ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

ADIUVANTIBUS

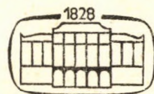
I. DÖMÖK, E. FARKAS, I. HORVÁTH

I. NÁSZ, K. RAUSS, F. B. STRAUB, J. SZIRMAI, L. VÁCZI, K. VAS, J. WEISSFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XVIII FASCICULUS I



AKADÉMIAI KIADÓ, BUDAPEST

1971

AMAHA 5 18(1) 1—74 (1971)

Acta Microbiol. Acad. Sci. Hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

Főszerkesztő

IVÁNOVICS GYÖRGY

akadémikus

Szerkesztő

LÁNYI BÉLA

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, Budapest, Keleti Postahivatal, Postafiók 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (Budapest V., Alkotmány utca 21. Bankszámla 05-915-111-46), a külföld számára pedig a „Kultúra" Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő utca 32. Bankszámla: 43-790-057-181) 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, Budapest, Keleti Postahivatal, Postafiók 64.

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

The rate of subscription is $ 16.00 a volume.

Orders may be placed with "Kultúra" Foreign Trade Company for Books and Newspapers (Budapest I., Fő utca 32. Account No. 43-790-057-181) or with representatives abroad.

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

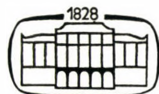
ADIUVANTIBUS

I. DÖMÖK, E. FARKAS, I. HORVÁTH,
I. NÁSZ, K. RAUSS, F. B. STRAUB, J. SZIRMAI, L. VÁCZI, K. VAS,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XVIII



AKADÉMIAI KIADÓ, BUDAPEST

1971

Acta microbiol. Acad. Sci. Hung.

INDEX

TOMUS XVIII

Fasciculus 1

Pallinger, Z., Pallinger-Szekeres, G.: Measles Immunity in Infants 1
Csányi, V., Mile, I., Ferencz, I., Szabó, E.: Some Properties of the Membrane-bound Penicillinase of *Bacillus cereus* 569 7
Hajdú, J., Ferencz, I., Mile, I., Csányi, V.: Effect of Urea on the Two Iodine Inactivation Reactions of *Bacillus cereus* Penicillinase 17
D. Tóth, F., Váczi, L., Berencsi, Cl.: Interferon and Antibody Production in Inbred Mice Infected with the Rauscher Virus 23
Gaál, V., Molnár, J., Ivánovics, G.: Relationship of Lysogeny and Megacinogeny in *Bacillus megaterium* 29
Mile, I., Csányi, V., Koczka, I., Badár, E., Horváth, I.: Effect of Pyrazocillin on the Induced Penicillinase Synthesis of *Bacillus cereus* 41
Kétyi, I., Vertényi, A., Financsek, I.: Lysogenic Conversion in the *Shigella flexneri* Group 47
Schlammadinger, J., Szabó, G.: The Effect of Theophylline upon Induced β-Galactosidase Synthesis in *Escherichia coli* 55
Szathmáry, J., Hegedűs, L., Koller, M.: Effect of Various Diluents on Stability of Vaccinia Virus Dilutions 61
Csányi, V., Mile, I., Szabó, É., Harmath, Gy., Ferencz, I.: Water Limited Reaction of *Bacillus cereus* Penicillinase in Nonaqueous Solvent 69

Fasciculus 2

Vághy, T.: Correlation between the Morphological Variability and Riboflavin Producing Capacity of *Eremothecium ashbyii* 75
Szabó Szűcs, J.: Phage Infection of Vitamin B_{12} Producing Rhizobium Cultures 81
Szita, J., Svidró, A.: Bacteriologic Diagnosis of *Yersinia pseudotuberculosis* 87
Hoch, V., Hérmán, G.: Phage Typing of D-Group Streptococci. I. Typing of Enterococci with Roumanian Phages 95
Hérmán, G., Hoch, V.: Phage Typing of D-Group Streptococci. II. Isolation of Supplementary Phages for Classification of Enterococci Untypable with Roumanian Phages 101
Molnár, J., Prágai, B.: Attempts to Detect the Presence of Teichoic Acid in *Bacillus anthracis* 105
D. Tóth, F., Váczi, L., Berencsi, Cl.: Effect of Interferon Treatment on the Tumour-Specific Antibody Response of BALB/c Mice Infected with Rauscher Virus 109
Szita, J., Káli, M., Rédey, B.: Serological Diagnosis of *Yersinia enterocolitica* 113
Hérmán, G., Milch, H.: Twenty Years Observation on the Stability of *Salmonella typhi* Phage Types 119
Ádám, M. M., Deák, S.: A New Salmonella Serotype: *Salmonella isaszeg* (48 : z_{10} : e, n, x) 133

Fasciculus 3

Csányi, V., Mile, I., Romhányi, T., Hortobágyi, P., Hatfaludi, F.: Irreversible Adsorption of Exopenicillinase on *Bacillus cereus* 569 Cells 137
Mérő, E., Ralovich, B.: Some Epidemiologic Problems of Human Listeriosis in Hungary 149
Vandra, E., Fodor, T.: Phage Typing of *Mycobacterium tuberculosis* Strains Isolated n Hungary 155

Váczi, L., Surján, M., Kiss, M. J., Füst, G., Rédai, I.: Bacterial Cardiolipins and their Serological Activity ... 159
Tenner, C., Rácz, P., Rédey, B.: Significance of the "Epithelial Phase" in Experimental Salmonella Conjunctivitis ... 167
Tenner, C., Rácz, P., Serényi, B.: Significance of "Intracellular Parasitism" in Experimental Shigella Cystitis ... 179
Geck, P.: India-ink Immuno-reaction for the Rapid Detection of Enteric Pathogens .. 191
Duc, N. D.: La recherche des Shigella dans les eaux par le test des anticorps fluorescents et par la réaction de Geck ... 197
Lantos, J.: Serological Grouping of Pseudomonas Typing Phages ... 207
Kétyi, I., Vertényi, A.: Host-Specificity Types in *Shigella flexneri* ... 213

Fasciculus 4

Tenner, C., Rácz, P., Mérő, E.: Significance of "Intracellular Parasitism" in Experimental Salmonella Cystitis ... 219
Pálfi, A. B.: Virus Content of Sewage in Different Seasons in Hungary ... 231
Serény, B., Tenner, C., Rácz, P.: Immunogenicity of Living Attenuated Shigellae 239
Weiszfeiler, G., Karasseva, V., Karczag, E.: A New *Mycobacterium* Species: *Mycobacterium asiaticum* n. sp. ... 247
Pellecuer, M., Galzy, P., Pasero, J.: Use of Mutations in Marking Industrial Yeast Strains 253
Losonczy, G., Tóth, L., Petrás, G., Bognár, S., Lányi, B., Csekes, J.: *Pseudomonas aeruginosa* Infections in an Artificial Respiratory Ward ... 261
D. Tóth, F., Karsai, T., Váczi, L.: Effects of Rauscher Leukaemia Virus on the Immune System in Susceptible and Resistant Mouse Strains ... 271
Szeri, H., Anderlik, P., Bános, S., Radnai, B.: Studies on Young Germ-free Mice. I. Stress Reaction of Germ-free Mice ... 279
Berek, L., Bános, S.: Studies on Young Germ-free Mice. II. Bone Changes in Germ-free Mice ... 283
Szathmáry, J., Hegedüs, L.: Effect of the Diluent on the Pock Count of Vaccinia Virus Dilutions ... 291
Rédai, I., Réthy, A., Sebessi-Gönczy, P., Váczi, L.: Lipids in *Staphylococcus aureus* and *Escherichia coli* Cultured in the Presence of Human Serum ... 297
Ádám, M. M., Kontrohr, T., Horváth, E.: Serological Studies on *Pseudomonas aeruginosa* O Group Lipopolysaccharides ... 307
Némedi, L., Lányi, B.: Incidence and Hygienic Importance of *Pseudomonas aeruginosa* in Water ... 319
Fornosi, F., Tálas, M., Weiszfeiler, G.: Effect of Tilorone Hydrochloride on the Experimental Rabies of Mice ... 327
Books Received ... 333

Acta microbiol. Acad. Sci. hung. 18, 1—6, 1971

MEASLES IMMUNITY IN INFANTS

By

Z. Pallinger and Gizella Pallinger-Szekeres

National Institute of Public Health (Director: T. Bakács), *Budapest*

(Received October 5, 1970)

Summary. Sera from 218 babies less than 1 year old were examined for measles antibodies by the haemagglutination inhibition test. The antibody level was found to decrease rapidly up to 5 months of age, and to persist at a low level until beyond 9 months of age it tended to rise again. These findings are in good accordance with the epidemiological curves for the age group distribution of measles, which show an abrupt increase in morbidity at 5—8 months of age.

Defining the youngest age group to be vaccinated against measles, consideration should be given to the specific antibody levels and the age distribution of incidence. Data for infants under one year of age have been reported from West Germany [1] and Japan [5, 6], while in Hungary serological screening of children 1—9 years old for measles antibodies has been described [2].

This paper is a report on the measles antibody status of infants less than 1 year old, as assessed by screening of sera from 218 babies.

Materials and methods

Antibodies were demonstrated by the haemagglutination inhibition (HI) test.

Antigen. Four strains of measles virus were used as antigens, the strains Leningrad-4, Edmonston-A, Prague-1 and the strain designated "5", isolated in this laboratory from the leukocytes of a measles patient. The infective titre of the virus strains was $10^{3.0} - 10^{4.2}$ $TCID_{50}$/ 0.1 ml in primary vervet monkey kidney epithelial cell cultures.

The haemagglutinating titre of the strains was determined individually for each lot of red blood cells. The average titre was $2^4 - 2^5$. Storage temperature was -20 °C.

Blood sera. Serum samples submitted for routine laboratory tests in a children's hospital were used throughout. Sera from patients with infectious disease or immune deficiency were excluded from the study. The sera were collected from February to July, 1967. Fresh samples were incativated at 56 °C for 30 minutes.

Haemagglutination inhibiton test. Each serum sample was tested against all the four antigens with the reaction described by Rosen [3], using Takátsy's microtitrator technique [4]. The criterion of positivity was the complete inhibition of haemagglutination of 4 antigen units by a serum dilution of at least 1 : 8. The behaviour of the serum samples was practically identical with all the four antigens.

Red blood cells freshly withdrawn from rhesus and vervet monkeys were used after washing in phosphate buffer solution pH 7.2 until de-proteinization of the supernatant, checked by the sulphosalicylic acid test.

Comparison of samples by different serological tests. At the beginning of the experiments, parallel with the HI test, 50 serum samples each were tested for virus neutralizing and complement fixing antibody contents. Results of the three tests were in good accordance.

Results

The quarterly distribution of the positive serological response of the examined 218 infants is shown in Fig. 1. The proportion of serological positivity was highest in the youngest, 0—3 month old age group, in which 28 of 38 serum samples contained antibodies to measles virus. The number of sero-

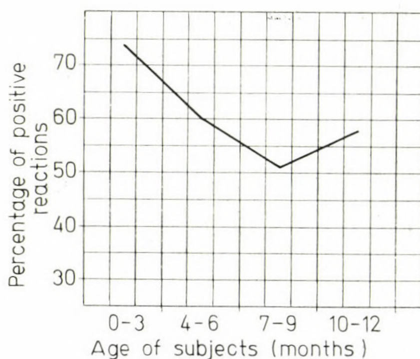


Fig. 1. Proportion of positive serological responses among babies 0—12 months old, as demonstrated by HI test

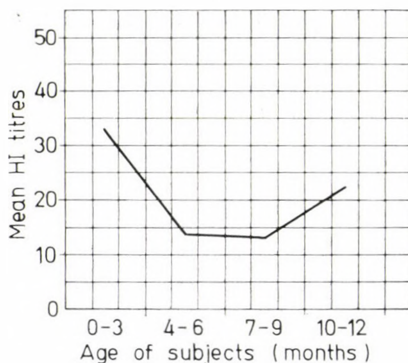


Fig. 2. Mean HI titres in serum samples from babies 0—12 months old

logically positive infants first tended to decrease with age, HI antibodies being found in only 26 of the 51 serum samples representing the 7—9 months age group, then it rose slightly as shown by the positivity of 37 from the 64 samples examined for the 10—12 months age group.

The geometric mean of the HI titres (Fig. 2) was also highest in the 0—3 months old group, showing a decreasing tendency in the second and third quarters and increasing tendency in the fourth quarter. The slopes of the curves in Figs 1 and 2 are similar, but the latter shows a steeper descent.

The percentual distribution of the HI titres is seen in Fig. 3. The proportion of serologically negative babies tended to rise up to 9 months of age, with a break at 7 months. The number of sera with titres of 1 : 64 or above tended to decrease up to 5 months; in the 6—7 months old group few serum samples had reached that level. Beyond 9 months of age the proportion of high titres tended to rise again.

Data in Fig. 3 correlate in every respect with those of Fig. 4, which show the age distribution of the reported cases of measles among 0—11 months old infants. An abrupt increase of incidence was noted at the age of 5 months when the proportion of seronegativity attained about 50%.

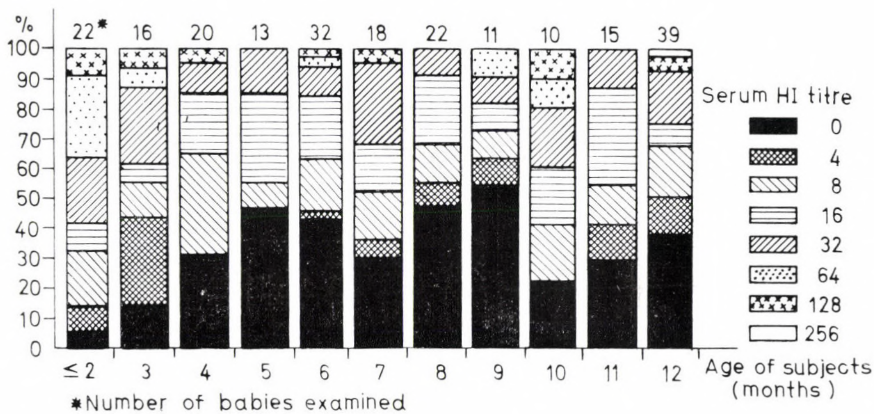


Fig. 3. Percentual distribution of HI titres in the examined age groups

Discussion

Most reports on the serological screening for measles antibodies were related to the potency testing of various measles vaccines. Examinations on infants less than 1 year old were reported by ENDERS-RUCKLE *et al.* [1], who tested sera from 180 infants in a survey of the population of West Germany. They demonstrated measles antibodies in 90% of the sera from babies less than 6 months old and in 10% of those from babies 7—12 months old. Similar findings have been described by UEDA *et al.* [5, 6] who found antibodies in the sera of all infants up to 2 months old and a subsequent decrease of the antibody level to zero at 8 months of age.

In this laboratory, a steady increase of negative serological responses was noted up to 9 months of age, with a corresponding decrease of the high, 1 : 64 or above, titre levels which were rare in the 5—8 months old group until from the 9th month on the titres tended to rise again. The increasing tendency of serological negativity and the decrease of the mean titre were very likely

related with the decline of maternal antibodies. The steep decrease of the mean titre, as shown in Fig. 2, indicated a reduction of titre levels which, however, did not always imply serological negativity. The reappearance of high titres after 9 months of age suggested a convalescent state in part of the cases, as confirmed also by the abrupt rise of the incidence of measles after 5 months of age (Fig. 4), when the proportion of serologically negative

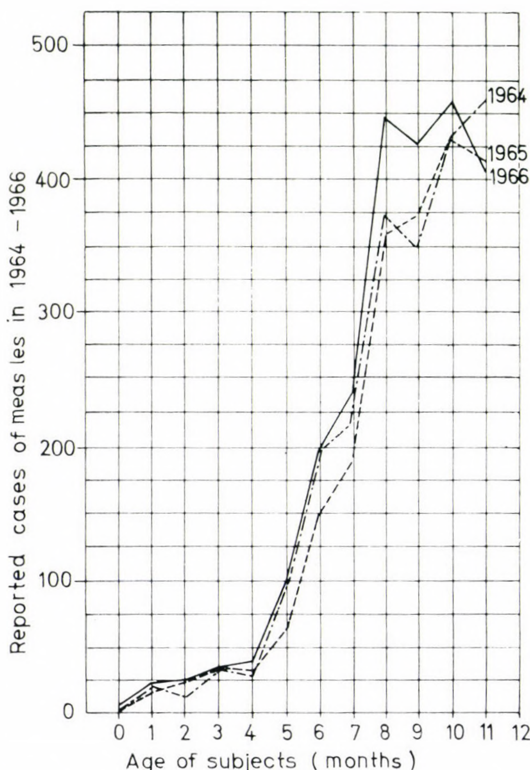


Fig. 4. Age distribution of reported cases of measles in 0—11 months old babies in the period 1964—66

individuals was highest — about 50% — in the population group studied. At 7—9 months of age the epidemiological curve showed a similar break as shown in Fig. 3. As already noted at 7—10 months of age the proportion of serologically negative babies was lower than expected; it was, therefore, checked whether the collection of the related samples had perhaps coincided with an epidemic period (latent infection, booster effect). However, no such correlation was demonstrable, as each supply of test material contained samples from all age groups examined in roughly uniform distribution. According to the epidemiological data, the period of maximum measles incidence is in April

and May, that of minimum incidence in August and September. Since the babies with a considerably reduced antibody level, *viz.* those older than 4—5 months, contracted measles in the period of maximum incidence, serum samples collected in May, June and July from 5—8 months old babies ought to show an elevated HI titre. Examining the mean HI titre assessed in the different age groups with respect to months of sampling (Fig. 5), the curves for the 0—3 and 10—12 months old age groups showed high values in February,

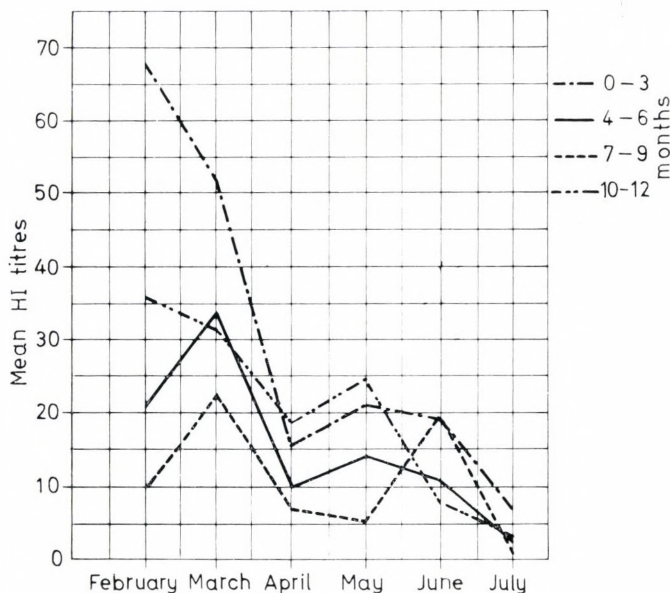


Fig. 5. Mean HI antibody titre to measles virus in sera from babies 0—11 months old in the different months of the year

fell steeply until April and after a slight rise in May, fell again until July. A similar rough parallelism was noted with the curves for the other two age groups, differing from the former only by a peak in March, after which they showed a decrease to a minimum in July.

The available literary data offer no explanation for the fluctuation of antibody levels in the different months of the year, neither for the relationship of these fluctuations with the seasonal changes of measles incidence. Nor is there information whether or not similar fluctuations occur in the other months of the year.

The slight dissimilarity of our findings to the cited literary data being possibly related to differences in the epidemiological conditions, we agree with Enders-Ruckle's view in that a serological survey of the measles status is of immediate interest in every country.

Acknowledgements. The authors are indebted to Dr. P. Baranyai, Head of the Central Laboratory, Heim Pál Children's Hospital, for supplying serum samples; and to Dr. O. Rudnai, Head of the Epidemiology Department, National Institute of Public Health, for epidemiological data.

REFERENCES

1. Enders-Ruckle, G., Siegert, R., Baum, U.: Dtsch. med. Wschr. **90**, 285 (1965).
2. Farkas, E., Rudnai, O., Csonka, É., Lenetay, K.: Hungarian Association of Microbiologists. Debrecen, September 1966.
3. Rosen, L.: Virology **13**, 139 (1961).
4. Takátsy, Gy.: Acta microbiol. Acad. Sci. hung. 3, 191 (1955).
5. Tuchinda, P., Jayavashu, J., Sangkawibha, N., Okuno, Y., Ueda, Sh., Bukkavesa, S., Mansuvan, P., Ketusingha, R., Viranuvatti, P., Bhangananda, P.: Biken's J. **10**, 135 (1967).
6. Ueda, Sh., Okuno, Y., Sangkawibha, N., Jayavashu, J., Tuchinda, P., Bukkavesa, S., Ketusingha, R., Mansuvan, P.: Biken's J. **10**, 129 (1967).

Address of the authors:

Zoltán Pallinger, Gizella Pallinger-Szekeres
National Institute of Public Health
Gyáli út 2—6, Budapest, IX., Hungary

Acta microbiol. Acad. Sci. hung. 18, 7—16, 1971

SOME PROPERTIES OF THE MEMBRANE-BOUND PENICILLINASE OF BACILLUS CEREUS 569

By

V. CSÁNYI, I. MILE, ILONA FERENCZ and ÉVA SZABÓ

Institute of Medical Chemistry (Head: F. ANTONI), Semmelweis University Medical School, Budapest

(Received August 1, 1970)

Summary. Three kinds of membrane-bound penicillinase, differing from one another in certain properties, were isolated by various methods from cells of the *Bacillus cereus* 569/H strain. The properties of one variant corresponded with those of the γ-penicillinase isolated earlier by POLLOCK. Conclusions drawn from experimental evidence concerning the mechanism of formation of the three kinds of bound penicillinase are presented.

B. cereus produces two kinds of penicillinase which are well distinguishable by immunological and chemical methods. The bulk of penicillinase is secreted into the surrounding medium (exopenicillinase), whereas about 10% of the total enzyme yield is cell-bound; the latter fraction has been termed by POLLOCK γ-penicillinase. He observed that while the exopenicillinase is relatively iodine-resistant, the cell-bound penicillinase is rapidly inactivated by elementary iodine [1]. Detailed investigations into the behaviour against iodine showed of the two enzyme fractions to be conformers of each other [2]. Previous studies in this laboratory revealed that the kinetics of induction of the membrane-bound enzyme and the exoenzyme are different [3], and that the iodine inactivation of the latter is a complicated reaction taking place in several steps [4—6].

This paper reports on investigations into the similarities and dissimilarities of the iodine inactivation reactions of membrane-bound and exopenicillinase.

Materials and methods

Exopenicillinase produced by the strain *B. cereus* 569/H was used after purification by a procedure described earlier [4]. The membrane-bound penicillinase was extracted from cells of the same strain by the method described under "Results". The conditions of culturing were the same as those applied for the synthesis of the exoenzyme [4].

Iodine treatment of the two enzyme fractions was carried out at pH 6.5 in 0.05 *M* phosphate buffer and at pH 9 in 0.05 *M* carbonate buffer, respectively, as described previously [4].

The exopenicillinase-antiserum was prepared in rabbits by the method of POLLOCK and KRÁMER [7].

The protamine sulphate used was a product of the Gedeon Richter Pharmaceutical Works, Budapest, and the trypsin of Reanal Co., Budapest.

Results

Purification and properties of membrane-bound penicillinase. The method of purification proposed by POLLOCK [1] consists of dissolution of the enzyme by prolonged autolysis of the cells and subsequent purification by fractionation with ammonium sulphate. The penicillinase thus obtained is characterized by a high iodine sensitivity, solubility in concentrated (up to 0.8 saturated) ammonium sulphate and practically complete resistance to inactivation by exopenicillinase-antiserum. Since, however, a substantial part of the enzyme was inactivated during autolysis, we elaborated a new procedure for its purification.

B. cereus 569/H cells were centrifuged, ground with powdered aluminium oxide of identical weight and resuspended in a pH 6.5 solution containing 1 *M* NaCl, 0.2 *M* Na-citrate and 0.05 *M* phosphate; the volume of the solution always corresponded to the wet weight of the cells in grammes. The suspension was homogenated, centrifuged at 20 000 *g* for 30 minutes and the supernatant was discarded; then fresh "washing" solution was added to the cell-aluminium oxide sediment of the supernatant v/v and centrifugation was repeated. Three such washings removed the exopenicillinase loosely bound to the cell surface and also the greater part of the sedimented cell membranes and cell walls was extracted by enzymic digestion, using 100 μg/ml trypsin in 0.05 *M* phosphate buffer pH 6.5 at 30 °C.

On incubation with trypsin for about 30 minutes, 15—20% of the bound enzyme was released and only little of it had been inactivated, while prolonged incubation resulted in inactivation of the greater part of the enzyme. In view of this, we interrupted the incubation at 30-minute intervals on 4—5 occasions, when the suspension was centrifuged and the supernatant was collected and replaced by a fresh trypsin solution. The supernatants, which contained a substantial part of the bound enzyme in a dissolved state, were pooled, protamine sulphate was added at 1 mg/ml concentration, the copious precipitate formed was removed by centrifugation at 10 000 *g* for 5 minutes and the fluid phase was fractionated with ammonium sulphate. As, according to POLLOCK, the membrane-bound enzyme precipitates at saturation degrees of 0.8—1.0, the corresponding precipitates were collected and chromatographed on a CM-cellulose column. The results of a typical experiment are shown in Table I.

As can be seen from Table I, the degree of purification was about 200-fold; the fraction thus obtained was named "bound enzyme I". The fraction obtained at a 0.8 degree of saturation was initially discarded according to the method of POLLOCK, but later it was collected as it still contained a considerable amount of penicillinase. We shall revert to this question later.

Table I

Treatment	Penicillinase		per cent	Specific activity U/mg N
	Supernatant	Sediment		
100 g bacteria in 100 ml buffer (1 M NaCl + + 0.2 M Na citrate + 0.05 M phosphate), pH 6.5	1,440,000		100	140
1st centrifugation	720 000	720 000		
2nd centrifugation	162 000	650 000	—	—
3rd centrifugation	38 000	610 000	—	—
Sediment in 0.05 M phosphate buffer of pH 6.5, treated with 100 μg/ml trypsin at 30 °C for 30 min.	112 000	490 000	—	—
2nd trypsin treatment	78 000	400 000	—	—
3rd trypsin treatment	180 000	270 000	—	—
4th trypsin treatment	90 000	90 000	—	—
Pool of trypsinized supernatants, 260 ml	390 000	—	27	—
1 mg/ml protamine sulphate, precipitate centrifuged	385 000	4 000	—	—
Saturated with ammonium sulphate to the degree of 0.8	181 000	204 000	—	—
Supernatant saturated to the degree of 1.0	—	168 000	11.5	4 700
Chromatography on CM-cellulose and elution with NaCl	102 000	—	7.0	33 000

0.05 M NaCl in 0.05 M phosphate buffer (pH 6.5) was conducted with the gradient technique under constant stirring into a bottle containing 250 ml 0.05 M phosphate of pH 6.5. The peak containing the enzyme eluted from the column at an 0.1—0.2 M concentration of NaCl.

Bound enzyme I was examined for certain properties; Figs 1 and 2 respectively, show its pH stability and thermostability, and Fig 3 shows its iodine sensitivity together with that of the exopenicillinase.

It was demonstrated earlier (4—6) that the iodine inactivation of exopenicillinase takes place in two independent steps, one requiring a pH of about 9 and an iodine concentration above 10^{-4} N, the other either a pH well above 9 or a much higher (10^{-1} N) iodine concentration at pH 9.

As can be seen from Fig. 3, the iodine inactivation of the bondu enzyme apparently did not include two reactions; this fraction was practically iodine resistant at pH 6, as was also the exoenzyme [4], and became completely inactivated at a low concentration (10^{-3} N) of iodine at pH 9. Thus the iodine-sensitivity and solubility in ammonium sulphate of the "bound enzyme I" corresponded in every respect with that of POLLOCK's isolate; as will be seen later also their serological properties were identical. In respect of other properties, however, our "bound enzyme I" and POLLOCK's γ-penicillinase were different as shown by further studies.

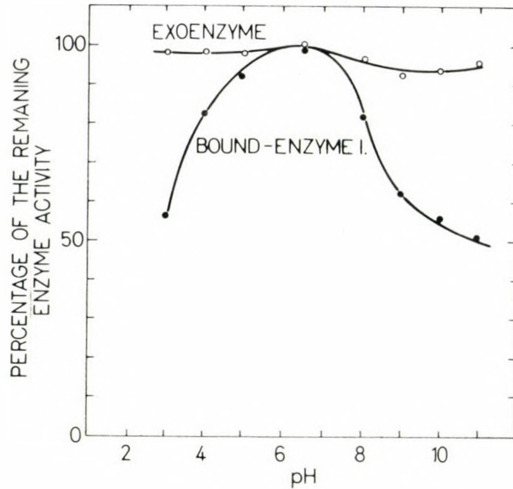


Fig. 1. pH stability of "bound enzyme I". 300 U exoenzyme or "bound enzyme I" were incubated for 20 minutes at 30 °C in two ml of 0.05 *M* phosphate buffer of a pH specified in the Figure, and the samples were subsequently assayed for enzyme activity. The results were expressed in per cents of the activity of the control sample in 0.05 *M* phosphate buffer, pH 6.5, at 0°C

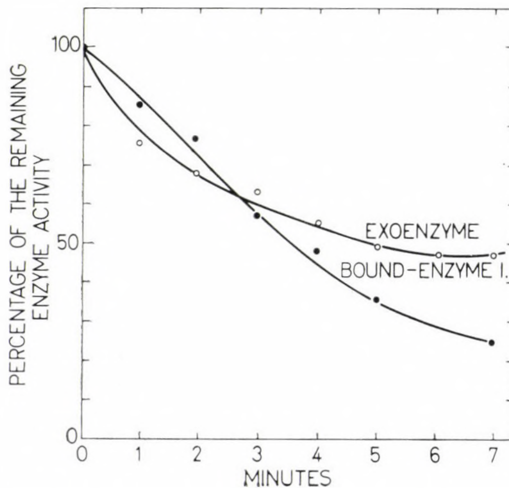


Fig. 2. Thermostability of "bound enzyme I". 300 U exoenzyme or "bound enzyme I" were incubated for different times at 50 °C in one ml of 0.05 *M* phosphate buffer of pH 6.5 and the samples were assayed for residual enzyme activity which was expressed in per cents of the activity of the control sample at 0 °C

Membrane-bound penicillinase of different properties. POLLOCK employed fractionation with ammonium sulphate to separate the bound enzyme from the exoenzyme present in the autolysate, as he did not remove the latter by washing prior to autolysis. For this reason he discarded the fraction obtained at 0.8 degree of saturation as one containing exopenicillinase.

As in the present experiments the loosely bound exoenzyme was completely removed prior to autolysis by washing with a concentrated electrolyte solution, the enzyme present in the fraction in question was supposed to be

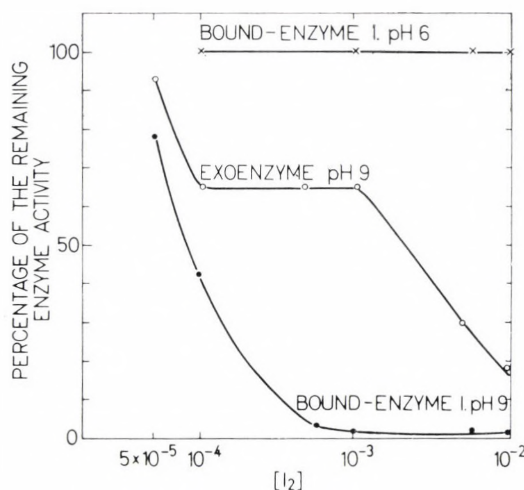


Fig. 3. Iodine sensitivity of "bound enzyme I". 500 U exoenzyme or "bound enzyme I" were treated with different concentrations of iodine for one minute at 0 °C, using as incubation medium three ml 0.05 *M* phosphate buffer of pH 6, or three ml 0.05 *M* carbonate buffer of pH 9. Residual enzyme activity was expressed in per cents of the activity of the untreated sample

cell-bound and accordingly termed "bound enzyme II". In iodine sensitivity tests, "bound enzyme II" was completely resistant at pH 6 (a fact not shown in Fig. 4), and still considerably resistant at pH 9, in contrast to the high sensitivity of "bound enzyme I" (POLLOCK's γ-penicillinase) (Fig. 3); it was even more resistant than exopenicillinase.

This does not, however, imply that the cell-bound penicillinase consists of two fractions differing in respect of iodine sensitivity and solubility in ammonium sulphate, because both POLLOCK and ourselves isolated the enzyme by a lytic enzymic procedure which may have produced an artefact by partial alteration of the bound enzyme, without changing its activity. An attempt was therefore made to elaborate a new method of isolation less likely to produce an artefact.

Isolation of bound enzyme by ultrasonic disintegration. Cells containing the bound enzyme were ground with aluminium oxide and washed in the concentrated electrolyte solution as before, to remove the exopenicillinase. The disrupted cells were resuspended in 0.05 M phosphate buffer, pH 6.5, and sonicated for 15 minutes at 0 °C, using a MSE 100 Watt Ultrasonic Disintegrator. Sonication was interrupted every two minutes and the mixture was cooled to 0 °C, to prevent enzyme inactivation by the rise of temperature.

Ultrasonic disintegration made it possible to extract 70—80% of the enzyme. In one experiment, the supernatant of the sonicated cell suspension

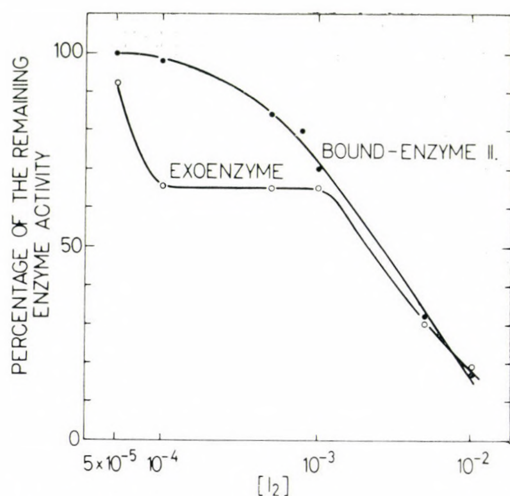


Fig. 4. Iodine sensitivity of "bound enzyme II". 500 U "bound enzyme II" or exopenicillinase were incubated in three ml 0.05 *M* carbonate buffer of pH 9 for 1 minute at 0 °C, with iodine added at the concentrations specified in the Figure. Subsequently, the iodine was removed by washing and the residual activity of the samples was measured and expressed in per cents of the control not treated with iodine

(125 ml) contained 197 000 U penicillinase, while that of the ammonium sulphate fraction (0.8) only 4000 U; 190 000 U were retained in the precipitate. Thus, the enzyme fraction obtained by sonication did not readily dissolve in ammonium sulphate (saturated to 0.8), while the corresponding fraction obtained by enzymic (lytic) disintegration covered almost 50% of the total cell-bound enzyme. The ultrasonically extracted (US) enzyme precipitated at 0.6—0.8 saturation degrees of ammonium sulphate and had a specific activity of 4000—5000 U/mg N. The specific activity of the chromatographic enzyme fraction varied from 29 000 to 37 000 U/mg N, depending on the purity of the preparation, i.e. its purity corresponded to that of "bound enzyme I". Testing the iodine sensitivity of the "US enzyme" at pH 9, it was found to be intermediate between the sensitivities of "bound enzyme I" and "bound enzyme II", obtained by the lytic method (Fig. 5).

The immune reaction towards exopenicillinase antiserum of the three bound enzyme fractions and the exopenicillinase fraction was the least distinct with "bound enzyme I" and the most distinct with "bound enzyme II", the response of the "US enzyme" being intermediate between the two; the bound enzyme fractions behaved in general quite differently from exopenicillinase (Fig. 6).

Attempts at transmutation of the cell-bound fractions have failed; trypsin treatment of the "US-enzyme" did not yield" "bound enzyme I" and sonication of the latter and of the "bound enzyme II" did not yield "US enzyme"; neither the iodine sensitivity, nor the immunological behaviour of any fraction could be altered once it had been isolated.

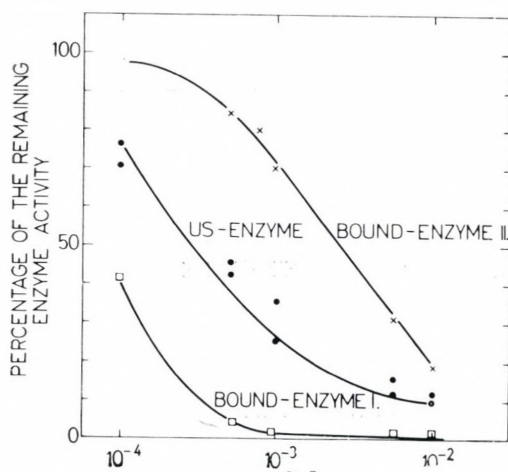


Fig. 5. Iodine sensitivity of the bound enzyme obtained by sonication 500 U enzyme pretreated in different manners were iodinated in three ml 0.05 *M* carbonate buffer of pH 9, at iodine concentrations specified in the Figure. Residual enzyme activity was measured and expressed in per cents of the activity of the untreated control

γ-penicillinase was prepared by POLLOCK's original method; this fraction, on the basis of its solubility in ammonium sulphate, its iodine sensitivity and immunological behaviour, can be regarded as identical with the fraction "bound enzyme I" isolated by us.

Discussion

About 10% of the penicillinase synthetized by *B. cereus* is membrane-bound [1, 3]. POLLOCK observed that his partially purified bound enzyme preparation differed from the exoenzyme in iodine sensitivity, in ammonium sulphate solubility and immunological behaviour. The clarification of the

relationship between the two kinds of penicillinase was expected to throw a light on the regulation of its biosynthesis. If CITRI and POLLOCK's [2] hypothesis that the difference between the bound enzyme and the exoenzyme is only conformational is true, the formation of the bound enzyme is easy to explain, at least at the messenger RNA level. If, however, the bound enzyme represents in fact an independent structural entity determined by a separate structural gene, the regulation of penicillinase biosynthesis will have to be

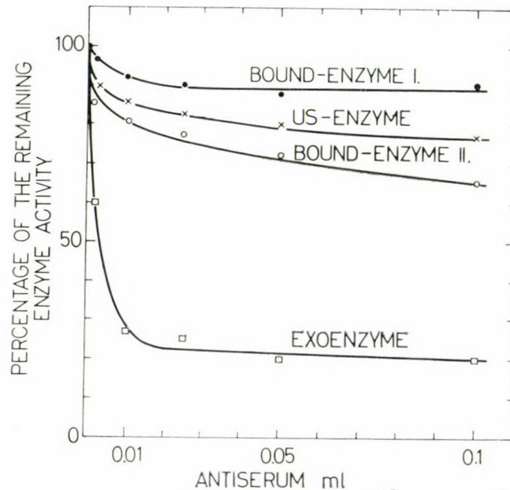


Fig. 6. Immunological reactions of the three bound enzyme preparations with exopenicillinase antiserum. 500 U enzyme were incubated in two ml 0.05 *M* phosphate buffer pH 6.5 for 15 minutes at 30 °C, with exopenicillinase antiserum added at concentrations specified in the Figure. Residual activity of the enzyme was expressed in per cents of the activity of the untreated controls

approached in a different way. Consideration should also be given to the fact that while the bound enzyme protects the cell wall directly against the action of penicillin, the exoenzyme confers only a "collective" protection upon the cell population in general.

Significant as it is, the bound enzyme has nevertheless been hardly studied. The reasons may have been its low concentration in the cell and the difficulty of its purification; the procedures proposed up to now for this purpose [1, 8, 9] are all based on its differentiation from the exoenzyme by the characteristic properties observed by POLLOCK (high iodine sensitivity, easy solubility in ammonium sulphate and immune response differing from that of the exoenzyme). The degree of purification never exceeded one hundredfold, to judge from the data available.

It was demonstrated in this laboratory that the autolytic procedure of extraction, the efficiency of which is low, can be replaced by trypsin treat-

ment which yields a bound enzyme fraction corresponding in various properties to the γ-penicillinase of POLLOCK. Analysing the trypsin procedure in more detail, it was found to yield two kinds of enzyme, both of which are membrane bound, but only one resembles POLLOCK's γ-penicillinase. Disintegration by sonication yielded a single homogeneous fraction, the properties of which differed from those of the two fractions obtained by lytic procedures. This suggested that one of the three enzyme preparations may have been an artefact. As the probability of artefact formation is greater with the procedures employing lytic enzymes, we believe that POLLOCK's γ-penicillinase cannot be regarded as a bound enzyme until proof is forthcoming from further experiments. It seems altogether more probable that the the true bound enzyme is that present in the sonicated preparation, whose properties differ from those of the exoenzyme and from those of the γ-penicillinase as well. With these considerations in mind it seems necessary to revise the hypothetical conclusion derived from certain conformational changes of the exoenzyme, *i.e.* that the primary structures of the cell-bound and exopenicillinase are identical [2].

Iodine inactivation of the exopenicillinase was shown to take place in two independent steps [4—6]. The iodine sensitivity tests of "bound enzyme I" and "bound enzyme II" obtained by trypsinization showed that each represents another extreme; at pH 9, "bound enzyme I" was extremely sensitive, while "bound enzyme II" was resistant, to iodine and the sensitivities of the exoenzyme and of the US enzyme prepared by sonication were intermediate between the two extremes.

From our investigations into the mechanism of action of the penicillin analogon pirazocillin, the hypothetical conclusion was drawn that the penicillinase molecule probably contains two independent active centres [10]. Correlating this with the observation that the US enzyme and the exoenzyme are similar in iodine sensitivity, it does not seem improbable that the bound enzyme is either identical with, or hardly differs structurally from, the exoenzyme and its different behaviour stems from its being fixed to the cell membrane in a given conformation which allows its being split by the lytic enzymes into two active parts, one of which might correspond to POLLOCK's γ-penicillinase, *i.e.* to our "bound enzyme I", and the other to our "bound enzyme II". The former fraction becomes inactivated in the "pH sensitive", the latter in the "pH resistant" step of the iodine inactivation reaction. These remain, naturally, only speculations until further purification and a more detailed structural analysis of the three enzyme preparations will throw more light on the problem.

Acknowledgements. The authors are indebted to Professor F. B. STRAUB for valuable advice and to Mr. I. BALÁZS for excellent technical assistance.

REFERENCES

1. Pollock, M. R.: J. gen. Microbiol. **15,** 154 (1956).
2. Citri, N., Pollock, M. R.: Advances in Enzymology **28,** 237 (1966).
3. Csányi, V.: Acta microbiol. Acad. Sci. hung. **17,** 91 (1970).
4. Csányi, V., Mile, I., Ferencz, I., Szabó, É.: Biochim. biophys. Acta (Amst.) **198,** 332 (1970).
5. Mile, I., Csányi, V., Ferencz, I.: Acta biochim. biophys. Acad. Sci. hung. **5,** 33 (1970).
6. Ferencz, I., Csányi, V., Mile, I.: Acta biochim. biophys. Acad. Sci. hung. In press.
7. Pollock, M. R., Krámer, M.: Biochem. J. **70,** 665 (1958).
8. Citri, N.: Bull. Res. Counc. Israel **9A,** 28 (1960).
9. Citri, N., Kalkenstein, A.: Arch. Biochem. **121,** 720 (1967).
10. Csányi, V., Mile, I., Koczka, I., Badár, É., Horváth, I.: Biochim. biophys. Acta (Amst.) **220,** 317 (1970).

Address of the authors:

Vilmos Csányi, Imre Mile, Ilona Ferencz, Éva Szabó
Institute of Medical Chemistry, Semmelweis University Medical School
Puskin u. 9, Budapest VIII., Hungary

Acta microbiol. Acad. Sci. hung. 18, 17—21, 1971

EFFECT OF UREA ON THE TWO IODINE INACTIVATION REACTIONS OF BACILLUS CEREUS PENICILLINASE

By

J. HAJDU, ILONA FERENCZ, I. MILE and V. CSÁNYI

Institute of Medical Chemistry (Head: F. ANTONI), Semmelweis University Medical School, Budapest

(Received August 1, 1970)

Summary. The presence of urea stimulated both steps of the iodine inactivation reaction of *Bacillus cereus* penicillinase and the degree of stimulation was proportional to the concentration of urea. Preincubation of the penicillinase with 7.5 *M* urea for 30 minutes altered the conditions of iodine inactivation, as both reactions took place at lower pH than normally. Combining urea with methicillin, each acted on one of the two steps, causing them to take place simultaneously, with the result that inactivation was practically complete at both pH grades tested.

CITRI *et al.* have shown that urea accelerates the iodine inactivation reaction of penicillinase [1, 2]. This reaction takes place in two independent steps, as shown earlier in this laboratory [3—5]. The first step, termed by us "pH sensitive", is the inactivation of 30—40% of the enzyme activity by 10^{-3} *N* iodine at 0 °C and pH 9, while the second, "pH resistant", step takes place in the pH range above 9, unless methicillin, a selective inhibitor of penicillinase, is added to the reaction mixture; this causes the accomplishment of the reaction already at pH 6.

The present experiments were undertaken to clarify which of the two steps of the iodine inactivation reaction of penicillinase is influenced by urea.

Materials and methods

Exopenicillinase produced by the 569/H strain of *B. cereus* and purified as described earlier [3] was used. Iodine treatment of the enzyme was carried out in glass cuvettes of 5 ml volume placed in a water bath of 0 °C, using as incubation medium 0.05 *M* phosphate buffer of pH 6 and 0.05 *M* carbonate-bicarbonate buffer of pH 9. After treatment, the excess iodine was removed by the addition of an equivalent quantity of thiosulphate (in 0.1 ml solution) and residual enzyme activity was estimated as described earlier [3].

Results

Effect of short-term urea treatment on the iodine inactivation reactions of penicillinase. Urea at different concentrations was dissolved in the iodine containing incubation medium and the reaction was started by addition of the enzyme to ensure that the urea should act only for the duration of iodine treatment which was one minute.

As can be seen from Fig. 1, inactivation by 10^{-3} *N* iodine was accelerated linearly with the elevation of the concentration of urea at both pH 6

and 9. In previous experiments [5, 6] the addition of compounds specifically influencing the iodine sensitivity (methicillin, polyethyleneglycol) resulted in higher reaction rates than might have been expected from the elevation of the iodine concentration. In the present study, elevation of the iodine level to 7×10^{-3} *N* failed to alter the linear relationship of the reaction rate with the concentration of urea added.

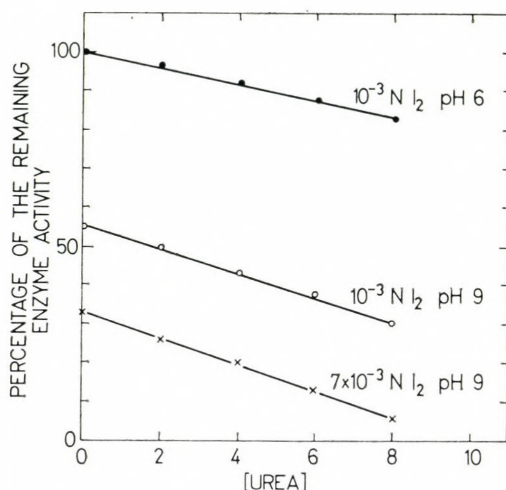


Fig. 1. Effect of the concentration of urea on the iodine inactivation of penicillinase. 300 U penicillinase was treated with iodine at different concentrations in the presence of urea in amounts specified in the Figure, using as incubation medium 1 ml phosphate buffer (0.05 *M*) of pH 6 or 1 ml carbonate buffer of pH 9. Iodination lasted for 1 minute at 0 °C, then the samples were diluted 20fold and assayed for residual enzyme activity which was expressed in per cents of the activity of the untreated controls

The same conclusion was drawn from experiments in which the level of urea was kept constant (7.5 *M*) and the iodine concentration was changed (Fig. 2). Inactivation of the enzyme was accelerated linearly with the elevation of the iodine level, without showing the abrupt increase which occured in the presence of methicillin or polyethylene glycol in the iodine concentration range above 10^{-3} *N*.

Effect of preincubation in urea on the iodine inactivation reactions of penicillinase. Penicillinase and 7.5 *M* urea were dissolved in 0.05 *M* phosphate buffer of pH 6 and incubated at 30 °C for different times. Samples taken after each incubation period were diluted 30fold and incubated with iodine at pH 6 and pH 9.

Results are shown in Fig. 3. Depending on the time of preincubation with urea, at pH 6 the rate of iodine inactivation increased, then persisted at the same level and did not exceed the 30—35% inactivation level common in the "pH sensitive" step. Preincubation with urea seemed to make the "pH

sensitive" reaction to take place already at pH 6, though it normally takes place at pH 9. Iodine treatment of the preincubated enzyme at pH 9 resulted in a slow inactivation of 90% of the activity. Apparently, in the presence of urea, the "pH resistant" step of the reaction took place already at pH 9.

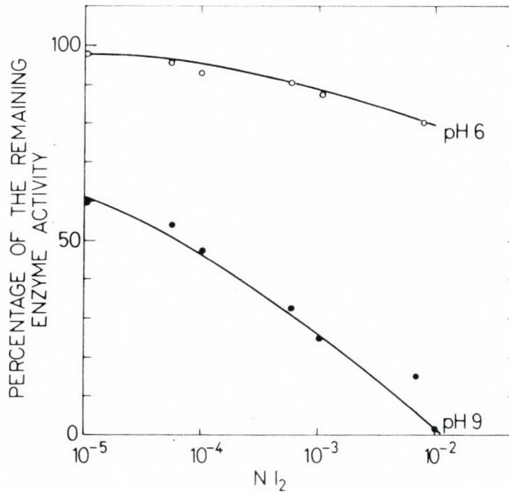


Fig. 2. Effect of the concentration of iodine on the inactivation of penicillinase in the presence of 7.5 *M* urea. 300 U penicillinase was treated with iodine at different concentrations in the presence of 7.5 *M* urea, using as incubation medium 1 ml phosphate buffer (0.05 *M*) of pH 6 or 1 ml carbonate buffer (0.05 *M*) of pH 9. Iodination lasted for 1 minute at 0 °C, then, samples were diluted 20fold and assayed for residual enzyme activity which was expressed in per cents of the activity of the untreated controls

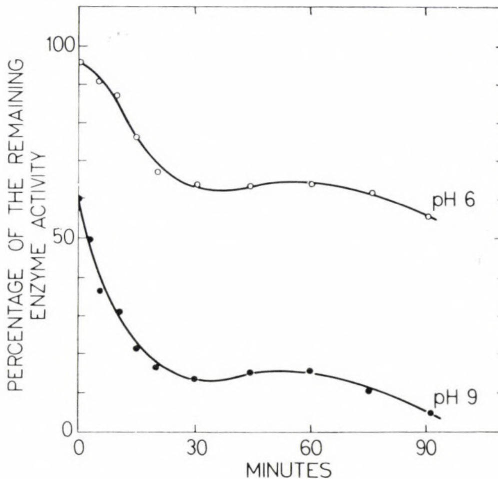


Fig. 3. Effect of preincubation with 7.5 *M* urea on the iodine sensitivity of penicillinase. 4500 U/ml penicillinase was incubated with 7.5 *M* urea in 0.05 *M* phosphate buffer pH 6 for various times at 30 °C. Samples taken at different intervals were diluted 30fold and assayed for iodine sensitivity (residual enzyme activity) by incubation with 10^{-3} *N* iodine in 3 ml 0.05 *M* phosphate buffer pH 6 or 0.05 *M* carbonate buffer pH 9, for 1 minute at 0 °C. Residual enzyme activity was expressed in per cents of the activity of the non-iodinated control

Combined effect of urea and methicillin on the iodine inactivation reaction of penicillinase. It was shown [5] that methicillin specifically accelerated the "pH resistant" step of the iodine inactivation reaction, but failed to influence the "pH sensitive" step. Correlating this with the observation that preincubation of the enzyme with urea caused the "pH sensitive" reaction to take place at pH 6, it has been concluded that the joint action of urea and methicillin caused both reactions to take place simultaneously at pH 6.

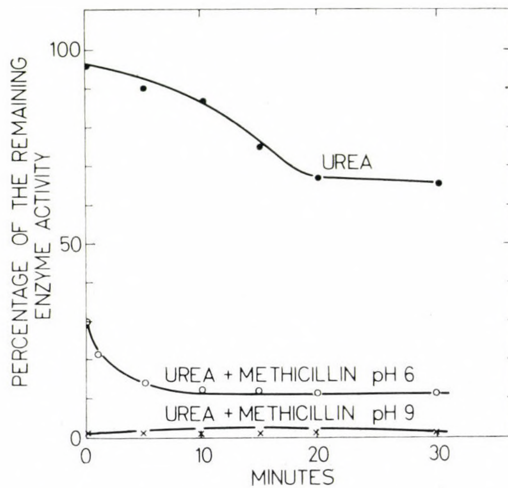


Fig. 4. Combined effect of methicillin and urea on the iodine inactivation reaction of penicillinase. 4500 U/ml penicillinase was incubated with 7.5 *M* urea in 0.05 *M* phosphate buffer pH 6 at 30 °C. Samples taken at different intervals were treated with 10^{-3} *N* iodine for 1 minute at 0 °C, either in 0.05 *M* phosphate buffer pH 6, or with 100 μg/ml methicillin added in 0.05 *M* phosphate buffer pH 6, and 0.05 *M* carbonate buffer pH 9. Residual enzyme activity was expressed in per cents of the activity of the non-iodinated controls

Experimental evidence was presented as follows. Penicillinase was incubated with 7.5 *M* urea and samples taken at different times were treated with iodine in the presence of methicillin at pH 6 and pH 9. As shown in Fig. 4, in the presence of methicillin 10^{-3} *N* iodine inactivated the enzyme completely at pH 9 and about 90% of it at pH 6.

This fact confirmed that in the given system the actions of urea and methicillin are additive.

Discussion

The iodine inactivation reaction of penicillinase is a suitable model for the study of conformational changes [2, 3]. Previous experiments in this laboratory [3, 4] showed this reaction to take place in two steps. The first reaction, involving the loss of 30—35% of the enzyme activity, has been termed "pH

sensitive" as it requires a pH of about 9; the direct cause of the decrease in activity is the substitution of iodine for one tyrosine side chain [7]. The other reaction, termed "pH resistant", requires a pH above 10 and involves the loss of 65—70% of the enzyme activity by some unidentified mechanism. In the present experiments, urea, known considerably to influence protein conformation, was examined for its effect on the two reactions with the result that its short-term action accelerated both steps of iodine inactivation and this effect increased linearly with the concentration. The fact that the increase in the reaction rate was moderate is explained by the short (1-minute) treatment with urea which might not have produced a notable conformational change.

However, preincubation with 7.5 *M* urea altered the pH requirements of both reactions, as the "pH sensitive" step took place at pH 6 and the "pH resistant" step at pH 9, in contrast to their normal requirements of pH 9 and above 10, respectively. The results suggest that the conformational change effected by urea involves the displacement of the iodine-reactive tyrosine side chain to the surface of the molecule.

In earlier studies methicillin was shown to cause the "pH resistant" step of iodine inactivation to take place at pH 6. The combined application of methicillin and urea in this study resulted in an almost complete inactivation of the enzyme at both pH 6 and pH 9. The additive effect of the two compounds manifested itself so that preincubation of the enzyme with urea caused the "pH sensitive" step to take place at pH 6 instead of pH 9 and methicillin caused the "pH resistant" step to take place at pH 6 instead of above pH 10. The result was a complete inactivation of the enzyme and the conclusion has been drawn that the conformation changing actions of urea and methicillin are entirely different.

Acknowledgements. The authors are indebted to Professor F. B. Straub for valuable advice and to Mr. I. Balázs for excellent technical assistance.

REFERENCES

1. Citri, N., Garber, N.: Biochim. biophys. Acta (Amst.) **30**, 664 (1958).
2. Citri, N., Garber, N., Sela, M.: J. biol. Chem. **235**, 3454 (1960).
3. Csányi, V., Mile, I., Ferencz, I., Szabó, É.: Biochim. biophys. Acta (Amst.) **198**, 332(1970).
4. Mile, I., Csányi, V., Ferencz, I.: Acta biochim. biophys. Acad. Sci. hung. **5**, 33 (1970).
5. Ferencz, I., Csányi, V., Mile, I.: Acta biochim. biophys. Acad. Sci. hung. In press.
6. Csányi, V., Ferencz, I., Székely, T., Lázár, J., Mile, I.: Acta biochim. biophys. Acad. Sci. hung. In press.
7. Csányi, V., Ferencz, I., Mile, I.: Biochim. biophys. Acta (Amst.) **236**, 619 (1971).

Address of the authors:

J. Hajdu, Ilona Ferencz, Imre Mile, Vilmos Csányi
Institute of Medical Chemistry, Semmelweis University Medical School
Puskin u. 9, Budapest VIII., Hungary

Acta microbiol. Acad. Sci. hung. 18, 23–27, 1971

INTERFERON AND ANTIBODY PRODUCTION IN INBRED MICE INFECTED WITH THE RAUSCHER VIRUS

By

F. D. TÓTH, L. VÁCZI and CLARA BERENCSI

Institute of Microbiology (Director: L. VÁCZI), *University Medical School, Debrecen*

(Received October 9, 1970)

Summary. Splenomegaly, as well as interferon and tumour-specific antibody production, were examined in inbred Balb/c, DBA/1 and C57B1/10Sn mice infected with the Rauscher virus. A direct relation was found between antibody production and resistance. In the medium-resistant DBA/1 mice, the Rauscher virus proved to be a good interferon inducer, in the other two strains interferon production was negligible.

It is well-known that the splenomegaly caused by the Rauscher leukaemia virus is considerable in Balb/c mice, whereas slight and transitory in C57B1 mice [1]. In the latter strain, high-titre cytotoxic antibodies are produced after introduction of the virus [2]. On the other hand, according to GLASGOW and FRIEDMAN [3] the serum interferon level in the infected CD-1 mice, a strain resistant to the Rauscher virus, is significantly higher than in infected Balb/c mice. Thus, both interferon and immune response may be responsible for the resistance, but it is not clear how general the role of each of the two factors is. The present work was undertaken to contribute to the clarification of this problem. Antibody and interferon production by Balb/c, DBA/1 and C57B1/10Sn mice infected with the Rauscher virus was studied.

Materials and methods

Viruses. The Rauscher leukaemia virus was kindly supplied by Professor V. M. BERGOLTS (Gretsen Institute for Oncology, Moscow) in infected Balb/c mice. The enlarged spleen of the animals was removed in the third week following infection, and its 20% suspension was centrifuged at 5000 *g* for 30 minutes, and the supernatant was stored at −70 °C until used. Experimental animals were injected with 0.2 ml of this suspension by the intraperitoneal route.

Vesicular stomatitis (VS) virus was propagated in HeLa cell cultures and the culture media containing virus were stored at −70 °C.

Mice. Inbred male 8-week-old Balb/c, C57B1/10Sn and DBA/1 mice obtained from the National Blood Supply Service, Budapest, were used in the experiments. Blood samples for antibody and interferon titration were taken from seven mice at the times indicated in the figures.

Fluorescent-antibody test. The indirect technique was applied in living cells, used by MÖLLER [4] and by KLEIN and KLEIN [5]. In the first phase of the reaction, 0.05 ml undiluted serum was added to 2×10^6 leukaemic Balb/c spleen cells. After thorough shaking the suspension was incubated for 20 minutes at 37 °C; in the meantime it was shaken three times. Subsequently, the suspension was washed three times with 4 ml Ringer solution, pH 7.2. In the second phase of the reaction, 0.05 ml swine anti-mouse gamma globulin labelled with fluorescein isothiocyanate (SEVAC Prague) was added, and the procedure described for the first phase was repeated. The cells were then resuspended in one drop of Ringer's solution and dropped

onto a slide. After the cells had sedimented the preparation was incubated at room temperature until complete drying. Finally, one drop of Ringer's solution mixed with an equal volume of glycerine pH 7.2 was dropped onto the cells and the preparation was covered with a coverslip and sealed with paraffin. In each preparation 200 intact cells were examined under the fluorescence microscope. From the percentage of the cells showing surface fluorescence, the fluorescence index was calculated as recommended by KLEIN and KLEIN [5].

The Ringer's solution used in the course of the procedure was ice-cold and the centrifugations were carried out at —4 °C. To exclude reactions to isoantigens, the C57B1/10Sn and DBA/1 serum samples were absorbed with Balb/c spleen cells; 10^7 cells were added to 1 ml serum and the suspension was kept for 5 minutes at 37 °C, then for 30 minutes at room temperature and, at last, for 60 minutes at —4 °C. During incubation the suspension was shaken several times.

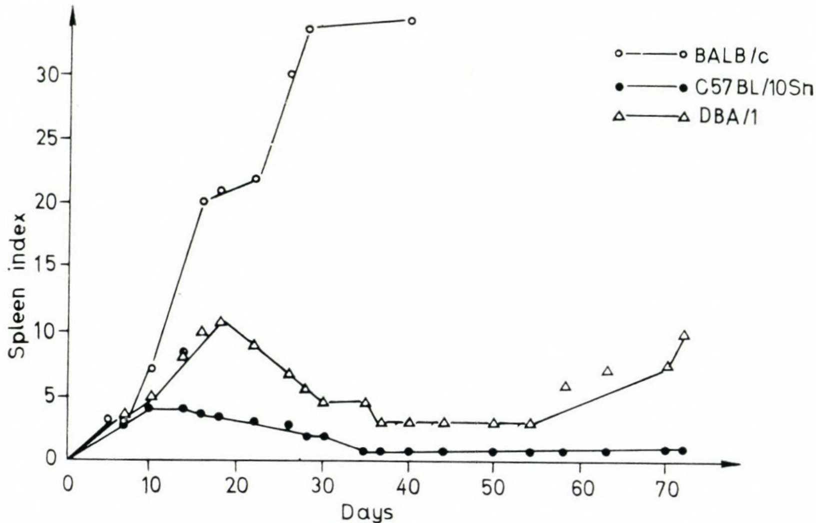


Fig. 1. Splenomegaly in different mouse strains following Rauscher virus infection. Mean spleen weight in normal animals = 1

Interferon titration. Sera were dialysed against 100 volumes of glycine-HCl buffer pH 2 for 96 hours, then the pH was re-adjusted to 7.4 by dialysing against PBS.

From each preparation a four-fold dilution series was prepared in Parker's medium No. 199, containing 2% calf serum, 0.5% lactalbumin hydrolysate and 0.125% sodium hydrocarbonate. From each dilution 1 ml was added to a monolayer culture of L cells, and the cultures were incubated overnight. Subsequently, the cultures were washed and infected with approximately 50 CPD_{50} of VS virus contained in 1 ml culture medium. The result was read as the degeneration in the control tubes had reached 70—100%. The highest virus dilution affording protection to 50% of the cells was considered the interferon titre.

Results

Fig. 1 shows the degree of splenomegaly as a function of time.

The spleen of Balb/c mice grew to 30—40-fold of the normal size within 4 weeks after infection; practically all the infected Balb/c mice died within 40 days.

For C57B1/10Sn mice the highest index of splenomegaly was 4, which began to decrease after the second week and reached the normal value by the

end of the 5th week; then it remained at the same level till the end of the observation period 70 days after infection. No specific death was observed in this strain.

The spleen weight of the DBA/1 mice showed a peculiar two-wave curve, which reached its first peak at the middle of the third week with an index between the maximum for the Balb/c mice and that for the C57Bl/10Sn mice. In the subsequent wave-through the index stagnated at a value of 3. It began to rise again at the 50th day. At the time of the first peak about 10% of the mice died. Most survivors died at the time of the second wave of splenomegaly.

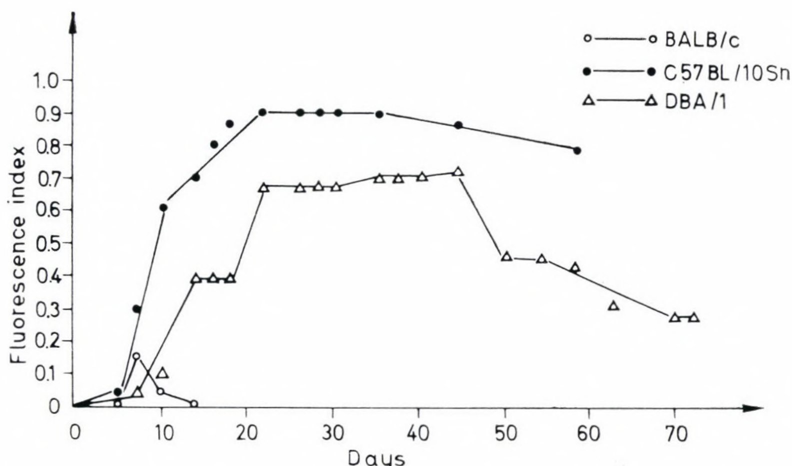


Fig. 2. Antibody production in different mouse strains following Rauscher virus infection

Fig. 2 shows the kinetics of the tumour-specific antibody production.

In the sera of infected Balb/c mice, initially some tumour-specific antibody was demonstrable; the titres in general did not reach the threshold of significance (0.3).

Strain C57Bl/10Sn was characterized by an excellent antibody production, whereas the immune response of the DBA/1 line was less intensive and the antibody titre tended to decline from the 40th day on.

In Fig. 3 the interferon levels in mice infected with the Rauscher virus are shown.

The Rauscher virus induced little interferon production in mice of the Balb/c and C57Bl/10Sn strains. In contrast, interferon production by the DBA/1 mice was intensive; the peak titre was 5 times higher than that for the other two mouse strains and the serum of the DBA/1 mice still contained interferon in the 64th hour after infection.

Discussion

The erythroleukaemia induced by the Rauscher virus was the most severe in the Balb/c mouse strain, and the least severe in the C57Bl/10Sn strain. A reaction of the same type as observed by us in DBA/1 mice was described by others [6] in BDF_1 mice infected with the Friend virus.

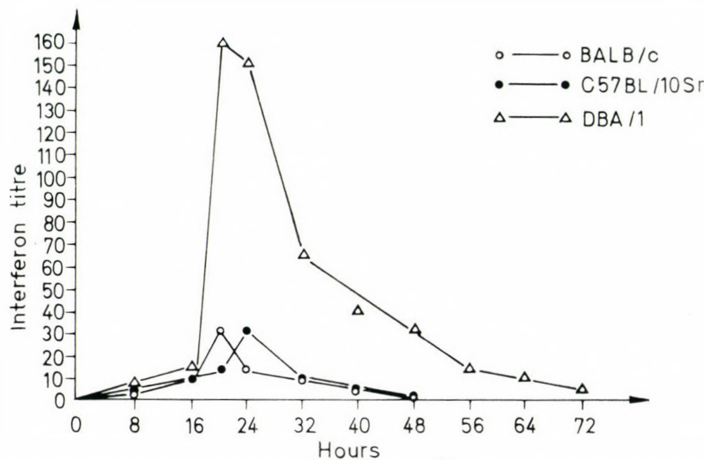


Fig. 3. Interferon production in different mouse strains following Rauscher virus infection

The resistance of the strains ran parallel with their antibody production. The role of the high-titre antibodies in the suppression of the pathological process is obvius. It may be attributed to the cytotoxic, virus-neutralizing and virolytic [7] activities of antibodies.

The strain-dependence of the immune response may be due to the strain-dependence of the immunosuppressive effect of the Rauscher virus [8—10]. According to Siegel and Morton [11], immunosuppression is a consequence of a competition between virus replication and antigenic stimulus, both of which are directed towards the same pluripotent type of target cell. This view seems to be supported by the observation that in Balb/c mice, charging of the immuno-apparatus by "indifferent" antigens considerably interferes with the progression of Rauscher leukaemia, if immunization precedes the infection with Rauscher virus [11]. In this case the protective effect may be attributed to a reduction in the number of the precursor cells capable of malignant transformation. The genetically determined equilibrium between the defensive and replicative functions of the immune system may alternatively be shifted in the direction of susceptibility. Pretreatment with complete Freund adjuvant enhances the transitory splenomegaly in the otherwise resistant C57Bl/6 mice [12], a phenomenon attributable to an increased re-

production of the virus [13]. At the same time the immune response is delayed [14].

The inhibition of the progression of viral leukaemia by interferon was first observed after the introduction of exogenous interferon. This inhibition could be explained by an inhibition of both virus reproduction and malignant transformation [15]. Using CD-1 mice as experimental animal, GLASGOW and FRIEDMAN [3] demonstrated that an intensive interferon production is one of the mechanisms responsible for resistance. We have, however, failed to demonstrate a parallelism between interferon response and resistance.

Taking into account the mechanism of development of immunosuppression as outlined above, a synergism between the two kinds of defence mechanism seems to be very likely; the good immune response characteristic of DBA/1 mice may be based on their good interferon production.

REFERENCES

1. RAUSCHER, F. J.: J. nat. Cancer Inst. **29,** 515 (1962).
2. OLD, L. J., BOYSE, E. A., STOCKERT, E.: Nature (Lond.) **201,** 777 (1964).
3. GLASGOW, L. A., FRIEDMAN, S. B.: J. Virol. **3,** 99 (1969).
4. MÖLLER, G.: J. exp. Med. **114,** 415 (1961).
5. KLEIN, E., KLEIN, G.: J. nat. Cancer Inst. **32,** 547 (1964).
6. DAWSON, P. J., FIELDSTEEL, A. H.: Cancer Res. **29,** 1206 (1969).
7. OROSZLAN, S., GILDEN, R. V.: Science **168,** 1478 (1970).
8. SIEGEL, B. V., MORTON, J. I.: Immunology **10,** 559 (1966).
9. SIEGEL, B. V., MORTON, J. I.: Proc. Soc. exp. Biol. (N.Y.) **123,** 467 (1966).
10. MILLIAN, S. J., SCHAFFER, M.: Cancer **21,** 989 (1968).
11. SIEGEL, B. V., MORTON, J. I.: Experientia (Basel) **25,** 186 (1969).
12. Ирлин, И. С., Тер-Григоров. В. С., Тихонова, З. Н., Цысина, Э., Н., Берголыц, В. М., Московкина, О Г.: Конференция по вирусологии и иммунологии опухолей. Тезисы докладов 20. Москва 1969.
13. Киселев, Ф. Л., Ирлин, И. С., Шаталова, Г. Г.: Конференция по вирусологии и иммунологии опухолей. Тезисы докладов 13. Москва 1969.
14. D. TÓTH, F.: Meeting of Hungarian Society of Microbiology, Budapest 1969.
15. GRESSER, I.: Rev. Europ. Étud. clin. biol. **15,** 23 (1970).

Address of the authors:

FERENC D. TÓTH, LAJOS VÁCZI, CLARA BERENCSI
Institute of Microbiology, University Medical School
Debrecen 12, Hungary

Acta microbiol. Acad. Sci. hung. 18, 29—39, 1971

RELATIONSHIP OF LYSOGENY AND MEGACINOGENY IN BACILLUS MEGATERIUM

By

Vera Gaál, J. Molnár and G. Ivánovics

Institute of Microbiology (Director: G. Ivánovics), University Medical School, Szeged

(Received October 23, 1970)

Summary. Megacinogenic *Bacillus megaterium* strain 216 was infected with host range mutant phage obtained from lysogenic strain 899. Spore clones developing on nutrient agar plates seeded with the infected bacteria were different in respect to prophage and megacin factor. As an effect of phage infection, the megacin factor may become labile and be lost. The non-megacinogenic segregants obtained in this manner may become lysogenic, but frequently no stabilization of the prophage occurs in the cells and thus in certain isolates lysogeny fails to develop after the loss of megacin factor. When the megacin factor had not disappeared, the derivatives showed labile or stable lysogeny. In stable isolates carrying the two plasmids the function of one of them predominated. These derivatives were lysed upon the addition of mitomycin; the kinetics of the lysis and the quantitative relationship of phage and megacin produced differed according to the predominance of the factors. In certain isolates the megacin factor completely repressed the induction of prophage. The two plasmids may, accordingly, hinder one another's replication and induction in different degrees.

Part of *Bacillus megaterium* strains occurring in nature shows lysis after ultraviolet irradiation. The lysates contain a narrow-spectrum antibacterial agent termed megacin [1]. The product exerted a bactericidal effect on all of a great number of *B. megaterium* strains by destroying the cytoplasmic membrane [2, 3]. A larger part of *B. megaterium* strains produced bacteriocins clearly distinguishable from the above agent on the basis of the manner of formation and mode of action. Holland and Roberts [4] classified these organisms into producers of megacin B and megacin C. The bacteriocin produced after induction and causing cytoplasmic destruction is termed megacin A. As the present paper deals with megacin A, in the following the agent will be referred to as megacin.

In the course of screening of about 400 *B. megaterium* strains we have isolated more than 24 megacin-producing cultures [6, unpublished data]. Strain 216, yielding the most effective lysates, was studied intensively. The lysates of this culture may be more active by several exponents than those prepared from other megacinogenic organisms. The megacin of strain 216 was isolated as a homomolecular protein [7] and it has been shown to be identical with phospholipase A [8]. It would appear that the genetic control of megacin is not located in the chromosome but is connected with a plasmid; this was supported by the high degree segregation of the megacin factor. The proportion of non-megacinogenic segregants in spore populations is usually 10^{-2} to

10^{-3}, but there is a strain which yields such segregants at a frequency as high as 10% [5, 6]. Induction of megacin production is very similar to the induction of typical lysogenic *B. megaterium* [9]. On the basis of the similarity it may be assumed that megacin production is controlled by a defective prophage [5]. This assumption, however, could not be confirmed by the experimental conversion of typical lysogenic cultures into megacinogenic organisms. Electron microscopic studies of the lysates of strain 216 and other cultures failed to show defective prophage structures [10 and Dr. BELIAEVA, personal communication].

The present paper reports on the production and study of lysogenic derivatives from strain 216.

Materials and methods

Bacterial strains. B. megaterium strain 216 produces megacin C in addition to megacin A [4]. The genotype of this organism is referred to as 216 MA^+C^+. For phage titration *B. megaterium* strain KM, for megacin assay phage resistant strain MUT-C were used. The latter was isolated in our laboratory from *B. megaterium* strain "Mutilate" [11]; strain MUT-C is about ten times as sensitive to megacin than strain KM.

Phage. The phage of *B. megaterium* lysogenic strain 899(1) was used. The phage was isolated and propagated to high titre on strain KM. As the phage exerted a slight activity on strains 216 and 216 MA^-C^+, its host range mutant (hp) was used. The e.o.p. value for the mutant was still lower to strain 216 than to strain KM (0.05 as related to the value for strain KM). The plaques of hp phage on culture KM were large and semi-clear, while on 216 MA^-C^+ cultures they were smaller and definitely turbid.

Media. YT (yeast extract tryptone) medium: tryptone (Oxoid), 10 g; yeast extract (Oxoid), 2.5 g; K_2HPO_4, 1 g; NaCl, 5 g; distilled water, 1000 ml; pH 7.5. YP (yeast extract peptone) medium was prepared as described previously [12].

Megacin and phage titration. YP broth culture of MUT-C ($O.D._{620} = 0.8$) was diluted 1 : 4 with YP medium and 1 ml was mixed with 1 ml molten (47 °C) nutrient agar and poured on a basic layer of the same medium. The basic layer was prepared with 1.5% agar, the upper soft layer contained half of this concentration. Serial dilutions of the lysates were prepared with saline containing 20% YP broth. By means of a loop equal amounts of the serial dilutions were placed onto the surface of the indicator plates. The reciprocal of the highest dilution causing clear zones or lysing at least half of the bacteria at the corresponding site was regarded as one unit of megacin/ml.

Cultivation of bacteria and induction of megacin factor and prophage. From a standing culture in YT medium incubated overnight 0.2 ml was transferred to 10 ml YT medium pipetted into a 100 ml Erlenmeyer flask. The flask was shaken in water bath at 37 °C. When an O.D. value of 0.25 had been reached (number of colony formers, 5×10^6/ml average chain length, 4—5), the cultures were supplemented with mitomycin (0.5 μg/ml) and the change in O.D. on further incubation was recorded.

Checking of lysogenicity and megacinogenicity of sporulating bacteria exposed to phage action was performed with indicator strains KM and MUT-C by the cross-streak method. If more isolates were examined, the heated spore suspensions were streaked onto YP agar so as to give 80—100 colonies. The young cultures were then transferred to KM and MUT-C indicator plates by means of velveteen stamps. Before incubation the replica plates were irradiated for 5 seconds with a low pressure mercury vapour lamp (7 erg/sec/mm²). The presence of megacinogenic clones was indicated by an activity on both indicator plates; in case of lysogeny, lysis occurred only on KM plates. Final examination of the isolates was carried out by mitomycin induction in liquid medium. If the lysates exhibited very low megacin activity, the differentiation from megacin C was performed by the use of anti-megacin A immune serum. The immune serum was prepared in rabbits by repeated injections of megacin A kindly supplied by Dr. I. B. HOLLAND. Megacin A neutralization test was performed as described in [13].

Preparation of spore suspension. Well-sporulating colonies or agar slant cultures were suspended in 0.02 *M* phosphate buffer (pH 7.2). After centrifugation the deposit was resus-

pended and incubated at 47 °C for a few hours to digest the cellular debris. After a second washing the suspension was centrifuged at 300 r.p.m. for 5 minutes then the supernatant, which contained mainly single spores, was collected and heated at 75 °C for 10 minutes.

Results

Lysogenization of strain 216 with hp phage. YT medium culture (O.D. = = 0.5; 8×10^6/ml colony former chains each consisting on the average of 8 rods) was mixed with hp phage (10 m.o.i.) and left to stand at room temperature for 1 hour. After centrifugation different amounts of the deposit were streaked to YT and YP plates. The cultures were incubated at 37 °C for 6—7 days. Colony morphology differed in various experiments. The following grouping was made:

1. Transparent colonies containing lysed bacteria which could not be subcultured.

2. Satellite colonies; small colonies along a concentric line at a distance of about 1 mm from the edge of a large, seemingly normal colony. On subculturing, the satellites grew as normal colonies. Mitomycin lysate of the colonies showed the exclusive presence of phages. The phage titre was high (10^{10}—10^{11}/ml); megacin was absent, but megacin C production was retained. These isolates were designated as 216 MA^-C^+ (hp).

3. Colonies of normal morphology characterized by stable plasmids. Heated spore suspensions of 25 colonies showed the presence of megacin A and phage. The megacin titre of the lysates varied between 10 and 10^4 units and the phage yields were in the range of 10^6—10^{10} PFU/ml. These isolates were designated as 216 MA^+C^+ (hp).

4. Colonies of normal morphology characterized by labile plasmids. In one lysogenization experiment the heated spore suspensions of 10 different colonies were subcultured in tubes of liquid medium. After the addition of mitomycin the cultures did or did not lyse. If the bacteria were lysed, the lysates were not identical. The behaviour of subcultures prepared from the 10 colonies may be classified as follows.

a) Subcultures of 3 colonies were not lysed after the addition of mitomycin. Other methods also failed to show the presence of prophage or megacin factor in the bacteria.

b) In the lysate of 4 colonies only megacin (approximately 10^4 units) was demonstrated, *i.e.* no lysogenization occurred.

c) Both phage and megacin were present in the lysate of 3 colonies, but these clones were not stable on subculturing.

Stability of the isolates. Isolates described under 2 and 3, 216 MA^-C^+ (hp) and 216 MA^+C^+ (hp), were stable and retained their original properties after several subcultures. In contrast, isolates described under 4c segregated on subculturing. The spore suspension of one colony of the last-mentioned iso-

lates was examined for phage and megacin production; of 90 colonies obtained by subculturing, 17 produced neither phage nor megacin, 73 lost their prophage but retained their megacin-forming capacity.

Accordingly, the relationship and stability of the two plasmids (prophage and megacin factor) may be variable in spore clones obtained by treating the megacinogenic strain with temperate phage. The lysogenized clone may contain stable bacteria possessing both properties (lysogenic and megacinogenic) so that one or the other character predominated. In other spores the prophage was unstable and segregated on germination. The lability of the prophage may involve the loss of megacin factor and thus both plasmids may be segregated from the cell. In contrast, the prophage may become stabilized but the megacin factor is lost.

Kinetics of the induction of various derivatives. The relationship and formation of products controlled by the two plasmids may be highly variable in isolates 216 MA^+C^+ (hp) and 216 MA^-C^+ (hp) derived from "wild" type strain 216 MA^+C^+ (Table I).

Table I

Characteristics of some derivatives of "wild" type strain 216

Strain	Product in the lysate*		Remarks
	megacin-A	phage	
216 MA^+C^+	+	—	"wild" type strain
216 MA^-C^+ (hp)	—	+	"satellite" colonies
216 MA^+C^+ (hp)-cll	+	+	high megacin yield
216 MA^+C^+ (hp)-b21	+	+	low megacin yield

* Megacin C occurring in insignificant amount in the lysates was disregarded in all experiments

The kinetics of the induction of "wild" type strain 216 MA^+C^+ and its lysogenized derivative 216 MA^-C^+ (hp) is shown in Fig. 1.

The lysogenic derivative, having lost megacin A factor, reached maximum growth soon after the addition of mitomycin and the rapid lysis of the culture resulted in a high phage titre. The "wild" type 216 MA^+C^+ strain grew less rapidly in the presence of mitomycin and reached the peak only after 50 minutes. Then a prolonged lysis followed which had not been complete by the end of the experiment. Megacin released from the culture reached a peak titre at the 150th minute. The characteristic behaviour of the strains was confirmed in three different experiments carried out under identical conditions (Table II).

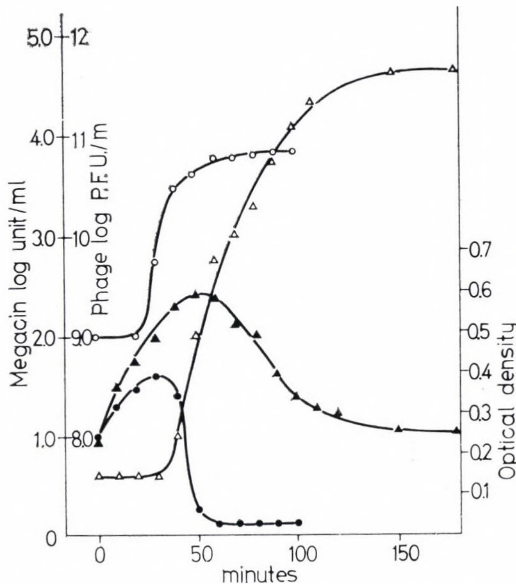


Fig. 1. Kinetics of the induction of strains 216 $MA^{+}C^{+}$ and 216 $MA^{-}C^{+}$ (hp). Logarithmic growth phase culture in YT medium was supplemented with mitomycin at 0 minute and reincubated. 216 $MA^{+}C^{+}$: ▲ optical density, △ megacin titre. 216 $MA^{-}C^{+}$ (hp): ● optical density, ○ phage titre

Table II

Results of three different experiments with strains 216 $MA^{+}C^{+}$ and 216 $MA^{-}C^{+}$(hp)

Experiment	Strain*	Maximum of growth curve		Onset of production		Maximum log titre	
		time (min)	O. D.	megacin	phage	megacin	phage
Exp. 1	$MA^{+}C^{+}$	57	0.65	55		4.0	
(21 06)	$MA^{-}C^{+}$ (hp)	35	0.35		20		10.4
Exp. 2	$MA^{+}C^{+}$	50	0.63	50		4.6	
(25 06)	$MA^{-}C^{+}$ (hp)	30	0.42		20		10.8
Exp. 3	$MA^{+}C^{+}$	60	0.62	45**		4.4	
(08 08)	$MA^{-}C^{+}$ (hp)	35	0.47		20		9.8

* Abbreviated symbols (see Table I).
** Estimated from the two intervals of assays.

For supplementing the data presented in Table II it should be mentioned that the phage titre before the induction of the lysogenic strain was already 5—6 log units or even higher, indicating that part of the population had undergone spontaneous induction before the addition of mitomycin. The megacinogenic culture contained slight amounts of bacteriocin before induction and lysis occurred considerably later than with the lysogenic strain.

The course of the induction of lysogenic and megacinogenic derivative 216 MA^+C^+ (hp)-cll differed definitely from that shown for the above cultures. As shown in Fig. 2, growth continued for a considerable time after mitomycin treatment and the culture reached a high O.D. value. The growing culture released phages as soon as after 40 minutes. The intensity of the rise in phage titre decreased at the 60th minute, then a second high degree phage release was observed. This double phage production was demonstrated

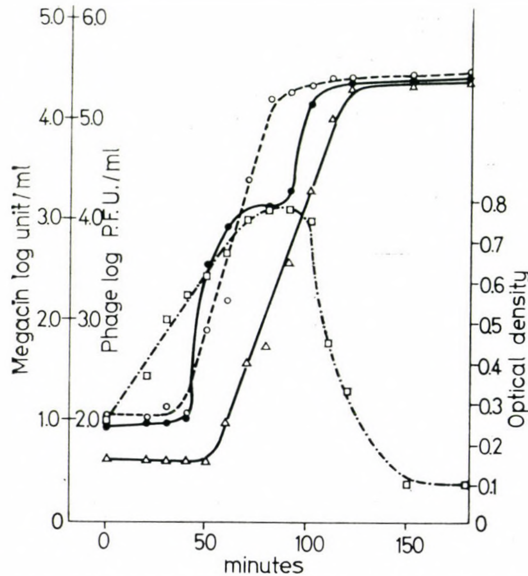


Fig. 2. Kinetics of the induction of isolate 216 MA^+C^+ (hp)-cll. The culture was supplemented with mitomycin at 0 minute and reincubated. □ optical density, ● phage titre, ○ phage titre after the addition of lysozyme, △ megacin titre

in all experiments. The second phase coincided with the accumulation of megacin. It was supposed that from part of phage-producing cells the virions were not released spontaneously and these cells were lysed only as an effect of megacin. To confirm this assumption, the following experiment was performed. Two parallel samples were taken from the induced culture of 216 MA^+C^+ (hp)-cll and one of them was treated with lysozyme (10 μg crystalline product per ml). The phage titres obtained are shown in Fig. 2.

It was questionable why lysozyme-treated cells yielded in the 50 and 60 minutes samples of poorer in phage than that present in the untreated control culture. There are several examples that phage replication is closely associated with certain structural elements of the cell. For example, replication of lambda-phage is associated with the cytoplasmic membrane from which DNA molecules enter the cytoplasm where their final maturation takes place

[14]. Lysozyme treatment in the early stage of growth acts by destroying the cellular structure on phage replication and is presumably the reason for the lower titre of infective virions.

