only a few suckling mice developed characteristic neurological symptoms. Death occurred at and after 10 days postinfection among one-week-old animals (C-LCM-I group) and 7 days postinfection in the group of two-week-old (C-LCM-II group) and six-week-old (C-LCM-III group) mice. Mortality rate was higher (80%) and death occurred earlier in the virus infected and vaccine treated one-week-old mouse group (P-LCM-I group) than in their untreated siblings (C-LCM-I group). Two-week-old animals subjected to pretreatment and infection (P-LCM-II group) displayed 100% mortality 7–18 days after virus infection in contrast to the 58% mortality observed in the sibling control group (C-LCM-II group). Neurological symptoms characteristic of virus infection were observed in animals dying early in both the P-LCM-I and P-LCM-II groups and in the brain of mice succumbing in these groups 7–8 days after virus infection lymphocytic infiltration of the leptomeninx was revealed by histological examination.

In contrast, the mortality rate/day was lower in the vaccine treated and virus infected 6-week-old animals (P-LCM-III group) than in the C-LCM-III group and 12% of the animals survived the virus infection.

From the brain of each animal surviving the virus infection by 21 days, virus could be reisolated, thus these mice were carriers. Neither disease nor death occurred in the groups not subjected to LCM virus infection.

Discussion

Development of the immune system at birth is different in the individual animal species. T lymphocytes appear gradually in the peripheral lymphoid organs of mice [9, 10] and the cellular immune response develops continuously in the postnatal weeks [11–13]. Our results also show that mice infected at different ages display different mortality rates and the proportion of survivors decreases with age.

In the present experiments, lymphocytosis and splenomegaly developed in consequence of pertussis vaccine treatment in both one (P-I group) and two-week-old (P-II) mice. Simultaneously, the course of LCM virus infection changed too in the vaccine treated animals. Death occurred earlier and its rate was higher in the P-LCM-I group than in their siblings not subjected to vaccine treatment (C-LCM-I group). Mortality was 100% in the P-LCM-II group. The appearance of characteristic neurological symptoms as well

as lymphocytic infiltration on the leptomeninx could be detected in animals dying early in both the P-LCM-I and the P-LCM-II groups.

Our results indicate that pertussis vaccine treatment contributed to the development of lethal meningitis following LCM virus infection. Comparing the course of LCM virus infection in vaccine pretreated one-week-old (P-LCM-I group) and not pretreated two-week-old (C-LCM-II group) mice, furthermore in the vaccine treated two-week-old (P-LCM-II group) and not pretreated 6-week-old mouse groups (C-LCM-III group), mice of the P-LCM-I group were found to react to LCM virus infection similarly as did the animals of the C-LCM-II group and the P-LCM-II and C-LCM-III mice also showed a similar reaction. This referred to an enhancing effect of vaccine treatment on the development of the cellular immune response in suckling mice. The way of the enhancing effect is not clear [3]. Cell proliferation was found in the spleen of adult mice with intact lymphoid system under the effect of pertussis vaccine [14, 15]. The observations that the proportion of cells reacting to phytohaemagglutinin stimulation rises in peripheral blood upon pertussis vaccine treatment [16] furthermore that no lymphocytosis occurs in thymectomized and thymus deficient (nude) mice [17–19] refer to the role of T cells in the lymphocytosis elicited by the vaccine.

Considering the significance of T lymphocytes in the development of meningitis after intracerebral LCM virus infection, one may assume that vaccine treatment enhances development of the cellular immune response of suckling mice by causing T lymphocyte proliferation. In our experiments the daily death rate was lower in 6-week-old vaccine treated and virus infected (P-LCM-III group) animals than in the C-LCM-III group and part of the animals survived the infection. Accordingly, the course of intracerebral virus infection showed a change similar to the immune depressive conditions induced in different ways [20]. This result agrees with earlier observations concerning the effect of pertussis vaccine treatment of adult mice infected with LCM virus. These findings revealed that intravenously given pertussis vaccine reduced the cellular immune response to heterologous antigens being present simultaneously in the organism [21]. The present results indicate that the pertussis vaccine reduced the cellular immune response to LCM virus infection in adult mice with developed immune system, whereas the effect increased the cellular immune response in suckling mice.

REFERENCES

1. Parfentjev, I. A., Manuelidis, E. E.: Fed. Proc. **15,** 607 (1965).
2. Morse, S. J.: J. Exp. Med. **121,** 49 (1965).
3. Finger, H.: In Neter, E., Milgrom, F. (eds): The Immune System and Infectious Diseases. Karger, Basel 1975, p. 132.
4. Schwab, J. H.: Bact. Rev. **39,** 121 (1975).
5. Hotchin, J.: Cold Spring Harbor Symp. Quant. Biol. **27,** 479 (1962).

6. Cole, G. A., Nathanson, N., Prendergast, R. A.: Nature (Lond.) **238,** 335 (1972).
7. Doherty, P. C., Zinkernagel, R. M.: Transplant. Rev. **19,** 89 (1974).
8. Hotchin, J.: In Melnick, J. L. (ed.): Monographs in Virology **3,** Karger, München 1971, p. 2.
9. Adler, W. H., Takiguchi, T., Marsh, B., Smith, R. T.: J. exp. Med. **131,** 1049 (1970).
10. Miller, J. F. A. P., Osoba, D.: Physiol. Rev. **47,** 437 (1967).
11. Bortin, M. M., Rimm, A. A., Saltzstein, E. C.: J. Immunol. **103,** 683 (1969).
12. Goldstein, A. L., Guha, A., Howe, M. L., White, A.: J. Immunol. **106,** 773 (1971).
13. Marker, O., Andersen, G., Thörner Volkert, M.: Acta path. microbiol. scand. Sect. C. **84,** 23 (1976).
14. Beneke, G., Finger, H., Emmerling, P.: Z. med. Mikrobiol. Immunol. **154,** 178 (1968).
15. Stöcker, E., Finger, H.: Wirchows Arch. Zellpath. **10,** 179 (1972).
16. Phanuphak, P., Moorhead, J. W., Claman, H. N.: Int. Arch. Allergy. Appl. Immunol. **43,** 305 (1972).
17. Kalpaktsoglu, P. K., Yunis, E. J., Good, R. A.: Clin. exp. Immunol. **5,** 91 (1969).
18. Wortis, H. H.: Clin. exp. Immunol. **8,** 305 (1971).
19. Finger, H., Mohr, W., Hof, H., Elekes, E., Plager, L.: Z. med. Mikrobiol. Immunol. **159,** 13 (1973).
20. Lehmann-Grube, F.: Lymphocytic choriomeningitis virus. In Gard, S., Hallauer, C., Meyer, K. F. (eds): Virology Monographs **10,** Springer Verlag, Wien 1971, p. 54.
21. Anderlik, P., Szeri, I., Bános, Zs., Földes, I., Radnai, B.: Experientia **28,** 985 (1972).

Address of the authors:

Zsuzsanna Bános, Piroska Anderlik, Ilona Szeri
Institute of Microbiology, Semmelweis University Medical School
Hőgyes Endre u. 7—9, H-1092 Budapest, Hungary

Béla Radnai
Department of Pathological Anatomy and Histopathology, István Hospital
Nagyvárad tér 1, H-1096 Budapest, Hungary

Acta microbiol. Acad. Sci. hung. 25, 285—289, 1978

INHIBITORY AND MUTAGENIC EFFECTS OF SODIUM NITROPRUSSIDE AND ITS ADENINE COMPLEX ON ESCHERICHIA COLI

I. SZILÁGYI and S. VITÁLIS

Institute of Biology, University Medical School, Debrecen

(Received December 19, 1977)

Sodium nitroprusside and its adenine complex were found to decrease the growth rate of exponentially growing *Escherichia coli* cultures, and the adenine complex to exert in addition a bactericidal effect. In mutation experiments the latter compound failed to induce base-pair substitutions in the *E. coli* strains tested but the results do not allow to exclude other mutagenic mechanisms.

In recent years several papers have discussed the toxicity of sodium nitroprusside but no data could be found concerning its mutagenicity.

On the other hand, nitroprusside was shown to react with nucleobases under biological conditions, and the product of its reaction with adenine was prepared in solid state [1]. The product will be designated as adenine complex. It was suggested that this reaction *in vivo* could lead to mutations [1]. Moreover, in plants it was found to be mutagenic (M. BECK and I. TAMÁSSY, personal communication).

The present report describes the effects of nitroprusside and its adenine complex on *Escherichia coli* and the results of mutation tests performed with the latter on two strains of the microorganism.

Materials and methods

Strains. Two *E. coli* strains were used, wild type K-12 and a B strain derivative, WP 2 uvrA Trp$^-$.

Media and growth. WP 2 uvrA cells were handled according to GREEN and MURIEL [2]. K-12 cells were grown in the following medium: KH_2PO_4, 13.6 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; glycerol, 2.0 g per litre; pH 7.4. An overnight culture of the cells was diluted in fresh medium at an optical density (OD) of about 0.150, measured at 570 nm. Twenty ml aliquots of this suspension were pipetted into 100 ml Erlenmeyer flasks, then the cultures were shaken at 250 rpm in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc.) at 37 °C for one hr. Subsequently the stock solution of the compounds to be tested was added to the cultures at the final concentration indicated. The cells were then grown for 4 hr and 2.0 ml samples were taken every hour. The samples were cooled in an ice bath and centrifuged in a refrigerated centrifuge at 3000 *g* for 20 min, and resuspended in 2.0 ml of fresh medium of the same composition as above but lacking glycerol. Optical density of the suspensions was determined at 570 nm.

Determination of survival. E. coli K-12 cells were grown as described above. After the dilution step, the stock solution of adenine complex was added to give a final concentration of 5×10^{-3} M. At zero, 3 and 5 hr, 2.0 ml samples were taken, centrifuged at 3000 *g* for 20 min

and resuspended in 2.0 ml of physiological saline. Viable counts were determined after dilution and plating on Oxoid Nutrient Agar plates [3].

Testing of mutagenicity. E. coli K-12. Exponentially growing cultures of K-12 cells were obtained as described above. After dilution to OD = 0.150 the cells were grown for 2.5 hr. Then the cultures were halved and 10^{-3} M uridine or 10^{-3} M uridine plus 5×10^{-3} M adenine complex were added to the new cultures. This time was considered the zero point of the experiment. Then the cultures were shaken at 37 °C for one hr, cooled and centrifuged. The culture treated with adenine complex was resuspended in the original volume of fresh medium while the control was diluted threefold in the same medium. Then the cultures were grown for 3 hr. The number of viable cells was determined at zero, 1 and 4 hr; streptomycin resistant mutants were counted in samples taken at zero and 4 hr. Streptomycin resistance was determined on Oxoid Nutrient Agar plates containing streptomycin sulphate (EGYT, Budapest) at a concentration of 15 μg per ml. Samples were centrifuged and resuspended in one tenth of their original volume; 0.2 ml of this suspension was spread on the selective plates and colonies were counted after incubation at 37°C for 48 hr.

WP 2 uvrA. The protocol described in the paper of GREEN and MURIEL [2] was followed. For spot tests, crystals of the compounds were placed directly onto the agar surface. In "treat and plate" tests adenine complex was used at a concentration of 5×10^{-3} M. Cells were incubated with the agent for 30 and 120 min.

Results

Sodium nitroprusside, adenine, adenine complex and adenine complex plus uridine were added to exponentially growing *E. coli* K-12 cultures. The decrease of growth rate constant (g.r.c.), λ in equation

$$N_t = N_0 \cdot e^{\lambda t}$$

was used to describe the inhibitory effect of the agents tested. Results are summarized in Table I.

The tested compounds were all found to decrease g.r.c. The inhibitory effect of adenine could be prevented by the addition of 10^{-3} uridine. The effect of the adenine complex was only moderated by uridine, and it was more effective than nitroprusside at the same concentration even in the presence of uridine. Therefore, in the following experiments adenine complex was used at a concentration of 5×10^{-3} M.

The effect of 5×10^{-3} M adenine complex on the survival of an exponentially growing K-12 culture is shown in Fig. 1. Adenine complex was found to kill approximately 60% of the cells by 3 hr, and the effect increased only slightly during subsequent incubation for 2 hr. In other experiments killing of WP 2 uvrA cells treated in buffer was found to reach 50% after 30 min.

Mutation tests were performed with two strains. In the first series of experiments it was tested whether the adenine complex increases the frequency of streptomycin resistant cells in a culture of wild type K-12 cells. There was no significant increase in the frequency of resistant cells after exposure to adenine complex (Table II). In the second series of experiments the test system of GREEN and MURIEL [2] was used. This system makes use of the reversion

Table I

Growth rate constant of E. coli K-12 cultures after exposure to the compounds indicated

Compound	Concentration, M	Growth rate constant	Per cent of control growth rate constant
Control	—	0.464	100.0
Sodium nitroprusside	2×10^{-2}	0.257	55.4
	10^{-2}	0.283	61.0
	5×10^{-3}	0.309	66.6
Control	—	0.487	100.0
Adenine	10^{-2}	0.083	17.0
	2×10^{-3}	0.212	43.5
	10^{-3}	0.323	66.3
Control (10^{-3} M uridine)	—	0.578	100.0
Adenine plus	10^{-2}	0.528	91.4
10^{-3} M uridine	5×10^{-3}	0.609	105.5
	10^{-3}	0.624	107.9
Control	—	0.442	100.0
Adenine complex	10^{-2}	0.075	17.0
	5×10^{-3}	0.131	29.6
	10^{-3}	0.198	44.8
	10^{-4}	0.366	82.8
Control (10^{-3} M uridine)	—	0.496	100.0
Adenine complex plus	10^{-2}	0.199	40.1
10^{-3} M uridine	5×10^{-3}	0.213	42.9
	10^{-3}	0.479	96.6

K-12 cells were grown and treated as described in Materials and methods. Growth rate constant was calculated from OD_{570} of the cultures at zero and 3 hr, using the equation

$$\lambda = \frac{\ln \frac{N_t}{N_0}}{t}$$

where $N_0 = OD_{570}$ at zero hr, $N_t = OD_{570}$ at 3 hr and $t = 3$ hr

to tryptophan prototrophy in strain WP 2 uvrA Trp^-. Reversion is due to base-pair substitution at the site of the original alteration or to the induction of ochre suppressors [4]. Spot tests and treat and plate tests were performed. In the spot test, neither nitroprusside nor its adenine complex increased the number of mutant colonies. (Control: 58 ± 5.3; nitroprusside: 22 ± 11.8; adenine complex: 44 ± 17.5.) In treat and plate tests the number of mutant colonies was either equal on control and treated plates, or on the latter it was approximately 50% of the control, after 30 and 120 min exposure, respectively.

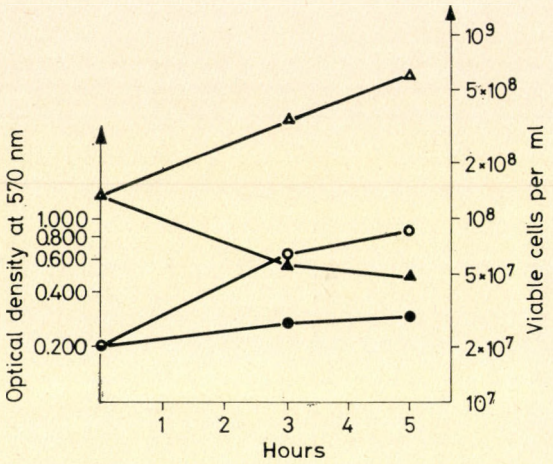


Fig. 1. Effect of 5×10^{-3} adenine complex on the survival of an *E. coli* K-12 culture. Exponentially growing cultures of *E. coli* were obtained and viable counts were determined as described in Materials and methods. OD of control: ○—○, treated: ●—●, viable cells per ml in coltrol: △—△, treated: ▲—▲

Table II

Frequency of streptomycin resistant mutants in adenine complex treated E. coli K-12

Time, (hr)	Control			Treated		
	a	b	c	a	b	c
0	4.46×10^8 $\pm 1.07 \times 10^8$	43.4 ± 16.0	4.9	4.43×10^8 $\pm 0.45 \times 10^8$	52.4 ± 10.7	5.9
1	1.05×10^9 $\pm 0.89 \times 10^9$	—	—	6.58×10^8 $\pm 1.30 \times 10^8$	—	—
4	9.09×10^8 $\pm 1.13 \times 10^8$	35.6 ± 10.6	2.0	1.82×10^9 $\pm 0.14 \times 10^9$	104.2 ± 12.4	2.9

a = number of viable cells per ml; b = number of streptomycin resistant mutants in 0.2 ml of tenfold concentrated sample; c = number of mutants per 10^8 viable cells

Exponentially growing cultures of *E. coli* were treated with 10^{-3} M uridine (control) or 10^{-3} M uridine plus 5×10^{-3} M adenine complex, for one hr. After centrifugation, the cells were resuspended in the original volume of fresh medium (treated culture) or were diluted threefold in the same medium (control). Cultures were let to grow for 3 hr. Samples were taken at the indicated points of time and the number of streptomycin resistant mutants and viable counts was determined as described in Materials and methods

Discussion

Sodium nitroprusside and its adenine complex were found to decrease the growth rate of exponentially growing *E. coli* cells. This effect of adenine complex was due in part to the inhibitory effect of its adenine component on

the *de novo* synthesis of pyrimidine nucleotides [5]. This component of the inhibition could be eliminated by the addition of 10^{-3} M uridine. The mechanism of the remaining inhibition was not investigated. Adenine complex, however, was more effective than sodium nitroprusside even in the presence of uridine. The higher nitroprusside content of the former [1] cannot alone account for this; the difference might be due to differences in uptake.

Adenine seems to play a significant role in the lethal effect of the adenine complex, as it was suggested by the data of Fig. 1 and Table II. Cells treated in the absence of uridine, either in culture medium or in buffer (for 3 hr and 30 min, respectively) showed approximately 50% killing. Incubation of a culture for 60 min in the presence of 10^{-3} M uridine plus 5×10^{-3} M adenine complex did not result in any decrease of viable count.

In mutation experiments no proof of mutagenicity was found. The small increase in the frequency of streptomycin resistant mutants after exposure to adenine complex cannot be regarded as a sign of mutagenic activity as the slight difference falls within the limits of error of the method. The sensitivity of the method of GREEN and MURIEL is also limited; the treat and plate test even under optimum conditions only revealed a considerable increase over the spontaneous mutation rate. In the experiments presented the increase should be about twentyfold, corresponding to the maximum sensitivity of the test. On the other hand, the spot test is insensitive unless the agent to be tested is volatile or a strong mutagen. It was used only to test a number of nitroprusside derivatives; none of them gave a positive result (unpublished data).

Thus, it is concluded that the adenine complex cannot have a high mutagenic activity in the *E. coli* tests employed and since one of our aims was to find a new strong mutagen, these experiments were discontinued. The data presented, however, do not permit to exclude a weak mutagenic action or the induction of frameshift mutations.

Acknowledgements. We are indebted to Professor M. BECK and Dr. L. DÓZSA (Institute of Physical Chemistry, Kossuth Lajos University, Debrecen) for samples of adenine complex and to Professor G. SZABÓ for helpful discussions. The strain WP 2 uvrA Trp$^-$ was kindly supplied by Dr. S. IGALI (National Research Institute of Radiobiology, Budapest).

REFERENCES

1. BECK, M. T., DÓZSA, L.: Bioinorg. Chem. **7**, 1 (1977)
2. GREEN, M. H. L., MURIEL, W. J.: Mutation Res. **38**, 3 (1976).
3. The Oxoid Manual. 3rd ed. Oxoid Ltd., London 1965, p. 199.
4. BRIDGES, B. A., DENNIS, R. E., MUNSON, R. J.: Genetics **57**, 897 (1967).
5. HOSONO, R., KUNO, S.: J. Biochem. **75**, 215 (1974).

Address of the authors:

ISTVÁN SZILÁGYI, SÁNDOR VITÁLIS
Institute of Biology, University Medical School
H-4012 Debrecen, P.O.B. 23, Hungary

Acta microbiol. Acad. Sci. hung. 25, 291—298, 1978

PLASMID CURING AND ANTIBACTERIAL EFFECTS OF SOME CHLORPROMAZINE DERIVATIVES IN RELATION TO THEIR MOLECULE ORBITALS

J. MOLNÁR and B. SCHNEIDER

Institute of Microbiology, University Medical School, Szeged

(Received January 5, 1977)

7-Hydroxychlorpromazine, 7,8-dihydroxychlorpromazine and 7,8-dioxochlorpromazine exerted a bacteriostatic effect on Gram-positive and Gram-negative bacteria, with the latter being more resistant. The three compounds also had a bactericidal effect on selected strains of *Escherichia coli* and *Staphylococcus aureus*. 7-Hydroxychlorpromazine was able to cure 2% of F'lac plasmid of the *E. coli* K-12 LE 140 strain at 60 μg/ml, while 7,8-dihydroxychlorpromazine and its quinone failed to produce such an effect at the same concentration. In the presence of 0.1 M magnesium sulphate the plasmid curing effect of CPZ and 7-OH-CPZ decreased markedly, while the MIC values of CPZ and its derivatives increased. It seems therefore that the relative ineffectivity of CPZ in the presence of magnesium ions was not due to its metal chelation.

It was shown earlier that chlorpromazine (CPZ) and three other phenothiazine compounds exerted an antibacterial effect on various bacteria and a plasmid curing action on the R factor and F'lac plasmid of *Escherichia coli* [1–3]. Furthermore, it was shown that chlorpromazine sulphoxide, a biologically active metabolite of CPZ, had neither the antibacterial nor plasmid curing effect of its parent drug [4]. It was evident that a small change in the molecular structure of CPZ led to the loss of biological activity. Human studies in various biological systems with known or possible metabolites of CPZ, such as 7-OH-CPZ (7-hydroxychlorpromazine), 7,8-$(OH)_2$-CPZ (7,8-dihydroxychlorpromazine) and 7,8-(O_2)-CPZ (7,8-dioxochlorpromazine), showed these compounds to possess an activity similar to or, in many of the test situations, greater than, chlorpromazine itself [5]. Our aim was therefore to study the antibacterial and the plasmid curing effects of these agents in comparison to those of CPZ.

Materials and methods

Drugs. Chlorpromazine HCl was obtained from EGYT Pharmaceutical Works, Budapest. 7-Hydroxychlorpromazine, 7,8-$(OH)_2$-CPZ and 7,8$(O)_2$-CPZ were kindly provided by Dr. ALBERT A. MANIAN of the Psychopharmacology Research Branch, National Institute of Mental Health, Rockville, Md. 20857, USA.

Bacterial strains. Bacillus anthracis VR, *Bacillus megatherium* 299 and *Escherichia coli* K-12 LE 140 strains were used in this study. The *E. coli* strain was kindly provided by Mr. P. A. MEACOCK. The *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*,

Streptococcus viridans, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* strains were isolated from clinical specimens in our laboratory.

Media. The antibacterial and plasmid curing effects of the drugs were studied in a tryptone-yeast extract (MTY) liquid medium [6]. For plating, MTY was supplemented with 1.5% agar (Difco). Eosin methylene blue (EMB) agar [7] was used in curing experiments to distinguish lac positive colonies from colonies formed by lac negative plasmidless bacteria.

Bacteriostatic effect. The drugs were tested on different bacteria by use of the tube dilution method in MTY liquid medium. Overnight precultures were diluted to 10^{-4} and 0.05 ml (approximately 8×10^2–5×10^3 bacteria) was inoculated in 5.0 ml of MTY broth containing 0–500 μg/ml of the drugs. In turn, the cultures were grown in the dark in the presence of the drug without shaking, at 37°C. The minimum inhibitory concentrations (MIC) were determined after 24 hr incubation.

Bactericidal action of CPZ and its derivatives. The drugs were added at various concentrations to exponential phase cultures (optical densities were 0.22–0.25 at 620 nm, containing $5–8 \times 10^8$ bacteria per ml) of *E. coli* K-12 LE 140 and *S. aureus* growing in MTY broth at 37 °C. After 60 min the number of survivors was determined by plating on MTY agar plates.

Elimination of plasmid. Elimination of F'lac plasmid was carried out by the method of MÁNDI *et al.* [2]. An overnight preculture of *E. coli* K-12 LE 140 was diluted to 10^3 bacteria per ml in MTY medium and CPZ and its derivatives were added to 5.0 ml of the diluted cultures at final concentrations of 0–140 μg/ml. The cultures were then incubated at 37°C without shaking. After a 24 hr incubation period the cultures were diluted in physiological saline and plated to obtain individual colonies on EMB agar. The plates were incubated at 37°C for an additional 24 hr and then the lac positive and lac negative colonies were counted.

Elimination of plasmid in the presence of chelate complexes. Magnesium chelate complexes of CPZ and its derivatives were prepared according to RAJAN *et al.* [8] in MTY medium. In preliminary experiments the MTY medium contained 0.1 M magnesium sulphate and was supplemented separately with CPZ and its derivatives to a final concentration of 500 μg/ml, then 5×10^3 bacteria were added to 5.0 ml of medium containing the complex. The samples were incubated at 37 °C for 24 hr and the curing efficiency was tested by plating on EMB agar.

Results

The minimal inhibitory concentrations of 7-OH-CPZ, 7,8-$(OH)_2$-CPZ and 7,8-$(O)_2$-CPZ were determined and compared to that of CPZ on some Gram-positive and Gram-negative bacteria. It can be seen in Tabe I that the Gram-positive bacteria were more sensitive to the drugs tested than the Gram-negative ones. The CPZ derivatives showed no apparent difference in bacteriostatic effect when tested on the same bacterial species. Among the Gram-positive organisms *S. pneumoniae* was the most sensitive; in the group of Gram-negative bacteria. *P. aeruginosa* was completely resistant to the drugs. In studies of the bactericidal action of CPZ derivatives on *S. aureus* and *E. coli*, increasing the concentration of the drugs gradually decreased the number of survivors. The CPZ derivatives exerted a somewhat weaker bactericidal effect on *E. coli* (Fig. 1) than on *S. aureus* (Fig. 2). The bactericidal effect of CPZ and 7-OH-CPZ was similar, while 7,8-$(OH)_2$-CPZ was less active. 7,8-Dioxochlorpromazine had an ever weaker bactericidal activity than the other three compounds.

Effective plasmid curing concentrations of curing compounds are somewhat below those of their MIC values. In our curing experiments, the subinhibitory concentrations of drugs allowed the multiplication of cells at a suboptimal rate. The cells of *E. coli* were grown in the presence of 0–140 μg/ml

Table I

Bacteriostatic effect of chlorpromazine, 7-hydroxychlorpromazine, 7-8-dihydroxychlorpromazine and 7,8-dioxochlorpromazine on bacteria using the tube dilution method

Bacterial strain	Minimum inhibitory concentration (μg/ml)			
	CPZ	7-OH-CPZ	7,8-$(OH)_2$-CPZ	7,8-$(O)_2$-CPZ
B. anthracis VR	20	10	15	10
B. megaterium 299	10	10	10	10
S. aureus	30	15	10	10
S. pyogenes	20	10	10	15
S. viridans	30	20	15	15
S. pneumoniae	5	5	5	5
E. coli K-12 LE 140	90	90	140	150
P. vulgaris	250	250	250	250
K. pneumoniae	250	150	250	250
P. aeruginosa	>500	>500	>500	>500

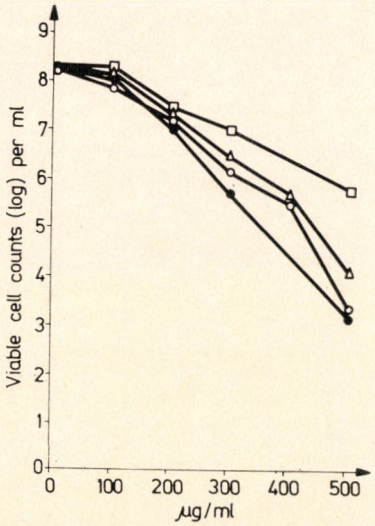


Fig. 1. Bactericidal effect of different concentrations of chlorpromazine, ● ——— ●; 7-hydroxychlorpromazine, ○ ——— ○; 7,8-dihydroxychlorpromazine, △ ——— △; and 7,8-dioxochlorpromazine, □ ——— □ on the *E. coli* K-12 LE 140 strain

Table II

Elimination of F'lac plasmid of E. coli K-12 LE 140 strain by chlorpromazine and 7-hydroxychlorpromazine

μg/ml	Per cent efficiency of curing			
	CPZ		7-OH-CPZ	
	Lac⁻ colonies, per cent	No. of colonies tested	Lac⁻ colonies, per cent	No. of colonies tested
0	0	1680	0	1720
20	1	1780	0.1	2110
30	5	2860	0.8	3050
40	20	2540	1	2100
50	22	2403	1.5	2285
60	29	2300	2	2103
70	—	—	—	—
80	—	—	—	—
100	—	—	—	—
120	—	—	—	—
140	—	—	—	—

— = no bacterial growth

Table III

Elimination of F'lac plasmid of E. coli K-12 LE 140 strain by 7,8-dihydroxychlorpromazine, 7,8-dioxochlorpromazine

μg/ml	Per cent efficiency of curing			
	7,8-$(OH)_2$-CPZ		7,8-$(O)_2$-CPZ	
	Lac⁻ colonies, per cent	No. of colonies tested	Lac⁻ colonies, per cent	No. of colonies tested
0	0	2390	0	1429
20	0	1850	0	2200
30	·	·	·	·
40	0.2	3000	0	2300
50	·	·	·	·
60	0.15	4500	0	1465
70	·	·	·	·
80	0.1	3360	0	2423
100	0.2	3315	0	1121
120	0.3	3445	0.1	2846
140	—	—	0.1	2673

· = not tested
— = no bacterial growth

CPZ and its derivatives at 37°C for 24 hr and the number of lac positive and lac negative, plasmidless bacteria were then determined by plating on EMB agar plates. The results are shown in Tables II and III. It is seen that 7-OH-CPZ was about ten times less effective than CPZ while 7,8-$(OH)_2$-CPZ and 7,8-$(O)_2$-CPZ had practically no plasmid curing activity. It was shown previously that discontinuation of pi-conjugation in the ring system led to loss of the plasmid curing effect of 7,8-dioxochlorpromazine; pi-conjugation is, however, not essential for the antibacterial effect.