A striking property of strain 216 MA^+C^+ (hp)-cll was that the phage titre in its lysate was lower by 3—4 exponents than in the lysate of 216 MA^-C^+ (hp)-cll.

Similar experiments were performed with isolate 216 MA^+C^+(hp)-b21. Induction of this derivative resulted always in very low megacin titre (5—100

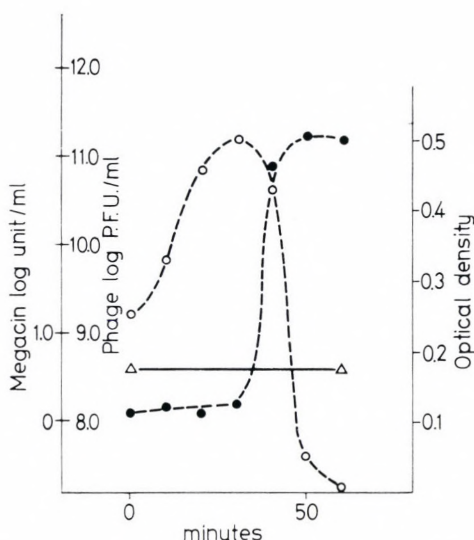


Fig. 3. Kinetics of the induction of isolate 216 MA^+C^+ (hp)-b21. ○ optical density, ● phage titre. Megacin titre (△) ranged between 1 and 10 units/ml in different assays. The solid line shows the course of megacin titre for an average value of 5 units/ml

units in various experiments). The antibacterial agent could be identified with specific immune serum as megacin A. The kinetics of the induction of this strain is shown in Fig. 3.

The megacin titre was very low, in the range of 5 units, throughout the experiment. This finding could not be explained by assuming that the megacin factor had considerably segregated from the cells of the isolate. Colonies grown from 2304 spores of isolate 216 MA^+C^+ (hp)-b21 were examined for the presence of megacin factor and there were only 6 colonies in which the factor was absent. The low megacin content of the lysate was probably due to a rapid induction of the prophage upon mitomycin treatment and the cells were lysed before the time necessary for the development of megacin production.

Determination of the single cell burst size. Experiments with strain 216 $MA^{-}C^{+}$ (hp) are summarized in Table III.

Table III

Single cell burst size experiments with spore suspension of strain 216 $MA^{-}C^{+}$(hp)

	Exp. 1	Exp. 2
No. of spores/tube (calculated)	0.3	0.5
No. of tubes inoculated	60	50
No. of tubes showing growth	15	35
No. of tubes inoculated + mitomycin	60	50
No. of tubes showing growth	0	0
No. of tubes with phage	14	35
PFU/tube maximum	890	966
PFU/tube minimum	49	88
Distribution of tubes according to phage yields		
PFU/tube 100	2	2
101—200	2	0
201—400	4	2
400	6	31
Burst size, average	364	708

Spore suspension of strain 216 $MA^{-}C^{+}$ (hp) was diluted with YT medium and used as inoculum at 0.5 ml (calculated No. of spores, 0.3—0.5) per tube. Before distribution into tubes the spore suspension was incubated at 37 °C for 1 hour to induce germination. Then the suspension was divided into two portions: one of them was supplemented with mitomycin (0.5 μg/ml), the other served as control. The suspensions were distributed at 0.5 ml amounts so that the calculated No. of spores was 0.3—0.5 per tube. All tubes with mitomycin remained clear and were used for phage counting by KM indicator. Part of mitomycin-free tubes showing growth served for the determination of actual spore counts.

The difference in average values and in distribution of phage yields according to tubes in the two experiments could not be attributed to the difference in the average number of spores. The Poisson distribution may only partly be responsible for the finding. Induction started after 1 hour germination had ended only 30—40 minutes later; this time sufficed for at least one division cycle. The induced bacteria showed not only an increase in size but also doubled their number [15]. It may be assumed that the actual number of virions released from lysogenic cells was lower than the value determined.

In similar experiments with isolate 216 $M^{+}C^{+}$ (hp)-b21 100 tubes were inoculated. Phages were detected in 14 tubes, in the rest there was no growth. The phage count in the positive tubes fell in the range 118—296; the average cell burst size was 200. Accordingly, the presence of megacin factor decreases

the value of burst size without exerting a significant effect on the kinetics of lysis.

In experiments with isolate 216 M^+C^+ (hp)-c11 the long induction time was taken into consideration: after the addition of mitomycin, inoculations were made into 60 tubes after each sampling performed at 1, 2 and 3 hours. After incubation for 24 hours none of the 180 tubes, each inoculated with an average of 0.54 spore, showed evidence of growth. The content of all tubes was mixed with soft agar containing the phage-sensitive indicator culture and poured into plates. None of the 180 plates showed plaques. Accordingly, induction of the calculated 108 spores failed to give rise to phage production.

To the control cultures no mitomycin was added. In 36 out of 60 control tubes growth was visible after incubation overnight. From 8 of the turbid cultures 0.1 ml aliquots were transferred into tubes with 10 ml YT broth and the cultures were induced with mitomycin in the usual manner. The lysates showed the presence of phages (10^5—10^6 PFU/ml) and megacin (10^3—10^4 units). Accordingly, in all spores of isolate 216 MA^+C^+ (hp)-c11 both plasmids were present. If a single cell is induced, it fails to release phages. If a population derived from one cell is induced, however, the products of both plasmids can be observed. The finding can be interpreted by assuming that the megacin factor represses the induction of prophage; larger populations may contain megacin-factor-free lysogenic segregants and thus on induction they may release phages. Strain 216 produces non-megacinogenic segregants at a rate of approximately 10^{-3} [6], in other words, the phage yields may be expected to be lower by 3 exponents than those for the above isolate. This difference agrees well with the experimental findings. Isolate MA^+C^+ (hp)-c11 was examined for the presence of megacin-factor-free spores. A total of 5037 individual spores all contained megacin factor. Consequently, the experiments failed to offer a direct proof of the assumed segregation. The failure may have had several reasons: 1. The number of spores was not sufficient for showing the expected 10^{-3} frequency of segregation; 2. bacteria after losing the megacin factor may undergo a spontaneous induction during sporulation and thus their proportion may be lower than expected. The high lability of the exclusively lysogenic strains indicates a spontaneous induction of the segregants.

Discussion

There are several examples that more than one extrachromosomal genetic element (plasmid) may exist in a single host. One of these factors may hinder the propagation of the other. Lysogenic strains are well-known to lose their prophages upon infection by homologous phage [16, 17].

Different sex factors in *E. coli* may hinder the replication of the others. The cell carrying the homologous sex factor fails to propagate the superinfecting sex factor [18, 19]. As an effect of R factor, introduced into *E. coli*

by conjugation, the culture loses its colicinogenic property [20, 21]. The presence of more plasmids may give rise to an unstable state in the cell, in consequence of which a segregation of these factors may occur during multiplication [22]. Introduction of a certain colicin factor into the cell may mobilize the otherwise latent R-determinant [23]. The mutual effect on *E. coli* cells and competition of sex factors and resistance factors are of special interest. The different factors may cause structural changes (specific pili) on the cell surface manifesting themselves in an alteration of phage sensitivity and of the organism's antigenic structure. In respect to this interesting problem we refer to the review of MEYNELL and DATTA [24], and only mention that the function of F factor is completely repressed by fi $^-$R factor introduced into the cell, while its fi $^+$R mutant exerts no such effect.

In our experiments the mutual relationship of *B. megaterium* plasmids was studied. For the genetic control of megacin A, which is identical with phospholipase A [8], a defective prophage appears to be responsible [5]. The stability of the hypothetical defective prophage, referred to as MA$^+$ plasmid, was highly influenced by superinfection with a phage obtained from *B. megaterium* strain 899(1). In our experiments strain 216 was infected by a high multiplicity phage, sporulated on agar plate for prolonged periods and analysed for megacin factor and prophage relationship. Unfortunately this method, in spite of its advantages, offered practically no data to allow for quantitative conclusions. The effect of phage treatment was marked, that is, on further incubation the majority of lysogenic colonies were lysed. The transient lysogenic state was probably very labile; similar observations were made for individuals of spore clones originating not unfrequently from lysogenic bacteria. During germination and multiplication of these the prophage was frequently lost. The loss of plasmids occurred in different manners as follows. (a) The prophage introduced failed to stabilize. (b) As an effect of phage infection, the megacin factor disappeared from the sporulated cells, but the bacteria became lysogenic and were stabilized in this state. (c) In spore clones derived from infected cells neither prophage nor megacin factor was present, *i.e.* as an effect of phage action the megacin factor disappeared from the infected cell without rendering the organism lysogenic. (d) Both plasmids were introduced into the cells in stable state.

Stable megacinogenic and lysogenic isolates were especially interesting in that, although both plasmids could be induced separately with mitomycin, their simultaneous occurrence had influenced one another's induction. There were isolates the induction of which ensued rapidly and the lysate characterized by high phage yields contained very slight amounts of megacin although the plasmid of the last-mentioned factor was present practically in all spores. An isolate, in which the megacin factor repressed the prophage in such a degree that its induction failed to take place, deserves special interest.

The finding that the repressor produced under the effect of megacin factor may completely repress the induction of prophage should also be stressed. The question arises of the specificity of the repressor in that case. Does the same repressor, in view of its high amount, act on the corresponding operator of both plasmids? If so, a less definite common specificity would also bring about the effect. It might of course be supposed that in these isolates a recombination of the corresponding sectors of the two plasmids occurred, which resulted in a repressor protein capable of exerting an action on both factors.

Finally, we wish to mention that we isolated about 24 strains producing megacin A. The lysates of these, except for strain 216, showed a slight or medium antibacterial activity. Although all megacin A lysates were identical in mode of action, they differed sharply in immunobiological specificity from the megacin of strain 216 [25]. Two of the isolates released very slight amounts of phages [6, unpublished data]. It would appear that in natural lysogenic derivatives of megacin A-producing strains prophage induction can be repressed by megacin factor.

REFERENCES

1. Ivánovics, G., Alföldi, L.: Nature (Lond.) **174**, 465 (1954).
2. Ivánovics, G., Alföldi, L., Nagy, E.: J. gen. Microbiol. **21**, 51 (1959).
3. Holland, I. B.: J. gen. Microbiol. **29**, 603 (1962).
4. Holland, I. B., Roberts, C. F.: J. gen. Microbiol. **35**, 271 (1964).
5. Ivánovics, G.: Zbl. Bakt. I. Abt. Orig. **196**, 318 (1965).
6. Ivánovics, G., Nagy, E.: J. gen. Microbiol. **19**, 407 (1958).
7. Holland, I. B.: Biochem. J. **78**, 641 (1961).
8. Ozaki, M., Higashi, Y., Saito, H., An, T., Amano, T.: Biken J. (Osaka), **9**, 201 (1966).
9. Marjai, E., Ivánovics, G.: Acta microbiol. Acad. Sci. hung. **11**, 193 (1964).
10. Ivánovics, G., Alföldi, L., Lovass, B.: Acta microbiol. Acad. Sci. hung. **4**, 295 (1957).
11. Ivánovics, G., Alföldi, L., Széll, A: Acta microbiol. Acad. Sci. hung. **4**, 333 (1957).
12. Csiszár, K., Ivánovics, G.: Acta microbiol. Acad. Sci. hung. **12**, 73 (1963).
13. Ivánovics, G., Nagy, E., Alföldi, L.: Acta microbiol. Acad. Sci. hung. **6**, 161 (1959).
14. Salivar, W. O., Gardiner, J.: Virology **41**, 38 (1970).
15. Ivánovics, G., Alföldi, L.: J. gen. Microbiol. **16**, 522 (1957).
16. Bertani, G.: Cold Spr. Harb. Symp. quant. Biol. **18**, 65 (1953).
17. Appleyard, R. K.: Genetics **39**, 440 (1954).
18. Scaife, J., Gross, J. D.: Biochem. biophys. Res. Comm. **7**, 403 (1962).
19. Maas, W. K.: Proc. X. Int. Congr. Microbiology, Mexico 1970. p. 203.
20. Kracméry, V., Hurwitz, C., Fredericq, P.: J. Bact. **102**, 521 (1970).
21. Kato, Y., Hanaoka, M., Amano, T.: Biken J. (Osaka) **5**, 77 (1962).
22. Driscoll-Zamenhof, R.: In: Gunsalus, J. C., Stanier, Y., The Bacteria. Vol. 5., p. 155. Academic Press, New York—London 1946.
23. Anderson, F. S.: Ann. Ref. Microbiol. 22, 131 (1968).
24. Meynell, E., Datta, N.: Ciba Foundation Symp. on Bacterial Episomes and Plasmids. Churchill, London (1969) p. 120.
25. Nagy, E., Alföldi, L., Ivánovics, G.: Acta microbiol. Acad. Sci. hung. **9**, 285 (1962).

Address of the authors:

Vera Gaál, József Molnár, György Ivánovics
Institute of Microbiology, University Medical School
Szeged, Hungary.

Acta microbiol. Acad. Sci. hung. 18, 41—45, 1971

EFFECT OF PYRAZOCILLIN ON THE INDUCED PENICILLINASE SYNTHESIS OF BACILLUS CEREUS

I. MILE, V. CSÁNYI, I. KOCZKA, ÉVA BADÁR and I. HORVÁTH

By

Institute of Medical Chemistry (Head: F. ANTONI*), Semmelweis University Medical School, and Section of Chemotherapy (Head:* I. KOCZKA*), Institute for Pharmaceutical Research, Budapest*

(Received October 29, 1970)

Summary. Pyrazocillin (1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl penicillin) induced a low-degree penicillinase synthesis in *Bacillus cereus* 569 cells. Its affinity to the "penicillin binding factor" of the cells was low in contrast to the high affinity of penicillin, but the binding was irreversible, like that of penicillin.

The semi-synthetic penicillin derivatives have three important properties *viz.* they exert a distinct antibiotic action, they induce penicillinase synthesis in inducible strains and they influence the conformation of the penicillinase protein [2]. Pyrazocillin,* containing a 1-(2,6-dichlorophenyl)4-methyl-5-pyrazolyl side chain, suppresses the growth of Gram-positive microorganisms at a low concentration and is, therefore, an excellent therapeutic agent [2].

Detailed studies by one of us [3] on the effect of pyrazocillin on the exopenicillinase of the *B. cereus* 569/H strain suggested that the compound influences the conformation of penicillinase differently from other penicillin analogons. In its presence, penicillinase was rapidly inactivated, this effect was reversible by dilution and not reversible by addition to the inactivated system of G-penicillin, the enzyme's natural substrate. Unlike G-penicillin and the known analogons, pyrazocillin is bound to penicillinase weakly and this seems to account for its failure to inactivate the enzyme if G-penicillin is present.

These extraordinary properties of pyrazocillin have initiated further investigations into its effect on the synthesis of induced penicillinase.

Materials and methods

Pyrazocillin (1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl penicillin) was used in the form of its sodium salt. The potassium salt of penicillin-G was a product of the Biogal Pharmaceutical Works (Debrecen, Hungary).

Cells of the *B. cereus* 569 strain were cultivated under the same conditions as described earlier [4]. The dry material content of the cells was assessed by photometry and their penicillinase activity was determined as reported earlier [5].

* The pyrazocillin preparation used in the experiments was supplied by the Institute of Pharmaceutical Research (Budapest)

Results

First, pyrazocillin was tested for ability to induce penicillinase synthesis. G-penicillin or pyrazocillin were added to *B. cereus* 569 cultures in the log phase of growth and the dry material contents and penicillinase production of the cells were determined. G-penicillin induced maximum enzyme synthesis at a concentration as low as 0.5 μg/ml [6], while one hundred times more (50 μg/ml)

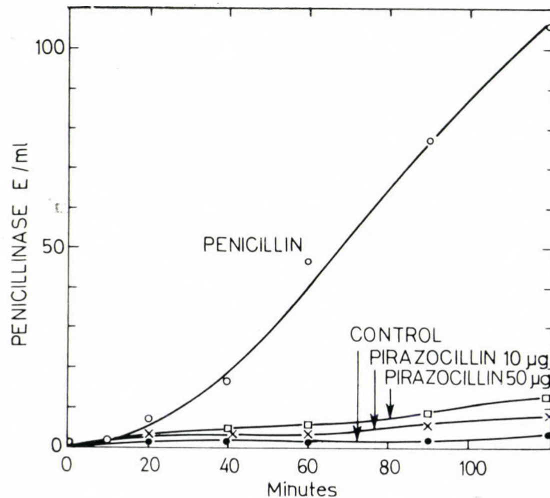


Fig. 1. Induction of penicillinase with pyrazocillin. G-penicillin (0.5 μg/ml) or pyrazocillin at various concentrations were added to cells in the log phase of growth (0.1 μg/ml dry material content), and the penicillinase activity of the systems was estimated at different points of time. The dry material contents of the differently treated samples did not differ notably by the end of the experiment

pyrazocillin induced the production of ten times less enzyme. This yield could not be increased by augmenting the concentration of pyrazocillin above 50 50 μg/ml; there was rather a certain decrease which may have been due to the enzyme-inhibitory action of the compound.

In the experiment shown in Fig. 1, the dry material content of the cultures did not change under the effect of pyrazocillin at 0 to 200 μg/ml concentration, but it decreased by about 10% at 250 μg/ml concentration.

Obviously, pyrazocillin is able to induce penicillinase like the other penicillin analogons, but this activity is very slight.

The next step was to clarify whether pyrazocillin is as irreversibly bound by the cells as G-penicillin is [6] and if so, how this would influence the induction by G-penicillin.

A suspension of *B. cereus* cells in the log phase of growth was divided into four parts. To the first suspension, pyrazocillin was added up to a final

concentration of 250 μg/ml, then it was incubated for 5 minutes at 30 °C, centrifuged and washed with cold (0 °C) growth medium. The washed cell sediment was resuspended in G-penicillin (0.5 μg/ml) containing warm medium (30 °C), allowed to stand for 5 minutes, centrifuged, washed with cold medium, and finally resuspended in fresh growth medium. The second suspen-

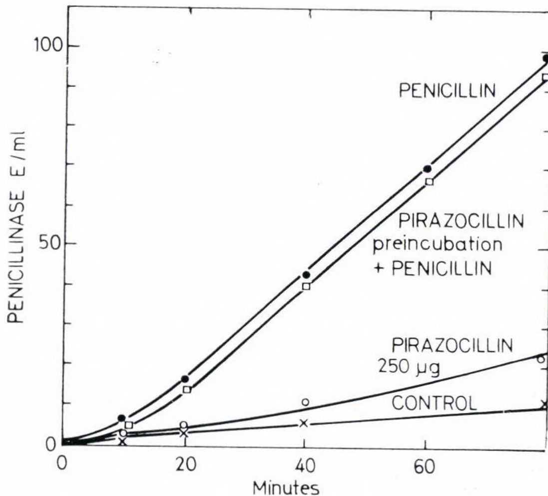


Fig. 2. Effect of pyrazocillin on the quantity of penicillinase induced by G-penicillin. *B. cereus* 569/H cells pretreated in different ways in the log phase of growth were centrifuged, resuspended in fresh growth medium and assayed for penicillinase activity. Pretreatments. × = Control, not pretreated. ○ = 250 μg/ml pyrazocillin was added to the cell suspension for 5 minutes, then it was removed by centrifugation and washing. □ = Preincubation with pyrazocillin as above +G penicillin, the washed cells were resuspended in an 0.5 μg/ml G-penicillin containing medium for 5 minutes, then washed and centrifuged. ● = Penicillin was added to the medium at a concentration of 0.5 μg/ml and was removed after 5 minutes by centrifugation and washing

sion was treated in the same manner, but G-penicillin was omitted. The third suspension was treated only with the 0.5 μg/ml G-penicillin containing medium, centrifuged, washed and resuspended. The fourth suspension served as an untreated control. All four suspensions were assayed for enzyme activity (Fig. 2).

As can be seen from Fig. 2, 5-minute pyrazocillin pretreatment had not notably affected the inducer activity of G-penicillin; the enzyme yields were almost the same in pretreated and not pretreated cultures if G-penicillin was kept at the same level. At a concentration of 250 μg/ml, pyrazocillin itself acted as an inducer despite the attempt to remove it by washing after 5 minutes; the fact that this action was more pronounced than that of 50 μg/ml retained in the system in the first experiment, suggests that pyrazocillin is irreversibly bound by the cells.

Discussion

Previous studies in this laboratory suggested a special influence of pyrazocillin on the conformation of penicillinase. Pyrazocillin was shown to inactivate the enzyme after a few minutes of incubation, and the inactivation was not reversible by G-penicillin, the specific substrate. The binding of pyrazocillin to penicillinase was by several orders weaker than that of G-penicillin [4].

It was supposed that cells capable of an inductive penicillinase synthesis possess in addition to the enzyme at least one protein which reacts specifically with penicillin or its derivative; this hypothetical protein was termed "penicillin binding factor". The G-penicillin complex of this factor seemed to be responsible for the increase in the rate of penicillinase synthesis during induction [6].

As in the present experiments pyrazocillin has been shown to be capable of inducing penicillinase synthesis, its linking with the "penicillin binding factor" seems very probable.

The affinity between them is, nevertheless, weak as 50 μg/ml pyrazocillin activated ten times less enzyme synthetizing capacity than did 0.5 μg/ml G-penicillin.

Despite their low affinity, the binding between pyrazocillin and the "penicillin binding factor" seems irreversible; cells preincubated with 250 μg/ml of the compound continued to synthetize penicillinase after the removal of unbound pyrazocillin by washing (Fig. 2).

The low affinity of pyrazocillin to both the "penicillin binding factor" and the penicillinase enzyme seems to suggest a great similarity of the respective components responsible for binding (active centres).

A dissimilarity, in contrast, was suggested by the fact that the presence of pyrazocillin caused no reduction of the amount of G-penicillin bound by the "penicillin binding factor", though such a decrease might have been expected from the inactivating effect of pyrazocillin on penicillinase. Cells preincubated with pyrazocillin were inducible with penicillin G to the same extent as the control cells if the unbound pyrazocillin had been removed by washing. This suggests that pyrazocillin eliminates the inhibitory effect of the "penicillin binding factor", in agreement with RICHMOND's hypothesis [7] that the regulatory systems of penicillinase consist of at least two units of protein nature, one of which links with the inducer and acts negatively. Our own experiments on the messenger stability of penicillinase also suggest that the positively acting protein cannot stabilize the messenger unless the negatively regulating "penicillin binding factor" has been "neutralized" by the inducer [8].

REFERENCES

1. Citri, N., Pollock, M. R.: Advances in Enzymology **28**, 237 (1966).
2. Koczka, I., Fehér, O., Vargha, L.: Pyrazocillin: A new semi-synthetic penicillin. Proc. Sixth Int. Congress for Chemotherapy, Tokyo 1969. Al 1.
3. Csányi, V.: Acta biochim. biophys. Acad. Sci. hung. **1**, 149 (1966).
4. Csányi, V., Mile, I., Koczka, I., Badár, É., Horváth, I.: Biochim. biophys. Acta (Amst.) **220**, 317 (1970).
5. Csányi, V.: Acta physiol. Acad. Sci. hung. **18**, 261 (1961).
6. Pollock, M. R.: Brit. J. exp. Path. **33**, 587 (1952).
7. Richmond, M. H.: J. molec. Biol. **26**, 357 (1967).
8. Gervai, J., Csányi, V., Mile, J.: Biochim. biophys. Acta (Amst). **240**, 430 (1971).

Address of the authors:

Imre Mile, Vilmos Csányi
Institute of Medical Chemistry, Semmelweis University Medical School
Puskin u. 9, Budapest VIII, Hungary
István Koczka, Éva Badár, István Horváth
Institute for Pharmaceutical Research
Szabadságharcosok útja 47—49, Budapest IV, Hungary

Acta microbiol. Acad. Sci. hung. 18, 47–54, 1971

LYSOGENIC CONVERSION IN THE SHIGELLA FLEXNERI GROUP

By

I. KÉTYI, ADÉL VERTÉNYI and I. FINANCSEK

Institute of Microbiology (Head: K. RAUSS), *University Medical School, Pécs*

(Received October 30, 1970)

Summary. A temperate phage capable of inducing antigen conversion was isolated from a *Shigella flexneri* type 4b strain. The isolate, designated P90, induced the appearance of type-specific antigen IV_1 in all examined strains except those carrying antigen complex IV. The antigen appearing in the converted strains was not identical but showed an a, b — a, c antigenic relationship with antigen IV_1.

Neutralization tests showed phage P90 to be serologically identical with phage φIV_1 of similar antigen-converting ability. Yet there was no cross immunity between the two phages and a double lysogenic derivative could be induced with them.

Lysogenic conversions of antigenic structure have been studied primarily with regard to the Salmonella group. Similar studies of *Sh. flexneri* were first reported by MATSUI [1], who described an alteration of the antigenic pattern by lysogenic conversion into type IV, using a phage isolated from a 4aA strain. More detailed examinations along this line were then performed by ISEKI and HAMONO [2]. OKADA *et al.* [3] isolated a phage which induced the appearance of type I antigen in many serotypes. GIAMMANCO [4] and GIAMMANCO and NATOLI [5] demonstrated the induction of antigens "II", "V" and "7, 8" by lysogenic conversion. Conjugation experiments by TIMAKOV *et al.* [6] showed the chromosomal localization of the information of all these antigens to be in linkage with the proline locus.

In this laboratory, examinations of lysogenic conversion in the *Sh. flexneri* group were undertaken to complement genetic studies performed on the same group with the conjugation technique. This paper reports on results obtained with a phage of antigen-converting ability isolated by us.

Materials and methods

Media. Meat broth, liquid and solidified by addition of 1.8% agar was used. Both the liquid and the solid medium contained 0.5% NaCl and 1% peptone.

Propagation of the phage in liquid medium was made in LOEB's [7] solution.

Strains. The strains representing various *Sh. flexneri* serotypes are listed in Table I, with their origins marked under the heading "Strains". Other strains used in the experiments were a *Sh. flexneri* 4b strain, defective in respect of antigen IV_1, designated "527", ISEKI's "variant 4c" strain lysogenic for phage φIV_1 and tester strain EW8, a *Sh. flexneri* type 1a strain, both supplied by the author.

Sera. Sh. flexneri type and factor sera prepared in this institute were used [8]. Antiphage sera were prepared in rabbits by treating them intravenously with 5 ml phage lysate

twice weekly for 3 weeks; the dose corresponded to about 10^7 e.o.p/ml. Five days after the last inoculation the animals were exsanguinated and the harvested serum was stored without preservation at -10 °C.

Antigen adsorption tests were performed with 1 : 10 diluted sera and the results were obtained by slide agglutination tests.

Phage techniques. Isolation of the temperate phage of antigen-converting ability was made from the single plaque formed by the phages released from the chloroform-killed lysogenic strain under covering with the tester strain. The block of agar containing the plaque was cut out from the plate, placed in broth, the bacteria were killed with chloroform and removed together with agar fragments by centrifugation. The isolation of a single plaque was repeated 3 times for appropriate purification.

Induction by UV, which failed, was made with a 25 W bulb at 50 cm distance, with the exposure varying between 10 seconds and 5 minutes.

Attempts to propagate the phage in liquid medium — broth or LOEB's solution — invariably failed, also with varied sizes of inoculum. Propagation was, therefore, made by washing off with broth the plaques formed on agar plates showing confluent lysis.

Phage titrations were made on the surface of agar plates, using serial dilutions of the lysate in broth and of about 10^7 germs of the added tester strain. Mean plaque counts calculated from the appropriate dilutions were expressed in terms of plating efficiency (e. o. p.)

In the neutralization tests, 10^7 e. o. p./ml phage was added to tenfold serial dilutions. of serum in broth (10^{-1} — 10^{-4}); such mixtures of the preheated (37 °C) components were incubated for 5, 10 and 15 minutes in a water bath of 37 °C. In samples immediately diluted to 1 : 100 the plaque count was estimated. By means of parallel titrations of the non-neutralized phage, the results were expressed as "k" value.

For lysogenic conversion, the bacterial strain was carried through 5 serial passages in broth to which about 10^6 e. o. p./ml of phage was added. The broth culture was streaked onto agar plates and, after appropriate incubation, at least 50 colonies were isolated and examined for change of antigenic pattern in the slide agglutination test using factor sera.

For the purpose of "disinfection", the lysogenic strain was carried through 1 : 10 diluted antiphage-containing broth, at least 5 times.

Mathematical-statistical analysis of the results was performed by STUDENT's *t* test [9].

Results

1. *Isolation of P90 phage.* A temperate phage was isolated from a lysogenic type 4b *Sh. flexneri* strain designated "90", by means of an *Sh. flexneri* 1a sensitive strain. With regard to the host strain, the isolate was designated P90. The phage was released spontaneously after the host strain had been killed by chloroform vapour; attempts to induce it with UV irradiation had failed. In the broth culture of the tester strain the phage did not cause lysis, nor a reduction of germ count. $CaCl_2$ did not increase spontaneous lysis. Propagation by washing off of plaques formed on agar plate resulted in a lysate with a value of about 5 × 10^7 e. o. p./ml. Phage P90 proved to be resistant to chloroform, but displayed low stability in saline. Plaques formed on the *Sh. flexneri* 1a strain used as tester were of the clear minute type (Table I), while on other strains reacting with lysis, the plaques were rather of turbid character (representant strains of the 1b, 3a and Y variant serotypes). Representant strains of the other serotypes except 4aA and 4aB, had adsorbed the phage as concluded from the resulting antigenic conversion.

2. *Conversion of antigen induced by phage P90.* Eventual conversion of antigen and the rate of conversion were assessed by slide agglutination test using factor sera. Of the strains reacting to the phage with lysis or adsorption,

Table I

Sensitivity to phage P90 of Shigella flexneri strains representing different serotypes

Serotype	Strain*	Sensitivity to phage P90
1a	Weil; 63—125—700	++
1b	Weil; 66—1—409	+
2a	Weil; 63—40	A
2b	Weil; 66—1—1268	A
3a	Weil; 63—38	+
3b	Ewing; 9124	A
4aA	Carpenter; Ratcliffe	—
4aB	Carpenter; 11D	—
4b	Rauss; 90	.
5	Weil; 63—149—119	A
6	Weil; 64—104—i	A
var. X	Weil; 66—1—411	A
var. Y	Weil; 63—143—4	++

* Origin and original designation
Explanations: ++ = clear minute type plaque; + = turbid minute plaque; A = adsorption of phage without lysis; — = phage resistant; . = not tested

in a frequency of 2—70% showed the phenomenon of antigenic conversion as assessed by examination of 50 colonies each. The frequency found was in itself exclusive of the phenomenon of transduction. In every case, conversion manifested itself by the appearance of a group IV (IV_1) specific antigen (Table II).

In the case of *Sh. flexneri* type 1a and 1b strains, the type specific "I" antigen disappeared at the appearance of type IV_1 specificity. Essentially the same occurred with serotypes 2a and 2b, but in this case conversion II → IV_1 was associated with the appearance of group antigen "3, 4". Group antigen "3, 4" could not be demonstrated in S-phase strains, its appearance being interpreted as the sign of R mutation taking place by group antigen "3, 4" of the specific antigens. In contrast, in the serorepresentant *Sh. flexneri* var. Y, group factor 3, 4 was converted into type specific antigen IV_1. Unlike the former, the converted type 3b strain was found to contain exclusively IV_1 antigen; group factors "3, 4" and "6" as well as type specific antigen "III" had disappeared. The representant strains of *Sh. flexneri* 3a, 5, 6 and var. X retained all antigenic factors originally present in them despite the actual antigenic conversion of IV_1.

It should be noted that the strains reacting to phage P90 by lysis (types 1a, 1b, 3a, var. Y) lost their sensitivity to this phage after lysogenization.

Table II

Antigenic conversions induced by phage P90 by agglutination test using factor sera

Original strain (serotype) and converted strain	Rates of conversion (No. of colonies examined /No. of colonies converted)	Agglutination in factor sera									
		3.4	6	7.8	I	II	III	IV_1	IV_2	V	VI
1a	3/150	—	.	.	++	.	.	—	.	.	.
1a/26*		—	.	.	—	.	.	++	.	.	.
1b	2/50	—	++	.	++	.	.	—	.	.	.
1b/39		—	++	.	—	.	.	++	.	.	.
2a	1/50	—	.	.	.	++	.	—	.	.	.
2a/41		++	.	.	.	—	.	++	.	.	.
2b	1/50	—	.	+	.	++	.	—	.	.	.
2b/49		++	.	++	.	—	.	++	.	.	.
3a	36/50	—	+++	++	.	.	++	—	.	.	.
3a/6		—	+++	++	.	.	++	++	.	.	.
3b	20/50	+	+++	—	.	.	++	—	.	.	.
3b/9		—	—	—	.	.	—	++	.	.	.
5	4/50	.	.	+	.	.	.	—	.	++	.
5/12		.	.	++	.	.	.	++	.	++	.
6	3/50	.	.	.	.	.	.	—	.	.	+++
6/9		.	.	.	.	.	.	++	.	.	+++
var. X	7/50	.	.	++	.	.	.	—	.	.	.
X/6		.	.	++	.	.	.	++	.	.	.
var. Y	11/50	+++	.	.	.	.	.	—	.	.	.
x/46		—	.	.	.	.	.	++	.	.	.

* Designation of the converted strain
Symbols used: +++, ++, + = agglutination
— = negative reaction
. = not tested

Type 4b *Sh. flexneri* strain "90" lost its IV_1 antigenic specificity upon treatment with anti-phage serum and on subsequent lysogenization with phage P90 it adsorbed this without lysis, with the reappearance of the specific antigen IV_1. In a *Sh. flexneri* type 4b strain ("527") which had previously lost its factor IV_1 this factor appeared after lysogenization with phage P90.

3. *Analysis by antigen absorption of the converted antigenic specificity IV_1.* These examinations were carried out by means of the seroconvertant *Sh. flexneri* 1a/26, using the factor sera prepared during this antigenic analysis to identify the converted antigens which uniformly proved to be of IV_1 specificity in the representant strains of all the serotypes examined.

Results are summarized in Table III. Despite its IV_1 antigenic character, the converted strain *Sh. flexneri* 1a/26 failed to absorb antibodies to IV specific (IV_2) and IV group factors (IV_1) from anti-4aA, -4aB or -4b sera. On the other hand, negative results of the absorption experiment with 1a type serum verified the loss of antigenic factor "I", which was not demonstrable by agglutination, either.

Table III

Serological analysis of IV_1 type specific antigen induced in Shigella flexneri 1a (1a/26) strain by lysogenization with phage P90

Strains	Agglutination with absorbed sera							
	4aA>1a/26*	4aB >1a/26	4b > 1a/26	1a > 1a/26	1a/26 > 4aA	1a/26>4aB	1a/26>4aB+3a	1a/26>4b(90)
1a	—	—	—	+++	—	—	—	—
1a/26	—	—	—	—	+++	+++	+++	+++
3a	—	—	+++	—	—	++	—	—
4aA	+++	—	—	—	—	+++	—	—
4aB	—	+++	+++	—	+++	—	—	—
4b	—	+++	+++	—	++	+	—	—
var. Y	—	—	—	—	—	—	—	—

* = 1 : 10 diluted serum and antigen used for absorption
Symbols used: +++, ++, + = agglutination
— = negative reaction

Reciprocal absorption experiments, too, have shown the failure of all strains carrying antigen IV_1, including the host strain of phage P90, to remove specific antibodies from the antiserum to the converted strain 1a/26.

The antigenic analyses have confirmed an a, b—a, c type relationship between factor IV_1 and the converted antigen.

4. *Comparative studies of phages P90 and φIV_1.* It seemed interesting to compare the phages P90 and φIV_1 [2] of similar converting ability. The lysogenic "variant IVc" strain carrying phage φ IV_1 and, for propagation, the *Sh. flexneri* type 1a strain EW8 were kindly supplied for the experiments by Dr. S. Iseki. Phage φIV_1 produced clear plaques of pinpoint rather than minute type in the EW8 strain as well as in our type 1a strain. The lysate obtained by propagation of the φIV_1 phage was of about the same order (10^7) as that obtainable with phage P90. Apart from the dissimilar plaque morphology, the two phages differed also in other respects, as strain EW8 was resistant to phage P90 and our *Sh. flexneri* type strains behaved dissimilarly against phage φIV_1. In addition to our 1a strain, only the 4b strain designated "527" which was defective in respect of antigen IV_1, reacted by lysis to phage φIV_1.

The latter phage was reported by ISEKI and HAMANO [2] to convert strains of 1a, 1b, 2a and 3b type whereas, as outlined in the foregoing, our phage P90 converted all examined strains except those carrying antigen complex IV.

The ability of phage φIV_1 to convert the *Sh. flexneri* 1a strain maintained in this laboratory was examined under the conditions previously employed for phage P90. The 50 colonies examined after lysogenization in the phage-containing broth showed a very high — 46/50 — rate of conversion. One of the converted strains (1a/1) was examined further; testing it with the factor sera obtained by antigenic analysis, the factor replacing antigen "I" was identified as a complete IV_1.

The converted strain 1a/1, lysogenic for phage φIV_1 retained its original sensitivity to P90. The other converted mutant of the 1a strain, 1a/26, lysogenic for phage P90, proved sensitive to φIV_1. Using the latter derivative and phage φIV_1, a double lysogenic strain could be produced. The proof of double lysogenicity was on the one hand the immunity to both phages, and on the other hand the presence of the antigenic factor specific for P90 together with a full antigenicity of IV_1, as demonstrated serologically.

Table IV

Serological comparison of phages P90 and φIV_1 by neutralization tests

Phages	k-values obtained with antiphage sera	
	P90	φIV_1
P90	66.4 ± 14.8*	59.9 ± 21.6
φIV_1	1250 ± 216	1480 ± 185

* = ± standard deviation

Finally, using antiphage sera prepared with the two phages, their serological relationship was examined by neutralization tests. Experimental results obtained in this series are summarized in Table IV. As seen from this Table, the average values yielded by cross neutralization tests varied within the limits of the standard deviation, *i. e.* the serological differences between the two phages were not significant statistically.

Discussion

Genetic information and immunochemistry of the antigenic structure of *Sh. flexneri* differ in certain respects from those of the Salmonella group which has been studied in more detail. In both *Salmonella* [10] and *Escherichia* [11], the locus of the O antigens was found to be in linkage with the histidine gene. As to *Sh. flexneri*, FORMAL *et al.* [12], using a *Sh. flexneri* 2a Hfr strain

obtained by terminal selection, found the group factor "3, 4" to be related to the histidine locus, while the type specific antigen "II" to loci linked with the proline gene. As a result of crosses performed with the *E. coli* K-12 HfrC strain as donor, TIMAKOV *et al.* [6] identified the sites of loci of the I, II, IV and V type-specific antigens and of the group factors "7, 8" as well, to be in a linkage group with the proline region. The proline region-linked O-antigen loci differ considerably from the histidine linkage, which is certainly the evolutionarily archaic one of its kind. This difference and contradiction appear to be virtual if it is considered that the formation of these antigens by lysogenic conversion has been verified.

In immunochemical respect, the findings of SIMMONS [13] are worth of consideration. Unlike the O-specific polysaccharide side chain of salmonellae, *Sh. flexneri* contains one primary and one so-called secondary side chain. The primary side chain consists of alternate rhamnose and acetyl-glucosamine molecules, its group-specific character being defined by the type of linkages. On this chain is superposed the so-called secondary side chain consisting of glucose or perhaps O-acetylglucose molecules, which is responsible for the type-specificity. As outlined in the foregoing, most of the type-specific antigenic information is linked with the proline locus. Thus, according to SIMMONS [13], a uniform explanation can be found for lysogenic conversions.

The phage with converting ability isolated in this laboratory appears interesting in several respects. First of all it induced antigenic conversion in all serotypes tested except the strains carrying the antigen complex IV. Examining the data for serological conversions in Table II from the angle of changes in the antigenic formula of the converted strains, it is obvious that the appearance of IV_1 specificity always took place with the loss of I (1a, 1b) and II (2a, 2b) type specific antigens. This did not, however, occur with group factor "7, 8" (2b, 3a, 5, var. X) and V (5) antigen (also, "proline-group-related"). The available data naturally do not suffice for conclusions on the allelic nature of I, II and IV antigenic informations. Appearance of IV_1 specificity did not involve the disappearance of the "histidine-related", *viz.* "6", "3, 4", "VI" or "III" antigens, except with the representant strains 3b or Y variant, for which it is difficult to find an explanation. An eventual masking effect remains to be confirmed by further antigen adsorption experiments.

Another inconsistent feature of antigenic conversion produced by phage P90 is the appearance of a serologically non-identical IV_1 specificity. This problem is most reasonably discussed in relation to phage φIV_1. Phage φIV_1, the ability of which to induce antigenic conversion of identical IV_1 specificity has also been confirmed in this laboratory, may be considered serologically identical with phage P90 on the evidence of cross neutralization tests. Yet there is no immunity (repression) between the two phages and even a double lysogenic derivative can be produced with them. Hypothetically, the explana-

tion may be offered that the chromosomal attachment sites of the two converting phages are not identical. Accepting LeMinor's [14] hypothesis in that the mechanism of lysogenic conversion is due to a functional modification by the attachment of the prophage to the chromosomal locus responsible for antigen synthesis, both the difference in antigen specificity and the absence of cross immunity between phages φIV and P90 seem reasonable.

The attachment sites of the prophages and the precise localization of the antigenic informations on the *Sh. flexneri* chromosome remain to be clarified by further studies.

Acknowledgement. The authors wish to express their thanks to Dr. S. Iseki (Dept. of Legal Medicine, School of Medicine, Gunma University, Maebashi, Japan) for kindly supplying the lysogenic strain "variant 4c" and the tester strain EW8 used for comparative examinations in this study.

REFERENCES

1. Matsui, S.: Jap. J. Microbiol. **2,** 153 (1958).
2. Iseki, S., Hamano, S.: Proc. Jap. Acad. Sci. **35,** 407 (1959).
3. Okada, S., Hoshino, S., Karato, T.: Science **30,** 90 (1960).
4. Giammanco, G.: Ann. Inst. Pasteur **114,** 63 (1968).
5. Giammanco, G., Natoli, D.: Ann. Inst. Pasteur **117,** 16 (1969).
6. Timakov, V. D., Petrovskaya, V. G., Bondarenko, V. M.: Ann. Inst. Pasteur **118,** 3 (1970).
7. Loeb, T., Zinder, N. D.: Proc. nat. Acad. Sci. (Wash) **47,** 282 (1961).
8. Rauss, K.: Acta physiol. Acad. Sci. hung **1,** 105 (1950).
9. "Student" Biometrika 6, 1—25 (1908).
10. Sanderson, K. E.: Bact. Rev. **31,** 354 (1967).
11. Taylor, A. L.: Bact. Rev. **34,** 155 (1970).
12. Formal, S. B., Gemski, P., Baron, L. S.: Bact. Proc. 106, M147 (1969).
13. Simmons, D. A. R.: Europ. J. Biochem. **11,** 554 (1969).
14. LeMinor, L.: Thesis, Paris 1965.

Address of the authors:

Iván Kétyi, Adél Verténvi, István Financsek
Institute of Microbiology, University Medical School
Szigeti út 12, Pécs, Hungary

Acta microbiol. Acad. Sci. hung. 18, 55–59, 1971

THE EFFECT OF THEOPHYLLINE UPON INDUCED β-GALACTOSIDASE SYNTHESIS IN ESCHERICHIA COLI

By

J. SCHLAMMADINGER and G. SZABÓ

Institute of Biology (Director: G. SZABÓ), University Medical School, Debrecen

(Received December 11, 1970)

Summary. Theophylline at concentrations of 3×10^{-3} M and higher was found to inhibit β-galactosidase synthesis in fully induced *Escherichia coli* K 12 cells to an extent depending upon the concentration employed. A competition between theophylline and cyclic AMP is assumed.

Cyclic adenosine 3',5'-monophosphate (cyclic AMP) plays an important role in various regulating mechanisms in animal tissues (for review, see ROBINSON, BUTCHER and SUTHERLAND [1]) and also in bacteria as it has recently been summarized by PASTAN and PERLMAN [2]. In inducible *E. coli* strains, transcription of the *lac* operon needs not only the presence of the inducer (*e. g.* methylthio-β-D-galactropyranoside, TMG) but also a sufficient intracellular amount of cyclic AMP. Mutants which do not produce cyclic AMP are not inducible with the proper inducer unless cyclic AMP is provided in the medium. Glucose (and other carbohydrates) which causes a rapid lowering of intracellular cyclic AMP concentration elicits a catabolite repression of induced β-galactosidase synthesis in *E. coli* and other bacteria [2].

In animal cells and in some bacterial strains, cyclic AMP is hydrolyzed to 5'AMP by a cyclic AMP phosphodiesterase. In mammalian material, BUTCHER and SUTHERLAND [3] reported the inhibition of this enzyme by methylxanthines, especially by theophylline. PASTAN and PERLMAN were not able to detect this effect of methylxanthines in *E. coli* [unpublished data, cit. 2]. Therefore, we have studied the effect of theophylline upon induced β-galactosidase synthesis in *E. coli* K 12 wild-type strain. Theophylline at concentrations of 3×10^{-3} M and higher was able to inhibit induced β-galactosidase synthesis.

Materials and methods

E. coli K 12 wild-type strain was used. Cells were grown in the following medium: NH_4Cl, 2.0 g; Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 3.0 g; $MgCl_2$, 0.04 g; Na_2SO_4, 0.116 g; Na-succinate, 10.0 g; and casamino acids, 1.0 g per litre, pH 7.4. An overnight culture was centrifuged in the morning and washed with a solution of the inorganic components of the medium and resuspended in fresh medium at an optical density (OD) of about 0.300, measured at 570 mμ.

The suspension of the bacterial cells was induced with 5×10^{-4} M TMG (Sigma). After the very rapid dissolving of the inducer (less than 15 second), aliquots of this suspension were pipetted into Erlenmeyer flasks containing theophylline in solid form (Ph. Hung. V) to give the final concentration wanted. The subcultures were placed onto a reciprocal shaker in a water bath of 37 °C and shaking was started (= 0 minute).

If the theophylline (or glucose) was added later, part of the control culture was pipetted nto an Erlenmeyer flask containing the required addition.

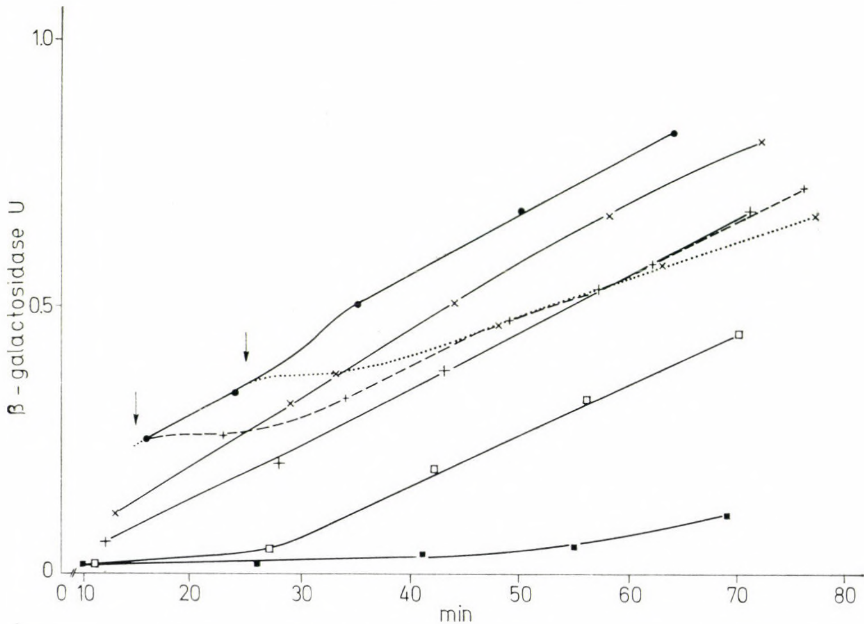


Fig. 1. An overnight culture of *E. coli* K 12 was collected and washed by centrifugation, resuspended in a fresh medium at an $OD_{570} = 0.300$ (approx.). To induce β-galactosidase synthesis, TMG was added at 0 minute to a final concentration of 5×10^{-4} *M*. Samples were immediately pipetted into Erlenmeyer flasks containing theophylline to give the concentration wanted and shaking was started in a water bath of 37 °C. At 15 and 25 minutes, as indicated by the arrows, samples were taken from the control culture and pipetted into theophylline-containing Erlenmeyer flasks and shaking was continued. 1.0 ml aliquots were taken at intervals and pipetted into a 0.1 ml solution of CTAB and chloramphenicol (1.0 mg/ml and 0.5 mg/ml, respectively). After vigorous mixing, the samples were placed into an ice bath. β-galactosidase activity was estimated on the basis of ONPG hydrolysis. 1.5 ml of a 1.0 mg/ml solution was given to the samples and incubated at 37 °C for 10 minutes. Hydrolysis was terminated by adding 1.0 ml of 1.0 *M* Na_2CO_3. The absorbance of the supernatant was read at 420 mμ. Control ●—●, theophylline 2×10^{-2} *M* ■—■, 10^{-2} *M* □—□, 6×10^{-3} *M* +—+, 3×10^{-3} *M* ×—× at 0 min. 6×10^{-3} *M* theophylline at 15 min. +---+, at 25 min. +...+.

Samples of 1.0 ml were taken at intervals and pipetted into 0.1 ml solution of cetyl-trimethyl-ammonium bromide (CTAB, Fluka) and chloramphenicol 1.0 mg and 0.5 mg per ml, respectively. After vigorous mixing the samples were placed into an ice bath.

For the estimation of β-galactosidase activity, samples were incubated with 1.5 ml of a 1.0 mg/ml solution of o-nitrophenyl-β-D-galactopyranoside (ONPG, Fluka) for 10 minutes at 37 °C and the reaction was terminated by adding 1.0 ml 1.0 *M* Na_2CO_3. Absorbance was read at 420 mμ in a Spektromom 201 spectrophotometer (MOM, Budapest). Enzyme activity was expressed as follows: 1 unit = 1 μmole ONPG hydrolyzed at 37 °C in 10 minutes by 1 ml bacterial suspension.

Growth of the cultures was followed by estimating ^{14}C-leucine incorporation (0.1 μC/ml) or by the determination of OD_{570}.

Results

Results are presented in Figs 1 and 2. Fig. 1 shows the effect of 2×10^{-2} M, 10^{-2} M, 6×10^{-3} M and 3×10^{-3} M theophylline, added at 0 minute of the induction, and that of 6×10^{-3} M, added at the 15th and 25th minute of the experiment. Synthesis of the enzyme was inhibited by theophylline and the extent of the inhibition depended on the concentration employed.

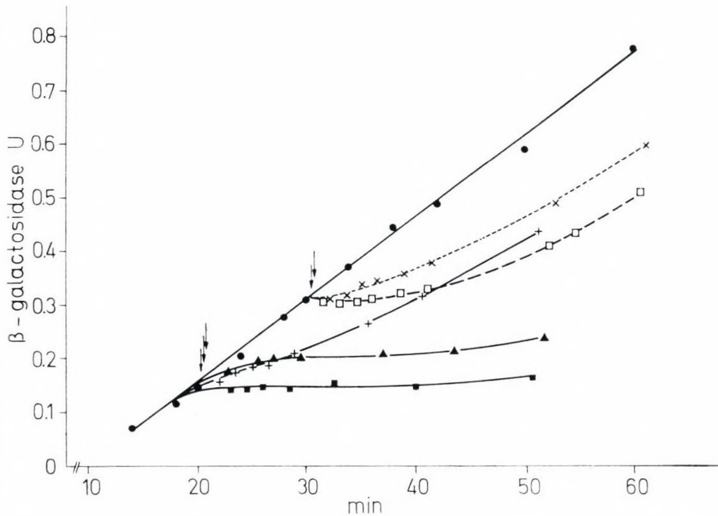


Fig. 2. Procedure essentially as in Fig. 1. Induction with 5×10^{-4} M TMG at 0 min. Of the induced culture aliquots were taken and pipetted into Erlenmeyer flasks containing solid theophylline to give the appropriate final concentrations at 20′25″, 20′30″, 30′28″ and 30′33″, as indicated by arrows. An aliquot of the control culture was pipetted into glucose to give a final concentration of 10^{-3} M at 20′40″. Control ●——●, theophylline 2×10^{-2} M ■——■, 6×10^{-3} M +---+, glucose 10^{-3} M ▲——▲; theophylline 10^{-2} M □——□, 6×10^{-3} M +——+

Fig. 2 shows the kinetics of the inhibition, after induction at 0 minute; addition of 2×10^{-2} M, 6×10^{-3} M theophylline and 10^{-3} M glucose at 20′25″, 20′30″ and 20′40″, respectively, and the early kinetics of the inhibition of β-galactosidase synthesis was followed. At 30′28″ and 30′33″ 10^{-2} M and 6×10^{-3} M theophylline, respectively, was added to flasks containing the induced culture. The effect of the various concentrations was similar to those presented in Fig. 1. In the first period of the inhibition there was a severe repression which was followed by a slight inhibition of enzyme synthesis. The differential rate of β-galactosidase synthesis in the presence of theophylline was lower than that of the control.

The differential rate of enzyme synthesis plotted against the incorporation of radioactive leucine showed a severe repression in the first period of the

experiment but after escape from this strongly repressed state the differential rate of β-galactosidase synthesis was similar to that of the control (not shown in the figure).

Table I

Specific activity of E. coli β-galactosidase in the presence of theophylline and cyclic AMP

Addition (s)	$\frac{\Delta\ \beta\text{-galactosidase U}}{\Delta OD_{570}}$
None (control)	0.025
10^{-2} *M* theophylline	0.019
$+10^{-3}$ *M* cyclic AMP	0.024
10^{-3} *M* cyclic AMP	0.030

Table I shows the specific activity of β-galactosidase at the 70th minute of an experiment in which the effect of cyclic AMP was tested upon the inhibition by theophylline of enzyme synthesis. The data obtained showed that theophylline inhibits β-galactosidase synthesis specifically and cyclic AMP can reverse the inhibitory effect of theophylline.

Discussion

Theophylline was found specifically to inhibit β-galactosidase synthesis in *E.coli*. The growth of the organism was also inhibited at a rate of about 34% but the inhibition of enzyme synthesis amounted to 52% and inhibition of the specific activity to 34% (Table I). Theophylline did not inhibit the enzyme itself.

β-Galactosidase synthesis is specifically inhibited by glucose and other carbohydrates by decreasing the intracellular amount of cyclic AMP. The kinetics of the inhibitory action of theophylline reminds of the catabolite repression by glucose (see for comparison Fig. 2). The enzyme activity curves in the presence of 10^{-3} *M* glucose and 2×10^{-2} theophylline were similar.

To explain the mode of action of theophylline one must consider the role of cyclic AMP regulating the transcription of the *lac* operon [2]. The degree of induction or repression depends upon the concentration of cyclic AMP in the cell. Cyclic AMP is formed of ATP by adenyl cyclase and is hydrolyzed to 5'AMP by a specific phosphodiesterase. According to our hypothesis, theophylline inhibits adenyl cyclase or competitively inhibits the attachment of cyclic AMP to CR protein which is a prerequisite of the effect of cyclic AMP upon β-galactosidase synthesis [4].

It does not seem probable that theophylline should inhibit cyclic AMP phosphodiesterase as it was shown in mammalian systems [3], since theophylline at low concentrations (5×10^{-4} M and 10^{-4} M) did not regularly increase enzyme synthesis, in agreement with the data of PASTAN and PERLMAN [2].

The above data did not allow to exclude a non-specific theophylline-inhibition of β-galactosidase synthesis by decreasing the rate of growth of the cells.

Acknowledgement. We are indebted to Dr. L. PÓLYA for calling our attention to theophylline, to Mrs. I. SZEKERES for skilful technical assistance, and to Mr. F. FÁBIÁN for the drawings.

REFERENCES

1. ROBINSON, G. A., BUTCHER, R. W., SUTHERLAND, E. W.: Ann. Rev. Biochem. **37,** 149 (1968).
2. PASTAN, I., PERLMAN, R.: Science **169,** 339 (1970).
3. BUTCHER, R. W., SUTHERLAND, E. W: J. biol. Chem. **237,** 1244 (1962).
4. EMMER, M., DECROMBRUBGHE, B., PASTAN, I., PERLMAN, R.: Proc. nat. Acad. Sci. (Wash.) **66,** 480 (1970).

Address of the authors:

JÓZSEF SCHLAMMADINGER, GÁBOR SZABÓ
Institute of Biology, University Medical School
Debrecen 12, Hungary

Acta microbiol. Acad. Sci. hung. 18, 61—67, 1971

EFFECT OF VARIOUS DILUENTS ON STABILITY OF VACCINIA VIRUS DILUTIONS

By

J. SZATHMÁRY, L. HEGEDŰS and M. KOLLER

Institute for Serobacteriological Production and Research "HUMAN" (Director: I. VETŐ), Budapest

(Received December 31, 1970)

Summary. — Vaccinia virus dilutions prepared with different diluents in the range of 10^{-5} — 10^{-7} showed loss of pock forming unit content when incubated at 37 °C. Pock titres were most stable in the skim milk containing buffer and next to it in the serum containing diluents. The presence of accompanying bacteria or tissue debris in the virus suspension did not appear to affect the dilution stability of the virus. Comparison of serial dilutions of the virus in plain and milk-containing buffer showed that the pock titres were always higher in the latter diluent.

The diluents commonly used for the infectivity titration of vaccinia virus suspensions are saline [1] or some kind of buffer solution [2] with serum [3], broth [4, 5] or milk [6, 7] alone or added for stabilization. The stability of the dilution is particularly important in neutralization tests which include an incubation of the virus-antibody mixture for 2 hours at 37 °C. Examining the stability of virus dilutions at 37 °C, BOULTER [6] found that the stabilizing effect varied with the diluent. Since the pox vaccines and other suspensions of vaccinia virus contain varying amounts of tissue debris and cutaneous bacterial flora from the host organism, it seemed worthwhile to examine whether there was a relationship between the quality and the dilution stability of such materials.

Materials and methods

Vaccinia virus strains. The "Budapest" strain of vaccinia virus, used in Hungary for vaccine production since 1927, and the "Elstree"-(Lister) strain recommended for this purpose by the WHO and adopted here as a vaccine strain since 1967 were used in the experiments. The properties of the two strains have been described in detail [8, 9].

Virus suspensions. The strains were propagated on chicken embryo chorioallantoic membrane (CAM) and on the skin of calves and rabbits. CAMs from 11—12 day old chicken embryos showing confluent lesions were collected 48—72 hours later. Cows and rabbits were inoculated by cutaneous scarification and the pustules were harvested on the fourth and third day, respectively. The virus containing tissues were homogenated in 1 : 5 McIlvaine buffer or 50% glycerol and the suspensions were stored at —15 °C until use. From the "Budapest" material a partially purified elementary body suspension was prepared by the routine procedure applied in this institute, *i. e.* after treatment with phenol and Frigen 113, centrifuged and resuspended in 5% peptone solution, distributed into ampoules in 0.2 ml volume and freeze-dried [10]. The counts of the accompanying bacteria were assessed by the pour-plate method.

Determination of pock counts was made by the method of WESTWOOD *et al.* [11]. The suspensions were diluted serially in threefold steps and each appropriate dilution was inoculated

in 0.1 ml doses onto the CAMs of 5—8 chicken embryos preincubated for 11—12 days. The pocks were counted after incubation at 37 °C and expressed in base 10 logarithms.

Diluents. (1) Phosphate-buffered saline, adjusted to pH 7.2; (2) 0.004 *M* McIlvaine buffer (citric acid + phosphate), pH 7.2; (3) peptone water (1—5% solution of Difco-Bacto peptone), adjusted to pH 7.6;

All diluents were sterilized in the autoclave and to each were added streptomycin and penicillin to a final concentration of 50 μg/ml and 500 U/ml, respectively.

Results

The stability of the dilutions was assessed by counting the number of pock forming units in 0.1 ml volume immediately after preparation and after incubation for two hours in a water bath of 37 °C. Two or three dilution steps, usually in the range 10^{-5} — 10^{-7}, were selected from those which were judged to be able to produce 10—60 easily readable pocks. Results are shown in Table I.

Table I

Decrease in per cent of pock counts after incubation for two hours at 37 °C in 10^{-5} — 10^{-7} vaccinia virus dilutions prepared with different diluents

Diluent	Crude pustule homogenate			Purified pustule homogenate		
	medium	min.	max.	medium	min.	max.
Phosphate-buffered saline	70.1	50.5	94.6	68.8	58.2	88.2
McIlvaine buffer	67.4	54.9	85.0	70.2	58.0	84.4
Peptone solutions, 1—5%	21.2	∅	50.6	26.4	9.7	54.2
Broth (bacteriological)	19.8	8.2	66.0	24.3	∅	74.5
McIlvaine buffer + 5% horse serum	5.4	∅	23.6	—*	—*	—*
McIlvaine buffer + 10% milk	4.0	∅	24.6	4.8	5.1	25.2

* Not tested

The stability of the dilutions did not significantly vary with the quality of the virus suspensions. The extent of pock count reduction was unrelated to the type of the strain (Budapest, Elstree), species of the host (chicken embryo, rabbit, cow) and the presence of no (CAM), few (glycerol-treated or elementary body suspensions) or many accompanying bacteria (pustule material in McIlvaine buffer) as well as to the degree of purification from microbes or tissue detritus. Table I shows only data for crude and purified pustule homogenated to promote a better understanding. Obviously, the dilutions prepared in plain buffer solution were the least stable, showing a pock count reduction by 50—95%, and those prepared in horse serum or milk containing McIlvaine buffer were the most stable, as found also by BOULTER. Surprisingly different results were obtained with broth, which stabilized very well in one part of the experiments but failed to stabilize in the other part, regardless whether it was used in itself undiluted or added to McIlvaine buffer at a concentration

of 10—50%. We scrutinized this particular diluent in great detail as it has been commonly used to dilute vaccinia virus. The stability data and the inactivation kinetics are shown in Table II.

Table II

Pock counts at 37 °C in stable and instable dilutions of vaccinia virus

Preparation	Diluent	Pock counts in 0.1 ml after			
		0 min.	30 min.	2 hrs.	4 hrs.
233	Phosphate-buffered saline	17.1	10.3	7.2	1.2
	Broth (bacteriological)	18.5	18.5	17.5	17.0
242	Phosphate-buffered saline	17.3	6.8	3.0	1.6
	Broth (bacteriological)	16.8	17.2	7.8	4.5

In the dilution prepared with buffered saline (10^{-6}) the number of pock forming units tended to decrease until it had fallen to 10% of the origina count by the 4th hour in both experiments. The broth dilution was stable fol 4 hours in the first experiment, but in the second experiment its stabilitr tended to decline after half an hour and more than 50% of the pock forminy units had deteriorated by the second hour.

Both the liquid glycerol and the freeze-dried pox vaccine contain microorganisms displaying a high enzyme activity. DOSTAL [12] observed that such germs influenced the thermostability of the vaccinia virus suspension. Enzymes originating from tissue detritus have a similar effect. Bacteria of high proteolytic or haemolytic activity isolated from vaccinia virus suspensions in this laboratory were inoculated into those virus suspensions which remained stable when diluted in broth, to examine whether one or another enzyme would destroy the virions at 37 °C. None of the organisms tested affected the stability of the virus dilutions; however, we may have chosen the wrong bacteria. These negative results did not, however, change our view that the enzyme content of the virus suspension may at least in part be responsible for the loss of stability. Experiments with phenol-treated virus suspensions are in support of this hypothesis. One half of pustule homogenates in McIlvaine buffer were treated with 1.0% phenol and allowed to stand at room temperature for 48 hours before the addition of an equal volume of glycerol, while to the other half glycerol was added without phenol pretreatment. When tested for dilution stability after storage at —15 °C for three months, the dilutions of the phenol-treated samples were considerably more stable than those of the untreated control samples which contained many accompanying germs and presumably also relatively much enzyme (Table III).

Table III

Stability at 37 °C of vaccinia virus suspensions with different germ counts diluted in broth

50% glycerol containing pustule homogenate	Preparation No. 244		Preparation No. 246	
	Bacterial count/ml	Reduction of pock count after 2 hr	Bacterial count/ml	Reduction of pock count after 2 hr
Not phenol-treated	26.650	62.6%	24.500	72.2%
Treated with 0.5% phenol	450	23.0%	77	18.0%

As the dilution stability was found to be good in the skim milk containing McIlvaine buffer, we have adopted this diluent instead of the plain McIlvaine buffer previously used for routine pock-titrations of vaccinia virus suspensions and pox vaccines, and continued to use the latter for control examinations. Titration results of the freeze-dried pox vaccine, used in this laboratory as a reference preparation, diluted in McIlvaine buffer with and without skim milk are shown in Table IV.

As can be seen from the last column of Table IV, one and the same virus suspension had a 2—5.8 times higher titre when diluted in milk-containing instead of plain McIlvaine buffer. Further experiments performed since the submission of this paper had similar results. The "milky" titre was never identical to or lower than, its "plain" counterpart.

Table IV

Pock titre of the freeze-dried pox vaccine No. L22

McIlvaine buffer $\times 10^8$/ml	McIlvaine buffer +10% milk $\times 10^8$/ml	R
0.34	2.0	5.8
0.3	0.6	2.0
0.5	2.2	4.4
0.44	1.3	2.9
0.7	1.4	2.0
0.3	1.6	5.3
Mean: 0.43	mean: 1.5	mean: 3.7

R = Quotient of pock counts established after incubation in buffer diluent with and without milk.

The stabilizing role of the milk may stem from its action either on the embryo or on the virus. Its influence on the embryo may be metabolic or one promoting the penetration of partially or completely mature virions into the

Table V

Effect on pock titres of vaccinia virus suspensions diluted in buffer of milk administration in different phases of egg inoculation

CAM dropped with	Time of addition of 0.1 ml diluent	Quality	Titre × 10^7/ml	R
M	—	—	3.2	1.4
P	—	—	2.2	
P	30 minutes prior to infection	M	2.6	1.1
P		P	2.3	
P	At infection	M	2.7	0.9
P		P	3.0	
P	30 minutes after infection	M	5.0	1.0
P		P	5.1	

P = Plain McIlvaine buffer
M = McIlvaine buffer containing 10% milk
R = Quotient of pock titres obtained with and without separate administration of milk

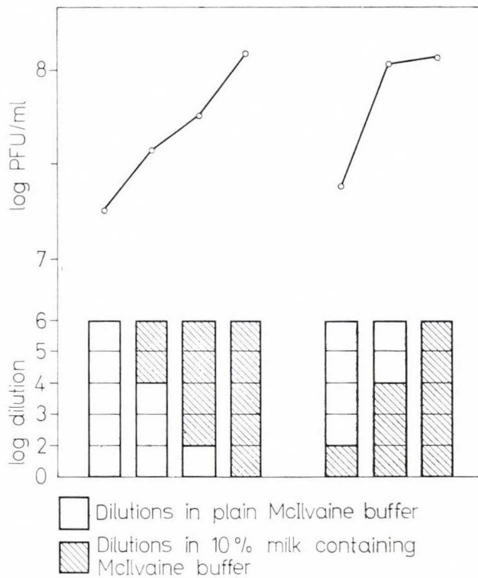


Fig. 1

CAM. Milk was therefore administered separately before, during and after the inoculation of virions diluted in plain buffer to study whether it caused an alteration of pock counts.

The titres obtained with and without milk were almost identical and the value of R was always about one. The number of pocks developing on CAM was clearly unrelated with the timing of milk administration into the egg.

As to the action of milk on the virions, it was supposed to prevent their aggregation during dilution. To confirm this experimentally, dilution series from 10^{-1} to 10^{-6} were so prepared that the plain and milk-containing buffer diluents were exchanged after a given number of steps, as shown in Fig. 1. The values shown in Fig. 1 represent means of 3—4 measurements.

Both diagrams in Fig. 1 suggest that the more dilution steps were prepared with milk-containing buffer, the higher was the pock titre.

Discussion

The infective titre of one and the same vaccinia virus suspension varies in a wide range in subsequent assays. The results of neutralization tests at 37 °C are particularly difficult to reproduce, owing probably to the thermosensitivity of the virus [13]. Though the presence of protein in the diluent increases the stability of the virus dilution, in cannot completely prevent the destruction of the virions. Pox vaccines, namely the liquid glycerol-treated preparations, contain a considerable amount of tissue detritus and cutaneous bacteria from the host organism; these unwanted components can be removed for the most part or completely by purification and freeze-drying. But the basic materials of the vaccine preparation, mainly the pustule homogenates, contain a rich accompanying bacterial flora, some components of which are enzymically highly active [12] and also the cells of the tissue detritus contain enzymes. These enzymes may affect the stability of the virus dilution, especially at 37 °C, thus influencing the reproducibility of the infective titre. We pursued the present investigations to obtain more information on this problem, but no convincing result has emerged from these studies. Nevertheless, the greater dilution stability of the phenol-treated preparation suggests that the probability of enzymic interference cannot be excluded.

The fluctuation of the infective titre depends to a certain extent not only on the quality of the virus suspension, but also on on that of the eggs. Though the eggs used in the experiments were procured from one and the same source throughout, the aspecific embryonic death rate before or after inoculation varied considerably with the individual lots. There were also differences in the individual eggs congenital malformations were frequent. We tried to minimize the error arising from these sources by repeated experiments. A certain virus preparation, for example, was tested for stability twenty times.

GALASSO and SHARP [14] and others observed that vaccinia virus particles are extraordinarily liable to aggregation. As a single aggregate consists of two to several hundred virions, the clumping considerably influences the number of demonstrable active units, in other words the infective titre. The variations in dilution stability with the different diluents at 37 °C can be explained by the diverse influence of the degree of dilution on aggregation at a given temperature. Protein in the diluent prevents aggregation to a certain extent. The evidence emerging from our experiments that the more dilution steps are prepared in milk-containing diluent, the higher is the number of the active units demonstrable on the CAM suggests that milk prevents either clumping on the whole or the formation of larger aggregates. Experiments are being carried on to substantiate this hypothesis.

REFERENCES

1. KOLB, R. W., COX, C. B., AYLOR, H. T.: Bull. WHO **25**, 19 (1961).
2. POLAK, M. F., BRANS, L. M., BEUNDERS, J. W., VAN DER WERFF, A. R.: Bull. WHO **27**, 311 (1962).
3. OVERMAN, J. R., TAMM, I.: J. Immunol. **76**, 228 (1956).
4. BAXBY, D.: J. Hyg. (Lond.) **67**, 637 (1969).
5. FULLER, V. J., KOLB, R. W.: Appl. Microbiol. **16**, 458 (1968).
6. BOULTER, E. A.: J. Hyg. (Lond.) **55**, 502 (1957).
7. CUTCHINS, E., WARREN, J., JONES, W. P.: J. Immunol. **85**, 275 (1960).
8. SZATHMÁRY, J.: Ann. Immunol. hung. **8**, 75 (1965).
9. NYERGES, G., SZATHMÁRY, J., BENKŐ, A.: Acta microbiol. Acad. Sci. hung. **15**, 199 (1968).
10. SZATHMÁRY, J., SZENTIRMAY, E., SIKOS, K., HEGEDŰS, L., KOLLER, M.: Ann. Immunol. hung. **13/14** (1969/1970).
11. WESTWOOD, J. C. N., PHIPPS, P. H., BOULTER, E. A.: J. Hyg. (Lond.) **55**, 123, (1957).
12. DOSTAL, V., BRANTER, H.: Z. Immun.-Forsch. **134**, 493 (1968).
13. DOWNIE, A. W., VINCENT, L. ST., RAO, A. R., KEMPE, C. H.: J. Hyg. (Lond.) **67**, 603 (1969).
14. GALASSO, G. J., SHARP, D. G.: J. Immunol. **88**, 339 (1962).

Address of the authors:

JÓZSEF SZATHMÁRY, LÁSZLÓ HEGEDŰS, MIKLÓS KOLLER
Institute for Serobacteriological Production and Research Human
Szállás u. 5—7. Budapest X, Hungary

Acta microbiol. Acad. Sci. hung. 18, 69–73, 1971

WATER LIMITED REACTION OF BACILLUS CEREUS PENICILLINASE IN NONAQUEOUS SOLVENT

By

V. Csányi, I. Mile, Éva Szabó, Gy. Harmath and Ilona Ferencz

Institute of Medical Chemistry (Head: F. Antoni), Semmelweis University Medical School, Budapest

(Received January 18, 1971)

Summary. It was found that *Bacillus cereus* penicillinase can be kept in glycerol without denaturation. Some features of the reaction catalysed by penicillinase were investigated in glycerol of low water content. The reaction was dependent on water and enzyme contents at the substrate saturation level.

Studies of the interaction of proteins and different nonaqueous solvents have revealed that most of the proteins undergo a series of marked conformational changes when the amount of water is limited [1]. Further studies have shown that under certain conditions enzyme activity and native conformation remain intact in spite of the limitation of water [2, 3].

In the present paper some experiments are described proving that the hydrolytic reaction catalysed by *B. cereus* penicillinase can be investigated in glycerol of low water content. Under these circumstances the rate of the reaction catalysed by penicillinase was dependent on the water content.

Materials and methods

Exopenicillinase produced by *B. cereus* strain 569/H was used in the experiments. Purification of the enzyme has been described earlier [4].

All the solutions were made from freshly distilled glycerol containing 1% water as determined by Karl Fischer's reagent.

The "buffer-penicillin" solution contained a mixture of potassium-dihydrogen phosphate and disodium-hydrogen phosphate dried at 110 °C. The ratio of the two compounds was maintained so that the pH of a sample of the mixture dissolved in water was 6.5. The reaction velocity in glycerol showed its maximum at the particular ratio of the two compounds. The solution also contained sodium-benzylpenicillin dried *in vacuo*. The amount of the buffer salts and penicillin was 5% in each of the final incubation mixtures containing penicillinase.

Lyophilized penicillinase was dissolved seperately in glycerol.

Measurement of the reaction. The water content requested in each experiment was adjusted 30 min before starting the experiments in "buffer-penicillin" and penicillinase solution separately. The reaction was started by vigorous mixing of the two prewarmed solutions at 30 °C. The mixtures were sealed, further stirring was not necessary. In order to stop the reaction at appropriate intervals, 0.5–1.0 ml samples were taken and transferred by a glass syringe into 5 ml of a solution consisting of 0.5 *M* sodium-wolframate and 1.0 *M* sodium acetate, pH 4.0. Penicilloic acid was then estimated iodometrically as described previously [5] and the result was expressed in ml of 0.005 $N\ I_2$. On the basis of preliminary experiments, concentration of buffer salts and penicillin were chosen so that the velocities measured during the incubation referred to maximum velocity.

Results

The work reported here was prompted by our observation that penicillinase could be dissolved and preserved for a long time in glycerol and isopropyl alcohol without any loss of the original activity measured after dilution of the organic solvents by excess water. These findings gave an opportunity to examine the catalytic properties of penicillinase dissolved in glycerol of low water content.

Fig. 1 shows the activity of penicillinase in different water-glycerol mixtures containing decreasing quantities of water. Approximately 50—60% water was necessary to maintain the velocity of the reaction at the level ob-

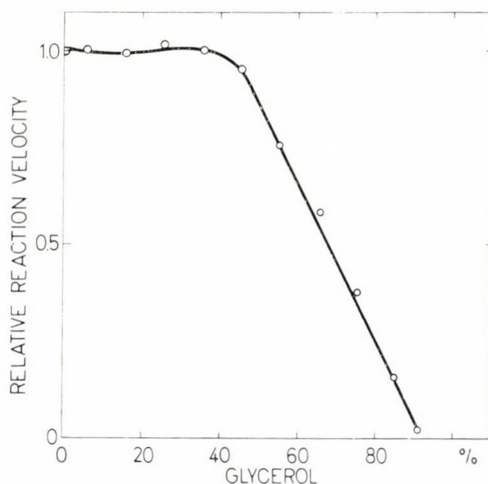


Fig. 1. Penicillinase activity measured in glycerol of decreasing water content. 500 U penicillinase and 0.06 g penicillin G were incubated in 5 ml of a 0.1 *M* mixture of phosphate buffer pH 6.5 and glycerol, for 5 min at 30 °C. (Reaction velocity 1.0 refers to 80 ml 0.005 N I_2)

served in pure water solution. If the water content of the incubation mixtures was less than 50%, the reaction rate decreased linearly with water content. The velocity of the reaction was practically zero at 10% water content. However, if the penicillinase content of the reaction mixtures was raised by several orders of magnitude, some charasteristic features of the remaining slow reaction (1—2% of the original velocity) could be investigated.

The experiments performed in the presence of the lowest water content (1%) penicillin hydrolysis proceeded at a limited rate (Fig. 2).

Raising the water content was followed by an increase in the reaction rate. This under 6—10% depended exponentially on the water content (Fig. 3).

Above 10% water, the reaction rate depended linearly on the water content as shown in Fig. 1. Several experiments showed that the reaction

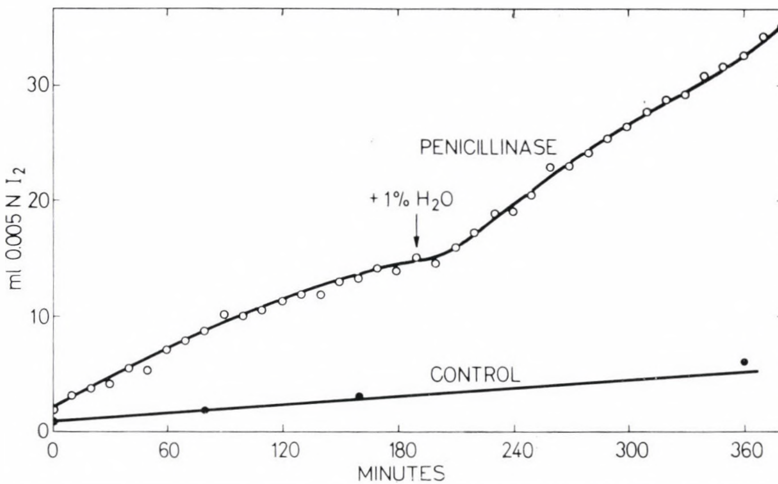


Fig. 2. Penicillinase activity in glycerol with one per cent water. 6800 U/ml penicillinase was incubated in a buffer-penicillin solution containing 1% water at 30 °C. At 190 min of the experiment the incubation mixture was supplied with another 1% water. The control samples did not contain penicillinase

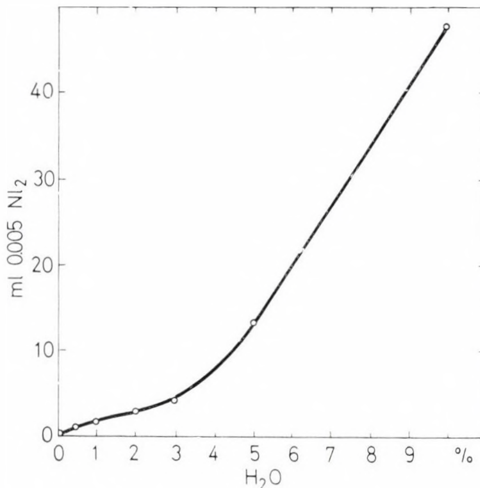


Fig. 3. Effect of water content on penicillinase activity. 10,300 U/ml penicillinase was incubated in buffer-penicillin mixtures of different water contents for 10 min at 30 °C

velocity was under double limitation, the limiting factors being water and enzyme concentrations. The concentration of substrate was at the saturation level measured in preliminary experiments. The rate of the reaction measured either at different water concentrations (Fig. 4), or at different enzyme concentrations (Fig. 5) was always linear.

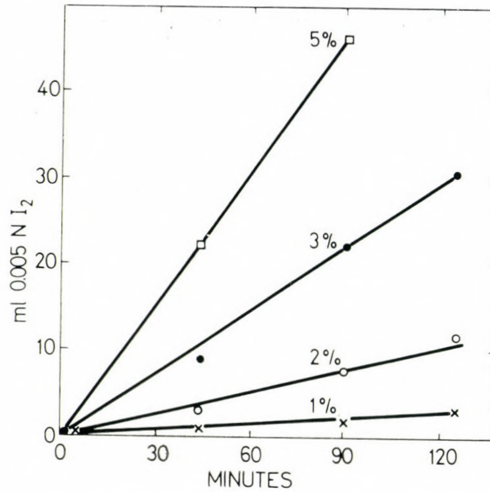


Fig. 4. Penicillin hydrolysis rate in mixtures of different water contents. 10,300 U/ml penicillinase was incubated in mixtures of various water contents at 30 °C

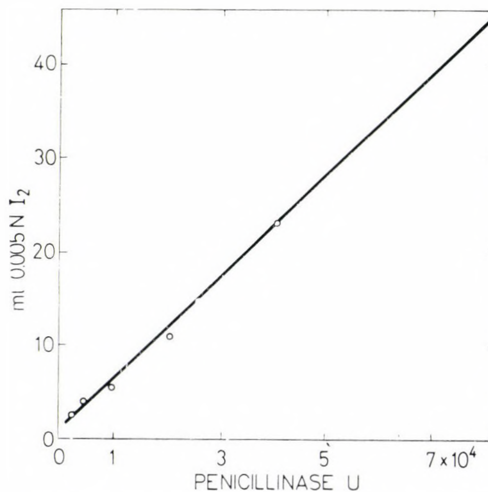


Fig. 5. Penicillin hydrolysis rate in mixtures of different penicillinase contents. Different amounts of penicillinase were incubated in buffer-penicillin solution with 2.5% water for 30 min.

Discussion

Penicillinase can be dissolved in glycerol of low water content without any sign of denaturation. In a mixture consisting of phosphate buffer and penicillin-G dissolved in glycerol, the hydrolytic reaction was slow. The rate of the reaction catalysed by penicillinase dissolved in glycerol and a few per cent of water was limited by the water content.

The findings have supplied a convenient tool for the further investigation of the reaction catalysed by penicillinase. Whether the cause of the slowing down of the reaction was in connection with the physico-chemical properties of the glycerol or with changes in the properties of the penicillinase molecule remain to be decided. However, similarly to water glycerol has an unusually high dielectric constant. Thus, high concentrations of buffer and penicillin can be dissolved in glycerol. The dissociation of some groups in the penicillinase molecule essential for enzyme did not seem to have severely altered in glycerol.

It is known from the results of TANFORD that a protein molecule can bind 100—1000 molecules of water as an indispensible part of the active protein structure [6]. SKUJINS and McLAREN found that the catalytic activity of dry urease depends on the absorbed water necessary for the reaction itself and, as perhaps also in our case, for shaping the catalytic conformation [7].

A plausible explanation is that in our experiments the role of the water was to maintain a catalytically active enzyme conformation. The water necessary for the hydrolytic reaction itself seemed to be abundant.

Acknowledgement. We are indebted to Professor F. B. STRAUB for helpful and valuable discussions.

REFERENCES

1. REES, E. D., SINGER, S. J.: Arch. Biochem. **63**, 144 (1956).
2. TANFORD, C., BUCKLEY, III, C. E., DE P. K., LIVELY, E. P.: J. biol. Chem. **237**, 1168 (1962).
3. KAY, C. M., BRAHMS, J.: J. biol. Chem. **238**, 2945 (1963).
4. CSÁNYI, V., MILE, I., FERENCZ, I., SZABÓ, É.: Biochim. biophys. Acta (Amst.) **198**, 332 (1970).
5. CSÁNYI, V.: Acta physiol. Acad. Sci. hung. **18**, 261 (1961).
6. TANFORD, C.: J. molec. Biol. **39**, 539 (1969).
7. SKUJINS, J., J., McLAREN, A. D.: Science **158**, 1569 (1967).

Address of the authors:

VILMOS CSÁNYI, IMRE MILE, ÉVA SZABÓ, GYÖRGY HARMATH, ILONA FERENCZ
Institute of Medical Chemistry, University Medical School
Puskin u. 9, Budapest VIII, Hungary

INDEX

Pallinger, Z., Pallinger-Szekeres, G.: Measles Immunity in Infants 1

Csányi, V., Mile, I., Ferencz, I., Szabó É.: Some Properties of the Membrane-Bound Penicillinase of *Bacillus cereus* 569 7

Hajdú, J., Ferencz, I., Mile, I., Csányi, V.: Effect of Urea on the Two Iodine Inactivation Reactions of *Bacillus cereus* Penicillinase 17

D. Tóth, F., Váczi, L., Berencsi, Cl.: Interferon and Antibody Production in Inbred Mice Infected with the Rauscher Virus 23

Gaál, V., Molnár, J., Ivánovics, G.: Relationship of Lysogeny and Megacinogeny in *Bacillus megaterium* 29

Mile, I., Csányi, V., Koczka, I., Badár, É., Horváth, I.: Effect of Pyrazocillin on the Induced Penicillinase Synthesis of *Bacillus cereus* 41

Kétyi, I., Vertényi, A., Financsek, I.: Lysogenic Conversion in the *Shigella flexneri* Group 47

Schlammadinger, J., Szabó, G.: The Effect of Theophylline upon Induced β-Galactosidase Synthesis in *Escherichia coli* 55

Szathmáry, J., Hegedűs, L., Koller, M.: Effect of Various Diluents on Stability of Vaccinia Virus Dilutions 61

Csányi, V., Mile, I., Szabó, É., Harmath, Gy., Ferencz, I.: Water Limited Reaction of *Bacillus cereus* Penicillinase in Nonaqueous Solvent 69

The Journal of
General Microbiology

Volume 66 Part 2 1971

J. THOMAS and K. A. V. DAVID: Studies on the Physiology of Heterocyst Production in the Nitrogen-fixing Bluegreen Alga *Anabaena* sp. L-31 in Continuous Culture

P. H. RAY and T. D. BROCK: Thermal Lysis of Bacterial Membranes and Its Prevention by Polyamines

S. A. PALIMBO, M. G. JOHNSON, V. T. RIECK and L. D. WITTER: Growth Measurements on Surface Colonies of Bacteria

Y. KOLTIN and I. CHORIN-KIRSCH: Alteration of Fungal Morphology Induced by a Substance from *Bacillus cereus*

I. KISS, I. BEREK and G. IVÁNOVICS: Mapping the δ-Aminolaevulinic Acid Synthetase Locus in *Bacillus subtilis*

C. E. CLARKSON and P. M. MEADOW: Diaminopimelic Acid and Lysine Auxotrophs of *Pseudomonas aeruginosa* 8602.

M. S. FULLER and L. W. OLSON: The Zoospore of Allomyces

A. B. COBET, G. E. JONES, J. ALBRIGHT, H. SPION and C. WIRSEN: The Effect of Nickel on a Marine Bacterium: Fine Structure of *Arthrobacter marinus*

S. NAGAI, Y. NISUIZAWA, M. ONODEPA and S. AIBA: Effect of Dissolved Oxygen on Growth Yield and Aldolase Activity in Chenostat Culture of *Azotobacter vinelandii*

K. NORDSTRÖM: Increased Resistance to Several Antibiotics by One Mutation in a P-factor, P 1a

A. J. VAN RENSBURG: Genetic Transformation in *Proteus mirabilis*

S. G. WILKINSON and K. A. CAPRY: Amino Sugars in the Cell Walls of Pseudomonas Species

M. MCLOUGHLIN and P. S. STEWART: Red Cell-sensitizing Antigens of Lactobacilli

SHORT COMMUNICATIONS

I. J. NIU and J. L. AMIEL: Fatty Acid Nature and Adjuvant Activity of Wax D from *Mycobacterium tuberculosis*

A. G. MILLER and K. BUDD: Chloride Uptake by Mycelium of *Neocosmospora vasinfecta* and Its Inhibition by Glucose

M. P. STRATFORD and A. J. GRIFFITHS: Excystment of the Amoeba *Hartmannella castellanii*

R. SMYTHE and G. E. ANDERSON: Electrophoretic Protein Spectra of Wild-type and Isogenic Monokaryons of *Coprinus lagopus*

Price £3.00 net ($9.00 in U.S.A.) Annual subscription £30 net ($100.00 in U.S.A.)

CAMBRIDGE UNIVERSITY PRESS

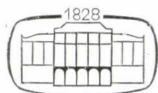
Bentley House, 200 Euston Road, London NW1 2DB
American Branch: 32 East 57th Street, New York, N.Y. 10022

T. Trencséni and B. Keleti

Clinical Aspects and Epidemiology of Haemorrhagic Fever with Renal Syndrome

The merit of this monograph lies in the fact that the authors give a detailed analysis of the clinical and epidemiological aspects of haemorrhagic fever with renal syndrome as well as its aetiology and pathology based on the nearly 130 cases they have encountered since 1953 in Hungary. All pertaining observations in the literature are also reported. Moreover, the epidemiological criteria of the epidemic and sporadic cases which have occurred in Hungary are discussed. Based on the knowledge of general epidemiology, recent data on haemorrhagic fevers concomitant with renal syndrome are examined with special regard to unsolved problems. The appendix provides information on various virus induced haemorrhagic fevers. Another valuable feature of this monograph is that there are 310 reference works in the Bibliography and another 100 in the Appendix.

In English . 247 pages . 17 × 25 cm . Cloth



Akadémiai Kiadó

PUBLISHING HOUSE OF THE HUNGARIAN ACADEMY OF SCIENCES
BUDAPEST

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója   Műszaki szerkesztő: Várhelyi Tsam
A kézirat nyomdába érkezett: 1971. VII. 7. — Terjedelem: 7 (A/5) ív, 31 ábra

71.72082 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, Budapest, Keleti Postahivatal, Postafiók 64.

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $ 16.00.

Bestellbar bei dem Buch- und Zeitungs-Außenhandels-Unternehmen »*Kultúra*« (Budapest I., Fő utca 32. Bankkonto Nr. 43-790-057-181) 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 de fascicules qui seront réunis en volumes.

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

Acta Microbiologica, Budapest, Keleti Postahivatal, Postafiók 64.

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

Le prix de l'abonnement est de $ 16.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur de Livres et Journaux «*Kultúra*» (Budapest I., Fő utca 32. Compte courant No. 43-790-057-181) 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, Budapest, Keleti Postahivatal, Postafiók 64.

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписная цена — $ 16.00 за том.

Заказы принимает предприятие по внешней торговле книг и газет «*Kultúra*» (Budapest I., Fő utca 32. Текущий счет № 43-790-057-181), или его заграничные представительства и уполномоченные.

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

ALBANIA

Drejtorija Qändrone e Pärhapjes
dhe Propagandimit tä Libri
Kruga Konferenca e Päzes
Tirana

AUSTRALIA

A. Keesing
Box 4886, GPO
Sydney

AUSTRIA

GLOBUS
Höchstädtplatz 3
A-1200 Wien XX

BELGIUM

Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
162, rue du vidi 5, Place St. Jean
100. Bruxelles

BULGARIA

HEMUS
11 pl Slaveikov
Sofia

CANADA

Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA

Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA

Artia
Ve Smečkách 30
Praha 2
Poštovní Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha 1
SLOVART A. G.
Gorkého
Bratislava

DENMARK

Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND

Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE

Office International de Documentation et Librairie
48, rue Gay-Lussac
Paris 5e

GERMAN DEMOCRATIC REPUBLIC

Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701
Zeitungsvertriebsamt
Fruchtstraße 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC

Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN

Blackwell's Periodicals
Oxenford House
Magdalen Street
Oxford
Collet's Subscription Import Department
Dennington Estate
Wellingsborough. Northants.
Robert Maxwell and Co. Ltd.
4—5 Fitzroy Square
London W. 1

HOLLAND

Swetz & Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA

Hind Book House
66 Babar Road
New Delhi 1

ITALY

Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze
Techna
Via Cesi 16.
40135 Bologna

JAPAN

Kinokuniya Book-Store Co. Ltd.
826 Tsunohazu 1-chome
Shinjuku-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central

KOREA

Chulpanmul
Phenjan

NORWAY

Tanum-Cammermeyer
Karl Johansgt 41—43
Oslo 1

POLAND

RUCH
ul. Wronia 23
Warszawa

ROUMANIA

Cartimex
Str. Aristide Briand 14—18
Bucureşti

SOVIET UNION

Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN

Almquist & Wiksell
Gamla Brogatan 26
S—101 20 Stockholm

USA

F. W. Faxon Co. Inc.
15 Southwest Park
Westwood Mass. 02090
Stechert Hafner Inc.
31. East 10th Street
New York, N. Y. 10003

VIETNAM

Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA

Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

25. XI. 1971 Index: 26.016

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

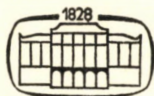
ADIUVANTIBUS

I. DÖMÖK, E. FARKAS, I. HORVÁTH
I. NÁSZ, K. RAUSS, F. B. STRAUB, J. SZIRMAI, L. VÁCZI, K. VAS,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XVIII FASCICULUS 2



AKADÉMIAI KIADÓ, BUDAPEST

1971

AMAHA 5 18(2) 75—136 (1971)

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

Főszerkesztő

IVÁNOVICS GYÖRGY

akadémikus

Szerkesztő

LÁNYI BÉLA

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, Budapest, Keleti Postahivatal, Postafiók 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (Budapest V., Alkotmány utca 21. Bankszámla 05-915-111-46), a külföld számára pedig a „Kultúra" Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő utca 32. Bankszámla: 43-790-057-181) 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, Budapest, Keleti Postahivatal, Postafiók 64.

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

The rate of subscription is $ 16.00 a volume.

Orders may be placed with "Kultúra" Foreign Trade Company for Books and Newspapers (Budapest I., Fő utca 32. Account No. 43-790-057-181) or with representatives abroad.

Acta microbiol. Acad. Sci. hung. 18, 75—80, 1971

CORRELATION BETWEEN THE MORPHOLOGICAL VARIABILITY AND RIBOFLAVIN PRODUCING CAPACITY OF EREMOTHECIUM ASHBYII

By

T. Vághy

Phylaxia Veterinary Biologicals and Feedstuffs Ltd. (Director: J. Molnár), Budapest

(Received February 10, 1971)

Summary. A total of 3484 individual colonies of the genetically instable fungus *Eremothecium ashbyii* was examined for a correlation between external properties (growth intensity, shape, colour, saltation, etc.) and riboflavin producing capacity. The ideal colony type distinguished by the highest riboflavin synthesizing capacity, was characterized by medium growth intensity, smooth surface covered by air mycelia, distinct chromogenic property and no sector formation.

Riboflavin is produced on a plant scale from submerged cultures of *Ashbya gossypii* or *Eremothecium ashbyii* microorganisms [5, 6]. The vitamin synthesizing capacity of the *E. ashbyii* strain used in this laboratory showed distinct fluctuations. The yield of one fermentor culture varied from 800 to 2000 μg/ml, without any change of the fermentation parameters. According to the adopted practice, the inocula were prepared in flasks seeded with individual colonies isolated from streak plates of spore suspensions. Many morphologically different colonies grew on the plates and their mixed use as inoculum might have accounted for the fluctuation of the vitamin yield. This suggested the responsibility of the genetic instability and marked spontaneous mutation of the strain and the examinations reported in this paper were carried out with the aim to obtain more information on the relationship between the colony morphology and vitamin producing capacity of *E. ashbyii.*

Colonies of dissimilar morphology were harvested from streak plates of spore suspensions on peptone-water agar. A total of 3484 colonies was examined for colony characteristics (growth intensity, colour, sector formation and surface) and riboflavin synthesizing capacity, in view of the fact that more than 1000 data are required to obtain results of high probability [3].

Material and methods

Monospore cultures were prepared with a Zeiss type 3041 micromanipulator, using 100 ml growth medium of the following composition (g per 1000.0 ml tap water): NaCl, 1; $MgSO_4$, 0.7; $CaCl_2$, 0.5; $FeSO_4$, 0.05; $MnSO_4$, 0.02; $ZnSO_4$, 0.02; corn steep liquor, 60; brewer's yeast (dry), 1.0; meat extract, 5.0; glucose, 10; molasses, 5.0; malt, 10; gibb. mycelia, 5.0; adjusted to pH 6.8.

The culture was shaken at 380 r.p.m. for 48 hours at 27°C, then about 50 heat-sterilized glass beads 4 mm in diameter were added to it under sterile conditions and, after additional shaking for 30 minutes, it was passed through an about 1 cm thick fibre-glass filter pre-sterilized with heat [7]. Spore counts were determined in the filtrate and adjusted to 100/ml with sterile saline. The diluted suspension was streaked onto peptone agar plates, each of which were seeded with about 40—50 spores. The peptone agar consisted of 2.0 g brewer's yeast, 3.0 g peptone, 10.0 g dextrose, 20.0 g agar-agar made up to 1000 ml with distilled water and adjusted to pH 6.5.

The plates were incubated at 27°C for 96 hours, then each colony was transferred into a flask containing the fluid medium and shaken for 120 hours as described above. Riboflavin was extracted by boiling with acetic acid from samples taken in the 72nd, 96th and 120th hour and determined quantitatively in a Pulfrich stepphotometer.

The colonies were classified according to the following criteria: 1. colony size: (a) small, 0—5 mm in diameter (Fig. 1), (b) medium size, 5—10 mm in diameter (Fig. 4), (c) large, 10 mm in diameter (Fig. 1); 2. colour: (a) colourless (achromogenic) (Fig. 2), (b) yellow (chromogenic) (Fig. 5), (c) intensive yellow (intensively chromogenic) (Fig. 3); 3. sectorial properties: (a) homogeneous colony (Fig. 5), (b) positive or negative sectors in colonies (Figs 2 and 6); 4. dissimilarity of shape: (a) colony without air mycelia, with radial folds (Fig. 1), (b) colony with air mycelia and without folds (Fig. 5), (c) colony with central protrusion (horny type).

In addition, mutation was induced in streak plate cultures of spore suspensions, using ultraviolet light as the mutagenic agent (Original Hanau lamp, Sterisol tube, type NN 30/89 burner, 220 V), at a FB distance of 50 cm for 3-minute exposure time.

Results

The data for colony size distribution and the corresponding riboflavin synthesizing activity are shown in Table I.

Table I

Distribution of colonies according to size and riboflavin yield

		Colony size			Total
		large	medium	small	
Colonies	number	114	2247	1123	3484
	per cent	3.54	64.06	32.4	100
Riboflavin level after 120 hours, μg/ml		843.7	968.3	931.2	

Table II shows colony distribution according to colour and the corresponding vitamin yields. Table III shows the homogeneity versus production data.

Table II

Distribution of colonies according to colour and riboflavin yield

Colour	Colonies		Riboflavin yield after 120 hours, μg/ml
	number	per cent	
Yellow	3112	89.10	1007.4
Dark yellow	158	4.58	1139.5
Colourless	214	6.32	634.4

Table III

Proportion of sectored and homogeneous colonies and the corresponding riboflavin yields

Colony type	Colony incidence		Riboflavin yield after 120 hours, μg/ml
	number	per cent	
Sectored	268	7.7	764.0
Homogeneous	3216	92.3	1058.5
Total	3484	100.0	

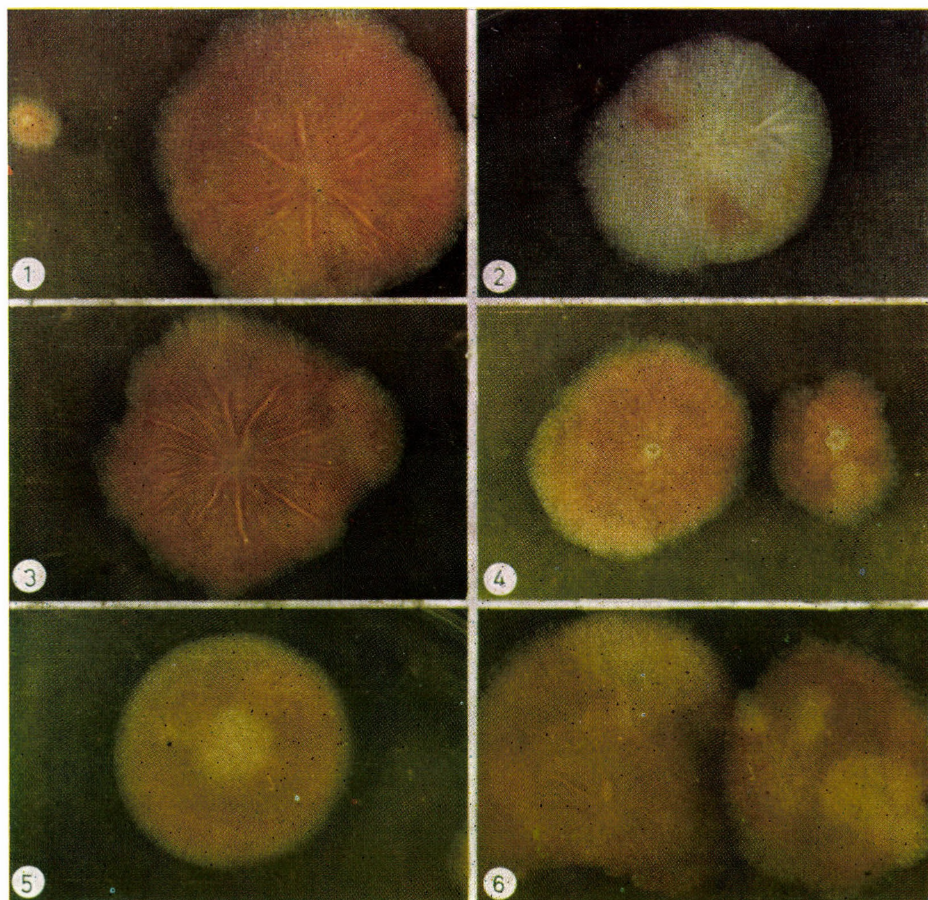


Fig. 1. Colonies of different size (×3)
Fig. 2. White (achromogenic) colonies (×2)
Fig. 3. Deep yellow (intensively chromogenic) colony with radial folds and without air mycelia (type "R")
Fig. 4. Yellow (chromogenic) colonies (×1.5)
Fig. 5. Smooth colony covered by air mycelia ("S" type) (×3)
Fig. 6. Sectored colonies (×3)

On further investigating the problem of sectorial distribution, the negative sectors of the colonies were segregated and inoculated into fluid medium to compare their riboflavin yields with those of the rest of the colonies, inoculated into another flask. The 120-hour mean yield of the achromogenic sectors was 632 μg/ml, a value distinctly inferior to that obtained for the "mother" colonies (943.5 μg/ml).

Then the proportions of sectored and homogeneous colonies were compared in spore suspension cultures from the "mother" and sectorial colonies. From the former, 16.1% of the colonies were sectored and from the latter 32.4%.

It was more difficult to evaluate the colonies according to morphological differences. Those bulging at the centre were so rare (0.2%) that they had to be disregarded, 36.2% of the colonies were smooth and covered with air mycelia and 63.6% were creased and bore no air mycelia. The colonies representing these two main categories were more difficult to distinguish than the rest, because their dissimilarities are inconspicuous; however, their riboflavin synthesizing capacity differed considerably (Table IV).

Table IV

Riboflavin yield for the two dominant colony shapes after 72, 96 and 120 hours incubation

Colony type	Riboflavin yield μg/ml, at		
	72 hours	96 hours	120 hours
Creased colony without air mycelia	597.2	866.0	998.7
Smooth colony with air mycelia	731.0	957.6	1124.5

Induced mutation experiments yielded surprising results. In contrast to spontaneous mutants, only very few (0.2%) of the UV-exposed colonies became achromogenic or sectored.

Discussion

According to the above findings, the *E. ashbyii* colony ideal for riboflavin production is characterized by (a) medium growth intensity (colony size), attaining 8—10 mm in diameter over a culturing period of 96 hours; (b) intensive yellow colour; (c) absence of sector formation; (d) smooth surface covered by air mycelia.

From the point of view of production, the so-called defective dwarfs occurring among the small colonies (less than 5 mm in diameter) are distinctly dangerous, because they carry a lethal effect and are indistinguishable from viable colonies. Colourless and sectored colonies should also be removed from the seed. The presence in the inoculum of colonies without air mycelia may cause a certain reduction of the riboflavin yield, but by no means will it render the fermentation improductive.

It seems that the fluctuation of the riboflavin yield can reasonably be prevented by an appropriate selection of the colony type used for seeding the inocula. Colonies not meeting the above criteria of optimum productivity must not be used.

An explanation for the greater liability of the fungus strain to spontaneous mutation should be sought in the spore itself or, more precisely, in the stage of spore formation. In this respect BUXTON's [1, 2] view on the spontaneous anastomosis of the hyphae and the consequent heterokaryosis does not seem acceptable. In the present experiments the spontaneity of the phenomena, above all that of saltation (sector formation) could be excluded, since

(a) the material having been derived from monospore suspensions, there was no possibility for hyphae of different origin to anastomose;

(b) on culturing of the altered and the remaining parts of sectored colonies there was a significant increase in the proportion of the altered ones;

(c) on mutagenic treatment only 0.2% of the new colonies turned out to be sectored or achromogenic.

As to the causes of the unusual variability of *E. ashbyii*, we offer the following hypothetical explanation [4]:

(1) Segregations stemming from exchange of genetic material between units of higher systematic position.

(2) Reduction of the information material characteristic of the species to a level still capable of producing more or less viable mutants.

(3) A probably unequal distribution of the nuclear substance (into normal and defective sectors) in the course of nuclear amitosis. The defective sector either does not produce any new individual (elimination), or it produces a mutant in the physiological or morphological sense if the defect is less pronounced.

The third point seems the most probable in relation to the strain in question. At the development of the sporangium spore, the nucleus of the sporangium divides until it has reached the number of nuclei characteristic of the genus (4—32, mean 10—14). Some of the relatively numerous nuclei probably carry the basis for a defect and perhaps also a heterokaryosis may temporarily be present within one and the same species. The nucleus gives rise to the spores and on their release the mature spores give rise to new individuals unless they carry a lethal defect.

In summary, there seem to be two ways of preventing the mutations responsible for the disadvantageous fluctuations of riboflavin yield on a plant scale, *viz.*

(i) induced mutation (*e.g.* with UV) to destroy the less viable (defective) individuals;

(ii) selection of the colonies used to seed the inoculum for optimum riboflavin yield, on the basis of growth intensity, colour, shape and homogeneity.

REFERENCES

1. Buxton, E. W.: Plant Pathology, Academic Press, New York 1954.
2. Buxton, E. W.: J. gen. Microbiol. **15**, 133 (1956).
3. Denkinger, G.: Valószínűségszámítás. Tankönyvkiadó, Budapest 1965.
4. Faludi, B.: Örökléstan. Tankönyvkiadó, Budapest 1963.
5. Foster, J. E.: Chemical Activities of Fungi. Academic Press, New York 1949.
6. Guilliermond, A.: C. R. Acad. Sci. Paris, **200**, 1556 (1935).
7. Nordström, K.: J. gen. Microbiol. **55**, 1 (1969).

Address of the author:

Tamás Vághy
Phylaxia, Veterinary Biologicals and Feedstuffs Ltd.
Szállás u. 5—7. Budapest X, Hungary

Acta microbiol. Acad. Sci. hung. 18, 81—86, 1971

PHAGE INFECTION OF VITAMIN B_{12} PRODUCING RHIZOBIUM CULTURES

By

J. SZABÓ SZŰCS

Phylaxia Veterinary Biologicals and Feedstuffs Ltd. (Director: J. MOLNÁR), Budapest

(Received February 17, 1971)

Summary. Two phage strains have been isolated from plant scale fermentor cultures fo a *Rhizobium melilotii* strain maintained in the laboratory for a long time as a vitamin B_{12} producer. Attention to phage infection had been attracted by the declining vitamin synthetizing capacity of the cultures. The two isolates differed in plaque size, bacterium contents and antigenicity, and have been used with success for the selection of phage resistant *Rhizobium* mutants of high vitamin B_{12} producing capacity. A correlation has been demonstrated between the B_{12} activity and capsule producing capacity of the selected bacterial mutants.

Phage infection of the cultures of various vitamin B_{12} producing microorganisms (*Bacillus megaterium*, actinomycetes) has been observed by GARIBALDI *et al.* [6], CARVAJAL [5] and RAUTENSTEIN [16].

The vitamin B_{12} producing capacity of rhizobia was first reported by BURTON and LOCCHEAD [3]. Later a method was elaborated and patented in this Institute [11] for the plant scale production of the vitamin from these microorganisms. In the course of production it was noted that in certain fermentor cultures the rhizobia either grew poorly or not at all, or showed an unusually long lag phase period. Also, the vitamin B_{12} contents of the cultures were sometimes unusually low without any apparent reason and the average titres fell gradually.

An explanation of this phenomenon was sought first in the medium, in the compressed air or some toxic substance and, as 20% of the fermentor cultures were found to have been infected with saprophytic organisms, even a bacterial antagonism was taken into consideration.

A phage infection was not suspected before all the above speculations had proven erroneous; in fact, there was not the slightest indication of the presence of a phage (plaques or failure of growth on agar slants), neither had been a phage infection of submerged rhizobium cultures reported in the literature. Anyhow, of 17 samples taken from different fermentors, 7 lysed the culture of the host cells completely or almost completely, and the bacterium-free filtrate of the lysed cultures gave rise to characteristic plaques in solid agar cultures of the producer strain.

This paper is a report of examinations of the phage infection of mass cultures of the B_{12} active *Rhizobium melilotii* strain.

Material and methods

The phage strains were isolated from fermentor cultures of *Rh. melilotii*. From each suspect culture 1 ml of the fluid phase was added to a 12—16-hour shaken culture of the host strain. After incubation for 16—24 hours, the strains were examined microscopically and those showing complete lysis or poor growth in comparison to the controls were qualified as infected with a *Rhizobium* phage. Samples secured from the lysed cultures were centrifuged and passed through a G—5 glass filter; the phage particle counts of the filtrates were then determined on agar plates in Petri dishes. According to the method of ADAMS [1], plates containing 2.5% agar were overlayered each with 3 ml 0.8% soft agar (42°C) to which 1.5 ml of a 16-hour phage sensitive bacterial culture and 0.5 ml of diluted phage filtrate had previously been added. The phage material had been diluted in tenfold serial steps, using a 10%, pH 7 peptone-water diluent. The cooled plates were then incubated for 24—36 hours at 27°C. The number of phage particles was expressed as plaque counts, taking into consideration the plaque sizes, to assess the identity or non-identity of the phage strains. The latter were cultivated from single morphologically dissimilar plaques, designating the small ones as P_1 and the large ones as P_2.

In addition to plaque size determination, the phage strains were identified by cross infection experiments and neutralization tests. The serum used for the latter was prepared in rabbits, by immunizing them with rising doses (0.5—4.0 ml) of undiluted (10^9/ml) phage suspensions on five occasions at 3-day intervals. The sera were inactivated at 56°C for 30 minutes and stored without preserving agent in the refrigerator (0—4°C). Sera for control tests were obtained in the same manner from rabbits immunized with the phage-free host strain. In the neutralization tests, 1 : 5 diluted serum was mixed with different dilutions of the phage strain in an equal volume, the mixture was incubated at 27°C for 30 minutes and titrated for phage content by the method of HORVÁTH and ALFÖLDI [8]. The reverse test was carried out by adding an equal volume of different serum dilutions to a known amount of phage (10^7/ml).

The pH-sensitivity of the phage strains was examined in the range of pH 5—9. A suspension containing 10^8/ml phage particles was mixed with a diluent adjusted to different pHs. The mixture was incubated for 1 hour at 27°C and titrated for phage content by the pour-plate technique. Thermosensibility was established from the titre decrease of a known phage suspension (10^8/ml) after incubation at 56°C for 5, 10, 20, 30 and 60 minutes.

Bacterial strain. Vitamin B_{12} was produced from a mutant (Rh 66/27) of *Rh. melilotii*, which served also as the host strain of phage P_1. Simultaneously with the isolation of the phage, also phage resistant mutants, designated as F_1, F_2 and F_{122}, were selected from the lysed culture of the strain. Later phage P_2 was isolated from the mutant strain F_{12}, which proved to be its host and was resistant to P_1. The mutants resistant to phage P_2 (F_{123}—F_{239}) were selected as above.

Phage resistance was examined with the droplet-test developed by LOVREKOVICH and JOHAN [13] for the checking of actinomycetes. B_{12} activity was estimated by the *E. coli*-test and the composition of the vitamin B_{12} product was analyzed by paper chromatography. To prevent phage carriership, the resistant strains were treated with specific antibody containing blood serum.

The composition of the medium pH 7.0 used in the experiments was (g/litre): sucrose, 20.0; sodium nitrate, 2.0; potassium iodide, 0.5; iron sulphate, 0.01. Solid media for maintenance of the strains and pour-plate technique were prepared by addition of 2% agar to the mixture.

Results

The titre of phage P_1 grown in shaken culture of the host bacterium attained 10^9/ml. The plaque diameter varied between 0.4 and 0.8 mm; the plaque base was clear and transparent and the margins were smooth and sharply contoured. The pH optimum was between 6.8 and 7.2, but heat was deleterious for phage activity even under such conditions. The phage was inactivated at 56°C in 20 minutes and at 0—4°C in 10—14 days. On the addi-

tion of 1% hydroxygen base or formaldehyde it lost its activity in a few minutes even at room temperature, but maintained it for as long as 1 year in the freeze-dried state (no activity tests were made beyond that period).

Since the various technological measures (method of sterilization, construction of the system, filters, etc.) can minimize bacterial contamination but do not protect against phage infection, certain physical or chemical procedures were scrutinized for their value in suppressing phage growth directly, without damage to the host bacterium. But as the heat and pH optima of the vitamin producing rhizobium strain corresponded with those of phage P_1 and the former was as sensitive to chemical agents as the latter, phage growth inhibitors (citrate, phosphate) were tested without any obvious result. Although the phage suspensions induced a specific antibody production in the rabbit, addition of such immune sera to the fermentor cultures was out of question. The only possible way of increasing the safety of vitamin B_{12} production seemed to be the selection of appropriately vitamin-active, but phage resistant strains. For this purpose, streak plates were prepared from the mutant strain Rh66/27 after exposure to the phage. On incubation for 3—5 days, characteristic small rhizobium colonies grew on the agar, which differed from one another and often also from the colonies of the parent strain. Between dry, slightly opalescent, small and slightly bulging colonies there appeared larger, flat, glistening, distinctly mucinous and often confluent ones which were whiter in colour and less transparent than the former type. The subcultures of the isolated colonies maintained their characteristics and all of them were resistant to phage P_1, but differed considerably in respect of B_{12} activity (from a few tenths to 5 μg/ml). Activity of non-mucinous mutants F_{10} and F_{12} was, however, consistently superior to that of the parent strain, while their *E. coli*-active product was in every respect similar to that synthetized by the latter. Strain F_{10} and later strain F_{12} were subsequently used with success for the production of vitamin B_{12} as selected P_1-resistant mutants. However, phenomena indicative of phage infection reappeared in the fermentor cultures after a production period of 4 months, although the strains were treated with specific anti-phage sera (Table I).

The isolate, phage P_2, differed from P_1 in colony morphology, giving rise to plaques 1.0—1.3 mm in diameter, larger than those of the latter. Strains resistant to P_1 were sensitive to P_2 and the host strain of the former (Rh 66/27) was resistant to the latter. Also, the antibodies specific to P_2 did not neutralize P_1. Of the many strains isolated from lysates of P_2 and P_1, that designated F_{222}, forming slightly mucinous, glistening, transparent colonies, was resistant to both phages and produced in shaken cultures 5—15% more vitamin B_{12} than did strain F_{12}. Since the composition of the *E. coli*-active substance did not change despite its higher titre, strain F_{12} was replaced by strain F_{222}, for plant scale production of the vitamin.

Table I

Some characteristics of fermentor cultures of phage-infected Rh. melilotii strains

Designation of fermentor	Fermentor culture		
	Time of culturing, hours	Bacterial count* x 0.9×10⁹	B_{12} titre *E. coli*-test, μg/ml
XXII	12	14	—
	15	22	±
	18	3**	0.6
XX	12	8	—
	15	13	1.8
	18	18	2.9
	21	2**	2.9
XVII	12	7	—
	15	11	1.9
	18	3**	2.1

* Culture diluted corresponding to 3rd grade nephelometric density (figures in Table), multiplied by 0.9×10^9

** Bacteriolysis

The different B_{12} activity of the P_1-P_2 phage resistant mutants and above all the higher vitamin synthetizing ability of certain strains have called for the use of the phage isolates for strain selection experiments. Colonies of dissimilar morphology were isolated from phage-exposed sensitive bacterial cultures and the resistant mutants were examined for B_{12} activity in 48 or 72 hour shaken cultures, by comparing them to the activity of the original

Table II

B_{12} activity of phage-resistant mutants

Rhizobium strain	Phage resistance			B_{12} activity, μg/ml
	P_1	P_2	$P_1 + P_2$	
Rh 66/27	S	S	.	5.25
F_{10}	R	S	.	5.95
F_{12}	R	S	.	6.31
F_{125}	R	R	.	3.10
F_{222}	R	R	.	7.21
F_{312}	R	R	.	0.55
F_{XIII}	R	R	R	0.73
F_{XX}	R	R	R	8.65

S = sensitive; R = resistant; . = not tested

producer strain. Table II shows data for some typical strains, to illustrate that simultaneously with the development of phage resistance another type of mutation (transduction) is taking place. The new properties induced by the phage (phage-resistance, alteration of B_{12} activity, morphological properties) proved to be hereditary, which was important from the point of view of production. It should be noted that a fairly large proportion of the mutants were B_{12}-positive; this was due probably to the primary selection for colony type (see later) among others. The most B_{12}-active mutant isolated up to now, F_{XX}, was also encountered in the course of this work.

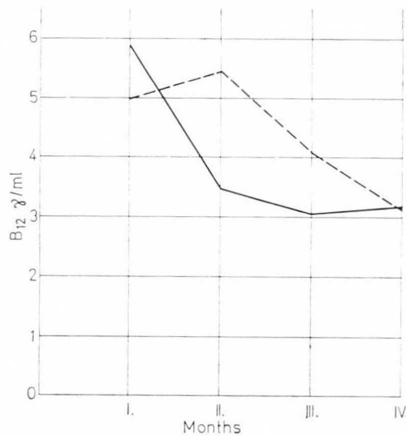


Fig. 1. Change of mean B_{12} titres over four months after phage infection. ——— Fermentor No. XIX, - - - - - Fermentor No. XVIII

Discussion

Of all speculations concerning the origin of phage P_1 (*Rhizobium* bacterial strain, surrounding soil, lysogenesis, etc.), its introduction with wild *Rhizobium* strains cultured in small fermentors for another purpose seemed most probable (Fig. 1). The observation that initially the phage infection was confined to certain fermentors only was in support of this assumption.

The origin of phage strain P_2 was also unclear. The experiments failed to reveal a mutation of phage P_1 or of bacterial strains F_{10} and F_{12} which are resistant to it. The probably lysogenic nature of these strains cannot be excluded either, although the dissimilarity of the plaques was against that possibility and so was the circumstance that on repeated treatment of the strains with UV or lithium chloride to increase their B_{12} activity, no lysis occurred in any subculture of the surviving strains. Also, there were no plaques in pour-plate cultures of mixed suspensions of the resistant host strains and certainly susceptible strains. It seemed therefore that the phage population

introduced into the plant in some way had been a mixed one from the beginning. Identification of the isolates as one or another of the known *Rhizobium* phages (PARKER and ALLEN [15]) is beyond the scope of this report.

The aimed application of *Rhizobium* phages for strain selection is a new method of great practical importance. The underlying fact is probably the occurrence of a mutation and of a subsequent selection, during which some individuals of the mixed bacterial strain might resist phage infection and it is probably these which are segregated by the isolation as resistant mutants. What matters is the inheritance of the latter property which is specific and originates from a change in the bacterial wall. The establishment of B_{12} activity in *Rh. melilotii* mutants is also related to the wall.

Polish investigators [10] have observed that highly mucinous strains are less active than "dry" ones. In fact, of the many phage resistant mutants isolated in the present study, those showing the highest B_{12} activity were certainly not mucinous; this correlation, nevertheless, requires further investigations.

REFERENCES

1. ADAMS, M. H.: Bacteriophages. Interscience Publishers, New York 1959.
2. BAKÁCS, T., FARKAS, E.: Orvosi Virológia, Medicina, Budapest 1965.
3. BURTON, M. O., LOCCHEAD, A. G.: Canad. J. Botany, **30**, 316 (1952).
4. BOYD, J. S. K.: J. Path. Bact. **63**, 445 (1951).
5. CARVAJAL, F.: United States Patent Office 2808364 (1957, Oct. 1).
6. GARIBALDI, J. A., SNELL, N. S., LEWIS, J. C.: Ind. Eng. Chem. **45**, 838 (1953).
7. HERSHEY, A. D.: Genetics **31**, 620 (1946).
8. HORVÁTH, I., ALFÖLDI, L.: Acta microbiol. Acad. Sci. hung. **1**, 495 (1954).
9. JACOB, F., WOLLMAN, E. L.: Ann. Inst. Pasteur **90**, 282 (1956).
10. JANICZKI, J., PAWELKIEWICZ, J., STAWICKI, K., ZODROW, K.: Acta microbiol. pol. **3**, 3 (1954).
11. JOHAN, B., LÁSZLÓ, É.: Hungarian Patent No. J. O.-86-146687 (1958).
12. JOHAN, B., SZABÓ SZÜCS, J., SZABÓ, A.: Acta microbiol. Acad. Sci. hung. **7**, 391 (1960).
13. LOVREKOVICH, I., JOHAN, B.: Personal communication.
14. LURIA, S. E.: Genetics **30**, 84 (1945).
15. PARKER, D. T., ALLEN, O. N.: Canad. J. Microbiol. **3**, 651 (1957).
16. Раутенштейн, И. И., Кофанова, Н. Д.: Микробиология **26**, 315 (1957).

Address of the author:

JÁNOS SZABÓ SZŰCS
Phylaxia Veterinary Biologicals and Feedstuffs Ltd.
Szállás u. 5—7, Budapest X, Hungary

Acta microbiol. Acad. Sci. hung. 18, 87—94, 1971

BACTERIOLOGIC DIAGNOSIS OF YERSINIA PSEUDOTUBERCULOSIS

By

J. SZITA and ANNA SVIDRÓ

National Institute of Public Health (Director: T. BAKÁCS), Budapest

(Received March 10, 1971)

Summary. (i) Six *Yersinia pseudotuberculosis* strains have been investigated. Five cultures were isolated from the mesenterial lymph gland of patients with Masshoff's disease, one strain was cultured from a removed appendix.

(ii) Resistance of the strains to heat and disinfectants was similar to that of *Enterobacteriaceae.*

(iii) Five strains belonged to serogroup IA, 1 strain to serogroup IB. All strains showed uniform biochemical reactions corresponding to characteristics described in the literature.

(iv) A total of 1214 sera from patients and 400 sera from healthy persons was tested for *Y. pseudotuberculosis* antibodies. It has been shown that titres of 1 : 100 should be regarded as doubtful, while titres of 1 : 200 or over as positive reactions.

The importance of human *Yersinia pseudotuberculosis* infection in Europe has been emphasized by MASSHOFF [1]. In Hungary, several authors have dealt with abscessing reticular mesenterial lymphadenitis known as Masshoff's disease [2—8]. A bacteriologically and serologically verified outbreak affecting 30 children was described by ZOLTAI *et al.* [9].

In this paper we present studies on the properties of *Y. pseudotuberculosis* strains isolated in Hungary and on the results of agglutination tests made with serum samples received in our Department during a 3-year period.

Materials and methods

Strains and bacteriological examination. Five strains were isolated from mesenterial lymph glands of 5 different patients. One strain was cultured in another laboratory from a surgically removed appendix and was sent for identification to our Department.

Cultural properties were studied on simple agar, chocolate, blood, eosin methylene blue, bismuth sulphite and desoxycholate citrate media [10]. Media described by BRIZIN [11] and PATERSON and COOK [12] were also used. Antibiotic sensitivity was examined by the disk method [10]. Bactericidal effect of disinfectants and thermoresistance were tested as described in [13—15]. O sera for slide agglutination (IA, IB, IIA, IIB, III, IV, V) were prepared from the following heated ($2\frac{1}{2}$ hours at 100°C) cultures: N.C.T.C. 1103, 824, 8477, 1779, 10278, 8580, 8579.

Antibody titres in human sera. A total of 1214 sera from patients was examined with Widal-type agglutination. Control sera were taken from 300 blood donors and from 100 persons screened for Wassermann reaction. Bacterial antigens I, II, III, IV and V were prepared from the following N.C.T.C. strains: 10275, 10277, 10278, 8580, 8579. Stable suspensions were obtained by culturing the organisms at room temperature for 48 hours in Roux flasks containing chocolate medium with an agar concentration 10% lower than the usual. The culture was killed with 0.5% formalin and adjusted to the density of a standard giving 74% transmittance at 1 cm light path in the Beckman DU spectrophotometer set at 0.03 mm slit

and 530 mμ wavelength. Suspensions prepared in this manner remained stable for at least 6 months; certain antigens (III and IV) showed no spontaneous agglutination even after 18—24 months.

For the agglutination test 0.05 ml antigen was added with Takátsy's pipette to 0.5 ml serum dilution. For antigen I, dilutions were put up ranging from 1 : 100 to 1 : 1600; for antigens II, III, IV and V the sera were tested in single tubes with dilutions 1 : 100 and serial dilutions were made only if positive reactions were observed. The tubes were incubated at 37°C overnight. In view of other workers' opinion [16, 17], 50 sera were tested parallel at 37°C and in water bath at 52°C overnight. The results were identical.

If a serum agglutinated *Y. pseudotuberculosis* antigens II or IV, in view of the known antigenic relationship between these groups and *Salmonella* O4,27 and O9,46 antigens, respectively, the sera were retested with *Y. pseudotuberculosis* antigens after absorption with the corresponding *Salmonella* antigen.

Results

Cultural properties. Y. pseudotuberculosis grows readily on simple, blood and chocolate agar media forming greyish, translucent, low convex colonies 2—3 mm in diameter. It fails to grow on several selective media (bismuth sulphite agar, eosin methylene blue agar with anionic detergent), and produces very small yellowish-pink colonies on desoxycholate citrate agar after 48 hours. All strains studied were isolated on blood or chocolate agar. As our efforts to isolate the agent from faeces have failed, in model experiments we tested the media described by Brizin [11] and Paterson and Cook [12]. On the former the organism produced greyish-black, on the latter faint violet colonies. These media were not sufficiently selective for faecal cultures and did not allow an easy distinction of *Y. pseudotuberculosis* colonies.

For differential diagnosis (especially for distinguishing from *Y. pestis*), motility testing is of importance. Our strains failed to migrate through U tubes incubated at 37°C for 14 days, but at 20°C motility was evident in 3—5 days.

Disinfectants exerted an effect similar to that observed with *Enterobacteriaceae*. All strains were killed in 1 minute by 5% phenol, 3% formalin, 0.1% chloramine, mercuric chloride, Sterogenol® (hexadecylpyridinium bromide), and 0.01% Famosept (phenyl mercuric borate).

Heat resistance. The strains survived exposure to 50°C for 6 hours, to 60°C for 1/2 to 1 hour. All strains were killed at 70°C in 3 minutes. In broth cultures stored at 4°C all strains were viable after 1 month.

Antibiotic sensitivity. All strains were sensitive to streptomycin, chloramphenicol, neomycin, moderately sensitive to tetracycline, resistant to penicillin, erythromycin, polymyxin B, and sulphonamides.

Biochemical reactions are presented in Table I. All strains behaved uniformly, only some differences were noted in the time before sugar fermentation had appeared. The strains conformed in biochemical reactions to data in the literature.

Serological typing. On the basis of thermostable O antigens, *Y. pseudotuberculosis* can be divided into 5 serological groups. Groups I, II and IV can

Table I

Biochemical reactions of Y. pseudotuberculosis

Adonitol	$+^{7-14}$	Xylose	+
Arabinose	+	Methyl red	+
Dulcitol	—	Voges—Proskauer	—
Galactose	$+^{1-2}$	Tryptophan deaminase	—
Glucose	+	Lysine decarboxylase	—
Glycerol	$+^{2-10}$	Arginine dihydrolase	—
Inositol	—	Ornithine decarboxylase	—
Laevulose	+	Gelatin liquefaction	—
Lactose	—	Urease	+
Maltose	$+^{1-2}$	Indole	—
Mannitol	$+^{1}$	Serum liquefaction	—
Raffinose	—	Nitrate reduction	+
Rhamnose	$+^{1}$	H_2S	—
Sucrose	—	Ammonium citrate utilization	—
Salicin	$+^{1-7}$	Catalase	+
Sorbitol	—	Oxidase	—
Trehalose	$+^{1-2}$	KCN	—

be classified into subgroups IA, IB, IIA, IIB, IVA and IVB. *Y. pseudotuberculosis* contains an R antigen identical in all groups and in *Y. pestis.* In view of the common partial antigens, the thermolabile H antigen is of no practical value in differentiation. Five strains isolated in this study from lymph glands belonged to subgroup IA; one strain cultured from appendix was classified into subgroup IB.

Antibody titres in human sera. Between June 2, 1966, and November 15, 1970, a total of 1214 serum specimens was received from patients with suspected *Y. pseudotuberculosis* infection. The agglutination test was negative in 902 sera. Distribution of reacting sera according to antigens and mean titres is presented in Table II.

The titre of 312 sera reacting with one of the 5 different antigens fell in the range 1 : 100 to 1 : 25 600. Titres of 1 : 100 or 1 : 200 were obtained for 58 out of 400 control sera. Accordingly, if sera reacting at titres of 1 : 100 or over are considered, the mean titre for patient sera is 1 : 226; for control sera, 1 : 111. It is interesting that the majority of patients' positive sera reacted with O antigen I, while most positive control sera with O antigen II. In respect to the known antigenic relationships, agglutination with O antigen II might indicate a history of *Salmonella* B group infection. After absorption with *Salmonella* B group strains these sera failed to agglutinate *Y. pseudotuberculosis* O group II antigen.

Table II

Distribution of human sera reacting with Y. pseudotuberculosis antigens I–V

Sera	Antigens I	II	III	IV	V	Total positive	Mean titre
1214 patient sera	263	29	6	8	6	312	1 : 226
400 control sera	4	51	—	2	1	58	1 : 111

$$\text{Mean titre} = \sqrt[N]{d_1^{n_1} \cdot d_2^{n_2} \ldots d_n^{n_n}}$$

$d_1, d_2 \ldots d_n$ = reciprocals of serum dilutions
$n_1, n_2 \ldots n_n$ = number of sera giving the corresponding titre
N = total number of sera

Table III shows the distribution of sera according to antigens and titres. As regards antigen I positive agglutinations were specific. A titre of 1 : 100 or over was displayed by 263 out of 1214 patient sera. The number of sera titered 1 : 200 or over was considerable in this group of persons. In contrast, only 4 out of 400 control sera showed O group I agglutination at a titre of 1 : 100. For O group II antigen the difference between the two groups of persons was not significant.

Table III

Distribution of human sera reacting with Y. pseudotuberculosis antigen, according to agglutination titres

Antigen	Sera	Titres 1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200*	Total no. of reacting sera	Mean titre
I	patients	111	84	42	13	5	8	263	1 : 202
	control	4	—	—	—	—	—	4	1 : 100
II	patients	15	8	3	2	1	—	29	1 : 178
	control	30	21	—	—	—	—	51	1 : 102
III	patients	3	1	2	—	—	—	6	1 : 252
	control	—	—	—	—	—	—	—	—
IV	patients	5	2	1	—	—	—	8	1 : 141
	control	1	1	—	—	—	—	2	1 : 141
V	patients	1	2	1	1	1	—	6	1 : 356
	control	1	—	—	—	—	—	1	1 : 100

* or higher

Discussion

The *Y. pseudotuberculosis* strains examined in this study were isolated on blood or chocolate agar. From mixed bacterial population (faeces, appendix, throat specimens) their isolation on these media may be difficult. For the isolation of the genus *Yersinia* different selective media have been described [11, 12, 19, 20]. Füzi's vancomycin medium [21] is recommended for the isolation of *Pasteurella* from Gram positive bacteria. Instead of selective media, the method of Rédey [22] using the ability of *Y. pseudotuberculosis* to cause keratoconjunctivitis in the guinea pig may be applied: a loopful of mixed bacterial culture is inoculated into the eyes of the animal and after the development of inflammation a subculture on non-selective media is prepared. Attempt to show *Y. pseudotuberculosis* by immunofluorescent tracing yielded contradictory results [23, 24].

Y. pseudotuberculosis cultures showed a resistance to disinfectants and heat similar to that of *Enterobacteriaceae.* Our strains were resistant to penicillin, Brizin's strains were also resistant to therapeutic concentrations of penicillin [25]. For the differentiation of *Y. pestis* and *Y. pseudotuberculosis,* testing of motility, urea, trehalose, sorbitol and rhamnose reactions are of value. Timofeva [26] emphasizes the importance of phage examinations. For the differentiation of *Y. enterocolitica* and *Y. pseudotuberculosis,* in addition to serological typing, ornithine decarboxylase production and sucrose, sorbose, melibiose and rhamnose fermentation are especially important.

On the basis of serotyping of strains and in view of the antibody content of human sera it may be concluded that, similarly to many countries in Europe [27—30], *Y. pseudotuberculosis* group I strains predominate also in Hungary. Before 1967, group V was the most frequent in Japan [33], while in the U.S.A. the majority of human infections are caused by group I [34]. In Hungary, Kemenes [35] reported that in hares group I was the one most frequently encountered.

All *Y. pseudotuberculosis* strains share a common antigen present also in *Y. pestis.* The relationship between the two species has been studied in antigenic analyses [36, 37], genetic experiments [38, 40] and antibody titration of animal sera obtained in endemic foci [41]. The relationship is indicated by the fact that animals having passed through natural or artificial pseudotuberculosis infection are resistant to bubonic plague. Although Knapp [42] pointed out that there were no proofs for the transformation of the two species into each other, the spread of *Y. pseudotuberculosis* among wild animals may extinguish plague in the corresponding area.

In addition to the aforementioned serological relationship between salmonellae and *Y. pseudotuberculosis,* Knapp described common partial antigens in *Y. pseudotuberculosis* IVA and *E. coli* O17 and O77. Wetzler [45] used haemagglutination for detecting a lipopolysaccharide-like antigen present in

Enterobacteriaceae and in *Y. pseudotuberculosis*. In view of this finding he recommended that *Y. pseudotuberculosis* should be excluded from the genus *Pasteurella* and classified in the new genus *Yersinia*.

Our absorbed typing sera gave no reaction with *Salmonella* groups B and D, human pathogenic *Y. enterocolitica* groups 3 and 9, *P. tularensis* and *Brucella* antigens. Antisera prepared against these organisms did not agglutinate *Y. pseudotuberculosis* groups I to V.

The fairly large number of sera sent to our laboratory for the detection of agglutinins against *Y. pseudotuberculosis* indicate that clinicians are needing more frequently a serological than a cultural examination. It was, therefore, considered important to produce storable antigens and to establish the normal titres by examining a sufficient number of sera from healthy individuals. The results showed that *Y. pseudotuberculosis* group I agglutinins are unfrequent in normal population (Table III). The large number of normal sera reacting with *Y. pseudotuberculosis* group II might in some of the cases be associated with a history of *Salmonella* B group infection. A negligible number of the control sera reacted with other groups of *Y. pseudotuberculosis*. In routine examinations serological relationships with other bacteria present no difficulties as sera reacting with such antigens are always retested after absorption with the corresponding antigen.

In comparing titres obtained for patients' and control sera (Table III), it seems justified that titres of 1 : 200 or over should be regarded as positive. This is in agreement with the opinion of Hudeman and Ezold [17] and of Splino *et al.* [46], whose data indicate that titres of 1 : 166 or higher are positive. According to other authors, values lower than the above should also be regarded as positive [16, 32, 47].

Antibodies to *Y. pseudotuberculosis* may persist for months; out of 26 patients with positive antibody titres the sera of 9 still reacted after 4 months and 1 patient was positive even after 13 months [9].

According to Mollaret [48] patients with *Y. pseudotuberculosis* infection give a positive cutaneous reaction. Our experiments on persons that had passed through typhoid fever or had been vaccinated against it and on tuberculous patients [49] verified the specificity of Mollaret's antigen.

Although human pseudotuberculosis is not a frequent disease, the bacteriologist should be ready for the examination of materials from suspected cases. The diagnosis is confirmed by the biochemical and serological identification of the agent cultured from lymph glands, faeces, appendix, blood, or pus, by the Widal-type agglutination test of the sera and by a positive cutaneous test.

REFERENCES

1. MASSHOFF, W.: International Symposium on Pseudotuberculosis, Paris 1967. Symp Series immunobiol. Standard. Karger, Basel—New York **9**, 12 (1968).
2. PODHRAGYAY, L., FODOR, I.: Orv. Hetil. **97**, 277 (1956).
3. GÁTI, B.: Orv. Hetil. **99**, 927 (1958).
4. HERCZEG, B., RUTKAI, P.: Zbl. Chir. **85**, 2432 (1960).
5. GYARMATI, L.: Orv. Hetil. **102**, 601 (1961).
6. HERCZEG, B., KÖVECS, G., BÁNHIDY, A.: Orv. Hetil. **106**, 1787 (1965).
7. FÁBIÁN, F.: Orv. Hetil. **109**, 446 (1968).
8. BAKSA, J., NAGY, L., LEY, G.: Orv. Hetil. **108**, 1368 (1967).
9. ZOLTAI, N., RUDNAI, O., SZITA, J., SVIDRÓ, A.: Orv. Hetil. **108**, 1441 (1967) and International Symposium on Pseudotuberculosis, Paris 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 85 (1968).
10. Módszertani Útmutató. Országos Közegészségügyi Intézet, Budapest 1969.
11. BRIZIN, B.: Zbl. Bakt. I. Abt. Orig. **189**, 543 (1963).
12. PATERSON, J. S., COOK, R.: J. Path. Bact. **85**, 241 (1963).
13. Official Methods of Analysis. Association of Organization of Agricultural Chemists. Washington D. C. 1960.
14. HOUSTON, T., MCCLOY, J. M.: Lancet **2**, 632 (1916).
15. DIBLE, J. H.: J. Path. Bact. **24**, 3 (1921).
16. WINBLAD, S., NILÉHN, B., STERNBY, N. H.: Brit. med. J. **2**, 1363 (1966).
17. HUDEMANN, H., EZOLD, R.: Z. ges. Hyg. **15**, 602 (1969).
18. THAL, E.: Schweiz. Arch. Tierheilk. **108**, 372 (1966).
19. THAL, E., CHEN, T. H.: J. Bact. **69**, 103 (1955).
20. MORRIS, E. J.: J. gen. Microbiol. **19**, 305 (1958).
21. FÜZI, M.: Kísérl. Orvostud. **20**, 558 (1968).
22. RÉDEY, B.: Personal communication.
23. KARLSSON, A., THAL, E.: International Symposium on Pseudotuberculosis, Paris 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 187 (1968).
24. SIZARET, P., MOLLARET, H. H.: *Ibid.* p. 197.
25. BRIZIN, B.: *Ibid.* p. 207.
26. TIMOFEVA, L.: *Ibid.* p. 233.
27. THAL, E.: Untersuchungen über Pasteurella pseudotuberculosis unter besonderer Berücksichtigung ihres immunologischen Verhaltens. Lund, Berlingska Boktryckeriet, 1954, p. 69.
28. GIRARD, G., CHEVALIER, A.: Ann. Inst. Pasteur **88**, 227 (1955).
29. MAIR, N. S.: J. Path. Bact. **90**, 275 (1965).
30. VAN HAAFTEN, J., POELMA, F. G., ZWART, P.: International Symposium on Pseudotuberculosis, Paris, 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 117 (1968).
31. JUSCENKO, G. V.: *Ibid.* p. 107.
32. RUSU, V., HORTNOG, E., ANDRONESCU, C., DUMITRIU, M.: *Ibid.* p. 99.
33. ITAGAKI, K., TSUBOKURA, M., SASAKI, Z., NAGAI, T.: *Ibid.* p. 27.
34. WETZLER, T. F., HUBBERT, W. T.: *Ibid.* p. 33.
35. KEMENES, F.: Personal communication.
36. GOLDENBERG, M., HUDSON, B. W.: International Symposium on Pseudotuberculosis, Paris, 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 303 (1968).
37. BURROWS, T. W.: *Ibid.* p. 145.
38. LAWTON, W. D., MORRIS, B. C., BURROWS, T. W.: *Ibid.* p. 285.
39. KOROBKOVA, E., LENSKAJA, G. N., SOLOLOVA, N. M.: *Ibid.* p. 245.
40. MARTINEVSKI, I. T.: *Ibid.* p. 265.
41. MARSHALL, J. D., CURRIE, J. A., QUY, D. V.: *Ibid.* p. 309.
42. KNAPP, W.: Münch. med. Wschr. **111**, 2633 (1969).
43. KNAPP, W.: Arch. Hyg. **117**, 369 (1963).
44. KNAPP, W.: International Symposium on Pseudotuberculosis, Paris, 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 179 (1968).
45. WETZLER, T. F., EITZEN, H. E., CURRIE, J. A., MARSHAL, J. D.: *Ibid.* p. 155.
46. SPLINO, M., PEYCHL, L., KYNTERA, F., KORTLIK, J.: Zbl. Bakt. I. Abt. Orig. **211**, 360 (1969).

47. KAREN-HEYBERGER, E.: International Symposium on Pseudotuberculosis, Paris, 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9,** 81 (1968).
48. MOLLARET, H. H.: Presse méd. **70,** 1923 (1962) and Path. Biol. **13,** 554 (1965).
49. SZITA, J., ERDŐS, L., ZOLTAI, N., RUDNAI, O., SVIDRÓ, A.: Congress of the Hungarian Association of Hygienists. Budapest 1970.

Address of the authors:

JÓZSEF SZITA, ANNA SVIDRÓ
National Institute of Public Health,
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 95—99, 1971

PHAGE TYPING OF D-GROUP STREPTOCOCCI

I. TYPING OF ENTEROCOCCI WITH ROUMANIAN PHAGES

By

VIOLA HOCH and G. HÉRMÁN

National Institute of Nutrition (Director: R. TARJÁN)
and National Institute of Public Health (Director: T. BAKÁCS), *Budapest*

(Received March 11, 1971)

Summary. Phage typing as an epidemiological marker of enterococci associated with food poisoning has been introduced. A total of 557 D-group streptococcal strains isolated from food specimens and other materials was typable in 53.0% with Roumanian phages.

In recent years, food hygienists have devoted increased attention to D-group streptococci. These organisms, in addition to being excellent indicators of faecal contamination, may cause food poisoning. Alimentary toxicoinfections associated with enterococci have been described from 1899 onward by several authors [9, 16]. Enterococci were isolated from meat specimens involved in food poisoning by TURNER and THORN [19], HART *et al.* [11] and LANG [13]. LÜÖND and GASSER [15] assumed that a similarity existed between staphylococcal and enterococcal food poisoning. This finding was confirmed by FUJIWARA *et al.* [10] who isolated toxins from two strains of *Streptococcus zymogenes* in connection with food poisoning. Biochemical examinations, human volunteer experiments and the classical Dolman test indicated that the organism produced a toxin similar in action to the enterotoxin of *Staphylococcus aureus.*

Owing to their high resistance, enterococci are good indicators of faecal contamination. Their presence in refrigerated or dried foods proves a contamination that had taken place long before the examination. Search for these organisms on the surface of fomites and hands of staff may be used in hygienic examinations [12]. As phage typing has been widely used as an epidemiological marker for a number of pathogenic bacteria, it seemed interesting to study the applicability of this method for D-group streptococci.

Material and methods

Food specimens or swabs were inoculated in LITSKY–MALLMANN enrichment medium [14]. Subcultures were streaked on SZITA's E_{67} agar [17]. The isolates were identified on the basis of SHERMAN's criteria, i.e. growth in the range of 10—45°C, in the presence of 6.5% NaCl, 40% bile and at pH 9.6 [18]. As recommended by PUSZTAI [18], TTC, potassium tellurite and sorbitol reactions were also tested.

Phage typing of enterococci was carried out with phages used in Roumania. The set contained phages acting on *Str. faecalis* var. *zymogenes* and var. *liquefaciens* (group I) and on

Str. faecium, durans and *bovis* (group II). For phage typing Todd—Hewitt broth and agar were used. On plates of the latter an abundant confluent growth most suitable for phage typing was obtained.

A 24-hour broth culture was inoculated into fresh broth. After 2 hours incubation, agar plates were flooded with the culture. The excess culture was pipetted off, the plates were dried at room temperature, then one drop of the appropriate dilution of each phage was placed on the plate. Readings were made after 24 hours incubation at 37°C. According to the lysis pattern, the strains were classified into group I or group II. Some strains were lysed by phages of both groups ("mixed phage group").

Results

A total of 557 D-group streptococci was typed (Table I). Typable cultures were encountered in 53.0%, the remaining 47.0% of strains showed no lysis with any phage of the set. Strains associated with food poisoning were typable in 64.2%. Other food specimens yielded typable strains in 51.2%, hygienic examinations in 47.4%.

Strains isolated from food specimens and from food poisoning belonged to group I in 59.7% and 46.3%, respectively; hygienic examinations yielded phage group I strains in 55.1%. Classification of strains into subspecies by phage typing corresponded in 95.7% to results obtained by combined bacteriological and serological examination. BIRZU *et al.* [5] examined 2000 strains isolated from food specimens in association with food poisoning and found *Str. faecalis* group strains typable in 56.32%. After a modification of the phage set they were able to raise the percentage of typable strains to 65.03. *Str. faecium* group strains were typable in 43.5%.

Table I

Typing with Roumanian phages of D-group streptococci isolated from various materials

Specimen	No. of identified strains	Untypable		Typable		Phage group distribution of typable strains					
						I		II		Mixed	
		No.	%	No.	%	No.	%	No.	%	No.	%
Food	256	125	48.8	131	51.2	78	59.7	50	38.1	3	2.2
Food poisoning	81	29	35.8	52	64.2	24	46.3	16	30.7	12	23.0
Hygienic examinations	169	89	52.6	80	47.4	44	55.1	33	41.2	3	3.7
Other	51	19	37.2	32	62.8	12	37.5	16	50.0	4	12.5
Total	557	262	47.0	295	53.0	158	53.8	115	38.9	22	7.3

Both *Str. faecalis* and *Str. faecium* group phages lysed 22 strains. These cultures were classified as "mixed phage group" strains. Such isolates were studied by BELOIU *et al.* [4] who showed by multiple colony examination that *Str. faecalis* and *Str. faecium* group strains were present in mixed culture. After proper isolation the cultures were lysed only by the corresponding group of phages.

Table II

Number of enterococcal strains lysed by phages of the Roumanian set

Total number of strains tested, 295

Origin of strains	Phage group I*										Phage group II**						"Mixed" strains (unclassifiable)
	1	2	4	5	6	7	10	13	19	41	8	139	670	867	963	1510	
Food	21	1	—	1	1	5	28	20	—	71	36	5	25	6	27	4	3
Food poisoning	7	1	—	1	1	1	7	5	—	23	12	2	9	3	11	1	12
Hygienic examinations	12	—	—	—	—	—	12	6	—	42	28	2	20	6	18	3	3
Other	3	—	—	—	—	—	1	4	—	9	10	1	9	5	10	1	4
Total	43	2	—	2	2	6	48	35	—	145	86	10	63	20	66	9	22

* *Str. faecalis* var. *zymogenes*, var. *liquefaciens*
** *Str. faecium, durans, bovis*

Table II shows the number of strains lysed by different phages. The majority of strains was lysed by group I phages 41, 10, 13 and 1. Phages 2, 5, 6 and 7 each lysed less than 10 strains. None of the strains reacted with phages 4 and 19. In group II the majority of strains were lysed by phages 8, 963 and 670. Phages 139 and 1510 lysed 10 and 9 strains, respectively. The number of "mixed" strains was 22. As the same strain was frequently lysed by more than one phage, the number of typable strains shown in Table I differs from the total number of isolates in Table II.

Discussion

Phages acting on enterococci were first isolated by Beckerich *et al.* [3], Bagger [1] and Dutton [8]. Ciuca *et al.* [7] showed that by the use of a suitable phage set *Str. faecalis* and its variants can be distinguished from *Str. faecium, durans* and *bovis*. Phage typing combined with Barnes' medium and potassium tellurite medium became an important means of differential diagnosis between the two groups of enterococci. Baldovin-Agapi and Balteanu [2] and Beloiu [4] improved the typing technique by introducing new phages. Brock [6] confirmed that *Str. faecalis* and *Str. faecium* were distinguishable by phages. He showed that phage sensitivity is determined by temperate phages of the bacteria and that phage sensitivity may be altered by mutations in the organism. This finding is supported by the fact that mutants acquiring resistance to certain growth factor analogues differ in phage sensitivity from the original strains.

The practical applicability of the method is proved by the observation of Birzu *et al.* [5] who described that from patients' and cooks' faeces involved in two different food poisonings with cream confectionery and ewe-cheese and from the incriminated food specimens, identical phage types of enterococci were isolated.

Acknowledgement. The authors are indebted to Dr. A. Pleceas, Cantacuzino Institute, Bucharest, and to Dr. H. Milch, National Institute of Public Health, Budapest, for supplying the phage set, and to Dr. S. Pusztai, University Veterinary School, Budapest, for serologically identified strains.

REFERENCES

1. Bagger, S. V.: J. Path. Bact. **29**, 225 (1926).
2. Baldovin-Agapi, C., Balteanu, E., Mihalco, F., Beloiu, I., Pleceas, P.: Arch. roum. Path. exp. Microbiol. **21**, 385 (1962).
3. Beckerich, A., Hauduroy, P.: C. R. Soc. Biol. (Paris) **86**, 881 (1922).
4. Beloiu, I., Pleceas, P., Caffe, I.: Arch. roum. Path. exp. Microbiol. **23**, 355 (1964).
5. Birzu, A., Pleceas, P., Aizicovici, H., Morosanu, V.: Arch. roum. Path. exp. Microbiol. **25**, 245 (1966).
6. Brock, D. T.: J. Bact. **88**, 165 (1964).
7. Ciuca, M., Baldovin-Agapi, C., Mihalco, F., Beloiu, I.: Arch. roum. Path. exp. Microbiol. **18**, 519 (1959).

8. DUTTON, L. O.: J. infect. Dis. **39**, 48 (1926).
9. EVANS, A. O., CRINN, A. L.: J. Bact. **54**, 495 (1947).
10. FUJIWARA, K., SEKIYA, K., BAMBA, K.: Jap. J. Bact. **11**, 411 (1956).
11. HART, C. J., SHERWOOD, W. W., WILSON, E.: Publ. Hlth Rep. (Wash.) **75**, 527 (1960).
12. JÁNOSSY, G.: Egészségtudomány **14**, 69 (1970).
13. LANG, K.: Arch. Lebensmitt. Hyg. **12**, 125 (1961).
14. LITSKY, W., MALLMANN, W. L., FIFIELD, C. W.: Amer. J. publ. Hlth **45**, 1049 (1955).
15. LÜÖND, H., GASSER, K.: Mitt. Lebensmitt. Hyg. **55**, 144 (1964).
16. ORMAY, L., NOVOTNY, T.: Zbl. Bakt. I. Abt. Orig. **215**, 84 (1970)
17. ORMAY, L.: Orvosi laboratóriumi asszisztensek kézikönyve. Medicina, Budapest 1967, p. 178.
18. PUSZTAI, S.: Magy. Állatorv. L. **11**, 593 (1969).
19. TURNER, THORN: cit. SHATTOCK, P. H. F.: Inc. Symposium of Food. Iowa State University, Iowa City 1962.

Address of the authors:

VIOLA HOCH
National Institute of Nutrition,
Gyáli út 3, Budapest IX, Hungary
GÁBOR HÉRMÁN
National Institute of Public Health,
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 101–104, 1971

PHAGE TYPING OF D-GROUP STREPTOCOCCI

II. ISOLATION OF SUPPLEMENTARY PHAGES FOR CLASSIFICATION OF ENTEROCOCCI UNTYPABLE WITH ROUMANIAN PHAGES

By

G. Hérmán and Viola Hoch

National Institute of Public Health (Director: T. Bakács) *and National Institute of Nutrition (Director:* R. Tarján), *Budapest*

(Received March 11, 1971)

Summary. D-Group streptococci untypable with the Roumanian set of phages were classified in 42.9% with phages of the present authors. Heat treatment of untypable strains increased the incidence of classifiable enterococcal cultures. The new set is constituted of virulent phages isolated from sewage and of phages released after UV induction by strains isolated by the authors. Instability of typing phages was decreased by the addition of 0.01 *M* magnesium sulphate, 10% human serum and 1 *M* sucrose, and by acidification. In model experiments the heat sensitivity of the typing phage and the possibility of increasing heat resistance have been examined.

Our study on the applicability of Roumanian phages for typing of enterococci [4] was continued to increase the number of classifiable strains by the use of new phages.

Materials and methods

Isolation of phages from sewage. An untreated sewage specimen was divided into 3 portions in order to compare the effectiveness of various methods of phage isolation.

The first portion was centrifuged at 3000 r.p.m. twice for 20 minutes each and the supernatant was tested for the presence of phages.

The second portion was shaken with 1% (v/v) chloroform at room temperature for 5 minutes, centrifuged at 3000 r.p.m. for 20 minutes, then examined for phage content.

The third portion was treated with 0.1% (v/v) toluol at 37°C for 30 minutes, centrifuged, then the supernatant was tested for phages.

The supernatants were spotted on plates seeded with a selected set of indicator strains. The plates were read after 18 hours incubation at 37°C. The plaques were distinguished morphologically. If the isolated plaques were homogeneous in morphology, the phage was propagated in broth and finally tested in R.T.D. Sometimes phages isolated from sewage failed to show a homogeneous morphology even after several passages.

Release of temperate phages. From enterococcal strains isolated in the course of routine examinations, 30 different temperate phages were obtained. Of these, 11 have been included in our typing set. Bacteria in the 5th hour of exponential growth were suspended in phosphate buffer (pH 7.2) and irradiated with a Tungsram Germicid lamp at 256 mμ wavelength from 20 cm distance for 30 seconds. Subcultures were prepared in Todd—Hewitt broth at 37°C for 16 hours. Then the culture was centrifuged (3000 r.p.m., 30 minutes) and the supernatant was tested on a selected set of indicator strains. After incubation at 37°C for 18 hours, plaque isolation, propagation and lysis spectrum determination were performed as described above.

D-Group streptococci were isolated from food specimens associated or not associated with food poisoning and in the course of hygienic examinations. The cultures were identified as described in [4]. The strains were subcultured on blood agar at 3-month intervals and stored on blood agar slants at 4°C.

Phages were designated so that the numerator indicated the propagating strains, the denominator showed the serial number of the phage. Phages bearing serial numbers 4, 5, 6, 8,

9, 10, 11, 15, 16, 17, 18, 21, 22 and 23 corresponded to virulent phages isolated from sewage, those designed with 1, 2, 3, 7, 12, 13, 14, 19, 20, 24 and 25 were released by UV irradiation.

Phage typing was carried out on Todd—Hewitt agar plates seeded with cultures grown in Todd—Hewitt broth at 37°C for 3 hours. Readings were made with hand lens (×10) at oblique light after 18 hours incubation at 37°C. In recording the results, phages giving confluent or semiconfluent lysis were listed unbracketed, phages showing less definite lysis were listed in brackets.

Heat sensitivity of phage 917/11. The stock phage suspension was diluted 10^{-3} then exposed in phosphate buffers of different pH, in distilled water and in various combinations of sucrose, magnesium sulphate and human serum in a water bath at 56°C for 30 minutes. Before and after heat treatment 0.01 ml aliquots were spotted with platinum loop on plates inoculated with the indicator strains. Phage action was read as described above. This semi-quantitative method was more suitable than the use of soft agar plates in which the developed plaques were small and indefinite.

Storing of phages. At first, the phages were stored at 4°C in Todd—Hewitt broth. Later Todd—Hewitt broth supplemented with 1 *M* sucrose, 0.01 *M* magnesium sulphate and 10% human serum and adjusted to pH 6.9 was used.

Heat treatment of untypable strains. Three-hour broth cultures were treated in a water bath at 56°C for 2 minutes, then cooled rapidly [1, 5]. Typing of untreated and treated bacteria was performed in parallel experiments.

Results

A total of 259 untypable strains was examined with 14 virulent and 11 temperate phages isolated during this study. With the new phage set, 111 strains were typable (42.9%), 148 strains remained untypable (57.1%). After heat treatment 71 of the latter cultures became typable with the supplementary phage set.

Table I

Lysis spectrum of phages

Propagating strain	434/1	434/2	434/3	434/4	434/5	434/6	434/7	434/8	434/9	917/10	917/11	917/12
434	scl	cl	cl	—	cl	cl	cl	cl	—	—	±	—
917	—	—	—	—	—	—	—	—	—	+++	+++	—
1169	—	—	—	—	—	+	+++	+++	—	+++	+++	—
$H33_V$	—	++	±	—	±	++	scl	+++	—	+++	+++	—
$H33_F$	—	—	—	—	—	—	—	—	—	—	—	—
25	scl	—	—	—	scl	scl	+++	+++	—	++	—	—
H9	—	—	++	—	++	+++	cl	cl	—	—	—	—
200	—	—	—	—	—	+++	scl	cl	—	—	—	—
4	—	—	—	—	+++	+++	+++	+++	—	—	±	—
8	—	—	—	—	scl	scl	—	—	—	—	—	—
22	—	—	—	—	—	+++	scl	cl	—	—	—	—

cl = confluent lysis; scl = semiconfluent lysis; +++ = 50—100 plaques; ++ =

Virulent phages isolated from sewage gave rise to a broad lysis spectrum. Phages 16, 17 and 18 were especially active. In agreement with data in the literature [2], the lytic spectrum of temperate phages was very narrow.

Table I shows the lytic activity of our phages on the propagating strains used as indicators. On storage of the typing phages at 4°C in Todd—Hewitt broth there was a considerable decrease in titre or the activity was lost. To elaborate a method for inhibiting spontaneous inactivation, the thermal denaturation of the phages was examined in model experiments. In these studies phage 917/11 was used.

The optimum protective effect against heating at 56°C for 30 minutes was observed in a solution containing 10% human serum, 1 *M* sucrose and 0.01 *M* magnesium sulphate. Phosphate buffers of alkaline reaction failed to exert any protective effect, while in acid phosphate buffers the phage was destroyed less readily. The size of plaques increased on raising the sucrose concentration. On the basis of these experiments we prepared the preserving solution for storing the phages at 4°C.

Discussion

Enterococcal strains untypable with Roumanian phages were classified in 42.9% by the use of our set of virulent and temperate phages. With heat treatment the effectiveness of typing increased. It may be assumed that the

isolated in the present study

1169/13	1169/14	$H33_V/15$	$H33_V/16$	$H33_F/17$	$H33_F/18$	25/19	25/20	H9/21	H9/22	200/23	4/24	8/25
—	—	—	scl	cl	cl	—	—	cl	scl	cl	cl	cl
—	—	—	—	scl	scl	—	—	—	—	—	—	—
cl	scl	—	—	—	—	—	—	+++	—	cl	—	—
—	—	cl	+++	cl	cl	—	—	scl	—	cl	—	—
—	—	—	+++	cl	cl	—	—	—	—	—	—	—
+	+	++	++	+++	+++	cl	cl	—	+++	scl	—	—
—	—	—	cl	cl	cl	—	—	cl	cl	cl	+++	+++
—	—	—	—	cl	—	—	—	cl	+	cl	+++	++
—	—	—	+++	cl	cl	—	—	+++	+++	cl	cl	+++
—	—	—	++	scl	+++	—	—	—	+++	cl	scl	cl
—	—	—	—	cl	—	—	—	cl	+	cl	++	+

20—50 plaques; + = 10—20 plaques; ± = 0—10 plaques

hypothesis concerning the mode of action of heat treatment of staphylococci [1, 5] is valid for enterococci. The phage set consisting of virulent and temperate phages may be considered an adequate combination, since only the simultaneous application of broad-spectrum virulent and narrow-spectrum temperate phages would allow a sufficient classification of enterococci.

Our virulent phages were unspecific in that they lysed 7 *Str. uberis* and some serologically untyped alpha-haemolytic streptococcal strains. The temperate phages failed to lyse the above cultures.

Inactivation of phages during storage may be explained by a spontaneous denaturation of their protein constituents. Experiments with phage 917/11 showed that the phage was heat sensitive. Accordingly, a slow denaturation of its protein cover at a velocity depending on the temperature may occur. Heat resistance of the phage is increased by protective colloids and membrane-stabilizing substances such as human serum, magnesium and sucrose. When for some reason filtration cannot be done, the propagating bacteria can be removed from the phage lysate by heating and centrifuging in the presence of the preserving solution.

Repeated phage typing of the strains indicated that the lysis pattern was reproducible. Slight instability was observed only with less definite lytic reactions.

Multiple colony examination and temperate phage experiments indicated that one of our untypable strains was a mixed culture. It contained a lysogenic, an untypable and a non-lysogenic variant. This finding indicates the type determining role of temperate phages [2].

The combined application of the Roumanian and our phage sets has been found suitable for studies of the role of enterococci in food poisoning. The studies of GROMAN [3] and ZABRISKIE [6] on diphtheria and scarlet fever have opened a new aspect for investigations into the pathogenicity of enterococci: the transfer of the genetic factor of pathogenicity may be associated with temperate phages.

REFERENCES

1. ASHESHOV, E. H., JEVONS, P. M.: J. gen. Microbiol. **31**, 97 (1963).
2. BROCK, D. T.: J. Bact. **88**, 165 (1964).
3. GROMAN, N. B.: J. Bact. **66**, 184 (1953).
4. HOCH, V., HÉRMÁN, G.: Acta microbiol. Acad. Sci. hung. **18**, 95 (1971).
5. MILCH, H., LÁSZLÓ, V.: Acta microbiol. Acad. Sci. hung. **14**, 287 (1967).
6. ZABRISKIE, J. B.: Fed. Proc. **21**, 457 (1962).

Address of the authors:

GÁBOR HÉRMÁN
National Institute of Public Health,
Gyáli út 2–6, Budapest IX, Hungary
VIOLA HOCH
National Institute of Nutrition,
Gyáli út 3, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 105–108, 1971

ATTEMPTS TO DETECT THE PRESENCE OF TEICHOIC ACID IN BACILLUS ANTHRACIS

By

J. MOLNÁR and B. PRÁGAI

Institute of Microbiology (Director: G. IVÁNOVICS), University Medical School, Szeged

(Received March 22, 1971)

Summary. Teichoic acid was absent from the cell wall of two strains and an adenine deficient mutant of *Bacillus anthracis.* Other authors failed to detect this compound in *Bacillus cereus.* The absence of polyphosphate from the cell wall indicates a further phylogenetic connection between the two related species.

Auxotrophic mutants of *Bacillus anthracis* requiring exogenic adenine were shown to be apathogenic for the mouse [1]. These mutants were deficient in adenylosuccinate synthetase or in adenylosuccinate lyase. These specific adenine-requiring mutants failed to multiply in mice even when the animals were treated with adenine after infection [2]. Studying this problem was made difficult by the fact that adenine is rapidly metabolized or excreted [3, 4] and is toxic in larger amounts [5, 6]. Although by giving high doses the required blood adenine level could be attained, adenine dependent bacteria injected intraperitoneally failed to invade the blood stream and to kill the animals. In the peritoneal exudate neither adenine nor guanine were detectable. When guanine deficient mutant cells were injected intraperitoneally, the bacteria multiplied gradually and then suddenly entered the circulation and killed the animals [2].

These observations indicated that the avirulence of adenine dependent mutants is not merely due to the absence of exogenic adenine but may be associated with an alteration in the cell wall structure of the mutant. This assumption seemed to be supported by the fact that, in contrast to wild-type strains and other auxotrophs, these mutants showed a change in alkaline phosphatase regulation [7]. Data for the cell wall structure of *B. anthracis* [8] are in agreement with the old observation that in autolysates of this organism there is a mucopeptide containing acetylglucosamine and galactose at equimolar amounts [9]. No data were available as to whether the cell wall of this organism contained teichoic acid, a substance usually present in other Gram-positive bacteria. It was supposed that this component played a part in the loss of virulence in adenine dependent mutants. To elucidate this problem we have attempted to detect teichoic acid in non-capsulogenic wild-type *B. anthracis* strains and in their auxotrophs.

Materials and methods

Bacterial strains. B. anthracis non-capsulogenic strain Vollum (VC^-), its adenine auxotroph ($23C^-ade^-$II-3) and the prototrophic revertant of the latter ($23C^-ade^+$) described by IVÁNOVICS *et al.* [2] were used. *B. anthracis* Sterne (CN 35—18) and *B. subtilis* 168 were also studied.

Medium. Yeast extract peptone medium [10] was supplemented with 0.01 *M* phosphate. The medium was hardened with 1.5% agar and distributed into Roux flasks.

Cultures. Spore suspensions were inoculated on agar slants and incubated overnight. The cultures were suspended in 25 ml saline and 4 ml aliquots were used to inoculate each Roux flask. Incubation was done at 37°C for 16 hours. The suspension was washed twice in 20 ml cold saline and filtered through 4 layers of gauze. By centrifugation at 3500 r.p.m. for 40 minutes the bacteria were washed 3 times in 400 ml portions of cold saline.

Cell wall preparation in Mickle disintegrator. One g washed bacteria was suspended in 6 ml cold distilled water and after the addition of 3 ml Ballotini glass beads the suspension was treated for 4×10 minutes in the disintegrator. At intervals between each treatment the bacteria were refrigerated for 10 minutes in ice and disintegration of the cells was examined under the phase microscope. By the end of the treatment less than 1% of the cells had remained intact. Glass beads were removed by filtration through G—3 filter. The cell wall suspension was heated at 70°C for 20 minutes, then 0.5% $NaHCO_3$ and 0.1% trypsin were added. After incubation at 37°C for 60—90 minutes the suspension was ultracentrifuged at 10 000 *g* for 30 minutes, washed twice in distilled water and dried in the exsiccator to constant weight.

Cell wall extraction. Two methods were employed: (a) 100 mg cell wall were suspended in 5 ml 10% trichloroacetic acid and refrigerated for 20 hours [11]. (b) 100 mg cell wall were suspended in 8 ml 0.5 *M* NaOH and left to stand at room temperature for 4 hours [12]. After centrifugation the supernatants were neutralized and dialysed against cold distilled water for 48 hours. Finally, the dialysed material was freeze-dried and the dry weight of the extract was determined.

Cell wall hydrolysis. To 50 mg cell wall preparation 3 ml 2 *N* HCl were added. The mixture was sealed in ampoules and heated at 100°C for 16 hours.

Analytical methods. Phosphorus content of the extracts was determined as described by CHEN and TORIBARA [13]. For showing ribitol and glycerol the specimens were hydrolyzed in 2 *N* HCl at 100°C for 16 hours and chromatographed on Kieselgur G thin layer by using n-butanol : acetone : pH 5.0 phosphate buffer (50 : 40 : 10) as solvent [14]; the hR_f values were 90 for glycerol and 77 for ribitol. The spots were detected with lead tetraacetate [15]. Quantitative determination of ribitol was performed with the periodate oxidation method [16]. Galactose and glucose were detected on Kieselgur G plate in the above solvent using the corresponding monosaccharides as controls. The spots were detected with aniline phthalate [17]. Quantitative determination of the two sugars was performed by eluting the corresponding spots [18]. Hexosamine was determined by the method of BOAS [19].

Agar gel immunoprecipitation. After neutralization the alkaline extract was tested against *B. anthracis* antipolysaccharide rabbit serum kindly supplied by Professor G. IVÁNOVICS. The plates were incubated at room temperature for 5 days.

Results

With cold trichloroacetic acid treatment from the cell wall of *B. anthracis* 1%, from the cell wall of *B. subtilis* 30% soluble material was obtained. Subsequently alkaline extraction was attempted, which yielded 5% soluble fraction for *B. anthracis* and 35% for *B. subtilis*. Alkaline extracts of *B. anthracis* cell wall failed to react with the polysaccharide antiserum. The amount of soluble fraction obtained for *B. subtilis* is in agreement with data in the literature [11], in that about 30% of the cell wall of this organism is extractible and that the extracted material is identical with teichoic acid. The amount of material extracted from *B. anthracis* was very small, indicating that the bacteria do not contain teichoic acid. By hydrochloric acid hydrolysis and thin

layer chromatography of alkaline extracts we have attempted to show ribitol and glycerol. These substances were present in *B. subtilis* but were absent from *B. anthracis* cell wall extracts.

In view of the failure of the alkaline extraction, the cell wall preparations were subjected to direct hydrochloric acid hydrolysis. The amount of extractible fractions obtained by different methods is shown in Table I.

Table I

Percentage of soluble fraction extracted with different methods from the cell wall of B. anthracis and B. subtilis

	Cold 10% TCA	0.5 *M* NaOH at room temp.	Hot 2 *N* HCl 16 hours*
B. anthracis VC⁻	1.2	5.5	88.0
B. anthracis 23C⁻ade⁻II-3	0.8	5.45	70.8
B. anthracis 23C⁻ade⁺	0.88	5.9	87.0
B. anthracis Sterne CN 35—18	1.5	5.3	83.0
B. subtilis 168 wild-type	30.0	35.0	89.7

* After hydrolysis in sealed ampoule the material was centrifuged and dried *in vacuo* over NaOH. The supernatant and deposit were analysed separately, then the sum of the two determinations was used for expressing the percentage of extractible fraction

Table II

*Products of direct hydrochloric acid hydrolysis of B. anthracis cell wall**

	Galactose	Glucosamine	Organic P	Ribitol	Glycerol
B. anthracis VC⁻	27.4	28.0	0.27	0	0
B. anthracis 23C⁻ade⁻II-3	31.0	34.0	0.29	0	0
B. anthracis 23C⁻ade⁺	28.0	28.9	0.28	0	0
B. anthracis Sterne CN 35—18	24.0	24.1	0.48	0	0

* The figures represent the average of 3 different analyses in percentage

Table II shows that direct hydrochloric acid hydrolysis yielded galactose glucosamine and organic phosphate but not ribitol or glycerol. The two monosaccharides were present in equimolar amounts corresponding to findings for mucopeptide isolated from *B. anthracis* autolysates.

Discussion

The wide-spread occurrence of teichoic acid in Gram-positive bacteria is well known. In a monograph, SALTON summarized the results for a large number of bacteria [20]. As to *B. anthracis*, we found no data in this respect. The absence of teichoic acid from this pathogenic agent was somewhat surprising, although the finding is not unparalleled, since *B. cereus* strain NCIB 2600 has been shown to lack this compound and to contain only intracellular glycerol teichoic acid [21].

B. cereus is phylogenetically closely related to *B. anthracis* and it has even been supposed that the latter is a pathogenic variant of the former and not a separate species [22]. As the criterion of, and the border between, species are debated problems, this conception has been rejected [23]. Nevertheless, the absence of teichoic acid from *B. anthracis* and from *B. cereus* is another proof for the close relationship of the two bacteria.

REFERENCES

1. IVÁNOVICS, G., MARJAI, E.: Zbl. Bakt. I. Abt. Orig. **193,** 363 (1964).
2. IVÁNOVICS, G., MARJAI, E., DOBOZY, A.: J. gen. Microbiol. **53,** 147 (1968).
3. BENNET, E. L.: Biochim. biophys. Acta (Amst.) **11,** 487 (1953).
4. SCHWARZ, M. R., RIEKE, W. O.: Lab. Invest. **12,** 92 (1963).
5. RASKA, S. B.: J. biol. Chem. **165,** 743 (1946).
6. BROWN, G. B., ROLL, P. M., PLENTL, A. A.: Fed. Proc. **6,** 517 (1947).
7. DOBOZY, A., MARJAI, E., IVÁNOVICS, G.: Acta microbiol. Acad. Sci. hung. **15,** 261 (1968).
8. LANTOS, J., IVÁNOVICS, G.: Acta microbiol. Acad. Sci. hung. **8,** 379 (1961).
9. IVÁNOVICS, G.: Z. Immun.-Forsch. **97,** 402 (1940).
10. CSISZÁR, K., IVÁNOVICS, G.: Acta microbiol. Acad. Sci. hung. **12,** 73 (1965).
11. ARMSTRONG, J. J., BADDILEY, J., BUCHANAN, J. G.: Biochem. J. **76,** 610 (1960).
12. ARCHIBALD, A. R., COAPES, H. E., STAFFORD, G. H.: Biochem. J. **113,** 899 (1969).
13. CHEN, P. S. JR., TORIBARA, T. Y., WARNER, H.: Anal. Chem. **28,** 1756 (1956).
14. WALDI, D.: J. Chromatogr. **18,** 417 (1965).
15. WRIGHT, J.: Chem. Ind. (Lond.) **11,** 1125 (1963).
16. HANAHAN, D. J., OLLEY, J. N.: J. biol. Chem. **231,** 813 (1958).
17. PESCHKE, W.: J. Chromatogr. **20,** 572 (1965).
18. PARK, J. T., JOHNSON, M. J.: J. biol. Chem. **181,** 149 (1949).
19. BOAS, N. F.: J. biol. Chem. **204,** 553 (1953).
20. SALTON, M. R. J.: The Bacterial Cell Wall. Elsevier Publ. Co., Amsterdam 1964.
21. BADDILEY, J.: *In* Heidelberger, M., Plescia, O. J., Day, R. A. (eds) Immunochemical Approaches to Problems in Microbiology. Rutgers Univ. Press, New Brunswick, N. J. 1961, p. 91.
22. SMITH, N. R., GORDON, R. E., CLARK, F. E.: Aerobic Sporeforming Bacteria. Agriculture Monograph No. 16, U.S. Dept. Agric., Washington 1952.
23. IVÁNOVICS, G., FÖLDES, J.: Acta microbiol. Acad. Sci. hung. **5,** 89 (1958).