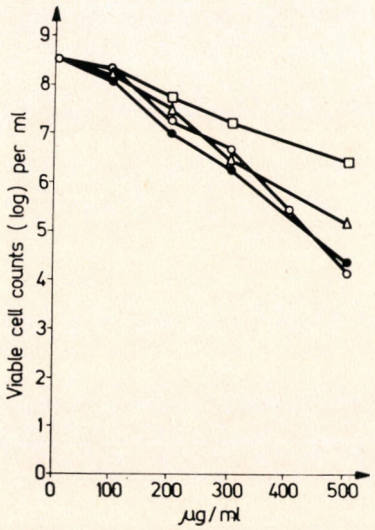


Fig. 2. Bactericidal effect of different concentrations of chlorpromazine, ●——●; 7-hydroxychlorpromazine, ○——○; 7,8-dihydroxychlorpromazine, △——△; and 7,8-dioxochlorpromazine, □——□ on *S. aureus*

The role of the free tertiary amino group of the side chain in the biological activities of chlorpromazine and its derivatives were studied by using chelate complexes. Under the conditions described, the plasmid curing effects of CPZ and 7-OH-CPZ were markedly decreased, while the MIC values of the CPZ derivatives increased in the presence of magnesium ions (Table IV). In other curing experiments the molar concentrations of CPZ and magnesium salt were gradually increased to study the role of metal chelation in the plasmid elimination by CPZ (Table V). As shown in Table V, magnesium ions did not prevent the antibacterial and plasmid curing effect of CPZ when both compounds were applied in the nearly equimolar concentration required for the metal chelation of CPZ.

Table IV

Elimination of F'lac plasmid of E. coli K-12 LE 140 strain by chlorpromazine and some of its derivatives in the presence of 0.1 M magnesium sulphate

Compound	μg/ml	Lac⁻ colonies, per cent	No. of colonies tested	Variable cells after 24 hr
Control	—	0	2390	7×10^8
CPZ	500	0.5	3060	2.0×10^6
7-OH-CPZ	500	0.4	2240	7.5×10^6
7,8-$(OH)_2$-CPZ	500	0	4400	4.3×10^8
7,8-$(O)_2$-CPZ	500	0	1190	9.7×10^7

Table V

Per cent efficiency of plasmid curing in the presence of various molar concentrations of chlorpromazine and magnesium sulphate

Chlorpromazine		Magnesium sulphate, M					
M	μg/ml	1×10^{-4}	1×10^{-3}	1×10^{-2}	3×10^{-2}	7.5×10^{-2}	1.5×10^{-1}
6.4×10^{-5}	20	0.5	0.8	0	0	0	0
9.6×10^{-5}	30	2.0	2.1	0	0	0	0
1.2×10^{-4}	40	11.0	18.0	0	0	0	0
1.6×10^{-4}	50	—	22.0	0	0.1	0	0
1.9×10^{-4}	60		29.0	0.5	0	0	0
2.5×10^{-4}	80		—	2.0	0.1	0	0
3.2×10^{-4}	100			1.5	0.4	0.2	0
6.4×10^{-4}	200			—	0	0.6	0.1
9.6×10^{-4}	300				0	2.0	2.8
1.2×10^{-3}	400				—	0	1.5
1.6×10^{-3}	500					0	0.5

0 = no plasmid curing
— = no bacterial growth

Discussion

Previously we have shown that CPZ, levomepromazine and promethazine had a bacteriostatic effect on Gram-positive bacteria at concentrations of 20–60 μg/ml and on Gram-negative bacteria at 130–180 μg/ml. The bacteriostatic effect was then tested on 10^5/ml of bacteria [1, 3]. The present experiments showed that the minimal bacteriostatic concentration is lower if a small number (approximately 10^3/ml) of bacteria is used.

In the present experiments CPZ was able to eliminate the R factor of an *E. coli* strain [1, 3] and Lon$^-$ mutants were selected by CPZ treatment [9]. Levomepromazine and promethazine also had a plasmic curing effect on another strain of *E. coli*, which was lac$^-$ on the chromosome but carried an F' plasmid containing the lac region [2]. On the other hand, chlorpromazine sulphoxide exerted neither an antibacterial nor a plasmid curing effect [7] despite of a small modification of the chemical structure of CPZ. Further 7-OH-CPZ, 7,8-$(OH)_2$-CPZ and 7,8-(O_2)-CPZ, which differ from each other in the substituents on the same aromatic ring of the CPZ molecule, were tested and despite of the steric modifications these compounds exerted both bacteriostatic and bactericidal effects. Their plasmid curing ability, however, was quite different. 7-Hydroxychlorpromazine showed a weaker plasmid curing effect than CPZ did and the two disubstituted CPZ derivatives were not able to eliminate the F'lac plasmid. On the basis of their plasmid curing activity, the CPZ derivatives with an antibacterial effect could be divided into two groups. Chlorpromazine and 7-OH-CPZ belong to the first group having antibacterial and plasmid curing effects. The second group consisting of 7,8-$(OH)_2$-CPZ and 7,8-$(O)_2$-CPZ is not able to eliminate the plasmid.

In the molecules of CPZ and 7-OH-CPZ the pi-electrons are in conjugation and the aromatic ring system has a well defined nucleophilic charge. In 7,8-(O_2)-CPZ the conjugation of the pi-electrons is broken at the position of carbon atoms 7 and 8 and, therefore, some change in polarization occurs in the ring system. The nucleophilic character of carbon atoms 7 and 8 is decreased. The reason for the ineffectiveness of 7,8-$(OH)_2$-CPZ may be an instability under the experimental conditions employed (in the presence of oxygen). It is known that the compound undergoes auto-oxidation in the presence of air, forming 7,8-dioxochlorpromazine and toxic species [10, 11]. We assume that in the case of the two disubstituted CPZ derivatives the discontinuation of conjugation of the pi-electron system is the reason for the absence of a plasmid eliminating effect.

The antibacterial and plasmid curing activities of the CPZ derivatives decreased in the presence of magnesium at high concentration. The metal chelation of CPZ and its derivatives is well known to occur in aqueous electrolytic media [8]. It is possible that in such complexes the nucleophilic character of the aromatic ring system is also altered and this might be connected with their relative ineffectiveness; however, the chelate complex of CPZ derivatives showed the same activity on bacteria as the derivatives themself.

Beside the mentioned mechanism the high magnesium concentration may stabilize the activity of different enzymes, thus the magnesium at high concentration prevents the binding of chlorpromazine derivatives to the structural components of the bacteria. There is some evidence of metal ions acting in such way as the manganese ions reversed the growth inhibitory action of

ethidium bromide on a *B. cereus* strain [12]. It was also observed that the presence of magnesium ions leads to an inhibition of tilorone binding to DNA [13].

Acknowledgements. The authors are indebted to Professor I. BÉLÁDI, Director of the Institute of Microbiology, University Medical School, Szeged, for her interest and to Professor A. A. MANIAN (Psychopharmacology Research Branch, National Institute of Mental Health, Rockville, Md., USA) for providing the chlorpromazine derivatives.

REFERENCES

1. MOLNÁR, J., KIRÁLY, J., MÁNDI, Y.: Experientia (Basel) **31,** 444 (1975).
2. MÁNDI, Y., MOLNÁR, J., HOLLAND, I. B., BÉLÁDI, I.: Genet. Res. **26,** 109 (1976).
3. MOLNÁR, J., MÁNDI, Y., KIRÁLY, J.: Acta microbiol. Acad. Sci. hung. **23,** 45 (1976).
4. MOLNÁR, J., MÁNDI, Y., HOLLAND, I. B., SCHNEIDER, GY.: Acta microbiol. Acad. Sci. hung. **24,** 1 (1977).
5. FORREST, I. S., CARR, C. J., USDIN, E. (eds): The Phenothiazines and Structurally Related Drugs. Raven Press, New York 1974.
6. ALFÖLDI, L., RASKÓ, I., KEREKES, E.: J. Bact. **96,** 1512 (1968).
7. CLOWES, R. C., HAYES, W.: Experiments in Microbiological Genetics. Blackwell, Oxford 1968.
8. RAJAN, K. S., MANIAN, A. A., DAVIS, J. M., SKRIPKUS, A.: In FORREST, I. S., CARR, C. J., USDIN, E. (eds): The Phenothiazines and Structurally Related Drugs. Raven Press, New York 1974, p. 571.
9. MOLNÁR, J., HOLLAND, I. B., MÁNDI, Y.: Genet. Res. **30,** 13 (1977).
10. HEIKKILA, R. E., COHEN, G., MANIAN, A. A.: Biochem. Pharmacol. **24,** 363 (1974).
11. TJIOE, S. A., MANIAN, A. A., O'NEILL, J. J.: Psychopharmacol. Comm. **1,** 373 (1975).
12. TOMCHIK, R., MANDEL, H. G.: J. gen. Microbiol. **36,** 225 (1964).
13. CHANDRA, P., WOLTERSDORF, M.: FEBS Letter **41,** 169 (1974).

Address of the authors:

JÓZSEF MOLNÁR, BÉLA SCHNIEDER
Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

Acta microbiol. Acad. Sci. hung. 25, 299—306, 1978

DEFECTIVE LIFE CYCLE AND LOW ANTIBIOTIC PRODUCTION IN SUBMERGED CULTURES OF STREPTOMYCES FRADIAE

I. BIOCHEMICAL CHARACTERIZATION

GY. VARGHA, S. VITÁLIS and G. SZABÓ

Institute of Biology, University Medical School, Debrecen

(Received January 9, 1978)

The life cycle of a *Streptomyces fradiae* strain producing high amounts of neomycin under industrial conditions has been investigated in liquid soybean medium where the production of antibiotic proved to be comparatively low. The changes occurring in the main macromolecular components and the enzyme activities of the mycelium during the life cycle and cytological observations proved that there was a block in the normal process of reproductive differentiation and a lack of exocellular alkaline phosphatase activity was found.

The life cycle of streptomycetes in submerged cultures was investigated most thoroughly by PROKOFIEVA-BELGOVSKAYA [1] who distinguished a vegetative and a reproductive phase each consisting of two further stages on the basis of cytological data, the growth character, the chemical composition and biological activity of the mycelium. This work has been extended in our laboratory using *Streptomyces griseus* strains which sporulate abundantly in submerged cultures [2]; in their life cycle the following stages have been differentiated. 1. The vegetative (growth) phase involves the stages of (a) germination, (b) vegetative (quasi-exponential) growth and (c) transitional stage (first lysis). 2. The reproductive phase (phase of differentiation) involves the stages of (a) reproductive growth, (b) spore formation, and second lysis (morphogenesis) [2, 3]. In these studies the changes in macromolecular components, marker enzyme activities and morphology of the cultures were related. Among the marker enzymes, peaks of beta-glucosidase and beta-galactosidase activity were characteristic of the vegetative phase, while alkaline phosphatase and protease activity was typical of the reproductive phase although they had maxima also in the vegetative phase. The two latter enzyme activities had been correlated with the sporulation in *Bacillus subtilis* [4–6]. Alkaline phosphatase activity was found to be directly linked with neomycin production in *Streptomyces* [7].

The antibiotic production of streptomycetes is in connection with the life cycle or differentiation of these organisms. The formation of spores by a *S. griseus* strain and the production of streptomycin thus show a close correlation [8]. There is a strain which in submerged culture does not produce

streptomycin but sporulates well, while another strain produces antibiotic but does not form spores in submerged culture (although some early steps occur towards reproductive differentiation) [9].

It is well known that the conditions of cultivation strongly influence the antibiotic production, the growth characteristics, metabolic activity and differentiation of antibiotic producing strains. Choosing a genetically good antibiotic producer strain it seemed interesting to examine the course of its life cycle under circumstances that suppress antibiotic biosynthesis, in order to obtain insight into connections between differentiation and secondary metabolism. For this reason in the following work we used an industrial *S. fradiae* strain synthetizing neomycin poorly under the conditions applied. In industrial fermentations the same strain produced many times more antibiotic. In this paper the results obtained by measuring the changes of the main macromolecular components and marker enzyme activities during cultivation are presented. The cytological findings will be published separately [10].

Materials and methods

Cultivation. S. fradiae LN 47 1-Gl spores were obtained from the surface of agar slants, suspended in 0.9% NaCl solution and added into 500 ml Erlenmeyer flasks each containing 100 ml of sterile filtered liquid soybean medium [11] plus 1 g of sterile $CaCO_3$ at about 10^6 spores/ml of culture medium. Cultivation was performed on a rotary shaker (stroke 50 mm, 230 rpm) at 27 °C. After 0, 24, 48, 72, 96, 120, 144 hr 3 cultures each were harvested by centrifugation at 4000 *g*, the mycelium was washed with 0.9% NaCl and frozen at −20 °C until use. The supernatant was also frozen and then used for measuring the extracellular enzyme activities. Two series of independent experiments each with 3 parallels were carried out in the same way. Because in two independent experiments the numerical values may vary, in the Figures always one of them is only shown.

Preparation of crude homogenate. The mycelium was suspended in 5 ml M/15 phosphate buffer pH 7.5. Cells were broken by sonication (20 kHz, Measuring and Scientific Equipment Ltd., Ultrasonic Power Unit No. 3000).

Determinations in the crude homogenate.

1. *DNA and RNA content.* Extractions were carried out by the slightly modified method of VALU and SZABÓ [12]. Cold acid soluble material was extracted with 5% perchloric acid (PCA) at 0°C for one hour. The precipitate was suspended in 1.5 N PCA, extracted at 70°C for 50 min and deoxypentose (for DNA) and aldopentose (for RNA) content was determined with diphenylamine [13] and phloroglucin [14], respectively. Nucleic acid contents of the cultures were expressed as μg DNA or RNA per ml of culture medium. The standards were chick blood DNA (Reanal, Budapest) and yeast RNA (Worthington).

2. *Protein content* was measured by the method of LOWRY *et al.* [15].

3. *Beta-galactosidase activity.* Substrate: 1 mg/ml *o*-nitrophenyl-beta-D-galactopyranoside (Fluka) in 0.1 M KH_2PO_4/K_2HPO_4 buffer pH 6.5. Assay mixtures contained 0.5 ml of crude homogenate plus 0.5 ml of substrate solution. After one hour of incubation at 27°C, 0.5 ml 1 M Na_2CO_3 was added and the assay tubes were immediately placed into water bath at 0°C. This was followed by centrifugation at 4000 *g* and the extinctions of the samples were measured at 420 nm. Activity was expressed as μ mole/ml DNA/hr.

4. *Beta-glucosidase activity* was measured in the same way as beta-galactosidase except that the substrate was 1 mg/ml *p*-nitrophenyl-beta-D-glucopyranoside (Merck) in 0.1 M KH_2PO_4/K_2HPO_4 buffer pH 7.2 and the incubation time 20 min.

5. *Alkaline phosphatase activity.* Substrate: 1 mg/ml *p*-nitrophenyl-phosphate (Koch–Light) in veronal-acetate buffer pH 8.5; 0.1 ml crude homogenate plus 0.4 ml substrate were incubated at 27°C for 20 min. The enzyme reaction was stopped by adding 0.1 ml formaldehyde,

the colour was developed with 0.5 ml 1 M Na_2CO_3. After centrifugation at 4000 *g* the extinction of the supernatant was measured at 420 nm.

6. *Protease activity* was determined by the method of BÉKÉSI *et al.* [16] and expressed as ΔE_{515}/mg DNA/hr.

Exocellular enzyme activities were measured in the cell-free culture medium (cells were sedimented by centrifugation) in the same way as in the crude homogenate.

Neomycin concentration in the culture medium was determined by the conventional agar diffusion method, using neomycin sensitive *Bacillus subtilis* ATCC 6633 strain.

Results

Growth characteristics of S. fradiae and neomycin production. As it can be seen in Fig. 1, the DNA content of the mycelium of *S. fradiae* increased intensely between 0–24 hr of growth, then rose further at a limited rate for two days and decreased slowly after 72 hr. The protein content of the mycelium increased similarly but only for 48 hr (Fig. 2). The amount of RNA was strikingly high at 24 hr after inoculation but later decreased (Fig. 1).

Both the RNA/DNA and the protein/DNA ratios were highest in the 24 hr old cultures of *S. fradiae*. These cultures were near the end of the quasi-exponential growth. After this time both ratios decreased and persisted at a comparatively low level. The definite decrease in the case of RNA/DNA ratio took place approximately 24 hr earlier than that of the protein/DNA ratio (Fig. 3).

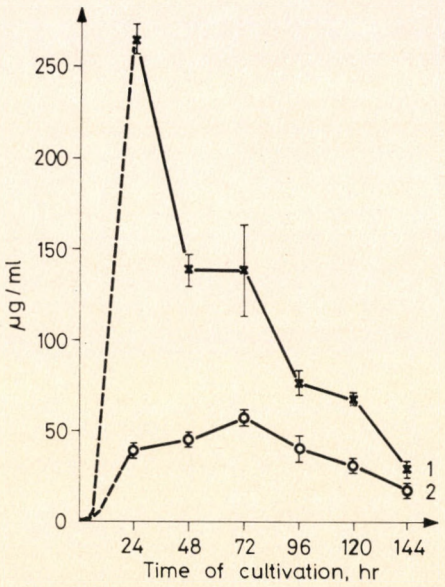


Fig. 1. RNA and DNA contents of the mycelium of *S. fradiae* during cultivation. Cultures were harvested at the times indicated after inoculation. Crude homogenates were prepared and the DNA and RNA contents were determined as described in Materials and methods. The DNA and RNA contents were calculated in µg per ml of culture. Points represent means of three parallels. 1 = RNA contents, 2 = DNA contents

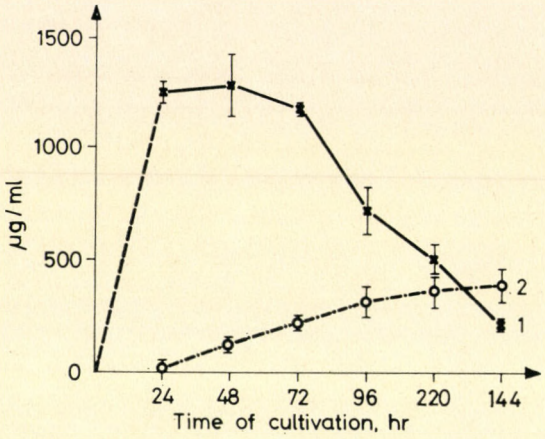


Fig. 2. Protein content of the mycelium of *S. fradiae* and the amount of neomycin produced. 1 = Cultures were harvested at the indicated times after inoculation. Crude homogenates were prepared as described in the Materials and methods. Protein contents determined by the method of LOWRY *et al.* [14] were expressed in μg per ml of culture. 2 = Cells were sedimented from the cultures at the indicated times after inoculation. The neomycin content of the cell-free medium was determined by the agar plate method and expressed in μg per ml of culture medium. Points represent means of three parallels

Neomycin production was poor although it rose between 48–144 hr at a nearly constant speed (Fig. 2).

Endocellular enzyme activities during cultivation. It has been found that each of the four enzyme activities measured in these experiments showed apparently different and characteristic levels at different stages of the life cycle. These changes were essentially similar in character when specific activ-

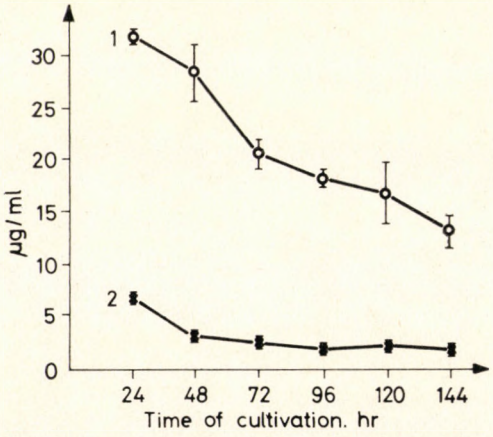


Fig. 3. RNA/DNA and protein/DNA ratios in the mycelium of *S. fradiae* during cultivation. The RNA, DNA and protein contents of the mycelium were determined as described in Materials and methods and in the legend of Figs 1 and 2. Points represent means of three parallels. 1 = Protein/DNA ratio, 2 = RNA/DNA ratio

ities were expressed as U/mg protein (data not shown) or as U/mg DNA (Fig. 4).

All of the four specific activities rose rapidly between 24–48 hr of cultivation to reach a comparatively sharp maximum at about 48 hr. After 48 hr of growth two types of changes were noticed. (i) Endocellular beta-glucosidase, beta-galactosidase and protease activities decreased and stayed at low levels which, however, were significantly higher than the zero values. (The specific activity of beta-glucosidase was always higher than that of beta-galactosidase.)

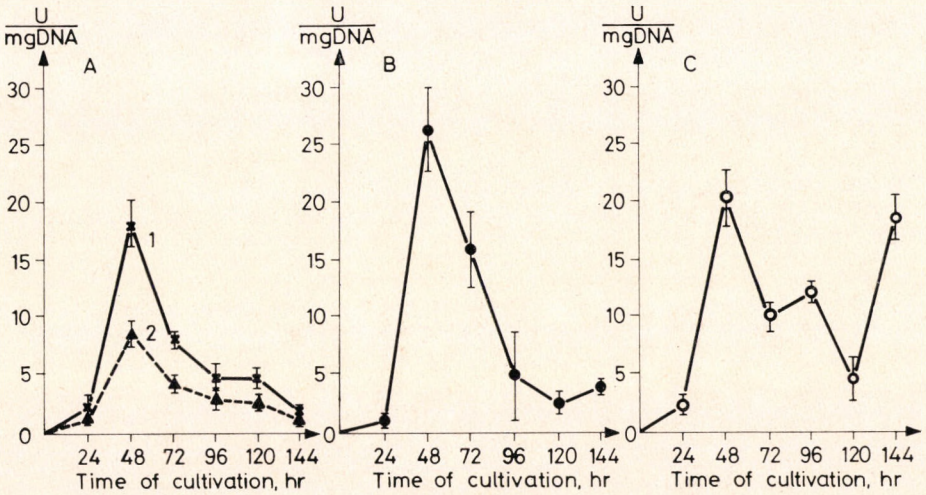


Fig. 4. Endocellular enzyme activities in the mycelium of *S. fradiae.* Cultures were harvested at the times indicated after inoculation. Crude homogenates were prepared and enzyme activities were determined as described in Materials and methods. A: straight line–beta-glucosidase activity, dotted line–beta-galactosidase activity; B: protease activity; C: alkaline phosphatase activity. On the abscissa, time of cultivation (hr); on the ordinate, U/mg DNA. In the case of protease activity: $U = \Delta E_{515}/hr$; in the case of other enzymes: $U = \mu mol/hr$. Points respresent means of three parallels

(ii) Alkaline phosphatase activity after the 48 hr maximum decreased till 72 hr, then persisted at the same level for a day, and after a minimum at 120 hr very quickly rose between 120 and 144 hr.

Exocellular protease activity during cultivation. Beta-glucosidase, beta-galactosidase and, surprisingly, alkaline phosphatase activities were not detectable in the culture medium. The exocellular protease activity was much higher than the endocellular one, and showed a different time course. It increased till 48 hr of cultivation, did not change between 48 and 96 hr and then increased to a high level at 144 hr (Fig. 5), while the endocellular protease activity showed a definite decreasing tendency between 48–120 hr of cultivation and stayed at a low level till 144 hr (Fig. 4).

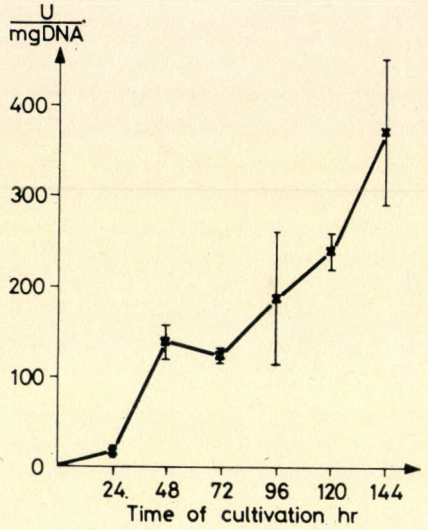


Fig. 5. Exocellular protease activity of *S. fradiae* during cultivation. Mycelium was sedimented by centrifugation at the times indicated and protease activity was determined in the cell-free supernatant by the method of BÉKÉSI *et al.* [16]. U = ΔE_{515}/hr. Points respresent means of three parallels

Discussion

The growth curves (Figs 1 and 2) show clearly that at 24 hr of cultivation the mycelium is at the end of the quasi-exponential stage of growth, or has passed it. After that time a smaller but definite increase of the DNA content takes place. Neomycin production begins in this period. Between 24 and 72 hr the RNA content and also the RNA/DNA and protein/DNA ratios decrease significantly (Figs 1 and 3). The changes may be due to the different rates of synthesis of RNA, DNA and proteins. The significant decrease in the absolute amounts of RNA between 24 and 48 hr (Fig. 1) and that of the endocellular enzymes between 48 and 72 hr (data not shown) indicate, however, that the increased degradation of specific RNAs and proteins within the hyphae might also be involved. A decreased RNA and protein synthesis may also play a role. In addition, some lysis and new vegetative hyphae are observed at 72 hr of cultivation [10]. After the decrease in RNA content of the mycelium (24–48 hr) the amount of RNA does not change in 48–72 hour-old hyphae; this may reflect a new growth of vegetative mycelium [10].

The life cycle period between 24 and 72 hr seems to correspond to the stage of limited growth or first lysis (on the basis of growth characteristics Figs 1 and 2) but this stage is much shorter if a normallly differentiated *Streptomyces* strain, as for example *S. griseus* No. 45-H having a normal

(complete) life cycle is cultivated under the same conditions [3]. It is assumed also on the basis of cytological observations, that the hyphae are prevented from entering into reproductive differentiation. There seems to be a block in the normal reproductive development in this medium [10].

Before discussing the changes in enzyme activity, it is necessary to emphasize that activity was calculated in units per DNA content, this value being proportional to the activity per genome.

The observed maxima of the endocellular marker enzyme activities at 48 hr fall into the prolonged transitional (first lytic) stage (see above). The exact points of time of these maxima could not be determined because of the few samplings. Similar maxima of the same enzyme activities were observed near the end of the quasi-exponential growth of *S. griseus* No. 45-H under the same circumstances but in this case alkaline phosphatase activity was measurable only in the culture medium [3]. The high activity of beta-glucosidase and beta-galactosidase seems to be characteristic of the vegetative phase, in good agreement with results obtained with *S. griseus* [3]. The high activity of these enzymes even after 48 hr of cultivation can be explained by the presence of newly grown young vegetative hyphae that appear till 120 hr [10]. After this time, when reproductive hyphae begin to appear, the beta-glucosidase and beta-galactosidase activities continue to decrease. Endocellular protease activity shows a similar time course. Alkaline phosphatase activity has maxima both in the vegetative phase and in the reproductive one. When measured separately, exocellular protease activity was many times higher than the endocellular one, and in contrast with the latter it was highest at 144 hr. This derepression of exocellular proteases may be in connection with the lysis beginning at 72 hr [10].

The different enzyme patterns in cultures of different ages reflect different regulation situations during the stages of development.

Neomycin accumulation in this medium begins at the same time as it does under industrial conditions, i.e. after or near the end of quasi-exponential growth, but it remains low until 144 hr of cultivation. Exocellular alkaline phosphatase activity is also lacking till 144 hr while under industrial circumstances, phosphatase activity is definitely measurable. The alkaline phosphatases were found to be essential in the last step of neomycin biosynthesis [7].

It is an important question to answer whether (i) the impaired differentiation [10], and/or (ii) the lack of exocellular alkaline phosphatase activity (a problem of enzyme regulation) are in any way connected with (iii) the abnormally low antibiotic production.

REFERENCES

1. PROKOFIEVA-BELGOVSKAYA, A. A.: Structure and Development of Actinomycetes. Publishing House of the USSR Academy of Sciences, Moscow 1963.
2. VITÁLIS, S., SZABÓ, G., VÁLYI-NAGY, T.: Acta biol. Acad. Sci. hung. **14,** 1 (1963).
3. VITÁLIS, S., VARGHA, GY.: In HERNÁDI. F., PAPP, S., SZABÓ, G., (eds): Proceedings of the 6th Conference of the Chemotherapeutical Section of the Hungarian Pharmacological Society, Vol. I., Debrecen, Hungary. 1978. pp. 29—40.
4. KAY, D., WARREN, S. C.: Biochem. J. **109,** 819 (1968).
5. WARREN, S. C.: Biochem. J. **109,** 811 (1968).
6. GLENN, A. R., MANDELSTAM, J.: Biochem. J. **123,** 129 (1971).
7. MAJUMDAR, M. K., MAJUMDAR, S. K.: Biochem. J. **122,** 397 (1971).
8. KHOKHLOV, A. S., ANISOVA, L. N., TOVAROVA, I. I., KLEINER, E. M., KOVALENKO, I. V., KRASILNIKOVA, O. I., KORNITSKAYA, E. YA., PLINER, S. A.: Z. Allg. Mikrobiol. **13,** 647 (1973).
9. SZESZÁK, F., SZABÓ, G., VITÁLIS, S.: Zbl. Bakt. Abt. II, **121,** 18 (1967.)
10. VITÁLIS, S., VARGHA, GY., SZABÓ, G.: Unpublished data.
11. SZABÓ, G., BARABÁS, GY., VÁLYI-NAGY, T.: Arch. Mikrobiol. **40,** 261 (1961).
12. VALU, G., SZABÓ, G.: Acta biol. Acad. Sci. hung. **21,** 99 (1970).
13. RICHARDS, G. M.: Anal. Biochem. **57,** 369 (1974).
14. DISCHE, Z., BORENFREUND, E.: Biochim. biophys. Acta (Amst.) **23,** 639 (1957).
15. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. J., RANDALL, R. J.: J. biol. Chem. **193,** 265 (1951).
16. BÉKÉSI, I., BIRÓ, S., VITÁLIS, S.: Kísérl. Orvostud. **29,** 16 (1977).