Address of the authors:

JÓZSEF MOLNÁR, BÉLA PRÁGAI
Institute of Microbiology, University Medical School
Dóm tér 10, Szeged, Hungary

Acta microbiol. Acad. Sci. hung. 18, 109—112, 1971

EFFECT OF INTERFERON TREATMENT ON THE TUMOUR-SPECIFIC ANTIBODY RESPONSE OF BALB/c MICE INFECTED WITH RAUSCHER VIRUS

By

F. D. TÓTH, L. VÁCZI and CLARA BERENCSI

Institute of Microbiology (Director: L. VÁCZI), *University Medical School, Debrecen*

(Received April 16, 1971)

Summary. Prolonged interferon treatment slows down the splenomegaly of BALB/c mice infected with Rauscher virus. Mice treated with interferon elicit an enhanced tumour-specific antibody response which precedes the suppression of the progress of leukaemia. The protective effect of interferon may be attributed to the prevention of the infection and of the malignant transformation of immunocompetent precursor cells.

The progress of viral mouse leukaemias can be slowed down by prolonged administration of interferon [1—3]. The protective effect of interferon is attributed to an inhibition of the virus multiplication [2] and of cell division [4]; in certain cases even a stimulation of phagocytic activity cannot be excluded [5].

Furthermore, leukaemia viruses possess a well-defined immunosuppressive activity [6] which is of decisive importance in preventing the protective reactions of the organism.

In the present study it has been studied whether the immunological defense mechanism, too, was enhanced by interferon. Besides, efforts were made to obtain data on the mechanism of the immunosuppressive effect of viruses.

Materials and methods

Viruses. Rauscher virus was passaged in BALB/c mice. Mice were inoculated intraperitoneally with 0.2 ml of the supernatant of 20% spleen suspension. The spleen suspension was stored at —70°C until used.

Vesicular stomatitis virus (VSV) was propagated in HeLa cell cultures. The nutrient medium containing virus was stored at —70°C.

Strain H of the Newcastle disease virus (NDV) was propagated in the allantois sac of the embryonated hen's egg, and the allantois fluids containing virus were stored at +4°C.

Mice. Eight-week-old inbred male BALB/c mice were used for both producing interferon and examining tumour-specific antibody production.

Each serum sample used for fluorescent antibody test was a pool collected from seven mice.

Fluorescent antibody test. The indirect test was applied in living cells. Our technique [7] was based on the methods described by MÖLLER [8] and KLEIN and KLEIN [9].

Production of interferon. Mice were injected with 0.2 ml NDV suspension, *i.e.*, $2 \cdot 10^8$ EID_{50} of virus, into the tail vein. The animals were exsanguinated 5—6 hours thereafter and the serum thus obtained was dialyzed against glycine-HCl buffer of pH 2 for 96 hours. The pH was then re-adjusted to 7.4 by dialyzing against PBS.

Interferon titration. L cell monolayers and, as challenge virus, 50 CPD_{50} of VSV were used. The highest dilution that prevented 50% of the cells from being infected was considered the virus titre.

Scheme of interferon treatment. Treatment was started in the 24th hour following infection with Rauscher virus. A daily dose of 0.3 ml, *i.e.* 900 units, was administered intraperitoneally to each mouse over a period of 14 days. A control group received normal serum pretreated with glycine-HCl buffer and PBS, another control group was not pretreated.

Results

The increase in spleen weight in interferon-treated and control animals is shown in Fig. 1 as a function of time.

Since there was no appreciable difference in spleen weight between the animals that had received normal serum and the untreated ones, the control data were illustrated by a single, combined curve.

From day 12 to day 20 after infection the curve for the animals treated with interferon is considerably less steep than the control curve, but after the 20th day the two curves run parallel.

The survival period of the interferon-treated animals was prolonged, the mean being 38 days in contrast to 30 days for the controls.

Fig. 2 illustrates the tumour-specific antibody response.

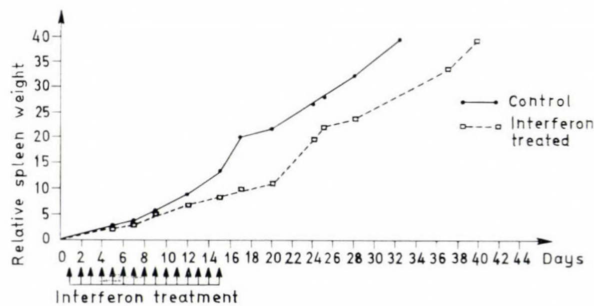


Fig. 1. Influence of prolonged interferon treatment on the progress of Rauscher leukaemia in BALB/c mice

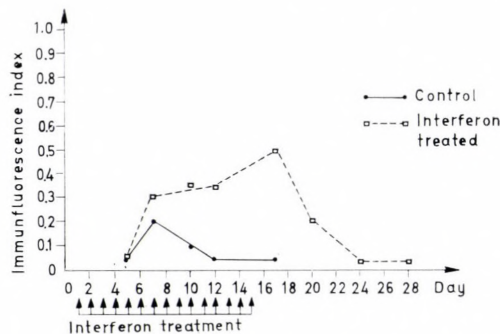


Fig. 2. Influence of prolonged interferon treatment on the tumour-specific immune response of BALB/c mice infected with Rauscher leukaemia virus

The sera of the mice that had received Rauscher virus alone or Rauscher virus and normal serum, contained no antibody except around the 7th day when the antibody level was below the level of significance (fluorescence index = 0.3). In the sera of the mice treated with interferon, on the other hand, the titre of the tumour-specific antibodies continuously increased after the 7th day until the 17th day when it reached a peak of 0.5. Subsequently, the titre declined suddenly.

Discussion

In the present study, interferon treatment was followed first by an enhanced antibody production and then by a suppression of tumour growth. The latter effect may be attributed first of all to the enhanced antibody production, for leukaemia and lymphoma cells are very sensitive to the lytic effect of cytotoxic antibodies [10]. This is well demonstrated by the fact that, although interferon treatment had been discontinued on the 15th day, the rate of spleen growth remained at a reduced level until the antibodies had disappeared. Subsequently, the spleen began to grow rapidly.

Svet-Moldavsky's [11] research group has shown that the cellular immune response to sarcoma cells is increased by interferon. Thus, among the components of the protective effect of interferon, the stimulation of tumour-specific immunity has an important role.

Since in the present study interferon treatment was started as late as 24 hours following virus infection, it could not influence the first cycle of virus multiplication. It cannot be supposed that interferon would be able to inhibit the division of tumour cells to a significant degree, for the rate of splenomegaly was the same in the interferon-treated mice as in the control animals up to the 9th day after virus inoculation.

Our data seem to be consistent with the suggestion of Siegel and Morton [12], who attribute the immunosuppressive effect of the leukaemia viruses to the identity with the immunoprecursors of the stem cells which are transformed by the viruses. According to Bennett and Steeves [13], on the other hand, the target cells are not identical with the immunocompetent precursors. However, in the immunoblasts of the spleen of mice infected with Rauscher virus, C-type particles have been demonstrated [14]. Thus, the immunosuppression nevertheless seems to be a consequence of the infection by leukaemia viruses. Our results seem to support this view.

REFERENCES

1. Gresser, I., Coppey, J., Falcoff, E., Fontaine, D.: Proc. Soc. exp. Biol. (N. Y.) **124,** 84, (1967).
2. Gresser, I., Berman, L., De-Thé, G., Brouty-Boyé, D., Coppey, J., Falcoff, E.: J. nat. Cancer Inst. **41,** 505 (1968).
3. Gresser, J. Coppey, J., Bourali, Ch.: J. nat. Cancer Inst. **43,** 1083 (1969).
4. Fontaine, D., Brouty-Boyé, D., Gresser, I., Macieira-Coelho, A., Rosenfeld, C., Thomas, M. T.: C. R. Acad. Sci (Paris) **269,** 406 (1969).
5. Gresser, I., Bourali, Ch.: J. nat. Cancer Inst. **45,** 365 (1970).
6. McCoy, V. L., Fefer, A., Glynn, I. P.: Cancer Res. **27,** 1743 (1967).
7. D. Tóth, F., Váczi, L., Berencsi, Cl.: Acta microbiol. Acad. Sci. hung. **18,** 23 (1971).
8. Möller, G.: J. exp. Med. **114,** 415 (1961).
9. Klein, E., Klein, G.: J. nat. Cancer Inst. **32,** 547 (1964).
10. Klein, E.: Europ. J. Cancer **6,** 15, (1970).
11. Chernyakhovskaya, I. Ju., Slavina, E. G., Svet-Moldavsky, G. J.: Nature (Lond.), **228,** 71 (1970).
12. Siegel, B. V., Morton, J. I.: Experientia (Basel) **25,** 186 (1969).
13. Bennett, M., Steeves, R. A.: J. nat. Cancer Inst. **44,** 1107 (1970).
14. Hanna, M. G., Szakal, A. K., Tyndall, R. L.: Cancer Res. **30,** 1748 (1970).

Address of the authors:

Ferenc D. Tóth, Lajos Váczi, Klára Berencsi
Institute of Microbiology, University Medical School,
Debrecen 12, Hungary

Acta microbiol. Acad. Sci. hung. 18, 113—118, 1971

SEROLOGICAL DIAGNOSIS OF YERSINIA ENTEROCOLITICA

By

J. SZITA, MARGARET KÁLI and B. RÉDEY

National Institute of Public Health (Director: T. BAKÁCS), *Budapest and Public Health Station (Director:* E. BEREND), *Veszprém*

(Received May 18, 1971)

Summary. (i) Studies on the preparation of *Yersinia enterocolitica* typing sera on the basis of WINBLAD's schema are described.

(ii) Serological typing of 41 strains indicated that in Hungary group 3 is the most frequent one. Group 3 was isolated from the faeces of 15, group 9 from the faeces of 3 persons.

(iii) Widal-type agglutination performed with sera of 1500 healthy individuals and of 249 patients showed that, in agreement with data in the literature, titres of 1 : 40 should be regarded as doubtful, titres of 1 : 80 or over as positive reactions.

(iv) Considering the serological relationship between *Y. enterocolitica* O9 and *Brucella abortus*, patient sera agglutinating O9 antigen should be tested against *Brucella* antigen. If the results are positive with both antigens, the serum should be re-examined after absorption and efforts should be made to isolate the causative agent.

Systematic studies on the incidence of *Yersinia enterocolitica* infection in Hungary have been carried out since 1969. Serological typing of the agent and Widal-type agglutination to detect the immune response of patients have been applied. The first isolation of *Y. enterocolitica* in this country has been reported recently [1, 2].

In this paper we give an account of observations on the preparation of *Y. enterocolitica* agglutinating sera and studies on the proper evaluation of immune response in infected patients.

Materials and methods

Preparation of antigens. WINBLAD's method was somewhat modified as follows. Type strains 348, 338, Wbdx, P76, Vache, P219, P413, 311 and 5385 were obtained from the Institute of Clinical Bacteriology, University of Lund, Malmö, Sweden. The strains were streaked on blood agar plates, incubated at room temperature for 48 hours and S colonies were transferred into broth. The tubes were again incubated at room temperature for 48 hours, then used for seeding Roux flasks containing soft chocolate agar. After incubation at room temperature for 48 hours the bacteria were harvested and centrifuged at 3000 r.p.m. for 30 minutes, then autoclaved at 120°C for 1 hour 15 minutes. Density was adjusted so as to correspond to a standard giving 70% transmittance at 1 cm light path in the Beckman DU spectrophotometer set at 0.03 mm slit and 530 mμ wavelength.

Immunization of rabbits was performed with the above-described antigen by injecting 6 graded intravenous doses ranging from 0.5 to 5 ml.

Absorption of sera was carried out on the basis of WINBLAD's schema. Usually 1 ml serum was absorbed with the same volume of antigen or mixture of antigens (Table I). The absorbed sera were checked with slide agglutination. Working dilution was adjusted so that the serum gave a definite, prompt reaction with the homologous strain, but failed to agglutinate heterologous cultures.

Detection of agglutinins in human sera. A total of 1500 serum specimens originating partly from Wassermann screening tests in the Serology Department of our institute, partly from donors of the National Blood Service were examined. From patients with suspected *Y. enterocolitica* infection 249 serum specimens were examined. All sera were inactivated at 56°C for 30 minutes and tested for agglutinins against O groups 3 and 9 which are the most frequently involved in human infection.

To 0.5 ml serum dilution 0.05 ml antigen prepared and adjusted as described under "Preparation of antigens" was added with Takátsy's pipette. For O3 antigen serial dilutions of the serum specimens were made in the range 1 : 10—1 : 160, for O9 antigen only two dilutions (1 : 10 and 1 : 20) were prepared. When a positive reaction was obtained, further dilutions were made to determine the end titre. The tubes were incubated overnight at 52°C as recommended by Winblad [4]. Parallel experiments with 100 specimens were carried out at 37°C; the results were the same at both incubation temperatures. In view of the serological relationship between *Y. enterocolitica* O9 and *B. abortus* [5], all sera reacting with the former were tested with *Brucella* suspension.

Table I

Slide agglutination reactions of Y. enterocolitica typing sera prepared on the basis of Winblad's schema

Sera	Group	1	2	3	4	5	6	7	8	9	Reciprocals of homologous titres	
	O antigens	1, 2, 3	2, 3	3	4	5	6	7, 8	8	9	before absorption	after absorption
	Type strains	P348	337	XWbd	P76	Vache	P219	P413	310	5385		
Anti-1 abs. by P338		+	—	—	—	—	—	—	—	—	1280	640
Anti-2, 3 abs. by XWbd		+	+	—	—	—	—	—	—	—	1280	640
Anti-1, 2, 3 abs. by P76		+	+	+	—	—	—	—	—	—	2560	1280
Anti-4 abs. by P348		—	—	—	+	—	—	—	—	—	1280	640
Anti-5 abs. by XWbd + P76 + P348		—	—	—	—	+	—	—	—	—	1280	640
Anti-6 abs. by XWbd + P348		—	—	—	—	—	+	—	—	—	1280	80
Anti-7, 8 abs. by P219 + 310		—	—	—	—	—	—	+	—	—	1280	640
Anti-8 abs. by XWbd		—	—	—	—	—	—	—	+	—	1280	640
Anti-9 abs. by XWbd + Vache		—	—	—	—	—	—	—	—	+	2560	1280

Results

Serological identification of Y. enterocolitica. Table I shows slide agglutination reactions obtained with our absorbed sera. The test strains represented the 9 different O groups described by Winblad. O groups 1, 2 and 7 contain complex antigens denoted by symbols 1, 2, 3 2, 3 and 7, 8, respectively. In order to avoid cross reactions, sera for other O groups were also absorbed. The original tube agglutination titre of the serum to the homologous strain decreased after absorption (right hand column in Table I). The absorbed sera gave slide reactions of adequate intensity at working dilutions 1 : 5—1 : 40. Our typing

sera were compared with those obtained from the *Yersinia enterocolitica* Reference Laboratory in Malmö. The results were identical.

In the course of these studies 41 strains of *Y. enterocolitica* were isolated in, or sent for identification to, our Department. The strains were isolated from 18 different patients. Thirty-four cultures belonged to O3, 7 cultures to O9 group. No other serogroups have so far been isolated in Hungary. Ten of the strains were sent to the *Yersinia enterocolitica* Reference Laboratory in Malmö, where our results of serotyping were confirmed.

Detection of agglutinins in sera of healthy individuals and of patients. In these experiments heated antigens for O groups 3 and 9 were used. Two-hundred sera were tested parallel with formalinized antigens, which proved to be more stable than heated suspensions.

As seen in Table II out of the 1500 specimens from healthy persons, 1348 and 1445 sera failed to react with antigens O3 and O9, respectively. With the former antigen 152 sera, with the latter 55 sera gave 1 : 10—1 : 160 titre agglutinations. The mean titre calculated by the use of the given formula was similar for both antigens (1 : 15.4 and 1 : 15.3).

From patients with suspected *Y. enterocolitica* infection 249 sera were received. With O3 antigen 66 sera reacted at titres 1 : 10—1 : 2560, with O9 antigen 21 sera showed agglutination in the range of 1 : 10—1 : 1280. The mean titre in patients was higher than in healthy persons: considering only the positive sera, for O3 antigen 1 : 88.7, for O9 antigen 1 : 45.6 values were calculated.

Table III shows titres in 10 patients suffering from clinically typical *Y. enterocolitica* infection and excreting the organism in their faeces. From the 4 additional patients in Table III the causative agent was not isolated, but positive serum titres, epidemiological data and clinical symptoms supported the history of *Y. enterocolitica* infection.

In case 1 an increased O9 titre was demonstrated in the serum and *Y. enterocolitica* of the same serogroup was isolated from the faeces. In cases 2, 8 and 9, despite the positive faecal culture and clinical symptoms, antibodies failed to appear. Whenever it was possible, we examined two different serum samples taken from the same patient at intervals. As seen in Table III, in cases 3, 5, 6, 7 and 10 the increase in titre supported the *Y. enterocolitica* aetiology of the diarrhoea.

In 2 patients with negative faecal culture (cases 11 and 14) increased or high titres were detected. At the time of examination patient 11 still showed clinical symptoms, while patient 14 had already recovered. In the family of patients 12 and 13 there were contacts with subacute diarrhoea the *Y. enterocolitica* aetiology of which was confirmed by faecal culture.

From Table III we omitted some patients yielding a high antibody titre and a positive culture because no clinical data were available. In the 14 cases

Table II

Distribution of control and patient sera according to

Sera	Antigen O3 (strain Wbdx)									
	<10	10	20	40	80	160	320	640	1280	2560
Control sera (healthy persons) 1500 samples	1348	78	57	15	1	1	—	—	—	—
Patient sera, 249 samples	183	8	14	12	5	6	6	6	7	2

Mean titre $= \sqrt[N]{d_1^{n_1} \cdot d_2^{n_2} \ldots d_n^{n_n}}$

$d_1, d_2 \ldots d_n$ = reciprocals of serum dilutions

demonstrated, faecal examination in view of the enteritis usually preceded the search for antibodies in the serum. In some of the cases the serum specimens were examined first, then, in view of their positive reaction, faecal samples were asked for. The present findings indicate that serum antibodies persisted at least for 5 weeks.

Table III

Antibody titre to Y. enterocolitica O3 and O9 antigens in sera from patients with and without positive faecal culture

Case	Age, years	Interval between two samplings, days	Antibody titres O3	Antibody titres O9	Faecal culture and group of strain	Symptoms
1	4		—	1 : 160	+ O9	Acute diarrhoea
2	2		—	—	+ O9	Spastic bronchitis + 2 putrid faeces
3	38	10	1 : 1280 1 : 20	—	+ O3	Acute diarrhoea
4	4		1 : 160	—	+ O3	Enteritis
5	10	37	— 1 : 80	—	+ O3	Prolonged diarrhoea
6	6	37	— 1 : 20	—	+ O3	Acute diarrhoea
7	5	37	1 : 10 1 : 20	—	+ O3	Acute diarrhoea
8	2		—	—	+ O3	Acute respiratory infection + 5—7 putrid faeces
9	2		—	—	+ O3	Prolonged diarrhoea
10	8 months	10	1 : 1280 1 : 160	—	+ O3	Enterocolitis
11	12	33	1 : 80 1 : 160	—	—	Prolonged diarrhoea
12	43	27	1 : 1280 1 : 160	—	—	Environment
13	42	33	1 : 40 1 : 40	—	—	Environment
14	29		1 : 1280	—	—	Enterocolitis

antibody titre against Y. enterocolitica O3 and O9 antigens

Antigen O9 (strain 5385)										Mean titre	
<10	10	20	40	80	160	320	640	1280	2560	O3	O9
1445	32	15	5	3	—	—	—	—	—	15.4	15.3
228	3	7	5	—	3	2	—	1	—	88.7	45.6

$n_1, n_2 \ldots n_n$ = number of sera giving the corresponding titre
N = total number of sera

Discussion

WINBLAD's antigenic schema is based on the O antigens of *Y. enterocolitica.** The specific O antigens of the species have been demonstrated neither in *Enterobacteriaceae* nor in *Y. pseudotuberculosis* and *Y. pestis.* WINBLAD [3] showed that immunization with heated *Y. enterocolitica* strains produced antibodies reacting with *Salmonella* groups A, B, C and E and with *E. coli* and *Proteus* strains. Living cultures failed to induce such antibodies. Our cross-agglutination experiments indicated no antigenic relationship between *Y. enterocolitica* and *Y. pseudotuberculosis* [9]. AHVONEN *et al.* [5] described a strong cross-reaction between *Y. enterocolitica* O9 and *B. abortus.* Our studies confirmed this relationship: after absorption by *B. abortus* a rabbit O9 serum contained only low titre *Y. enterocolitica* agglutinins (1 : 80).

Similar findings were demonstrated for patient sera reacting with *Y. enterocolitica* O9. Accordingly, every human serum agglutinating *Y. enterocolitica* O9 should be tested with *Brucella* antigen and efforts should be made to isolate the causative agent. In contrast to some other countries, for example to Finland [6], where brucellosis has been eradicated, in Hungary we still have to consider the incidence of human *Brucella* infections.

According to NILÉHN [7] in human cases *Y. enterocolitica* O3 strains predominate. Our findings in Hungary were similar: O3 strains were cultured from the faeces of 15, while O9 strains from the faeces of only 3 persons. The higher incidence of O3 strains was indicated by the frequency of human sera containing antibodies to this group.

In testing human sera with O3 antigen, WINBLAD [4] regards 1 : 40 titres as doubtful, 1 : 80 or higher titres as positive reactions. RUSU *et al.* [8] similarly consider 1 : 40 as a border value. Our findings confirmed these data. Considering only positive sera, in healthy individuals 1 : 15.4—1 : 15.3, in

* While this paper was in preparation a report by WAUTERS *et al.* (WAUTERS, G., LE MINOR, L., CHALM, M. M.: Ann. Inst. Pasteur *120,* 631 (1971)), who supplemented the Winblad schema and distinguished 17 different O groups, attracted our attention.

patients 1 : 88.7—1 : 45.6 mean titres were obtained for the two most common serogroups.

We were unable to collect a sufficient number of data as to the appearance, maximum titre and persistence of antibodies in human infections. From the literature [3] it seems that the antibodies may persist even for 9 months and their titre rises 6—12 days after the onset of symptoms. For a proper serological diagnosis it is, therefore, recommended to examine paired sera (one at the time of onset and one 6—10 days later).

The present study may be regarded as the first successful step in detecting a pathogenic agent so far unknown in Hungary.

Acknowledgement. The authors are indebted to Professor S. Winblad and Dr. B. Niléhn (Institute of Clinical Bacteriology, University of Lund, Malmö, Sweden) for advice in the production of absorbed sera and for checking typing results.

REFERENCES

1. Szita, J.: Egészségtudomány **15**, 92 (1971).
2. Rédey, B., Szita, J., Vörös, S., Gacs, M., Oláh, B., Káli, M.: Egészségtudomány, Suppl. **15**, 94 (1971).
3. Winblad, S.: Int. Symposium on Pseudotuberculosis, Paris, 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 337 (1968).
4. Windblad, S., Niléhn, B., Sternby, N. H.: Brit. med. J. **2**, 1362 (1966).
5. Ahvonen, P., Jansson, E., Aho, K.: Acta path. microbiol. scand. **75**, 291 (1969).
6. Huhtala, E.: Bull. Off. Int. Epid. **60**, 447 (1963).
7. Niléhn, B.: Acta path. microbiol. scand. Suppl. **206**, (1969).
8. Rusu, V., Hortnog, E., Andronescu, C., Dumitriu, M.: Int. Symposium on Pseudotuberculosis, Paris 1967. Symp. Series immunobiol. Standard. Karger, Basel—New York **9**, 99 (1968).
9. Szita, J., Svidró, A.: Acta microbiol. Acad. Sci. hung. **18**, 87 (1971).

Address of the authors:

József Szita, Margit Káli
National Institute of Public Health,
Gyáli út 2—6, Budapest IX, Hungary
Barnabás Rédey
Public Health Station,
József Attila u. 30, Veszprém, Hungary

Acta microbiol. Acad. Sci. hung. 18, 119–131, 1971

TWENTY YEARS OBSERVATION ON THE STABILITY OF SALMONELLA TYPHI PHAGE TYPES

By

G. Hérmán and Hedda Milch

National Institute of Public Health (Director: T. Bakács), Budapest

(Received May 19, 1971)

Summary. A total of 3776 chronic and transient carriers of *Salmonella typhi* has been examined. The order of incidence of phage types in Hungary is described. Loss of Vi antigen was observed in most phage types; Vi negative strains appeared more frequently in carriers excreting phage types A, D_1 and E_{1a} than in carriers of types C_1 and F_1. Phage types other than the original type or antigenic alteration influencing type determination appeared in 15.9% of the carriers. Excretion of different types by the same person was assumed to be due mainly to infection with more than one type and less frequently to change in phage type.

In the last 30 years, phage typing has been an important method of epidemiological studies of typhoid fever. The method introduced by Craigie and Yen [1, 2] has been systematically developed and based on well-defined genetic grounds [3–10]. Phage types determined according to the typing schema have usually proved stable and allowed the application of the method in epidemiological investigations [2, 4]. The stability of phage types is somewhat limited. Spontaneous alterations in phage type may occur under laboratory conditions [4] and many phage types can artificially be transformed into other — but not to any — phage type by lysogenization or "curing" [12]. Changes in phage type were observed during prolonged carriership [13]; phage type A was isolated from a carrier harbouring types T and O [11]. Helmer *et al.* isolated phage types F_1 and F_2 from the blood of a patient with typhoid fever [14]. The simultaneous appearance of different phage types was described by Boyd [15], Eörsi [16] and Rische and Rohne [17]. Borecká investigated *in vivo* changes and simultaneously appearing different *S. typhi* phage types [18].

The above data and our own observations have induced us to study the frequency of type alterations on a large material and to follow up changes in Vi antigen content and xylose reaction of the strains.

Materials and methods

Phage typing of *S. typhi* was carried out under internationally standardized conditions. Type phages were obtained from the WHO International Centre of Enteric Phage Typing, London. Vi antigen was detected with slide agglutination in the appropriate working dilution of anti-Vi serum prepared by the Department of Bacteriology of our institute. Xylose fermentation was examined in 1% peptone water containing 1% substrate; incubation lasted for 6 days at 37°C.

Chronic and transient carriers were grouped according to phage type of *S. typhi* cultured on the first examination. Typing of strains obtained on repeated examinations was also considered. Xylose fermentation was tested only for part of the strains; the percentage distribution of xylose positive and negative strains was determined for each phage type. Carriers excreting Vi negative, rough, minute or phage carrier strains on the first faecal culture were classified separately.

The incidence of carriers harbouring phage types different from their original type and the association between different types present in the same carrier were also studied. Carriers examined for more than one *S. typhi* colony in each faecal culture were analysed separately.

Results

The incidence of phage types in Hungary is presented in Table I. Type A was the most frequent, types E_{1a}, D_1, F_1, C_1, etc., were next in order. The data were collected from results for 3776 carriers yielding positive cultures on 1, 3, 5, 10 or more occasions. Although rough, phage carrier and Vi negative strains do not represent phage types; in view of their influence on the evaluation and applicability in epidemiological tracing, they were included in Table I. In 15.8% of carriers the first examination yielded cultures unclassifiable into phage types (untypable, degraded, Vi negative, phage carrier and rough strains).

Table II shows xylose fermentation by our strains. As shown by a long experience, the ability to ferment this sugar is a stable property. As to xylose fermentation by strains falling into different phage types, our results corresponded to other authors' findings. *S. typhi* strains supposed to have undergone a phage type alteration in carriers always retained their original xylose reaction.

Table III shows the incidence according to phage types and carriers of Vi negative, rough, phage carrier and minute strains in persons harbouring different phage types. In accordance with RISCHE and ROHNE [17], we showed that strains producing minute colonies occur most frequently in persons excreting phage type A. (The number of B_3 carriers was not sufficient for drawing conclusions.)

Phage carrier strains were frequently observed among cultures from Vi negative carriers. Table IV summarizes findings concerning type alteration within phage types and the appearance of new types. In carriers grouped according to the phage type of the first isolate, the appearance of a different phage type was regarded as an alteration. The criterion for a change in carriers with untypable (I—IV positive) cultures was the appearance of degraded strains. In carriers of rough strains the development of O antigen positive, in Vi negative carriers the appearance of Vi positive typable, degraded or untypable organisms was regarded as an alteration.

The incidence of phage type alteration in carriers excreting frequent types B_2 and A was 16.2 and 9.6%, respectively. Lower percentages were shown for C_1 (8.8%), D_1 (8.5%) and F_1 (6.8%) carriers. Among commoner phage types the lowest value (50%) of appearance of a new phage type was

Table I

Distribution of 3776 chronic and transient carriers examined over a 20 year period (1948—1968) according to phage type of S. typhi isolated on first examination

Phage type	Number of carriers yielding					Total	
	1	3	5	10	10	No.	per cent
	positive cultures					of carriers	
A	162	202	121	158	93	736	19.49
B_1	7	7	2	5	2	23	0.60
B_2	34	41	37	29	19	160	4.23
B_3	5	4	8	2	2	21	0.55
C_1	88	75	43	52	23	281	7.43
C_2	3	2	1	2	—	8	0.21
C_4	2	2	1	5	—	10	0.26
D_1	147	153	101	120	65	586	15.51
D_2	6	8	4	1	7	26	0.68
D_4	7	7	6	6	1	27	0.71
D_5	1	2	—	—	—	3	0.07
D_6	—	—	2	—	8	10	0.26
D_7	—	—	—	1	—	1	0.02
D_9	—	1	2	—	1	4	0.10
D_{10}	—	—	—	—	1	1	0.02
E_{1a}	179	180	109	151	99	718	19.01
E_{1b}	6	9	—	4	3	22	0.58
E_2	1	—	1	—	—	2	0.05
E_4	—	—	—	1	—	1	0.02
F_1	105	131	71	85	62	454	12.02
F_2	1	—	1	2	2	6	0.15
J_1	—	—	1	1	—	2	0.05
N	1	2	3	1	2	9	0.23
T	5	5	3	8	12	33	0.87
27	1	4	—	—	—	5	0.12
28	6	6	1	3	—	16	0.42
38	3	2	1	1	—	7	0.18
40	2	—	—	—	—	2	0.05
43	—	1	—	—	—	1	0.02
46	1	1	—	—	—	2	0.05
Nt	37	44	20	43	38	182	4.80
Degraded	62	76	37	35	18	228	6.03
Vi negative	44	31	30	26	25	156	4.13
Phage-carrier	3	3	3	2	4	15	0.39
Rough	1	4	4	7	2	18	0.45
Total	920	1003	613	751	489	3776	100.00

Table II

Distribution of carriers according to phage type and xylose reaction of S. typhi

Phage type	Xylose positive, per cent	Xylose negative, per cent	Xylose reaction unknown*
A	32.33	56.65	11.01
B_1	73.79	8.69	17.39
B_2	61.87	30.62	7.50
B_3	33.33	61.90	4.76
C_1	87.54	1.06	11.38
C_2	—	100.00	—
C_4	100.00	—	—
D_1	80.20	10.92	8.87
D_2	19.23	61.53	19.23
D_4	88.88	—	11.11
D_5	33.33	—	66.66
D_6	—	100.00	—
D_7	100.00	—	—
D_9	100.00	—	—
D_{10}	100.00	—	—
E_{1a}	88.16	0.27	11.55
E_{1b}	100.00	—	—
E_2	50.00	—	50.00
E_4	—	—	100.00
F_1	89.20	0.66	10.13
F_2	83.33	—	16.66
J_1	100.00	—	—
N	—	100.00	—
T	96.96	3.03	—
27	100.00	—	—
28	81.25	18.75	—
38	71.42	28.57	—
40	50.00	50.00	—
43	—	100.00	—
46	100.00	—	—
Nt	66.48	24.72	8.79
Degraded	43.43	46.49	10.08
Vi negative	71.15	23.07	5.76
Phage-carrier	60.00	26.66	13.33
Rough	61.11	27.77	11.11
Total	68.86	21.18	9.96

* Test not performed. The method was introduced in 1951.

Table III

Incidence of Vi negative, rough, minute and phage carrier strains in subjects harbouring different phage types of S. typhi

Phage type	Vi negative		Rough		Minute		Phage-carrier	
	No.	per cent	No.	per cent	No.	per cent	No.	per cent
A	80	10.8	8	1.0	35	4.7	20	2.7
B_1	1	43.4	—		—		—	
B_2	12	7.5	—		3	1.8	3	1.8
B_3	1	4.7	—		2	9.5	—	
C_1	14	4.9	1	0.3	—		2	0.7
C_2	—		—		—		—	
C_4	—		—		—		—	
D_1	54	9.2	8	1.3	2	0.3	13	2.2
D_2	1	3.8	—		—		1	3.8
D_4	1	37.0	—		—		—	
D_5	—		—		—		—	
D_6	3	30.0	1	10.0	—		1	10.0
D_7	—		—		—		—	
D_9	1	25.0	—		—		—	
D_{10}	—		—		—		—	
E_{1a}	61	8.4	3	0.4	7	0.9	24	3.4
E_{1b}	2	9.0	—		—		1	4.5
E_2	—		—		—		—	
E_4	—		—		—		—	
F_1	19	4.1	3	0.6	4	0.8	3	0.6
F_2	1	16.6	—		—		—	
J_1	—		—		—		1	50.0
N	—		—		—		—	
T	6	18.1	2	6.0	—		1	3.0
27	—		—		—		1	20.0
28	1	6.2	—		—		—	
38	—		—		—		—	
40	—		—		—		—	
43	—		—		—		—	
46	—		—		—		—	
Nt	20	10.9	2	1.0	1	0.5	4	2.1
Degraded	10	4.3	2	0.8	4	1.7	4	1.7
Vi negative	/		7	4.4	1	0.6	10	6.4
Phage-carrier	3	20.0	2	13.3	—		/	
Rough	3	16.6	/		—		—	
Total	294	7.7	39	0.1	59	0.1	89	0.2

Table IV

Incidence of phage type alteration in 3776 carriers

(Figures indicate number of carriers)

Phage type	Carriers excreting one phage type	Carriers excreting two or more phage types						All carriers
		in 3	5	10	10	total		
		positive cultures				No.	per cent	
A	665	14	13	25	19	71	9.64	736
B_1	15	2	1	4	1	8	24.70	23
B_2	134	—	9	12	5	26	16.20	160
B_3	15	—	3	1	2	6	28.50	21
C_1	256	3	6	10	6	25	8.80	281
C_2	5	1	—	2	—	3	37.50	8
C_4	9	—	1	—	—	1	10.00	10
D_1	536	7	11	19	13	50	8.50	586
D_2	25	—	—	—	1	1	3.80	26
D_4	17	3	4	2	1	10	37.10	27
D_5	3	—	—	—	—	—	—	3
D_6	5	—	—	—	5	5	50.00	10
D_7	1	—	—	—	—	—	—	1
D_9	4	—	—	—	—	—	—	4
D_{10}	—	—	—	—	1	1	100.00	1
E_{1a}	678	4	6	14	16	40	5.50	718
E_{1b}	16	5	—	1	—	6	27.20	22
E_2	1	—	1	—	—	1	50.00	2
E_4	1	—	—	—	—	—	—	1
F_1	423	6	7	10	8	31	6.80	454
F_2	1	—	1	2	2	5	83.30	6
J_1	2	—	—	—	—	—	—	2
N	4	1	2	—	2	5	55.50	9
T	12	1	2	8	10	21	63.60	33
27	5	—	—	—	—	—	—	5
28	14	—	1	1	—	2	12.50	16
38	6	—	1	—	—	1	14.20	7
40	2	—	—	—	—	—	—	2
43	—	1	—	—	—	1	100.00	1
46	2	—	—	—	—	—	—	2
Nt	106	12	8	29	27	76	41.70	182
Degraded	130	31	22	28	17	98	42.90	228
Vi negative	74	16	22	22	22	82	52.50	156
Phage-carrier	3	3	3	2	4	12	80.00	15
Rough	5	1	4	7	1	13	72.70	18
Total	3175	111	128	199	163	601	15.90	3776

observed for type E_{1a}. New phage types emerged in carriers excreting untypable strains in 41.7%, degraded strains in 42.9% and Vi negative strains in 52.9%. Strains belonging to new phage types were frequently isolated on repeated examinations from F_2, N and T type carriers. This finding, although the data represent a small number of strains, was remarkable.

As regards the total material comprising 20 years' data for 3776 carriers, phage types or Vi and O antigen variants different from those encountered on the first examination emerged in 15.9% of carriers. If the 189 carriers with Vi negative, phage carrier and rough strains are disregarded, new phage types appeared in 13.7% of 3587 carriers.

For a further analysis of type alterations the behaviour of strains isolated on periodical examinations from each of our carriers was examined. On the basis of characteristic forms of alteration the following 4 groups were distinguished (Table V).

(1) *Stable monotype carriers.* On serial examination the lytic pattern corresponds to the given phage type or shows negligible changes. Carriers belonging to this group harboured mainly phage types E_{1a}, F_1, D_2 and C_1.

(2) *Oscillating monotype carriers.* Repeated examinations showed forms transient into degraded phage types. Degradation appeared mainly in different B types, certain D types as well as in types A, T and 28. The high incidence of degraded phage type was observed in carriers excreting Vi negative strain on the first examination.

(3) *Ditype carriers.* Two different phage types are harboured which are often simultaneously present in the faecal sample. This group of carriers excreted mainly B, certain D, C_1 and A types. The appearance of new phage types in excreters of untypable strains occurred frequently. (Systematic introduction of new typing phages allowed the classification into new phage types of strains which had previously been considered untypable cultures.)

(4) *Polytype carriers.* Three or more phage types are excreted simultaneously or at subsequent samplings. A number of carriers of types F_2, T, D and untypable strains belonged to this group. The occurrence of three or more types was also observed in type C_1 and E_{1a} carriers.

Table VI shows the incidence of new phage types emerging in ditype carriers. In carriers of common phage types (E_{1a}, F_1, D_1, C_1, B_2) phage type A was the most frequent as a second type. In carriers of type A, mainly types B_2, D_1 and E_{1a} appeared. Type B_2 was very frequently followed by type A. In type C_1 carriers the appearance of type A predominated.

Untypable strains were encountered usually with phage type D_1. In carriers of unfrequent types D_4 and D_6 some D_1 strains were isolated. In group E the simultaneous excretion of E_{1a} and E_{1b} strains was characteristic. In F_1 carriers, as well as in C_1 carriers, the occurrence of type A was the most frequent; in 3 cases type F_3 emerged. In carriers of degraded strains, in addition to

Table V

Distribution of carriers according to the characteristics of phage type alteration

Phage type	Stable monotype, per cent	Oscillating monotype, per cent	Ditype, per cent	Polytype, per cent
A	77.74	12.64	7.34	2.30
B_1	52.18	13.04	34.78	—
B_2	30.00	53.76	13.12	3.12
B_3	23.81	47.61	23.81	4.76
C_1	81.82	9.22	8.16	0.80
C_2	50.00	12.50	25.00	12.50
C_4	90.00	—	10.00	—
D_1	75.77	15.69	6.66	1.88
D_2	84.62	11.53	3.85	—
D_4	44.44	18.52	22.22	14.82
D_5	100.00	—	—	—
D_6	20.00	30.00	50.00	—
D_7	—	100.00	—	—
D_9	—	100.00	—	—
D_{10}	—	—	100.00	—
E_{1a}	88.72	5.72	4.45	1.11
E_{1b}	72.73	—	27.27	—
E_2	50.00	—	50.00	—
E_4	—	100.00	—	—
F_1	85.25	7.92	5.50	1.32
F_2	16.67	—	16.67	66.66
J_1	50.00	50.00	—	—
N	22.22	22.22	33.34	22.22
T	3.03	33.33	36.36	27.28
27	100.00	—	—	—
28	31.25	56.25	12.50	—
38	57.15	28.57	14.28	—
40	100.00	—	—	—
43	—	—	100.00	—
46	50.00	50.00	—	—
Nt	55.50	2.75	30.22	11.53
Degraded	57.02	32.46	6.14	4.38
Vi negative	47.43	42.30	5.13	5.13
Phage-carrier	20.00	66.66	13.34	—
Rough	27.77	55.55	16.68	—
Total	72.57	15.78	8.77	2.88

Table VI

Phage type alterations of ditype character in carriers

Phage type detected on first examination	Phage types detected on subsequent examinations; figures in brackets indicate the number of carriers
A	B_1(3), B_2(15), B_3(3), C_1(3), C_4(1), D_1(12), D_4(1), D_6(1), E_{1a}(7), F_1(2), T(1), 28(1), 46(1), Nt(3)
B_1	A(6), D_1(1), 28(1)
B_2	A(15), C_1(1), E_{1a}(1), N(1), T(1), 28(1), Nt(1)
B_3	A(4), N(1)
C_1	A(12), B_2(1), D_1(4), E_{1a}(3), F_1(1), 38(1), Nt(1)
C_2	A(2)
C_4	E_{1a}(1)
D_1	A(15), B_1(1), B_2(1), D_7(1), D_9(2), E_{1a}(5), F_1(2), N(1), NT(11)
D_2	D_4(1)
D_4	D_1(4), E_{1a}(1), Nt(1)
D_5	Nt(1)
D_6	A(3), D_1(2)
D_{10}	D_9(1)
E_{1a}	A(7), C_1(2), D_1(3), E_{1b}(14), E_9(1), F_1(1), Nt(4)
E_{1b}	E_{1a}(5), Nt(1)
E_2	E_{1a}(1)
F_1	A(6), C_1(2), C_4(2), D_4(2), D_6(3), F_3(3), Nt(7)
F_2	F_1(1)
N	B_3(1), D_1(1), Nt(1)
T	C_1(3), 28(5), 46(1), Nt(3)
28	A(1), Nt(1)
38	A(1)
46	Nt(1)
nt	A(9), B_3(2), C_1(1), C_2(2), C_4(2), D_1(7), E_{1b}(6), F_1(4), F_2(2), F_4(1), N(1), T(1), 27(2), 28(7), 38(1), 40(5), 45(2)
Degraded	A(21), B_2(11), B_3(4), C_1(7), C_2(1), D_1(20), E_{1a}(3), 27(1), 46(1), Nt(3)
Vi negative	A(13), B_2(4), C_1(2), D_1(12), E_{1b}(19), F_1(4), F_7(1), Nt(5), Degraded (6)
Phage-carrier	A(1), B_3(1), D_1(2), E_{1a}(4), F_1(1), Nt(1)
Rough	A(6), D_1(2), E_{1a}(1), F_1(1)

type A, type D_1 was frequent. Untypable strains were, in addition to common types, often accompanied by types 28 and 40. Carriers harbouring Vi negative strains on the first examination frequently yielded E_{1a}, A and D_1 cultures afterwards; types F_1 and C_1 were found in a strikingly low number in these subjects.

Some interesting combinations of polytype carriership and the order of appearance of new types were as follows.

A. 1. D_1-D_8-D_{10}-D_9
2. D_1-D_4-D_{11}
3. E_{1a}-Vi negative-E_7-nt-E_7-phage carrier
4. F_1-nt-F_2
5. T-degraded-28-T-A
6. T-nt-T-degraded-nt-degraded-A
7. T-degraded-nt-28-degraded-28

In other polytype carriers the following "irregular" changes were recorded.

B. 1. F_1-A-D_1-A
2. T-T+F_1 degraded (multiple colony examination)-F_1-Vi negative-T-A-T-Vi negative-T-T-degraded-28 degraded
3. T-nt-F_2 degraded-F_2T degraded-28-T-T degraded-28 degraded-28
4. F_1-E_{1a}-F_1 degraded (3 colonies)+E_{1a} (7 colonies)-E_{1a} (9 colonies)+ +F_2 (1 colony)
5. E_{1a}-A-A+E_{1a}(multiple colony examination)-E_{1a}

Phage typing of cultures prepared from several isolated colonies grown from the same specimen was useful for analysing the appearance of different phage types. Characteristic findings are presented in the following (typings are shown in chronological order, figures with multiplication mark before types indicate the number of consecutive samples yielding the corresponding type).

Case 1. $1\times$A degraded, $1\times$ { degraded (5 colonies) / A (5 colonies) } $8\times$A

Case 2. $1\times$A, $1\times$ { Vi negative (9 colonies) / A (1 colony) } $3\times$A

Case 3. $1\times$A, $4\times D_1$ (10 colonies), $1\times$A, $1\times$ { A (5 colonies) / D_4(5 colonies) } $4\times D_4$

Case 4. $1\times$A, $4\times D_6$ $1\times$ { A (several colonies) / D_6 (several colonies) } $3\times D_6$

Case 5. $2 \times$ nt, $1 \times F_1$, $3 \times$ nt, $1 \times$ nt
F_1 (3 colonies)
(2 colonies)
F_2 (1 colony)

Case 6. $1 \times$ Vi negative, 1×46, $1 \times$
46 (1 colony)
nt (4 colonies)

Case 7. $5 \times E_{1a}$, $2 \times A$, $1 \times$
A (several colonies)
E_{1a} (several colonies)
$1 \times E_{1a}$

Case 8. $2 \times F_1$, $1 \times E_{1a}$, $1 \times$
F_1 degraded (3 colonies)
E_{1a} (7 colonies),
$1 \times$ F_2 (1 colony)
E_{1a} (1 colony)

Discussion

The incidence of phage types in chronic and transient carriers registered in Hungary over a 20 year period has been presented. The data do not reflect the present situation, as systematic investigations reveal every year a number of carriers, and many carriers die or cease to harbour typhoid bacteria. Yet, collection of such data is of great importance. When the phage type distribution of *S. typhi* in different parts of the world will be known, it will be possible to estimate the source of a new type appearing first in a country.

In the present studies, alterations in Vi antigen content of the strains which limit the use of phage typing in epidemiological investigations have also been studied. On repeated examination of the same carrier, Vi negative or rough strains in the majority of cases were followed by typable cultures. As regards frequent phage types, the incidence of Vi negative strains was the less frequent in C_1 and F_1 carriers. A frequent alternative incidence of Vi positive and Vi negative strains was observed in E_{1a} carriers. There was no definite association between the loss of Vi antigen and certain phage types. The appearance of Vi negative or phage carrier strains was frequent in persons harbouring types A_1, D_1 and E_{1a}; these strains were less common with phage types C_1 and F_1. This finding indicates that the loss of Vi antigen may be associated with phage action.

The appearance of one or more phage types on subsequent examination of the same carrier may be explained as follows. Data for polytype carriers indicate that a mixed infection had occurred and that different phage types

present in the carrier were isolated simultaneously or alternatively (see example B/1—5 for polytype carriers). This explanation is supported by the fact that carriers, who mainly belong to older age groups, had been passing part of their lives under unsatisfactory hygienic conditions and many of them had acquired typhoid fever in the course of water-borne outbreaks. In the literature, EÖRSI [16] in 1955 was the first to report on a water-borne epidemic caused by *S. typhi* strains belonging to different phage types. In her paper she also mentioned that, as indicated by later examination of carriers harbouring different phage types, a water-borne outbreak in 1929 had also been due to mixed infection. NICOLLE [19] reported on a possibly water-borne outbreak of typhoid fever caused by phage types D_1 and F_1. BOYD [15] described mixed *S. typhi* phage type infections in a crowded prisoner of war camp. Conditions during the first and second world wars yielded an ample chance for mixed infections in Hungary.

Ditype and polytype carriers as well as multiple colony examinations revealed the simultaneous appearance of phage types, the alternation of which cannot be explained by an uptake or loss of the type-determining phage. The frequency of ditype and polytype carriers harbouring a wide scale of phage types indicates that in the majority of carriers a mixed infection had taken place (see examples 7 and 8 for multiple colony examinations and example B for polytype carriers). In a smaller part of carriers the phage types may undergo alterations associated with type-determining phages (see examples 3, 4, 5 and 6 for multiple colony examinations and example A for polytype carriers). For the appearance of pairs A-D_1, D_4-D_1, D_6-A, E_{1a}-E_9, T-28 and 28-A in ditype carriers probably the type-determining phage is responsible, although a reinfection might also have occurred. Alteration E_{1a}-E_{1b} belongs also to this group. This assumption is supported by the opinion of CRAGIE and FELIX that the appearance of phage type A does not necessarily mean a mixed infection.

The majority of carriers excreting two types (pairs A-C_1, A-F_1, A-E_{1a}, etc.) had probably been infected with two different phage types. It is also possible that the carrier had subsequently been reinfected with a different phage type.

When a new phage type different from the original one appears in a carrier who has so far been excreting one single type, the following possibilities may be considered.

(1) If the alteration can be explained by an uptake or loss of the type-determining phage, an *in vivo* change may be assumed.

(2) If the alteration cannot be associated with a type-determining phage, mixed infection or reinfection should be suspected. A history of long monotype carriership makes the latter probable. The finding suggests that in the environment of the carrier another, yet undetected, carrier may be present;

accordingly, epidemiological investigations and screening tests are recommended.

For supplementing the changes listed and analysed above, it should be mentioned that a phage type alteration associated with type-determining phages may occur in persons who have become carriers after contracting a mixed infection. In such cases the analysis of type alteration may be difficult. (Case 8 for multiple colony examinations presents a good example.)

A survey of our 20 year material and epidemiological experience indicates that, in view of the stability of phage types and xylose fermentation, *S. typhi* phage typing introduced in Hungary by Eörsi in 1948 has become an essential means of search for infective foci. When antigenic alterations make typing difficult, repeated and multiple colony examinations usually reveal typable strains.

When, rarely, a type different from the original one emerges in a carrier, a cooperative genetic and epidemiological analysis will solve the problem.

REFERENCES

1. Craigie, J.: Yen, C. H.: Canad. J. publ. Hlth **29,** 448 (1938).
2. Craigie, J.: Yen, C. H.: Canad. J. publ. Hlth **29,** 484 (1938).
3. Craigie, J.: Bact. Rev. **10,** 37 (1946).
4. Craigie, J., Felix, A.: Lancet **I,** 823 (1947).
5. Anderson, E. S.: J. Hyg. **49,** 458 (1951).
6. Felix, A., Anderson, E. S.: Nature (Lond.) **167,** 603 (1951).
7. Anderson, E. S., Felix, A.: J. gen. Microbiol. **9,** 65 (1953).
8. Anderson, E. S.: Nature (Lond.) **175,** 171 (1955).
9. Anderson, E. S., Fraser, A.: J. gen. Microbiol. **13,** 519 (1955).
10. Anderson, E. S.: Ann. Inst. Pasteur **102,** 379 (1962).
11. Craigie, J.: Canad. J. publ. Hlth **31,** 4 (1940). Abstr.
12. Read, K. S., Ferguson, W. W.: J. Bact. **81,** 474 (1961).
13. Felix, A., Anderson, E. S.: J. Hyg. (Lond.) **49,** 349 (1951).
14. Helmer, D. E., Kerr, D. E., Dolman, C. E., Ranta, L. E.: Canad. J. publ. Hlth **31,** 433 (1940).
15. Boyd J. S. K.: Brit. med. J. **1,** 719 (1943).
16. Eörsi, M.: Zbl. Bakt. I. Abt. Orig. **168,** 509 (1957).
17. Rische, H., Rohne, K.: Z. ges. Hyg. **7,** 236 (1961).
18. Borecká, J.: Zbl. Bakt. I. Abt. Orig. **200,** 436 (1966).
19. Nicolle, P.: Colloquium über Fragen der Lysotypie. Zentrallaboratorium für Lysotypie, Wernigerode (Harz) 1956, p. 64.

Address of the authors:

Gábor Hérmán, Hedda Milch
National Institute of Public Health,
Gyáli út 2–6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 133—135, 1971

A NEW SALMONELLA SEROTYPE: SALMONELLA ISASZEG (48 : z_{10} : e, n, x)

By

MARIA M. ÁDÁM and SUSAN DEÁK

National Institute of Public Health (Director: T. BAKÁCS), *Budapest*

(Received July 6, 1971)

Summary. A new *Salmonella* subgenus I serotype: *S. isaszeg* (48 : z_{10} : e, n, x) isolated from surface water is described.

A new *Salmonella* serotype has been isolated from the water of brook Rákos near the town Isaszeg (pronounced i'shahseg) in the year 1969. Above the sampling point there was an inlet of a canal carrying the sewage of Gödöllő town.

Materials and methods

Isolation of the culture was performed by passing one litre of brook water through membrane filter and transferring the filter into RAPPAPORT's medium [4].

Biochemical and serological methods were as described in [1—3].

Results

As seen in Table I, according to biochemical reactions the strain belongs to *Salmonella* subgenus I.

Serological analysis of the strain is presented in Table II. The O antigen of the culture is identical with that of *S. dahlem* (O48). The first phase of its H antigen is identical with the z_{10} antigen of *S. glostrup*. In the second H phase there is a minor difference between the isolate and *S. abortus-equi* (e, n, x).

The above antigenic structure of the new serotype has been confirmed in the International Salmonella Centre, Paris, with the completion that its O antigen is a complex of $O48_1 48_2$ and the second phase of the H antigen corresponds to e, n, x, z_{16}.

The new serotype has been included in the Hungarian National Collection of Medical Bacteria, Budapest, under accession number 10253. Our attempts at isolating more strains with the same antigenic structure from the same geographical area have failed.

Table I

Biochemical reactions of strain 10 253

Adonitol	—	D-Xylose	+
L-Arabinose	+	Aesculin	—
Dulcitol	+	Dextrin	+
D-Fructose	+	Starch	+[7]
D-Galactose	+	ONPG	—
D-Glucose (gas) 37°	+	Methyl red 37°	+
22°	+	22°	+
Glycerol	+[5]	Voges—Proskauer 37°	—
m-Inositol	—	22°	—
Inulin	—	Urea	—
Lactose	—	Indole	—
D-Maltose	+	H_2S	+
D-Mannitol (gas) 37°	+	Gelatin	—
22°	+	Ammonium citrate 37°	+[2]
D-Mannose	+	22°	+[2]
Raffinose	—	D-Tartrate	+[3]
L-Rhamnose	+	Malonate	—
Salicin	—	Lysine decarboxylase	+
D-Sorbitol	+	Ornithine decarboxylase	+
Sucrose	—	Arginine dihydrolase	—
D-Trehalose	+	KCN	—

Table II

Serological analysis of strain 10 253

O antigens	O serum *S. dahlem* (O48)		O serum strain 10 253	
	—	absorbed by strain 10 253	—	absorbed by *S. dahlem*
S. dahlem	1280	—	5120	—
Strain 10 253	1280	—	5120	—

H antigens	H serum *S. glostrup* phase 1 (z_{10})		H serum strain 10 253 phase 1		H serum *S. abortus-equi* (*e, n, x*)		H serum strain 10 253 phase 2	
	—	abs. by 10 253	—	abs. by *S. glostrup*	—	abs. by 10 253	—	abs. by *S. abortus-equi*
S. glostrup phase 1	5120	—	5120	—	.	.	.	.
Strain 10 253 phase 1	5120	—	5120	—	.	.	.	.
S. abortus-equi	.	.	.	.	10 240	—	10 240	—
Strain 10 253 phase 2	.	.	.	.	10 240	—	10 240	320

Acknowledgement. The authors are indebted to Professor L. Le Minor, International Salmonella Centre, Paris, for checking and completing the results.

REFERENCES

1. Edwards, P. R., Ewing, W. H.: Identification of Enterobacteriaceae. Burgess, Minneapolis 1962.
2. Kauffmann, F.: The Bacteriology of Enterobacteriaceae. Munksgaard, Copenhagen 1966.
3. Le Minor, L.: Le diagnostic de laboratoire des Entérobactéries. La Tourelle, Saint-Mandé 1969.
4. Rappaport, F., Konforti, N., Navon, B.: J. clin. Path. **9**, 261 (1956).

Address of the authors:

Mária M. Ádám, Zsuzsanna Deák
National Institute of Public Health,
Gyáli út 2—6, Budapest IX., Hungary

INDEX

Vághy, T.: Correlation between the Morphological Variability and Riboflavin Producing Capacity of *Eremothecium ashbyii* 75

Szabó Szűcs, J.: Phage Infection of Vitamin B_{12} Producing Rhizobium Cultures 81

Szita, J., Svidró, A.: Bacteriologic Diagnosis of *Yersinia pseudotuberculosis* 87

Hoch, V., Hérmán, G.: Phage Typing of D-Group Streptococci. I. Typing of Enterococci with Roumanian Phages 95

Hérmán, G., Hoch, V.: Phage Typing of D-Group Streptococci. II. Isolation of Supplementary Phages for Classification of Enterococci Untypable with Roumanian Phages 101

Molnár, J., Prágai, B.: Attempts to Detect the Presence of Teichoic Acid in *Bacillus anthracis* 105

D. Tóth, F., Váczi, L., Berencsi, Cl.: Effect of Interferon Treatment on the Tumour-Specific Antibody Response of BALB/c Mice Infected with Rauscher Virus 109

Szita, J., Káli, M., Rédey, B.: Serological Diagnosis of *Yersinia enterocolitica* 113

Hérmán, G., Milch, H.: Twenty Years Observation on the Stability of *Salmonella typhi* Phage Types 119

Ádám, M. M., Deák, S.: A New Salmonella Serotype: *Salmonella isaszeg* (48 : z_{10} : e, n, x) 133

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója
Műszaki szerkesztő: Várhelyi Tamás
A kézirat nyomdába érkezett: 1971. XI. 24. — Terjedelem: 5.5 (A/5) ív, 9 ábra, 6 színes

71.72744 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, Budapest, Keleti Postahivatal, Postafiók 64.

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $ 16.00.

Bestellbar bei dem Buch- und Zeitungs-Außenhandels-Unternehmen »*Kultúra*« (Budapest I., Fő utca 32. Bankkonto Nr. 43-790-057-181) 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 de fascicules qui seront réunis en volumes.

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

Acta Microbiologica, Budapest, Keleti Postahivatal, Postafiók 64.

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

Leprixde l'abonnement est de $ 16.00 par volume.

On peut s'abonner à l'Entreprise pour le Commerce Extérieur de Livres et Journaux «*Kultúra*» (Budapest I., Fő utca 32. Compte courant No. 43-790-057-181) 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, Budapest, Keleti Postahivatal, Postafiók 64.

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписная цена — $ 16.00 за том.

Заказы принимает предприятие по внешней торговле книг и газет «*Kultúra*» (Budapest I., Fő utca 32. Текущий счет № 43-790-057-181), или его заграничные представительства и уполномоченные.

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

ALBANIA
Drejtorija Qändrone e Pärhapjes
dhe Propagandimit tä Librit
Kruga Konferenca e Päzes
Tirana

AUSTRALIA
A. Keesing
Box 4886, GPO
Sydney

AUSTRIA
Globus
Höchstädtplatz 3
A-1200 Wien XX

BELGIUM
Office International de Librairie
30, Avenue Marnix
1050 Bruxelles
Du Monde Entier
162, rue du Midi
1000 Bruxelles

BULGARIA
Hemus
11 pl Slaveikov
Sofia

CANADA
Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA
Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA
Artia
Ve Smečkách 30
Praha 2
Poštovní Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha I
Slovart A. G.
Gorkého
Bratislava

DENMARK
Ejnar Munksgaard
Nörregade 6
Kopenhagen-K

FINLAND
Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki 10

FRANCE
Office International de Documentation
et Librairie
48, rue Gay-Lussac
Paris 5

GERMAN DEMOCRATIC REPUBLIC
Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701
Zeitungsvertriebsamt
Fruchtstraße 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC
Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart I

GREAT BRITAIN
Blackwell's Periodicals
Oxenford House
Magdalen Street
Oxford
Collet's Subscription Import
Department
Dennington Estate
Wellingsborough. Northants.
Robert Maxwell and Co. Ltd.
4—5 Fitzroy Square
London W. I

HOLLAND
Swetz and Zeitlinger
Keizersgracht 471—478
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA
Hind Book House
66 Babar Road
New Delhi I

ITALY
Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze
Techna
Via Cesi 16.
40135 Bologna

JAPAN
Kinokuniya Book-Store Co. Ltd.
826 Tsunohazu 1-chome
Shinjuku-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central 100-31

KOREA
Chulpanmul
Phenjan

NORWAY
Tanum-Cammermeyer
Karl Johansgtan 41—43
Oslo I

POLAND
Ruch
ul. Wronia 23
Warszawa

ROUMANIA
Cartimex
Str. Aristide Briand 14—18
Bucureşti

SOVIET UNION
Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN
Almquist and Wiksell
Gamla Brogatan 26
S—101 20 Stockholm

USA
F. W. Faxon Co. Inc.
15 Southwest Park
Westwood Mass. 02090
Stechert Hafner Inc.
31. East 10th Street
New York, N. Y. 10003

VIETNAM
Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA
Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

31. III. 1972

Index: 26.016

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

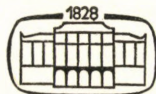
ADIUVANTIBUS

I. DÖMÖK, E. FARKAS, I. HORVÁTH
I. NÁSZ, K. RAUSS, F. B. STRAUB, J. SZIRMAI, L. VÁCZI, K. VAS,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XVIII FASCICULUS 3



AKADÉMIAI KIADÓ, BUDAPEST

1971

AMAHA 5 18(3) 137—218 (1971)

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

Főszerkesztő

IVÁNOVICS GYÖRGY

akadémikus

Szerkesztő

LÁNYI BÉLA

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, Budapest, Keleti Postahivatal, Postafiók 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (Budapest V., Alkotmány utca 21. Bankszámla 05-915-111-46), a külföld számára pedig a „Kultúra" Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő utca 32. Bankszámla: 43-790-057-181) 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, Budapest, Keleti Postahivatal, Postafiók 64.

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

The rate of subscription is $ 16.00 a volume.

Orders may be placed with „Kultúra" Foreign Trade Company for Books and Newspapers (Budapest I., Fő utca 32. Account No. 43-790-057-181) or with representatives abroad.

Acta microbiol. Acad. Sci. hung. 18, 137—148, 1971

IRREVERSIBLE ADSORPTION OF EXOPENICILLINASE ON BACILLUS CEREUS 569 CELLS

By

V. Csányi, I. Mile, T. Romhányi, P. Hortobágyi and F. Hatfaludi

Institute of Medical Chemistry (Director: F. Antoni), Semmelweis University Medical School, Budapest

(Received January 18, 1971)

Summary. Under appropriate experimental conditions, a small proportion of exopenicillinase was capable of adsorption onto the surface of *Bacillus cereus* cells and other adsorbents. The most favourable experimental conditions needed for irreversible adsorption were studied. It was found that in the penicillinase fraction incapable of irreversible adsorption, a form capable of adsorption appeared under the effect of strong conformation-altering agents.

The irreversibly adsorbed penicillinase could be redissolved by incubation with concentrated bases. Immunological studies of the γ-penicillinase formed during induction and of the irreversibly adsorbed exoenzyme revealed close similarities between the two enzymes.

Bacillus cereus 569 is known to produce two kinds of penicillinase, *viz.* exopenicillinase which is secreted into the surrounding medium, and γ-penicillinase which represents about 10% of the total enzyme yield and is irreversibly bound to the cells, *i.e.* cannot be removed by concentrated electrolyte solutions. The two kinds of enzyme differ in several chemical properties. These differences are, however, supposed to be due to differences in the conformation of the enzymes [1, 2].

Studying the formation of the γ-enzyme, Pollock and Krámer [3] have demonstrated that it does not serve as a precursor of exopenicillinase. Studies performed in this laboratory [4] have shown that it is probably the γ-enzyme which is formed from exopenicillinase through secondary adsorption.

According to Citri [8] exopenicillinase is readily adsorbed onto various surfaces such as glass, quartz, cells, etc. and the conformation of the adsorbed penicillinase is different from that of exopenicillinase. The present experiments were performed in order to study the process of adsorption and the role of this in the formation of bound γ-penicillinase.

Materials and methods

Cultivation of B. cereus 569 has been described previously [5].

The method of exopenicillinase preparation from *B. cereus* 569 cells was the same as that used for strain 569/H [6]. Enzyme preparations not older than five days were used in these experiments.

Separation and estimation of the activity of the bound enzymes were performed as reported earlier [4].

Exopenicillinase antiserum was prepared by the method of Pollock and Krámer [3].

Results

The bound enzyme content of non-induced *B. cereus* 569 cells varied between 0.2—0.5 U/mg dry material. The exopenicillinase binding capacity of the cells in an electrolyte deficient medium was studied by the following method. The cells were suspended in aliquots of 5 ml distilled water (2.5 mg cells/aliquot) and then purified exopenicillinase was added in growing con-

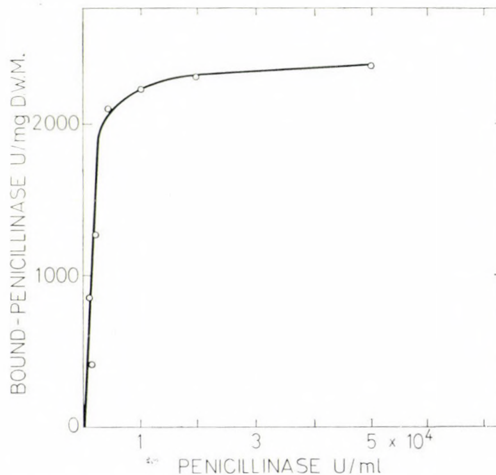


Fig. 1. Adsorption of penicillinase to *B. cereus* 569 cells in electrolyte poor medium. The amount of adsorbed penicillinase is plotted against the penicillinase content of the adsorbed mixture. Experimental conditions are described in the text

centration to each of the aliquots. After incubation at 0 °C for 15 minutes, the cell suspension was centrifuged and the cells were washed with five changes of 10 ml distilled water, then resuspended in 3 ml 0.5% gelatin solution and the amount of penicillinase bound to the cells was estimated. As shown in Fig. 1, the cells were binding large quantities of exopenicillinase in an electrolyte poor medium.

A similar technique was used for studying the adsorption of exopenicillinase to diatomite celite 535. The adsorption kinetics showed similar characteristics and the maximum values of the bound enzyme were around 100 U/mg celite.

When instead of distilled water 1% NaCl was used during adsorption and for the subsequent washings, the qualitative course of the adsorption process was similar to that described above and only the adsorbed amounts of the enzyme showed values lower (40—60%) than in the experiments with distilled water. Penicillinase bound to the cells in this way is termed "loosely bound" penicillinase which is easily removed from the cells by treatment

with concentrated electrolyte solutions [1]. Studies were performed to decide whether it was possible to remove all the "loosely bound" exopenicillinase from the cells if the concentrated electrolyte solutions were applied after the completion of adsorption, as such solutions leave only the irreversibly bound penicillinase on the cell surface [1].

For the adsorption procedure, 2 mg cells were suspended in each of 1 ml NaCl solution aliquot, and exopenicillinase was added in growing concentra-

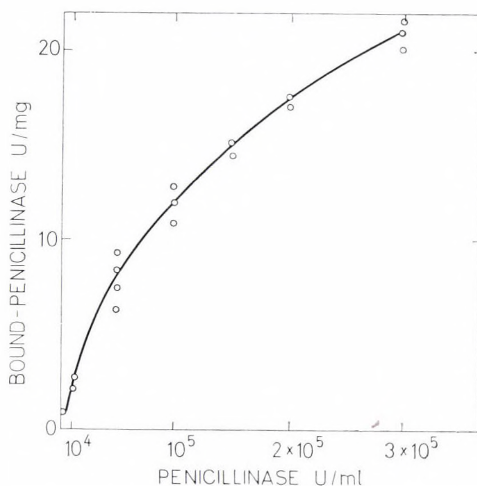


Fig. 2. Irreversible adsorption of exopenicillinase to *B. cereus* 569 cell. The amount of irreversibly adsorbed enzyme is plotted against the penicillinase content of the experiment (see text)

tion to each of them. After incubation at 0 °C for 15 min. the cells were centrifuged and washed five times with the elution fluid (1 *M* NaCl + 0.2 *M* Na-citrate, pH 0.8) used also for the separation of the γ-enzyme formed during induction [4]. The amount of cell-bound enzyme not removable by concentrated electrolyte solutions was estimated. In order to demonstrate the high reproducibility of the experiments, the results of experiments performed at different points of time are shown in Fig. 2. The cells were able to adsorb several tenfolds of their original bound enzyme content. The shape of the adsorption curve is, however, different from that found for the loosely bound enzyme in electrolyte-free medium.

In the following, experiments are presented which have been performed to determine the most favourable experimental conditions needed for irreversible adsorption.

The effect on irreversible adsorption of the concentration of the washing solution and of the number of washings is presented in Fig. 3 and Fig. 4, respectively.

It was found that the preliminary addition of the washing fluid inhibited the occurrence of adsorption. This finding offered a possibility for studying the time course of the process of adsorption; the results are shown in Fig. 5.

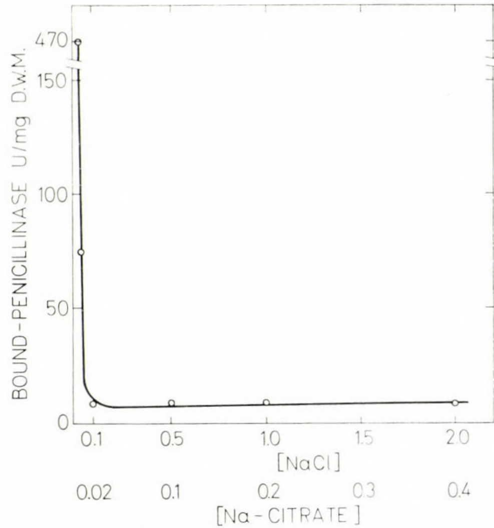


Fig. 3. Effect of the concentration of the washing solution on the amount of irreversibly adsorbed penicillinase. *B. cereus* 569 cells (3.5 mg) were suspended in 5 ml 5% NaCl containing 5×10^4 U exopenicillinase. After an incubation period of 15 minutes, the cells were centrifuged and washed 5 times with washing fluid at the concentration shown. The cells were finally suspended in 3 ml 1.5% gelatin solution and the quantity of bound enzyme was estimated

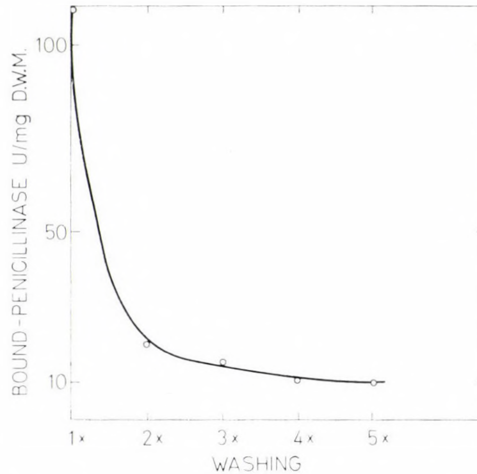


Fig. 4. Effect of the number of washings with electrolyte solution on the quantity of irreversibly adsorbing penicillinase. The cells (2.5 mg) were suspended in 5 ml 1% NaCl containing 5×10^4 U penicillinase, incubated at 0 °C for 15 minutes and then centrifuged. The samples were washed 5 times. The cells were finally suspended in 0.5% gelatin solution and the amount of adsorbed enzyme was estimated

Incubation temperature in the range of 0—40 °C was found to have little influence on irreversible adsorption.

Experiments were performed to decide whether the amount of the irreversibly adsorbed penicillinase was dependent on the concentration of the exo-enzyme as found by us in several experiments on "loose" adsorption. The results of these experiments unequivocally showed that the amount of the

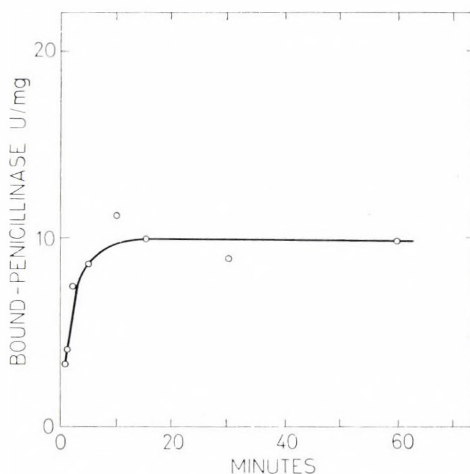


Fig. 5. Time course of the irreversible adsorption of exopenicillinase. The cells (2.5 mg) were suspended in 5 ml 1% NaCl containing 5×10^4 U penicillinase. The mixture was incubated at 0 °C and adsorption was interrupted by a mixture of 5 ml 2 *M* NaCl and 0.4 *M* Na-citrate at different points of time. The cells were centrifuged, washed five times and the amount of bound enzyme was estimated

irreversibly adsorbed penicillinase depends on the quantity instead of the concentration of exopenicillinase used during incubation (Figs 6 and 7).

This finding led to the assumption that only a definite fraction instead of all of the penicillinase molecules present in the solution might be involved in irreversible adsorption. In other words, the exopenicillinase solution can be "depleted", *i.e.*, its molecules capable of adsorption can be completely removed.

To test this hypothesis, a "depletion" experiment was performed in the following way. After the centrifugation of an incubation mixture of a given amount of exopenicillinase and a given amount of bacterial cells, fresh cells were added to the supernatant containing the enzyme. The mixture was re-incubated and then centrifuged four times. The amounts of the loosely and irreversibly adsorbed enzymes were estimated after each centrifugation. The amount of the loosely bound enzymes was about the same after each centrifugation. On the other hand, a significant decrease in the quantity of the irreversibly adsorbed enzyme could be observed after each centrifugation, so

that practically no irreversible adsorption was detectable after the third or fourth centrifugation (Fig. 8).

If the enzyme capable of adsorption forms only a small fraction of exopenicillinase, it is questionable whether it is indeed the exopenicillinase or eventually another enzyme exhibiting penicillinase activity that is involved

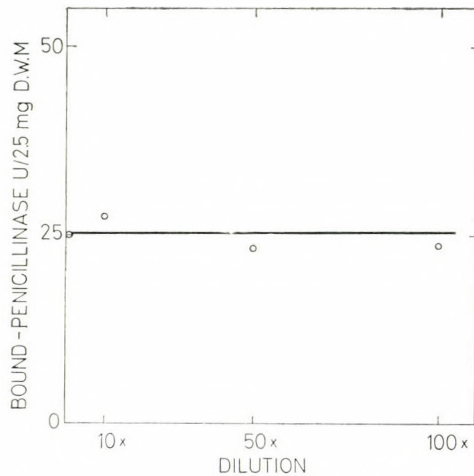


Fig. 6. Effect of dilution on the irreversible adsorption of penicillinase. To 1, 10, 50 and 100 ml of 1% NaCl each, 2 mg cells and 5×10^4 U penicillinase were added. The samples were incubated at 0 °C for 15 minutes. After centrifugation, the cells were washed five times and the amount of bound enzyme was estimated

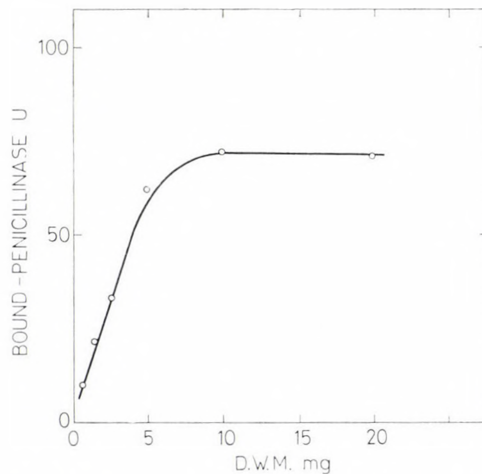


Fig. 7. Dependence of the amount of irreversibly bound enzyme on that of the adsorbent. The cells (in amounts shown in the Figure) were incubated with 5×10^4 U penicillinase in 5 ml 1% NaCl at 0 °C for 15 minutes, centrifuged and washed five times. The amount of bound enzyme was estimated

in the adsorption. It seemed reasonable to suppose that the exopenicillinase preparation used by us contained a small amount of γ-penicillinase present originally in a cell bound state and this was the factor that had the specific capacity of adsorption. Therefore, we studied the adsorption capacity of the γ-penicillinase described by POLLOCK [1] and of the bound enzyme I, bound enzyme II and US-enzyme isolated by us [4]. The results of these studies unequivocally showed that the adsorption capacity of these preparations was

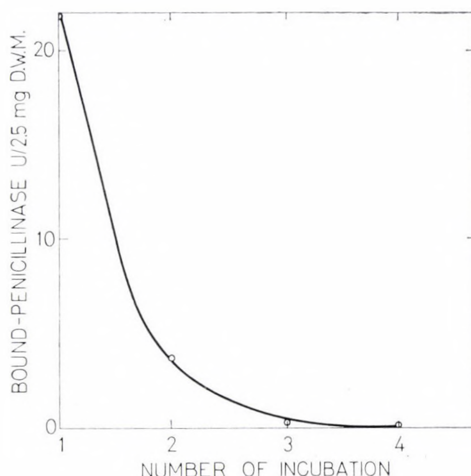


Fig. 8. "Depletion" by repeated incubation of the enzyme fraction capable of adsorption. The cells (2.5 mg) were incubated with 5×10^4 U penicillinase in 5 ml 1% NaCl at 0 °C for 15 minutes, centrifuged and washed five times. The amount of bound enzyme was estimated. To the supernatant of the incubation mixture, further 2.5 mg cells were added and centrifuged as above. The amount of enzyme bound to the cells used for subsequent incubations is plotted against the number of incubations. The total enzyme content of the incubation mixture decreased by about 10% after the 4th incubation.

much lower than that of the exopenicillinase. The originally cell bound γ-penicillinase thus has no special adsorption capacity.

The next problem was that if the fraction capable of adsorption can be removed from the exoenzyme preparation, is it retained during isolation when celite chromatography is applied by which irreversible adsorption is otherwise well detectable? It could be assumed that the raw mixture contained the irreversibly bound fraction at a high concentration. Therefore, the proportion of the enzyme adsorbed from the supernatant of cells induced by penicillin onto cells irreversibly not induced was estimated. The proportion of the irreversibly adsorbed fraction was found to be around 0.1%, a value similar to that for purified enzyme preparations.

This experiment showed that the amount of the enzyme capable of adsorption is about the same both before and after the isolation procedure

though adsorption chromatography applied during isolation should obviously have resulted in differences. The contradiction could only be explained by supposing the existence of a kind of balance between the fractions capable and incapable of adsorption, *i.e.*, the formation of the fraction capable of adsorption from that incapable of adsorption without the occurrence of any conformational changes.

Accepting this supposition as a working hypothesis, it must immediately be added that the presumed balance must be a very special one since no data indicating the fast occurrence of an equilibrium were obtained during our studies on the conditions of adsorption (on the effect of time, temperature, etc.).

Our first attempts to enable the "depleted" enzyme preparation incapable of adsorption to regain its capacity for irreversible adsorption, failed serially. If an enzyme preparation was completely "depleted" with repeated incubations with cells, no newly formed fraction capable of irreversible adsorption could be detected even after several weeks storage in solution at 0 °C. It was thus concluded that the effect of a conformation-altering agent must be tested.

It has been shown in previous studies that exopenicillinase does not lose its activity in 0.1 *N* base at 0 °C, but undergoes conformational changes [11]. Attempts were therefore made to apply alkaline treatment for producing the conformation capable of irreversible adsorption. The method proved to be successful as in a completely "depleted" exoenzyme preparation the enzyme fraction capable of irreversible adsorption reappeared two minutes after alkali treatment (Table I).

Further studies revealed great differences in the amount of the enzyme capable of irreversible adsorption obtained after alkali treatment (from 3—4 U

Table I

Production from "depleted" enzyme of irreversibly adsorbing penicillinase by alkali treatment

For details, see text

Treatment	Units irreversibly adsorbed enzyme 2.5 mg cells
Before depletion (50 000 U)	7.2
After total depletion (50 000 U)	0.0
50 000 U enzyme before depletion — 50 000 U enzyme after depletion	8.6
Depleted enzyme after alkali treatment (50 000 U)	18.9

to 100 U/10^4 U "depleted" enzyme). The phenomenon may be explained as follows.

The enzyme capable of irreversible adsorption is present at relatively high concentrations in freshly isolated enzyme preparations and, after removal of the enzyme by depletion, it is possible to restore high enzyme concentrations by alkali treatment. However, the ageing of the enzyme preparation results in a parallel decrease both in the amount of the fraction capable of adsorption and in the restoration rate.

Estimates of the irreversibly adsorbing enzyme content of the fresh enzyme preparations showed that this fraction represents about 0.1% of the total enzyme activity. This value falls to about one fifth during one week by primary kinetics. Depleted fresh enzyme preparations can be reactivated by alkali treatment or even by precipitation with concentrated ammonium sulphate solution and amounts two or three times higher than the original of the irreversibly adsorbed fraction may thus be obtained. Preparations one week old or older can be "activated" to a small extent. The factors responsible for this special "ageing" are not known.

The irreversibly adsorbed enzyme could be redissolved by 0.1 *N* alkali solution. The enzyme adsorbed to celite could also be redissolved with a minimum decrease of activity by treatment with 0.1 *N* NaOH for one minute.

This finding offered a possibility for studying the immunological properties of both the γ-penicillinase formed during induction and the irreversibly adsorbed enzyme prepared by us, and also the relation between the two enzymes.

For the following experiment, samples were prepared in four different ways. (i) The bound γ-enzyme was set free from the penicillin-induced cells by ultrasonic treatment (US-γ-enzyme). (ii) After removal of cell detritus by centrifugation, part of the US-γ-enzyme was incubated at pH 13 at 0 °C for 1 minute and then reacidified to pH 6 (US-γ-enzyme + NaOH). (iii) The exoenzyme was adsorbed irreversibly to a large quantity of cells and after washing with elution fluid, the cells were sonicated by the method used for induced cells. In this way, the adsorbed enzyme was redissolved (adsorbed enzyme). (iv) Part of the adsorbed enzyme in solution was submitted to alkali treatment and then reacidified (adsorbed enzyme + NaOH). The immune response to exo-antiserum of each of the four samples as well as that of the purified exopenicillinase was then tested.

The results of the immune reactions are summarized in Fig. 9. The response to antiserum of the exoenzyme redissolved by ultrasonic treatment and of the redissolved preparation of the irreversibly adsorbed enzyme was much weaker than that of the exoenzyme. Alkali treatment resulted in an increase in the intensity of the reaction with exo-antiserum of both fractions though these values were still lower than those obtained for the exoenzyme.

In other experiments when adsorption to celite was studied, the reaction with antiserum of the enzyme eluted by alkali and that of the exoenzyme was similar.

When evaluating the data obtained, it must be taken into account that the immune serum specificity of the enzyme preparations increased after alkali treatment.

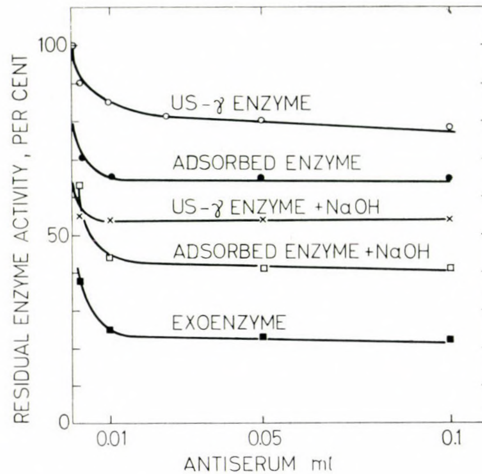


Fig. 9. Immune response to exopenicillinase of the bound and the irreversibly adsorbed enzyme. Of the enzymes, 300—400 U were added to 10 ml 0.05 *M* phosphate buffer and incubated with exopenicillinase antiserum for 15 minutes. Residual enzyme activity was expressed in per cent of the activity of the serum-free control samples

Discussion

It has been shown that exopenicillinase from *B. cereus* is adsorbed in two ways. First, the enzyme is adsorbed "loosely" onto the cells or other structures and is readily removed from the surface on treatment with concentrated electrolyte solutions. Second, the adsorption is "irreversible" when washing of the cells with electrolyte solutions does not result in a release of the enzyme. About 0.1% of the enzyme content of the raw ferment juice or of the exoenzyme preparations prepared by us is capable of irreversible adsorption. Repeated adsorption results in a complete removal from the exopenicillinase preparation of the enzyme fraction capable of irreversible adsorption. In fresh enzyme preparations, strong conformation altering agents such as concentrated ammonium sulphate or precipitation by 0.1 *N* alkali may restore the capacity for irreversible adsorption of part of the "depleted" enzyme preparation. Irreversible adsorption may be prevented and the adsorbed enzyme redissolved by base treatment. The immune response of different enzyme preparations to exopenicillinase antiserum showed that the

"native bound enzyme" found during induction as well as the irreversibly adsorbed penicillinase give a weak reaction with exo-antiserum. The specific response of either of these two preparations can be reinforced by alkali treatment.

The mechanism of bound penicillinase formation is a much discussed problem [1, 3]. Earlier induction experiments have made us to conclude that

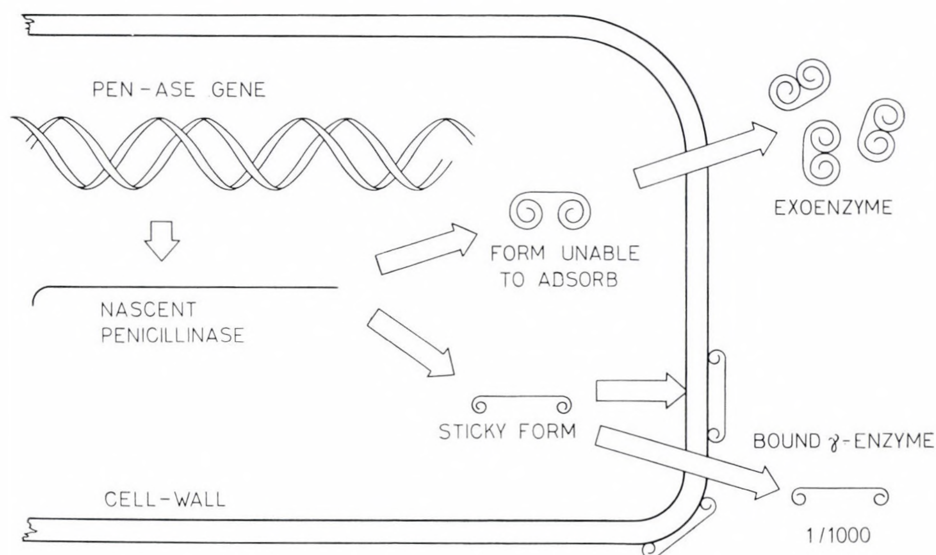


Fig. 10. Formation of bound penicillinase

bound γ-penicillinase produced during induction was formed by secondary adsorption.