Address of the authors:

GYÖRGY VARGHA, SÁNDOR VITÁLIS, GÁBOR SZABÓ
Institute of Biology, University Medical School
H-4012 Debrecen, P.O.B. 17, Hungary

Acta microbiol. Acad. Sci. hung. 25, 307—317, 1978

MOUSE LUNG OEDEMA CAUSED BY A TOXIC SUBSTANCE OF ESCHERICHIA COLI STRAINS*

I. Kétyi, L. Emődy, T. Kontrohr, Adele VerténYi, S. Pácsa, T. A. Avdeeva, N. V. Safonova, and N. K. Golutova

Institute of Microbiology, University Medical School, Pécs and Institute of Epidemiology and Microbiology, Pasteur Institute, Leningrad, USSR

(Received February 1, 1978)

Some *Escherichia coli* strains isolated from patients and instilled into the nostrils of mice cause a rapidly developing fatal, haemorrhagic lung oedema. Lung toxic strains were found in different serogroups, with the predominance of O4, O6 and O18. The toxic material seems to be bound to the cells; the toxin yield is poor by different methods of extraction. The toxic principle causes toxic, haemorrhagic oedema in mouse foot pad test and shows cytotoxicity for AV-3 cells. The "lung toxin" is heat labile and after Sephadex fractionation has a molecular weight of about 100 000 dalton. There is a possible identity with a toxic haemolysin.

One of the authors (T.A.A.) when investigating the pathogenicity of *Escherichia coli* strains isolated from infants suffering from acute enteritis realized that some of the strains did not belong to any serogroups of known nosological units and displayed infrequent and low sign of enterotoxigenity. In the lung test introduced by Voino-Yasenetskaya [1] some of the above mentioned unclassified *E. coli* strains caused in mice a rapid, haemorrhagic lung oedema (not interstitial pneumonia) indicating the toxic activity of the material [2].

In this paper some features of the lung toxic material are described.

Materials and methods

Strains. E. coli strains were isolated from faecal samples of infants suffering from acute enteritis. Pure cultures were tested with OB antisera representing the known serogroups responsible for infantile gastroenteritis and for dysenteriform enteritis of adults. Strains not belonging to these serogroups were tested for invasivity (Serény-test) and for production of either heat-labile (CHO and Y-1 cell lines), or heat-stable (suckling mouse test) enterotoxins. A total of 72 strains not belonging to well known nosological units were analysed serologically, determining mostly their O antigen only.

* Supported in part by the Scientific Research Council, Hungarian Ministry of Health (3-10-0305-03-1/K).

Some investigations presented in this paper dealt only with a few representative strains. Among them were the standard K type strains of serogroup O4 (I. and F. ØRSKOV, Statens Serum Institut, Copenhagen), a strain designated "Kreka" with the antigenic structure O4 : K12 : H5, and a few others. Two strains of the serogroup O139, causing oedema disease in swine, were kindly sent by Dr. B. NAGY (Szombathely, Hungary). An *E. coli* strain (No. 281/54) carrying a Hly plasmid (serologically not determined) was isolated in the Pécs laboratory.

Media. Bacto broth medium with or without 1.6% of agar, and modified Sakazaki medium for shaking cultures [3] were used. Modification means the use of Levinthal broth instead of "Myosat". Shaking was performed in the Psychrotherm apparatus (New Brunswick) at 37°C for 18 hr.

Blood agar plates containing 2.5% of defibrinated sheep blood were used.

Bacterial extracts, lysates. For obtaining the toxic material from bacteria the following methods were applied. Alternative freezing and thawing 10 times, ultrasonic treatment at 0 °C for 30 min (MSE, 500 W, "full energy setting") and the extraction method of VAN HEYNINGEN and GLADSTONE [4] with alkali treatment at 37°C instead of 57°C in view of the heat lability of the toxic material.

All lysates and extracts were dialysed against tap water for 2 days, and against distilled water for 2–3 days, membrane-filtered (Membranfilter Gesellschaft, Göttingen, No. 1121) and freeze-dried. The freeze-dried materials were distributed in vials, dried further over P_2O_5, then filled with N_2 gas and stored at −20°C.

Test for bacterial invasiveness was performed according to SERÉNY [5]; the guinea pig eyes were observed for 5 days.

Tests for enterotoxigenicity. The presence of heat-labile (LT) enterotoxin was demonstrated in CHO or Y-1 cultures [6, 7], using plastic plates (Linbro, 96-F8-TC, FLOW) in the micromethod applied. In most cases culture filtrates were tested, but in the case of representative strains concentrated ultrasonic lysates were investigated with other LT specific tests including ligated rabbit loop [8], blueing [9], and mouse foot oedema tests [10].

Materials were tested also for the presence of heat-stable (ST) enterotoxin in suckling mice [11].

Lung test [1, 2]. Mice of our own breed or from the BALB/c line weighing 10–12 g were used. They were anaesthetised superficially with ether and 0.05 ml of the cultures, filtrates, or extracts diluted in PBS were instilled into their nostrils.

In positive cases convulsions developed, with signs of acute asphyxia, and foam, sometimes tinged with blood, appeared in the nostrils. Death mostly occurred 1–4 hr after infection; in the case of very active material the animals died in the first hour. Post-mortem examination revealed haemorrhagic lung oedema. In negative cases the mice survived or died in a few days with interstitial pneumonia.

Cytotoxicity of filtrates, extracts, or fractionated materials was estimated by a micromethod [12] in a cell line of human amniotic origin (AV-3).

Tests for haemolytic activity. Screening of cultures was made on blood agar plates. Supernatants, filtrates, or extracts were tested for haemolytic activity by TAKÁTSY's method [13] using 1% sheep red blood cells. The type of haemolysin [14] was determined by the overlayer method; the cultures were killed by chloroform vapour for 20 min, and the chloroform was evaporated for 45 min, the killed culture was overlayered with 2.5–3 ml medium containing 0.6% agar and 5% sheep erythrocytes.

Molecular weight of the toxic material was determined by fractionation of a Sephadex G–100 column of 1.5 × 90 cm for 200 mg freeze-dried ultrasonic lysate. The material was dissolved in 0.005 M Tris/HCl buffer pH 7.6 containing 0.001 M EDTA used also as eluent. The eluation speed was 20 ml/hr and samples of 5 ml each (50 fractions) were collected. Optical density was measured at 280 nm (Uvicord II, LKB). Fractions were stored at −20 °C. Using the same column, other materials were also fractionated, e.g. materials containing *E. coli* LT.

Antiserum was produced by immunizing rabbits according to CALLAHAN *et al.* [15]. Fractions of Sephadex G–100 containing the highest activity were mixed 1 : 1 with Freund (Difco) adjuvant and rabbits weighing 2.5–3.5 kg were inoculated subcutaneously 4 times with 0.5 ml, and thereafter with 1 ml of the mixture weekly, into the axillary and inguinal region. One week after the last injection the animals were bled, sera were collected, sterilized by filtration and stored at −20°C without chemical preservative.

Mutagenesis. Some strains were mutagenized by ethyl-methane sulphonate (EMS) [16]. Non-haemolysing clones were selected and isolated on blood agar.

Statistical analysis. LD_{50} values were calculated according to KÄRBER [17].

Results

Some *E. coli* strains isolated from infants with enteritis did not belong to any known entity. In other words, they failed to react with OB antisera, prepared by causative agents of infantile enteritis or dysenteriform disease, they were negative in the Serény test and did infrequently produce LT or ST. In the lung test, a number of these strains, instead of causing a slowly developing interstitial pneumonia [1] gave rise to a rapidly fatal haemorrhagic lung oedema. These observations indicated that the strains in question produced some toxic material(s). Efforts were made to elucidate this assumption.

1. *Serological distribution of the lung toxic strains.* According to data summarized in Table I, lung toxic *E. coli* strains were found in 26 different serogroups. Strains isolated from cases with clinical symptoms and partly belonging to known nosological units, being negative in the lung test, served as controls in respect of serological distribution.

Among the lung toxic strains, the serogroups O4, O6 and O18 predominated (Table I) but such serogroups occurred also in the control group.

The marked predominance of serogroup O4 led us study some standard K type strains. Data demonstrated in Table II showed that three of these old subcultures still possessed a toxic effect.

2. *Production of the lung toxic material.* The lung toxic effect of live bacteria was not influenced by cultural conditions. Not less than 10^7 germs were needed to evoke fatal mouse lung oedema regardless of the age (between 6 and 48 hr) and the method of cultivation (with or without agitation, with or without thioglycolate). Furthermore the effectivity the same was found when bacteria were washed off from agar plates. Alkaline meat extraction [18] had no inductive effect.

Supernatants of broth, modified Sakazaki liquid cultures (with or without agitation) and of washed agar cultures did not contain toxic material.

Difficulties arose with extraction of toxic material from the cells. Approximately the same low yield was obtained by ultrasonic lysis, freezing and thawing 10 times, and by alkaline extraction [4]. Heat treatment at 57°C for 30 min or at 60°C for 15 min destroyed all toxic activity of both living bacteria and extracts.

To avoid these difficulties, mostly ultrasonic lysates concentrated by freeze-drying were used.

3. *Testing the lung toxic substance with other methods.* The lung toxic *E. coli* strains were selected on the basis of their lack of enterotoxicity, among others. A lung toxic strain of *E. coli* designated "Kreka" (O4 : K12 : H5) was investigated in detail. Apart from culture filtrates, ultrasonic lysates and concentrated material from the latter lysate were investigated in several tests. No toxic effect was observed in CHO, Y-1 cells, and no positive blueing was observed

Table I

Serological distribution of lung toxic E. coli strains

Serogroup	Number of lung toxic strains	Number of lung negative strains[1]	Serogroup	Number of lung toxic strains	Number of lung negative strains[1]
O1	2	—	O111	—	4[4]
O2	1	—	O112a, b	2	—
O4	25	2	O112a, c	—	1[4+3]
O5	1	—	O115	1	2
O6	10	1[2]	O119	—	1
O15	—	2[2]	O123	1	—
O18	10	7	O124	—	1[3]
O25	1	3[3]	O128	—	1
O26	—	1[2+4]	O129	—	1[3]
O28	1	1[3]	O135	—	1[3]
O30	1	—	O136	—	1[3]
O32	—	1[3]	O138	1	1
O44	—	1	O141	1	1
O45	1	—	O142	—	1
O50	1	—	O143	—	1[3]
O62	1	1	O144	—	1[3]
O63	1	—	O148	—	3
O71	1	—	O151	—	3
O75	2	1	O152	—	1[3]
O78	—	1[2]	O153	—	3
O82	1	—	O158	—	1
O83	1	—	O159	—	1
O85	1	1	O162	—	6
O86	1	—	not deter-	2	2
O91	1	—	mined	—	—

Total number of lung toxic strains, 72 (26 serogroups)
Total number of lung negative strains, 63 (32 serogroups)
[1] control strains isolated from enteric cases; [2] strains producing enterotoxin; [3] Serény-test positive strains: [4] strains belonging to serogroups associated with infantile enteritis

in the rapid or in the delayed test [19]. On the other hand, 18 hr after intradermal inoculation, necrotic lesions developed in the rabbits skin. This test as an assay method showed no advantage over the mouse lung test, and was less sensitive than the latter. No toxic effect could be detected in the rabbit ligated loop (index below 0.48) and the suckling mice oral test (index below 0.06) even with concentrated material.

Table II

Lung toxicity of standard (Copenhagen) K type strains belonging to serogroup O4

Designation		Antigenic structure	Mouse lung test	
original	local		survivor infected	mean survival, hr
U 4/41	2891	O4 : K4(L) : H5	2/9	2.28
Bi 7457/41	3082	O4 : K6(L) : H5	6/9	10.33
Su 65-42	3088	O4 : K12(L) : H5	6/10	10.33
A 103	3127	O4 : K52(L) : -	9/9	—

Ultrasonic lysates or living cells did not cause fluid accumulation in the mouse foot pad test (index with undiluted ultrasonic lysate: 1.1), but a marked haemorrhagic necrosis, with frequent death of the animals could be observed. According to data summarized in Table III of a concentrated freeze-dried material with a LD_{50} of 1600 μg about 125 μg was needed to evoke the necrotic effect. Similar results were obtained with living bacteria. Sensitivity of the foot pad test was somewhat lower than the lung test (Table III), therefore it had no advantage.

A cell culture microtest was found useful in testing the cytotoxic capacity. Among several cell lines (HeLa, HEp-2, mouse fibroblast) tested, the human amniotic cell line AV-3 proved to be the most sensitive one. Testing freeze-dried material, about a dose of 125 μg showed a cytotoxic end point (12–16% of the cells remained unaffected). Similar results were obtained with other extracts.

4. *Molecular weight range of the lung toxic material.* Of the freeze-dried ultrasonic lysate 200 mg was fractionated on Sephadex G-100 column. Fractions were tested for cytotoxicity in AV-3 cells (Table IV, Fig. 1). The low toxicity obtained led us to test the positive fractions qualitatively in a modified microtest. About 10^4 cells in 0.1 ml of medium were mixed with 0.1 ml of undiluted Sephadex fraction. After incubation overnight at 37°C the wells were emptied, washed and the remaining cells were fixed with methanol. Then 0.1 ml of 4-nitrophenyl-phosphate (1 g/litre) in 10% diethanolamine buffer was added to each well. After 30 min at room temperature, extinctions expressing phosphatase activity were determined by a SPECOL spectrophotometer at 400 nm. As it is seen in Table IV and Fig. 1, a sharp peak of cytotoxic activity characterized the fractions 13–15. Comparing it with other materials separated on the same Sephadex column (e.g. *E. coli* LT), it indicates a high molecular weight (100 000 daltons) of the lung toxic material.

In view of the low toxicity of the fractions, it was not possible to test them in the mouse lung. Nor could we test their antigenicity, as the neutralizing capacity of antibodies produced in rabbits with the fractions 13–15 could not

Table III

E. coli lung toxin in mouse lung and foot pad tests

Toxic agent	Amount	Test	Survivors/ inoculated	Mean time of death, hr	Necrosis/ inoculated
Living bacteria, strain "Kreka"	3.7×10^8	lung	0/5	6.2	.
	0.9×10^8		0/5	5.2	.
	4.7×10^7		1/5	8.25	.
	2.3×10^7		4/5	20.00	.
	killed 60°C 30 min		5/5	.	.
	$LD_{50} = 3.0\times10^7$ germs				
	3.7×10^8	foot pad	4/5	20.0	3/5
	0.9×10^8		4/5	44.0	3/5
	4.7×10^7		5/5	—	2/5
	2.3×10^7		5/5	—	1/5
	killed 60°C 30 min		5/5	—	0/5
Concentrated ultrasonic lysate, strain "Kreka"	2000 μg	lung	2/5	40.0	.
	1000 μg		3/5	60.0	.
	500 μg		3/5	60.0	.
	250 μg		3/5	84.0	.
	125 μg		4/5	96.0	.
	58°C — 30 min		5/5	—	.
	$LD_{50} = 900$ μg				
	2000 μg	foot pad	1/5	48.0	5/5
	1000 μg		5/5	—	5/5
	500 μg		5/5	—	4/5
	$LD_{50} = 1600$ μg				

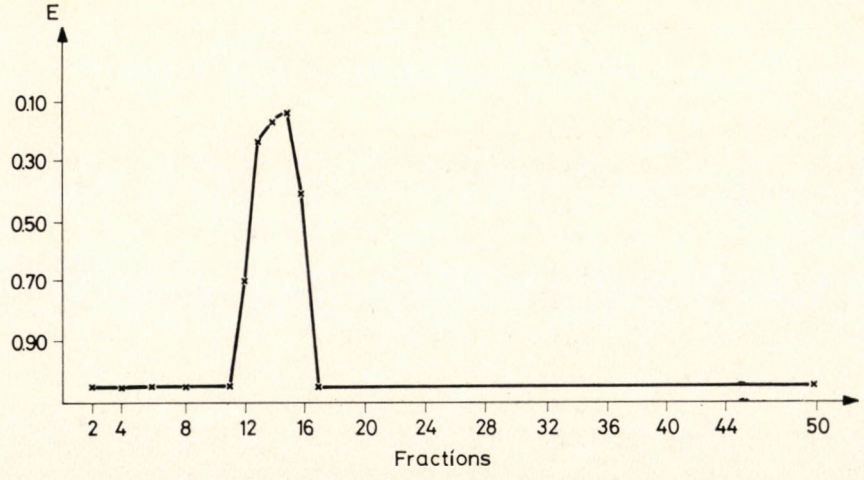


Fig. 1. Cytotoxicity in AV-3 cells, Sephadex G-100 fractions of ultrasonic lysate of strain "Kreka" (O4 : K12 : H5). E = extinction values for phosphatase activity of cells

Table IV

Cytotoxicity in AV-3 cells of Sphadex G-100 fractions of ultrasonic lysates of strain "Kreka" (O4: H5), as evaluated by phosphatase activity

Material (fracction No.)	Cytotoxicity	Extinction at 400 nm
1–12	—	.
3	—	1.20
12	+	0.70
13	++	0.24
14	++	0.18
15	++	0.15
16	+	0.41
17–50	—	1.20
Not fractionated material	++	0.20
S. dysenteriae 1 crude filtrate	++	0.64
Cell control	—	1.40

be measured with a material of low toxicity. No cross neutralization was observed in experiments carried out with *Shigella dysenteriae* 1 enterotoxin in AV-3 cells, or with *E. coli* LT in CHO cells with their corresponding antitoxins.

5. *Correlation between lung toxic activity and haemolytic capacity.* Since the isolated substance exerted a haemorrhagic effect in both the lung test and the foot pad test and as a toxic haemolysin [18] caused oedema when given intravenously into mice, the possibility of a certain connection between toxic action and haemolysis was assumed.

Investigating the haemolytic character of the lung toxic strains, a striking correlation was found. Data presented in Table V demonstrate that all the 72 lung toxic strains investigated were haemolytic, whereas all the 73 lung negative strains were non-haemolytic. Furthermore, 5 subcultures of originally lung positive strains which had lost their mouse lung toxicity, were also lacking a haemolytic capacity.

For the presence of haemolysin, in supernatants of their broth culture 24 strains were tested. The haemolytic activity was weak with titres between 1 : 2 and 1 : 32. A similarly weak activity was found in ultrasonic lysates, too. Due to the low yield of haemolysin, we have no clear proof whether the cytotoxin is identical with haemolysin. Fractions with positive cytotoxicity showed a weak haemolytic activity, and this prevented reliable evaluation.

In order to overcome these difficulties we have tried to isolate spontaneous non-haemolytic derivatives from a few strains. These efforts were unsuccessful. Experiments to cure plasmids by acridine dyes, sodium dodecyl

Table V

Correlation between mouse lung toxicity and haemolytic capacity among E. coli strains

Lung toxicity	Number of strains		
	total	haemolytic	non haemolytic
Lung toxic strains	72	72	none
Lung non toxic strains	73	none	73
Subcultures had lost lung toxicity	5	lost	5

Table VI

Mouse lung toxicity of haemolytic E. coli strains and their derivatives with impared haemolytic activity

Wild-type strains	Lung toxicity, survivors/ infected	Derivatives	Yield by	Lung, toxicity, survivors/ infected	Haemolysis	
					blood agar	over-layer
2891		2891/3		1/20	—	++
O4 : K3 : H5		2891/4		1/10	—	++
K type strain	4/20	2891/5		3/10	—	++
		2891/6		9/10	—	—
		2891/7		0/10	—	++
3082		3082/1		10/10	—	—
O4 : K6 : H5	2/20	3082/3		10/10	—	—
K type strain		3082/8	EMS	14/15	—	—
3088						
O4 : K12 : H5	2/15	3088/6	treatment	2/5	—	++
K type strain		3088/10		15/15	—	—
3976	5/15	3976/2		15/15	—	—
O4 strain		3976/3		5/5	—	—
		659/1		5/5	—	—
659		659/2		5/5	—	—
O4 : H5	2/5	659/3		5/5	—	—
strain		659/4		5/5	—	—
		659/5		5/5	—	—
Bp-S-57* O139	5/5	.		.	+++	+++
M-S-15* O139	5/5	.		.	+++	+++
281/54** O4	4/10			.	++	—
		281/54/Hly−	plasmid curing	5/5	—	—

* Swine oedema disease strain
** *E. coli* strain, carrying Hly plasmid which informs haemolysin

sulphate and etidium bromide have also failed. Therefore these strains were mutagenized by EMS and non-haemolytic colonies were selected. These derivatives, together with their wild-type parents, were tested in the mouse lung. With some mutants an incongruency was observed between the loss of the haemolytic character and the loss of mouse lung toxicity. Therefore all the clones were tested further for haemolysin production with the sensitive overlayer method. In this way it was found that the 12 mutants had no haemolytic and lung toxic activity. These data are summarized in Table VI. Data of control strains included in Table VI suggest that caution is needed to consider the haemolytic character the sole indicator of lung toxicity. Two strains of *E. coli* serogroup O139, freshly isolated from swine oedema disease, showed a well expressed alpha-type haemolytic activity with weak lung toxicity (crepitation was observed only). On the other hand, the *E. coli* strain No. 281/54 produced haemolysin, informed by a Hly plasmid. This strain was toxic for the mouse lung in the presence of the Hly plasmid, but the toxicity disappeared after effective plasmid curing. Unfortunately, the transferred Hly plasmid is highly labile in *E. coli* K-12 host and this may explain why the lung toxicity cannot be demonstrated by Hly^+ K-12 derivative.

Discussion

In addition to the well known nosological units of pathogenic *E. coli* the existence of other units with a novel pathomechanism has to be expected as causative agents of enteral or extraintestinal diseases. There are data [20, 21] that *E. coli* strains isolated from human meningitis have meningococcal cross-reacting antigens. Other data suggested the pathogenic role of colicin V [22]. The *E. coli* strains isolated in the present study carried some toxic material easily demonstrable by the mouse lung model. The same toxic material had a cytotoxic effect in AV-3 cell cultures, caused necrosis in the rabbit skin after intracutaneous inoculation, and haemorrhagic necrosis with oedema in the mouse foot pad.

The lung toxic strains belonged to different serogroups with the predominance of O4, O6 and O18. Three of the lung positive K type strains of serogroup O4 carried different K antigens (K3, K6 and K12) but identical H (H5) antigens. The negative strain (O4 : K52 : —) was not motile. These limited data are not conclusive either in connection with antigen O4 or with H5.

The low yield and the cell-bound nature of the toxic material caused difficulties in setting up experiments. Culture supernatants were practically non-toxic, and an effective extraction method is lacking. Concentrated ultrasonic lysates passed through Sephadex G-100 column allowed to determine that the toxin has a molecular weight of more than 100 000 daltons, which

suggests that the heat-labile toxin has antigenicity. Due to the low toxicity we were unable to demonstrate any neutralizing effect of the antiserum produced. No cross neutralization was observed with the lung toxin and *E. coli* LT and *S. dysenteriae* 1 enterotoxin or antitoxins.

SMITH [18] described an alpha-type toxic haemolysin, which evoked lung oedema in mice after intravenous infection. This observation and theoretical considerations have made us to investigate the relationship between the lung toxin and haemolysins. It was then found that all the lung toxic strains were haemolytic. Strains which had lost their lung toxicity during subcultivation were negative for haemolytic activity. Five lung toxic, haemolytic strains were mutagenized by EMS and Hly$^-$ mutants were selected. All of them were negative in the lung test.

Our data suggest an identity of the toxin and the haemolysin, but the question needs further investigation. It is clear that not every alpha-type haemolysin is connected with lung toxicity, as it was shown by the strains of swine oedema disease origin. On the other hand, there is a possibility that Hly informed haemolysins are connected with lung toxicity, as proved by plasmid curing.

Our data are not conclusive enough to establish a new nosological unit of *E. coli*, or the human pathogenicity of the mouse lung toxic strains. Further investigations are needed before the pathogenetic role of this type of toxin will be clarified.

Acknowledgements. We are indebted to Drs IDA and F. ØRSKOV (Copenhagen, Denmark) and Dr. B. NAGY (Szombathely, Hungary) for providing strains.

REFERENCES

1. ВОЙНО-ЯСЕНЕТСКАЯ, М. К.: ЖПЗИ **4.** 65 (1957).
2. АВГЕВА, Т. А., СТИРНОВА, Л. А., АРБУЗОВА, В. А., ДРАГУНСКАЯ, Е. М., ОЗЕРИАНОВА, А. Г.: ЖМЗИ **2,** 20 (1973).
3. SAKAZAKI, R., TAMURA, K., NAKAMURA, A., KUWATA, T., GHODA, A., TAKEUCHI, S.: Jap. J. med. Sci. **27,** 19 (1974).
4. VAN HEYNINGEN, W. E., GLADSTONE, G. P.: Brit. J. exp. Path. **34,** 202 (1953).
5. SERÉNY, B.: Acta microbiol. Acad. Sci. hung. **4,** 367 (1957).
6. DONTA, S. T., SMITH, D. M.: Infect. Immun. **9,** 500 (1974).
7. GUERRANT, R. L., BRUNTON, L. L., SCHNAITMAN, T. C., REBHUN, L. I., GILMAN, A. G.: Infect. Immun. **10,** 320 (1974).
8. DE, S. N., BATTACHARYA, K., SARKAR, J.: J. Path. Bact. **71,** 201 (1956).
9. EVANS, D. G., EVANS, D. J. JR., GORBACH, S. L.: Infect. Immun. **8,** 715 (1973).
10. LEXOMBOON, U., GOTH, A., FINKELSTEIN, T. A.: Res. Comm. Path. Pharmacol. **2,** 245 (1971).
11. JACKS, T. M., WU, B. J.: Infect. Immun. **9,** 342 (1974).
12. PÁCSA, S., HANKOVSKY, O., HIDEG, K.: Eur. J. Cancer **10,** 193 (1974).
13. TAKÁTSY, GY.: Acta microbiol. Acad. Sci. hung. **3,** 191 (1955).
14. VALTON, J. R., SMITH, D. H.: J. Bact. **98,** 304 (1969).
15. CALLAHAN, L. T., III., RYDER, R. C., RICHARDSON, S. H.: Infect. Immun. **7,** 611 (1971).
16. CLARK, A. J.: J. Cell. Physiol. **70,** 165 (1967).
17. KÄRBER, G.: Arch. exp. Path. Pharmak. **162,** 480 (1931).

18. SMITH, H. W.: J. Bact. Path. **84,** 197 (1963).
19. SANDEFUR, P. D., PETERSON, J. W.: Infect. Immun. **14,** 671 (1976).
20. GLODE, M. P., SUTTON, A., MOXON, E. R., ROBBINS, J. B.: Infect. Immun. **16,** 75 (1977).
21. GLODE, M. P., ROBBINS, J. B., LIU, T-Y., GOTSCHLICH, E. C., ØRSKOV, I., ØRSKOV, F.: J. Infect. Dis. **135,** 94 (1977).
22. SMITH, H. W., HUGGINS, M. B.: J. gen. Microbiol. **92,** 335 (1976).

Addresses of the authors:

IVÁN KÉTYI, LEVENTE EMŐDY, TIVADAR KONTROHR, ADELE VERTÉNYI, SÁNDOR PÁCSA
Institute of Mirobiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

TATJANA A. AVDEEVA, N. V. SAFONOVA, N. K, GOLUTOVA
Institute of Epidemiology and Microbiology, Pasteur Institute of Leningrad
Ul. Mira 14, Leningrad, 197101, USSR

Acta microbiol. Acad. Sci. hung. 25, 319—325, 1978

ENTEROTOXIN PRODUCTION BY SHIGELLA FLEXNERI TYPE 2A, STRAIN No. M42–43*

I. Kétyi, Adele VerténYi, S. Pácsa and B. Kocsis

Institute of Microbiology, University Medical School, Pécs

(Received February 8, 1978)

Enterotoxin produced by *Shigella flexneri* type 2a, strain M42–43, is similar to "Shiga-like" cytotoxic enterotoxin and shares common features with that of other *S. flexneri* strains. On the basis of molecular filtration and neutralization experiments it is suggested that the same molecule carries these biological characters.

Shigella flexneri type 2a strain M42—43 isolated and characterized by O'Brien *et al.* [1] produces a "Shiga-like" cytotoxic enterotoxin. Keusch and Jacewicz [2] showed that neutralizing antibodies against *Shigella dysenteriae* 1 enterotoxin could be detected in sera of patients after infection by *S. dysenteriae* 1 or other shigellae. They concluded that such enterotoxins might widely be produced by *Shigella* species.

It has been shown previously [3, 4] that nearly all of the investigated *S. flexneri* strains produced a heat-stable (ST) enterotoxin with the unique features of a high molecular weight and antigenicity. In this report we present data regarding the relationship between such ST and the "Shiga-like" enterotoxin produced by the strain M42—43.

Materials and methods

Strains. M42–43 of *S. flexneri* type 2a was kindly provided by Dr. S. B. Formal (Walter Reed Army Research Institute, Washington, D.C.). *S. dysenteriae* 1 No. 16 (P2 relevator strain) and *S. flexneri* type 3a No. 140 originated from our collection.

Medium and cultivation. Sakazaki medium [5] modified by us [6] was used for production of enterotoxins: Levinthal broth 1%, mannitol 0.1%, NaCl 0.35%, K_2HPO_4 0.368%, KH_2PO_4 0.132%, pH 7.6. Cultures were incubated at 37°C in a shaker (New Brunswick, Psychrotherm) for 18 or 48 hr (in case of Heyningen extraction).