The experiments presented give further support to this hypothesis. Our attempts at demonstrating the irreversible adsorption of exopenicillinase have been successful. Bound γ-enzymes adsorbed irreversibly or formed during induction showed a weak reaction with anti-exopenicillinase. The immune response of both enzymes was enhanced by alkali. Our experiments performed thus far suggest that the exopenicillinase molecule carries an "attachment centre" which — in the case of exopenicillinase — is buried deep in the molecule. However, another conformation of exopenicillinase exists when the "attachment centre" reaching the surface allows an intensive adsorption to any suitable surface. Similar attachment centres have been suggested also for other proteins [12].

Penicillinase is formed during biosynthesis in a conformation from which both types — capable and incapable of adsorption — can develop. It is the intracellular conditions that determine the ratio of the enzyme produced to

develop into bound γ-enzyme and of the enzyme leaving the cell. A small proportion, 0.1%, of the extracellular enzyme exhibits the conformation allowing for adsorption. The presence of electrolytes results in a decrease of the amount of bound enzyme [4] since electrolytes are inhibiting the adsorption. The suggested mechanism is shown in Fig. 10.

REFERENCES

1. Pollock, M. R.: J. gen. Microbiol. **15**, 154 (1956).
2. Citri, N., Pollock, M. R.: Adv. Enzymol. **28**, 237 (1966).
3. Pollock, M. R., Krámer, M.: Biochim. J. **70**, 665 (1958).
4. Csányi, V.: Acta microbiol. Acad. Sci. hung. **17**, 91 (1970).
5. Csányi, V.: Acta biochim. biophys. Acad. Sci. hung. **1**, 149 (1966).
6. Csányi, V., Mile, I., Ferencz, I., Szabó, É.: Biochim. biophys. Acta (Amst.) **198**, 332 (1970).
7. Csányi, V., Mile, I., Ferencz, I., Szabó, É.: Acta microbiol. Acad. Sci. hung. **18**, 7 (1971).
8. Citri, N.: Biochim. biophys. Acta (Amst.) **27**, 277 (1958).
9. Zittle, C. A.: Adv. Enzymol. **14**, 319 (1953).
10. Ullman, R., Koral, J., Eirich, F. R.: Proc. 2nd Int. Congress Surface Activity **3**, 485 (1952).
11. Mile, I., Csányi, V., Ferencz, I.: Acta biochim. biophys. Acad. Sci. hung. **5**, 33 (1970).
12. King, C. A.: Biochim. biophys. Acta (Amst.) **154**, 269 (1968).

Address of the authors:

Vilmos Csányi, Imre Mile, Tibor Romhányi, Pál Hortobágyi, Ferenc Hatfaludi
Institute of Medical Chemistry, Semmelweis University Medical School
Puskin u. 9, Budapest VIII, Hungary

Acta microbiol. Acad. Sci. hung. 18, 149–154, 1971

SOME EPIDEMIOLOGIC PROBLEMS OF HUMAN LISTERIOSIS IN HUNGARY

By

E. MÉRŐ and B. RALOVICH

National Institute of Public Health (Director: T. BAKÁCS), *Budapest, and Institute of Microbiology (Director:* K. RAUSS), *University Medical School, Pécs*

(Received April 26, 1971)

Summary. Examinations of the faeces of healthy slaughter-house workers, healthy persons of other employments, pregnant and postpartum women and enteritis patients were carried out with the aim to estimate the occurrence of *Listeria monocytogenes* in Hungary.

The faecal samples were cultivated at 4 °C using Holman's cooked-meat medium and a selective serum agar containing trypaflavine and nalidixic acid.

The organism was isolated from 3.5% of the nearly 3900 samples examined. The strains were serotype 1, 3, 4 and also non-typable strains were encountered.

It is concluded that human carriers of *L. monocytogenes* are not infrequent in Hungary and that they may be responsible in the spread of disease.

The fact that some animals may suffer from *Listeria monocytogenes* infection is known since 1911 [1]. The first human case was reported by NYFELDT in 1929 [2]. The pathogenesis of the infection is not clear and its epidemiology has not been investigated sufficiently. Apart from some rare outbreaks human illness occurs sporadically. The number of bacteriologically confirmed cases hitherto diagnosed comes to several thousands.

In Hungary the study of listeriosis was initiated by veterinarians MÓCSY and SÁLYI [3] reporting on listeriosis in sheep. Later the organism was demonstrated by several authors from the goose, cattle, pig, rabbit and guinea pig [4–8].

As regards human infection, the first observation was published by PÁLÓCZY [9]. Investigations with the aim to obtain further informations on the epidemiology and pathogenesis of the organism have been carried out since 1957 [10–37]. A human case of purulent meningitis was reported by RODLER and SZEMES in 1966 [38] and the authors presented bacteriological evidence of its aetiology. In 1960, RÉDEY [39] isolated listeria from a food sample. RALOVICH *et al.* [34] cultivated *L. monocytogenes* from the throat of a symptomless shepherd in 1968. In the same year FAZEKAS *et al.* [28] diagnosed *L. monocytogenes* as the causative agent of a miscarriage and a premature birth. BODNÁR *et al.* [33] reported the infection of a pregnant woman. Since then five additional bacteriologically proven cases have been described.

At the Bacteriological Department of the National Institute of Public Health we have examined the occurrence of *L. monocytogenes* in sewage and surface water. Forty per cent of the sewage samples proved to contain

L. monocytogenes and even 0.5% of surface water samples was bacteriologically positive. This observation, especially if one compares the figures to those obtained by the veterinary service, calls for particular attention and suggests that listeria is widespread in this country. This alone may well justify a study of the importance of *L. monocytogenes* infection in human populations.

In the present report we wish to give an account on our investigations for the demonstration of *L. monocytogenes* in human faecal samples, and attempt to give an estimate on the incidence of the organism, based in part on authentic isolations carried out till now in Hungary.

Materials and methods

Faecal samples were examined in the following groups of persons. 1. Healthy slaughter-house workers. 2. Healthy persons of other employments. 3. Pregnant and postpartum women. 4. Gastroenteritis patients.

Cultivation, isolation and identification of the strains. A small quantity of faeces was inoculated in Holman's cooked meat medium. The tubes were kept at 4 °C and after an incubation period of fourteen days, one month and three months, subcultures were made on selective serum agar containing trypaflavine and nalidixic acid (TNSA) [40]. The plates were examined by Henry's oblique lighting method, suspect colonies were isolated and identified according to the principles described previously [40].

As listeriosis is not notifiable in Hungary, the data on human cases have been collected from the literature or are based on personal communications.

Results and discussion

We were successful in isolating the organism from 3.5% of the nearly 3900 specimens examined. This incidence is in accordance with the finding of several authors, who stated that *L. monocytogenes* can often be detected in human faeces. Most of the strains isolated were serotype 4, or belonged to the group of non-typable (NT) strains (32.4 and 33.5%, respectively). Less frequent were serotype 1/2 strains (23.3%), and serotype 3 isolates were encountered only in 10.5%. In Table I the strains referred to as NT types morphologically and biochemically behaved as typical *L. monocytogenes* and shared some antigen components characteristic of listeriae, but they could not be typed according to the accepted antigenic scheme. WELSHIMER and DONKER-VOET [42] also reported that such strains were often isolated from sewage and faecal samples, and that they proved to be avirulent for laboratory animals (no reaction after the conjunctival infection of guinea pigs, and mice were not killed by intraperitoneal injection of the cultures). The pathogenetic role and epidemiological importance of NT strains is not sufficiently clear.

We cultivated *L. monocytogenes* from the faeces of 86 slaughter-house workers (an incidence of 4.2%). That the high occurrence of *L. monocytogenes*

Table I

Incidence of L. monocytogenes carriers in Hungary 1970—71

Groups	Number of samples examined	Serotypes				Total	
		1/2	3	4	NT*	No. of cases	Per cent
1. Healthy slaughter-house workers	2055	20	10	33	23	86	4.2
2. Other employees (healthy)	212	2	—	3	1	6	2.8
3. Pregnant and postpartum women (healthy)	1164	8	4	4	18	34	2.9
4. Enteritis patients	399	1	—	3	3	7	1.8
Total	3830	31	14	43	45	133	3.5

* Non-typable strains according to the accepted antigenic scheme.

is correlated with direct contact with animals is suggested also by the findings of BOJZEN-MØLLER [43] who found the organism in 4.8% in the faeces of healthy slaughter-house workers (55 excreters out of 1150 persons investigated).

Among healthy individuals of other employments *L. monocytogenes* carriers were found in 2.8%. This group comprised persons who were submitted to employment cultural test or subject to the regular bacteriological examination of their faeces (employees in the catering trade or in children's communities, etc.). In this group *L. monocytogenes* was detected from the faeces of a shop assistant and from that of a canning factory worker, and even a mother milk donor was found to be excreter. These observations indicate that in addition to milk and dairy products other foodstuffs may play a significant part in the spreading of listeriosis.

Thirty-four of 1164 pregnant and postpartum women harboured *L. monocytogenes* but no disorders of any kind were observed either in the affected mothers or their infants. Though pregnant women with a positive bacteriological finding were treated with antibiotics, two of them still excreted the organism at the control laboratory examination carried out after parturition.

In the group of 399 gastroenterities patients *L. monocytogenes* was isolated in 7 cases (1.7%). Other enteropathogenic bacteria were not cultivated from the faeces of *L. monocytogenes* excreters. Similar results were reported by BOJZEN-MØLLER [43], who had found the organism in 6 cases (1%) out of 620 enteritis patients. According to all evidence available the aetiological bearing of *L. monocytogenes* upon enteric infections is out of question.

We may conclude from the above findings and from the literature on the subject that human carriers play an important part in the spread of

L. monocytogenes infection of man. It seems likely, on the other hand, that the organism passing into the intestinal tract under normal circumstances exerts no harmful effect upon the host organism. Special conditions, however, of which we ignore the nature, may give rise to the variable manifestations of listeriosis.

From our investigations evidence has accumulated to suggest that *L. monocytogenes* is not less common in Hungary than in other countries. It appears, moreover, that as regards the source of infection (ill animal, healthy human and animal excreters) and the factors responsible of its transmission (foodstuffs, stools, infected slopwater) the same is valid in this country as in the U.S.A. or in Germany.

In the last 6 years in Hungary nine bacteriologically confirmed human cases of listeriosis were on record eight of which have dated from the recent four years. The cases were found in counties Tolna, Zala, Szabolcs-Szatmár, Veszprém and in the town of Budapest. Five of the affected persons were males and four were females. Six of the infections were lethal, five of them as a result of meningitis and one in the form of perinatal death. From the small number of findings no conclusions can be drawn concerning the age distribution of the disease; newborn infants, young and aged adults were equally affected. Six times the causative organism was demonstrated from cerebrospinal fluid, three isolations were carried out from other sources (spleen, liver, lung, placenta, umbilical blood, lochia). Seven strains were serotype 1/2, two isolates belonged to serotype 4 (Table II).

Clearly, the prevention of listeriosis is a complex public health problem, which has both veterinary and food-hygienic aspects. Defence presents much

Table II

Bacteriologically confirmed cases of listeriosis in Hungary 1965—71

Site of the isolation	1965	1966	1967	1968	1969	1970	1971	Total No. of cases	References
County Tolna (Szekszárd)	1[a]	—	—	1[b]	—	—	—	2	[a] [38] [b] [44]
County Szabolcs-Szatmár (Szokoly, Nagykálló)	—	—	—	2	—	—	—	2	[33] [28]
County Veszprém (Herend)	—	—	—	—	1	—	—	1	[45]
County Zala (Lenti, Lovászi)	—	—	—	—	—	2	—	2	[46]
Budapest	—	—	—	—	—	1[a]	1[b]	2*	[a] [47] [b] [48]
Total	1	—	—	3	1	3	1	9	

* both strains were serotype 4b.

difficulty as we almost completely ignore the epidemiology of the disease. There is, however, a considerable amount of evidence to show that in the dissemination of the infection both human and animal symptomless excreters may play some part. As pointed out by GRAY and KILLINGER [49] it is not clear whether human listeriosis is due chiefly to a direct contact with ill animals or animal carriers. KAMPELMACHER and NOORLE JANSEN [50] concluded that a direct contact with animals does not play a major part in determining the spread of the disease. To what extent are foodstuffs of animal origin concerned in the transference of listeriosis is still open to investigation. Infected soil, water and sewage may probably also act as means of spreading the disease. Mention should be made of the observations of SEELIGER *et al.* [51] who suggested that both human and animal outbreaks could survive in themselves as separate recurrences. They succeeded in isolating *L. monocytogenes* from the faeces of a woman who had a history of four abortions, from her husband's faeces, from the soil of their garden and from the sewage with which they had manured the garden. A possible chain of infection might be as follows: faeces containing *L. monocytogenes* — pollution of the soil — contamination of salad or vegetable with soil or excreta (sewage) — oral infection of man.

On the basis of other authors' findings [42, 50] and our observations there is every reason to believe that the causative organism is excreted in the faeces by a high percentage of the affected persons; hence the insistence on a high standard of personal hygiene in all who are in direct contact with animals, or who are engaged in preparing food, employed in children's communities is of outstanding importance. Sanitary education and hygienic information among pregnant women and in rural population is an additional precaution.

As a prophylactic means against the listeriosis of pregnants and against perinatal death the search for *L. monocytogenes* in the faeces and in the vaginal specimen of pregnant women is advisable. In the case of a positive bacteriological finding, or of fever of uncertain origin during pregnancy, the administration of specific antibiotics is recommended.

REFERENCES

1. MAGUIRE, B. J., RILEY, H. D.: Amer. J. med. Sci. **254,** 421 (1967).
2. NYFELDT, A.: C. R. Soc. Biol. (Paris) **101,** 590 (1929).
3. MÓCSY, J., SÁLYI, G.: Magy. Állatorv. Lap. **7,** 172 (1952).
4. CSONTOS, L., DERZSY, D., TÓTH BARANYI, I.: Magy. Állatorv. Lap. **10,** 110 (1955).
5. CSONTOS, J., PESTI, L.: Magy. Állatorv. Lap. **11,** 204 (1956).
6. CSONTOS, L., SZEMERÉDY, G.: Magy. Állatorv. Lap. **13,** 245 (1958).
7. VETÉSI, F., KEMENES, F.: Magy. Állatorv. Lap. **20,** 406 (1965).
8. FORRAY, A.: Personal communication.
9. PÁLÓCZY, J.: Orv. Hetil. **97,** 689 (1956).
10. PATAKY, L.: Adatok a csírasejtek, az embrio és a magzati károsodáshoz. Komárom megyei Orvosnapok közleménye. 1957.
11. PATAKY, L., KEMENES, F.: Orv. Hetil. **109,** 3489 (1968).
12. PATAKY, L., KEMENES, F.: Orv. Hetil. **110,** 1409 (1969).

13. FÜZI, M., PILLIS, I.: J. Bact. **81**, 155 (1961).
14. FÜZI, M., PILLIS, I.: Zbl. Bakt. I. Abt. Orig. **186**, 556 (1962).
15. FÜZI, M., PILLIS, I.: Nature (Lond.) **196**, 195 (1962).
16. FÜZI, M., PILLIS, I.: Kísérl. Orvostud. **15**, 148 (1963).
17. FÜZI, M., PILLIS, I.: Kísérl. Orvostud. **15**, 280 (1963).
18. FÜZI, M.: J. Path. Bact. **85**, 524 (1963).
19. FÜZI, M., PILLIS, I.: Kísérl. Orvostud. **18**, 169 (1966).
20. FÜZI, M., CSUKÁS, Z.: Orv. Hetil. **109**, 1854 (1968).
21. FÜZI, M., PILLIS, I.: Kísérl. Orvostud. **22**, 261 (1970).
22. DÖMÖTÖRI, J., SZEMERÉDI, G., GIMES, R.: Orv. Hetil. **103**, 1210 (1963).
23. DÖMÖTÖRI, J., KISZEL, J.: Magy. Nőorv. Lap. **30**, 348 (1967).
24. DÖMÖTÖRI, J., KISZEL, J.: Zbl. Gynäk. **89**, 714 (1967).
25. CZEIZEL, E., BOGNÁR, Z., HANCSÓK, M.: Orvosképzés **38**, 472 (1963).
26. CZEIZEL, E., BOGNÁR, Z., HANCSÓK, M.: Egészségtudomány **8**, 353 (1964).
27. HANCSÓK, M., CZEIZEL, E.: Magy. Nőorv. Lap. **30**, 126 (1967).
28. FAZEKAS, Á., BODNÁR, L., KEMENES, F.: Orv. Hetil. **109**, 2323 (1968).
29. FAZEKAS, Á., BODNÁR, L., ERTNER, I., KEMENES, F.: Gyermekgyógyászat **20**, 131 (1969).
30. FAZEKAS, Á., BODNÁR, L., KEMENES, F.: Egészségügyi felvilágosítás **10**, 114 (1969).
31. FAZEKAS, Á., BODNÁR, L., KEMENES, F.: Gyermekgyógyászat **20**, 185 (1969).
32. BODNÁR, L., FAZEKAS, Á., KEMENES, F.: Magy. Nőorv. Lap. **32**, 182 (1969).
33. BODNÁR, L., FAZEKAS, Á., KEMENES, F.: Orv. Hetil. **109**, 2500 (1968).
34. RALOVICH, B., VÖRÖS, S., MÁLOVICH, I., DOSZPOD, J., GÁTI, I., FORRAY, A.: Egészségtudomány **13**, 345 (1969).
35. RALOVICH, B., FORRAY, A., MÉRŐ, E., MÁLOVICH, I.: Orv. Hetil. **111**, 1583 (1970); Zbl. Bakt. I. Abt. Orig. **241**, 231 (1970).
36. BOGNÁR, Z., CZEIZEL, E., HANCSÓK, M., HEGYESSY, G.: Orv. Hetil. **102**, 2079 (1962).
37. RÁCZ, P., TENNER, K., MÉRŐ, E.: Egészségtudomány **14**, 379 (1970).
38. RODLER, M., SZEMES, F.: Orv. Hetil. **107**, 2041 (1966).
39. RÉDEY, B.: Personal communication.
40. RALOVICH, B., FORRAY, A., MÉRŐ, E., MÁLOVICH, I., SZÁZADOS, I.: Egészségtudomány **14**, 305 (1970); Zbl. Bakt. I. Abt. Orig. **216**, 88 (1971).
41. SEELIGER, H. P. R.: Problem der Listerioseforschung. Symposion mit internationaler Beteiligung, Mai 1968, Leipzig. Thieme, Leipzig 1969.
42. WELSHIMER, H. J., DONKER-VOET, J.: J. Appl. Microbiol. **21**, 516 (1971).
43. BOJZEN-MØLLER, J.: Occurrence of *Listeria monocytogenes* in feces from healthy and sick persons. Proc. Scand. Congr. Pathology and Microbiology, Norwegian Universities Press, Oslo 1964.
44. KRASSÓY, K., SZEPES, J.: Orv. Hetil. **111**, 1766 (1970).
45. RÉDEY, B.: Personal communication.
46. LACZKÓ, M.: Personal communication.
47. TELEGDY, L., CSUKÁS, ZS., HAMMER, S.: Orv. Hetil. **112**, 2585 (1971).
48. BÁN, É.: Personal communication.
49. GRAY, M. L., KILLINGER, A. H.: Bact. Rev. **30**, 309 (1966).
50. KAMPELMACHER, E. H., NOORLE JANSEN, L. M.: Zbl. Bakt. I. Abt. Orig. **211**, 353 (1969).
51. SEELIGER, H. P. R., WINKHAUS-SCHINDL, I., ANDRIES, L., VIEBAHN, A.: Path. et. Microbiol. (Basel) **28**, 590 (1965).

Address of the authors:

ENDRE MÉRŐ
National Institute of Public Health
Gyáli út 2—6, Budapest IX, Hungary

BÉLA RALOVICH
Institute of Microbiology, University Medical School
Szigeti út 12, Pécs, Hungary

Acta microbiol. Acad. Sci. hung. 18, 155—158, 1971

PHAGE TYPING OF MYCOBACTERIUM TUBERCULOSIS STRAINS ISOLATED IN HUNGARY

By

EDITH VANDRA and T. FODOR

"Korányi" National Institute for Tuberculosis and Pulmonology (Director: I. HUTÁS), Budapest

(Received April 27, 1971)

Summary. The phage type distribution of 152 *Mycobacterium tuberculosis* strains has been examined by the use of phages DS6A, GS4E, BG1, D-34 and BK1. The incidence of strains according to the grouping of BATES *et al.* was: A, 8.5%; B, 16.5%; C, nil; intermediate 75%.

In recent years a new progress has been achieved in the classification of mycobacteria. By the use of phages, BAESS [1], BATES and FITZHUGH [3], TOKUNAGA, MARUYAMA and MUROHASHI [15] and SULA and REDMOND [11] distinguished several subgroups in *Mycobacterium tuberculosis*. The incidence of the subgroups has been surveyed in several countries. The present study is a contribution to this work.

Materials and methods

Strains. All 152 *M. tuberculosis* strains were isolated from tuberculous patients. Out of the strains 108 were cultured from sputum, 24 from urine and 20 from gynaecological material. With the standard method [12] each specimen was cultured on 2 modified SULA media [14] and on 2 Löwenstein—Jensen media. One of the latter contained PAS (100 μg/ml) for the differentiation of atypical strains. Cultures isolated from patients with pulmonary tuberculosis were all sensitive to INH, those cultured from urine and gynaecological specimens were partly sensitive, partly resistant to INH. Drug sensitivity was determined by the method of CANETTI *et al.* [5] as modified by SZABÓ [13]. On the basis of biochemical tests and PAS sensitivity all strains belonged to *M. tuberculosis.*

Biochemical methods. Nitrate reductase was detected as described by VIRTANEN [17]. One loopful of a 4-week culture was suspended in 2 ml 1% KNO_3 dissolved in phosphate buffer (pH 7.0, 1/15 *M*). After 2 hours incubation at 37 °C, 0.5 ml Griess—Ilosvay reagent was added. The change in colour was recorded after 10 minutes standing at room temperature. KONNO's niacin test [7] was performed as modfied by MEDVECZKY [8] using 3 weeks old Löwenstein—Jensen cultures and 3% alcoholic benzidine with concentrated cyanogen bromide.

Phage typing was performed by the method of REDMOND and WARD [10]. Phages DS6A, GS4E, BG1 of REDMOND [9], D-34 of FROMAN *et al.* [6] and BK1 of BAESS [1] were used at routine test dilutions (1 : 1000 for DS6A, GS4E and BK1, 1 : 100 for BG1 and 1 : 10 for D-34). Cultivation of the strains and typing were performed on the medium described by REDMOND and WARD [10]. The degree of lysis (0—4) was read after 1 week incubation.

The results are shown on the basis of the schema of BATES *et al.* [3, 4], who distinguished subgroups A, B and C and intermediate group. Table I shows subgroups A, B and C as determined by phages DS6A, GS4E, BG1 and D-34 and the intermediate group divided by us into units a, b, c and d. The action of phage BK1 on subgroup A, B and C strains and on intermediate cultures is presented on the basis of experience gained in the study of BATES and MITCHISON [4].

Table I

Classification of M. tuberculosis with phages

Phage types		Phages				
		DS6A	GS4E	BG1	D-34	BK1
A		+	—	—	—	—
B		+	+	+	—	+
C		+	+	+	+	·
Intermediate	a	+	+	—	—	—
	b	+	—	+	—	—
	c	+	+	+	—	—
	d	+	—	+	—	+

· = no data

Results

Fig. 1 shows that 83 strains (54.6%) fell into subgroup Intermediate b. According to the grouping of BATES *et al.* only 25% of the strains belonged to subgroups A and B, the remaining strains could be classified only as intermediate cultures. None of our strains belonged to subgroup C.

Comparison of the origin and phage type of the isolates is shown in Table II. As indicated by the χ^2 test, the difference in distribution of phage types in sputum, urine and gynaecological specimens was not significant.

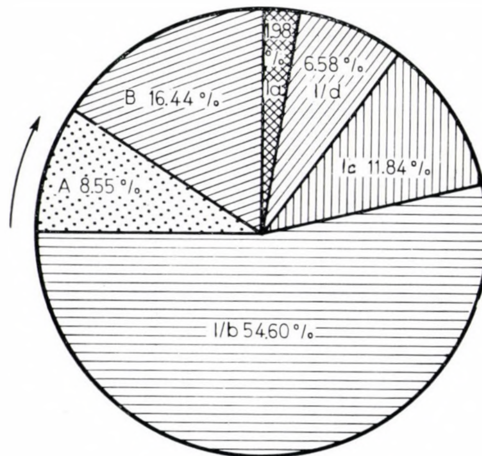


Fig. 1. Incidence of phage subgroups

Table II

Distribution of M. tuberculosis phage types according to source

Source	Phage subgroups							Total
	A	B	C	Intermediate				
				a	b	c	d	
Sputum	11	15	—	2	61	11	8	108
Urine	1	7	—	—	14	1	1	24
Gynaecological specimens	1	3	—	1	8	6	1	20
Total No.	13	25	—	3	83	18	10	152
Per cent	8.55	16.44		1.98	54.60	11.84	6.58	100.00

Discussion

The purpose of phage typing of *M. tuberculosis* strains was to obtain data as to the incidence of phage subgroups in Hungary.

Baess [1] was the first to use the phage sensitivity of *M. tuberculosis* as an epidemiological marker. Although a great part of the isolates was resistant to phage BKl, Baess concluded that the method was suitable for the division of the species into subgroups.

Bates and Fitzhugh [3] used 4 phages and were able to classify 92 strains into 3 different subgroups. Subgroup A was the most frequent in tuberculous patients.

Tokunaga *et al.* [15] showed that *M. tuberculosis* strains isolated from epidemiologically connected cases were identical in phage sensitivity. Isolates originating from different foci showed no such association. Later, Bates and Mitchison [4] reported on the typing by 5 phages of 255 strains collected from patients with pulmonary tuberculosis in countries of different geographical situation. Subgroup A showed the highest incidence in all countries (Hong Kong 89%, Rhodesia 78%, Madras 54%, England 50%). In addition to subgroups A, B and C they distinguished an "intermediate" group. They described this group as inhomogeneous and not well definable by phage typing.

Only 25% of our 152 *M. tuberculosis* strains fell into subgroups A and B. In contrast to the above findings group A was encountered infrequently (8.5%). As about 75% of our strains belonged to the intermediate subgroup, we attempted to make a subdivision. As shown in Table I, we established 4 new units termed a, b, c and d. The lysis patterns of these subgroups showed an intermediary position between subgroups A and B. The majority of our

isolates (83 strains, 54.6%) fell into intermediate b. In incidence subgroup B and intermediate c were next in order. This distribution was characteristic of all kinds of specimens examined.

From patients with pulmonary tuberculosis only INH sensitive strains were studied since INH resistance may cause difficulties in distinguishing *M. tuberculosis* and *M. bovis*. In view of the small number of strains isolated from urine and gynaecological specimens, both INH sensitive and resistant strains were used if other tests doubtlessly indicated *M. tuberculosis*. Phage sensitivity is independent of drug resistance [3, 16] and thus the latter did not influence our results.

The incidence of phage subgroups varies according to countries [4] and authors. The difference may be due partly to geographical, partly to methodical reasons. Let it suffice to mention the results of BAESS [2] which varied with different phage concentrations. Elucidation of this problem needs further studies.

REFERENCES

1. BAESS, I.: Amer. Rev. resp. Dis. **93,** 622 (1966).
2. BAESS, I.: Acta path. microbiol. scand. **76,** 464 (1969).
3. BATES, J. H., FITZHUGH, J. K.: Amer. Rev. resp. Dis. **96,** 7 (1967).
4. BATES, J. H., MITCHISON, D. A.: Amer. Rev. resp. Dis. **100,** 189 (1969).
5. CANETTI, G., RIST, N., GROSSET, J.: Rev. Tuberc. **27,** 217 (1963).
6. FROMAN, S., WILL, D. M., BOGEN, E.: Amer. J. publ. Hlth **44,** 1326 (1954).
7. KONNO, K.: Science **124,** 985 (1956).
8. MEDVECZKY, E.: Amer. Rev. resp. Dis. **81,** 757 (1960).
9. REDMOND, W. B.: Excerpta med. Int. Congr. Ser. **63,** 317 (1963).
10. REDMOND, W. B., WARD, D. M.: Symposia in Mycobacteria and Experimental Tuberculosis. Tuberculosis Research Institute, Prague 1965.
11. SULA, L., REDMOND, W. B.: Proceedings on the Sixth Symposium on Isolation, Classification and World-wide Distribution of Mycobacteria. Tuberculosis Research Institute, Prague 1970.
12. SZABÓ, I.: Tuberk. Kérd. **8,** 92 (1955).
13. SZABÓ, I.: Beitr. klin. Tuberk. **136,** 299 (1967).
14. TÁRNOK, I., SZABÓ, I.: Z. Tuberk. **120,** 74 (1963).
15. TOKUNAGA, T., MARUYAMA, Y., MUROHASHI, J.: Amer. Rev. resp. Dis. **97,** 469 (1968).
16. VANDRA, E., GÁL, F.: Acta microbiol. Acad. Sci. hung. **5,** 43 (1958).
17. VIRTANEN, S.: Acta tuberc. scand. Suppl. 48 (1960).

Address of the authors:

EDIT VANDRA, TAMÁS FODOR
"Korányi" National Institute for Tuberculosis and Pulmonology
Pihenő út 1, Budapest XII, Hungary

Acta microbiol. Acad. Sci. hung. 18, 159—165, 1971

BACTERIAL CARDIOLIPINS AND THEIR SEROLOGICAL ACTIVITY

By

L. Váczi, Margaret Surján, Jolán M. Kiss, G. Füst and I. Rédai

Institute of Microbiology (Director: L. Váczi), *University Medical School, Debrecen and National Institute of Public Health (Director:* T. Bakács), *Budapest*

(Received May 17, 1971)

Summary. *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Clostridium novyi* and *Clostridium tetani* were analysed for total lipid and cardiolipin content. Cardiolipin constituted 0.2—0.7%, total lipid 3.1—9.5% of the dry weight of bacteria. Alteration in cultural conditions resulted in a change in total lipid and phosphatide content but not in cardiolipin content.

Cardiolipins varied not only quantitatively but also qualitatively in the organisms tested; glycerol : phosphorus ratio determinations indicated structural differences.

Bacterial cardiolipins were equivalent in sensitivity and specificity to international standard products. After 1 year storage all specimens were stable.

A considerable part of bacterial lipids consists of phospholipids. These substances are partly functional partly structural constituents of the bacterial cell. Among phospholipids, disphosphatidyl glycerol (cardiolipin) is an especially important substance present in Gram-negative and in Gram-positive bacteria.

The purpose of the present investigations was to compare quantitatively and qualitatively the cardiolipin content in 5 different bacteria. In order to obtain data for the suitability of bacterial cardiolipins in the serological diagnosis of syphilis, the activity of cardiolipin preparations was compared to international standard products.

Materials and methods

Strains. Escherichia coli strain 30006 and *Proteus vulgaris* strain 61001 were obtained from the Hungarian National Collection of Medical Bacteria, National Institute of Public Health, Budapest. *Pseudomonas aeruginosa* strain 21 was isolated in the Institute of Microbiology, Debrecen. Anaerobic bacteria were represented by *Clostridium novyi* and *Clostridium tetani* Harvard strains.

Culture media. Gram-negative bacteria were cultured in the following media. *Medium I.* Casitone (Difco), 10 g; NaCl, 5 g; Na_2HPO_4, 5 g; KH_2PO_4, 5 g; $Fe(NH_4)_2 SO_4$, 0.03 g; $MnSO_4$, 0.01 g; $MgSO_4$, 0.01 g; distilled water, 1000 ml. *Medium II* was prepared by adding 1% yeast extract (Difco) to medium I. *Medium III* was prepared by adding 1% yeast extract (Difco) and 10% human serum to medium I. *Medium IV* was prepared by adding 10% human serum to medium I. The media were adjusted to pH 7.2. All ingredients were analytical grade reagents. The inoculated media were shaken at 120 r.p.m. for 18 hours at 37 ± 1 °C.

Cl. novyi was cultured in a medium containing yeast extract (Difco), 5 g; trypcasin, 15 g; glucose, 5 g; sodium thioglycollate (Schuchardt), 0.5 g; NaCl, 2.5 g; 0.2% methylene-blue solution, 0.5 ml; distilled water, 1000 ml. Standing cultures were incubated for 18 hours at 37 °C. *Cl. tetani* culture was prepared in Fisek—Mueller—Miller medium by the Institute for Serobacteriological Production and Research Human, Budapest.

Extraction and analysis of lipids. The bacteria were harvested by centrifugation at 3600 r.p.m., washed in saline and dried in acetone (bacterial cultures in 1000 ml medium were suspended in 35 ml acetone).

After refrigeration at 4 °C for 24 hours, the bacteria were separated by filtration, dried *in vacuo* over phosphorus pentoxide and homogenized. Extraction of lipids was performed as described by VORBECK and MARINETTI [1].

Preparation of cardiolipin. Cardiolipins were prepared by two different methods. The first method was as described in [14]. The other method was as follows. The lipid extract was dried over phosphorus pentoxide then dissolved in 96% ethanol. The ethanol-insoluble fraction was separated by centrifugation at 10 000 r.p.m. at —10 °C. The supernatant, containing the main part of neutral lipids and very slight amount of polar lipids, was discarded. The deposit with the main part of polar lipids was dissolved in chloroform and fractionated on silicic acid column (length, 20 cm; diameter, 1.7 cm; contents, 4 parts 40—50 mesh and 1 part 100 mesh Merck silicic acid). The following eluants (100 ml each) were used: 1. chloroform; 2. 2% methanol in chloroform; 3. 4% methanol in chloroform; 4. 8% methanol in chloroform; 5. 15% methanol in chloroform; 6. 25% methanol in chloroform; 7. methanol.

The fractions were concentrated by evaporation. Thin-layer chromatography was performed on 500μ Silicagel G layer in chloroform : methanol : water (70 : 22 : 3) solvent. Cardiolipin was present in fractions 4 and 5. These were united, dissolved in ethanol and centrifuged as described above. The deposit soluble in chloroform : methanol (2 : 1) corresponded to pure cardiolipin.

Purified cardiolipins were analysed by determining their phosphorus content [2], glycerol content [3] and nitrogen content [4]. Measurements were performed by the use of a Joben Yvon spectrophotometer. Fatty acid constituents were determined as described in [5].

Preparation of bacterial cardiolipin for syphilis serological tests. Dried preparations of known dry weight were stored in ampoules. Test antigens were prepared immediately after dissolving the content of the ampoule in 1 ml chloroform : methanol mixture (2 : 1). To the bacterial cardiolipin specimen, internationally standardized lecithin and cholesterol were added. Control antigens were prepared with international standards of cardiolipin and lecithin. Kolmer antigens for 50% haemolysis and micro-complement test were prepared from 0.03% cardiolipin, 0.05% lecithin and 0.3% cholesterol. VDRL antigens contained 0.03% cardiolipin, 0.22% lecithin and 0.9% cholesterol.

50% haemolysis test was performed according to the WHO method [10] modified as described in [11].

Comparison of antigens. Kolmer antigens prepared from bacterial cardiolipins and from the standard antigen were compared with micro-complement test [12]. In some experiments the cardiolipin preparation was checked as VDRL antigen [12].

Statistical methods. Reactions recorded by crosses were expressed in $\log_{10}$ titres according to KÄRBER [13]. Significance of the difference in average titres between the examined and the standard antigens was tested by analysis of variance.

Results

Table I shows the total lipid content of *E. coli, P. vulgaris* and *Ps. aeruginosa* after 18 hours cultivation in basal medium and in the presence of 10% serum. The results indicated that in bacteria grown with serum the total lipid content was higher.

In subsequent experiments the proportion of neutral and polar lipids was examined. Within polar lipids the cardiolipin content was also determined. In bacteria grown in the basal medium polar lipids were present in greater proportion than neutral lipids, but in serum medium the amount of the latter increased. The cardiolipin content of the three bacteria ranged between 0.17 and 0.65% and was not significantly influenced by cultural conditions.

In comparing phosphorus, glycerol and nitrogen content of pure bacterial cardiolipins and standard beef heart cardiolipin the following results

Table I

Lipid content of Gram-negative bacteria cultured in different media

	Medium*	Percentage for dry weight of bacteria**			
		Total lipid	Neutral lipid	Polar lipid	Cardiolipin
E. coli	1	3.8	0.5	3.3	0.17
	2	4.7	2.1	2.6	0.22
P. vulgaris	1	5.4	0.5	4.9	0.20
	2	7.0	3.0	4.0	0.18
Ps. aeruginosa	1	7.2	1.7	5.5	0.65
	2	9.5	6.0	3.5	0.56

* 1 = basal medium; 2 = basal medium supplemented with 10% serum
** Average of 3 determinations

were obtained. Beef heart cardiolipin contained 4.1—4.3% phosphorus, 18.5—22% glycerol and less than 0.01% nitrogen; the phosphorus—glycerol ratio was 2 : 3. In *E. coli* and *Ps. aeruginosa* cardiolipins the phosphorus—glycerol ratio was the same as in beef heart cardiolipin, but for *P. vulgaris* a slightly different value was found. In serum-supplemented medium the bacterial cardiolipins showed a higher glycerol content and a somewhat lower phosphorus content as compared to beef heart cardiolipin.

Table II

Main fatty acids in bacterial and beef heart cardiolipins

Cardiolipins	Percentage distribution of fatty acids*						
	C_{16}	$C_{17\Delta}$	$C_{18:2}$	$C_{18:1}$	$C_{19\Delta}$	Total unsaturated	Total cyclo-propane
E. coli	40	22	—	8	20	12	42
P. vulgaris	42	30	—	+	20	1	50
Ps. aeruginosa	40	5	—	30	16	42	21
Beef heart	+	—	78	8	—	96	—

* Average of 3 determinations

In Table II are presented the main fatty acids in the three above bacteria. While in bacterial cardiolipins the majority of fatty acids corresponded to saturated or cyclopropane compounds, in beef heart cardiolipin unsaturated fatty acids predominated. In beef heart cardiolipin unsaturated double-bond fatty acid $C_{18:2}$ was present in 78%. In bacterial cardiolipins this compound was absent and the main fatty acid component was palmitic acid.

Table III shows the lipid composition of the two anaerobic bacteria. Main fatty acids in cardiolipins of these organisms are presented in Table IV. Similarly to Gram-negative bacteria, in anaerobic organisms palmitic acid was the main component amounting to 50% of all fatty acids. Myristic acid was detected also in high amounts. It was especially interesting that *Cl. tetani* cardiolipin contained no unsaturated fatty acids. In *Cl. novyi* cardiolipin the phosphorus—glycerol ratio was 2 : 3, in *Cl. tetani* a slight difference was noted from the above value.

Table III

Lipid content of clostridia

	Percentage for dry weight of bacteria*			
	Total lipid	Neutral lipid	Polar lipid	Cardiolipin
Cl. novyi	3.1	1.3	1.8	0.65
Cl. tetani	3.7	1.7	2.0	0.70

* Average of 3 determinations

Table IV

Main fatty acids in clostridial cardiolipins

Cardiolipins	Percentage distribution of fatty acids*						
	C_{14}	$C_{16:1}$	C_{16}	$C_{18:1}$	C_{18}	Total saturated	Total unsaturated
Cl. novyi	23	9	51	4	5	84	16
Cl. tetani	45	+	50	—	—	100	—

* Average of 3 determinations

The serological activity of 14 bacterial cardiolipin preparations was examined in micro-complement fixation test. Strains used for producing cardiolipins and average titres obtained with Kolmer antigens prepared from them are shown in Table V.

In serological activity 12 out of 14 cardiolipin preparations were identical with the international standard cardiolipin. Probably because of a decomposition during preparation, two cardiolipins made from *E. coli* and *P. vulgaris* showed practically no serological activity.

VDRL antigen was prepared from 3 bacterial cardiolipins (IV/P, I/P and I/PO). These were equivalent in activity with the standard VDRL antigen.

The serological activity of Kolmer antigen prepared from bacterial cardiolipin I/P was compared with the standard Kolmer antigen by examining 21 positive sera with the 50% haemolysis test. On the basis of sequential analysis, in view that the difference of G-values was higher than 0.070 only

Table V

Serological activity of bacterial cardiolipins

Cardiolipin preparation		Positive sera		Average of $\log_{10}$ titres		Significance	
		Total No.	No. reactive	test antigen	control antigen	t	P
E. coli I	I/C*	17	15	1.2183	1.2302	0.154	0.8
E. coli II	II/C	17	17	1.1321	1.2302	1.272	0.2
E. coli III	III/C	17	2	—	—	—	—
E. coli IV	IV/C	17	17	1.1801	1.2302	0.663	0.5
Ps. aeruginosa I	I/P	18	18	1.2850	1.3044	0.193	0.8
P. vulgaris I	I/V	18	18	1.2495	1.3044	0.540	0.5
P. vulgaris IV	IV/V	6	1	—	—	—	—
Ps. aeruginosa II	IV/P	18	18	1.2090	1.3044	0.945	0.3
Ps. aeruginosa	I/PO**	21	21	1.023	1.119	0.763	0.4
E. coli	I/CO	24	22	0.7840	0.8170	0.423	0.6
P. vulgaris	I/VO	24	22	0.6150	0.8170	1.239	0.2
Ps. aeruginosa	I/PO. 2.	24	24	0.8270	0.8170	0.080	0.9
Cl. novyi		12	12	0.8083	0.7288	0.828	0.4
Cl. tetani		12	11	0.7347	0.7288	0.061	0.9

* Cardiolipins were prepared according to the first method [14]
** Cardiolipins were prepared according to the second method (abbreviations in text)

in one serum, bacterial cardiolipin was considered suitable as a constituent of syphilis antigen.

Kolmer and VDRL antigens prepared from bacterial cardiolipins were tested with 50 and 150 negative sera, respectively. No aspecific reactions were obtained.

Kolmer antigen prepared from cardiolipin I/PO and a standard Kolmer antigen made at the same time were stored for 1 year, then the two antigens were compared with a freshly prepared standard Kolmer antigen. There was no significant difference in mean titres obtained with the three antigens.

Discussion

The present findings indicated that cultural conditions influence the lipid constitution of *E. coli, P. vulgaris* and *Ps. aeruginosa.* In serum-free medium polar lipids predominated and neutral lipids were present only in 10—25%. In serum-supplemented medium the ratio of polar lipids increased and a quantitative alternation of these substances was also evident. *E. coli* and *P. vulgaris* synthesized a great amount of triglycerides in the presence

of serum but failed to produce them in serum-free medium. In the lipids of *Ps. aeruginosa* there was an increase in diglycerides if the medium was supplemented with serum.

As to polar lipids, the appearance of a new component was not observed after completing the medium with serum and there was no alteration in the cardiolipin content of dry bacteria. This finding indicates that diphosphatidyl glycerol is a structural component.

The variability of phosphorus—glycerol ratio, especially the low phosphorus and high glycerol content in *P. vulgaris*, were suggestive of structural differences between bacterial cardiolipins.

The most striking difference was noted in fatty acid chains. The main fatty acid component (structural fatty acid) was palmitic acid in all bacterial cardiolipins. In other fatty acid components there was a considerable variability. It was especially interesting that *Cl. tetani* cardiolipin contained only saturated fatty acids. FAURE and MORELEC [6] and DE HAAS and VAN DEENEN [7] showed that cardiolipins including natural and synthetic preparations differed mainly in their fatty acid chains. KATAOKA and NOJIMA [8] reported similar results in actinomycetes. INOUE and NOJIMA [9] showed that natural and synthetic cardiolipins containing fatty acids varying in the degree of saturation had different R_f values. Natural cardiolipins characterized by saturated fatty acids showed lower R_f values than beef heart cardiolipins containing unsaturated fatty acids. In our experiments thin-layer chromatographic R_f values for bacterial cardiolipins were identical, with the exception of *Cl. tetani* cardiolipin which migrated less rapidly than beef heart cardiolipin.

Serological checking of purified bacterial cardiolipins indicated that they were equivalent in sensitivity and specificity with internationally standardized beef heart products. There was no difference in the serological activity of cardiolipins prepared from different bacteria. The loss of serological activity in two specimens was probably due to errors made during preparation. It may, therefore, be concluded that bacterial cardiolipins are suitable for the serological diagnosis of syphilis. This finding is of interest partly theoretically, partly from the practical point of view, since mass fermentation of bacteria is relatively inexpensive and large amounts of bacteria are available as byproducts of exotoxin preparation.

REFERENCES

1. VORBECK, M. L., MARINETTI, G. V.: J. Lipid Res. **6**, 3 (1965).
2. BARTLETT, G. R.: J. biol. Chem. **234**, 466 (1959).
3. RENKONEN, O.: Biochim. biophys. Acta (Amst.) **56**, 367 (1962).
4. LANG, C. A.: Anal. Chem. **30**, 1692 (1958).
5. VÁCZI, L., MAKLEIT, J. K., RÉTHY, A., RÉDAI, I.: Acta microbiol. Acad. Sci. hung. **11**, 383 (1964/65).
6. FAURE, M., MORELEC, C.: Ann. Inst. Pasteur **104**, 246 (1963).

7. De Haas, G. H., van Deenen, L. L. M.: Nature (Lond.) **206**, 935 (1965).
8. Kataoka, T., Nojima, S.: Biochim. biophys. Acta (Amst.) **144**, 681 (1967).
9. Inoue, K., Nojima, S.: Chem. Pharm. Bull. **16**, 76 (1968).
10. Pangborn, M. C.: Cardiolipin Antigens. WHO Palais des Nations, Geneva 1955. No. II.
11. Surján, M., Füst, G.: Z. Immun.-Forsch. **136**, 303 (1968).
12. Surján, M., Füst, G.: Orv. Hetil. **109**, 1499 (1968).
13. Kärber, G.: cit. Gaddum, J. H. in Spec. Rep. Ser. No. 183. Med. Res. Coun. London 1933.
14. Kiss, J., Rédai, I.: Kísérl. Orvostud. **24**, 84 (1972).

Address of the authors:

Lajos Váczi, Jolán M. Kiss, Imre Rédai
Institute of Microbiology, University Medical School
Debrecen 12, Hungary

Margit Surján, György Füst
National Institute of Public Health
Gyáli út 2–6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 167—177, 1971

SIGNIFICANCE OF THE "EPITHELIAL PHASE" IN EXPERIMENTAL SALMONELLA CONJUNCTIVITIS

By

CLARA TENNER, P. RÁCZ and B. RÉDEY

National Institute of Public Health (Director: T. BAKÁCS), *Budapest and Public Health Station (Director:* E. BEREND), *Veszprém*

(Received May 22, 1971)

Summary. Conjunctivitis running a regular course was induced by inoculating 10^9 bacteria of different *Salmonella typhi-murium* and *Salmonella enteritidis* strains into the conjunctival sac of guinea pigs. The development of the infective process has been followed histologically for 28 days. It was found that an "epithelial phase" precedes the penetration of salmonellae into the macrophage-type cells situated in deeper parts of the organ. During the "epithelial phase", part of the bacteria not only passes through the intercellular space but invading the conjunctival epithelial cells multiplies there.

The morphogenesis of conjunctivitis salmonellosa is compared with that of keratoconjunctivitis shigellosa. While the short "epithelial phase" observed in salmonella conjunctivitis is followed by a long "macrophage phase" during which the centre of the infection is transferred from the epithelial area to the macrophage-type cells, in keratoconjunctivitis shigellosa the shigellae are localized throughout the course of the disease to the conjunctival and corneal epithelial cells. They are rarely seen in macrophage-type cells and then only for a very short period. This difference is related to the basic biological properties of the two pathogens.

Successful induction of keratitis in guinea pigs by the local inoculation of *Salmonella typhi* has been reported by SEDAN and HERRMAN [1] in 1924.

It has been confirmed by several investigators that conjunctivitis can be induced in the guinea pig by different *Salmonella* strains [2—8]. According to certain literary data not only conjunctivitis but also keratoconjunctivitis can be induced with certain strains [9—11]. Other investigators reported on negative results [12—14]. Only one detailed work has been published [15] on the histology of salmonella conjunctivitis; even this work failed, however, to deal with the interaction between the corneal or conjunctival epithelial cells and the pathogenic salmonellae. An analysis of this phase of pathogenesis was made timely by the morphological studies of keratoconjunctivitis shigellosa [16—21], and keratoconjunctivitis listeriosa [22—24], which revealed that the invasion of the pathogen into the live cells and its multiplication must have a leading role in the pathogenesis of these experimental diseases. We have suggested to term this stage "epithelial phase" within the conceptual sphere of intracellular parasitism [25].

The purpose of the present experiments was to study the significance of the "epithelial phase".

Materials and methods

Culture preparation. Eight different strains were used for inoculation. Five of them were provided by Dr. G. ISTRATI (Dr. I. Cantacuzino Institute, Bucharest) with the following designations: *S. typhi-murium* 818, *S. typhi-murium* cobai II, *S. typhi-murium* 114, "Salmonella gärtneri" cobai II, and "Salmonella gärtneri" 374. These strains had induced kerato-conjunctivitis in guinea pigs in 1960, and were maintained in Dorset culture medium by yearly transfer till 1968. According to ISTRATI [26] their capacity to induce keratitis had been lost by this time. A *S. typhi-murium* and a *S. enteritidis* strain was isolated from a patient in the laboratory of the László Hospital for Infectious Diseases, Budapest. The animals infected with the above-mentioned strains were observed for 5 days after infection, and then histological examinations were performed.

Our main experiment which included bacteriological examinations was carried out with a *S. typhi-murium* strain freshly isolated from man by Dr. B. RÉDEY. The infective process was followed for 28 days after inoculation. Eighteen-hour agar cultures were used for infection in each experimental line.

Technique of infection. Guinea pigs of both sexes, weighing 250—300 g, from the breed of the Institute of Public Health were used. Groups of 3 animals were infected with about 10^9 organisms by means of a platinum loop into the conjunctival sac. The animals were sacrificed by cervical dislocation 1, 3, 6, 9, 12, 18, 24, 36, 48, 60 and 72 hours after infection, further on every other day up to 28 days. Exudate was taken from the left eye previous to killing, smears were prepared and stained with Giemsa. Bacterial count was determined from the conjunctival exudate of the same eye. The right eye-ball was removed together with the eyelids and the content of the bony orbit. This material was fixed in Maximov fixative and after embedding it in combined celloidin—paraffin, 3μ thin sections were prepared at 18 levels from each eye. Staining was performed with haematoxylin—eosin and thionin—methylene-blue.

Bacteriological examinations. Besides the bacterial count of the conjunctival exudate, bacteriological examinations were carried out from heart blood, liver, spleen and kidney. Results of the examinations will be reported elsewhere.

Results

The course of the pathological process was essentially identical in each experimental line, except some deviations in intensity. Thus, results only of the main experiment will be presented here.

One hour after inoculation the majority of the salmonellae was found extracellularly. However, a great number of phagocyting polymorphonuclear leucocytes were visible. Some of them had a well retained cytoplasmic structure; these contained small amounts of salmonellae. The organisms were situated in a circumscribed cytoplasmic area (Plate I, Fig. 1). Other polymorphonuclear leucocytes showed pycnotic nuclei, and their cytoplasm was almost filled with bacteria (Plate I, Fig. 2). In some cases even the desquamated conjunctival epithelial cells contained a few salmonellae. Epithelial cells with few bacteria in their cytoplasm were found occasionally, in different areas of the epithelial coat of the eyelid conjunctiva, when 1-hour sections were examined (Plate II, Fig. 1). Although there was no general rule in localization of the infected cells, it was striking that the epithelial cells containing the greatest number of salmonellae were found around a circumscribed lymphoreticular growth, situated under the epithelial coat of the conjunctiva (Plate III, Fig. 6, Figs 4 and 1). Polymorphonuclear leucocytes were often detected in the neighbourhood of the infected cells (Plate I, Figs 4 and 2).

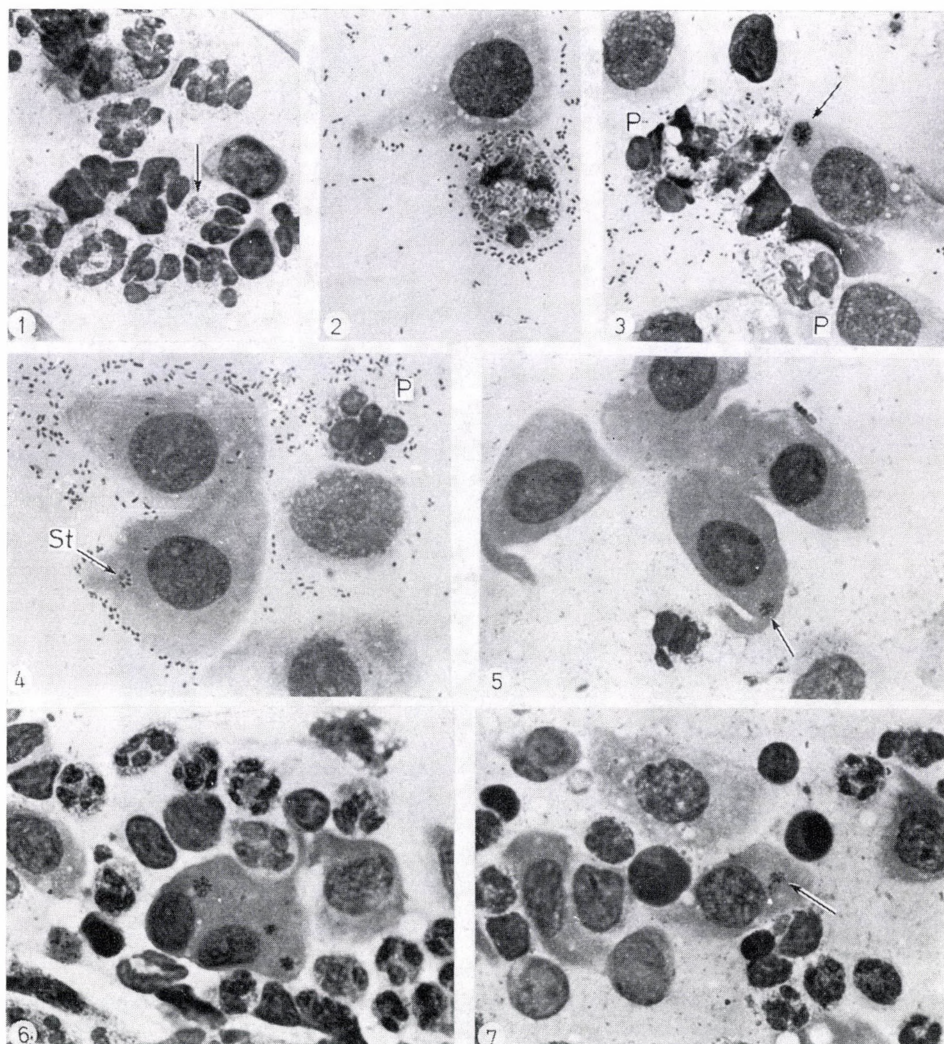


Plate I. Smears prepared from the exudate of the conjunctival sac at different intervals following inoculation (May—Grünwald—Giemsa stain, ×1200)

Fig. 1. Phagocyting polymorphonuclear leucocyte, salmonellae localized in a circumscribed area of the cytoplasm (arrow). Maintained nuclear ultrastructure, one hour after inoculation

Fig. 2. Desquamated conjunctival epithelial cell. Below is a polymorphonuclear leucocyte with pycnotic nucleus. The cytoplasm is almost completely filled with salmonellae, and a number of them are situated extracellularly. One hour after inoculation

Fig. 3. Relatively big salmonellae in polymorphonuclear leucocytes (P). "Salmonella star" in the cytoplasm of a desquamated conjunctival epithelial cell (arrow). Nine hours after inoculation

Fig. 4. Phagocyting polymorphonuclear leucocyte (P). "Salmonella star" (St) in a desquamated conjunctival epithelial cell. Nine hours after inoculation

Fig. 5. "Salmonella star" in a desquamated conjunctival epithelial cell (arrow). Nine hours after inoculation

Fig. 6. Desquamated conjunctival epithelial cells in the middle of the figure; "salmonella stars" in their cytoplasm. Twelve hours after inoculation

Fig. 7. "Salmonella star" in a desquamated conjunctival epithelial cell (arrow). Twelve hours after inoculation

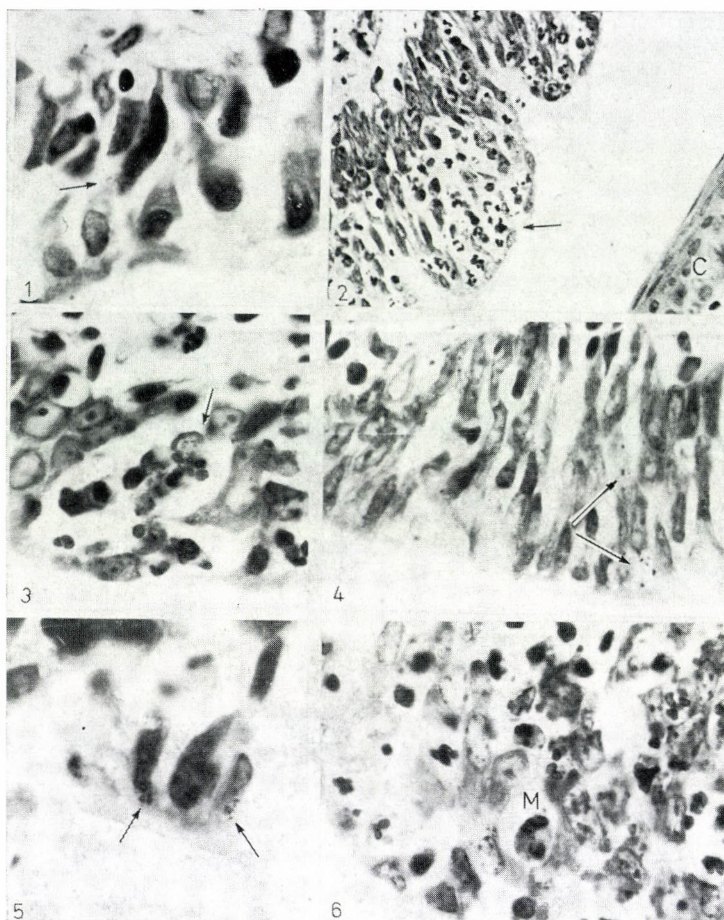


Plate II

Fig. 1. Conjunctival epithelium. Intensely staining salmonella (arrow) in cytoplasm of an epithelial cell. One hour after infection (thionin—methylene-blue stain, ×1200)

Fig. 2. "Intraepithelial abscess" (arrow) in conjunctival epithelium. Corneal epithelium (C) intact. Three hours after inoculation (thionin—methylene-blue stain, ×480)

Fig. 3. Phagocyting polymorphonuclear leucocyte in lumen of "intraepithelial abscess". Twelve hours after inoculation (thionin—methylene-blue stain, ×1200)

Fig. 4. Numerous salmonellae (arrow) in conjunctival epithelial cells. Twelve hours after inoculation (thionin—methylene-blue stain, ×1120)

Fig. 5. "Salmonella stars" (arrow) in conjunctival epithelial cells. Twelve hours after inoculation (thionin—methylene-blue stain, ×1640)

Fig. 6. Macrophage-type cell (M) in loosened conjunctival epithelium. Numerous salmonellae in the neighbouring epithelial cells. Twelve hours after inoculation (thionin—methylene-blue stain, ×1120)

Three hours after the inoculation micro-abscesses developed in the epithelium of the eyelid conjunctiva. Polymorphonuclear leucocytes gathered in foci among the epithelial cells, in the area of the infected cells (Plate III, Fig. 2). Salmonellae were visible also in the polymorphonuclear leucocytes (Plate II, Fig. 3). The calix cells were excreting increased amounts of mucus.

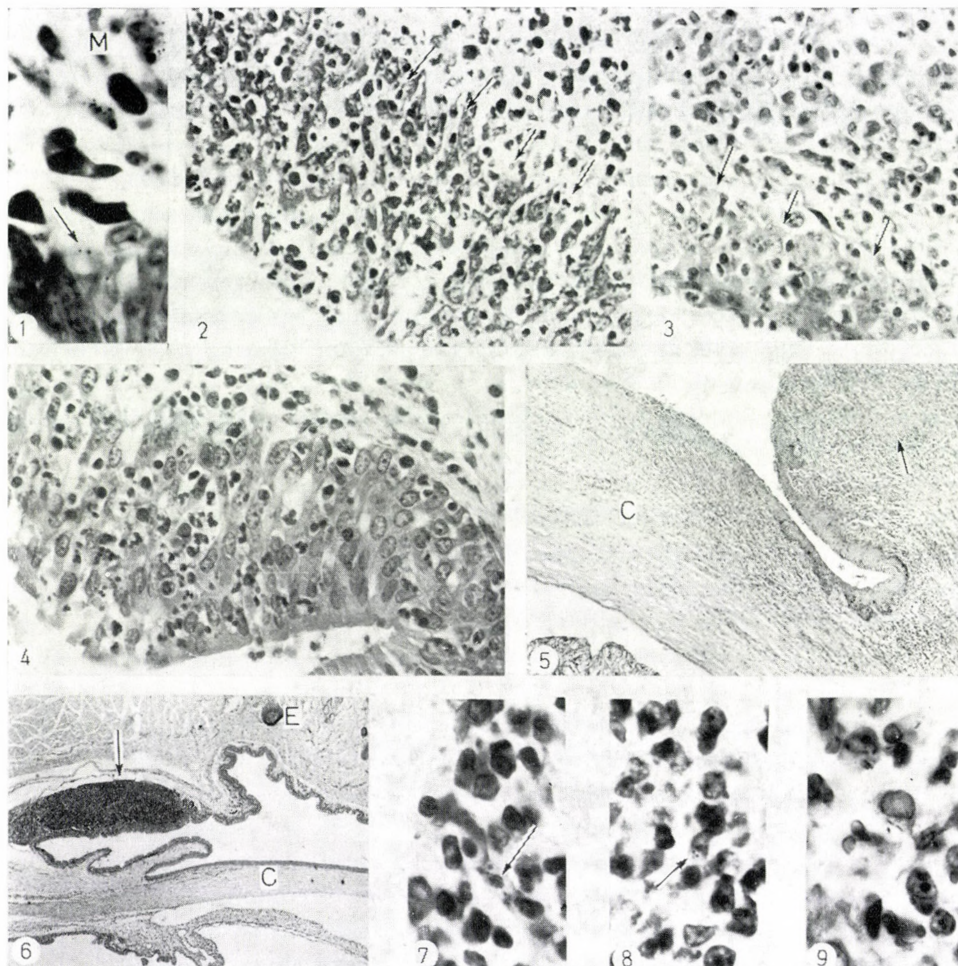
The number of the epithelial cells which contained bacteria increased gradually 6, 9 and 12 hours after inoculation (Plate II, Figs 4 and 5), and the occurrence of infected cells in the areas above the lymphoreticular tissue, *i.e.*, in other parts of the conjunctival epithelium, had became more apparent. Macrophage-type cells appeared among the cells infiltrating the epithelium (Plate II, Fig. 6). Salmonellae were frequent in these cells. Macrophage-type cells containing salmonellae were observed also in the subepithelial lymphoreticular tissue (Plate III, Figs 1, 7, 8, 9).

An unusual phenomenon was observed in the smears of epithelial cells, 9 to 12 hours after infection. Salmonellae, arranged in foci appeared in small circumscribed areas of the cytoplasm of epithelial cells, which failed to show morphological lesions: salmonella-stars developed (Plate I, Figs 3, 4, 5, 6, 7). Macrophage-type cells also appeared containing salmonellae.

Conjunctival ulceration could not be observed 24 hours after inoculation. The changes were most marked in the conjunctival area corresponding to the lymphoreticular system. The epithelium became loosened and formed a reticulum-like structure, the reticular spaces contained few leucocytes, the infiltration consisted mainly of macrophage-type cells (Plate III, Fig. 2). The number of cells containing salmonellae decreased significantly, while numerous macrophage-type cells containing bacteria appeared in the lymphoreticular tissue. Cells containing bacteria became decomposed in some areas and intensely staining extracellularly situated salmonellae as well as polymorphonuclear leucocytes could be observed around them.

Thirty-six, 48 and 72 hours after the inoculation the number of epithelial cells containing bacteria decreased rapidly and the infective process was gradually shifting to the lymphoreticular tissue, near the conjunctival fornix. A detailed analysis of this finding would need further experimental work and now only the observations connected with the "epithelial phase" will be described. It seemed of importance that great numbers of plasma cells and Russel bodies appeared after the 36th hour, near the epithelium and even among the epithelial cells. Besides, an activation of the local cells, phagocyting macrophage-type cells could be seen in the area of the eyelids (Plate III, Fig. 3). The infiltrate consisted prevalently of polymorphonuclear leucocytes only in the circumscribed epithelial areas, where bacteria persisted in the cells (Plate III, Fig. 4).

The corneal epithelium remained intact in each experimental animal till the last day of observation, 28 days after the infection. No infiltration



could be observed in the substantia propria. One animal represented an exception displaying keratitis (Plate III, Fig. 5); this animal was sacrificed 10 days after inoculation. The corneal epithelium did not show signs of destruction in this case either. At the time of the examination the corneal epithelial cells as well as the cells infiltrating the substantia propria, mostly lymphoid cells, did not contain any bacteria.

Discussion

Conjunctivitis with a regular course was successfully induced in each experimental series by introducing 10^9 organisms of *S. typhi-murium* and *S. enteritidis* into the conjunctival sac of guinea pigs. Mild keratitis developed in one animal.

The salmonellae within one hour enter the epithelial cells of the conjunctiva. Nine to 12 hours after the inoculation the number of salmonellae increases in the infected cells, which refers to the multiplication of the pathogen in the epithelium.

The penetration of salmonellae into the epithelium was reported also in connection with other organs. FLOREY [27] in 1933 applied a thick suspension of *S. typhi-murium* on the surface of the ileal loop of guinea pigs, and as a result coccoid and weakly staining salmonellae could be observed at some sites in the epithelial cells. In the opinion of this author, part of the pathogens is destroyed in the cells, while the rest may pass through the cells. The epithelial cells which contained salmonellae failed to show histological signs of cell death. Salmonellae were observed in the endothelium after experimental infection also by BELYANIN [28]. TAKEUCHI [29] confirmed by electron microscopic examinations that salmonellae enter the reactive, living epithelial cells of the endothelium; in his experiments guinea pigs preconditioned by starving and opium were infected orally with *S. typhi-murium*. In our experiments with salmonella-cystitis, the penetration and multiplication of *S. typhi-murium* and *S. enteritidis* were studied in the living epithelial cells of the bladder [30]. ARIEL and BERNOVSKAYA [31] observed salmonellae in the bronchial epithelium of some mice with experimentally induced salmonella-pneumonia.

The question arises of the way of entry of salmonellae into the conjunctival epithelium. In our opinion the process is similar to that observed in keratoconjunctivitis listeriosa [24], thus it may be a special form of endocytosis.

Plate III

Fig. 1. Salmonellae in conjunctival epithelial cells (arrow). Salmonella-phagocyting macrophage-type cell (M) in lymphoreticular tissue. Twelve hours after inoculation (thionin—methylene-blue stain, ×240)

Fig. 2. Few polymorphonuclear leucocytes in the spaces of the loosened conjunctival epithelium. The infiltrate consists mostly of macrophage-type cells. The borders of the epithelium and of the areas under the epithelium are indicated by arrows. Twenty-four hours after inoculation (thionin—methylene-blue stain, ×480)

Fig. 3. Active macrophage-type cells in the connective tissue under the conjunctival epithelium. The borders of the epithelium are indicated by arrows. Thirty-six hours after inoculation (haematoxylin—eosin stain, ×448)

Fig. 4. Infiltrate consisting of polymorphonuclear leucocytes in a circumscribed area of the conjunctival epithelium. Thirty-six hours after inoculation (haematoxylin-eosin stain, ×448)

Fig. 5. Cellular infiltrate of the substantia propria of the cornea (C). Granuloma (arrow) in the area under conjunctival epithelium. Ten days after inoculation (haematoxylin—eosin stain, ×67.5)

Fig. 6. Circumscribed lymphoreticular growth (arrow) in the eyelid of healthy guinea pig. Cornea (C), eyelid (E)

Fig. 7. Salmonellae (arrow) in the cells situated in the area of the lymphoreticular growth. Twelve hours after inoculation (thionin—methylene-blue stain, ×1200)

Fig. 8. Macrophage-type cells containing salmonellae in the area of the lymphoreticular growth (arrow). Twelve hours after inoculation (thionin—methylene-blue stain, ×1200)

Fig. 9. Macrophage-type cells containing salmonellae in the area of the lymphoreticular growth. Twelve hours after inoculation (thionin—methylene-blue stain, ×1240)

By comparing the human diseases caused by *S. typhi* with the experimental salmonella-conjunctivitis, it is of interest that in the latter case the greatest number of bacteria was found in the epithelium covering the lymphoreticular tissue. Similarly to the relations of the Payer-plaques in the intestine, here too, close connections were observed between the epithelial cells and the

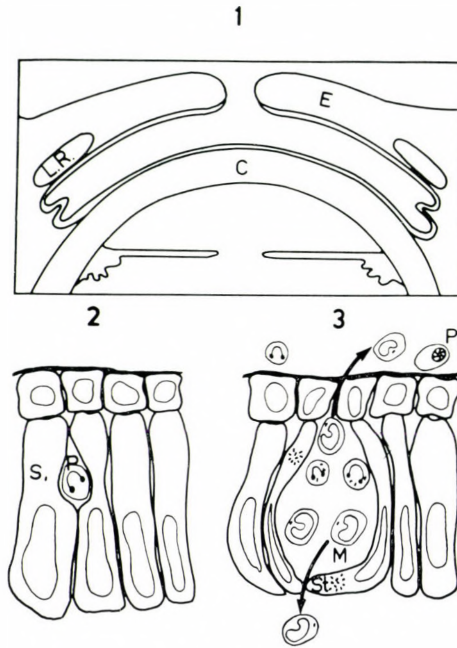


Plate IV

Fig. 1. Anatomical scheme showing the histological appearance of the eye of the guinea pig. Cornea (C), lymphoreticular tissue (L. R.), eyelid (E)

Fig. 2. One hour after inoculation. Salmonella (S) in conjunctival epithelial cell. Polymorphonuclear leucocyte (P) in the neighbourhood of the epithelial cell

Fig. 3. Late "intraepithelial abscess". Besides polymorphonuclear leucocytes, phagocyting macrophage-type cells in the lumen, the latter may enter later the subepithelial tissue or the conjunctival cavity, carrying with them the infection (the arrow shows the direction of their migration). "Salmonella star" (St) in the epithelial cells. Plasma cell (Pl) in the subepithelial infiltrate

lymphoreticular tissue. The salmonellae gain entrance through the epithelium into the macrophage-type cells of the lymphoreticular tissue and multiply there on the first days after inoculation. Later, regular typhoid nodes develop at these sites (Plate III, Fig. 5). There might exist a possibility that the macrophages containing salmonellae migrate from the lymphoreticular tissue, thus from the opposite direction, through the epithelial barrier into the conjunctival sac. Salmonella-conjunctivitis can serve as a suitable model for the study of the morphogenesis of the typhoid granuloma. This problem will be the subject of another paper.

The polymorphoncular leucocytes are of special importance from the point of view of the "epithelial phase". They appear at an early phase in the epithelium containing salmonellae. Focal cell groups and "microabscesses" develop, similarly to those observed in keratoconjunctivitis listeriosa [22]. The salmonellae are intensively phagocyted by the polymorphonuclear leucocytes. The polymorphonuclear leucocytes become often destroyed during the phagocytosis and, in our opinion, the released toxic material destroys the epithelial cells. The salmonellae persisting in the epithelial cells seem to affect the cells only slightly and the epithelial cells containing bacteria, both in the exudate of the conjunctival sac and in the histological sections, showed maintained nuclear and cytoplasmic ultrastructures.

Striking is the phenomenon of the "stars" consisting of bacteria in the epithelial cells of the smears. We do not know of similar observations reported in the literature. Two possible explanations can be found concerning their origin; either a great number of bacteria enter the cell simultaneously staying together in the phagosome, or else a group of bacteria multiplying in the host cell stays together for a certain period. The latter possibility seems to be the more likely one.

What is the significance of the "epithelial phase" from the point of view of systemic resistance, on the basis of these observations? The desquamation of the cells with the exudate through the palpebral fissure decreases the number of bacteria which gain entrance into the deeper parts of the organism. In this sense the desquamation of the infected cells has a role in the defence of the host.

The significance of the "epithelial phase" is not restricted to the desquamation of cells containing bacteria. The polymorphonuclear leucocytes, macrophage and lymphoid type cells appearing among the cells of the epithelial barrier or directly under it, as well as the cells of the lymphoreticular tissue showing a tonsil-like arrangement near the fornix of the conjunctiva, form together a functional unit. The constituents of the epithelial barrier undergo significant changes during the infective process. The functional and morphological changes which have been observed in experimental listeria keratoconjunctivitis [24] might occur also in the course of a salmonella infection of the conjunctival epithelial cells. This, however, needs further confirmation by ultrastructure studies. We use the term "activation of the epithelial cells" for these changes, considering the phenomenon to be similar to that described by North and MacKaness [32, 33] in connection with the macrophages. As regards the morphological relations, this activation manifests itself in the increase of the lysosomes and in the hypertrophy both of the Golgi-apparatus and of the endoplasmic reticulum, in the epithelial cells similarly to that observed in the macrophages. The appearance in salmonella conjunctivitis of plasma cells and Russel bodies among the epithelial cells and directly

under them, belongs to the immune-morphological changes of the epithelial barrier.

To understand the "barrier" function of the epithelium in conjunctivitis salmonellosa, it seemed useful to compare the morphogenesis of the disease with that of the keratoconjunctivitis caused by the "epithelial parasite" shigella. One of the most important characteristics of the virulent shigella is its ability to penetrate and multiply in the conjunctival and corneal epithelial cells. However, they seldom penetrate into the subepithelial areas. In our histological studies performed on about 1500 eyes during 10 years, shigellae were observed only exceptionally in the macrophage-type cells of the lymphoreticular tissue under the conjunctival epithelium. According to SERÉNY [34] an incidental bacteriaemia following the infection is only of transitory significance, and of no importance from the pathogenetic point of view. The process does not become generalized, septicaemia is rare and develops only when the resistance of the organism is artificially reduced.

Histological examinations showed that the infection proceeds from numerous foci situated all over the conjunctiva, these foci develop quickly and large ulcerations are formed. The infection spreads rapidly to the corneal epithelium and with this the process is practically transferred to the cornea [18].

In contrast, in salmonella conjunctivitis the process is not so uniformly distributed on the conjunctival epithelium as in the case of shigellae. It manifests itself more intensively in the areas above the lymphoreticular tissues. Thus the centre of the process fails to be transferred to the corneal epithelium but the pathogens penetrate into the areas under the conjunctival epithelium, and the process often becomes generalized.

The functional condition of the epithelial barrier is of great importance in salmonella-conjunctivitis, this being the anatomical area where the pathogen is prevented from penetrating into the organism. This explains the finding that in the efficacy of vaccines differences were found in the salmonella infections induced by oral or parenteral administration of the pathogen. The works of McLEOD [35] and GREENWOOD, TOPLEY and WILSON [36] refer also to this observation. These authors found that mice immunized with heat-killed *S. typhi-murium* vaccine, showing partial resistance against intraperitoneal infection, failed to be protected against oral infection.

The conclusions drawn concerning the role of the epithelial barrier in salmonella conjunctivitis might be of universal validity, and apply to all those infections in which the salmonellae gain entrance into the organism through the epithelial barrier.

All these emphasize the importance of the local factors in the immune prophylaxis of this group of salmonelloses as well as perhaps in the human diseases caused by *S. typhi.*

REFERENCES

1. Sedan, J., Herrman, R.: C. R. Soc. Biol. (Paris) **90**, 293 (1924).
2. Pandalai, N. G.: Indian J. med. Res. **21**, 625 (1934).
3. Moore, B.: J. Hyg. (Lond.) **55**, 414 (1957).
4. Feng, C. S., Fang, K., Zia, S. A.: Nat. med. J. China **44**, 329 (1958).
5. Serény, B.: Zbl. Bakt. I. Abt. Orig. **178**, 325 (1960).
6. Кацнельсон, И. А.: Lab delo **4**, 39 (1961).
7. Instrati, B., Ciufeco, C., Cimpeanu, I.: Arch. roum. Path. exp. **21**, 89 (1962).
8. Розет, Е. Л.: Материалы 11 науч. конф. Ленинградского вете ринарского и-та. Ленинград 48. 1962.
9. Stenzel, W.: Z. Hyg. **148**, 512 (1962).
10. Баймарова, Э.: Эдрав. Туркменистана, **7**, 3 (1963).
11. Istrati, G., Szégli, L., Ciufeco, C., Filipesco, S., Dobre, M.: Arch. roum. Path. exp. **22**, 101 (1963).
12. Gekker, V. D., Belaja, J. A., Melnik, E. G.: J. Hyg. Epidem. (Praha) **1**, 70 (1957).
13. Manolov, D. G.: J. Hyg. Epidem. (Praha) **1**, 322 (1957).
14. Mackel, D. C., Langley, L. F., Venice, L. A.: Amer. J. Hyg. **73**, 219 (1961).
15. Глейберман, С. Е., Баймарова, Э., Степанян, Е. Г., Юрно, Л. П.: В кн.: Труды Ашхабадского ин-та эпидемиол. и гигиены. Ашхабад, т. VII. 61. (1964).
16. Piéchaud, M., Szturm-Rubinsten, S., Piéchaud, D.: Ann. Inst. Pasteur **94**, 298 (1958).
17. Rácz, P., Serény, B.: Morph. Ig. Orv. Szle **2**, 272 (1962).
18. Rácz, P.: Thesis, Budapest—Leningrad 1962.
19. Rácz, P., Tenner, K.: Verh. dtsch. Ges. Path. 47. Tagung. Gustav Fischer Verlag, Stuttgart 1963, p. 403.
20. Rácz, P.: Acta morph. Acad. Sci. hung. Suppl. XII, 1 (1963).
21. Wessel, W., Rácz, P.: Virchows Arch. path. Anat. **342**, 109 (1967).
22. Rácz, P., Pesthy, L.: 3rd Int. Congr. on Pathology of Infection, Bucharest 1962, p. 915.
23. Теннер, К., Рац, П., Щерень, Б.: Арх. пат. **11**, 12 (1970).
24. Rácz, P., Tenner, K., Szivessy, K.: Acta microbiol. Acad. Sci. hung. **17**, 221 (1970).
25. Rácz, P., Tenner, K., Mérő, E.: Virchows Arch. Abt. B. **8**, 1971. In press.
26. Istrati, G.: Personal communication.
27. Florey, H. W.: J. Path. Bact. **37**, 283 (1933).
28. Белянин, В. Л.: Дисс. канд.: Ленинград, (1968).
29. Takeuchi, A.: Amer. J. Path. **50**, 109 (1967).
30. Bakács, T., Rácz, P., Rédei, A., Tenner, K.: Egészségtudomány, **14**, 94 (1970).
31. Ариель, Б. М., Берновская, А. А.: Арх. пат. **11**, 17 (1970).
32. North, R. J., MacKaness, G. B.: Brit. J. exp. Path. **44**, 601 (1963).
33. North, R. J., MacKaness, G. B.: Brit. J. exp. Path. **44**, 608 (1963).
34. Serény, B.: Acta microbiol. Acad. Sci. hung. **4**, 367 (1957).
35. MacLeod, D. R. E.: J. Hyg. (Lond.) **52**, 9 (1954).
36. Greenwood, M., Topley, W. W. C., Wilson, J.: J. Hyg. (Lond.) **31**, 257 (1931).

Address of the authors:

Klára Tenner, Pál Rácz
National Institute of Public Health
Gyáli út 2—6, Budapest IX, Hungary

Barnabás Rédey
Public Health Station, József Attila utca 30, Veszprém, Hungary

Acta microbiol. Acad. Sci. hung. 18, 179—189, 1971

SIGNIFICANCE OF "INTRACELLULAR PARASITISM" IN EXPERIMENTAL SHIGELLA CYSTITIS*

By

Clara Tenner, P. Rácz and B. Serény

National Institute of Public Health (Director: T. Bakács), *Budapest*

(Received June 24, 1971)

Summary. Cystitis displaying a regular course has been induced by introducing 2×10^8 *Shigella flexneri* 3a, and in another experimental series the same number of *Shigella sonnei*, into the urinary bladder.

Detailed histological studies of Bingel's model concerning the biological properties of shigellae were leading to the same results as the histological examination of keratoconjunctivitis shigellosa did: shigellae are able to penetrate and multiply in the epithelial cells, their cytotoxic effect on the epithelial cells is slight, they are localized during the infective course to the epithelial barrier, polymorphonuclear leucocytes destroy them, and during this event toxic materials are released and are injuring the epithelial cells. Shigellae are rarely seen in the cytoplasm of macrophage-type cells.

The morphogenesis of ulcerations on the surface of the bladder corresponds to those observed in the conjunctival epithelium. In keratoconjunctivitis shigellosa, focal ulcerations proceed from a few infected epithelial cells, the foci are growing and become confluent. Extensive epithelial defects develop during the infective process.

The corresponding phenomena observed in two different experimental models refer to the basic properties of shigellae.

Successful induction of cystitis showing a regular course by the intravesical introduction of an appropriate number of shigellae was reported by Bingel [1] in 1943.

On the basis of the results, Bingel came to the conclusion that virulent shigellae are attacking the epithelium of the bladder. Bingel applied histological techniques only as subsidiary examinations, and could not confirm his statements with suitable morphological evidence. Letterer and Seybold [2] then repeated the experiments, but failed to confirm the result of pathohistological examinations and asserted that the site of primary lesions was the vascular system.

It was subsequently Piéchaud and Szturm-Rubinsten [3] and Stenzel [4, 5] who studied cystitis shigellosa from a bacteriological point of view.

The cystitis model of Bingel has not been applied widely in bacteriological diagnosis, it became, however, of great theoretical importance as soon as it was found that keratoconjunctivitis can be induced in guinea pigs and rabbits without any pretreatment using virulent shigellae [6]. It was found

* Work performed in collaboration with the Institute of Experimental Medicine of the Academy of Medical Sciences of the USSR.

by light and electron microscopic examinations [7—12] that the pathogenicity of virulent shigellae was connected with their ability to penetrate and multiply in the corneal and conjunctival epithelium. Histological studies then clarified the mechanism of the development of ulcerations on the conjunctival and corneal epithelium. Concerning the biological properties of shigellae it is therefore important to establish how far the conclusions drawn from the studies of keratoconjunctivitis would apply to the epithelium of other organs.

Considering the fact that BINGEL's model has not been subjected to histological examinations, the purpose of the present experiments was to perform detailed histological studies of that model.

The aim therefore was to study the ability of penetration and multiplication of live shigellae in the epithelial cells of the bladder as well as the toxic effect on the invaded host cell. In addition the question has been studied whether the shigellae were localized throughout the infective process into the epithelium or gained entrance into the subepithelial tissues. On this basis an attempt was made to form an opinion in the controversy between BINGEL and LETTERER concerning the pathogenesis of ulcerations in the urinary bladder.

Materials and methods

Culture preparations. Sh. flexneri 3a and *Sh. sonnei* strains were used for inoculation. *Sh. flexneri* 3a was isolated some years ago from a patient with dysentery and its virulence was maintained by animal passages. The strain consisted mainly of virulent colonies as observed by oblique illumination [13]. The *Sh. sonnei* strain was freshly isolated from a patient. Prior to the experiment the pathogenicity of both strains was checked by the keratoconjunctivitis test. Bouillon cultures incubated for 18 hours at 37 °C were used for inoculation, containing 2×10^8/ml bacteria.

Technique of infection. Female guinea pigs weighing 250—300 g of the breed of our Institute were used in the experiments. The inoculation was performed by obtaining through a glass catheter urine from the bladder and introducing into it 1 ml of broth culture. Groups of three animals were sacrificed by cervical dislocation 1, 3, 6, 9, 12, 24, 36, 48, 72, 96 and 144 hours after infection.

Five untreated animals were used as control to observe the normal anatomical appearance, while another experimental group of 18 animals was used for controlling the technique of infection. These animals were treated with 1 ml sterile broth using the above-described technique. These animals formed similarly groups of three and were sacrificed by cervical dislocation 1, 3, 6, 12 and 24 hours after infection.

Light microscopic examination. After gross inspection of the internal organs, the urinary bladder was removed together with the organs of the small pelvis. In the case of *Sh. flexneri* infection, the organs were fixed in formalin while in the case of *Sh. sonnei* infection formalin and CARNOY's fluid were applied. In formalin were fixed furthermore appropriate fragments of kidney, spleen, liver and heart. After embedding in paraffin, sections of 3 to 4 μ were prepared and stained with haematoxylin—eosin, thionin, methyl green pyronine and with the technique of MASSON.

Bacteriological examination. Bacteriological examinations were carried out from the urinary bladder, kidney, adrenal, liver and spleen, of each animal infected with *Sh. sonnei.* Tissue fragments were incubated in 6 ml of sterile broth at 37 °C for 24 hours, and if no turbidity could be observed after one day, for 48 hours. The examination was considered negative if the broth remained clear after 48 hours. Agar plates, desoxycholate-citrate and triple sugar culture media were inoculated from the tubes showing turbidity and an agglutination test was performed with the developed colonies.

Results

Macroscopic changes corresponded from the point of view of chronology and appearance to those described by BINGEL [1]. Thus the oedema of the bladder wall became visible to the naked eye and punctiform haemorrhages were found at some sites of the mucous membrane after 6 hours. Later the oedema of the bladder was spreading towards the small pelvis and to the periureteric tissues. Beginning from the 24th hour, areas of pinhead size deprived of epithelium became visible even macroscopically.