Lysates and extracts. Ultrasonic treatment was performed at 0°C (MSE, 500 W, full energy setting). For enterotoxin extraction from *S. dysenteriae* 1 and in some cases from M42–43, the alkaline extraction method of Van Heyningen and Gladstone [7] was used. Lysates and extracts were dialysed against tap water for 2 days and against distilled water for 2–3 days, membrane filtered (Membranfilter Gesellschaft, Göttingen, No. 1121) and freeze-dried. The freeze-dried materials were distributed in vials, dried further over P_2O_5, then filled with N_2 gas and stored at —20°C.

* Supported by the Scientific Research Council, Hungarian Ministry of Health (3-10-0305-03-1/K).

Sephadex G-100 fraction was performed on a 1.5×90 cm column with 200 mg of freeze-dried material dissolved in a buffer used also as eluent: 0.005 M Tris/HCl buffer pH 7.6, containing 0.001 M EDTA. Eluation speed was 20 ml/hr and samples of 5 ml each were collected (50 fractions). The same column was used for all materials. Fractions were stored at −20°C. In cases of low toxicity, fractions were concentrated after dialysis by freeze-drying and diluted 1 : 10.

Antitoxic antisera were produced by immunizing rabbits according to CALLAHAN *et al.* [8]. Active Sephadex G-100 fractions were mixed with Freund (Difco) adjuvant (1 : 1) and rabbits weighing 2.5–3.5 kg were inoculated subcutaneously into the axillary and inguinal region with 0.5 ml four times and thereafter with 1 ml of the mixture at weekly intervals. One week after the fourth injection the animals were bled, sera were collected, sterilized by filtration and stored at −20°C without chemical preservative.

Enterotoxigenicity. 1. *Suckling mouse* test was performed according to JACKS and WU [9] using 3-day-old mice. Fluid accumulation was measured 3 hr after inoculation.

2. *Blueing test.* The "rapid" and "delayed" forms were carried out according to SANDEFUR and PETERSON [10].

3. *LT activity* was tested in CHO cells [11] grown in Linbro plastic plates in HEPES-buffered minimal essential medium. Elongation of cells was determined after an overnight incubation with the fractions of enterotoxic material. Choleragen served as the positive control.

4. *Cytotoxicity* was tested in a human amniotic cell line (AV-3) by a micromethod [12].

Neutralization. 1. *For suckling mouse test* 0.05 ml of crude enterotoxin was mixed with 0.05 ml of serum dilution and incubated at 37°C for 30 min and at 4°C for 1 hr. With each mixture 4–5 animals were inoculated. After 3 hr, body and bowel weights of the groups of suckling mice were measured. For evaluation the last negative (under index 0.07) and the first positive dilutions of a given serum were used. The neutralizing dose limit of a serum was determined by graphical plotting.

2. *In AV-3 cell culture* was determined the highest dilution of sera still inhibiting the cytotoxic effect of 1–3 tissue culture cytotoxic doses.

Results

1. *Enterotoxic activity of ultrasonic lysate from strain M42—43.* As described in Materials and methods, ultrasonic lysate as well as Heyningen extract concentrated by freeze-drying produced a cytotoxic effect similar to that of *S. dysenteriae* 1 enterotoxin, and was active in the suckling mice test, too (Table I). Tests specific for LT (delayed type of blueing and CHO) were neg-

Table I

Comparison of enterotoxin containing substances concentrated by freeze-drying

Tests	Minimal quantities of crude enterotoxin causing reaction		
	S. flexneri 2a M42-43 ultrasonic	*S. flexneri* 3a 140 ultrasonic	*S. dysenteriae* 1 16 Heyningen
Cytotoxic effect in AV-3 cells	937 μg	—	58 μg
Cytotonic effect in CHO cells	—	—	—
Suckling mice bowel/body weight index: 0.07	1200 μg	1600 μg	—
Blueing-test (rapid)	600 μg	800 μg	—
Blueing-test (delayed)	—	—	—

— Means negative reaction with high doses

ative, while a rapid PF activity could be demonstrated in all *S. flexneri* lysates.

Comparing the toxic activity of M42—43 with the control materials of *S. dysenteriae* 1 and *S. flexneri* 3 No. 140, it was concluded that in suckling mice the toxic activity of *S. flexneri* materials is approximately the same, but the cytotoxicity of M42—43 is much weaker than that of *S. dysenteriae* 1.

The M42—43 enterotoxic material retained its toxicity for suckling mice after heat treatment (100°C for 30 min), but a heat treatment of 65°C for 30 min destroyed its cytotoxicity.

2. *Molecular weight range.* In these experiments 200 mg of Heyningen extracts of M42—43 and *S. dysenteriae* 1, ultrasonic lysate of strain 140, all concentrated by freeze-drying, were fractionated on Sephadex G-100 column. Fractions were tested for enterotoxic activity. In case of M42—43 fractions, due to their low toxicity, it was necessary to concentrate these fractions (see Materials and methods).

Data are summarized in Table II and Fig. 1. Results of these experiments showed two peaks of cytotoxic activity. Among the fractions of M42—43, 14–16, 38 and 39 were found to be enterotoxic. The first, high molecular weight fractions (14–16) were toxic, while the second peak fractions (38, 39) of low molecular weight were not toxic for suckling mice. The first peak fractions were, like the only peak of *S. flexneri* 3 ST in the molecular weight range of 100 000 as determined earlier with *E. coli* LT [4]. There are data to show that this activity was in the retention of AMICON XM100A ultrafiltration. The cyto-

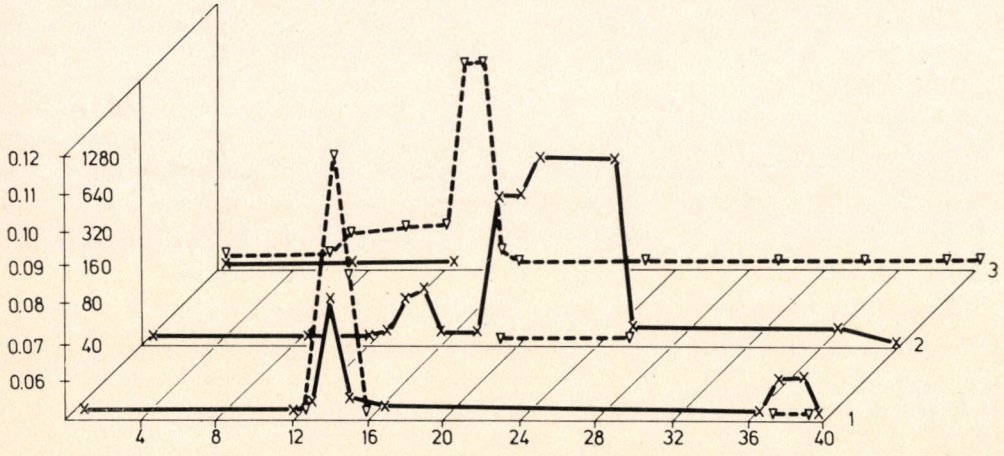


Fig. 1. Toxicity of Sephadex G-100 fractions of freeze-dried ultrasonic lysates or Heyningen extracts. 1. *S. flexneri* 2a M42–43 (dialysed fractions of Heyningen extract concentrated to 1 : 10 by freeze-drying); 2. *S. dysenteriae* 1 No. 16 (Heyningen extract); 3. *S. flexneri* 3a No. 140 (ultrasonic lysate); ×———× cytotoxicity in AV-3 cell culture, ▽— — —▽ suckling mice bowel/body weight index. Abscissa: number of fractions, odinate: curves of cytotoxic titres or suckling mice indexes

Table II

Comparison of Sephadex G-100 fractions of concentrated ultrasonic lysates or Heyningen extracts

Fraction No.	*S. flexneri* 2a M42-43 Heyningen extract*		*S. flexneri* 3a 140 ultrasonic lysate suckling mice index	*S. dysenteriae* 1 16 Heyningen extract cytotoxicity titre (AV-3)
	cytotoxicity titre (Av-3)	suckling mice index		
1–12	—	.	—	—
13	6**	.	0.088	10±
14	100	0.119	0.088	40±
15	50	0.092	—	40±
16	12	0.076	—	40±
17	6	.	—	10
18	—	.	—	10
19–20	—	.	—	160
21–25	—	.	—	320
26–36	—	0.06–0.066***	—	10±
37	6	.	—	10±
38	24	0.058	—	—
39	24	0.059	—	—
40–50	—	.	—	—

* Fractions were dialysed and concentrated 1 to 10 by freeze-drying
** Reciprocal titres
*** Only fractions 32 and 34 were tested
— Means cytotoxic titre below 1 : 6, or suckling mice index below 0.060
. Not tested

toxic activity of *S. dysenteriae* 1 material showed a wide distribution; it may be due to its higher toxic concentration, inhibiting sharper separations.

3. *Neutralization.* Table III shows the result of neutralization experiments carried out in AV-3 cell culture. The highly antitoxic antiserum against *S. dysenteriae* 1 neutralized the homologous enterotoxin effectively, but was of low titre against M42—43 fractions. It was striking that anti-*S. flexneri* ST serum should have neutralized M42—43 fractions. The antitoxin content of this serum is low, about the same homologous neutralization titres were observed in earlier experiments [4].

Results of neutralization experiments performed in suckling mice are summarized in Table IV. This model allowed to test the heat-treated M42–43 enterotoxin, too. Results with native toxin were in agreement with those of tissue culture neutralization experiments. The *S. dysenteriae* 1 antitoxin had

Table III

Neutralization of S. dysenteriae 1 enterotoxin and toxic fractions of S. flexneri 2a M42-43 in AV-3 cell culture

Antitoxin	Reciprocal titre of neutralization against		
	S. dysenteriae 1 fractions 21–25*	*S. flexneri* 2a M42-43	
		fraction 14**	fraction 38***
Antitoxin against *S. dysenteriae* 1	256	8	8
Antitoxin against *S. flexneri* ST	—	8	16
Antitoxin against *E. coli* LT	—	.	.

* Fraction with high cytotoxicity titre (1 : 320)
** High molecular weight fraction causing cytotoxic effect (1 : 100) and fluid accumulation in suckling mice bowel (index 0.119)
*** Low molecular weight fraction with cytotoxicity (1 : 24), but without suckling mice activity
— Titre below 1 : 4
. Not tested

Table IV

Neutralization of S. flexneri 2a M42-43 enterotoxin in suckling mouse test

Antitoxin against	Antitoxin dilution	*S. flexneri* 2a M42-43 crude enterotoxin(5 mg)	
		native index*	100°C, 30 min index
S. dysenteriae 1	1 : 2	0.056	0.069
	1 : 4	0.056	0.078
	1 : 8	0.069	0.078
	1 : 16	0.079	0.081
	1 : 32	0.092	.
	control	0.114	0.078
End point titre**		1 : 8.8	1 : 2?
S. flexneri ST	1 : 2	0.056	0.058
	1 : 4	0.056	0.059
	1 : 8	0.062	0.068
	1 : 16	0.102	0.087
	control	0.114	0.085
End point titre**		1 : 9.6	1 : 9.6

* Bowel/body weight
** Antitoxin titres reaching the 0.07 end point; for end point determination see text

an expressed neutralizing capacity but at low titre. Antiserum produced against *S. flexneri* ST showed the same effect. After heat treatment, which caused a loss of cytotoxicity, no neutralization was observed by *S. dysenteriae* 1 antitoxin. On the other hand, the neutralizing capacity of *S. flexneri* ST-antitoxin did not change.

Discussion

In previous studies [3, 4] we presented data suggesting that all the *S. flexneri* strains tested, including M42–43, were capable of producing a heat-stable enterotoxin. This enterotoxin has certain unique features regarding its heat and acid stability and its toxic effect is carried by a molecule of high molecular weight with antigenicity. Its molecular weight is approximately 100 000 on the basis of Sephadex G-100 fractionation and Amicon ultrafiltration.

Sephadex fractionation of M42–43 lysate or extract revealed two cytotoxicity peaks. One is in the high molecular weight range with toxic activity for suckling mice and the second in the low molecular weight range, without toxicity to suckling mice. Similar observations were made by KEUSCH and JACEWICZ [13] with fractionation of *S. dysenteriae* 1 enterotoxin. The second peak in their experiments was cytotoxic but caused no fluid accumulation in the ligated rabbit loop. Such a phenomenon was not observed by us with *S. dysenteriae* 1 toxin. If the wide range of molecular distribution is not a similar phenomenon, the occurrence of toxic subunits may explain the situation.

From data obtained by Sephadex fractionation and neutralization experiments it was assumed that a single molecule carries both cytotoxicity and ST. Therefore, this kind of enterotoxin might not be identical with the *S. dysenteriae* 1 enterotoxin. This is supported by the fact that the cytotoxicity can be neutralized with antitoxin in AV-3 cell culture only partially. On the other hand, this kind of toxin shows all characters unique for *S. flexneri* ST.

The antigenic relationships between choleragen, *E. coli* LT, *S. flexneri* ST, and this cytotoxic enterotoxin, furthermore the biological relations of enterotoxins suggest that the phenomenon is governed by modulation of one *tox* gene. The role of plasmid elements in this evolutionary divergence is an open question.

Acknowledgements. We are indebted to Dr. S. B. FORMAL (Washington, D.C., USA) for providing *S. flexneri* 2a strain M42–43 and to Dr. B. ROWE (London, England) for the CHO cell line.

REFERENCES

1. O'Brien, A. D., Thompson, M. R., Gemski, P., Doctor, B. P., Formal, S. B.: Infect. Immun. **15,** 796 (1977).
2. Keusch, G. T., Jacewicz, M.: Nature new Biol. **241,** 31 (1973).
3. Kétyi, I., Málovics, I., VerténYi, A., Kontrohr, T., Pácsa, S., Kuch, B.: Acta microbiol. Acad. Sci. hung. **25,** 165 (1978).
4. Kétyi, I., VerténYi, A., Málovics, I., Kontrohr, T., Pácsa, S.: Acta microbiol. Acad. Sci. hung. **25,** 219 (1978).
5. Sakazaki, R., Tamura, K., Nakamura, A., Kuwata, T., Ghoda, A., Takeuchi, S.: Jap. J. med. Sci. **27,** 19 (1974).
6. Kétyi, I., Czirók, É., VerténYi, A., Málovics, I., Pácsa, S.: Acta microbiol. Acad. Sci. hung. **25,** 23 (1978).
7. Van Heyningen, W. E., Gladstone, G. P.: Brit. J. exp. Path. **34,** 202 (1953).
8. Callahan, L. T. III., Ryder, R. C., Richardson, S. H.: Infect. Immun. **7,** 611 (1971).
9. Jacks, T. M., Wu, B. J.: Infect. Immun. **9,** 342 (1974).
10. Sandefur, P. D., Peterson, J. W.: Infect. Immun. **14,** 671 (1976).
11. Guerrant, T. L., Brenton, L. L., Schaitman, T. C., Rebhun, L. I., Gilman, A. G.: Infect. Immun. **10,** 320 (1974).
12. Pácsa, S., Hankovszki, O., Hideg, K.: Eur. J. Cancer **10,** 193 (1974).
13. Keusch, G. T., Jacewicz, M.: J. infect. Dis. **131,** 33 (1975).