The urinary bladder of the guinea pig is covered by an intermediate epithelium. LETTERER and SEYBOLD [2] gave a detailed description of its histological appearance. Our observations were in full agreement with their data.

One hour after infection a few epithelial cells appeared in different areas of the mucous membrane of the bladder, showing maintained structures and a few well-stained shigellae in their cytoplasm (Plate I, Fig. 1). A few polymorphonuclear leucocytes could be observed in their neighbourhood. Polymorphonuclear leucocytes were found in the lamina propria, directly under the epithelium in both the infected animals and the animals treated with sterile broth. The number of polymorphonuclear leucocytes increased also in the lumen of the small veins passing through the area. Slight oedema appeared prevalently in the lamina propria.

The number of shigellae increased in the cells 3 hours after infection (Plate I, Fig. 2) and in some cases a few shigellae could be detected in the cytoplasm of the epithelial cells surrounding the infected cells.

Six hours after infection the most striking finding was the gross increase of the oedema; its extent was identical in both the infected and the control animals. In the infected animals, under low power the otherwise well retained

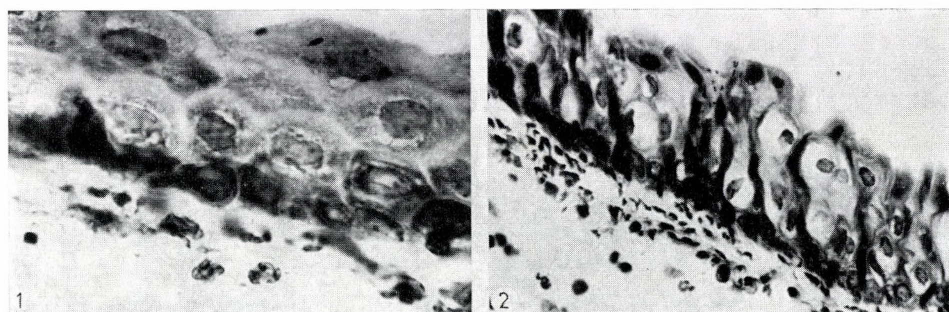


Plate I, Fig. 1. Intermediate epithelium of the bladder. Two shigellae at different levels in an umbrella cell situated in the centre of the surface. Polymorphonuclear leucocytes in the lamina propria. 1 hour after infection with *Sh. flexneri* 3a (thionin staining, ×1200)

Plate I, Fig. 2. Focus formed by epithelial cells containing shigellae. Infiltrate consisting of polymorphonuclear leucocytes in the lamina propria; 3 hours after infection with *Sh. flexneri* 3a (thionin staining, ×500)

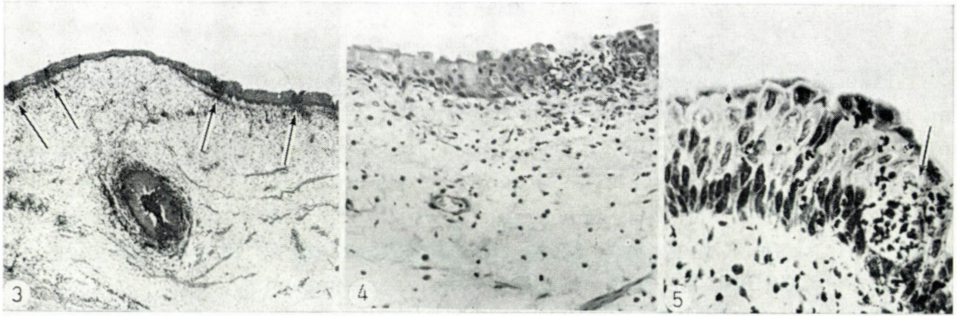


Plate I, Fig. 3. Epithelium covering the bladder is well retained, at some sites circumscribed dense areas (arrows). Pronounced oedema and cellular infiltration in the lamina propria. Numerous polymorphonuclear leucocytes in the lumen of the ureter; 6 hours after infection with *Sh. flexneri* 3a (thionin staining ×30)

Plate I, Fig. 4. Circumscribed ulceration on the surface of the bladder. Epithelium around the ulceration intact, pronounced oedema in the lamina propria; 6 hours after infection with *Sh. sonnei* (haematoxylin—eosin staining, ×200)

Plate I, Fig. 5. Part of Plate I, Fig. 3. Intraepithelial abscess (arrow). Numerous shigellae in the cytoplasm of the epithelial cells surrounding the abscess; 6 hours after infection with *Sh. flexneri* 3a (thionin staining, ×400)

epithelium contained small circumscribed dense areas (Plate I, Fig. 3). Increasing the magnification, three types of changes were discernible in these areas.

1. Epithelial cells displaying a cytoplasm filled with shigellae. They were situated mostly in the centre. Some cells had decomposed and released the shigellae. These cells were surrounded by polymorphonuclear leucocytes (Plate I, Figs 6 and 7).

2. In other areas intraepithelial micro-abscesses developed due to increase of oedema fluid between the epithelial cells containing shigellae, and polymorphonuclear leucocytes migrated into their lumen (Plate I, Fig. 5).

3. The infected epithelial cells which surrounded the micro-abscesses from the side of the vesical lumen became destroyed, thus the micro-abscesses

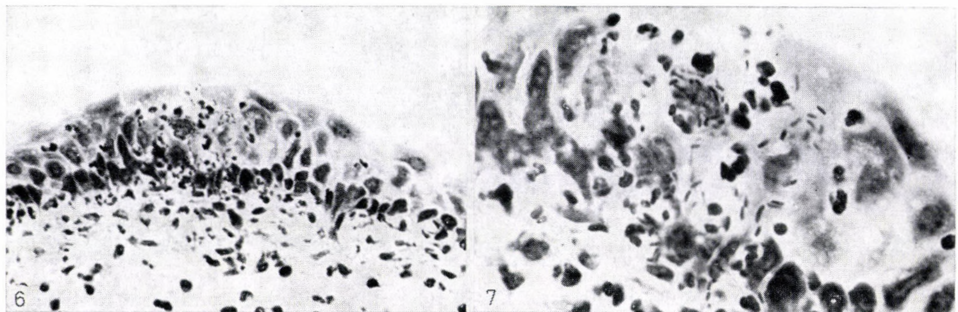


Plate I, Fig. 6. Epithelial cell showing destruction, in the centre of the epithelial focus, with cytoplasm almost filled with shigellae and polymorphonuclear leucocytes around it; 6 hours after infection with *Sh. flexneri* 3a (thionin staining, ×400)

Plate I, Fig. 7. High power view of epithelial focus in Plate I, Fig. 6 (×1200)

opened up and small erosions and ulcerations developed (Plate II, Fig. 4).

Polymorphonuclear leucocytes were found in the ureter passing through the bladder wall and shigellae were present in the epithelial cells of the ureter (Plate I, Fig. 3).

Numerous macrophage-type cells appeared among the polymorphonuclear leucocytes infiltrating the subepithelial area. Activation of local cells could also be observed.

Larger ulcerations become visible 9 hours after infection. Epithelial cells on the periphery of the ulcerations contained shigellae. New foci developed beside the old ones in the areas where the epithelial cells contained a great number of shigellae. The new foci were similar in appearance to those developed in the first hours after infection (Plate II, Fig. 1). The epithelium between the ulcerations was usually intact except for some intracellular oedema near the ulcerations. A vacuole like halo can be seen around the intermediary epithelial cells (Plate II, Fig. 1).

Twenty-four hours after the infection the majority of the "micro-abscesses" becomes open (Plate II, Fig. 2) and a few epithelial cells with their cytoplasm full of shigellae can be observed on the basis of smaller and larger ulcerations developed directly above the lamina propria (Plate II, Figs 3 and 4).

Forty-eight hours after infection extensive ulcerations can be seen. The oedema of the bladder wall increases. The epithelium retains its layers at the periphery of the ulcerations, in other cases it becomes, however, flat and umbrella-type cells creep to the bare surface.

The inflammatory infiltrate of the subepithelial tissue is of focal nature, similarly to the ulcerations, and is prevalent in the areas deprived of epithelium (Plate II, Fig. 5), or where the epithelium contains micro-abscesses.

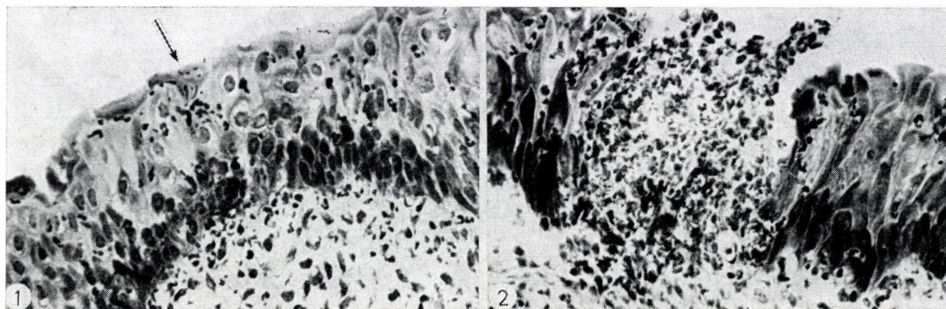


Plate II, Fig. 1. New focus of infected epithelial cells (arrow), polymorphonuclear leucocytes in their neighbourhood; 9 hours after infection with *Sh. flexneri* 3a (thionin staining, ×400)
Plate II, Fig. 2. Circumscribed ulceration in the epithelium of the bladder. Numerous polymorphonuclear leucocytes on the surface of the ulceration; 24 hours after infection with *Sh. sonnei* (thionin staining, ×450)

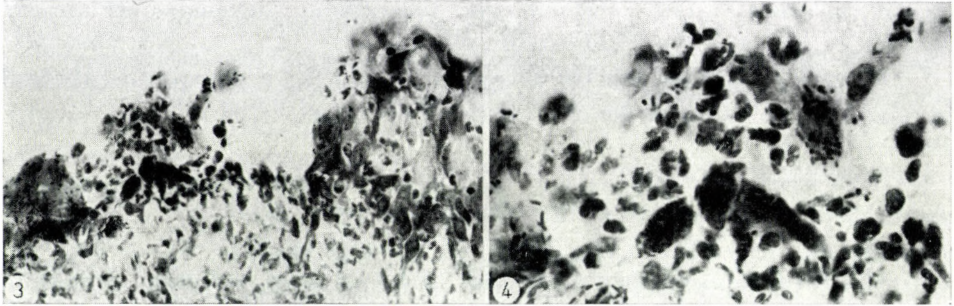


Plate II, Fig. 3. Ulceration with sharp borders on the mucous membrane of the bladder, epithelial cells showing destruction on the surface of the ulceration, cytoplasms filled with shigellae; 24 hours after infection with *Sh. flexneri* 3a (thionin staining, ×400)
Plate II, Fig. 4. High power view of the ulceration surface in Plate II, Fig. 3 (×1200)

Shigellae were usually not observed in the lamina propria, and macrophage-type cells containing shigellae were found only in exceptional cases, on the base of the ulcerations in the subepithelial tissue.

As observed in animals sacrificed at a later stage of the infective process, the ulcerations continued to spread despite the regeneration of the epithelium observed at some sites. Intraepithelial abscesses in random arrangement appeared at various sites of the epithelium. Neither the pycnotic polymorphonuclear leucocytes in their lumen, nor the epithelial cells surrounding them were fround to contain bacteria (Plate II, Fig. 6). Despite the extensive ulcerations, in their neighbourhood, the epithelium retained its normal morphological structure.

Six days after infection nearly the entire surface of the bladder becomes ulcerated and as seen in other phases, new foci develop in the still intact areas of the epithelium.

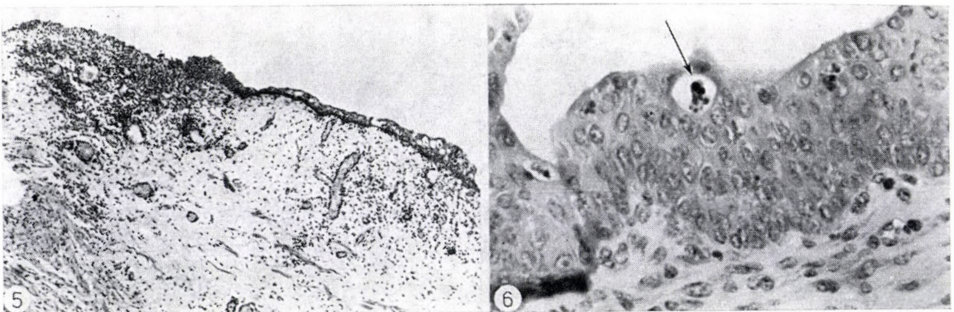


Plate II, Fig. 5. Extensive ulceration on the surface of the bladder. The inflammatory cellular infiltrate of the lamina propria is pronounced in the areas deprived of epithelium; 48 hours after infection with *Sh. sonnei* (haematoxylin—eosin staining, ×30)
Plate II, Fig. 6. Subsided intraepithelial abscess in the epithelium of the bladder (arrow). No bacteria in the epithelial cells, pycnotic cells in the lumen of the abscess; 3 days after infection with *Sh. sonnei* (haematoxylin—eosin staining, ×500)

From the organs subjected to histological examination a few data concerning the kidney are presented here. While only a few shigellae were detectable in the umbrella cells of the intermediate epithelium covering the renal pelvis 3 hours after infection, 9 hours after infection the epithelium of the renal pelvis showed intraepithelial micro-abscesses and ulcerations in each animal. The number of epithelial cells containing bacteria, and the number of the bacteria in one cell rose significantly (Plate III, Fig. 1). The urine in the lumen of the renal pelvis contained great numbers of polymorphonuclear leucocytes, detached epithelial cells and bacteria. Inflammatory oedema and a leucocytic infiltration appeared in the connective tissue (Plate III, Fig. 2). The same picture was observed 12 hours after infection (Plate III, Fig. 3). In animals sacrificed at a later period the intensity of the pyelitis decreased and more or less simultaneously the inflammatory changes of the ureter in the bladder wall subsided (Plate III, Fig. 4).

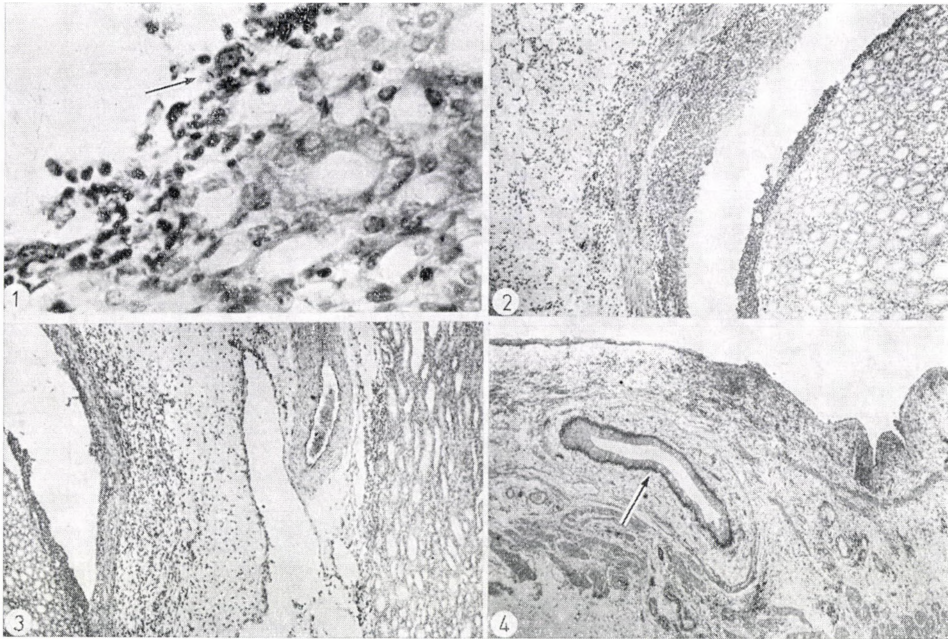


Plate III, Fig. 1. Shigellae in the epithelium covering the renal papilla (arrow); 9 hours after infection with *Sh. flexneri* 3a (thionin staining, $\times 600$)

Plate III, Fig. 2. Pronounced polymorphonuclear leucocytic infiltration and oedema in the connective tissue under the epithelium of the renal pelvis; 9 hours after infection with *Sh. flexneri* 3a (haematoxylin—eosin staining, $\times 120$)

Plate III, Fig. 3. Numerous polymorphonuclear leucocytes in the lumen of renal pelvis. Pronounced oedema in connective tissue under the epithelium of renal pelvis; 12 hours after infection with *Sh. flexneri* 3a (thionin staining, $\times 120$)

Plate III, Fig. 4. Extensive ulceration on the surface of the bladder. The part of the ureter in the bladder-wall (arrow) fails to show inflammatory changes; 3 days after infection with *Sh. sonnei* (haematoxylin—eosin staining, $\times 30$)

Table I

Occurrence of positive findings obtained by the vesical introduction of virulent Shigella sonnei culture after 24 and 48 hours incubation

Hours/number of animals	Urinary bladder		Kidney		Adrenal gland		Liver		Spleen	
	24	48	24	48	24	48	24	48	24	48
1/1	+		+		+		−	−	+	
1/2	+		+		−	−	−	−	+	
1/3	+		+		−	−	−	+	+	
3/1	−	−	−	−	−	−	−	−	+	
3/2	−	−	−	−	−	−	−	−	+	
3/3	+		−	−	−	−	−	−	−	−
6/1	−	−	−	−	−	−	−	−	−	−
6/2	+		−	−	−	−	−	−	−	−
6/3	+		−	−	−	−	−	−	−	−
9/1	+		−	−	−	−	−	−	−	−
9/2	+		−	−	−	−	−	−	−	+
9/3	+		+		+		−	−	−	−
12/1	+		−	−	−	−	−	−	−	+
12/2	+		+	−	−	−	−	−	−	−
12/3	+		−	−	−	−	−	+	−	−
24/1	+		+		+		−	−	+	
24/2	+		−	−	−	−	−	−	−	−
24/3	+		+		+		+		+	
36/1	+		+		+		+		+	
36/2	+		−	−	−	−	−	−	−	−
36/3	+		−	−	−	−	+		+	
48/1	+		−	−	−	−	−	−	−	−
48/2	+		+		−	−	−	−	−	−
48/3	+		+		+		+		+	
72/1	+		−	−	−	−	−	−	−	−
72/2	+		+		+		+		+	
72/3	+		+		−	−	+		+	
96/1	+		−	−	−	−	−	−	−	−
96/2	+		+		−	−	−	−	−	−
96/3	+		−	−	−	−	−	−	−	−
144/1	+		−	−	−	−	−	−	−	−
144/2	+		−	−	−	−	−	−	+	
Contr/1	−	−	−	−	−	−	−	−	−	−
Contr/2	−	−	−	−	−	−	−	−	−	−
Contr/3	−	−	−	−	−	−	−	−	−	−

Shigellae were localized all through to the level of the epithelium and did not spread to the renal parenchyma. A small amount of infiltrate consisting of polymorphonuclear leucocytes was found around the efferent canaliculi near the renal pelvis in animals sacrificed 9 and 12 hours after infection.

Bacteriological results are summarized in Table I.

Discussion

The histological examinations confirmed that shigellae are able to penetrate and multiply in the epithelial cells of the urinary bladder. In our opinion the failure of LETTERER and SEYBOLD [2] in finding bacteria in the epithelial cells may be explained by the fact that they did not apply histological staining techniques suitable for the demonstration of bacteria in the tissues. The same might apply also to the work of AWATAGUCHI *et al.* [14] who could not detect shigellae in histological sections stained with haematoxylin—eosin.

LEVENBUK and ANDREIEVA [15] confirmed on the basis of histological studies the supposition concerning the intracellular parasitism of shigellae. No detailed conclusions can be drawn, however, from their work concerning the dynamics of the process, they having used a limited number of animals to study the given periods following the infection. (With 4 different *Shigella* strains, 25 animals were infected and 15 of them died.) MANOLOV and KOSTURKOV [16] also supported the view of an intraepithelial multiplication of shigellae.

SZTURM-RUBINSTEN and PIÉCHAUD [17] confirmed the intraepithelial parasitism of shigellae in cystitis shigellosa in examinations of the urinary sediment and mucous membrane scrapings taken from the fundus and cervix of the urinary bladder. Studies of the cells isolated from the urinary sediment could not reveal the correlations which might be highlighted only by the analysis of connective tissue structures and by detailed pathohistological studies of cystitis shigellosa.

The present experiments, based on an analysis of the interaction between the pathogenic agent and the host cell, provided several new data to understand the pathogenesis of cystitis shigellosa. The study showed that the process started from numerous foci situated in different areas of the mucous membrane. At an early stage after the infection only a few infected epithelial cells were found in the area of these foci with 1—2 shigellae in their cytoplasm. At a later phase the number of infected cells increased, and being situated close to each other their position indicated that the process had spread from the originally infected cell. The number of shigellae in the cells belonging to these foci showed a regularity. Cells situated on the surface and in the centre of the focus contained considerably more shigellae than those at the periphery, which refers also to the mode of spreading described.

Two ways may serve for shigellae to pass from one epithelial cell into the other. In keratoconjunctivitis listeriosa [18] and shigellosa [11, 12] it was observed that listeriae and shigellae may pass together with the cytoplasmic particles of the originally infected cell from one corneal epithelial cell into the other, avoiding the intracellular space. This mechanism might apply also to cystitis shigellosa. This remains, however, to be confirmed by electron microscopic examinations. The other mode is the destruction of the infected cells. Shigellae situated in the cytoplasm are very slightly toxic, and epithelial cells which contain a great number of shigellae and show at the same time a well retained morphological structure are often seen; the extreme multiplication of shigellae, however, causes finally the destruction of the epithelial cell. During this event large quantities of shigellae become released and may infect the neighbouring cells. This phenomenon appears for the first time 6 hours after infection.

The development of ulcerations cannot be explained solely by the examination of the infected epithelial cells, as polymorphonuclear leucocytes appear at a very early stage, next to the infected cells. Micro-abscesses develop soon in the epithelium, as observed in the conjunctival epithelium in keratoconjunctivitis shigellosa [9] and keratoconjunctivitis listeriosa [10] and in the epithelium of the urinary bladder in cystitis listeriosa [19]. The number of polymorphonuclear leucocytes in the micro-abscesses increases in the period when the epithelial cells which contain great numbers of shigellae, decompose. The polymorphonuclear leucocytes are phagocyting shigellae released from the epithelial cells. During this event part of them is destroyed and the toxic material thus released lesions the neighbouring epithelial cells. The destroyed and perishing epithelial cells desquamate rapidly, the micro-abscesses open up, and ulcerations develop. The borders of the ulcerations are formed by infected epithelial cells. The desquamated epithelial cells which contain bacteria might again become attached to other areas on the surface of the bladder and may serve as starting points for a new infection. If the proportion of the destruction of bacteria and the dynamics of phagocytosis is favourable, the small focus becomes sterile.

The histological studies of cystitis shigellosa have confirmed our observations on two biological characteristics of virulent shigellae which have been observed also in keratoconjunctivitis shigellosa [9]. Shigellae are localized throughout the infective process to the epithelial barrier. A prompt and pronounced reaction, consisting of polymorphonuclear leucocytosis follows their appearance 24—48 hours after the infection, in the period of extensive ulceration, in the loose and fibrous subepithelial tissue. The polymorphonuclear leucocytes destroy the pathogenic agents.

It might occur that shigellae enter also other areas of the organism, as shown by the bacteriological examinations. Our observations have, however,

confirmed the data of BINGEL [1] in that shigellae cannot be regularly cultured from the organs, which indicated that after invading a tissue other than the epithelium, they become destroyed. Most frequently they can be detected in the kidney. Histological examinations revealed that shigellae persist in the intermediate epithelium of this organ. Our explanation for the bacteria invading the renal pelvis is that they are introduced into the bladder and from there ascend into the renal pelvis, or else, the infection spreads from the bladder through the ureter into the kidney but not by haematogenic route.

The observation that a pronounced macrophage reaction appears 24 hours after infection and that the macrophages very seldom contain shigellae refers to another biological property of the pathogen.

It is of interest to compare these two characteristics with those of *Listeria monocytogenes*, a facultative intracellular parasite. In cystitis listeriosa [19], the pathogen arrives soon into the lower parts of the bladder and multiplies first in the cytoplasm of the macrophage-type cells.

The detailed pathohistological study of cystitis shigellosa has confirmed the theory of BINGEL [1] who considered the epithelium of the bladder wall to be the primary site of lesion. Consequently, the pathogenesis of the process can be understood only by starting from a study of the interaction between shigellae and the epithelial barrier.

A detailed analysis of the pathological events in cystitis shigellosa developing in the kidneys, spleen, myocardium and liver will be discussed in another work.

REFERENCES

1. BINGEL, K. F.: Z. Hyg. Infekt.-Kr. **125**, 110 (1943).
2. LETTERER, E., SEYBOLD, G.: Z. Hyg. Infekt.-Kr. **129**, 466 (1949).
3. PIÉCHAUD, D., SZTURM-RUBINSTEN, S.: Ann. Inst. Pasteur **97**, 511 (1959).
4. STENZEL, W.: Z. Hyg. Infekt.-Kr. **147**, 123 (1960).
5. STENZEL, W.: Zbl. Bakt. I. Abt. Orig. **187**, 195 (1962).
6. SERÉNY, B.: Acta microbiol. Acad. Sci. hung. **2**, 293 (1955).
7. PIÉCHAUD, M., SZTURM-RUBINSTEN, S., PIÉCHAUD, D.: Ann. Inst. Pasteur **94**, 298 (1958).
8. RÁCZ, P., SERÉNY, B.: Morph. Ig. Orv. Szle **2**, 272 (1962).
9. RÁCZ, P.: Thesis, Budapest—Leningrad 1962.
10. RÁCZ, P., TENNER, K.: Verh. dtsch. Ges. Path. 47. Tagung. Gustav Fischer Verlag, Stuttgart 1963, p. 403.
11. WESSEL, W., RÁCZ, P.: Virchows Arch. path. Anat. **342**, 109 (1967).
12. Теннер, К., Рац., П., Шерень, Б.: Арх. пат. **2**, 12 (1970).
13. SERÉNY, B.: Acta microbiol. Acad. Sci. hung. **6**, 179 (1959).
14. AWATAGUCHI, S., KAWANO, Y., KOJIMA, A., SAKUMA, S.: Jap. J. Microbiol. **13**, 325 (1969).
15. Левенбук, И. С., Андреева, З. М.: Вестн. Акад. мед. Наук **17**, 70 (1962).
16. MANOLOV, D. G., KOSTURKOV, G. B.: J. Hyg. Epidem. (Praha) **9**, 455 (1965).
17. SZTURM-RUBINSTEN, S., PIÉCHAUD, D.: Ann. Inst. Pasteur **105**, 785 (1963).
18. RÁCZ, P., TENNER, K., SZIVESSY, K.: Acta microbiol. Acad. Sci. hung. **17**, 221 (1970).
19. RÁCZ, P., TENNER, K., MÉRŐ, E.: Virchows Arch. path. Anat. Abt. B. In press.

Address of the authors:

KLÁRA TENNER, PÁL RÁCZ, BÉLA SERÉNY
National Institute of Public Health
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 191—196, 1971

INDIA-INK IMMUNO-REACTION FOR THE RAPID DETECTION OF ENTERIC PATHOGENS

By

P. GECK

Hungarian Army Medical Corps, Budapest

(Received August 9, 1971)

Summary. A new serodiagnostic method has been elaborated for the rapid identification of pathogenic bacteria. Model experiments with pathogenic *Escherichia coli* serogroups, *Shigella* and *Salmonella* indicated that the India-ink immuno-reaction (IIR) is practically equivalent in sensitivity with cultivation and in specificity with immunofluorescent tracing.

The new method is especially useful for replacing immunofluorescent examinations. Smears are treated with India-ink and immune serum for 5 minutes, then read under simple light microscope. The method unites the advantages of direct, indirect and modified indirect stainings.

In modern bacteriology there is a trend of introducing diagnostic methods which are more rapid, simple, sensitive and specific than the existing ones. In recent years a great number of express tests, micromethods and immune reactions have been described for the determination of the characteristics of various bacteria.

Rapid laboratory diagnosis is especially advantageous when there is need for the detection of a pathogenic agent simultaneously with the admission of the patient or in case of outbreaks.

Technical difficulties in immunofluorescent tracing have greatly hindered the wide-spread application of this method. It seemed desirable to elaborate a simple and rapid method equivalent in sensitivity and specificity with immunofluorescent examination. As a result of studies with a wide range of bacteria, the India-ink reaction presented in this paper has been found adequate for the purpose.

Materials and methods

Organisms and faecal specimens. Escherichia coli O26 : B6, O55 : B5, O86 : B7, O111 : B4, O112 : B11, O119 : B14, O124 : B17, O125 : B15, O126 : B16, O127 : B8, O128 : B12, *Shigella flexneri* 2a, *Sh. sonnei, Sh. dysenteriae* 2, *Salmonella paratyphi-A, S. typhi-murium* and *S. typhi* were used.

Faecal specimens which had been shown by cultivation to be free from enteric pathogens and had not reacted with IIR were used in the model experiments. A pea-sized amount of the specimen was suspended in 3 ml saline and sedimented in a hand centrifuge. To 1 ml of the supernatant 1 ml of a tenfold serial dilution of each test organism cultured on agar slants was added so as to give cell counts ranging from 10^3 to 10^9 per ml.

Cultural methods. Culturing and identification of enteric pathogens were performed as described in the Standard Methods of Hungarian Public Health Bacteriology Laboratories [1]. The suspensions were plated immediately after preparation.

India-ink reaction. Preparation of faecal specimens. Faecal specimens prepared as described above are suitable not only for model experiments but also for routine examinations, when the presence of a great number of pathogenic organisms may be expected to be present in the specimen (enterocolitis). In patients with less definite enteric symptoms it is advisable to centrifuge at 3000 r.p.m. the supernatant of the sedimented specimen and to prepare smears of the deposit.

Smears. A loopful of the supernatant of the sedimented specimen or of the deposit obtained by centrifugation is smeared on a defatted slide. When low cell counts are expected, a pea-sized circle is drawn with wax pencil on the centre of the slide, then a loopful of the specimen is spotted at the corresponding site of the reverse surface. The smears or spots are left to dry then fixed over flame.

Working dilution of diagnostic sera. The sera should be three times more concentrated than the working dilution for slide agglutination. The sera are diluted with saline containing 0.5% phenol. To *Salmonella* polyvalent O serum an equal amount of phenolized saline is added. Sensitivity and specificity of the reaction highly depend on the quality of sera. In order to avoid aspecific reactions, antibodies reacting with heterologous bacteria should be removed by adsorption.

India-ink immuno-reaction. Eachs mear (corresponding in number to the kinds of antisera used) is covered with 1 drop of liquid India-ink ("Liquid Chinese Holló ink", Politur and Vegyi-termék Co., Budapest), then 1 drop of each of the suitably diluted sera is added to each smear. Then India-ink and serum are mixed with a loop taking care to avoid the transfer of one serum to another. The slides are placed horizontally in a wet chamber (Petri dish turned upside down) for 5 minutes at room temperature.

The smears are then washed off with a solution prepared from 1 part of physiological saline and 2 parts of tap water. If its pH is under 7, the solution is previously adjusted to neutral with sodium carbonate. All kinds of water are suitable for a washing-off fluid if to 1 litre of the above mixture 0.1 ml of 1% $FeCl_3$ solution is added. Washing off is made with a 10 ml wide orifice pipette provided with a rubber ball. The slide is kept at oblique angles and washed off with a medium-strength stream of the above mixture. It is important that precipitated India-ink visible to the naked eye should not remain on the smear. The smear is dried by blotting it with filter paper and examined under the light microscope with an oil-immersion objective (×800).

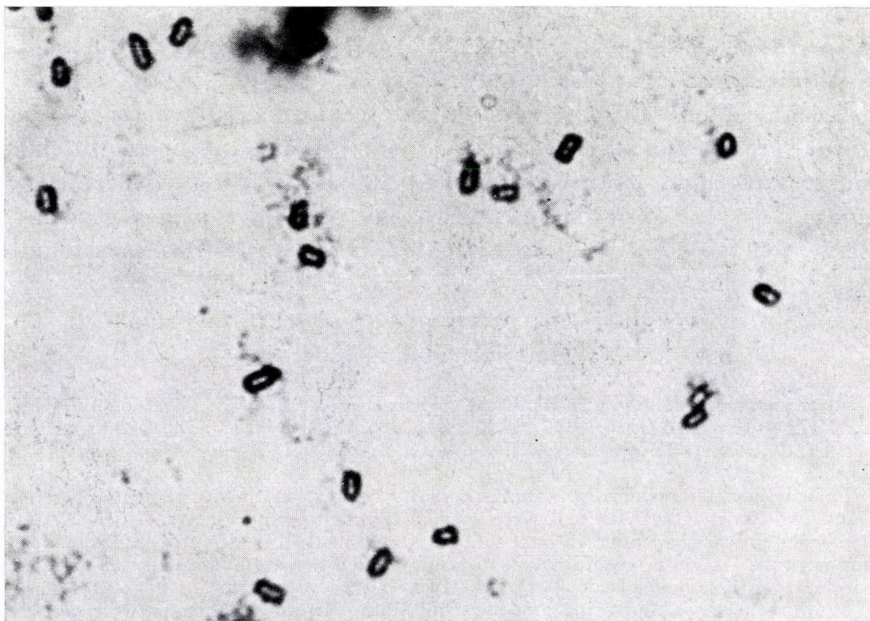


Fig. 1. India-ink immuno-reaction with *E. coli* 111:K58(B4)

Reading. Homologous binding of agglutinins (positive reaction) is indicated by the presence of a definite black contour corresponding in shape and size to the organism examined; the inside of the cell is ash-grey in colour. The black contour is analogous to the fluorescent halo of the cell seen in immunofluorescent staining. In the absence of agglutinin binding (negative result) bacteria are not visible, only remains and aggregates of India-ink particles are seen, but these are easy to distinguish from bacteria showing homologous binding (Fig. 1).

Diagnostic sera frequently contain natural antibodies against cocci, aerobic and anaerobic sporebearing bacteria. In view of differences in morphology and size, aspecific bindings with these organisms can easily be distinguished from specific reactions of Gram negative enteric bacteria.

Results

A total of 1320 faecal specimens was examined. After artificial contamination of 10^3 to 10^9 cells of pathogenic bacteria, the specimens were examined with IIR and with cultural methods.

Table I

Model experiments for comparing the effectiveness of cultural method and India-ink immuno-reaction

Test organisms: pathogenic *E. coli* strains, 10^3—10^9 cells/ml

Cultivation	India-ink immuno-reaction		Total
	Positive	Negative	
Positive	555 (80.4%)	8 (1.2%)	563 (81.6%)
Negative	72 (10.5%)	55 (7.9%)	127 (18.4%)
Total	627 (90.8%)	63 (9.2%)	690 (100.0%)

Table I shows the examination of 690 specimens contaminated with *E. coli* serogroups associated with infantile enteritis. It is evident that the majority of specimens (80.4%) was positive with both culturing and IIR. As shown by the number of specimens yielding negative cultures but positive IIR, the latter was more effective than the former (10.5%). Negative results were obtained with both methods mainly for specimens containing low numbers of pathogens (10^3—10^4/ml).

Table II

Model experiments for comparing the effectiveness of cultural method and India-ink immuno-reaction

Test organisms: *Shigella flexneri* and *Sh. sonnei*, 10^3—10^9 cells/ml

Cultivation	India-ink immuno-reaction		Total
	Positive	Negative	
Positive	219 (78.2%)	6 (2.2%)	225 (80.4%)
Negative	28 (10.0%)	27 (9.6%)	55 (19.6%)
Total	247 (88.2%)	33 (11.8%)	280 (100.0%)

Table II presents experiments with shigellae. The results were similar to those for *E. coli:* both methods gave positive results in 78.2%; positive IIR but negative culturing was recorded for 10.0% of the specimens.

Table III

Model experiments for comparing the effectiveness of cultural method and India-ink immuno-reaction

Test organisms: *Salmonella* strains, 10^3—10^9 cells/ml

Cultivation	India-ink immuno-reaction		Total
	Positive	Negative	
Positive	223 (63.7%)	18 (5.1%)	241 (68.8%)
Negative	16 (4.6%)	93 (26.6%)	109 (31.2%)
Total	239 (68.3%)	111 (31.7%)	350 (100.0%)

Table III shows the results for salmonellae. The specimens were contaminated with strains belonging to 3 different serogroups. The experiments were performed with *Salmonella* O polyvalent and with the corresponding group sera. The number of positive results with both methods was considerably lower than for *E. coli* and *Shigella* (63.7%); positive IIR with a negative cultural examination was obtained in 4.6%.

Discussion

Several years experience has shown that IIR is a simple, rapid, inexpensive and reliable method. Our model experiments have thrown some light on the value of cultivation and IIR in diagnostic bacteriology.

Comparative studies indicated that successful culturing of pathogens present in low numbers (10^3—10^4) is hindered by several conditions such as the time elapsing between taking the specimen and inoculation, the pH and bacterial constituents of faeces, the presence of specific phages, number of dead bacteria, quality of the medium, the mode of inoculation and reading. In our studies, in spite of optimum conditions, the lower limit of 100% positivity was in the range of 5×10^4—10^5 cells per ml.

In the model IIR experiments presented in this paper the chance for aspecific reactions was entirely excluded, as each specimen had previously been examined by culturing and IIR and only the negative specimens were used. It is evident from the results presented in the Tables that the sensitivity of IIR depends on the number of pathogens. Another important factor is the specificity of the serum. High titre homospecific sera highly increased the sensitivity and specificity of IIR. Certain *E. coli* antisera gave aspecific

bindings with some faecal streptococci and aerobic spore-bearing bacteria in 1 : 8 dilution; but not in 1 : 64 dilution.

Beyond the model experiments presented in this paper cultural methods, IIR and immunofluorescent staining were compared in a large routine material of several thousands of faecal specimens. These studies showed that IIR and immunofluorescent staining were practically equivalent.

The mechanism of IIR is not quite clear. In basic mechanism there is a great difference between the seemingly similar IIR and immunofluorescent staining. IIR may be regarded as an immune adsorption method. One valency of the globulin molecule of the immune serum is bound to the cell fixed on the slide, while a coal particle absorbs its other valency so that it cannot be removed by washing. If homologous antiglobulin is previously bound to the free valency, IIR fails to develop. The specific binding of coal particles does not occur when conditions other than physiological ones prevail; for example, if the pH of the washing fluid is under 6 or over 8, or if distilled water adjusted to pH 7 with sodium hydroxide is used.

As compared to the simple washing fluid used, other solution of more complicated constitution had no special advantage. The mixture of 1 part saline and 2 parts of tap or well water containing traces of complex-forming metal ions at pH 7 meets all requirements.

The brand of India-ink is of special importance: in these studies only one product, as indicated under "Materials and methods" gave constantly reproducible results, although several other Hungarian and foreign brands have been tested.

The purpose of IIR is to present a rapid diagnostic method suitable for replacing immunofluorescent tracing. It is evident from this paper that IIR has several advantages over the latter: it requires neither a fluorescent microscope nor conjugates and unites the advantages of direct, indirect and modified indirect methods. In comparison to cultivation it is superior in that it shortens the 24—72-hour incubation time to 1—2 hours and allows the saving of large amounts of culture media.

As to the aspects of IIR, it should be emphasized that the reaction is primarily suitable for the examination of patients with acute enteric infections and for the rapid detection of the pathogen at the beginning of an outbreak when the organisms responsible are present in great numbers in the faeces. Faecal samples positive with IIR should only be subjected to cultural examination in order to identify the agent and to test its antibiotic sensitivity.

Our experience indicates that IIR is suitable for the rapid diagnosis of every microorganism the antigens of which are capable of producing immune sera. By fixing known bacteria on slides, IIR may be used for the determination of antibody titres in convalescent patients. In research it can be applied

for checking the effect of bacterial vaccination, rapid detection of natural antibodies, studies on antigenic relationships, determination of O, K and H antigens of bacteria and for the study of intracellular parasitism.

REFERENCE

1. Módszertani Útmutató. Országos Közegészségügyi Intézet, Budapest, 1969.

Address of the author:

Péter Geck
National Institute of Public Health
Gyáli út 2–6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 197—205, 1971

LA RECHERCHE DES SHIGELLA DANS LES EAUX PAR LE TEST DES ANTICORPS FLUORESCENTS ET PAR LA RÉACTION DE GECK

par

N. D. Duc

Institut National d'Hygiène Publique (Directeur: T. Bakács), *Budapest*

(Manuscrit reçu le 19 mai, 1971)

Résumé. L'auteur rend compte de l'application du test des anticorps fluorescents et de la réaction du Geck pour la démonstration des *Shigella* dans les différentes sources d'eau en Hongrie. Cette recherche a été effectuée sur les frottis de sédiment, de filtrat et de la culture enrichie provenant de 60 échantillons d'eau examinés. Le test des anticorps fluorescents a révélé la présence des *Shigella* (ensemble des 4 sérotypes) dans 38.3 p. 100 des échantillons d'eau examinés, la réaction de Geck a fait apparaître dans 41,1 p. 100, tandis que les *Shigella* ont été isolées dans 3,3 p. 100 par la méthode de culture directe. Toutes les trois techniques susmentionnées ont toujours donné les résultats positifs presque identiques. Soulignant la valeur réelle de la réaction de Geck en comparant ses résultats avec ceux obtenus par le test des anticorps fluorescents et par la méthode bactériologique, la réaction d'immuno-encre de Chine s'est montrée très sensible, aussi certainement spécifique et plus rapide que les autres techniques indirectes usuelles. Elle est aussi une méthode très simple, bon marché, et d'un grand intérêt épidémiologique, qu'on peut appliquer et réaliser facilement dans tous les laboratoires bactériologiques.

La microscopie à l'immunofluorescence qu'on a employé pour l'identification des bactéries en très petit nombre dans les milieux extra-entériques, surtout dans les cas épidémiques dus aux agents pathogènes d'*Enterobacteriaceae* [2, 4—7, 13, 18—21, 27, 35, 36, 39, 44, 45] est une méthode plus sensible et plus rapide que celle de culture directe.

L'application de cette technique pour le diagnostic des *Shigella* a été étudiée par LaBrec *et coll.* [29—31], Hornung [26], Geck *et coll.* [20], Taylor [38], Thomason *et coll.* [39—41], Cherry *et coll.* [4, 5], Akiyoshi [1], etc. [20, 26, 29—32, 37, 38, 40, 41].

En particulier, l'expérience de modèle et la recherche des Vibrio-cholériques, des *Escherichia coli* ainsi que des *Shigella* dans les eaux souillées, la technique des membranes filtrantes associée au test des anticorps fluorescents, occupaient une place à part pour les auteurs comme Danielsson, Folsenfeld, Hammarström, etc. [8—12, 17, 24, 25].

Récemment il y a un certain nombre des bactériologistes qui ont l'intention de se procurer les autres techniques de diagnostic dont les contenus sont non sulement sensibles, spécifiques, mais encore plus rapides, plus simples et plus bon marché que les techniques usuelle. Cette intention-là vise le but de donner une grande possibilité aux bactériologistes afin de dépister le plus tôt possible l'étiologie de l'épidémie, et Geck [22, 23] a réussi à trouver une

nouvelle réaction séro-diagnostique nommée réaction d'immuno-encre de Chine [22, 23].

Dans cette note présente, nous exposons les résultats comparatifs de deux techniques des anticorps fluorescents et de la réaction de GECK avec notre constatation sur la valeur réelle de l'application de ces techniques dans le diagnostic des *Shigella* existant dans les différentes sources d'eau polluée.

Matériel et méthodes

Technique bactériologique .La méthode que nous avons appliquée pour la culture directe des *Shigella* survivant dans l'eau, consiste de trois techniques associées: empêcher la bactériophagie par le tampon salé glycériné à 30 p. 100, — concentrer un petit nombre des *Shigella* dans une grande quantité d'eau examinée par la technique des membranes filtrantes, — enrichir les *Shigella* à l'aide du milieu solide d'enrichissement d'ISTRATI. L'isolement des *Shigella* a été effectué sur les milieux sélectifs d'isolement tels que la gélose de desoxycholate-citraté-lactosé et la gélose d'éosine-bleu de méthylène [15, 28]. Toutes les colonies suspectes ont été repiquées sur le milieu de Russel et soumises ensuite aux recherches utiles pour leur identification (agglutination sur lames avec les sérums agglutinants: anti-sérum *Sh. sonnei* I, II; anti-sérums polyvalents et monovalents de *Sh. flexneri;* recherche de la mobilité, de l'indole et de la réaction biochimique comme la fermentation du mannite, dulcite, rhamnose, sorbite, xylose etc.).

Technique des anticorps fluorescents. Après avoir concentré les germes dans l'échantillon d'eau avec de différentes méthodes (voir ci-dessous) nous avons réalisé la préparation des frottis sur lames en les fixant par flambage rapide sur une flamme d'un bec de Bunsen. Des globulines anti-lapin produites dans la chèvre étaient conjugées par l'isothiocyanate de fluoresceine. Nous ajoutons 2 mg de fluochrome au 100 mg de protéine, celle-ci est purifiée ensuite par Sephades-G 25. La recherche des *Shigella* existant dans les eaux souillées a été effectué par la méthode indirecte du test des anticorps fluorescents avec mise en réalisation des contrôles convenables. Nous avons utilisé le microscope à fluorescence Leitz—Ortholux avec la lumière bleue en employant la lentille à immersion.

Les anti-sérums polyvalents et monovalents de *Shigella* (*Sh. dysenteriae* 1,2, *Sh. flexneri* 1-6,X,Y., *Sh. sonnei* I,II) que nous avons utilisé pour cette étude sont préparés par l'Institut National d'Hygiène publique de Hongrie.

Réaction d'immuno-encre de Chine (réaction de GECK). Pour l'exécution de la réaction de GECK nous avons fait la préparation des frottis sur lames avec les matières à examiner en séchant à l'air et en fixant par flambage. Les antisérums de *Shigella* avec de diverses dilutions convenables que nous avons utilisé dans cette réaction sont identiques aux anti-sérums employés dans la technique des anticorps fluorescents. Chaque frottis a été couvert par une goutte d'une encre de Chine commerciale et a reçu ensuite une goutte d'anti-sérum dilué convenable en se mêlant soigneusement avec une anse de platine. Placer la lame sous le couvercle d'une boîte de Petri doublé par un papier filtre imbibé d'eau pour éviter la dessication. Après 5 minutes de contact à la température de laboratoire, le frottis a été lavé par une solution salée (3 g de NaCl + 1,000 ml d'eau du robinet, le pH doit être ajusté à 7 par adjonction de carbonate de sodium de 5 p. 100) à l'aide d'une pipette de 10 ml. La préparation est finalement séchée entre deux papiers buvards. L'examen est mis en réalisation avec l'objectif à immersion d'un microscope ordinaire.

La réaction est positive si le contour coloré en couleur noire est conforme à la morphologie et à la dimension de la bactérie. Par contre, le résultat est négatif dans les cas où les bactéries sont inobservables sur plusieurs champs microscopiques.

Cependant une cause d'erreur peut exister: les sporulés aérobies ou anaérobies ayant la forme des bacilles sont aussi colorés par l'effet des anticorps naturels, mais leur morphologie et leur dimension permettent de les différencier facilement à l'*Enterobacteriaceae.*

Procédé de centrifugation, de filtration et d'enrichissement (Technique soi-disante «Trépied»). 1,000 ml d'eau de surface et 300 ml d'eau d'égouts ont été centrifugés à 1,000 t/mn pendant une $^1/_2$ heure et ensuite:

1. Le sédiment est lavé au moins trois fois avec la solution tamponnée pH 7,2 (T.S.).
2. Une moitié de surnageant est portée à la filtration avec les Black non-fluorescent membranes filtrantes (B.M.F.) Millipore HABP 047. Ensuite on lave les dépôts bactériens existant sur la surface de B.M.F. avec 50 ml de T.S.

3. Une autre moitié de surnageant est filtrée par les membranes filtrantes ordinaires avec pores de 0,45 μ. Celles-ci sont aussi lavées par 50 ml de T.S. et placées ensuite sur le milieu solide d'enrichissement d'ISTRATI [28]. Après une incubation à 37 °C durant 10 heures, nous avons récolté la population bactérienne développée sur la surface supérieure de la membrane en lavant avec T.S.

Tous les trois: sédiment, matière filtrante, population bactérienne enrichie sont mis en préparation des frottis pour la technique des anticorps fluorescents et pour la réaction de GECK.

Sources d'eau examinée. Les échantillons d'eau de surface sont prélevés d'un certain point du fleuve Danube et du lac Balaton. Les échantillons d'eau d'égouts provenaient des différentes régions de Budapest, de Mátra, et d'Eger en Hongrie.

Résultats

La démonstration des *Shigella* sur 60 échantillons d'eau de surface et d'égouts a été effectuée à la fois par la technique des anticorps fluorescents (A.F.), par la réaction de GECK (R.G.), et par la méthode bactériologique (M. bac). Avec la technique des A.F. et R.G. nous avons trouvé dans les eaux

Tableau I

Diverses Shigella dans 60 échantillons d'eau de surface et d'égouts provenant de différentes régions

Sources d'eau examinée	Méthode bactério-logique	*Shigella* trouvées par la technique des A. F. (+) et de la R. G. (++)		
		sédiment	filtrat	culture enrichie
Eau de surface	—	—	—	*Sh. sonnei* II
Eau d'égouts	*Sh. flexneri* 2a *Sh. sonnei* II	*Sh. dysenteriae* 2 *Sh. flexneri* 2a *Sh. flexneri* 3a *Sh. sonnei* II	*Sh. dysenteriae* 2 *Sh. flexneri* 2a *Sh. flexneri* 3a *Sh. sonnei* II	*Sh. dysenteriae* 2 *Sh. flexneri* 2a *Sh. flexneri* 3a *Sh. sonnei* II

N. B.: — = introuvable
(+) = technique des anticorps fluorescents
(++) = réaction de GECK

surtout celles d'égouts les *Shigella* suivantes: *Sh. dysenteriae* 2, *Sh. flexneri* 2a, *Sh. flexneri* 3a et *Sh. sonnei* phase 2 (tableau I, figure 1, 2). Sur 35 échantillons d'eau d'égouts examinés 20 cas étaient positifs (57,1 p. 100) par l'A.F. et 18 cas positifs par la R.G. (51,4 p. 100). Tandis qu'il y avait 2 cas seulement où nous avons réussi à isoler les *Shigella* (1 *Sh. flexneri* 2a et 1 *Sh. sonnei* II) par la M. bac. Naturellement dans tous les deux cas où la M. bac. a donné le résultat positif, l'A.F. et la R.G. le donnaient aussi. Sur un total de 60 échantillons d'eau de surface et d'égouts examinés, les cas positifs des *Shigella* atteignaient une proportion de 38,3 p. 100 par l'A.F. et 41,6 p. 100 par la R.G. (tableau II, figure 3).

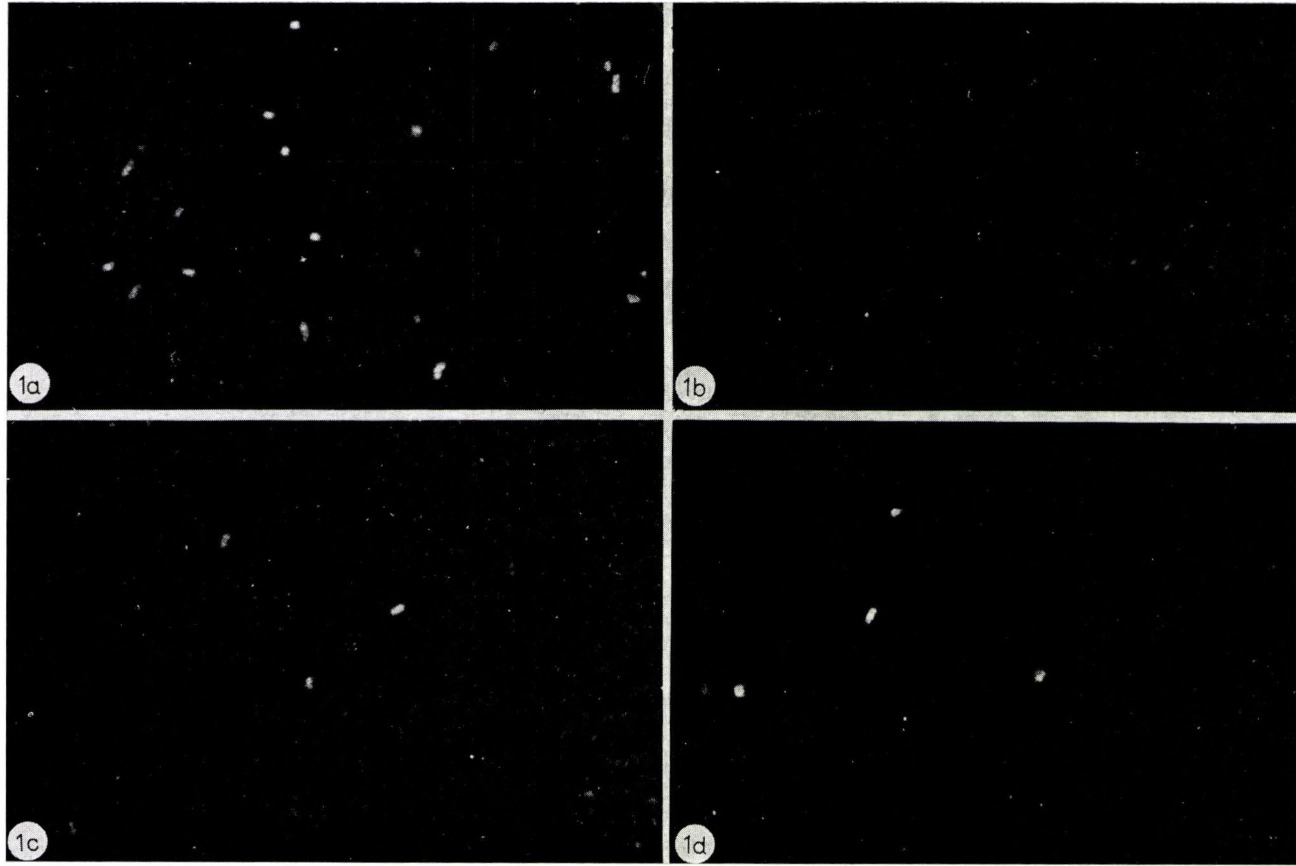


Fig. 1. Résultats obtenus sur les échantillons d'eau examinés par le test des anticorps fluorescents. a — contrôle positif, b — contrôle négatif, c — *Sh. flexneri* 2a, d — *Sh. sonnei* II

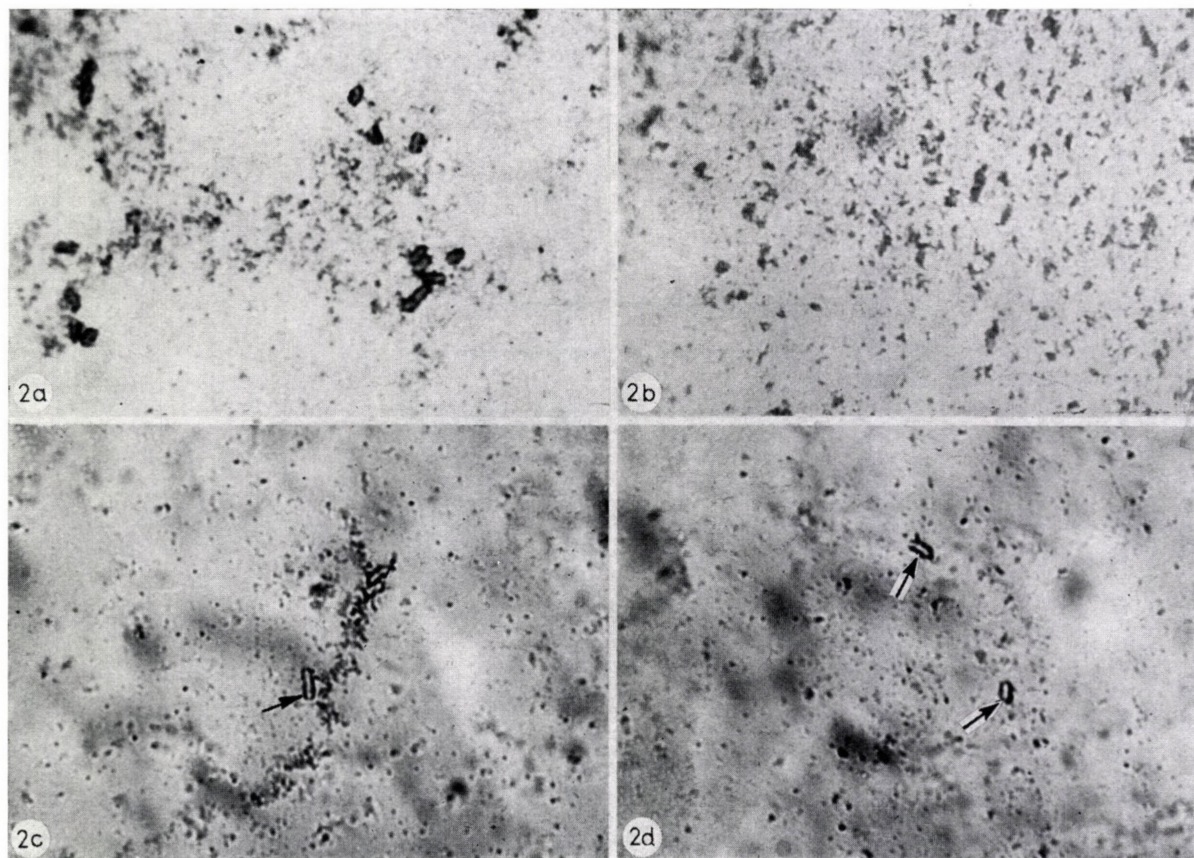


Fig. 2. Shigella trouvés dans les eaux par la réaction de GECK. a — contrôle positif, b — contrôle négatif, c — *Sh. flexneri* 2a, d — *Sh. sonnei* II

Parmi les *Shigella* trouvées dans les eaux de surface et d'égouts provenant de différentes régions, les souches de *Sh. sonnei* II atteignaient la proportion la plus élevée de 38,3 p. 100 [23] à 41,1 p. 100 [25], ensuite les *Sh. flexneri* 2a, 3a de 21,6 p. 100 [13] à 28,3 p. 100 [17] et enfin de 10,0 p. 100 [6] à 13,3 p. 100 [8] avec les *Sh. dysenteriae* 2 (tableau III). Tout cela est conforme à la

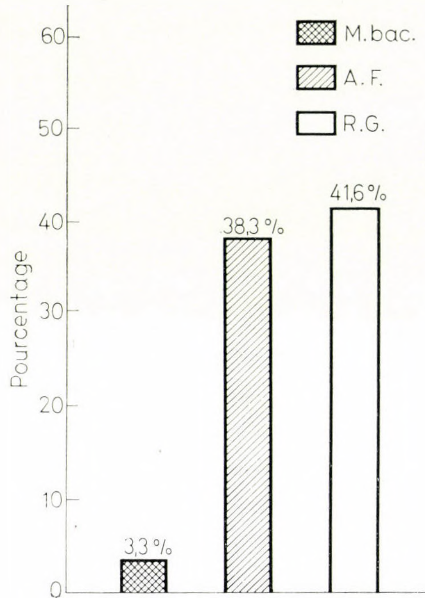


Fig. 3. Pourcentage des échantillons d'eau positive suivant la méthode bactériologique (Mb. ac.), le test des anticorps fluorescents (A. F.) et la réaction de GECK (R. G.)

situation des maladies dysentériques et tout comme à la tendance d'augmentation de la proportion des épidémies dues aux *Sh. sonnei* dans les années récentes [34].

Tableau II

Comparaison des résultats positifs sur les trois techniques: culture directe, test des anticorps fluorescents et la réaction de Geck

Sources d'eau examinée	Nombre des échantillons d'eau examinés	Échantillons d'eau positive (*Sh. dysenteriae* 2, *Sh. flexneri* 2a, *Sh. flexneri* 3a, *Sh. sonnei* II)		
		culture directe	test des anticorps fluorescents	réaction de GECK
Eau de surface	25	0 (0 %)	3 (12 %)	7 (28 %)
Eau d'égouts	35	2 (5,7 %)	20 (57,1 %)	18 (51,4 %)
Total	60	2 (3,3 %)	23 (38,3 %)	25 (41,6 %)

Tableau III

Diverses souches des Shigella trouvées sur 60 échantillons d'eau examinée par le test des anticorps fluorescents et par la réaction de Geck

Sources d'eau examinée	Nombre des échantillons d'eau examinés	Test des anticorps fluorescents					
		Sh. dysenteriae 2		*Sh. flexneri* 2a *Sh. flexneri* 3a		*Sh. sonnei* II	
		nombre	%	nombre	%	nombre	%
Eau d'égouts	35	6	17,1	12	30,7	20	57,1
Eau de surface	25	0	0	1	4,0	3	12,0
Total	60	6	10,0	13	21,6	23	38,3

Sources d'eau examinée	Nombre des échantillons d'eau examinés	Réaction de Geck					
		Sh. dysenteriae 2		*Sh. flexneri* 2a, *Sh. flexneri* 3a		*Sh. sonnei* II	
		nombre	%	nombre	%	nombre	%
Eau d'égouts	35	8	22,8	16	45,7	18	51,4
Eau de surface	25	0	0	1	4,0	7	28,0
Total	60	8	13,3	17	28,3	25	41,1

Discussion

Différence entre les résultats de la méthode bactériologique et des techniques indirectes. La démonstration des *Shigella* dans les eaux par la culture directe en se contentant des milieux électifs d'isolement est vraiment insuffisante. Nous avons eu l'occasion d'expliquer ces difficultés dans les notes antérieures [15, 16]. De cette fois nous avons réussi à isoler une seule souche de *Sh. flexneri* 2a et une seule souche de *Sh. sonnei* II sur 60 échantillons d'eau examinée (3,3 p. 100). Ces résultats susdits sont obtenus après l'enrichissement à l'aide du milieu d'enrichissement d'Istrati. L'application de la technique des A.F. en l'associant au procédé de centrifugation, de filtration et d'enrichissement (Trépied) a permis une grande possibilité de démontrer les *Shigella* existant dans les eaux (38,3 p. 100 des cas positifs). Ici nous ne voulons pas parler encore de la sensibilité et de la spécificité de cette technique qui a été appliquée pour la recherche des *Enterobacteriaceae* en général et des *Shigella* en particulier, parce que sur ce sujet il y avait beaucoup d'auteurs qui ont déjà discuté dans les diverses communications: Cherry *et coll.* [4—6], Thomason *et coll.* [40—43], LaBrec *et coll.* [30, 31], Taylor *et coll.* [37, 38], Danielsson *et coll.* [10, 11], Akiyoshi [1] etc.

La technique des A.F. a été appliquée par nous pour la recherche des *Shigella* dans les eaux, pendant que nous ne possédons pas encore des milieux liquides d'enrichissement convenables d'une valeur assez grande surtout dans les cas où l'on suspecte l'eau d'être la cause d'une épidémie de dysenterie bacillaire. Toutefois cette technique ne remplace pas les épreuves par l'isolement des souches de *Shigella* par la méthode bactériologique.

Sensibilité et spécificité de la réaction de Geck *et leur valeur d'utilisation pour la recherche des Shigella existant dans les eaux.* Ici, nous limitons cette réaction dans la démonstration des *Shigella* dans les eaux, parce que nous pensons que cette réaction est tout à fait neuve au point de vue de mécanisme et on peut l'appliquer efficacement pour le diagnostic des autres bactéries [23]. La réaction de Geck a révélé la présence des *Shigella* (ensemble des *Sh. dysenteriae* 2, *Sh. flexneri* 2a, 3a, *Sh. sonnei* II) dans 41,1 p. 100 des cas sur 60 échantillons d'eau examinée. Par la technique des A.F. les *Shigella* ont été mis en évidence dans 38,3 p. 100 des cas. Dans tous les cas où le test les A.F. était positif, la R.G. l'était aussi.

Soulignant la valeur réelle de la réaction de Geck, en comparant les résultats qu'elle fournit et ceux qui sont obtenus par l'A.F. et par les autres techniques indirectes usuelles:

— elle se montre aussi suffisamment spécifique et aussi certainement sensible;

— elle est pratique et aussi rapide. En temps d'épidémie dans tout lieu y compris sur le terrain où l'épidémie est en train de se couler, à l'aide de cette réaction nous pouvons dépister dans les moindres délais (pendant 10—20 minutes) l'étiologie de l'épidémie, les malades, et les porteurs de germes. Elle a surtout un intérêt épidémiologique;

— un fait assez important de cette technique est sa simplicité et son bon marché. Elle n'exige pas les réactifs dont la préparation doit être rigoureuse. Pour elle, le microscope ordinaire, l'encre de Chine commerciale et les sérums agglutinants sont suffisants. C'est pourquoi on peut l'appliquer et la réaliser très facilement dans tous les laboratoires bactériologiques.

Remerciements. Nous adressons nos sincères remerciements aux Drs Zs. Deák, J. Szita pour leur amabilité et tout particulièrement au Dr. P. Geck de l'Institut National d'Hygiène Publique, Budapest, pour leur aide dans notre étude. La photographie s'est faite dans le laboratoire de Mme M. Villányi. Nous l'en remercions vivement.

BIBLIOGRAPHIE

1. Akiyoshi, K. J.: Fluorescent Antibody Techniques and Their Applications. University of Tokyo Press, Tokyo 1969.
2. Bissett, M. L., Powers, C., Wood, R. M.: Appl. Microbiol. **17,** 507 (1969).
3. Burkhardt, F.: Zbl. Bakt. I. Abt. Orig. **212,** 177 (1969/70).

4. Cherry, W. B., Thomason, B. M.: Publ. Hlth Rep. **10,** 887 (1969).
5. Cherry, W. B., Moody, M. D.: Bact. Rev. **29,** 222 (1965).
6. Cherry, W. B., Thomason, B. M., Pomales-Lebrón, A., Ewing, W. H.: Bull. Org. mond. Santé **25,** 159 (1961).
7. Closs, O.: Acta path. microbiol. scand. **72,** 412 (1968).
8. Danielsson, D.: Acta path. microbiol. scand. **63,** 597 (1965).
9. Danielsson, D., Laurell, G.: Acta path. microbiol. scand. **63,** 604 (1965).
10. Danielsson, D., Laurell, G., Nordbring, F., Sandler, O.: Acta path. microbiol. scand. **72,** 118 (1968).
11. Danielsson, D., Laurell, G.: Acta path. microbiol. scand. **72,** 251 (1968).
12. Danielsson, D., Laurell, G.: Acta paediat. (Stockh.) **53,** 49 (1964).
13. Demissie, A.: Acta path. microbiol. scand. **67,** 393 (1966).
14. De Repentigny, J.: Rev. Canad. Biol. **17,** 492 (1959).
15. Duc, N. D., Deák, Zs.: Ann. Inst. Pasteur (Lille) Données inédites.
16. Duc, N. D.: Egészségtudomány **15,** 131 (1971).
17. Folsenfeld, O., Rokkanu, W.: J. Bact. **72,** 869 (1956).
18. Geck, P., Horváth, S., Karasszon, D.: Kísérl. Orvtud. **15,** 513 (1963).
19. Geck, P., Szántó, R.: Acta microbiol. Acad. Sci. hung. **11,** 211 (1964/65).
20. Geck, P., Osváth, P., Voltay, B., Backhausz, R., Losonczy, G., Vigh, G., Bognár, S.: Acta microbiol. Acad. Sci. hung. **10,** 1 (1963).
21. Geck, P., Gágó, G., Kovács, S.: Orv. Hetil. **106,** 1171 (1965).
22. Geck, P.: Módszertani levelek. OKI, OÉTI, OMI. **5,** 21 (1970).
23. Geck, P.: Egészségtudomány **15,** 87 (1971).
24. Guthrie, R. K., Reeder, D. J.: Appl. Microbiol. **17,** 399 (1969).
25. Hammarström, E., Ljutow, V.: Acta path. microbiol. scand. **35,** 365 (1954).
26. Hornung, J. E.: Amer. J. med. Techn. **31,** 239 (1965).
27. Hovnanian, H. P., Breunan, T. A., Botan, E. A.: J. Bact. **87,** 473 (1964).
28. Istrati, G., Ciufeco, C., Cimpeanu, I.: Arch. roum. Path. microbiol. exp. **21,** 89 (1962).
29. LaBrec, E. H., Formal, S. B., Schneider, H.: J. Bact. **78,** 384 (1959).
30. LaBrec, E. H., Formal, S. B.: Bact. Proc. 133 (1960).
31. LaBrec, E. H., Formal, S. B.: J. Immunol. **87,** 562 (1961).
32. Markuse, I., Thilov, W., Deutsch, J.: Z. ges. Hyg. **15,** 537 (1969).
33. Riggs, J. L.. Seinwald, R. J., Burchatter, J., Down, C. M., Metcalf, T. G.:Amer. J. Path. **34,** 1081 (1958).
34. Rudnai, O.: Siófoki Közegészségügyi Napok, 1970.
35. Stuberg, C. S., Caldweld, W. J., Kennedy, D. W., Caldroney, G.: Amer. J. Epidem. **83,** 518 (1966).
36. Szántó, R., Geck, P.: Orv. Hetil. **102,** 45 (1961).
37. Taylor, C. E. D., Lea, G. B., Tombinson, D. J. H.: J. clin. Path. **17,** 225 (1964).
38. Taylor, C. E. D., Heimer, G. V.: Brit. med. J. **2,** 165 (1964).
39. Thomason, B. M., MacWhorter, A. C.: Bull. Org. mond. Santé **33,** 681 (1965).
40. Thomason, B. M., Nahmias, A. J., Mathiew, A. S.: Appl. Microbiol. **15,** 912 (1967).
41. Thomason, B. M., Cowart, C. S., Cherry, W. B.: Appl. Microbiol. **13,** 605 (1965).
42. Thomason, B. M., Cherry, W. B., Davis, B. R., Pomales-Lebrón, A.: Bull. Org. mond. Santé **25,** 137 (1961).
43. Thomason, B. M., Cherry, W. B., Pomales-Lebrón, A.: Bull. Org. mond. Santé **25,** 153 (1961).
44. Thomason, B. M., Cherry, W. M., Edward, P. R.: J. Bact. **77,** 478 (1959).
45. Wagner, M.: Fluoreszierende Antikörper und ihre Anwendung in der Mikrobiologie. Gustav Fischer Verlag, Jena 1967.

Addresse de l'auteur:

Nguyen Dang Duc
Institut National d'Hygiène et d'Epidémiologie, Hanoi, Viet-nam

Acta microbiol. Acad. Sci. hung. 18, 207—211, 1971

SEROLOGICAL GROUPING OF PSEUDOMONAS TYPING PHAGES

By

JUDITH LANTOS

Public Health Station (Director: J. VETRÓ), *Szeged*

(Received September 1, 1971)

Summary. Serological grouping of the standard *Pseudomonas aeruginosa* phage set has been performed. By the use of 17 different antiphage sera the phages were classified into 10 groups.

Serological relationship was confirmed by artificial lysogenization of model strains. Cultures obtained in this manner changed their phage pattern and, with exception of members of phage group B, developed immunity to phages belonging to the corresponding serogroup.

Since 1961, several methods of phage typing of pseudomonads have been described [1—7]. As the overwhelming majority of isolates are lysogenic, obtaining of phages presents no difficulty and a suitable set allows the typing of strains in 90%. Most of the authors worked with different phage sets. SUTTER, HURST and FENNEL [5] supplemented the set of POSTIC and FINLAND with phages used by PAVLATOU and KAKLAMANI and with phages isolated at Colindale. SJÖBERG [7] used the phages of LINDBERG, who had collected them from various continents and selected a set containing virulent, temperate, specific and broad-spectrum phages with different lysis spectra [3]. All results indicate that *Ps. aeruginosa* can be divided into a number of phage types, the grouping of which is as yet unsolved and far less simple than that of *Staphylococcus aureus*. As in the literature there are no systematic studies on the serological examination of pseudomonad phages, it seemed desirable to elaborate a classification which would allow the characterization of strains according to serogroups of lytic phages instead of recording a long list of individual phages.

Materials and methods

Strains and phages. Twenty *Ps. aeruginosa* strains and phages were obtained from the Central Public Health Laboratory, Colindale, London: 7, 16, 21, 24, 31, 44, 68, 73, F7, F8, F10, 109, 119x, 352, 1214, M4, M6, C11, C18, C21. Of these phages 17 originated from LINDBERG's collection, 1 from Greece and 2 were isolated at Colindale. Phages 2 and C188 and their propagating strains were received from Dr. SJÖBERG. The phages were propagated with the soft agar technique and the lysis spectrum was checked on the test strains. Typing was carried out at routine test dilution.

Medium. Witte peptone, 1%; beef extract (Central Slaughter-house, Budapest), 0.75%; agar, 1.2%; the pH was adjusted to 7.2 with sodium carbonate.

Preparation of phage antisera. As described by ADAMS, rabbits weighing approximately 2.5 kg were injected twice weekly. As a first dose 0.5 ml, as subsequent doses 2.0 ml aliquots

of phage containing 10^9—10^{10} p.f.u./ml were given. The animals were bled one week after the last injection if a blood sample showed sufficiently high titres. Before use the sera were heated at 56 °C for 30 minutes.

Neutralization test was also made according to ADAMS. To 0.9 ml 1 : 100-diluted serum 0.1 ml phage was added, incubated at 37 °C for 20 minutes and cooled rapidly. Then 10-fold serial dilutions of the neutralization test mixture and of control phage were prepared. Velocity constants (K) were calculated by the formula

$$K = \frac{2.3 \times D}{t} \log \frac{P_0}{P},$$

where P_0 = phage particle counts at zero time, P = phage particle counts at time t, D = final dilution of serum in the phage—serum mixture.

Lysogenization. Three-hour broth cultures of the strains examined were flooded over Witte peptone agar plates, dried and spotted with lysogenizing phages. Secondary colonies growing within the plaques were subcultured and tested for immunity to the lysogenizing phage. The identity of the phage released from the lysogenized strain and of the lysogenizing phage was checked. Only strains corresponding to the above criteria were regarded as artificially lysogenized cultures.

Results

Anti-phage serum was prepared against 17 out of the 22 phages of the typing set. The phages were weak antigens and the K values for many of

Table I

Velocity constants (K values)

Serum \ Phage	2	7	C21	16	44	109	352	1214	F8	21
2	23	17	7							
7	27	46	12							
C21	17	14	24							
16				57	46	34	42	46	44	
352				46	48	44	50	48	48	
1214				54	46	44	46	57	48	
F8				54	46	46	46	58	58	
21										23
24										
M4										
68										
C18										
F7										
119x										
F10										
M6										
C188										

them were very low. A faint immune response was obtained for phages F10 and 119x even after 5 weeks immunization. With 5 phages there was no need to prepare immune sera as these could be classified by the use of other antiphage sera. Table I shows the velocity constants (K values) of sera for different phages of the set.

Phages showing a similar degree of titre lowering in the neutralization test with homologous and heterologous sera and giving cross-reactions and K values of the same order were classified into one serological group. Less intensive reactions were observed with 31 phages. These were probably due to the absence of certain partial antigens or differences in antigenic components of the phages. Serological grouping of phages is summarized in Table II.

Subsequently some of the broad-spectrum *Ps. aeruginosa* strains were lysogenized in order to show the effect of immunity against the lysogenizing phage on the phage pattern.

Table III shows the results. Strains used for lysogenization are characterized by the serogroups of the lytic phages. Lysogenization was performed with several phages of each of the phage groups (A, B, D, E). The lysogenized

for antiphage sera

24	M4	C11	68	C18	73	F7	119x	F10	M6	C188	31
											12
46	38	28									
46	57	26									
			34	12							
			18	18							
					17	35					
							6				
								6			
									19		
										34	28

strains usually acquired immunity to all phages of the same serogroup. Serogroup B was an exception, as although against 6 lysogenizing phages of this group the strains had always become resistant, against other B group phages they remained sensitive. It may be assumed that within this phage group there are fine serological differences.

Table II

Serological grouping of phages

A	B	C	D	E	F	G	H	I	K
2	16	21	24	68	73	119x	F10	M6	C188
7	44		M4	C18	F7				31
C21	F8		C11						
	109								
	352								
	1214								

Table III

Phage pattern alteration of broad phage spectrum Ps. aeruginosa strains after lysogenization

Phage spectrum of strain before lysogenization	Serogroup of lysogenizing phage	Immunity developed against serogroup of phages	Phage spectrum of strain after lysogenization
ABCDEG	A	A (B)	(B) CDEG
	B	BDG	ACE
	C	CEG	ABD
	D	D	ABCEG
	E	CEG	ABD
	G	CG	ABDE
ABDEG (F8)	B	(B) G	A (B) DE
	D	DAG	BE
	G	GE	ABD
BCEFIK	B	(B) CE	BFIK
	F	FCEI	BK
	E	ECFI	BK
	I	ICEF	BK
BD	B	B	D
	D	D	BCE

It was frequently observed that the strain examined developed immunity not only to phages of one, but also to another serogroup. Such relationships were observed with members of serogroups C, E, F, G and I. The cross-immunity caused, of course, a narrowing of the phage spectrum. The opposite of this finding was observed with one strain, as it developed immunity to the lysogenizing phage group, but became sensitive to another phage group.

Discussion

Serological classification performed in the present work has resulted in establishing 10 phage groups. The serological relationships observed have been proved by artificial lysogenization of model strains.

The weak antibody response against many of the phages presented some difficulty in these studies. It is known [10—12] that administration of lipopolysaccharide-type endotoxins of Gram-negative bacteria together with, and especially prior to, the antigenic stimulus, decreases the degree of antibody production. As most pseudomonads produce pyocine, it may be assumed that the unpurified phage lysate used for immunization contained toxic substances the immunosuppressive effect of which may have been responsible for the low antibody titre against certain phages. In subsequent studies an attempt will be made to remove toxins and bacterial disintegration products from the phage preparation. In this manner immune sera more suitable for finer classification can probably be expected.

Acknowledgement. The author is indebted to Mrs. E. Tóth and Miss A. Böröcz for skilled technical assistance.

REFERENCES

1. Gould, J. G., McLeod, J. W.: J. Path. Bact. **79**, 295 (1960).
2. Postic, B., Finland, M.: J. clin. Invest. **40**, 2064 (1961).
3. Lindberg, R. G., Latta, R. L., Brame, R. E., Moncrieff, J. A.: Bact. Proc. **15**, 81 (1964).
4. Meitert, E.: Arch. roum. Path. exp. Microbiol. **24**, 439 (1965).
5. Sutter, V. L., Hurst, V., Fennel, J.: Health Lab. Sci. **2**, 7 (1965).
6. Grün, L., Pillich, J., Heyn, U.: Arch. Hyg. Bakt. **151**, 641 (1967).
7. Sjöberg, L., Lindberg, A. A.: Acta path. microbiol. scand. **74**, 61 (1968).
8. Farmer, J. J. III, Lloyd, G. H.: Appl. Microbiol. **18**, 760 (1969).
9. Pavlatou, M. P., Hassikou-Kaklamani, E.: Ann. Inst. Pasteur **102**, 914 (1961).
10. Finger, H., Fresenius, H., Angerer, M.: Experientia (Basel) **27**, 456 (1971).
11. Condie, R. M., Zak, S. J., Good, R. A.: Fed. Proc. **14**, 459 (1955).
12. Bradley, S. G., Watson, D. W.: Proc. Soc. exp. Biol. (N. Y.) **117**, 570 (1961).

Address of the author:

Judit Lantos
Public Health Station
Tolbuhin sgt. 57, Szeged, Hungary

Acta microbiol. Acad. Sci. hung. 18, 213—218, 1971

HOST-SPECIFICITY TYPES IN SHIGELLA FLEXNERI

By

I. KÉTYI and ADELE VERTÉNYI

Institute of Microbiology (Director: K. RAUSS), University Medical School, Pécs

(Received September 11, 1971)

Summary. A total of 179 *Shigella flexneri* serotype 1a, 1b, 2a, 3 and 6 strains has been examined by the use of modified T5 phage lysates. The strains were divided into two different "host-specificity types". No association was found between antigenic structure and host-specificity types. The taxonomic value of the finding is discussed.

It has been shown earlier [1] that *Shigella flexneri* isolates differed in host-specific modification and restriction properties from *Escherichia coli* K-12 or B. In addition, the examined *Sh. flexneri* strains failed to form a homogeneous group in this respect.

Host-specific modification and restriction ensure the genetic stability of a given biological system and thus it may be assumed that these properties characterize the species more objectively than certain arbitrarily chosen biochemical reactions or the antigenic structure. In an attempt at finding a new taxonomic characteristic for *Sh. flexneri,* our studies were extended to 179 strains representing the most frequent serotypes.

Materials and methods

Strains. The following strains were used: 11 *Sh. flexneri* 1a, 51 *Sh. flexneri* 1b, 36 *Sh. flexneri* 3, 44 *Sh. flexneri* 6 and 37 *Sh. flexneri* 2a isolates kindly supplied by the Public Health Stations of Pécs, Debrecen, Veszprém and Szombathely and by the Department of Bacteriology, National Institute of Public Health, Budapest. Before use all strains were checked for biochemical reactions and antigenic structure.

Host-specificity was examined with phage T5 used in previous studies [1]. The phage was subjected to a few passages then it was propagated on the strain examined. Finally its titre for the propagating strain and for the other cultures tested was determined. The data were expressed in relative efficiency plating (e.o.p.) taking plaque counts for the propagating strain 1. Whether or not there was a difference in host-specificity between the propagating strain and the tested cultures, phage T5 was further propagated on certain strains chosen as representants and reciprocal e.o.p. determinations were again performed.

Titration was carried out by the method of ADAMS [2]: an agar plate was overlayered with 3 ml 0.6% agar containing 0.1 ml phage dilution and approximately 10^7 cells of the strain examined. Phage lysates were prepared as described by HERSHEY *et al.* [3]. The modified phage lysates were designated according to the principle of ARBER and DUSSOIX [4]; for example "T5.205" means phage T5 modified with *Sh. flexneri* strain 205.

Results

1. Sh. flexneri serotype 3. On the basis of fine antigenic differences the 36 strains were classified as follows: serotype variant 3a = 6; 7,8; III (7 strains), serotype 3c according to Ewing = 6; —; III (24 strains), variants 3^- = 6; —; — (5 strains). Cultures designated with 3^- undoubtedly represented a degraded antigenic variant, although they were not R mutants, since they retained the specificity of the primary polysaccharide side chain [5]. They may have derived from serotype 1b or 4b.

Phage T5 had been modified earlier with serotype 3c strain UP3042 and the lysate was used in the present experiments for examining all *Sh. flexneri* serotype 3 isolates. The results almost uniformly indicated the absence of host-specific restriction: the number of plaques produced by phage T5.3042 was similar for the tested cultures and for the original host. The only exception was strain 293, a 3^- (degraded) culture showing a slight plaque count reduction. In subsequent experiments phage T5 was modified with this culture and with strain UP249 (6; 7,8; III), which showed no restriction. *Sh. flexneri* 4b strain UP4042 (showing restriction in previous experiments) and phage T5 modified by it were also included in these examinations.

Table I

Host-specificity of strains representing Sh. flexneri serotype 3

Serotype	Strain	Relative e.o.p. obtained with phage lysates			
		T5.3042	T5.249	T5.293	T5.4042
3c	UP3042	1	1	1.6×10^{-1}	0.5×10^{-1}
3a	UP249	1	1	1.2×10^{-1}	0.8×10^{-1}
3	UP293	1.2×10^{-4}	8.9×10^{-3}	1	1
4b	UP4042	1.2×10^{-4}	9.8×10^{-3}	1	1

Table I shows reciprocal e.o.p. values determined in cross-tests. It is evident that strains UP3042 and UP249 were identical in host-specificity, but the degraded strain UP293 was similar to *Sh. flexneri* 4b strain UP4042. This finding, as pointed out later in this paper, did not allow to conclude that strain UP293 had derived from serotype 4b.

2. Sh. flexneri serotype 1b. Fifty-one freshly isolated strains were tested first with phage T5.3042 then the phage was modified with strain UP1000 of identical host-specificity and used for checking the experiments. Forty strains were similar in host-specificity to *Sh. flexneri* serotype 3. The remaining 11 strains showed well-defined restriction with phages T5.3042 and T5.1000. Of the latter cultures isolate UP129 was chosen and used for the modification

of phage T5. Cross-e.o.p. examinations including *Sh. flexneri* 4 strain UP4042 and phage T5 modified by it are shown in Table II.

Table II

Host-specificity of strains representing Sh. flexneri serotype 1b

Sero-type	Strain	Relative e.o.p. obtained with phage lysates			
		T5.3042	T5.1000	T5.129	T5.4042
3c	UP3042	1	1	2.5×10^{4}	1
1b	UP1000	1	1	1.8×10^{4}	1
1b	UP129	2.0×10^{-4}	5.2×10^{-4}	1	1
4b	UP4042	1.5×10^{-4}	1.2×10^{-4}	1	1

For serotype 1b strain UP129 phages T5.3042 and T5.1000 showed a 4-exponent e.o.p. reduction, which corresponded to that obtained for the representant strain of serotype 4b. Phage T5.4042 showed practically no restriction for any of the hosts. Phage T5 modified with *Sh. flexneri* 1b strain UP129 behaved unexpectedly: with strain 4b it showed no relative e.o.p. reduction, but with strains UP3042 and UP1000 it produced e.o.p. values increased by 4 exponents. This observation can only be interpreted in the sense that in restriction strain UP129 was similar to serotype 4b (UP4042) and not to strain 3c (UP3042) or to strain 1b (UP1000). Phage T5 propagated on strain UP129 showed no alteration, as the strain exerted no modifying capacity and was, accordingly, a so-called $m^{-}r^{+}$ mutant.

3. Sh. flexneri serotype 1a. In host-specifity the examined 11 *Sh. flexneri* 1a strains were not homogeneous: with lysates T5.3042 and T5.1000 7 strains failed to show restriction; 4 strains behaved similarly to the representant strain of serotype 4b. The results were confirmed with two T5 lysates (T5.673 and T5.551) representing the two host-specificity types.

4. Sh. flexneri serotype 6. With lysate T5.3042 none of the 44 isolates showed restriction. The results were checked with phage T5 propagated on *Sh. flexneri* serotype 6 strain UP205. Reciprocal titrations confirmed the absence of restriction and indicated the correctness of the finding that all serotype 6, 3a, and 3c strains were identical in host-specificity.

5. Sh. flexneri serotype 2a. A total of 37 freshly isolated strains was examined with modified T5 phages. With phage T5.3042 restriction was not observed for 25 strains, while for 12 isolates the phage lysate showed reduced e.o.p. Control experiments were made with phage T5 modified with strain UP80.

Table III shows the results obtained with strain UP80 and with some other cultures. In modification-restriction properties *Sh. flexneri* 2a strain UPA was identical with *Sh. flexneri* 3 strain UP3042, but strain UP80 (sero-

Table III

Host-specificity of strains representing Sh. flexneri serotype 2a

Sero-type	Strain	Relative e.o.p. obtained with phage lysates			
		T5.3042	T5.A	T5.80	T5.4042
3c	UP3042	1	1	3.2×10^{-3}	1.5×10^{-1}
2a	UPA	1	1	5.8×10^{-3}	1
2a	UP80	2.1×10^{-4}	2.8×10^{-2}	1	1
4b	UP4042	1.0×10^{-4}	5.6×10^{-3}	1	1

type 2a) showed a host-specificity characteristic of the culture representing serotype 4b.

6. Host-specificity of Sh. flexneri. In this respect only serotypes represented by a sufficient number of freshly isolated strains were considered. As summarized in Table IV, the examined 179 strains fell into not more than 2 different host-specificity types. It is evident that host-specificity is independent of antigenic structure: different serotypes may show identical host-specificity, while strains belonging to the same serotype may differ in modification restriction characteristics.

Table IV

Distribution of 179 Sh. flexneri strains according to host-specificity

Serotype	Total No. of strains	No. of strains falling into "host-specificity type"	
		A	B
3a and 3c	31	31	—
3⁻*	5	4	1
1b	51	40	11
1a	11	7	4
6	44	44	—
2a	37	25	12
Total	179	151	28

* Antigenically defective strain (6; —; —)

Discussion

The principle of host-specific modification and restriction is that the bacterial host "labels" its DNA in a specific manner by methylating certain bases. The cell "recognizes" foreign bacterial, viral or plasmid DNA and with specific endonuclease decomposes it or modifies it to its own specificity.

This procedure inhibits recombination between *Escherichia* [6], *Salmonella* [7] and *Sh. flexneri* [1] and hinders transduction or plasmid transfer. On the basis of its recombination-inhibiting effect the phenomenon may be regarded as a mechanism ensuring the genetic stability of the host cell. This assumption suggested that in analogy to species definition in higher organisms on the basis of fertility-studies host-specificity may reveal a utilizable, objective means for the taxonomic determination of bacterial species.

As in many other bacterial groups, in *Sh. flexneri* the category of species is a highly debated problem. KAUFFMANN [10] regards the Flexner group as a subgenus and claims that each individual serotype or serogroup is an individual species. In contrast, in the conception of EDWARDS and EWING [11] *Sh. flexneri* corresponds to one species within the *Shigella* group which may be regarded as a subgenus of *Escherichia.* A high degree of biochemical homogeneity and a multiple antigenic relationship are undoubtedly characteristic of members of this group. As regards immunospecific polysaccharides, SIMMONS [5] distinguished 3 basic types in the constituents of the so-called primary (non-lysogenic in origin) side chain: Y_1 (in serotypes 1a, 2a, var. Y), Y_2 (in serotypes 4a, 3a, 5, var. X) and a third type including *Sh. flexneri* 6. Although there are differences in the 3 types, all of them are characterized by N-acetyl-glucosamine-rhamnose-rhamnose repeating units.

Our studies on 5 serotypes including all 3 "chemotypes" showed only two different host-specificity types. These were independent of either the antigenic structure or the "chemotype". It cannot, of course, be stated that studies on more serotypes and on a greater number of strains would not reveal further "host-specificity types". However, it should not be neglected that informations for host-specificity may be carried by extrachromosomal factors as prophage or RTF-2 [12]. It would appear that this assumption explains the presence of different host-specificity types in *Sh. flexneri*. Experimental confirmation is not an easy task, to a large extent and variably defective prophages or plasmids may also retain this function.

As to our original assumption inspiring these experiments we may conclude that host-specificity is not associated with antigenic structure or with constituents of the polysaccharide side chain. Accordingly, the results have failed to indicate that it is justified to use the latter properties as species-determinants. In view of the fact that only two different host-specificity types have been found in *Sh. flexneri,* the results do not allow a new or more rational classification of *Sh. flexneri* and do not prove that members of this group belong to one species.

Our genetic studies on *Sh. flexneri* chromosome [13] have now been supplemented with the experience that our conjugation system should not be restricted to one serotype.

REFERENCES

1. Kétyi, I., Ørskov, F.: Acta path. microbiol. scand. Sect. B. **78**, 51 (1970).
2. Adams, M. H.: Meth. med. Res. **2**, 1 (1950).
3. Hershey, A. D., Kalmanson, G., Bronfenbrenner, J.: J. Immunol. **46**, 267 (1943).
4. Arber, W., Dussoix, D.: J. molec. Biol. **5**, 18 (1962).
5. Simmons, D. A. R.: Europ. J. Biochem. **11**, 554 (1969).
6. Boyer, H.: J. Bact. **88**, 1652 (1964).
7. Okada, M., Watanabe, T., Miyake, T.: J. gen. Microbiol. **50**, 241 (1968).
8. Wood, W. B.: J. molec. Biol. **16**, 118 (1966).
9. Arber, W., Morse, M. L.: Genetics **51**, 137 (1965).
10. Kauffmann, F.: The Bacteriology of Enterobacteriaceae. Munksgaard, Copenhagen 1941.
11. Edwards, P. R., Ewing, W. H.: Identification of Enterobacteriaceae. Burgess, Minneapolis 1967.
12. Arber, W.: The Molecular Biology of Viruses. University Press, Cambridge 1968.
13. Kétyi, I.: Acta microbiol. Acad. Sci. hung. **17**, 167 (1970).

Address of the authors:

Iván Kétyi, Adél Vertényi
Institute of Microbiology, University Medical School
Szigeti út 12, P. O. Box 99, Pécs, Hungary

INDEX

Csányi, V., Mile, I., Romhányi, T., Hortobágyi, P., Hatfaludi, F.: Irreversible Adsorption of Exopenicillinase on *Bacillus cereus* 569 Cells 137

Mérő, E., Ralovich, B.: Some Epidemiologic Problems of Human Listeriosis in Hungary 149

Vandra, E., Fodor, T.: Phage Typing of *Mycobacterium tuberculosis* Strains Isolated in Hungary 155

Váczi, L., Surján, M., Kiss, M. J., Füst, G., Rédai, I.: Bacterial Cardiolipins and their Serological Activity 159

Tenner, C., Rácz, P., Rédey, B.: Significance of the "Epithelial Phase" in Experimental Salmonella Conjunctivitis 167

Tenner, C., Rácz, P., Serény, B.: Significance of "Intracellular Parasitism" in Experimental Shigella Cystitis 179

Geck, P.: India-ink Immuno-reaction for the Rapid Detection of Enteric Pathogens 191

Duc, N. D.: La recherche des Shigella dans les eaux par le test des anticorps fluorescents et par la réaction de Geck 197

Lantos, J.: Serological Grouping of Pseudomonas Typing Phages 207

Kétyi, I., Vertényi, A.: Host-specificity Types in *Shigella flexneri* 213

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Várhelyi Tamás

A kézirat nyomdába érkezett: 1972. III. 6. — Terjedelem: 7,5 (A/5) ív, 26 ábra

72.73219 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

The Journal of General Microbiology

Volume 69, Part 3, December 1971

DEVELOPMENT AND STRUCTURE

P. DRAPER, The Walls of *Mycobacterium lepraemurium:* Chemistry and Ultrastructure.

PHYSIOLOGY AND GROWTH

J. F. PEBERDY and R. K. GIBSON, Regeneration of *Aspergillus nidulans* Protoplasts.

J. A. COLE and S. C. RITTENBERG, A Comparison of Respiratory Processes in *Spirillum volutans, Spirillum itersonii* and *Spirillum serpens.*

D. G. MACLENNAN, J. C. OUSBY, R. B. VASEY and N. T. COTTON, The Influence of Dissolved Oxygen on *Pseudomonas* AM 1 Grown on Methanol in Continuous Culture.

BIOCHEMISTRY

M. KISUMI, S. KOMATSUBARA, M. SUGIURA and I. CHIBATA, Properties of Isoleucine Hydroxamate-resistant Mutants of *Serratia marcescens.*

H. E. WADE, H. K. ROBINSON and B. W. PHILLIPS, Asparaginase and Glutaminase Activities of Bacteria.

M. P. STEPHENSON and E. A. DAWES, Pyruvic Acid and Formic Acid Metabolism in *Sarcina ventriculi* and the Role of Ferredoxin.

C. A. GODFREY and M. J. DILWORTH, Haem Biosynthesis from [^{14}C]-δ-Aminolaevulinic Acid in Laboratory-grown and Root Nodule *Rhizobium lupini.*

J. D. BU'LOCK and D. J. WINSTANLEY, Trisporic Acid Production by *Blakeslea trispora* and its Promotion by Barbiturate.

GENETICS AND MOLECULAR BIOLOGY

C. COLSON and A. M. COLSON, A New *Salmonella typhimurium* DNA Host Specificity.

A. VIVIAN, Genetic Control of Fertility in *Streptomyces coelicolor* A3(2). Plasmid Involvement in the Interconversion of UF and IF Strains.

S. BAUMBERG and E. ASHCROFT, Absence of Polar Effect of Frameshift Mutations in the *E* Gene of the *Escherichia coli argECBH* Cluster.

P. C. MCMAHON, High-frequency Gene Transfer in *Pasteurella pseudotuberculosis.*

ECOLOGY

N. WILDING, Discharge of Conidia of *Entomophthora thaxteriana* Petch from the Pea Aphid *Acyrthosiphon pisum* Harris.

SHORT COMMUNICATIONS

A. R. FERGUSON and A. P. SIMS, *In vivo* Inactivation of Glutamine Synthetase and NAD-specific Glutamate Dehydrogenase: Its Role in the Regulation of Glutamine Synthesis in Yeasts.

J. W. PATCHING and A. H. ROSE, Effect of Growth Temperature on Cold Osmotic Shock in *Escherichia coli* ML 30.

B. M. LUSCOMBE and T. R. G. GRAY, Effect of Varying Growth Rate on the Morphology of *Arthrobacter.*

Books Received

Price £4.00 net ($12.00 in U.S.A.). Annual subscription £32.00 net ($106.00 in U.S.A.)

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London NW1 2DB
American Branch: 32 East 57th Street, New York, N.Y. 10022

Die *Acta Microbiologica* veröffentlichen Abhandlungen aus dem Bereiche der Mikrobiologische in deutscher, englischer, granzösischer 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, Budapest, Keleti Postahivatal, Postafiók 64.

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $ 16.00.

Bestallbar bei dem Buch- und Zeitungs-Außenhandels-Unternehmen »*Kultúra*« (Budapest I., Fő utca 32. Bankkonto Nr. 43-790-057-181) oder bei seinen Auslandsvertretungen und Komissionären.

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

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

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

Acta Microbiologica, Budapest, Keleti Postahivatal, Postafiók 64.

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

Le prix de l'abonnement est de $ 16.00 par volume.

On peur s'abonner à l'Entreprise pour le Commerce Extérieur de Livres et Journaux «*Kultúra*» (Budapest I., Fő utca 32. Compte courant No. 43-790-057-181) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют грактаты 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, Budapest, Keleti Postahivatal, Postafiók 64.

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — $ 16.00 за том.

Заказы принимает предприятие по внешней торговле книг и газет «*Kultúra*» (Budapest I., Fő utca 32. Текущий счет № 43-790-057-181), или его заграничные представительства и уполномоченные.

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

ALBANIA
Drejtorija Qändrone e Pärhapjes dhe Propagandimit tä Librit
Kruga Konferenca e Päzes
Tirana

AUSTRALIA
A. Keesing
Box 4886, GPO
Sydney

AUSTRIA
GLOBUS
Höchstädtplatz 3
A-1200 Wien XX

BELGIUM
Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
5, Place St.-Jean
Bruxelles

BULGARIA
HEMUS
11 pl Slaveikov
Sofia

CANADA
Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA
Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA
Artia
Ve Směčkách 30
Praha 2
Poštovní Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha 1
SLOVART A. G.
Gorkého
Bratislava

DENMARK
Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND
Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE
Office International de Documentation et Librairie
48, rue Gay-Lussac
Paris 5

GERMAN DEMOCRATIC REPUBLIC
Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701
Zeitungsvertriebsam
Fruchtstraße 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC
Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN
Blackwell's Periodicals
Oxenford House
Magdalen Street
Oxford
Collet's Subscription Import Department
Denington Estate
Wellingborough, Northants.
Robert Maxwell and Co. Ltd.
4—5 Fitzroy Square
London W. 1

HOLLAND
Swetz and Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA
Hind Book House
66 Babar Road
New Delhi 1

ITALY
Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze
Techna
Via Cesi 16.
40135 Bologna

JAPAN
Kinokuniya Book-Store Co. Ltd.
826 Tsunohazu 1-chome
Shinjuku-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central

KOREA
Chulpanmul
Phenjan

NORWAY
Tanum-Cammermeyer
Karl Johansgt 41—43
Oslo 1

POLAND
RUCH
ul. Wronia 23
Warszawa

ROUMANIA
Cartimex
Str. Aristide Briand 14—18
Bucureşti

SOVIET UNION
Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN
Almquist and Wiksell
Gamla Brogatan 26
S—101 20 Stockholm

USA
F. W. Faxon Co. Inc.
15 Southwest Park
Westwood Mass 02090
Stechert Hafner Inc.
31. East 10th Street
New York, N. Y. 10003

VIETNAM
Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA
Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

30. VI. 1972 Index: 26.016

ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

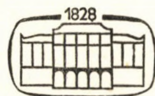
ADIUVANTIBUS

I. DÖMÖK, E. FARKAS, I. HORVÁTH,
I. NÁSZ, K. RAUSS, F. B. STRAUB, J. SZIRMAI, L. VÁCZI, K. VAS,
J. WEISZFEILER

REDIGIT

G. IVÁNOVICS

TOMUS XVIII FASCICULUS 4



AKADÉMIAI KIADÓ, BUDAPEST

1971

AMAHA 5 18(4) 219—333 (1971)

Acta microbiol. Acad. Sci. hung.

ACTA MICROBIOLOGICA

A MAGYAR TUDOMÁNYOS AKADÉMIA MIKROBIOLÓGIAI KÖZLEMÉNYEI

KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY UTCA 21.

Főszerkesztő

IVÁNOVICS GYÖRGY

akadémikus

Szerkesztő

LÁNYI BÉLA

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, Budapest, Keleti Postahivatal, Postafiók 64.

Ugyanerre a címre küldendő minden szerkesztőségi levelezés.

Megrendelhető a belföld számára az Akadémiai Kiadónál (Budapest V., Alkotmány utca 21. Bankszámla 05-915-111-46), a külföld számára pedig a „Kultúra" Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő utca 32. Bankszámla: 43-790-057-181) 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, Budapest, Keleti Postahivatal, Postafiók 64.

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

The rate of subscription is $ 16.00 a volume.

Orders may be placed with "Kultura" Foreign Trade Company for Books and Newspapers (Budapest I., Fő utca 32. Account No. 43-790-057-181) or with representatives abroad.

Acta microbiol. Acad. Sci. hung. 18, 219—229, 1971

SIGNIFICANCE OF "INTRACELLULAR PARASITISM" IN EXPERIMENTAL SALMONELLA CYSTITIS*

By

CLARA TENNER, P. RÁCZ and E. MÉRŐ

National Institute of Public Health (Director: T. BAKÁCS), Budapest

(Received October 28, 1971)

Summary. Detailed histological studies of BINGEL's salmonella cystitis were performed. Bacteria entered the epithelial cells of the bladder and passed through regular cycles there during the first phase of the process ("epithelial phase"). Subsequently and partly parallel to the first phase, salmonellae moved to the subepithelial region and proliferated the cytoplasm of macrophage type cells. Degenerative bacterial forms were observed in the cytoplasm of polymorphonuclear leucocytes indicating that they were the main killers of salmonellae. Inflammation often gave rise to a phlegmon. Studies of other organs revealed the generalization of the process.

Light [1] and electron-microscopic [2] studies on keratoconjunctivitis induced by the facultative intracellular parasitic bacterium, *Listeria monocytogenes* [3], and the light microscopic analysis of cystitis listeriosa [4], have led to the assumption that the "epithelial phase" might have a role in the pathogenesis of diseases caused by other intracellular parasitic bacteria. The term "epithelial phase" has been suggested in order to denote that period during which the pathogenic agent entering the organism from the environment penetrates the epithelial barrier protecting the internal milieu from the external environment, and it starts multiplying within the living epithelial cells [4].

Since our experiments on experimental salmonella conjunctivitis in the guinea pig have proved this assumption for one *Salmonella* group [5], the purpose of the present paper was to investigate whether an "epithelial phase" occurred with other epithelial barriers.

BINGEL in 1943 reported that the introduction of *Salmonella* strains into the bladder of the guinea pig resulted in cystitis [6]. This observation was later confirmed by STENZEL [7], but no detailed histopathological studies on this model disease have been performed.

In the present study the following problems, important with respect to the "epithelial phase", have been investigated in salmonella cystitis: (1) are living salmonellae capable of penetrating into the epithelial cells of the bladder; (2) what interactions may develop between the infective agent and the host cell; and (3) whether the salmonellae remain in the epithelium or move into the deep subepithelial tissues during the course of the infectious process.

* This work is part of a scientific collaboration plan between the National Institute of Public Health, Budapest, and the Research Institute of Experimental Medicine, Academy of Medical Sciences, Leningrad, U.S.S.R.

Materials and methods

Bacterial strains. S. typhi-murium 26772 was isolated by Dr. RÉDEY (Public Health Station, Veszprém) from infected patients. The freshly isolated strain induced keratoconjunctivitis in the guinea pig. A culture of the same strain was propagated in agar for 3 years. The following strains were isolated at the Department of Bacteriology of this institute from infected patients: *S. give* 63618, *S. derby* 63612, *S. stanley* 63622, *S. newport* 63616, *S. blockley* 63874, *S. bareilly* 63613.

For the infection of guinea pigs, broth cultures incubated at 37 °C for 18 hours and containing 10^9 germs per ml were used.

Infection. The bladder was emptied through a glass catheter and 1 ml broth culture was introduced into the bladder.

Experiment No. 1: 42 female guinea pigs of our own breed, weighing 200—250 g, were infected with virulent *S. typhi-murium* strain 26772 and 3 animals each were sacrificed 1, 3, 6, 9, 12, 24, 36, 48, 72, 96 and 120 hours after the infection.

Experiment No. 2: 18 guinea pigs weighing 200—250 g, were infected with virulent *S. typhi-murium* strain 26772 and 3 animals each were sacrificed 1, 3, 6, 9, 12 and 24 hours after the infection. In order to prevent a mechanical lesion of the epithelium, special head-tipped glass catheters with a side outlet were used for infection.

Experiment No. 3: 20 guinea pigs weighing 700—800 g, were infected with virulent *S. typhi-murium* strain 26772. The animals were sacrificed 1, 2, 3, 5, 6 and 8 days after the infection.

Experiment No. 4: 42 guinea pigs, weighing 200—250 g, were infected with *S. typhi-murium* laboratory strain 26772. The animals were sacrificed 1, 3, 6, 9, 12, 24, 36, 48, 72, 96 and 120 hours following infection.

Experiment No. 5: a total of 10 guinea pigs, weighing 200—250 g, were infected with *S. derby*, *S. stanley*, *S. newport*, *S. blockley*, and *S. bareilly*. Two of these animals were studied for each strain, one at 9 hours and one at 24 hours after the infection.

Experiment No. 6: 21 guinea pigs, weighing 200—250 g, were used as control animals. The bladder was emptied and 1 ml sterile broth was introduced. The animals were sacrificed 1, 3, 6, 9, 12, 24 and 48 hours after the treatment.

Light microscopic studies. The animals were killed by cervical dislocation. The bladder of two animals of each experimental group was fixed in formalin while the third animal's bladder in CARNOY's fluid. Specimens of the kidneys, liver, spleen, and mesenterial lymph node from each animal were fixed in formalin.

After embedding in paraffin, 3—4 μ thick sections were prepared and stained with haematoxylin-eosin, thionin and by the method of MASSON.

Results

The infection of young animals with freshly isolated *S. typhi-murium* strain 26772 resulted in a swelling of the vulvar region in 12 hours, and urinary incontinence and bristling of the fur over the whole body in 24 hours. Spontaneous death was frequent: of the 42 animals studied, 15 were sacrificed for experimental purposes while of the remaining animals 7, 1 and 2 animals died spontaneously on the first, second and fourth day of the experiment, respectively.

In the group of animals weighing 700—800 g, no spontaneous death occurred during the first days of the experiment and it was only by the 6th day that two animals died. Swelling of the perineal region and urinary incontinence developed in this group only by the 3rd day after the infection. Severe general symptoms were observed only in the two animals which died on the 6th day, and only on the last two days.

Autopsy revealed an oedema of the bladder wall as soon as 6 hours after the infection. The oedema then spread over the connective tissue of the lesser

pelvis, but the connective tissue located around the ureters remained unaffected. Pinpoint haemorrhages appeared on the mucous membrane of the hyperaemic bladder, occasionally also on the serosa, in 1—6 days. On the other hand ulceration was observed in one animal only which was sacrificed 5 days after the infection.

Essentially the same though less intense symptoms were observed in the older animals; the spleen displayed a marked enlargement and a dark purple colour in both groups 24 hours after the infection.

The severity of the clinical picture showed significant variations within the groups.

The bladder of the guinea pig is covered by transitional epithelium, the histological picture of which has been described by LETTERER and SEYBOLD [8]. The present studies yielded fully consistent findings.

A few infected epithelial cells could be found in the bladder epithelium in each experimental group one hour after the infection (Plate 1, Figs 1, 2 and 3). These cells were located either one by one or in smaller groups in different regions of the epithelium (Figs 4 and 5). The epithelial cells containing salmonellae showed normal nuclear and cytoplasmic structure. In the cytoplasm occasional vacuoles were observed (Plate 1, Fig. 2). Most infected epithelial cells contained one or two salmonellae, but cells with 5—6 bacteria were also observed. The number of salmonellae within the individual cells showed a slight increase 3 hours after the infection. The continuity of the epithelial envelop of the bladder was unimpaired 6, 9 and 12 hours after the infection. Clusters of infected epithelial cells could be observed in different regions. The epithelial cells containing salmonellae exhibited various morphological changes. Some cells had a normal nuclear and cytoplasmic structure. Others contained foci with numerous small bacteria in the cytoplasm. In some cells, part of the bacteria was enclosed in well-defined vacuoles while part of them was located free in the cytoplasm (Plate 1, Fig. 5). Dividing bacterial forms were also observed within the cells. In the close vicinity of the cells containing dividing bacterial forms, epithelial cells full of large, well staining salmonellae occurred (Plate 1, Fig. 6). Part of these salmonellae had already undergone division. In other salmonella-containing cells, the cytoplasm was slightly swollen and the nucleus showed chromatolysis (Plate 1, Fig. 4). Epithelial cells containing bacteria formed more distinct foci enclosing either large or small bacteria. At this stage, some of the epithelial cells the cytoplasms of which were full of bacteria, were already disintegrating. These cells were surrounded by polymorphonuclear leucocytes engulfing bacteria released from the cells (fresh intraepithelial microabscesses). In this region, oedema could be observed among the epithelial cells (Plate 1, Fig. 7).

A large proportion of the infected epithelial cells located in the uppermost layer desquamated into the lumen of the bladder. Groups of several dis-

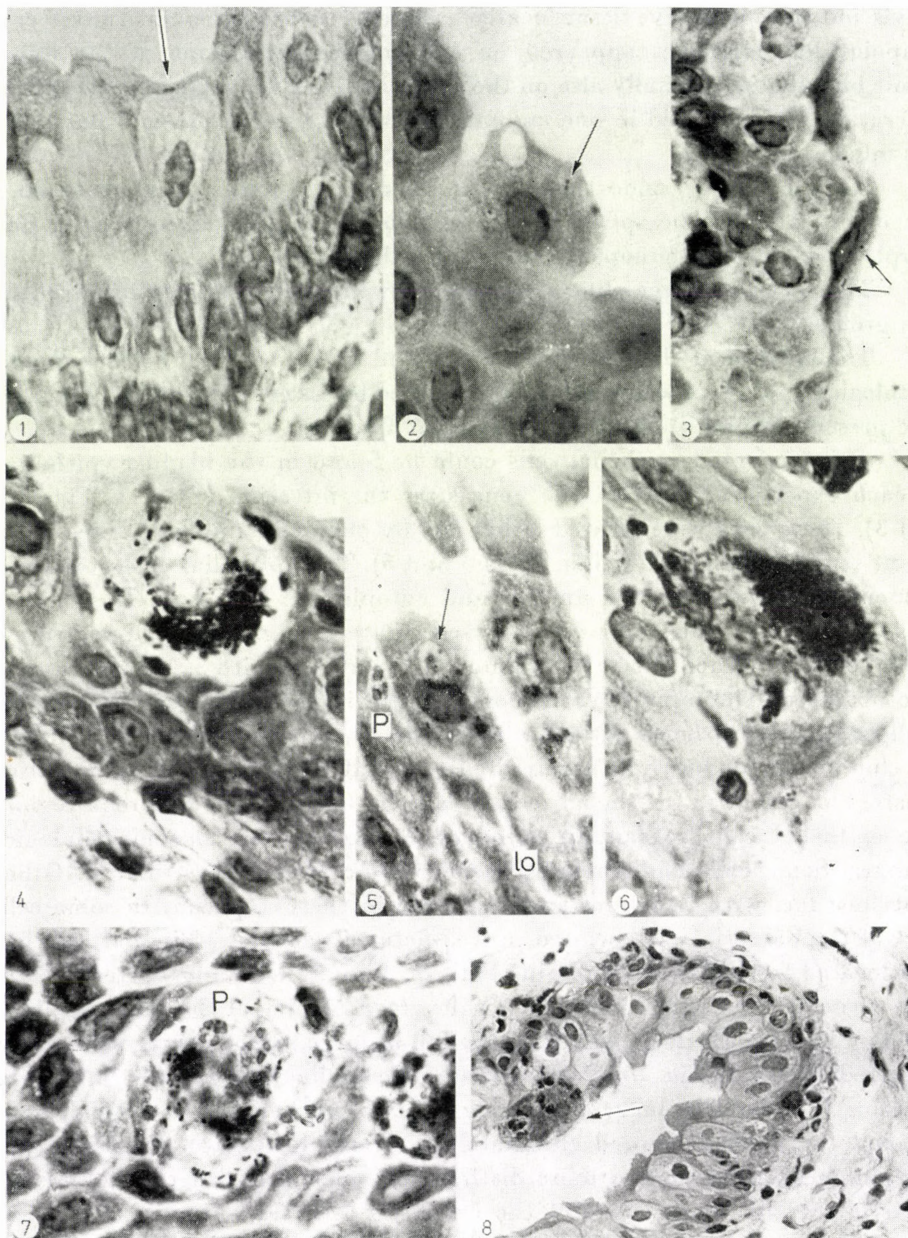


Plate 1

Fig. 1. Bacterium (arrow) in a superficial cell of the bladder epithelium. The nuclear and cytoplasmic structure of the epithelial cells is well retained. Experiment No. 1, one hour after *S. typhi-murium* 26772 infection. Thionin staining, ×1200

Fig. 2. Bacterium (arrow) in a superficial cell of the bladder epithelium. Note the vacuole in the cytoplasm of the infected epithelial cell. Experiment No. 2, one hour after infection with *S. typhi-murium* 26772. Thionin staining, ×1240

integrating epithelial cells, that lay in the close vicinity of the cytoplasm almost completely filled with salmonellae, were often separated — like a microinfarct — by polymorphonuclear leucocytes from the neighbouring epithelial cells (Plate 1, Fig. 8). Coinciding with their desquamation, mitoses were frequently observed, mainly in the basal epithelial cells.

From the 12th hour on, a growing number of cells of the macrophage type appeared in the microabscesses.

Intraepithelial clusters of macrophages and polymorphonuclear leucocytes free of salmonellae were found in several instances as early as 24 hours after the infection. Later, only a cavity containing cell detritus and fluid was present in the place of the microabscesses (Plate 2, Fig. 5). Some new groups of a few infected epithelial cells were observed in another area of the bladder surface. The structure of these clusters was the same as in the first hour.

The number of bacterium-containing epithelial cells increased until the 24th hour of the experiment, then decreased gradually. Epithelial cells containing bacteria were still found on the 8th day of the experiment. The foci were usually composed of 3—4 infected epithelial cells; their disintegration did not result in extensive ulceration, except in a single case (Plate 2, Fig. 6).

One hour after the infection, the number of polymorphonuclear leucocytes present in the lumen of the small blood vessels running in the subepithelial connective tissue immediately under the epithelium, showed a slight rise in both the infected and the control animals treated with sterile broth injected into the bladder cavity. Some of these leucocytes were located outside the blood vessels in the form of diffuse subepithelial infiltration. A slight oedema developed in the region of the lamina propria (Plate 2, Fig. 1). In the infected

Fig. 3. Bacterium (arrow) in a superficial cell of the bladder epithelium. The nuclear and cytoplasmic structure of the epithelial cells is well retained. Experiment No. 3, one hour after infection with *S. typhi-murium* 26772. Thionin staining, ×1100

Fig. 4. Epithelial cell containing a high number of salmonellae in the transitional epithelium of the bladder. The cytoplasm of the infected epithelial cell is swollen and stains lighter. Salmonellae are located mainly near one of the poles of the nucleus; this is also swollen and shows intense chromatolysis. Experiment No. 1, 12 hours after infection with *S. typhi-murium* 26772. Thionin staining, ×1300

Fig. 5. Cluster of infected epithelial cells. The nuclear and cytoplasmic structure of the infected cells is relatively well retained. Numerous small bacteria are present in the cytoplasm within the cluster. Part of the bacteria is enclosed in a confined vacuole (arrow), others are present free in the cytoplasm. Intercellular oedema between the epithelial cells. Experiment No.2, 9 hours after infection with *S. typhi-murium* 26772. Thionin staining, ×1240

Fig. 6. Infected epithelial cell almost completely filled with bacteria. There are few salmonellae in the neighbouring cells. Experiment No. 2, 9 hours after infection with *S. typhi-murium* 26772. Thionin staining, ×1300

Fig. 7. Two "microabscesses" in the vesical epithelium. There are remnants of a disintegrated epithelial cell, numerous salmonellae and polymorphonuclear leucocytes in the abscesses. Experiment No. 1, 9 hours after infection with *S. typhi-murium* 26772. Thionin staining, ×1240

Fig. 8. A cluster (arrow) of several infected epithelial cells. Their cytoplasm is filled with a high number of salmonellae. The group of epithelial cells containing salmonellae is demarcated by polymorphonuclear leucocytes. Experiment No. 1, 12 hours after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, ×480

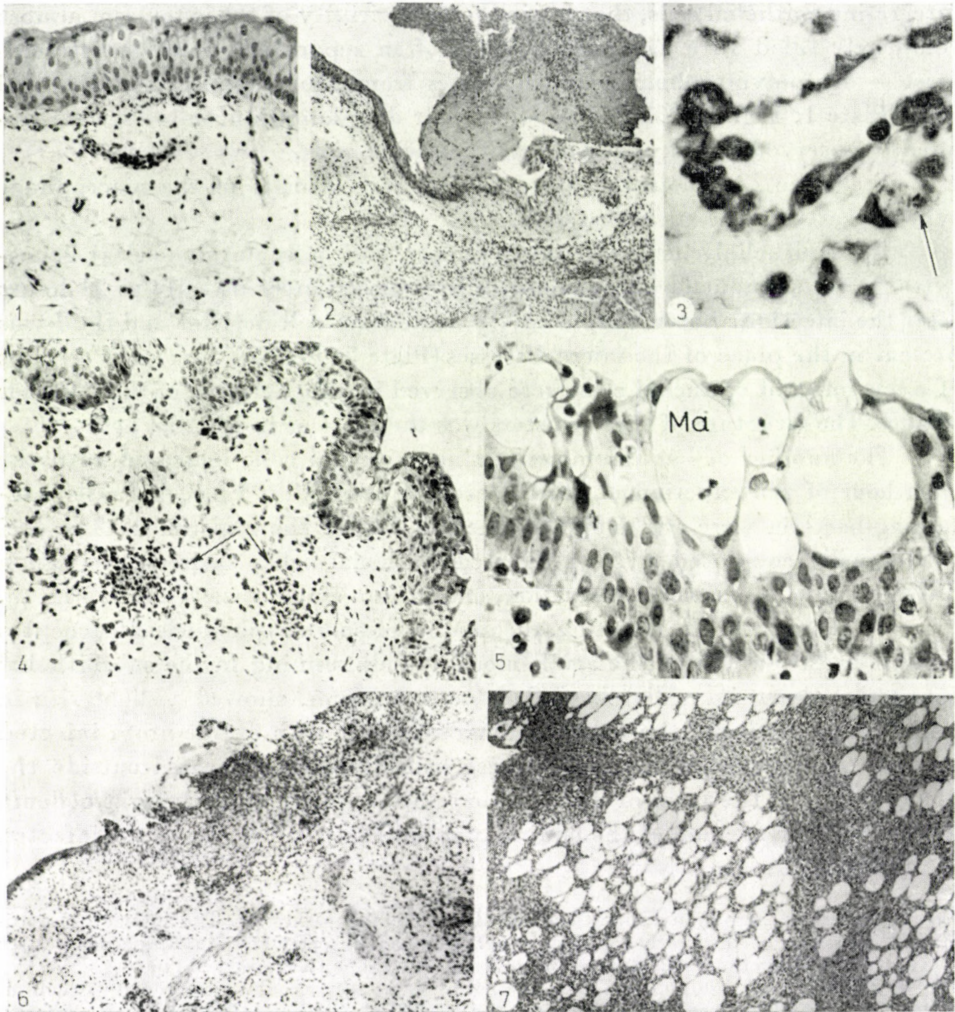


Plate 2

Fig. 1. Intact vesical epithelium. Light oedema in the submucosa. Inflammatory infiltration immediately under the epithelium. Experiment No. 2, one hour after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, ×169

Fig. 2. Erosion of the bladder surface. There is a thrombus-like blood clot in a location corresponding to the erosion. Oedema of the submucosa and infiltration with polymorphonuclear leucocytes. Experiment No. 1, 3 hours after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, ×30

Fig. 3. Cell of the macrophage type containing salmonella in the vicinity of a small blood vessel in the submucosa. The cytoplasm of the cell is swollen. Experiment No. 1, 24 hours after infection with *S. typhi-murium* 26772. Thionin staining, ×1240

Fig. 4. Salmonella (arrow) in the submucosa of the bladder. Experiment No. 1, 96 hours after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, ×169

Fig. 5. Cavities containing cell detritus and lightly staining amorphous material in bladder epithelium. Experiment No. 1, 24 hours after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, ×480

animals, the subepithelial infiltration composed of polymorphonuclear leucocytes assumed a focal form and a position corresponding to the epithelial areas containing bacteria.

One of the animals displayed a deepithelized area in a defined region of the bladder. On the surface of this area, a blood clot with thrombus-like structure was present (Plate 2, Fig. 2). In the vicinity of the area, marked oedema and an infiltration of polymorphonuclear leucocytes were found in the subepithelial tissue. The infiltration was most marked above the lamina propria. Oil immersion studies of this area revealed a high number of salmonellae partly in extracellular location among the polymorphonuclear leucocytes, partly in the polymorphonuclear leucocytes as a result of phagocytosis. Most of the salmonellae inside the polymorphonuclear leucocytes were swollen and stained poorly *viz.* showed degenerative phenomena.

Oedema of varying severity was present in the subepithelial tissue in each of the animals 6, 9 and 12 hours after the infection. Infiltration of the connective tissue by inflammatory cells became more marked and besides the polymorphonuclears, cells of the macrophage type appeared in growing number. Of the inflammatory cells, some polymorphonuclear leucocytes and macrophages contained salm onellae. An infiltration consisting mainly of polymorphonuclear leucocytes and corresponding to a phlegmon, was found above the muscular layer in 1, 2 and 2 of the animals sacrificed after 6, 9 and 12 hours, respectively.

After the 24th hour following the infection, most of the salmonellae present in the subepithelial tissue were located in cells of the macrophage type. Although the majority of the macrophages containing salmonellae showed no degeneration, some of them had a swollen cytoplasm (Plate 2, Fig. 3). The development of macrophage granulomas was also observed (Plate 2, Fig. 4). Part of the macrophages containing salmonellae disintegrated later when polymorphonuclear leucocytes appeared among the cells in the granulomas.

After the 3rd day, a marked infiltration of the perivesical fat tissue by polymorphonuclear leucocytes was found in one or two of the animals (Plate 2, Fig. 7). The presence of salmonellae in these regions could also be observed.

After the 6th hour of infection, the presence of salmonellae in the subepithelial tissue could be demonstrated also in those animals which had been infected through special catheters.

After the 9th hour of infection, pyelitis with morphological features resembling those characteristic of cystitis, developed in most animals. In one

Fig. 6. Extensive lesion of the vesical surface. Inflammatory infiltration in the submucosa especially corresponding to the lesion. Experiment No. 1, 120 hours after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, $\times 30$

Fig. 7. Extensive inflammatory infiltration in perivesical fat tissue. Experiment No. 1, 72 hours after infection with *S. typhi-murium* 26772. Haematoxylin–eosin staining, $\times 30$

of them, a severe inflammation occurred in the peripelvic connective tissue and also in the renal cortex 24 hours after the infection. After the 3rd day, focal lesions could be found in the other organs, too, especially in the spleen. First, small clusters of polymorphonuclear leucocytes containing salmonellae, later focal necrosis were observed.

Histological pictures essentially similar to those described above were seen after infection with other *Salmonella* strains. Clusters of infected epithelial cells occurred in the bladder epithelium. Salmonellae penetrated into the submucosa and later into the perivesical connective tissue. Salmonella granulomas consisting mainly of macrophages also developed by the 24th hour in each experiment. Infection with the laboratory strain of *S. typhi-murium* resulted in a significantly lower number of infected epithelial cells and in a less severe oedema and cellular infiltration in the submucosa than in the case of wild strains.

Discussion

Every *Salmonella* strain studied in these experiments was found to induce cystitis in the guinea pig, by penetrating into the epithelial cells and to undergo division there. Introducing large doses of *S. typhi-murium* into the small intestine of guinea pigs, FLOREY [9] found bacteria surrounded by lighter protoplasm ("vacuole") in part of the epithelial cells of the intestine. In contrast to the bacteria present in the intestinal lumen, those within the cells showed coccoid forms and stained lighter.

Based on morphological and staining characteristics, FLOREY considered the coccoid forms to represent degenerating salmonellae. This was partly confirmed by our electron microscopic studies revealing a disintegration of part of the bacterium within the epithelial cells [10]. On the other hand, the epithelial cells of the bladder were found in our experiments to offer especially good conditions for the reproduction of salmonellae. Each of the *Salmonella* strains studied underwent regular developmental cycles inside the cells. First, small poorly stained coccoid forms usually enclosed into vacuoles, later large, well staining forms normally occurring free in the cytoplasm were observed in the cells. Dividing forms of both *Salmonella* strains were also found. The duration of the first developmental cycle was 6 hours in the newly isolated *S. typhimurium* strain. The disintegration of epithelial cells full of large well-stained bacteria was first observed at this point of time. The duration of the following developmental cycles could not be estimated exactly owing to the continuous infection of the epithelial cells. The occasional presence of clusters of large and small bacteria within the same cell suggested the possible occurrence of reinfection of the infected cells.

Several authors [5, 9, 11–15] found in various model diseases that salmonellae could penetrate into the epithelial cells. Nevertheless evidence for

the above-described developmental cycle of salmonellae was first obtained in histological studies of experimental cystitis. This developmental cycle resembled the modification process described by VOINO-YASENETSKY [16] in experimental salmonella pneumonia of the mouse, when salmonellae underwent morphological changes during their development in cells of the macrophage type.

Bacteria released from the disintegrating cells were engulfed by polymorphonuclear leucocytes and by macrophage-like cells. However, while salmonellae within the macrophage cytoplasm usually retained their morphological characteristics, those in polymorphonuclear leucocytes usually showed disintegration during the early stages of infection.

Part of the salmonellae released from the epithelial cells reentered other epithelial cells. On the other hand, the epithelium may have been infected from the direction of the lumen of the bladder since part of the superficial epithelial cells containing bacteria had desquamated. The majority of the latter cells was eliminated with the urine while the rest might have become a source of infection when attached to an intact region of the bladder surface.

In contrast to shigella cystitis [17], no extensive epithelial defects were observed after infection with salmonellae. This phenomenon may have been due to either of the following two processes. (1) The histological picture suggested that polymorphonuclear leucocytes killed salmonellae at a higher rate than they killed shigellae. (2) Transfer of the process from one epithelial cell to the other may have occurred at a lower rate in salmonella than in shigella infection, supporting the demarcating action of the polymorphonuclear leucocytes on the process.

The role played by the microabscesses representing a special form of epithelial protection was the same in salmonella cystitis as in salmonella conjunctivitis [5].

The *Salmonella* strains studied propagated not only in the epithelial cells, but also in the cells of the subepithelial regions as the centre of the procesg moved into these areas. In the vicinity of the subepithelial areas containins extracellular salmonellae, polymorphonuclear leucocytes with disintegrating bacteria in their cytoplasm were always observed. Division of bacteria occurred primarily in the cytoplasm of macrophage-like cells in the subepithelial regions ("macrophage phase"). Macrophages formed definite clusters *viz.* "salmonellomas" as early as the 24th hour of the experiment. The cytoplasm of the macrophages contained bacteria in some of the salmonellomas while no bacteria could be observed in the macrophages of other salmonellomas. In the latter case, the formation of macrophages was induced probably as a response to an endotoxin effect.

The process often led to phlegmon development. Since in the main experiment epithelial defects were occasionally found (Plate 2, Fig. 2) an additional

experiment with the use of special catheters (experiment No. 2) was performed in order to exclude the possibility of any mechanical lesion caused by the catheter. However, bacteria soon moved into the subepithelial regions also in the latter experiment and the development of a phlegmon involving in most cases the perivesicular connective tissue, too, was observed. A predisposition for phlegmonous diffusion was described by BINGEL [6] in *S. enteritidis* infection.

Histological studies of various organs, mainly of the spleen, showed a generalization of the process by indicating that the bacteria did not remain in the bladder region. The predisposition for a generalization of the process was not a characteristic feature of all *Salmonella* strains. BINGEL showed that the intravesical introduction of *S. typhi* and *S. paratyphi-B* failed to lead to the generalization of the process in guinea pigs.

The severity of experimental salmonella cystitis was influenced by several factors. Infection with the same strain resulted in more severe disease leading to earlier death in the young than in the old animals (experiments Nos 1 and 3). The freshly isolated *S. typhi-murium* strain studied had lost part of its virulence under laboratory conditions (experiments Nos 1 and 4). Studying experimental cystitis induced by five freshly isolated and five laboratory strains, STENZEL [7] showed that pathogenicity was high with freshly isolated *S. enteritidis* and *S. java*, moderate with *S. typhi-murium* and *S. newington* and low with *S. bareilly*. Thus, the pathogenicity was significantly lower in the old laboratory strains than in the freshly isolated ones. As the primary purpose of the present study was to investigate the "epithelial phase", the number of the animals infected with the different strains — except *S. typhi-murium* — was not high enough to allow any comparison as to the severity of the induced disease (Experiment No. 5).

In conclusion, salmonella-induced cystitis in the guinea pig is a suitable experimental model for studying some of the characteristic biological properties of salmonellae. The "epithelial phase" in this experimental model is especially important for the pathogenesis of the salmonella infection. The prolongation of the "epithelial phase" and the characteristic developmental stages of the bacteria within the cells are special features when compared to those found in salmonella conjunctivitis [5]. All these indicate that studies of the biological properties of facultative intracellular parasitic bacteria must not be based on a single experimental model. As to the "epithelial phase", the epithelial barriers, though showing several similar characteristics, naturally exhibit differences depending on the metabolism and the morphological features of their cells. Experimental salmonella cystitis seems to be especially suitable for studying the interaction between epithelial cells and bacteria. It is of great importance in human pathology to understand the developmental cycle of bacteria observed in the epithelial cells since, though FRÄNKEL [18] had shown

in 1900 already that the presence of large well-staining bacteria detectable in corpses is the result of a post mortem propagation of salmonellae, their localization *in vivo* and their morphological forms are not yet known.

Further details of the modification of salmonellae in the vesical epithelium will be reve aled by electron-microscopic studies.

REFERENCES

1. Suter, E.: Bact. Rev. **20,** 94 (1956).
2. Rácz, P., Pesthy, L.: 3rd Int. Congr. on the Pathology of Infection, Bucharest 1962, p. 915.
3. Rácz, P., Tenner, K., Szivessy, K.: Acta microbiol. Acad. Sci. hung. **17,** 221 (1970).
4. Rácz, P., Tenner, K., Mérő, E.: Virchows Arch. path. Anat. Abt. B. Zellpath. **8,** 96 (1971).
5. Tenner, K., Rácz, P., Rédey, B.: Acta microbiol. Acad. Sci. hung. **18,** 167 (1971).
6. Bingel, K. F.: Z. Hyg. Infekt.-Kr. **125,** 110 (1943).
7. Stenzel, W.: Z. Hyg. **147,** 123 (1960).
8. Letterer, E., Seybold, G.: Z. Hyg. Infekt.-Kr. **129,** 466 (1949).
9. Florey, A. W.: J. Path. Bact. **37,** 283 (1933).
10. Tenner, K., Rácz, P.: Unpublished data.
11. Goodpasture, E. W., Anderson, K.: Amer. J. Path. **13,** 149)1937).
12. Kent, T. H., Formal, S. B., LaBrec, E. H.: Arch. Path. **81,** 501 (1966).
13. Takeuchi, A.: Amer. J. Path. **50,** 109 (1967).
14. Takeuchi, A., Sprinz, H.: Amer. J. Path. **51,** 137 (1967).
15. Ариель, Б. М., Берновская, А. А.: Арх. Пат. **32,** 17 (1970).
16. Войно-Ясенецкий, М. В.: Труды Института Эпидемиологии и Микробиологии им. Пастера **27,** 106 (1964).
17. Tenner, K., Rácz, P., Serény, B.: Acta microbiol. Acad. Sci. hung. **18,** 179 (1971).
18. Fränkel, E.: Z. Hyg. Infekt.-Kr. **34,** 482 (1900).

Address of the authors:

Clara Tenner, Pál Rácz, Endre Mérő
National Institute of Public Health,
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 231–237, 1971

VIRUS CONTENT OF SEWAGE IN DIFFERENT SEASONS IN HUNGARY

By

ÁGNES B. PÁLFI

National Institute of Public Health (Director: T. BAKÁCS), *Budapest*

(Received November 12, 1971)

Summary. From 336 sewage samples collected in 1969, 317 virus strains were isolated Of these, 62 proved to be poliovirus of vaccine origin. Of the remaining 255 strains, 43% belonged to reovirus type 1, 21% to echovirus type 7, and 18% to echovirus type 11. These three types of virus showed a prevalence all over the year. The other strains were identified as coxsackievirus B1, B3 or B4 or echovirus type 1, 6, 12, 14, 19 or 20. Most strains were isolated in August, followed by October, November and September; the isolation rate was lowest in February and March, *i.e.*, in the periods following compulsory vaccination campaigns. In 1968, reovirus 1, coxsackievirus B1 and B3 as well as echovirus 6 and 7 strains occurred in the same seasons as in 1969.

It is almost generally accepted [1–3, 11, 13–15, 17, 18, 21, 23, 24, 31] that, on the zones of temperate climate, enterovirus circulation has its peak in late summer and early autumn. This opinion is based on the fluctuation of the virus isolation rate and of the number of isolated strains. There are few exceptions, *e.g.*, RIORDAN [24] found the highest isolation rate in January, WALLIS and MELNICK [32] in June and April and FAULKNER and VAN ROOYEN [9] in October. (The last authors isolated, numerically, most strains in August.)

It is more difficult to develop a general view on the occurrence of individual types of enteroviruses. The results are greatly influenced, in addition to climatic factors, by the kind of sample taken for virus isolation, the method of sampling, the virological methods applied, the system of vaccination against poliomyelitis, *etc.*

In Hungary, DÁNIEL and DÖMÖK [7] were the first to point out that virological examination of sewage might present information on the occurrence of enteroviruses in the local population. The object of the present work was to collect data, on the basis of large-scale examination of sewages, on the enterovirus types circulating in Hungary and on the seasonal fluctuation of their occurrence. The investigations were conducted systematically, over a calendar year.

Materials and methods

Three hundred and thirty-six sewage samples were collected in 1969. The majority of the samples were obtained from the Budapest region (South-Pest sewage treatment plant; lifting-over plant, Ferencváros, Ferihegy), and from several sewage treatment plants in the country, *viz.*, Aggtelek, Bükkszék, Hajdúszoboszló, Hévíz, Keszthely, Ózd, Mátrafüred, Pécs, Szigetszentmiklós, Vecsés and Visonta.

Samples were taken either by dipping in sterilized 1-litre flasks which were then rubber-stoppered or by the gauze-pad method [21]. Swabs exposed to sewage for 3 or 4 days were transported to the laboratory in plastic bags.

The pH of the grab samples (approx. 1 litre each) was adjusted to 8 with N NaOH solution and the samples were centrifuged for 30 minutes at 3000 r.p.m. To each swab in plastic bag 10 ml Hanks' balanced solution was added, the pH of the fluid was adjusted with N NaOH to 8, the fluid (50—100 ml) was pressed out of the swab and centrifuged. The pH of the supernatant was adjusted to 6 with N HCl. Subsequently, of a suspension containing 50 mg/ml $CaHPO_4$ [27], 1 ml was added to each dip sample and 0.5 to each sample obtained by the gauze-pad method. The mixtures were shaken for 30 minutes, then centrifuged for 30 minutes at 3000 r.p.m. The sediment was resuspended in 0.5 ml 0.5 M phosphate buffer (pH 7.5), shaken for 30 minutes, and centrifuged for 10 minutes at 2000 r.p.m. The supernatant, mixed with 1 ml Parker's No. 199 medium and 0.3 ml chloroform, was left to stand at 4 °C overnight. Then the supernatant was removed and stored in the frozen state until used.

In order to isolate virus, three primary and three continuous [25] monkey kidney cell cultures in tubes were inoculated with 0.1 ml of each concentrated sample. As maintenance medium Parker's No. 199 was used for the primary cultures, the same medium enriched with human erythrocyte receptor [26] for the continuous ones. The cultures were incubated at 37 °C for 14 days, with a medium exchange on the 7th day. On the 14th day, a passage or blind passage was made and the subcultures were observed for another 14 days for cytopathic effects.

The medium from positive cultures was tested for haemagglutination (HA), applying TAKÁTSY's Microtitrator system [29]. The haemagglutinating strains were identified by the HA-inhibition (HAI) test, the others by the neutralization test.

Results

Table I shows that 317 virus strains were isolated from the 336 sewage samples taken in the calendar year 1969. Sixty-two strains proved to be poliovirus vaccine strains. Among the remaining 255 isolates, reovirus type 1 (43%)

Table I

Distribution of isolates by virus type and month of isolation

Month	No. of samples	No. of isolates	Distribution of isolates by type													Not tested
			Poliovirus	Coxsackie B			Echovirus								Reo 1	
				1	3	4	1	6	7	11	12	14	19	20		
Jan.	27	20	7					1		2					9	1
Feb.	23	25	17						2						6	
March	33	25	11					1	8						5	
Apr.	18	20	10						8						2	
May	27	30	5						11	6					8	
June	26	15				1			6	2	1				5	
July	29	20			4			3	2	9					2	
Aug.	29	30			3			5	3	6		1		1	11	
Sep.	23	25		1					2	9	1		2		10	
Oct.	29	34			2			5	2	4	2				19	
Nov.	28	35		1				3	9	4		1			17	
Dec.	44	38	12	1			5			4					16	
Total	336	317	62	3	9	1	5	18	53	46	4	2	2	1	110	1

was the most frequent. This was followed by echovirus 7 (21%), echovirus 11 (18%), and echovirus 6 (7%).

Due to the compulsory poliomyelitis vaccination campaigns which are carried out in Hungary during the winter months, poliovirus was very common in the sewage samples collected in the period from February to April. In addition, only echovirus 7 and reovirus 1 strains and an echovirus 6 strain were isolated in that period. Reovirus 1 strains were isolated in each month of the year, in greatest numbers in the autumn. Echovirus type 7 could be isolated in each month except January and December, echovirus type 11 in each month except the three months of the heaviest poliovirus excretion.

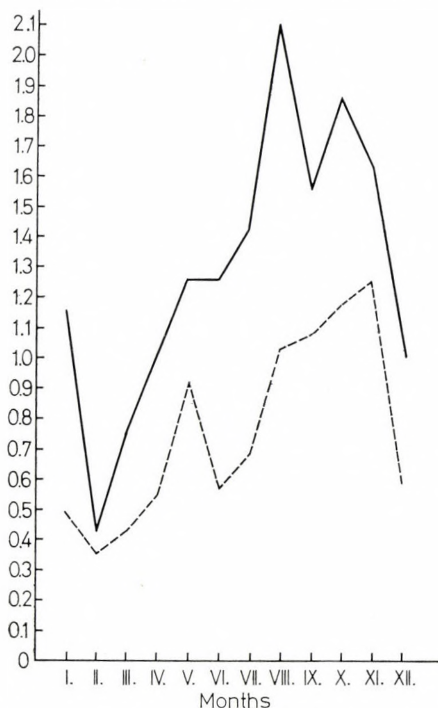


Fig. 1. Fluctuation of the isolation rate (isolate/sewage sample) by month. ——— isolation rate for raw sewage by swab samples, - - - - - isolation rate for all samples

To estimate the extent of virus circulation, we divided the number of isolated nonpolio virus strains per month with the number of samples examined in the same month (Fig. 1). Accordingly, the isolation rate was the highest for November and October and these months were followed by September and August. The lowest rates were obtained during the months of heaviest poliovirus excretion. These rates do not, however, reflect the real excretion quite correctly because the samples examined in different months were heterogeneous in origin, method of sampling and degree of purity. For a more precise analysis,

we selected a relatively homogeneous group of swab samples which had been taken from the raw sewage of the South-Pest sewage treatment plant. This plant receives both domestic and industrial sewage. Due to its character, this kind of samples is expected to provide the highest isolation rate [4]. Ninety-four samples, *i.e.*, approx. 30% of the total number of samples, belonged in this group. From the 94 samples, 122 virus strains were isolated (an isolation rate of 1.3 *vs.* the 0.75 calculated for all the 336 samples).

Taking only these samples into consideration, the maximum in late summer and autumn was more pronounced than in the curve representing the unselected material (Fig. 1).

The isolation rate was highest in August [2, 11], followed by October, November and September. The selected samples also showed that virus excretion was lowest in the winter months and in early spring.

Systematic sampling was carried on in 1968 in four months, *viz.* May, August, September and December. In May, reovirus type 1 was isolated both in 1968 and 1969, whereas echovirus type 7 and 11 strains only in 1969 (Table II). In August, coxsackievirus B3, echovirus 6 and 7 and reovirus 1

Table II

Virus types demonstrated in the same months of two successive years

Month	Types of virus trains isolated in	
	1968	1969
May	Reovirus 1	Echovirus 7, 11 Reovirus 1
August	Coxsackievirus B2, B3, B4, B5 Echovirus 6, 7, 12 Reovirus 1	Coxsackievirus B3 Echovirus 6, 7, 11, 14, 20 Reovirus 1
September	Coxsackievirus B2, B3, B4, B5 Echovirus 7 Reovirus 1	Coxsackievirus B1 Echovirus 7, 11, 12, 19 Reovirus 1
December	Coxsackievirus B1, B5 Echovirus 7, 11, 12, 14 Reovirus 1	Coxsackievirus B1 Echovirus 1, 11 Reovirus 1

strains were isolated in both years, whereas coxsackievirus B2, B4 and B5 and echovirus 12 strains were isolated only in 1968, and echovirus types 11, 14 and 20 strains only in 1969. In September echovirus 7 and reovirus type 1 strains were isolated in both years, coxsackievirus B2, B3, B4 and B5 strains only in 1968 and coxsackievirus B1, and echovirus 11, 12 and 19 strains only in 1969. In December, coxsackievirus B1, echovirus 11 and reovirus 1 strains were isolated in both years, coxsackievirus B5, and echovirus 7, 12 and 14 strains only in 1968 and echovirus 1 strains only in 1969.

Discussion

In the present study, the maximum of virus excretion occurred in August and October. This is in agreement with most of the literary data [1–3, 9, 11, 13–15, 17–19, 21, 23, 24, 31].

Recently, enterovirus circulation has been intensively influenced by the widely applied immunization against poliomyelitis. Several authors [13, 24] have observed that wild enteroviruses even stopped circulating at the time of the heavy excretion of poliovirus vaccine strains. We, too, observed a reduced excretion, but even in the periods immediately following vaccination campaigns, wild strains (entero- and reoviruses together) made up 36% of the isolates. In this period, strains belonging to reovirus type 1 and three echovirus types (types 6, 7 and 11) were isolated. The first coxsackieviruses appeared soon after excretion of the vaccine strains had ceased. This, however, does not mean that coxsackieviruses were absent in the vaccination period. They might have suppressed in the cell cultures by the vaccine strains, which destroy cell cultures rapidly.

Of echoviruses, type 7 and 11 strains were isolated most frequently; they even supplemented each other in different seasons (Fig. 2). Echovirus type 7 was especially predominant from March to June, echovirus 11 from July to September. Strains belonging to these two types amounted to more than a half of the isolates in those months. A similar dominance of echovirus 7 and 11 strains was observed by LAPINLEIMU and PENTTINEN [18] in Helsinki in 1960–1961 and later by KAZANTSEVA and BAGDASARYAN [14] in Moscow,

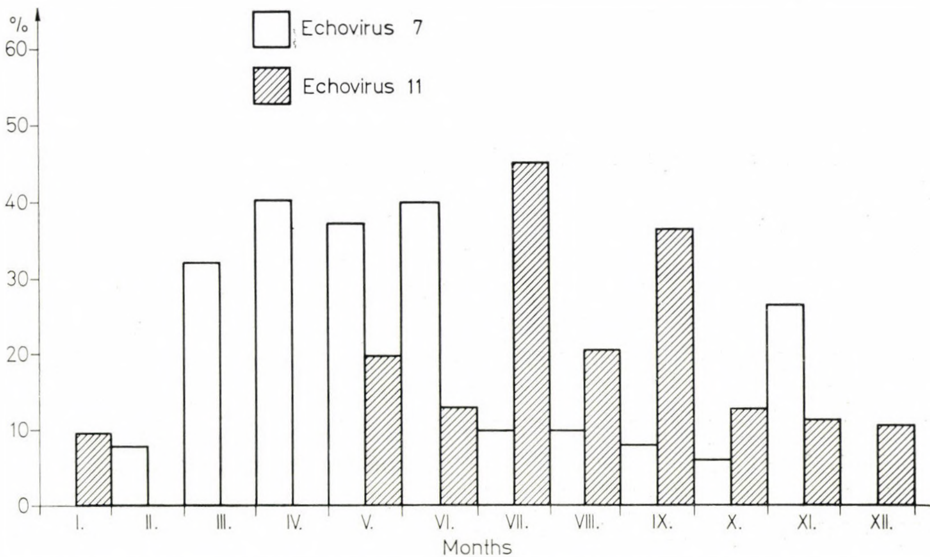


Fig. 2. Monthly occurrence of echovirus types 7 and 11 in 1969 as expressed in per cent of isolates

Wallis and Melnick [32] in Houston, Welke [33] in Potsdam and Hartmann [12] in Berlin.

Fig. 3 shows that echoviruses, which could be isolated in every month of the year, showed the highest percentage of the isolates in April, the lowest in January and February. Echoviruses therefore seem to be circulating all over the year.

Coxsackie B viruses were the rarest (4–20%) among the isolates. They were isolated with a maximum in July and August, in the period when poliomyelitis vaccination campaigns were not carried on. These results are in good accordance with data published by other investigators [2, 9, 12, 19, 31, 33].

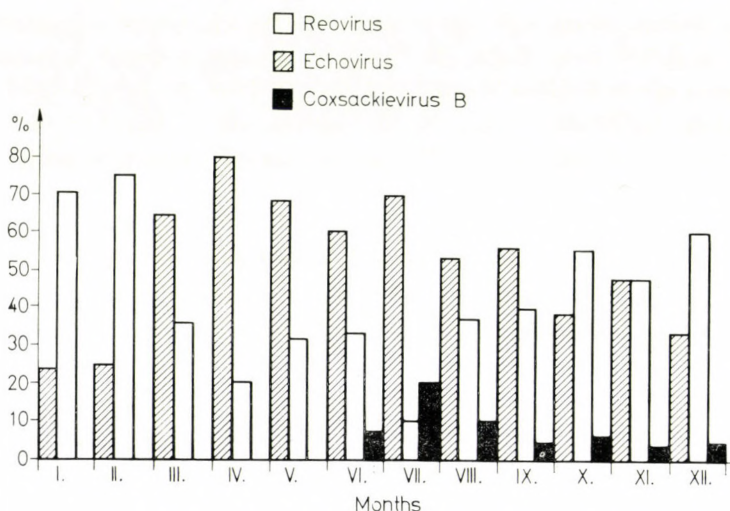


Fig. 3. Percentage distribution of coxsackievirus B, echovirus and reovirus isolates in different months of the year 1969

Our isolation systems were inadequate for the isolation of coxsackie A viruses.

The frequent occurrence of reoviruses in sewage has called the attention of numerous authors. Lamb *et al.* [17] were the first to isolate reovirus from sewage, namely, from the sewage of Chicago, where, as well-known, there are large slaughterhouses. They found that reovirus was the dominant virus in that sewage in 1962. It could be isolated even from the river that received the sewage. Many reovirus strains were isolated from slaughterhouse sewage by South-African [20] and German [16] authors. All authors agree in that reovirus can be isolated all over the year, even during vaccination periods, from 24–93% of sewage samples [6, 8, 13, 28]. We succeeded in isolating reovirus from 33% of the samples. Reoviruses amounted to 26–57% of the strains isolated from sewage by different authors [5, 8 13, 20]. In the present work, the corresponding percentage was 43%. England *et al.* [8] succeeded in isolating reo-

viruses more frequently from purified than from raw sewages. Our experience is in accordance with these: we isolated reovirus from 25% of raw sewage and 38% of purified one.

Serological investigations have also proved the wide dissemination of reoviruses. Serum antibody to reovirus type 1 was found [30] in 73% of samples representative of the population of Hungary. A wide occurrence of reoviruses in domestic animals has also been shown [10]. The presence of large amounts of reoviruses in sewage may be attributed to their ubiquitous presence, on the one hand, and to their high resistance to various effects, on the other.

REFERENCES

1. Belian, W., Böthig, B., Käppel, M.: Dtsch. Gesundh. Wes. **21,** 1036 (1966).
2. Bendinelli, M., Ruschi, A., Equi, A.: Riv. ital. Igiene **27,** 253 (1967).
3. Bloom, H. H., Mack, W. N., Krueger, B. J., Mallmann, W. L.: J. infect. Dis. **105,** 61 (1959).
4. B. Pálfi, Á., Simon, M., Schulek, E.: Egészségtudomány **14,** 127 (1970).
5. Chin, T. D. Y., Mosley, W. H., Robinson, S., Gravelle, C. R.: In: Transmission of viruses by the water route (Ed.: Berg, G.), Interscience, London 1967, p. 389.
6. Craske, J., Poynter, S. B.: Preliminary results of the examination of sewage in London for the presence of reoviruses. XII. Europ. Symp. Polio and allied Diseases, Bucharest 1969, p. 205.
7. Dániel, M., Dömök, I.: Acta microbiol. Acad. Sci. hung. **9,** 251 (1962).
8. England, B., Leach, R. E., Adame, B., Shiosaki, R.: In: Transmission of viruses by the water route (Ed.: Berg, G.), Interscience, London 1967, p. 401.
9. Faulkner, R. S., Van Rooyen, C. E.: Canad. J. publ. Hlth **60,** 345 (1969).
10. Fukumi, H., Takeuchi, Y., Ishida, M., Saito, H.: Jap. J. med. Sci. Biol. **22,** 13 (1969).
11. Gelfand, H. M.: Progr. med. Virol. **3,** 193 (1961).
12. Hartmann, D.: Zbl. Bakt., I. Abt. Orig. **207,** 141 (1968).
13. Jopkiewicz, T., Krzeminska, K., Stachowska, Z.: Przegl. Epidem. **22,** 521 (1968).
14. Kazantseva, V. A., Bagdasaryan, G. A.: J. Hyg. Epidem. (Prague) **11,** 9 (1967).
15. Kelly, S. M., Winsser, J., Winkelstein, W.: Amer. J. publ. Hlth **47,** 72 (1957).
16. Knocke, K. W., Pittler, H., Höpken, W.: Zbl. Bakt. I. Abt. Orig. **203,** 417 (1967).
17. Lamb, C. A., Chin, T. D. Y., Scarce, L. E.: Amer. J. Hyg. **80,** 320 (1964).
18. Lapinleimu, K., Penttinen, K.: Arch. ges. Virusforsch. **13,** 72 (1963).
19. Lund, E., Hedström C. E.: Water Res. **3,** 823 (1969).
20. Malherbe, H. H., Strickland-Cholmley, M.: In: Transmission of viruses by the water route (Ed.: Berg, G.), Interscience, London 1967, p. 347.
21. Melnick, J. L., Emmons, J., Opton, E. M., Coffey, J. H.: Amer. J. Hyg. **59,** 185 (1954).
22. Moore, B.: Mthly Bull. Minist. Hlth Lab. Serv. **7,** 241 (1948).
23. Pittler, H., Höpken, W., Knocke, K. W.: Zbl. Bakt. I. Abt. Orig. **204,** 33 (1967).
24. Riordan, J. T.: Yale J. Biol. Med. **34,** 512 (1962).
25. Ruzicska, P.: Acta morph. Acad. Sci. hung. **12,** 275 (1964).
26. Simon, M., Dömök, I.: Acta microbiol. Acad. Sci. hung. **13,** 229 (1966).
27. Simon, M., B. Pálfi, Á.: Egészségtudomány **14,** 13 (1970).
28. Stachowska, Z., Krzeminska, K.: Przegl. Epidem. **22,** 529 (1968).
29. Takátsy, Gy.: Acta microbiol. Acad. Sci. hung. **3,** 191 (1956).
30. Tóth, M., Honty, A.: Acta microbiol. Acad. Sci. hung. **13,** 119 (1966).
31. Турищева, Г. Г., Медведев, М. П., Иванова, Л. П., Каменский, В. А.: Гиг. и Санит. **34,** 119 (1969).
32. Wallis, C., Melnick, J. L.: Bull. Wld Hlth Org. **36,** 219 (1967).
33. Welke, G.: Z. ges. Hyg. **15,** 894 (1969).

Address of the author:

Ágnes B. Pálfi
National Institute of Public Health
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 239—245, 1971

IMMUNOGENICITY OF LIVING ATTENUATED SHIGELLAE

By

B. Serény, Clara Tenner and P. Rácz

National Institute of Public Health (Director: T. Bakács), *Budapest*

(Received November 26, 1971)

Summary. Colonial variants of virulent *Shigella* strains grown on dye-supplemented media were selected at oblique illumination and inoculated into the eyes of guinea pigs. As shown in keratoconjunctivitis model experiments, the selected avirulent cultures varied in immunogenic capacity. Pre-treatment with variants exerting high immunogenicity protected the eyes in about 50% against superinfection with homologous or heterologous *Sh. flexneri* types.

In recent years, many kinds of vaccine have been prepared from living *Shigella* cultures. Studies on this problem were begun by Istrati *et al.* [1—10]. They used a *Shigella* culture attenuated by serial passages on agar. This culture and a laboratory strain failed to cause experimental conjunctival infection in guinea pigs. Human volunteer experiments indicated that they were avirulent to man. The cultures produced a definite local and a moderate general immunity in the guinea pig conjunctival model. They were harmless to man and remained stable in serial human passages. The live vaccine gave favourable results in children with chronic shigella excretion.

Belaya *et al.* [11—13] confirmed the results of Istrati's team. Shigellae attenuated in serial passages caused no gross changes in the eyes of the guinea pig, although they invaded the epithelial cells of the conjunctiva and cornea and gave rise to a well-demonstrable, rapidly recovering inflammatory reaction. The bacteria were unable to multiply intracellularly. The live dysentery vaccine proved to carry a hazard in monkey experiments and multiple doses protected most animals against large numbers of virulent bacteria. Studies on a small group of humans [14] showed that the vaccine strain retained its original properties after human passages.

Formal's research group used two different *Sh. flexneri* 2a strains for oral immunization of monkeys [15—17]. One of the cultures was a spontaneous avirulent mutant, which had lost the ability to enter the epithelium either in the Formal model [18] or in conjunctival infection. The other attenuated culture, a hybrid of *E. coli* K-12 and *Sh. flexneri* 2a induced a rapidly recovering enteritis in the Formal test and caused keratoconjunctivitis in guinea pigs. As live oral vaccines, both strains protected monkeys against infection with the virulent homologous strain. Later Formal *et al.* [15] crossed *E. coli*

K-12 with *Sh. flexneri* 1b, 2a and 3 and *Sh. sonnei*. Oral vaccination with these cultures also resulted in a significant immunity. After freeze-drying the cultures retained their biological properties including immunizing capacity.

RAUSS, KÉTYI and ANGYAL [19] showed that FORMAL's mutant, which had lost its penetrating power, was not more protective than vaccines containing killed shigellae. The mutant which retained the penetrating capacity immunized better than the avirulent culture or the killed vaccine, but was not superior to Boivin extracts.

MEL *et al.* [20—23] used streptomycin-dependent avirulent *Sh. flexneri* strains as oral vaccines. First they demonstrated the protective effect in the FRETER model, later they experimented on groups of military personnel. They showed that shigella excretion was not influenced by vaccination in this manner.

SERGEYEV *et al.* [24—27] using the method of MEL's team demonstrated the protective effect of streptomycin-dependent shigellae in the conjunctival test. On monkey experiments they showed that no clinical symptoms developed after consuming the vaccine.

Our studies on live shigella vaccines have been presented in [28].

Materials and methods

Strains. Virulent cultures of *Sh. flexneri* 1b, 2a and 3a were freshly isolated from human, strain *Sh. flexneri* 4b from monkey, faecal specimens. The cultures were identified with the usual methods and were tested for virulence with the conjunctival test [29, 30].

Selection of avirulent mutants. As polyvalent shigella-phage was not satisfactory for the purpose, the following technique based on our dye-sensitivity method [40] was applied. A pinpoint amount of virulent culture grown on agar was transferred to solid and into liquid media supplemented with various dyes as follows. On the surface of previously dried agar plates 0.1 ml 0.07% aqueous safranin or 0.1 ml 0.25% aqueous basic fuchsin solution was spread. For liquid media 0.002 and 0.004% safranin, 0.01 and 0.02% basic fuchsin, 0.00025% acridine orange, 0.025% acridine red or 0.002% crystal violet were dissolved in broth and distributed in test tubes at 5 ml portions.

During 4 days incubation at 37 °C the culture from the dye-supplemented media was inoculated daily onto simple agar plate. The agar subculture was examined at oblique light [31—33]. Each colony different from the original culture was picked up, passaged several times on agar in order to obtain a homologous culture, then tested for virulence in the eyes of 3 guinea pigs. Cultures not causing gross changes were maintained in the refrigerator on Dorset medium; some cultures were freeze-dried.

Immunogenicity. Guinea pigs weighing 200—300 g were infected into the conjunctival sac twice weekly. Each of the 5 doses given contained 2×10^9 cells of the 24-hour agar culture of the variants. Ten days after the last dose, 4—5 M.I.D. ($4-5 \times 10^7$ living cells) of the virulent *Shigella* strain was inoculated into the eyes of the animals. The conjunctival test was regarded negative if no gross change had developed. The same number of untreated animals served as controls. In preliminary experiments 10 eyes of 5 animals were checked for resistance. In the main experiments 40—60 pretreated eyes of 20—30 animals were tested for each variant.

Histological examination. Highly immunogenic *Sh. flexneri* 2a variant 493/39 and weakly immunogenic *Sh. flexneri* variant 493/48 were used. An apathogenic *E. coli* O1:K11:H7 strain wasalso studied. *Shigella* variants were regarded highly immunogenic if pre-treatment in the above-described manner protected at least 8 eyes out of 10 against the development of gross changes after challenging with $4-5 \times 10^7$ virulent bacteria. Variants exhibiting a less definite but still appreciable protective effect were termed weakly immunogenic.

The guinea pigs were infected into each conjunctival sac with 2 loopful of a 24-hour agar culture. Groups consisting of 3 animals each were sacrificed after 1, 3, 6, 9, 12, 18, 24, 48 and 72 hours by cervical dislocation. The right eye was examined histologically, the left one

was sampled for bacteriological examination. The right eyeball together with the bony orbit was fixed in Maximov's solution for 5 hours. Then the bone was removed, the eye was halved by a sagittal cut. After a subsequent fixation for 1 hour, the eye was embedded in celloidin–paraffin. Sections 4—5 μ thick were stained with haematoxylin–eosin and thionin.

Results

Selection of avirulent variants. Culturing on dye-supplemented media yielded a great number of colonies different in structure from those of the parent strain. Freshly isolated strains formed low convex, circular colonies with entire edge, 1—2 mm in diameter. At oblique light they showed a fluorescence and, apart from a faint granular formation, they were homogeneous in structure. Weakly fluorescent or non-fluorescent colonies appeared in varying numbers after 1—2 days incubation on nutrient agar seeded with cultures grown in dye-supplemented media. Colonies with a concentric ring or net-like structure characterized by a definite fluorescence were virulent or weakly virulent. Homogeneous, slightly refractive, non-fluorescent colonies as well as colonies with a non-fluorescent concentric ring or net structure were usually less frequent. Bacteria forming such colonies produced no macroscopic change in the conjunctival test.

Part of the slightly virulent or avirulent variants reverted to the virulent form after a few subcultures. An interesting type of dissociation was when a *Sh. flexneri* 3 variant culture 493/37 was isolated from an agar plate subculture of crystal violet broth. A colony with concentric and net structure yielded on further subcultures partly strongly refractive, fluorescent colonies, partly colonies with a concentric ring and net-like structure. In the course of 60 subcultures the homogeneous colony type remained stable; the latter type showed a regular dissociation into homogeneous fluorescent and into concentric-structure colonies. Both variants gave a positive conjunctival test. Cultures made from the infected eyes showed that the homogeneous colony type failed to dissociate, but the concentric colony type split off homogeneous and concentric colonies *in vivo*.

Biochemical and serological examinations. Variants not causing gross changes in the conjunctival test showed insignificant biochemical differences from the parent strains. The properties tested were: fermentation of glucose, mannitol, lactose, rhamnose, sorbitol, arabinose, xylose, adonitol, dulcitol, inositol, salicin; urease and indole production; alkali production in amino acid media (glycine, L-alpha-alanine, DL-alpha-alanine, L-serine, DL-serine, D-serine, L-threonine, D-threonine, DL-threonine, DL-tryptophan, L-histidine, L-aspartic acid, L-glutamic acid, L-lysine) [32]. The growth curve for 4 variants was identical with that for the corresponding parent strains. The avirulent variants showed no spontaneous agglutination in 0.09% and in 0.3% sodium chloride solution. Agglutination test in 0.2% trypaflavine was usually negative,

but in 0.3% trypaflavine solution most strains showed agglutination. In tube agglutination with absorbed sera all antigens of the corresponding serotype were detected, but at different titres.

Immunogenicity. Table I shows that the avirulent variants differed in immunogenic capacity. In preliminary experiments the highest protective effect was obtained when vaccination was performed at daily intervals. This schedule being unsuitable for the vaccination of humans, was not used in further experiments. The finding that upon raising the challenging dose the number of resistant eyes decreased, indicated that immunity was relative: after challenging with 5 M.I.D., 72% of the vaccinated eyes remained intact, while 10 M.I.D. yielded immunity only in 55%.

Table I

Preliminary experiments with Sh. flexneri variants

Serotype	Number of variants			No. of variants tested for immunogenicity	No. of eyes remaining intact after challenging 10 eyes
	Isolated	Tested on guinea pig eyes	Avirulent for guinea pig eyes		
1b	3	—	—	—	
2a	15	9	5	—	
3a	123	93	15	25	0— 8
4b	113	113	41	8	2—10
6	11	11	7	—	

In the main experiment the avirulent variant of *Sh. flexneri* 4b was used for immunization. Challenging with the homologous virulent culture showed that 60% of the eyes were resistant. A similar degree of immunity was found if individual animals were considered. If the eyes immunized with *Sh. flexneri* 4b avirulent mutant were challenged with *Sh. flexneri* 3 virulent culture, a similar degree of immunity was found (61%).

In order to check the persistence of the immunogenic capacity, the avirulent *Sh. flexneri* 4 variant was stored at 4 °C on Dorset medium for 1 year. Immunization with a subculture of this strain and challenging with *Sh. flexneri* 3 indicated no significant change (55%).

Histological examination. Strain 493/39, exhibiting a definite immunogenic power, caused in 1 hour after conjunctival administration the appearance of a few polymorphonuclear leucocytes in and under the epithelium. The leucocytic reaction increased at the 3rd hour and after 6 hours the infiltration was definite both in the epithelium and in the subepithelial connective tissue. After 9 hours a few intraepithelial microabscesses developed. Cellular infiltration at that

stage was of the focal type, but after 12 hours it became diffuse and more intense. In the conjunctival sac there was an exudate containing leucocytes and mucous fibres. A similar histological picture was observed after 18 hours. The intensity of inflammation decreased gradually from the 24th hour onward, but normal histological conditions had not been restored till the latest examination (72 hours). At the site of the conjunctiva a mild, focal-type inflammation persisted; erosions or deep ulcers had never developed. The cornea was free from inflammation in every animal.

Bacterial staining revealed 3 hours after infection numerous extracellular, faintly stained rods in the conjunctival sac (Fig. 1). Intracellular bacteria appeared as thick, intensively stained rods (Fig. 2). There was no increase in

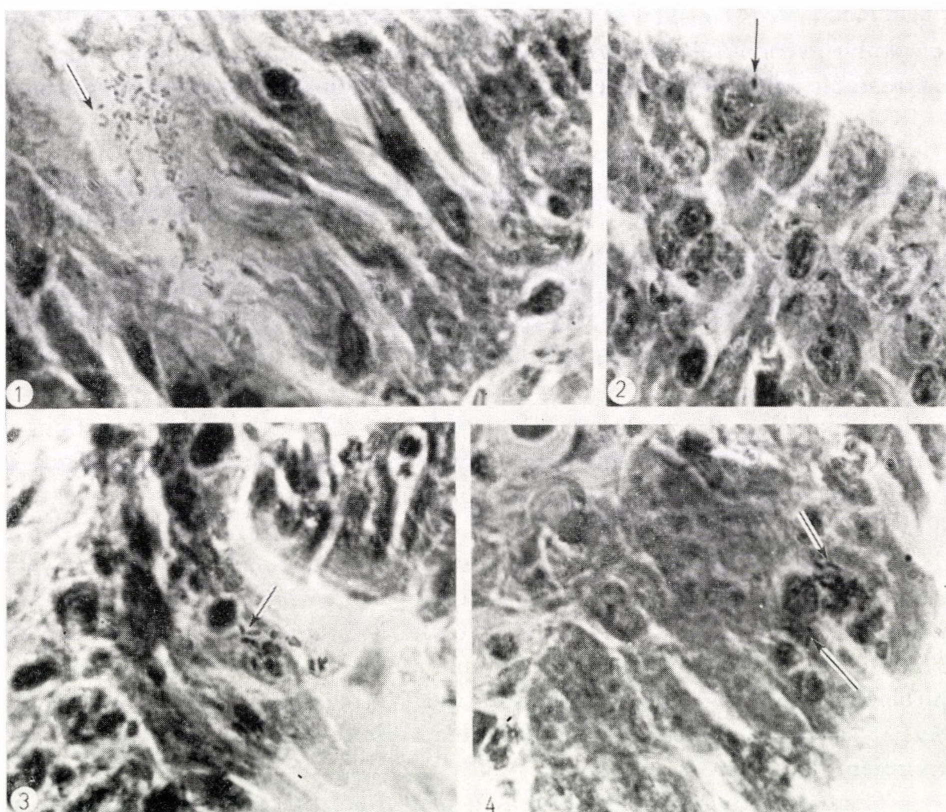


Fig. 1. Extracellular, faintly stained shigellae in the conjunctival sac (arrow), 3 hours after infection (thionin–methylene blue staining, $\times 1200$)

Fig. 2. Thick, intensively stained shigellae in conjunctival epithelial cells, 3 hours after infection (thionin–methylene blue staining, $\times 1200$)

Fig. 3. Intensively stained intracytoplasmic shigellae in the conjunctival epithelium, 6 hours after infection (thionin–methylene blue staining, $\times 1200$)

Fig. 4. Intensively stained shigellae in conjunctival epithelial cells (arrow) surrounded by polymorphonuclear leucocytes; 9 hours after infection (thionin–methylene blue staining, $\times 1200$)

the number of infected cells at the 6th hour and the number of intracellular shigellae remained low (1—2 bacteria per cell), as seen in Fig. 3. The nucleus and cytoplasm of the infected cells retained their normal staining. After 9 hours several foci appeared; these contained shigellae in increased numbers and some leucocytes (Fig. 4). After 12 hours the number of infected cells decreased and intracellular shigellae showed faint staining. From the 18th hour onward neither extracellular not intracellular bacteria were seen.

After the administration of weakly immunogenic strains no intracellular bacteria were demonstrated. The conjunctiva showed a slight oedema and a leucocytic reaction ceasing after 24 hours.

Culturing on media supplemented with dyes resulted in a dissociation of shigellae into various colony variants. If in the conjunctival test the absence of gross changes is considered the criterion of virulence, some of these cultures were stable avirulent derivatives. In addition, a great number of weakly virulent and unstable cultures were encountered, corresponding to data of other authors [34, 35].

In conjunctival model experiments the selected variants exhibited a protective effect against challenge with not only the homologous virulent, but also with virulent, heterologous serotypes. However, the guinea pig eye model is not suitable for demonstrating immunity against dysentery in man [36]. Thus, further investigations are needed to show the effectiveness of the live *Shigella* vaccine for the immunization of humans. The present experiments have shown that pre-treatment with avirulent shigellae increased the resistance to other serotypes. ISTRATI *et al.* [7], who also used the guinea pig model, came to a similar conclusion. In contrast, MEL *et al.* [21—23], in mass vaccination of humans, were able to demonstrate only a type-specific immunity. Thus it remains open whether aspecific immunity is restricted to the eyes of the guinea pig.

Histological studies indicated that shigellae, termed avirulent on the basis of the above criterion, caused a rapidly recovering inflammation in the guinea pig's conjunctiva. In contrast to the behaviour of virulent shigellae [37, 39], the highly immunogenic variant studied did not enter the corneal epithelium and failed to multiply intensively in the conjunctival epithelium. Thus it seems more justified to term this variant "attenuated" rather than "avirulent".

The correlation between immunogenicity and intracellular parasitism in shigellosis, that is, the association between immunity and fine cellular changes after the invasion of shigellae is at present unknown. According to SERGEYEV *et al.* [25] local antibodies are concerned. Our studies indicate that in addition to these factors the ability of bacteria to invade the epithelial cells may play a role. Antigens of the vaccine strain may block enzyme systems in the epithelial cells.

REFERENCES

1. Istrati, G.: Arch. roum. path. exp. **20,** 53 (1961).
2. Istrati, G., Istrati, M.: Arch. roum. Path. exp. **23,** 87 (1964).
3. Istrati, G., Istrati, M.: Arch. roum. Path. exp. **23,** 91 (1964).
4. Istrati, G., Istrati, M.: Arch. roum. Path. exp. **23,** 289 (1964).
5. Istrati, M., Istrati, G.: Arch. roum. Path. exp. **25,** 55 (1966).
6. Istrati, G., Istrati, M., Meitert, T., Ciufeco, C.: Arch. roum. Path. exp. **22,** 531 (1963).
7. Istrati, G., Meitert, T., Ciufeco, C.: Arch. roum. Path. exp. **24,** 677 (1965).
8. Istrati, G., Istrati, M., Meitert, T., Ciufeco, C.: Arch. roum. Path. exp. **24,** 867 (1965).
9. Istrati, G., Meitert, T., Ciufeco, C.: Arch. Immunol. Therap. exp. **16,** 333 (1968).
10. Istrati, G., Meitert, T., Ciufeco, C., Filipesco, S.: Arch. roum. Path. exp. **23,**1 41 (1964).
11. Белая, Ю. А.: ЖМЭИ **4,** 14 (1968).
12. Белая, Ю. А., Кавтарадзе, Р. Н., Геккер, В. Д., Джидзе, Е. К.: ЖМЭИ **2,** 22 (1970).
13. Левенбук, И. М., Белая, Ю. А., Хигиринский, А. А.: ЖМЭИ **4,** 37 (1968).
14. Рубцев, И. В., Белая, Ю. А., Мельник, Е. Г., Берлин, Е. И.: ЖМЭИ **7,** 58 (1970).
15. Formal, S. B., Kent, T. H., May, H. C., Palmer, A., Falkow, S., LaBrec, E. H.: J. Bact. **92,** 17 (1966).
16. Formal, S. B., LaBrec, E. H., Kent, T. H., May, H. C., Loeventhal, J. P., Borman, S.: Proc. Soc. exp. Biol. (N.Y.) **124,** 284 (1967).
17. Formal, S. B., LaBrec, E. H., Palmer, A., Falkow, S.: J. Bact. **90,** 63 (1965).
18. Formal, S. B., Dammin, G., LaBrec, E. H., Schneider, H.: J. Bact. **75,** 604 (1958).
19. Rauss, K., Kétyi, I., Angyal, T.: Acta microbiol. Acad. Sci. hung. **14,** 143 (1967).
20. Mel, D. B., Arsic, B. L., Nikolic, B. D., Radovanic, M. L.: Bull. Wld Hlth Org. **39,** 375 (1968).
21. Mel, D. M., Papo, R. G., Terzin, A. L., Vuksic, L.: Bull. Wld Hlth Org. **32,** 637 (1965).
22. Mel, D. M., Terzin, A. L., Vuksic, L.: Bull. Wld Hlth Org. **32,** 633 (1965).
23. Mel, D. M., Terzin, A. L., Vuksic, L.: Bull. Wld Hlth Org. **32,** 647 (1965).
24. Сергеев, В. В., Фролова, М. А., Проташова, О. В., Елкина, С. И., Лустер, Б. Ю.: ЖМЭИ **5,** 126 (1950).
25. Сергеев, В. В., Михайлов, И. Р., Глейберман, С. С., Юбецкая, Н. М., Кавтарадзе, К. М.: ЖМЭИ **11,** 26 (1968).
26. Сергеев, В. В., Тараторина, Г. М., Елкина, С. И.: ЖМЭИ **9,** 28 (1967).
27. Фролова, М. А., Сергеев, В. В., Голубева, О. С., Проташова, О. И., Елкина, С. И., Лустер, Б. Ю.: ЖМЭИ **4,** 96 (1970).
28. Tenner, K., Serény, B., Rácz, P., Orbán, I.: Bacteriological and Virological Section, Hungarian Association of Microbiologists, Budapest 1969.
29. Serény, B.: Acta microbiol. Acad. Sci. hung. **2,** 293 (1955).
30. Serény, B.: Acta microbiol. Acad. Sci. hung. **4,** 367 (1957).
31. Kerekes, L.: Acta microbiol. Acad. Sci. hung. **9,** 123 (1962).
32. Serény, B.: Acta microbiol. Acad. Sci. hung. **6,** 179 (1959).
33. Serény, B.: Acta microbiol. Acad. Sci. hung. **8,** 159 (1961).
34. Schneider, H., Falkow, S.: J. Bact. **88,** 682 (1964).
35. Stenzel, W.: Zbl. Bakt. I. Abt. Orig. **216,** 67 (1971).
36. Serény, B.: Acta microbiol. Acad. Sci. hung. **9,** 55 (1962).
37. Rácz, P.: Thesis. Budapest—Leningrad 1962.
38. Rácz, P.: Acta morph. Acad. Sci. hung. Suppl. **12,** 1 (1963).
39. Rácz, P., Tenner, K.: Verh. dtsch. Ges. Path. 47. Tagung. Gustav Fischer Verlag, Stuttgart 1963, p. 403.
40. Serény, B.: Acta microbiol. Acad. Sci. hung. **14,** 375 (1967).