Address of the authors:

Iván Kétyi, Adele Vertényi, Sándor Pácsa, Béla Kocsis
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

Acta microbiol. Acad. Sci. hung. 25, 327—329, 1978

NOTE

OCCURRENCE OF TRICHOPHYTON VANBREUSEGHEMII IN HUNGARY

J. GALGÓCZY

National Institute of Hygiene, Budapest

(Received October 3, 1977)

The occurrence of *Trichophyton vanbreuseghemii* in Hungary is reported. The micromorphological characteristics of the species are discussed.

World-wide investigations of soil samples have shown that among the species regarded as dermatophytes, *Microsporum gypseum*, *Trichophyton terrestre* and *T. ajelloi* can be isolated from soil at high frequency. *M. cookei* is less common, though this species, too, is of world-wide occurrence. The remaining geophil dermatophytes occur rarely. *T. vanbreuseghemii*, one of the rare species, was described by RIOUX *et al.* in 1964 [1]. Its perfect form was designated as *Arthoderma gertleri* by BÖHME in 1967 [2]. The same species was isolated by ALTERAS and EVOLCEANU [3] and PADHYE [4] from soil samples collected in Roumania and Canada, respectively. Considering the rare occurrence of *T. vanbreuseghemii* it seems justified to report on its isolation from soil samples collected in Hungary.

Materials and methods. Using the hair-bait method, we have regurarly examined soil samples collected from different parts of Hungary. The fungus grown on hair is transferred on to Sabouraud glucose agar plates containing 50 μg/ml chlorampenicol and 0.5 mg/ml cycloheximide. To analyse micromorphological properties, preparations of microcultures were stained with lactophenol–cotton blue.

Results. A fungus different from the commonly occurring species was cultured from a soil sample collected from a ditch in Tököl, Central Hungary.

Macroscopic picture of the 5 to 6-day-old culture: floccose and granular cream-coloured colonies 3 cm in diameter, yellowish-brown from the reverse side.

Microscopic picture: among a great number of pyriform microconidia, clusters of multiseptate, smooth-walled macroconidia were noticed. Thus, the clusters appeared hedgehog-like. Being three-dimensional, the most characteristic clusters, 35–55 × 7–8 μm in size, could not be photographed. In several fields spirals were also seen (Fig. 1). In lactophenol–cotton blue preparations, advanced fragmentation of macroconidia was observed (Fig. 2).

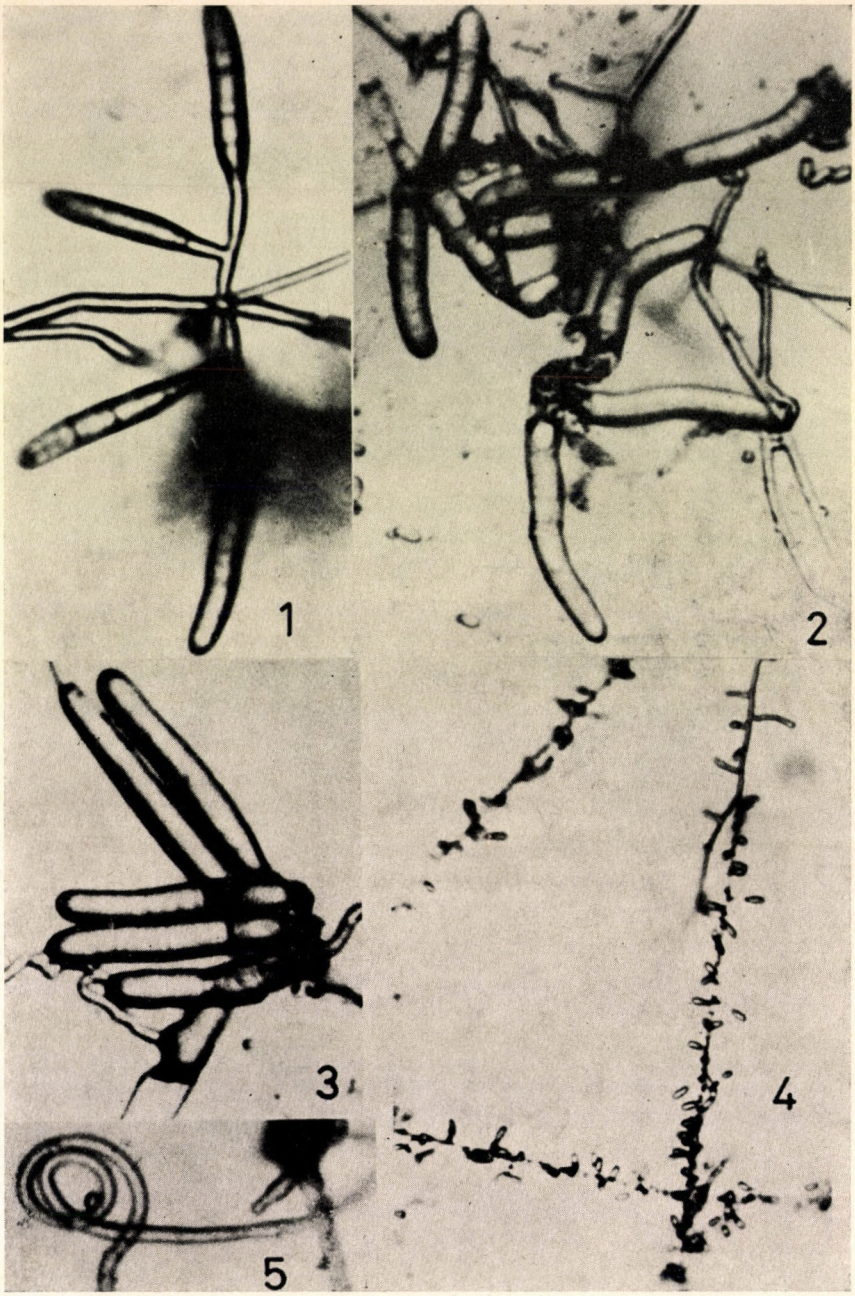


Fig. 1. T. vanbreuseghemii. Clusters of macroconidia *in situ* (1); clusters of macroconidia *in situ* under coverslip (2 and 3); microconidia and a spiral *in situ* (4 and 5). Original magnification, ×160

Discussion. The characteristic clusters of radially ramifying macroconidia and their disintegration into compartments, supported by the size of the macroconidia and the occurrence of spiral forms, prompted us to identify the strain as *T. vanbreuseghemii*. We have failed to develop sexual forms because of the lack of mating types.

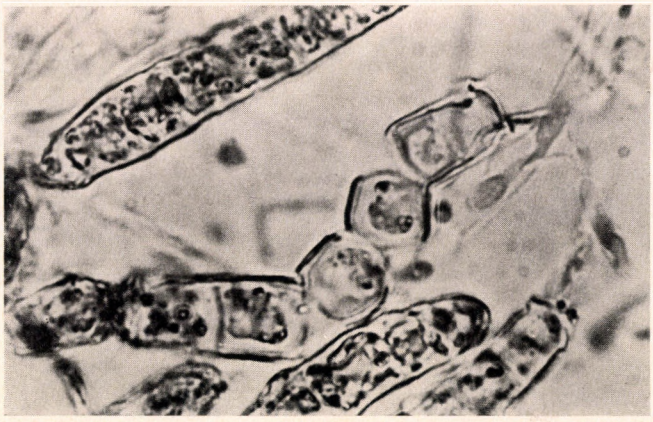


Fig. 2. T. vanbreuseghemii. A macroconidium fragmented into compartments. Lactophenol–cotton-blue staining. Original magnification, ×400

REFERENCES

1. Rioux, J. A., Jarry, D. T., Juminer, B.: Naturalia Monspel. Sér. bot. **16,** 153 (1964).
2. Böhme, H.: Mykosen **10,** 247 (1967).
3. Alteras, I., Evolceanu, R.: Mykosen **10,** 585 (1967).
4. Padhye, A. A.: Mycologia **61,** 414 (1969).

Address of the author:

József Galgóczy
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

BOOKS RECEIVED

SCHLESSINGER, D. (ed.): Microbiology. 1977. American Society for Microbiology, Washington, D.C. 1977. pp. ix + 593. Price U.S. $22.00.

HOBSON, D., HOLMES, K. K. (eds): Nongonococcal Urethritis and Related Infections. American Society for Microbiology, Washington, D. C. 1977. pp. xvi + 391. Price U.S. $14.00.

SCHLESSINGER, D. (ed.): Microbiology. 1978. American Society for Microbiology, Washington, D.C. 1978. pp. x + 449. Price U.S. $22.00.

INDEX

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária
A kézirat nyomdába érkezett: 1978. VII. 20. — Terjedelem: 9,10 (A/5) ív, 34 ábra

78.6107 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologie in deutscher, englischer, französischer und russischer Sprache.

Die *Acta Microbiologica* erscheinen in Heften wechselnden Umfanges. Mehrere Hefte bilden einen Band.

Die zur Veröffentlichung bestimmten Manuskripte sind an folgende Adresse zu senden:

Acta Microbiologica, Staatliches Institut für Hygiene, H-1966 Budapest, P.O.B. 64, Ungarn

An die gleiche Anschrift ist jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $36.00.

Bestellbar bei »Kultura« Außenhandelsunternehmen (H-1389 Budapest 62, P.O.B. 149, Bankkonto Nr. 218-10990) oder seinen Auslandsvertretungen.

Les *Acta Microbiologica* paraissent en français, allemand, anglais et russe et publient des travaux du domaine de la microbiologie.

Les *Acta Microbiologica* sont publiés sous forme des fascicules qui seront réunis en volumes.

On est prié d'envoyer les manuscrits destinés à la rédaction à l'adresse suivante:

Acta Microbiologica, Institut National d'Hygiene Publique, H-1966 Budapest, P.O.B. 64, Hongrie

Toute correspondance avec la rédaction doit être envoyée à cette même adresse.

Le prix de l'abonnement: $36.00 par volume.

On peut s'abonner à l'Entreprise du Commerce Extérieur « Kultura » (H-1389 Budapest 62, P.O.B. 149, Compte courant No. 218-10990) ou chez représentants à l'étranger.

«*Acta Microbiologica*» публикуют трактаты из области микробиологии на русском, немецком, английском и французском языках.

«*Acta Microbiologica*» выходят отдельными выпусками разного объема. Несколько выпусков составляют один том.

Предназначенные для публикации рукописи следует направлять по адресу:

Acta Microbiologica, Институт Здравоохранения, H-1966 Budapest, P.O.B. 64, Венгрия

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — $36.00 за том.

Заказы принимает предприятие по внешнец торговле «Kultura» (H-1389 Budapest 62, P.O.B. 149, Текущий счет № 218-10990), или его заграничные представительства и уполномоченные.

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

AUSTRALIA
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE
Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 145 Ackland Street, *St. Kilda (Melbourne), Victoria 3182*

AUSTRIA
GLOBUS, Höchstädtplatz 3, *1200 Wien XX*

BELGIUM
OFFICE INTERNATIONAL DE LIBRAIRIE,
30 Avenue Marnix, *1050 Bruxelles*
LIBRAIRIE DU MONDE ENTIER, 162 Rue du Midi, *1000 Bruxelles*

BULGARIA
HEMUS, Bulvar Ruszki 6, *Sofia*

CANADA
PANNONIA BOOKS, P.O. Box 1017, Postal Station "B", *Toronto, Ontario M5T 2T8*

CHINA
CNPICOR, Periodical Department, P.O. Box 50, *Peking*

CZECHOSLOVAKIA
MAD'ARSKÁ KULTURA, Národní třida 22, *115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 46, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

DENMARK
EJNAR MUNKSGAARD, Norregade 6, *1165 Copenhagen*

FINLAND
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128, *SF-00101 Helsinki 10*

FRANCE
EUROPERIODIQUES S. A., 31 Avenue de Versailles, *78170 La Celle St.-Cloud*
LIBRAIRIE LAVOISIER, 11 rue Lavoisier, *75008 Paris*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac, *75240 Paris Cedex 05*

GERMAN DEMOCRATIC REPUBLIC
HAUS DER UNGARISCHEN KULTUR, Karl-Liebknecht-Straße 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT, Straße der Pariser Kommüne 3—4, *DDR-104 Berlin*

GERMAN FEDERAL REPUBLIC
KUNST UND WISSEN ERICH BIEBER,
Postfach 46, *7000 Stuttgart 1*

GREAT BRITAIN
BLACKWELL'S PERIODICALS DIVISION, Hythe Bridge Street, *Oxford OX1 2EU*
BUMPUS, HALDANE AND MAXWELL LTD., Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate, *Wellingborough, Northants MN8 2QT*
W.M. DAWSON AND SONS LTD., Cannon House, *Folkestone, Kent CT1 5EE*
H. K. LEWIS AND CO., 136 Gower Street, *London WC1E 6BS*

GREECE
KOSTARAKIS BROTHERS, International Booksellers, 2 Hippokratous Street, *Athens-143*

HOLLAND
MEULENHOFF-BRUNA B.V., Beulingstraat 2, *Amsterdam*
MARTINUS NIJHOFF B.V., Lange Voorhout 9—11, *Den Haag*
SWETS SUBSCRIPTION SERVICE, 347b Heereweg, *Lisse*

INDIA
ALLIED PUBLISHING PRIVATE LTD., 13/14 Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD., Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTD., Books Import Division, Chandralok, 36 Janpath, *New Delhi 110001*

ITALY
EUGENIO CARLUCCI, P.O. Box 252, *70100 Bari*
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 45, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58, *20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

JAPAN
KINOKUNIYA BOOK-STORE CO. LTD., 17-7 Shinjuku-ku 3 chome, Shinjuku-ku, *Tokyo 160-91*
MARUZEN COMPANY LTD., Book Department, P.O. Box 5050 Tokyo International, *Tokyo 100-31*
NAUKA LTD. IMPORT DEPARTMENT, 2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

KOREA
CHULPANMUL, *Phenjan*

NORWAY
TANUM-CAMMERMEYER, Karl Johansgatan 41—43, *1000 Oslo*

POLAND
WĘGIERSKI INSTYTUT KULTURY, Marszałkowska 80, *Warszawa*
CKP I W ul. Towarowa 28 *00-958 Warsawa*

ROMANIA
D. E. P., *Bucureşti*
ROMLIBRI, Str. Biserica Amzei 7, *Bucureşti*

SOVIET UNION
SOJUZPETCHATJ — IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

SPAIN
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

SWEDEN
ALMQVIST AND WIKSELL, Gamla Brogatan 26, *S-101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB, Box 346, *401 25 Göteborg 1*

SWITZERLAND
KARGER LIBRI AG, Petersgraben 31, *4011 Basel*

USA
EBSCO SUBSCRIPTION SERVICES, P.O. Box 1943, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC., 15 Southwest Park, *Westwood, Mass. 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC., 140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC., 7250 Westfield Avenue, *Pennsauken N. J. 08110*

VIETNAM
XUNHASABA, 32, Hai Ba Trung, *Hanoi*

YUGOSLAVIA
JUGOSLAVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišića 1, *21000 Novi Sad*

28. XII. 1978 HU ISSN 0001—6187 Index: 26.016