Address of the authors:

Béla Serény, Klára Tenner, Pál Rácz
National Institute of Public Health
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 247—252, 1971

A NEW MYCOBACTERIUM SPECIES: MYCOBACTERIUM ASIATICUM N. SP.

By

G. Weiszfeiler, Valentina Karasseva and Erika Karczag

Microbiological Research Group (Director: G. Weiszfeiler), Hungarian Academy of Sciences, Budapest

(Received November 29, 1971)

Summary. Four out of 18 strains isolated from monkeys as *Mycobacterium simiae* were identified later as belonging to a slowly growing, photochromogenic, facultatively pathogenic new species named *Mycobacterium asiaticum*. The organism is virulent to the mouse and differs in biochemical and serological properties and in the type of sensitin from *M. simiae*.

Up-to-date taxonomic analysis of mycobacteria has shown that Bergey's classification [1] needs a revision. Systematic studies of atypical mycobacteria isolated from man and animals have led to the differentiation of several new, facultatively pathogenic species.

The presence of atypical mycobateria in monkeys was reported first by Weiszfeiler, Karasseva and Holland [2]. Karasseva, Weiszfeiler and Krasznay [3] examined 66 *Macacus rhesus* and 3 *Cercopithecus aethiops* monkeys, 44 of which were positive for atypical mycobacteria. As in 8 cases two or more organs contained mycobacteria, a total of 50 strains, including 46 slowly growing ones, were isolated. Part of the strains was photochromogenic species other than *M. kansasii* or *M. marinum*. The organism was named *M. simiae* n.sp.

Some of the photochromogenic strains proved to be niacin positive. Several strains formed non-pigment-producing, slowly growing, niacin positive cultures. These organisms were virulent to mice and were able to spread from infected to healthy animals and to cause spontaneous infection manifesting in pathomorphological changes and *Mycobacterium* positive cultures from the organs [4].

At a round table conference held by the Microbiological Research Group of the Hungarian Academy of Sciences in 1969 [5—11], it has been concluded that these organisms should not be regarded as subspecies of *M. simiae*, but in view of characteristic differences, some have to be regarded as strains belonging to a new species.

Materials and methods

The cultures were maintained by freeze-drying. Transfers were made on Löwenstein—Jensen medium. Morphology of the cells was examined after Ziehl—Neelsen staining. Colony morphology and time of growth were studied at 20, 28, 37 and 43 °C. Pigment production was recorded after incubation in dark and after illumination for 1 to 24 hours at diffuse sun-

light. Biochemical reactions performed were: niacin production, catalase, peroxidase, nitrate reductase, lipase, acid phosphatase, arylsulphatase, alpha and beta esterase and amidase series [12]. Immune sera for antigenic analysis were prepared in sheep and in rabbits [13]. Agar gel diffusion was performed as described by JÓKAY and KARCZAG [14]. Absorption of sera was made by the gradual method with antigens of heterologous strains. Sensitins were prepared by the method of MAGNUSSON. Sensitin RS 839 was prepared by Dr. M. MAGNUSSON from strain 61 and kindly supplied to us.

Results

Strains 61, 32, 27a and 64a described in our previous papers as subgroups of *M. simiae* have been included on the basis of biochemical and antigenic properties into a new species, *M. asiaticum*. Strain 61, as type strain for the new species, was deposited at the American Type Culture Collection where it received accession number 25276.

M. asiaticum forms acid-fast coccoid cells. It is definitely photochromogenic, niacin and nitrate reductase negative, but lipase, alpha and beta esterase, acid phosphatase and arylsulphatase positive. On Löwenstein–Jensen medium, *M. asiaticum* grows slowly at room temperature; at 37 °C it produces dysgonic growth in 15—21 days. When given intravenously, it gives rise to focal lesions in the mouse lung. Strain 64 causes infection fatal in 30—60 days. Sensitin prepared from *M. asiaticum* produces specific reaction in animals infected with the organism, but exerts a definite allergic reaction also in animals injected previously with BCG or with other mycobacteria [16].

M. simiae is represented by type strain 29 catalogued as strain ATCC 25275. Examination of this culture and 13 other *M. simiae* strains (20, 25, 14, 53, 57, 58, 59, 63, 68, 3, 51, 55 and 54) indicated that *M. simiae* grows slowly, produces dysgonic colonies and forms coccoid cells or very short rods. Part of the strains is photochromogenic, but this property is unstable and decreases gradually during serial passages; several non-photochromogenic cultures were encountered. Most of the strains are niacin positive. All cultures produce urease; one of the strains (No. 14) is nicotinamidase, pyrazinamidase and succinamidase positive. None of the strains produce lipase, nitrate reductase and acid phosphatase. Arylsulphatase reaction proved variable, alpha and beta esterase positive. After intravenous injection, the organisms cause severe changes and fatal infection in mice. *M. simiae* is resistant to streptomycin, isoniazide and PAS (10 μg/ml), sensitive to cycloserine and ethioninamide (20 μg/ml).

Table I shows the principal properties of the two new species as compared to those of *M. kansasii* and *M. avium*.

In antigenic structure studies *M. simiae* and *M. asiaticum* were compared with the related organisms *M. avium*, *M. kansasii*, *M. marinum* and *M. tuberculosis*. A comparison of the number of precipitation lines appearing in homologous and heterologous systems is indicative of the degree of antigenic rela-

Table I

Properties of mycobacterial strains

	M. asiaticum strain 61	*M. simiae* strain 29	*M. kansasii*	*M. avium*
Photochromogenicity	+	±	+	—
Niacin	—	+	—	—
Nitrate reductase	—	—	+	—
Lipase	+	—	+	—
Esterase alpha	+	+	—	+
beta	+	+	—	+
Phosphatase	+	—	+	—
Amidases	—	3(5, 6, 9)	3, 5	5, 6
Arylsulphatase	+	±	±	+
Virulence mouse	+	+	+	+
guinea pig	±	±	.	±
Sensitin*	+	+	.	.
Agglutination**	+	+	.	.
Resistance cycloserine 20 μg/ml	s	s	s	s/r
ethioninamide 20 μg/ml	s	s	s	r

* Cutaneous reaction in guinea pigs infected with homologous culture to species-specific sensitin
** Prof. G. MEISSNER's results
. = not tested
s = sensitive, r = resistant

Table II

Number of precipitation lines in homologous and heterologous immunodiffusion systems

Immune serum	Antigen					
	M. asiaticum 61	*M. simiae* 29	*M. avium*	*M. kansasii*	*M. marinum*	*M. tuberculosis* H37Rv
61	15	7	4	7	4	3
61 absorbed by k + s	6	—	—	—	—	—
29	7	16	8	4	4	5
29 absorbed by a + k + s	—	6	—	—	—	—
avium	4	8	11	5	5	5
kansasii	7	4	5	12	6	6
marinum	4	4	5	6	12	5
H37Rv	4	5	5	6	5	12

Abbreviations: a = avium, k = kansasii, s = smegmatis.
The difference in the number of precipitation lines shown in Table II and in the Figures is due to the fact that in Table II the maximum number of lines is shown.

tionship between the organisms tested. Common antigens can be identified after absorption of the type sera with heterologous antigens. In experiments shown in Table II antigens kansasii (k), smegmatis (s) and avium (a) were used for absorption.

Antiserum *M. asiaticum* gives 15 lines with the homologous, but only 3—7 lines with the heterologous antigens. This finding indicates that *M. asiaticum* differs considerably from other mycobacteria in antigenic structure. The

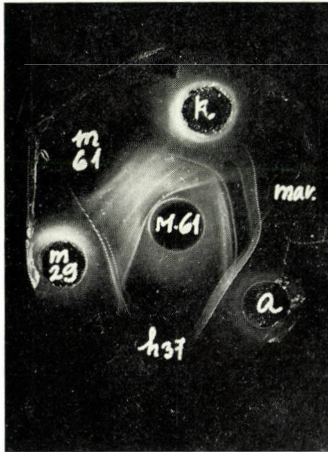


Fig. 1. Gel diffusion test with *M. asiaticum* (M 61) immune serum (central well) and reference antigens kansasii (k), marinum (mar), avium (a), tuberculosis (h37), simiae (29) and asiaticum (61)

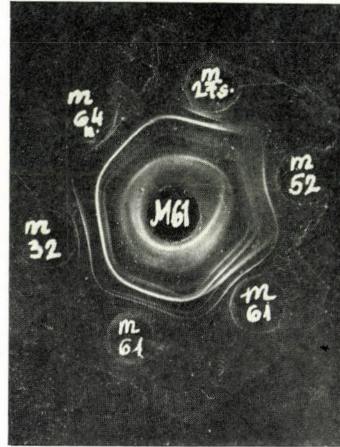


Fig. 2. Gel diffusion test with *M. asiaticum* (61) immune serum (central well) and *M. asiaticum* antigens 27, 52, 61, 32 and 64

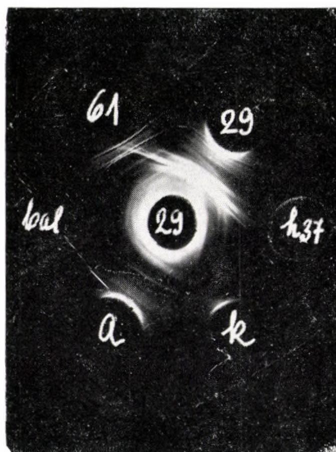


Fig. 3. Gel diffusion test with *M. simiae* (29) immune serum (central well) and antigens *M. simiae* (29), tuberculosis (h37), kansasii (k), avium (a), marinum (bal) and *M. asiaticum* (61)

fact that antiserum *M. asiaticum* 61 fails to react with antigens prepared from other slowly growing species after absorption with kansasii + smegmatis antigen, indicates that common mycobacterial antigens are present in *M. kansasii* and in *M. smegmatis*. The antigenic structure of *M. simiae* 29 differs definitely from that of other mycobacteria, but it shares a greater number of common antigenic components with *M. avium* than with other mycobacteria. In enzyme reactions, *M. asiaticum* strains behaved similarly to *M. avium* and, accordingly, a limited degree of relationship may exist between the two species. Gel precipitation reactions of the mycobacteria tested are shown in Figs 1—3.

Discussion

In view of the heterogeneity of cultures named *M. simiae*, we divided the species into subgroups [17]. The present experiments have shown that 4 cultures differed in basic properties from *M. simiae* type strain and it seemed justified to classify them into a new species, *M. asiaticum*.

M. simiae and *M. asiaticum* deserve special interest as facultatively pathogenic bacteria highly adapted to multiplication in the body. Their occurrence in nature and their potential parasitism in higher animals is analogous to the ability of atypical mycobacteria to invade man under suitable conditions. It is especially interesting that according to MAGNUSSON [6], the sensitin of atypical mycobacterial strain 127 isolated from a patient in New Guinea is identical with the sensitin of *M. asiaticum*, which may mean that the former belongs to the species *M. asiaticum*. Our examinations have confirmed this finding [16]. The sensitins of *M. simiae* and *M. asiaticum* are highly specific substances, they give an intensive reaction only in animals infected with the corresponding species. Thus it may be assumed that mycobacteria occurring in monkeys are capable of multiplying and causing pathological lesions in man. From our studies some conclusions can be drawn as to the evolution of pathogenic mycobacteria from saprophytes: slowly growing facultatively pathogenic mycobacteria may represent an intermediary step in evolution, as conditions for their survival are more favourable within the body than in nature.

REFERENCES

1. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore 1957. Pp. 695—706.
2. WEISZFEILER, J. G., KARASSEVA, V., HOLLAND, J.: Acta microbiol. Acad. Sci. hung. **11,** 403 (1964/65).
3. KARASSEVA, V., WEISZFEILER, G., KRASZNAY, E.: Acta microbiol. Acad. Sci. hung. **12,** 275 (1965).
4. KARASSEVA, V., WEISZFEILER, J. G.: Zbl. Bakt. I. Abt. Orig. **210,** 524 (1969).
5. WEISZFEILER, J. G., KARASSEVA, V., KARCZAG, E.: Proc. Microbiol. Res. Group **3,** 31 (1970).
6. MAGNUSSON, M.: Ibid. p. 71.

7. SCHRÖDER, K.: Ibid. p. 71.
8. KÄPPLER, W.: Ibid. p. 71.
9. MAKAREVICH, N. M.: Ibid. p. 57.
10. SAKELLARIDES, E.: Ibid. p. 63.
11. TACQUET, A.: Ibid. p. 73.
12. MEISSNER, G.: Mykobakterien und mykobakterielle Krankheiten. Fischer, Jena 1968. Vol. 4, pp. 149—239.
13. WEISZFEILER, J. G., JÓKAY, I., KARCZAG, E., ALMÁSSY, K., SOMOS, P.: Acta microbiol. Acad. Sci. hung. **15**, 69 (1968).
14. JÓKAY, I., KARCZAG, E.: Proc. Microbiol. Res. Group **2**, 81 (1968).
15. MAGNUSSON, M.: Amer. Rev. resp. Dis. **83**, 57 (1961).
16. KARASSEVA, V., WEISZFEILER, J. G.: Atypical Mycobacteria. Akadémiai Kiadó, Budapest. In press.
17. WEISZFEILER, J. G.: Biologie und Variabilität des Tuberkelbakteriums und die atypischen Mykobakterien. Akadémiai Kiadó, Budapest 1969. Pp. 227—253.

Address of the authors:

GYULA WEISZFEILER, VALENTINA KARASSEVA, ERIKA KARCZAG
Microbiological Research Group, Hungarian Academy of Sciences
Pihenő út 1, Budapest XII, Hungary

Acta microbiol. Acad. Sci. hung. 18, 253—259, 1971

USE OF MUTATIONS IN MARKING INDUSTRIAL YEAST STRAINS

By

MONIQUE PELLECUER, P. GALZY and J. PASERO

Laboratoire de Recherches de la Chaire de Génétique — INRA — ENSA, Montpellier and Division de Microbiologie, Société Française des Pétroles BP, Lavéra

(Received December 20, 1971)

Summary. Two yeast strains, a *Candida tropicalis* able to grow on hydrocarbons and *Saccharomyces cerevisiae* cultivated on glucose, were tested for their resistance to some antibiotics and ions reputed to be toxic for yeast. Actidione and canavanine were tentatively checked but the best results were obtained with cadmium, cobalt, copper and arsenate. It was possible to isolate ion-resistant mutants which could be used as marked strains to follow the behaviour of a given organism within the relatively mixed population of an industrial culture. Preliminary results showed that this mutation did not generally impair the viability of the organisms under investigation.

Yeast populations involved in industrial broth are rarely homogeneous. Very often, one species is dominant, then in most cases taxonomic tests make it easy to detect the contaminant species. On the other hand it is usually difficult to recognize strains belonging to the same species and differing only in physiological or technological criteria. In the same way, when one particular strain has been studied and used for inoculating the culture, it is difficult to follow its behaviour within an industrial broth.

Various authors have tried to utilize mutations in marking strains but they have rarely succeeded. For example, canavanine-resistant mutant strains require well-defined physiological conditions to grow with a good yield in the presence of this antibiotic [1, 2]. We have attempted to perform a study with the aim of marking industrial strains by the resistance to some toxic chemicals and the results obtained are given in this paper. It appears that mutants can be isolated provided a suitable marker is found for every particular case. The marked strains so selected could be used for the above industrial purpose.

Materials and methods

Strains. A strain of *Candida tropicalis* (CAST) Berkhout, isolated and purified by the SFP BP laboratory, was used. This strain is able to grow at a good rate using glucose or gas oil as a carbon source. We also used a haploid strain of *Saccharomyces cerevisiae* Hansen No. 6153 C_9P_1, α sign, marked through the ad^-_{3-3}, ad^-_6 and ur^-_1 genes.

Culture media. Two liquid culture media were used. Gas oil medium: yeast extract, 2 g; peptone, 5 g; gas oil (emulsified in water), 10 g; distilled water, 1000 ml. Glucose medium: yeast nitrogen base, 6.7 g; glucose, 5 g; distilled water, 1000 ml. A solution containing adenine, histidine and uracil was added to these media when they were used for the above strain of *S. cerevisiae.* Both media were sterilized for 40 minutes at 110 °C. The solid media were prepared by the addition of 30 g/litre of agar; their gas oil content was adjusted to 50 g/litre.

The inhibitors used were either antibiotics (actidione, canavanine) or ions reputed to be toxic for yeast (cadmium, cobalt, copper, arsenate) [3]. They were incorporated into the

media at various concentrations as indicated later; a sterile concentrated solution of a given inhibitor was added as the Petri dishes were prepared.

(a) The liquid media with glucose were used in shake-flasks filled to one tenth of their volume (80 oscillations per minute, with a 7.5 cm amplitude, working at 22 °C).

(b) The liquid media containing gas oil were shaken under the same conditions except that special vessels offering a large contact area with air were used. Growth was evaluated by direct microscopic counts.

(c) Resistance to inhibitors of the two yeast species was tested on solid media according to two techniques. In the first case, two aliquot quantities of a liquid culture were spread in parallel onto a base medium and on the same medium containing the inhibitor to be examined. In the second case, inoculation was performed by spreading onto the base medium, and after growth, the colonies were transferred through replica plating onto the medium containing the inhibitors. After inoculation, the solid media were incubated at 27 °C and the development of colonies was checked regularly.

(d) In the case of two media used in parallel, a selective concentration must be found, allowing the elimination of the wild type strain and a quantitative growth of the mutant. This means that less than 1% of the wild cells should give a colony, whereas the cells of mutants must form colonies in 100% of the cases. Nevertheless, it happens that for intermediary concentrations, the percentage of colonies given by the mutant varies from one experiment to another, according to the physiological state of the cells.

(e) If replica plating is used, the wild strain must not have a residual growth. The resistant mutant strains always give 100% growth as several cells are involved during the transfer of each colony.

Results

A. *Study of Candida tropicalis (CAST) Berkhout*

1. *Isolation of mutants.* Table I gives the results obtained with various ions and antibiotics expected to be toxic for yeast. In each case, three concentrations of the products were used and the tests were carried out on heavily inoculated Petri dishes (10^6-10^7 cells per test). The appearance of a few colonies corresponded to the growth of mutants which were isolated and studied to check their resistance.

The strain of *C. tropicalis* readily resists relatively high amounts of actidione and canavanine, antibiotics shown to be efficient on *S. cerevisiae.* These compounds are not suitable for meeting the proposed aim. On the other hand, mutants resistant to cadmium, cobalt, copper and arsenate have been found.

2. *Study of the behaviour of the mutants during vegetative multiplication.* One clone was picked up among the few colonies which developed on a Petri dish containing the inhibitor and was stored on a medium free of toxic material. A large number of subcultures spread at regular intervals allowed to check whether the resistance character was stable during vegetative multiplication.

3. *Study of the resistance thresholds.* The behaviour of the wild and mutant strain cultivated in the presence of each inhibitor at different concentrations was studied. This was done by spreading a given number of cells in parallel on Petri dishes with and without the inhibitor, and by the determination of the "survival percentage", which is the percentage of cells making up a colony. Actually, only one mutant was studied for each toxic substance except cobalt. Results are given in Table II.

Table I

Search for mutants of C. tropicalis and S. cerevisiae resistant to various inhibitors

Chemicals used	Concentrations	Culture of *C. tropicalis* on		Culture of *S. cerevisiae* on glucose
		gas oil	glucose	
Antibiotics (ppm):				
Actidione	1	+	+	—
	10	+	+	—
	100	+	+	—
Canavanine	100	+	+	*
	1 000	+	+	—
	10 000	+	+	—
Inhibitors ($\times 10^{-3}$ g ions/litre):				
Cadmium	0.64	—	+	*
	2.47	—	*	*
	3.2	—	—	*
Cobalt	0.41	+	+	+
	2.1	—	*	*
	4.2	—	—	—
Copper	0.5	+	*	+
	2.5	*	—	*
	5.1	—	—	—
Arsenate	3.2	+	+	+
	32	*	*	*
	64	—	—	—

+ abundant growth
* light growth (a few colonies)
— no growth

(a) The growth of both wild and mutant strains is completely inhibited by cadmium when gas oil is used as a carbon source. On glucose, a selective concentration of 2.5×10^{-3} g ions/litre can stop the growth of the wild strain whereas this amount has practically no effect on the mutant.

(b) In the presence of cobalt, two types of mutants were obtained, the colonies being white and brown, respectively. In the absence of cobalt, the colonies were always white. On glucose, the selective concentrations for these mutants were in the range of 2.1 to 4.2×10^{-3} g ions/litre, the wild strain was no longer able to grow while the survival percentage reached 90% for the mutants. With gas oil, the wild and mutant organisms were sensitive to lower concentrations.

Table II

Survival percentage of C. tropicalis and S. cerevisiae in the presence of various inhibitors

Inhibitors or antibiotic	Concentrations $\times 10^{-3}$ g ions/l	*C. tropicalis*				*S. cerevisiae*	
		culture on gas oil		culture on glucose		culture on glucose	
		wild	mutant	wild	mutant	wild	mutant
Nil	—	100	100	100	100	100	100
Cadmium	0.7	0	3	70	90	0	100
	1.75	0	0	65	90	0	90
	2.5	0	0	2	50	0	80
	3.25	0	0	0	50	0	50
	6.5	0	0	0	10	0	10
Cobalt	1.05	0	70	70	100	100	100
	2.1	0	70	0	90	2	70
	3.15	0	0	0	90	0	0
	4.2	0	0	0	90	0	0
	8.4	0	0	0	40	0	0
Copper	0.25	80	90	40	100	100	100
	0.5	60	90	25	100	80	90
	1.25	—	—	—	—	0	80
	2.5	1	90	0	0	0	20
	5.1	0	1	0	0	0	0
Arsenate	16	3	100	10	100	—	—
	24	2	90	2	100	—	—
	32	2	75	1	90	—	—
	64	0	75	0	80	—	—
	168	0	2	0	1	—	—
Actidione (ppm)	0.5	—	—	—	—	2	100
	1	—	—	—	—	0	95

(c) With copper as inhibitor, the opposite behaviour was observed: both wild and mutant strains were more sensitive in the presence of glucose than with gas oil as carbon source. In the latter case, the selective concentration was 2.5×10^{-3} g ions/litre.

(d) The mutant resistant to sodium arsenate grows fairly well in the presence of 32×10^{-3} g ions/litre (survival percentage, 80%) whereas only 1% of the wild strain can grow under such circumstances. Media made up with glucose or gas oil can be used.

4. *Characteristics of the mutants.* The action of a concentration considered selective has been studied for each mutant. The tests were carried out on glucose media except for copper. Actually, there was a greater difference in the resis-

tance to copper between the wild and mutant strains when gas oil was used (Table II).

(a) Mutant resistant to cadmium

(i) On glucose, when spread in the presence of 2.5×10^{-3} g ions/litre of cadmium (as nitrate), the wild strain gave rise to a few (2%) colonies only after a 5-day incubation instead of 50% in the case of the mutant. The results were rather variable for the survival percentage of the mutant strain.

(ii) On the contrary, this survival percentage was reproducible in the case of replica plating; the results available after 2 days of incubation gave 0 and 100% for the wild and the mutant strains, respectively.

(b) Mutant resistant to cobalt (white colonies)

(i) On glucose, when spread in the presence of 2.1×10^{-3} g ions/litre of cobalt (as chloride), the wild strain did not grow after 4 days of incubation while the mutant had a survival percentage of 100%.

(ii) With replica plating and a higher concentration of inhibitor (4.2×10^{-3} g ions/litre), the mutant reached a survival percentage of 100% after 2 days, whereas the wild strain gave 0%.

(c) Mutant resistant to cobalt (brown colonies)

On glucose, in the presence of 4.2×10^{-3} g ions/litre of cobalt (as chloride), this mutant had a survival percentage of 100% within 4 days after spreading and only 2 days with replica plating.

(d) Mutant resistant to copper

(i) On gas oil, when spread in the presence of 2.5×10^{-3} g ions/litre of copper (as sulphate), the wild strain gave rise to a few (2%) colonies after 3 days of incubation whereas 90% of the mutant cells were able to grow.

(ii) With replica plating, with the same concentration of copper sulphate, both wild and mutant strains grew abundantly. Thus, this technique is not suitable for differentiating wild cells from mutant cells resistant to copper.

(e) Mutant resistant to arsenate

(i) On glucose, when spread in the presence of 32×10^{-3} g ions/litre of arsenate (as sodium salt), the cells of the wild strain showed poor survival (1%) instead of 50% for the mutant cells, but unfortunately this value is rather variable from one test to another (readings after 6-day incubation).

(ii) Better results were obtained after 2 days using replica plating, *i.e.* 0% for the wild strain and 100% for the mutant.

B. *Study of Saccharomyces cerevisiae*

1. *Isolation of mutants.* A similar study was carried out with *S. cerevisiae* Hansen 6153 C_9P_1. Results are given in Table I. Only glucose media could be used in this case.

Mutants which were stable during vegetative multiplication have been obtained. They are resistant to canavanine, actidione, cadmium, cobalt and

copper. In the presence of arsenate (32×10^{-3} g ions/litre), the strain 6153 C_9P_1 gave many very small colonies. There was no difference in behaviour with respect to arsenate between the clones which were picked up, and the wild strain. In fact, the mutants resistant to canavanine were ignored in this work because previous studies have dealt with this phenomenon [1, 2].

2. *Characteristics of the mutants*

(a) Mutant resistant to cadmium [4]

(i) On spread plates, the wild strain was unable to grow in the presence of 0.7×10^{-3} g ions/litre of cadmium whereas the mutant strain had a survival percentage of 90% at concentrations higher than 1.75×10^{-3} g ions/litre.

(ii) Replica plating could be applied at this last concentration; the mutant cells gave 100% of the expected colonies while the wild strain did not grow.

(b) Mutant resistant to cobalt

(i) On spread plates, the wild strain resisted to 0.41×10^{-3} g ions/litre of cobalt but its survival percentage was low (2%) in the presence of 1.05×10^{-3} g ions/litre. On the contrary, the mutant strain gave a good growth at the same concentration.

(ii) Replica plating was performed with the same amount of cobalt; 100% of the expected colonies of the mutant strain appeared whereas a weak residual growth of doubtful significance was observed with the wild strain.

(c) Mutant resistant to copper [5]

(i) When spread, the wild strain grew well in the presence of 0.5×10^{-3} g ions/litre of copper but was no longer able to grow under the effect of 1.25×10^{-3} g ions/litre (no colony). At this concentration, about 80% of the spread mutant cells formed a colony.

(ii) These results were confirmed by replica plating at the above concentrations; a survival percentage of 0 and 100% was obtained with the wild and mutant strains, respectively.

(d) Mutant resistant to actidione [6]

(i) When spread, the wild strain was sensitive to 0.5 ppm of actidione; 10^8 cells inoculated gave only a few colonies. One of them picked up was checked as a mutant; its survival percentage was 95% in the presence of 1 ppm of inhibitor.

(ii) By replica plating and with the latter concentration the wild strain gave no colonies (0%) whereas all the mutant strains did (100%).

Discussion

The method described above allows the marking of a strain of yeast and makes it possible to recognize it within a mixed population including the mutant and the wild type. Thereby, it should be possible to follow the behaviour of a particular strain used for inoculating industrial system.

The two strains studied in this work behave differently *vis à vis* the inhibitors. This means that a particular investigation has to be performed in every case. However, we were able to mark the strains tested.

Two techniques were used comparatively and with each inhibitor studied the best results were obtained with one or the other. Spreading in parallel onto a basal medium and on the same medium containing the inhibitor allowed the tested strain to be counted within a mixture containing the wild strain in the following cases:

C. tropicalis: mutants resistant to cobalt, copper and arsenate; *S. cerevisiae:* mutants resistant to cadmium, copper and actidione.

On the other hand, replica plating was satisfactory in the following cases:

C. tropicalis: mutants resistant to cadmium, cobalt and arsenate; *S. cerevisiae:* mutants resistant to cadmium, copper and actidione.

It is obvious that this kind of marking can only be applied to industrial strains provided their vitality is not impaired by the mutation. The growth rates have been evaluated in liquid media containing gas oil for *C. tropicalis*, and glucose for *S. cerevisiae.*

Although it was not possible to check comparatively the various mutants in laboratory continuous fermenters, it appears from preliminary results obtained on batch cultures that the growth rates of the mutants remain comparable to those of the wild strains, except for the copper and cobalt resistant mutants of *C. tropicalis* (brown colonies) where some decrease was observed.

In conclusion, a genetic marking of the two yeasts tested has been possible. Several markers allow the recognition of the strain studied within a mixed population of several strains of *C. tropicalis*. These mutations do not alter the vitality of the ion-resistant strain.

Acknowledgement. The authors wish to thank the British Petroleum Company Limited for financial support and for permission to publish this paper.

REFERENCES

1. Galzy, P.: Progr. Agr. et Vit. **95** (1956).
2. Galzy, P., Dupuy, P.: Ann. Technol. **2,** 235 (1957).
3. Lindegren, C. C.: Antimicrobial Agents Annual (1960).
4. Middlekauff, J. E., Seiichi Hino, Sing Ping Yang, Lindegren, G., Lindegren, C. C.: J. Bact. **72,** 796 (1956).
5. Brenes-Pomales, A., Lindegren, G., Lindegren, C. C.: Nature (Lond.) **176,** 841 (1957)
6. Middlekauff, J. E., Seiichi Hino, Sing Ping Yang, Lindegren, G., Lindegren, C. C.: Genetics **42,** 66 (1957).

Address of the authors:

Monique Pellecuer, Pierre Galzy
Laboratoire de Recherches de la Chaire de Génétique — INRA — ENSA, Montpellier, France

Jean Pasero
Société International de Recherche BP, 28-Epernon, France

Acta microbiol. Acad. Sci. hung. 18, 261—269, 1971

PSEUDOMONAS AERUGINOSA INFECTIONS IN AN ARTIFICIAL RESPIRATORY WARD

By

G. Losonczy, L. Tóth, G. Petrás, S. Bognár, B. Lányi and Judith Csekes

Infectious Diseases Hospital "László" (Director: A. Kátay) and National Institute of Public Health (Director: T. Bakács), Budapest

(Received December 23, 1971)

Summary. Studies on 156 patients over a two-year period showed that single and multiple iatrogenic infections were common in artificially respirated (255 in 101) and less frequent among non-respirated (26 in 55) patients. Fatal cases occurred among respirated patients (37 died of the original disease, 18 of iatrogenic infections). Patients with tetanus were especially affected.

From the majority of the infections in patients subjected to artificial ventilation *Pseudomonas aeruginosa* was isolated (40 out of 65 infections with one single organism and from 46 out of 55 mixed infections). In the rest of the infections *Klebsiella, Escherichia coli, Staphylococcus aureus* and *Proteus* were involved. Serological typing of *Ps. aeruginosa* showed that strains belonging to three different serogroups (O4a, 4d, O7a, 7b and O3a, 3d) were responsible for the majority of the infections. The same serogroups were isolated from the environment and staff. The route of the infections and preventive measures have been discussed.

Artificial respiration was introduced in Hungary about twenty years ago mainly for the treatment of poliomyelitis victims. Since the eradication of this disease the respirators have successfully been used in other conditions such as encephalitis, meningitis, polyradiculitis, myasthenia gravis, toxicosis and tetanus.

Patients whose lives are saved by artificial respiration are often prone to a serious hazard of bacterial infection. Gotzmann and Whitby [1] showed pathogenic microorganisms in the trachea of 18 out of 23 patients within 3 days after tracheostomy; *Ps. aeruginosa* was present in 5 patients. Phillips and Spencer [2] described that 8 out of 13 such patients acquired respiratory *Ps. aeruginosa* infection. Tunewall [3] showed that *Staph. aureus*, coliform bacteria and *Ps. aeruginosa* colonized the respiratory tract of respirated polio patients. According to Dupont *et al.* [4] patients with tracheostomy harboured *Ps. aeruginosa* in 80%. Csernohorszky and Ádám [5, 6] demonstrated the risk of *Ps. aeruginosa* infection in patients subjected to intratracheal anaesthesia.

The most dangerous complications of artificial respiration are tracheitis, pneumonia and septicaemia. A number of other treatments (catheterization, infusion, transfusion, injections, artificial nutrition, drainage, etc. [7]) also involve an ample chance for hospital infections.

The purpose of the present work was to study the spread and prevention of these infections.

Materials and methods

Patients admitted to the artificial respiration unit for acute diseases have been examined over a period of 2 years. Nasal, throat, tracheal, urinary and other specimens were cultured and the isolated bacteria were identified as described in the Standard Methods of Hungarian Public Health Laboratories [8]. In October, November and December of the years 1964 and 1965 samples were taken at 14-day intervals from instruments and environment (endotracheal tube and its gauze pad, inspiratory and expiratory tubing, aspirators, catheters, stethoscopes, tables, washing basins, taps, etc.). The swabs were inoculated into usual media and in addition into merthiolate broth designed for the selective cultivation of *Ps. aeruginosa* [9]. Air samples were taken by exposure of blood agar plates for 15 minutes. *Ps. aeruginosa* isolates were serotyped as described by LÁNYI [10].

Results

Patients. A total of 209 patients were admitted, 53 of whom died or were discharged within 3 days. The remaining 156 patients were studied for the occurrence of hospital infection. Of these patients, 101 were subjected to artificial respiration. Their age distribution was: infants 15.4%, children (under 15) 26.9%, adults 57.5%. Fifty-five out of the 156 patients died (37 from the original disease, 18 from iatrogenic infections). Of the recovered patients 67 had been in serious condition. Mortality was the highest among patients above

Table I

Distribution of iatrogenic infections

Original disease	No. of patients	Artificial respiration	Number of iatrogenic infections								
			Tracheal	Thrombophlebitis	Urinary	Abscess	Eye infection	Enteric infection	Decubitus	Other	Total
Tetanus	41	31 yes	29	29	18	7	5	2	6	2	98
		10 no	—	4	3	—	—	—	—	—	7
Polyradiculitis	10	6 yes	5	4	5	—	3	1	3	1	22
		4 no	—	2	—	—	—	—	—	—	2
Encephalitis	26	20 yes	17	7	6	5	6	2	3	2	48
		6 no	—	—	2	—	1	—	—	—	3
Meningitis	21	17 yes	14	8	7	2	4	2	3	2	42
		4 no	—	2	1	—	—	—	—	—	3
Miscellaneous*	58	27 yes	19	4	5	2	4	4	2	5	45
		31 no	—	4	1	3	1	1	—	1	11
Total	156	101 yes	84	52	41	16	22	11	17	12	255
		55 no	—	12	7	3	2	1	—	—	26

* Toxicosis, diphtheria, multiple sclerosis, degenerative conditions of the nervous and locomotor system, etc.

70 years of age (7 out of 9). The distribution of patients according to original disease and the incidence of iatrogenic infections are shown in Table I.

Incidence of iatrogenic infections. As shown in Table I many of the patients were affected at two or more sites and thus the total number of hospital infections amounted to 281 (255 in 101 patients with, and 26 in 55 patients without, artificial respiration). There was no important association between the incidence and site of hospital infections and the different types of original diseases.

All severe and lethal infections fell into the group subjected to artificial respiration. Patients of the non-respirated group suffered usually from infections of slight or medium degree. According to their course, the 281 infections could be classified as mild, 120 (42.7%); moderately severe, 106 (37.7%); severe, 37 (13.2%); fatal, 18 (6.4%).

In addition to the 18 patients who died of iatrogenic infections, further 24 patients were lost in whom hospital-acquired infection may have more or less definitely contributed to the fatal outcome of the original condition. Patients with tetanus were the most frequently affected by lethal and severe infections (20 severe and 13 lethal cases).

Among the other patients subjected to artificial respiration only 5 lethal and 17 severe infections were recorded.

The severity of respiratory infections due to artificial respiration was indicated by the fact that death was due to tracheal infection in 15 out of the 18 patients (in 11 out of the 13 tetanus cases). The cause of death was in all patients a septicaemia developing from tracheitis, genito-urinary infection, thrombophlebitis and other inflammatory complications.

Causative agents. Bacteria isolated from various conditions were regarded as causative agents only when they were repeatedly shown in pure culture or in large numbers and when they disappeared from the site of infection after healing.

In view of these restrictions the responsibility of bacteria could be ascertained in 120 out of the 255 infections affecting respirated patients and in 9 out of the 26 infections affecting non-respirated patients.

Among the respirated patients, infections were characterized by one single causative agent in the following distribution: ***Ps. aeruginosa*** 40, ***E. coli*** 8, ***Proteus*** 6, ***Staph. aureus*** 6, ***Klebsiella*** 5.

In 55 infections developing in patients subjected to artificial respiration, two or more pathogens were isolated from each specimen. The distribution of bacteria present in such mixed infections was: ***Ps. aeruginosa*** 46, ***Klebsiella*** 41, ***E. coli*** 20, ***Staph. aureus*** 15, ***Proteus*** 11, not identified organism 1. In mixed infections, ***Ps. aeruginosa*** was present usually in larger numbers than the other organism(s) and ***Ps. aeruginosa*** could be isolated from the majority of severe and lethal infections.

Table II

Distribution of bacteria cultured

	Specimens	Total No. of specimens	Total No. of positive specimens	Number		
				Ps. aeruginosa		
				3ade	5ab	4ad
Environment	Endotracheal tube and gauze pad	11	11	1	—	2
	Respirators and other instruments	63	33	—	1	4
	Furniture	67	35	—	—	1
	Total	141	79	1	1	7
Patients	Nose	68	53	4	5	2
	Throat	67	41	3	1	1
	Ear	2	2	—	—	—
	Trachea	57	39	3	2	12
	Faeces	10	2	2	—	—
	Total	204	137	12	8	15
Staff	Nose	36	26	—	—	—
	Throat	36	9	—	—	—
	Hands	33	22	—	—	2
	Dresses	37	21	—	—	—
	Total	142	78	—	—	2
Total	No.	487	294	13	9	24
	per cent	100.0	60.4	3.4	2.4	6.3

No significant difference was shown in the distribution of bacteria as to the original disease.

Source of the infections. In order to investigate the spread of the infections, especially those caused by *Ps. aeruginosa*, two surveys, each lasting for three months, were made. In these we included in addition to the bacteriological examination of patients, staff and environment, the serological typing of *Ps. aeruginosa* strains. During the two 3-month periods a total of 1046 specimens were obtained. The results are shown in Tables II and III.

In comparing the results, a considerable difference can be seen between the two periods as to the presence of bacteria in fomites as well as in the staff. In contrast, the number of positive cultures from patients remained practically at the same level.

Alterations in the species distribution of microorganisms were also noticeable. In 1965, the incidence of *Staph. aureus* in the environment and patients decreased as compared to 1964. There was also some decrease in the incidence of *Str. pyogenes* in patients. The frequency of *E. coli* isolations decreased

in October—December, 1964

of strains							
serogroups			*Staph. aureus*	*Str. pyogenes*	*E. coli*	*Proteus*	*Klebsiella*
7ab	other	total					
—	2	5	4	—	2	1	3
2	9	16	13	—	17	1	8
—	2	3	9	—	11	1	2
2	13	24	26	—	30	3	13
2	14	27	38	1	4	—	6
2	17	24	17	6	5	—	—
—	—	—	2	—	2	2	—
4	8	29	15	—	6	1	2
—	2	4	—	—	—	—	—
8	41	84	72	7	17	3	8
1	—	1	18	—	9	—	5
—	—	—	1	—	5	—	3
1	—	3	8	—	10	1	6
—	3	3	5	—	11	—	6
2	3	7	32	—	35	1	20
12	57	115	130	7	82	7	41
3.1	14.9	30.1	34.0	1.8	21.5	1.8	10.8

markedly in the environment and staff. The increase in the incidence of *Ps. aeruginosa* was evident in almost all materials.

There was a considerable change in the distribution of *Ps. aeruginosa* serogroups. In 1964, strains belonging to serogroups 4a, 4d and 7a, 7b were frequently isolated from patients, staff and environment (Table II).

In the last three months of 1965, strains 3a, 3d appeared in addition to the above two frequently occurring serogroups. In this period, 202 out of 241 *Ps. aeruginosa* isolates belonged to the three nosocomial serogroups (Table III).

During the two 3-month surveys from 8 patients nasal, pharyngeal and tracheal specimens were taken at daily intervals. In most of them the first bacteriological examination carried out after admission was negative for *Ps. aeruginosa.* Positive findings became frequent after the third or fourth examination when the organism was usually present in all three kinds of specimen. In one and the same patient frequently two different serogroups were encountered (4a, 4d and 7a, 7b). Other patients carried one single serogroup throughout

Table III

Distribution of bacteria cultured

	Specimens	Total No. of specimens	Total No. of positive specimens	Number *Ps. aeruginosa* 2	3ad	4ad
Environment	Endotracheal tube and gauze pad	5	4	—	—	—
	Respirators and other instruments	77	15	—	—	2
	Furniture	29	3	—	—	1
	Total	111	22	—	—	3
Patients	Nose	128	97	5	14	16
	Throat	122	71	7	15	12
	Trachea	109	87	3	19	25
	Sputum	1	1	—	—	1
	Gastric lavage	7	5	—	1	—
	Faeces	27	16	—	1	4
	Urine	13	11	—	—	1
	Total	407	288	15	50	59
Staff	Nose	20	5	—	—	—
	Hands	21	6	—	—	—
	Total	41	11	—	—	—
Total	No.	559	321	15	50	62
	per cent	100.0	57.4	3.1	11.1	13.8

6—11 examinations. One patient carried only serogroup 4a, 4d throughout 11 samplings; then this organism disappeared and gave way to serogroup 7a, 7b which grew afterwards in pure culture throughout 7 subsequent examinations.

A total of 28 air samples failed to yield *Ps. aeruginosa*. The average number of colonies on plates exposed for 15 minutes was 50. Of bacteria associated with hospital infections only *Staph. aureus* was cultured from two plates.

Discussion

The present studies confirmed data in the literature that patients with tracheostomy and artificial respiration are especially prone to hospital-acquired infections. In our material the overwhelming majority of infections occurred in these patients. This might have been due to the fact that artificial respiration is applied usually in severe cases who receive for long periods of time a

in October—December, 1965

of strains							
serogroups			*Staph. aureus*	*Str. pyogenes*	*E. coli*	*Proteus*	*Klebsiella*
7ab	other	total					
2	—	2	1	—	—	—	1
4	6	12	2	—	2	—	—
—	—	1	1	—	1	—	—
6	6	15	4	—	3	—	1
38	5	78	45	1	7	4	8
13	—	47	28	8	14	3	7
24	7	78	24	—	15	—	5
—	—	1	—	—	—	—	1
2	—	3	2	—	1	1	—
4	—	9	1	—	—	3	5
1	5	7	—	—	6	2	—
82	17	223	100	9	43	13	26
—	—	—	5	1	—	—	—
2	1	3	2	—	1	—	—
2	1	3	7	1	1	—	—
90	24	241	111	10	47	13	27
20.1	5.4	53.5	24.7	2.2	10.5	2.9	6.0

number of other kinds of medical treatment involving the hazard of iatrogenic infection. Our studies showed that the incidence and severity of iatrogenic infections are generally not associated with the nature of the original disease but are influenced by the intensity of medical treatment and especially by the presence or absence of artificial respiration.

Patients with tetanus suffered definitely more severely and were more frequently subject to lethal infection than other patients.

The causative agent encountered most frequently and held responsible for the most severe infections was *Ps. aeruginosa.* In the ward, three different *Ps. aeruginosa* serogroups were predominant. As other serogroups which had frequently been found in pathological materials from other hospitals [10] were less frequently isolated or were entirely absent, it was concluded that *Ps. aeruginosa* serogroups 3a, 3d, 4a, 4d and 7a, 7b constituted the characteristic nosocomial organisms of the unit. These serogroups were frequently encountered not only in patients but also in the staff and the environment.

In comparing the two examination periods it was evident that the number of *Staph. aureus*, *E. coli* and *Klebsiella* isolations from staff and environment decreased in the second as compared to the first survey. However, the number of *Ps. aeruginosa* positive specimens remained practically at the same level in the environment and increased in patients.

The incidence of *Staph. aureus*, *E. coli*, *Proteus* and *Klebsiella* isolations in all other departments was similar to that shown in Tables II and III. In contrast, the incidence of *Ps. aeruginosa* was much lower in other units, namely, 0—3% as contrasted to the 23.6% (Table II) and 43.1% (Table III) frequency in the respiratory unit.

The reason for the difference between the two survey periods is not clear. The technique of bacteriological examination, care and treatment of patients were similar in both periods. The only change was that in 1965 the hygienic measures were more rigorously controlled than in the previous year. The more effective control might have contributed to the decrease in the incidence of less resistant bacteria in the environment, but at the same time perhaps it promoted the spread of *Ps. aeruginosa*.

As to the mode of transmission of *Ps. aeruginosa* infections, mainly the role of insufficiently sterilized or disinfected instruments and the hands of the staff may be assumed. The high degree of pollution in the environment was due probably to the following factors. First of all the insufficient number of nursing staff, which according to PETRI [7] is a main factor in the spread of hospital infections. It is generally accepted that a 14-bed intensive-care unit should be staffed with about 35 persons. Our 10-bed unit was staffed with less than half of this number. The second factor was that freshly tracheostomized patients could not always be separated from infected patients. Finally, it was evidently disadvantageous that, owing to the understaffing, doctors and nurses worked often in the respiratory unit for chronic cases and *vice versa*. The unit for chronic diseases was also colonized by *Ps. aeruginosa* and other facultatively pathogenic microorganisms.

A careful, continuous control of the sterilization or disinfection of instruments and hands has been introduced. However, sterilization of the respirators presented many difficulties. *Ps. aeruginosa* and other bacteria were frequently cultured after the routine cleansing and disinfecting of tubings, humidifiers, pumps, etc. Ethylene oxide treatment has so far been proved to be the best method for effective sterilization of the respirators.

On the basis of the present studies the following measures are thought advisable for preventing iatrogenic infections in the course of artificial respiration. (1) Isolation of patients (freshly admitted and infected). (2) Reliable disinfection of respirators (ethylene oxide). (3) Careful administration of broad spectrum antibiotics. (4) Rigorous control of hygienic conditions and of sterility of instruments.

REFERENCES

1. Gotzmann, M. S., Whitby, J. L.: Thorax (Lond.) **19**, 89 (1964).
2. Phillips, I., Spencer, G.: Lancet **2**, 1325 (1965).
3. Tunewall, G.: Acta med. scand. **154**, Suppl. **316**, 91 (1956).
4. Dupont, V., Lissac, J., Amstutz, P.: Rev. Prat. (Par.) **12**, 1909 (1962).
5. Csernohorszky, V., Ádám, M. M.: Zbl. Chir. **86**, 1622 (1961).
6. Csernohorszky, V., Ádám, M. M.: Zbl. Chir. **86**, 1651 (1961).
7. Petri, G.: Orv. Hetil. **108**, 2401 (1967).
8. Módszertani Útmutató, Országos Közegészségügyi Intézet, Budapest, 1969.
9. Lányi, B., Gregács, M., Ádám, M. M.: Acta microbiol. Acad. Sci. hung. **13**, 319 (1966/67).
10. Lányi B.: Acta microbiol. Acad. Sci. hung. **13**, 295 (1966/67).

Address of the authors:

György Losonczy, László Tóth, Győző Petrás, Sándor Bognár, Judit Csekes
Infectious Diseases Hospital "László", Gyáli út 5–7, Budapest IX, Hungary

Béla Lányi
National Institute of Public Health, Gyáli út 2–6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 271—278, 1971

EFFECTS OF RAUSCHER LEUKAEMIA VIRUS ON THE IMMUNE SYSTEM IN SUSCEPTIBLE AND RESISTANT MOUSE STRAINS

By

F. D. Tóth, T. Karsai and L. Váczi

Institute of Microbiology (Director: L. Váczi*) and*
Institute of Biochemistry (Director: B. Tankó*), University Medical School, Debrecen*

(Received January 6, 1972)

Summary. Infection of BALB/c, DBA/1 and C57BL/10 Sn mice with Rauscher leukaemia virus is followed by an increase in the levels of α_1, α_2 and β_2 globulin. The increase is in direct relation to the susceptibility of the mouse strain. The gamma-globulin level declines in strains BALB/c and DBA/1 parallel with the progress of the leukaemia, whereas in C57BL/10 Sn mice it increases and remains at an elevated level persistently. These changes can be attributed partly to the effects of the Rauscher virus on the immune system, partly to the production of new proteins by tumour cells. Multiplication of the virus in the lymph nodes, being in direct relation to the susceptibility of the mouse strain, results in a parallel decrease in the production of immuno-specific globulins.

In previous experiments it has been shown that in C57BL mice infected with the Rauscher leukaemia virus (RLV) the antibodies induced by the type-specific virus antigen, due to their cytotoxic effect, inhibit the progress of leukaemia [1], and that the resistance to RLV of a mouse strain is in direct relation to its tumour-specific antibody production [2]. In the present work, we have attempted to study the factors determining the degree of the humoral immune response.

Materials and methods

Virus. Spleens from BALB/c mice infected with RLV when 8-week-old were ground 16—18 days after infection to make a 20% suspension in PBS. The suspension was centrifuged at 5000 g for 15 minutes at a temperature of +4 °C and stored at −70 °C until used as virus preparation. Animals were inoculated with 0.2 ml of this suspension intraperitoneally. The inoculum contained 10^4 focus-forming units.

Mice. Inbred BALB/c, DBA/1 and C57BL/10 Sn mice were purchased from the experimental animal house of the National Blood Supply Service, Budapest. Eight-week-old males were used. Materials (serum, spleen and lymph nodes) were obtained from 7 mice at each time indicated. Cervical, axillar, inguinal and retroperitoneal lymph nodes were examined.

Production of immune serum. Anti-RLV serum was prepared as recommended by Fink and Rauscher [3]. The virus was kept in 0.1% formaldehyde solution at +4 °C for 21 days and shaken up each day. It was then emulsified with an equal volume of complete Freund adjuvant (Difco). BALB/c mice 8 weeks of age were injected intraperitoneally with 0.5 ml of the emulsion. Three weeks later, the animals were injected with 0.1 ml of the formalinized virus without any adjuvant. After further 10 days, the animals were exsanguinated.

Immunofluorescence studies. The tumour-specific antibodies were determined by indirect surface fluorescence. The method and the technique of absorption have been described in detail [2].

Multiplication of RLV in the lymph nodes was examined by direct membrane fluorescence, using the fluorescein-isothiocyanate-labelled globulin fraction of the anti-RLV serum. This method was used because there is only group-specific antigen in the cytoplasm of the RLV-producing cells while the immune serum produced in the same species contains only type-specific antibodies. For indirect immune fluorescence, fluorescein-isothiocyanate-labelled anti-mouse swine globulin (Sevac, Prague) was used.

Serum electrophoresis. Celogel (Chemetron, Milan) stripes 2.5×18 cm in size were used as described by AARONSON and GRÖNWALL [4]. Electrophoresis was carried out in Tris–boric acid–EDTA buffer of pH 8.6 at an intensity of 0.5 mA/cm. Electrophoretograms were stained with a 1% solution of amidoschwarz (Serva) in a 5 : 4 : 1 mixture of methanol, water and acetic acid. The spots corresponding to protein fractions were dissolved in acetone and examined in the Beckman spectrophotometer at 620 mμ. The levels of each serum fraction are expressed in relative value, *i.e.*, compared to the respective level for healthy mice of the same strain and age.

Results

Fig. 1 shows the splenomegalic response of BALB/c, DBA/1 and C57BL/10 Sn mice. The splenomegalic response is a good indicator of the degree of susceptibility of a mouse strain.

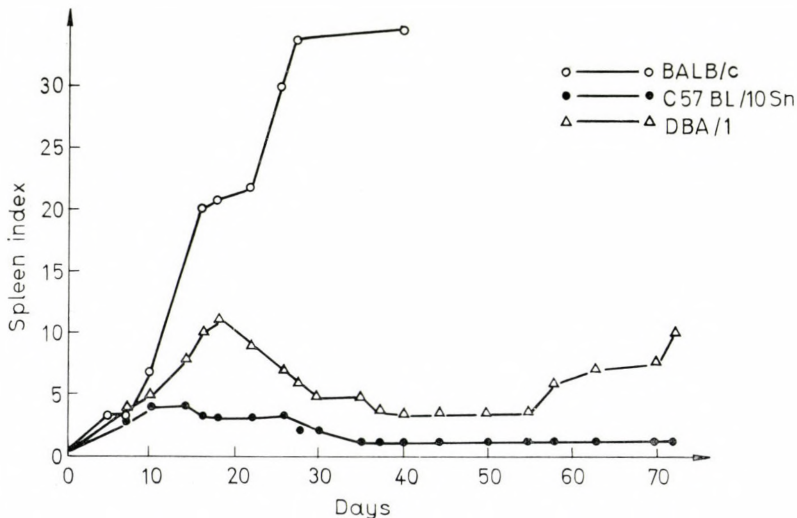


Fig. 1. Splenomegalic response of different mouse strains after Rauscher virus infection

In BALB/c mice, the leukaemia progressed rapidly, whereas in the C57BL/10 Sn mice splenomegaly was transient. In the DBA/1 strain, the illness was biphasic; most of the mice survived the first phase and after partial remission died in the second phase.

Among the serum globulin fractions, the alpha$_1$, alpha$_2$, beta$_2$ and gamma globulins were altered. No quantitative change could be demonstrated in the beta$_1$ globulin level for any of the mouse strains.

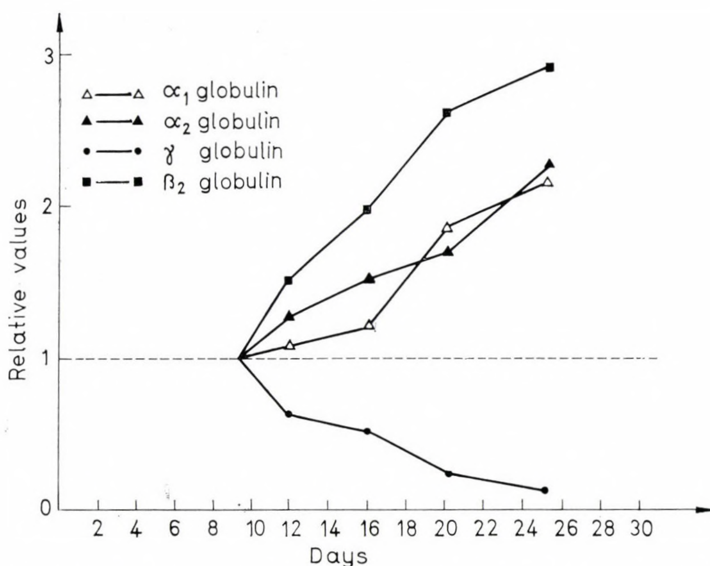


Fig. 2. Alteration of different globulin fractions in sera of BALB/c mice after Rauscher virus infection

In the RLV-infected BALB/c mice, the alpha$_1$, alpha$_2$ and beta$_2$ globulin levels increased with the progress of the leukaemia. On the other hand, the gamma-globulin level declined (Fig. 2).

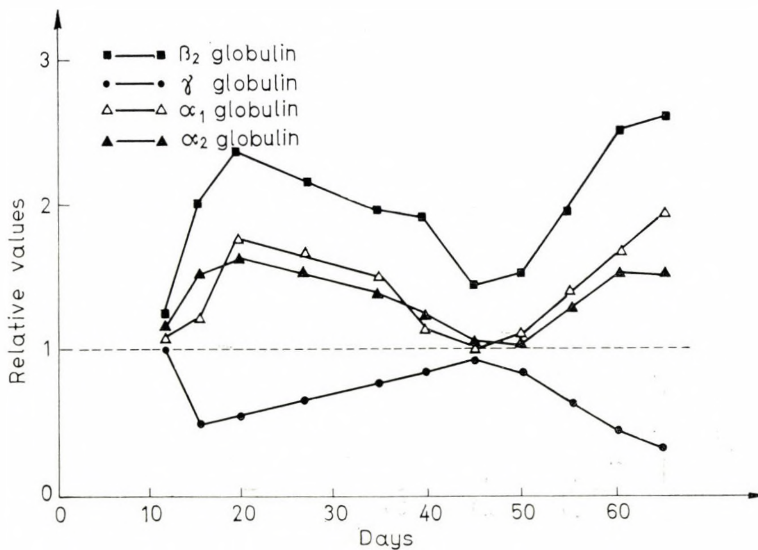


Fig. 3. Alteration of different globulin fractions in sera of DBA/1 mice after Rauscher virus infection

The tendencies were the same for the DBA/1 mice, but the values returned to normal levels during remission and deviated again during the second phase (Fig. 3).

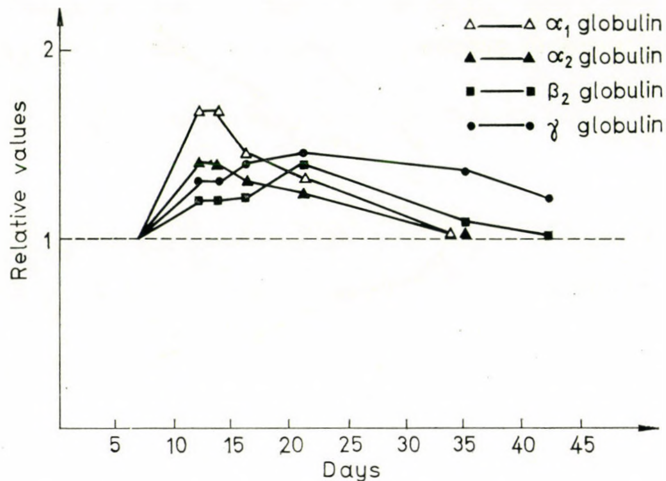


Fig. 4. Alteration of different globulin fractions in sera of C57B1/10 Sn mice after Rauscher virus infection

In C57BL/10 Sn mice the $alpha_1$, $alpha_2$ and $beta_2$ globulin levels increased but much less than in the other two strains. In contrast, gamma-globulin also increased in amount and persisted at an elevated level (Fig. 4).

The quantity of globulin bound to the cell surface was examined in cell suspensions prepared from lymph-node and spleen specimens (Figs 5, 6).

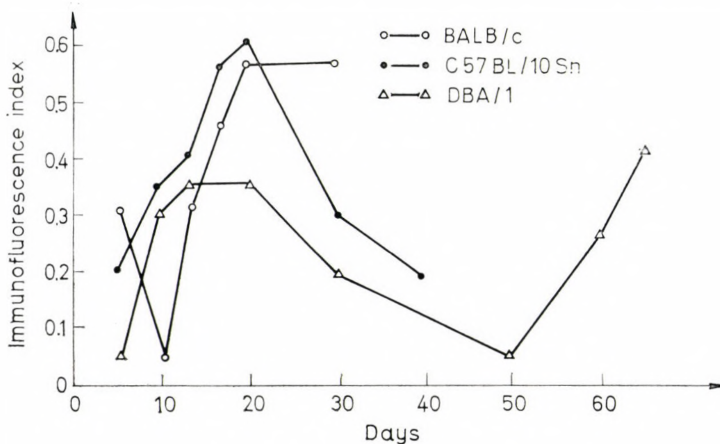


Fig. 5. Change in the ratio of anti-globulin-binding lymph node cells in different strains of mice after Rauscher virus infection

The ratio of lymph-node cells binding fluorescent anti-mouse globulin increased irrespective of the mouse strain, as compared to the uninfected control values. In C57BL/10 Sn mice, the increased fluorescence was apparent between the 10th and 30th days after infection, with a peak about the 20th day; in the case of strains BALB/c and DBA/1, the increase was interrupted by a transient decline, which was of short duration in the BALB/c mice,

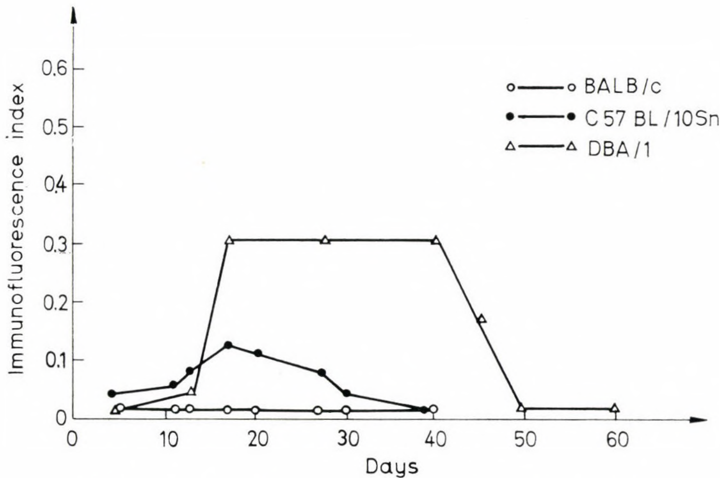


Fig. 6. Detection of globulin bound to the surface of spleen cells in different strains of mice infected with Rauscher virus

whereas in the case of the DBA/1 strain, the first peak corresponded to the initial progressive phase and the second peak to the exacerbation; during the partial remission, the fluorescence values agreed well with the control values.

No fixed globulin could be detected on the surface of the spleen cells of infected BALB/c mice (Fig. 6). In the C57BL/10 Sn and DBA/1 mice, on the other hand, splenomegaly was accompanied by an increase in the number of cells bearing globulin. The increase was more pronounced in the DBA/1 mice which displayed an elevated level between the first splenomegalic peak and the beginning of the second phase.

In infected C57BL/10 Sn mice, the tumour-specific serum antibody increased in amount abruptly and persisted at a high level. In DBA/1 mice, the response was somewhat delayed, and the peak was lower and was followed by a gradual decline. In the serum of BALB/c mice, tumour-specific antibody could not be detected except during a short period about the 7th day after infection (Fig. 7).

Multiplication of RLV in the lymph nodes was in direct relation to the susceptibility of the mouse strain (Fig. 8).

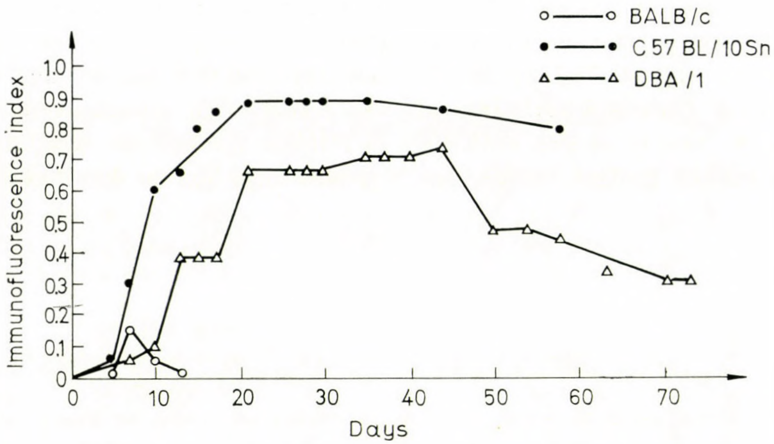


Fig. 7. Production of tumour specific antibodies in different mouse strains infected with Rauscher virus

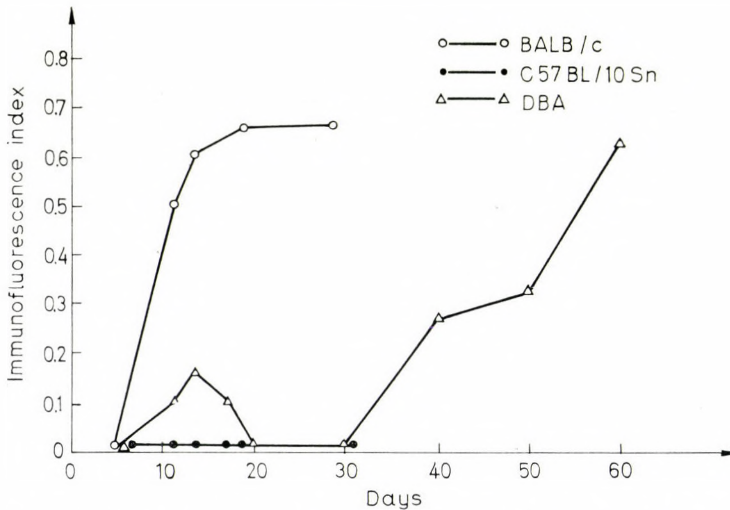


Fig. 8. Detection of Rauscher virus on the surface of lymph node cells after Rauscher virus infection

No virus could be detected in the lymph nodes of C57BL/10 Sn mice. In DBA/1 mice, after a low peak the virus disappeared early in the period of remission and reappeared some days before the exacerbation period. During the second wave, while the bulk of the DBA/1 mice died, a peak was reached as high as the peak shown by the BALB/c mice from the 10th day on. The high fluorescence indexes cannot be attributed to metastases, as Rauscher leukaemia does not give rise to lymph node metastases [5].

Discussion

In the present experiments, the serum gamma-globulin level in mice infected by RLV decreased parallel with the progress of leukaemia. This decrease was preceded by an intensive multiplication of the virus in lymph nodes. The quantitative changes in the gamma-globulin ran parallel with the tumour-specific antibody level in the serum. It seems reasonable to suppose that the tumour-specific antibody response, due to a competition between virus multiplication and antibody production, is dependent on the suppression of the production of IgG-type antibodies. This assumption seems to be supported by the electron-microscopic evidence that RLV appears in the blastic elements of the lymphoid system earlier than in the erythroid target cells [6]. It should also be taken into consideration that newborn rats infected with Moloney virus show a reduced serum IgG level as early as in the latency phase and their IgG antibody response was impaired at the same time, whereas the synthesis of IgM-type immunoglobulins appeared to be normal in infected rats [7].

It may be supposed that the tumour-specific antibody appearing in the serum of BALB/c mice around the 7th day after RLV infection is of the IgM type. Simultaneously, the globulin fixed on the surface of the lymph-node cells of these mice increased in amount. Besides, antibody could not be detected at a later time even by procedures resulting in the separation of antibodies from antigen–antibody complexes [8]. In the DBA/1 strain, high-titre tumour-specific antibodies were present in the serum during the initial progressive phase while the gamma-globulin level was declining. Taking, however, into consideration that the serum of such mice also contains antigen–antibody complex [8], and this is demonstrable on the surface of the tumour cells as well, there is no doubt that the loss of gamma globulin was not real. This view is supported by the fact that, subsequently, the gamma-globulin level was steadily rising until the remission ensued. In the terminal phase of the lethal exacerbation (days 70—80), on the other hand, there was neither free nor bound tumour-specific antibody in the serum [8], and the surface of the tumour cells bound no fluorescein-labelled anti-globulin.

It may be concluded that the production of tumour-specific antibodies of all classes are blocked by the RLV multiplying in the lymphoid system. Nevertheless, IgM synthesis is more resistant than IgG synthesis. The globulins produced after the total blocking of immune-specific globulin synthesis are supposedly paraproteins. It is partly from these that the excess $beta_2$ globulin in the serum of leukaemic mice may derive. It is an analogous observation that the tumour cells of patients with chronic lymphoid leukaemia produced IgM-type globulin [9] and, at the same time, there was a severe immunodeficiency.

The excess $alpha_1$ and $alpha_2$ globulins may have originated from tumour cells and partly proteins of the embryonic type, partly soluble tumour-specific antigens may come into consideration. It is well-known that the spleen of BALB/c mice with Rauscher leukaemia contains embryonic type protein [10] and, further, that the embryonic type globulin produced by hepatoma cells is of $alpha_1$ electrophoretic mobility [11]. Soluble viral antigens were demonstrated in the serum of mice with Friend leukaemia by LILLY [12]. The excess $beta_2$ globulin in the RLV-infected C57BL/10 Sn mice with an intact immune system suggests that tumour celi proteins may appear in the $beta_2$ fraction as well.

REFERENCES

1. Тер-Григоров, В. С., Московкина, О. Я., Тот, Ф., Ирлин, И. С., Иевлева, Е. С., Шевелов, Б. И., Бергольц, В. М.: Вопр. онкол. **17**, 70 (1971).
2. D. TÓTH, F., VÁCZI, L., BERENCSI, GL.: Acta microbiol. Acad. Sci. hung. **18**, 23 (1971).
3. FINK, M. A., RAUSCHER, F. J.: J. nat. Cancer Inst. **32**, 1075 (1964).
4. AARONSON, T., GRÖNWALL, A.: Scand. J. clin. lab. Invest. **9**, 338 (1957).
5. SEIDEL, H. J.: Verh. dtsch. Ges. Path. 2—6 April, 1968, p. 398.
6. HANNA, M. G., SZAKAL, A. K., TYNDALL, R. L.: Cancer Res. **30**, 1748 (1970).
7. CREMER, N. E.: J. Immunol. **99**, 71 (1967).
8. D. TÓTH, F., VÁCZI, L.: Unpublished data.
9. JOHANSSON, B., KLEIN, E.: Clin. exp. Immunol. **6**, 421 (1970).
10. HANNA, M. G., TENNANT, R. W., COGGIN, J. H.: Proc. nat. Acad. Sci. (Wash.) **68**, 1748 (1971).
11. ABELEV, G. I., PEROVA, C. D., KHRAMKOVA, N. J., POSTNIKOVA, Z. A., IRLIN, I. S.: Transplantation **1**, 174 (1963).
12. LILLY, F., NATHENSON, S. G.: Transplant. Proc. **1**, 85 (1969).

Address of the authors:

FERENC D. TÓTH, LAJOS VÁCZI
Institute of Microbiology, University Medical Schoo
Debrecen 12, Hungary

TAMÁS KARSAI
Institute of Biochemistry, University Medical School
Debrecen 12, Hungary

Acta microbiol. Acad. Sci. hung. 18, 279—282, 1971

STUDIES ON YOUNG GERM-FREE MICE

I. STRESS REACTION OF GERM-FREE MICE*

By

HELEN SZERI, PIROSKA ANDERLIK, SUSAN BÁNOS and B. RADNAI

Institute of Microbiology (Director: Z. ALFÖLDY*), Semmelweis University Medical School and István Hospital (Director:* I. KATONA*), Budapest*

(Received January 26, 1972)

Summary. The response to cold stress of germ-free mice differed from that of control animals. Germ-free mice exhibited increased susceptibility to cold and neither lymphopenic reaction nor loss of lymphoid organ weight occurred in them. The disturbances were similar to those observed in previous studies on non-germ-free mice with impaired lymphoid system.

It has been shown that mice with an impairment of the thymus dependent lymphoid system are developing general adaptational disturbances [1—3]. The degree and reversibility of the functional and morphological changes associated with general adaptation were found directly correlated with the degree and duration of impairment.

The underdevelopment and immunological areactivity of the lymphoid system of germ-free animals are due to the deficient antigenic stimuli of their environment [4—11].

It seemed interesting to study the problem whether the adaptational disorders observed in mice with impaired lymphoid system might be found also in germ-free mice with underdeveloped lymphoid system.

Materials and methods

Animals. Twenty CD^{R}-1 germ-free and 20 mouse-pathogen-free CD^{R}-1 COBS (Cesarean Originated Barrier Sustained) male mice (Charles River Breeding Laboratories Inc., Wilmington, Mass., U.S.A.) 28 days of age were used. The germ-free condition was maintained throughout the experiments.

Cold stress. Ten germ-free and 10 COBS mice were exposed to 4 °C temperature for 4 hours. Ten germ-free and 10 COBS mice served as non-stressed controls.

Lymphopenic reaction. To study the lymphopenic reaction, the absolute lymphocyte count was determined under standardized conditions in blood samples obtained from the caudal vein immediately before and 6 hours after stress [12].

Determination of spleen and thymus index. The ratio of spleen and thymus weight to body weight (relative organ weight) was determined at the end of the experiment in each group. The ratio of relative organ weight in the stressed group to that in the non-stressed group represented the index for the lymphoid organs. The loss of lymphoid organ weight, which is characteristic of the alarm reaction following stress, was estimated on the basis of spleen and thymus index values [13, 14].

Histology. The lymphoid organs were fixed in formalin and sections were stained with haematoxylin-eosin.

* Presented at the 28th Conference of the Immunological Section, Hungarian Society of Microbiology, Szolnok, 1971.

Results

Significant morphological and functional differences were found between germ-free and COBS mice.

Mean values for non-stressed COBS mice and germ-free mice are compared in Fig. 1.

The data indicate that mean body weight of germ-free mice was only 50% of that of the controls. The circulating lymphocyte count was remarkably low in germ-free mice. Also, mean relative spleen and thymus weight was considerably lower in germ-free animals than in the controls, indicating an underdevelopment of the lymphoid organs of germ-free mice. This was obvious even at gross examination (Fig. 2).

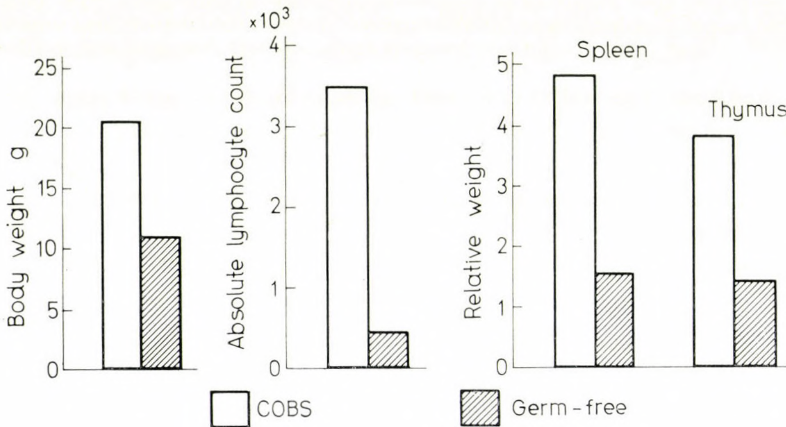


Fig. 1. Comparison of data for COBS and germ-free mice

Fig. 2. Gross picture of spleen and thymus from germ-free (a) and COBS (b) mice

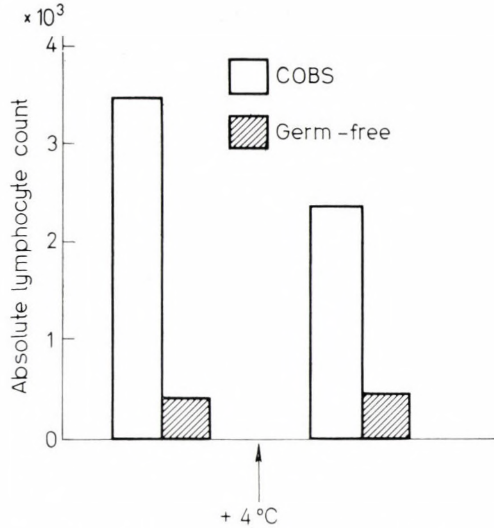


Fig. 3. Mean absolute lymphocyte count in COBS and germ-free mice before and after cold stress

Differences were found in the response to cold stress of COBS and germ-free mice. A lymphopenic reaction developed in COBS mice but not in germ-free mice (Fig. 3).

Spleen and thymus indexes of germ-free mice were 1, and those of COBS mice lower than 1, six hours after exposure to cold. The values indicate that no loss of lymphoid organ weight had occurred in germ-free mice. Four mice of the germ-free group died 4 hours after exposure to cold. No death occurred in the stressed COBS group, in the non-stressed germ-free and non-stressed COBS groups (Table I).

Table I

Effect of exposure to cold on COBS and germ-free mice 6 hours after stress

Group of mice	Spleen index	Thymus index	Death
COBS	0.85	0.84	0
Germ-free	1	1	40 %

Histology revealed a normal structure of the lymphoid organs of COBS mice; their spleen contained numerous germinative centres. Depletion of lymphoid elements could be observed in the lymphoid organs of germ-free mice. Germinative centres were practically absent in 13 spleens, a few of them were present in 7 spleens (4 of the non-stressed and 3 of the stressed group), indicating that the germ-free environment was not perfectly free from antigens. No germinative centre was found in the spleen of animals succumbing to cold

stress, while those possessing germinative centres survived it. An increased sensitivity to cold appears to be associated with the immunological areactivity of the lymphoid system.

Discussion

According to the present results, germ-free mice are more sensitive to cold than are the controls, and they differ also in the course of the alarm reaction. These findings refer to a disturbance of general adaptation.

The general adaptational disorders in germ-free animals were similar to those observed in non-germ-free animals with a lymphoid system impaired by neonatal thymectomy, antilymphocyte serum treatment or a graft *vs.* host reaction [1—3].

No active injury was applied in the germ-free mice; their breeding differed from that of the control animals only in the lack of antigenic stimuli of their environment. Underdevelopment of the thymus and peripheral lymphoid organs as well as the immunological areactivity were consequences of this lack.

The fact that general adaptational disturbances observed in non-germ-free mice with impaired lymphoid system occurred also in germ-free mice, indicates that a hypofunction or lacking function of the lymphoid system and not some other affected functions are responsible for the disorder. Underdevelopment and hypofunction of the thymus dependent lymphoid system seem to result in the above-described disorders.

It has been presumed on the basis of earlier experiments that not only immunological adaptation but also general adaptation depends on the sound and proper function of the thymus dependent lymphoid system. The presented findings support this theory.

REFERENCES

1. Szeri, H., Anderlik, P., Bános, S.: Acta microbiol. Acad. Sci. hung. **15**, 1 (1968).
2. Anderlik, P., Bános, S., Szeri, H.: Acta microbiol. Acad. Sci. hung. **15**, 327 (1968).
3. Anderlik, P., Bános, Zs., Szeri, I., Koltay, M., Virág, I.: Experientia (Basel) **26**, 94 (1970).
4. Gordon, H. A.: Ann. N. Y. Acad. Sci. **78**, 208 (1959).
5. Miyakawa, M.: Ann. N. Y. Acad. Sci. **78**, 221 (1959).
6. Thorbecke, G. J.: Ann. N. Y. Acad. Sci. **78**, 237 (1959).
7. Bauer, H., Horowitz, R. E., Levenson, S. M., Popper, H.: Amer. J. Path. **42**, 471 (1963).
8. Wilson, R., Bealmear, M., Sobonya, R.: Proc. Soc. exp. Biol. (N. Y.) **118**, 97 (1965).
9. Bealmear, M., Wilson, R.: Anat. Rec. **154**, 261 (1966).
10. Olson, G. B., Wostmann, B. S.: J. Immunol. **97**, 267 (1966).
11. Dukor, P., Miller, J. F. A. P., Sacquet, E.: Clin. exp. Immunol. **3**, 191 (1968).
12. Harlow, C. M., Selye, H.: Proc. Soc. exp. Biol. (N. Y.) **36**, 141 (1937).
13. Selye, H.: Nature (Lond.) **138**, 32 (1936).
14. Selye, H.: Endocrinology **21**, 169 (1937).

Address of the authors:

Ilona Szeri, Piroska Anderlik, Zsuzsanna Bános
Institute of Microbiology, Semmelweis University Medical School
Hőgyes Endre u. 7—9, Budapest IX, Hungary

Béla Radnai
"István" Hospital
Nagyvárad tér 1, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 283—289, 1971

STUDIES ON YOUNG GERM-FREE MICE

II. BONE CHANGES IN GERM-FREE MICE*

By

L. Berek and Susan Bános

Department of Orthopaedics (Director: A. Glauber) and Institute of Microbiology (Director: Z. Alföldy), Semmelweis University Medical School, Budapest

(Received January 26, 1972)

Summary. Comparative studies were performed on the skeletal system of germ-free and non-germ-free young mice. Retardation of skeletal system was observed in germ-free mice with underdeveloped lymphoid system. Hypofunction of the thymus dependent lymphoid system was considered responsible for the disturbance of osseal growth.

Several papers have been published on the characteristics of germ-free animals dealing mainly with the morphological and functional changes of the immune system. Underdevelopment of the lymphoid system including the thymus, as well as the low circulating lymphocyte count in germ-free animals are well known [1—8].

Results of comparative studies on the skeletal system of germ-free and non-germ-free mice of identical sex and age will be presented in this paper.

Materials and methods

Animals. Ten CD^R-1 germ-free and 10 CD^R-1 mouse pathogen-free COBS (Cesarean Originated Barrier Sustained) male mice (Charles River Breeding Laboratories Inc., Wilmington, Mass., U.S.A.) 28 days of age were used. The germ-free condition was maintained throughout the experiments.

Absolute lymphocyte count was estimated under standardized conditions in blood samples taken from the orbital sinus.

Calcium, phosphorus and alkaline phosphatase levels were determined in pooled blood samples obtained by orbital puncture from germ-free and COBS mice [9].

Radiomicrometric measurements. Radiographs were taken of germ-free and COBS mice in pairs at identical exposition times. The method of measurement has been published earlier [10].

Histology. Thymus, spleen and femur were subjected to histological study. As fixative, 10% neutral formalin in phosphate buffer was used. Bones were decalcified in EDTA solution. Haematoxylin–eosin and alcian blue PAS staining were used to study the mucopolysaccharide content of the intercellular substance in the distal femoral epiphyseal plates.

Results

Germ-free mice are comparatively small, their fur is moderately ruffled and their posture is hunched. Their average body weight and circulating lymphocyte count was lower than that of the control mice (Fig. 1).

* Presented at the 27th Conference of the Immunological Section, Hungarian Association of Microbiology, Debrecen, 1971.

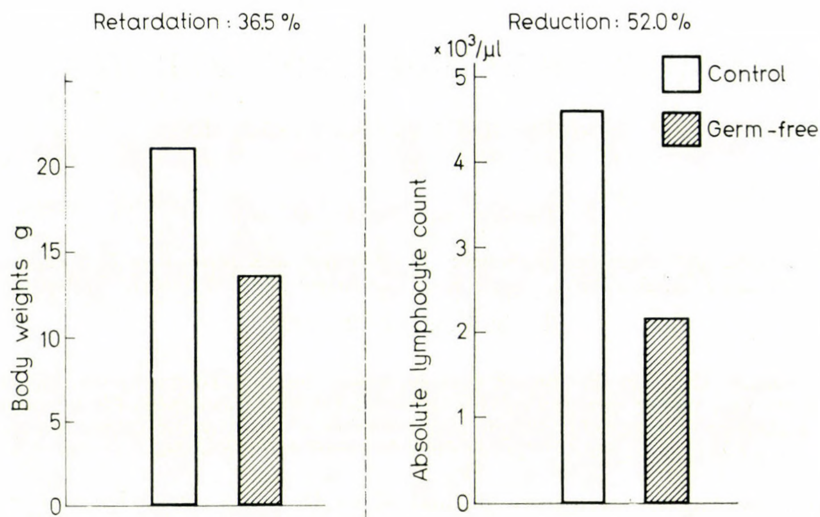


Fig. 1. Mean body weight and absolute lymphocyte count of germ-free and control mice

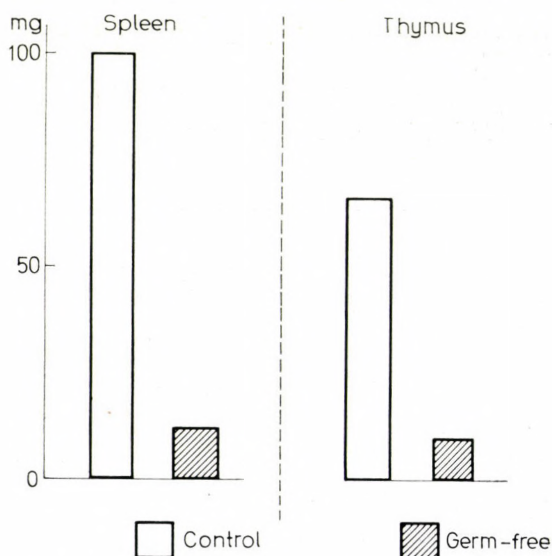


Fig. 2. Mean spleen and thymus weight of germ-free and control mice

Spleen and thymus weight was reduced and the lymphoid system was underdeveloped (Fig. 2).

Histology revealed a normal structure of the thymus and spleen or control mice. In the thymus, thinning of the cortex and medullary cytopenia could be observed in germ-free mice. A similar cytopenia was found in their spleen and the number of follicles decreased significantly.

X-rays revealed shorter tubular bones, thinner cortices and usually a lower calcium content in germ-free mice than in the controls (Fig. 3).

According to radiometric measurements, the retardation of longitudinal growth amounted to 14% and of diametric growth to 10%. There was a considerable (42.5%) retardation in the femoral cortical layer of germ-free mice (Fig. 4).

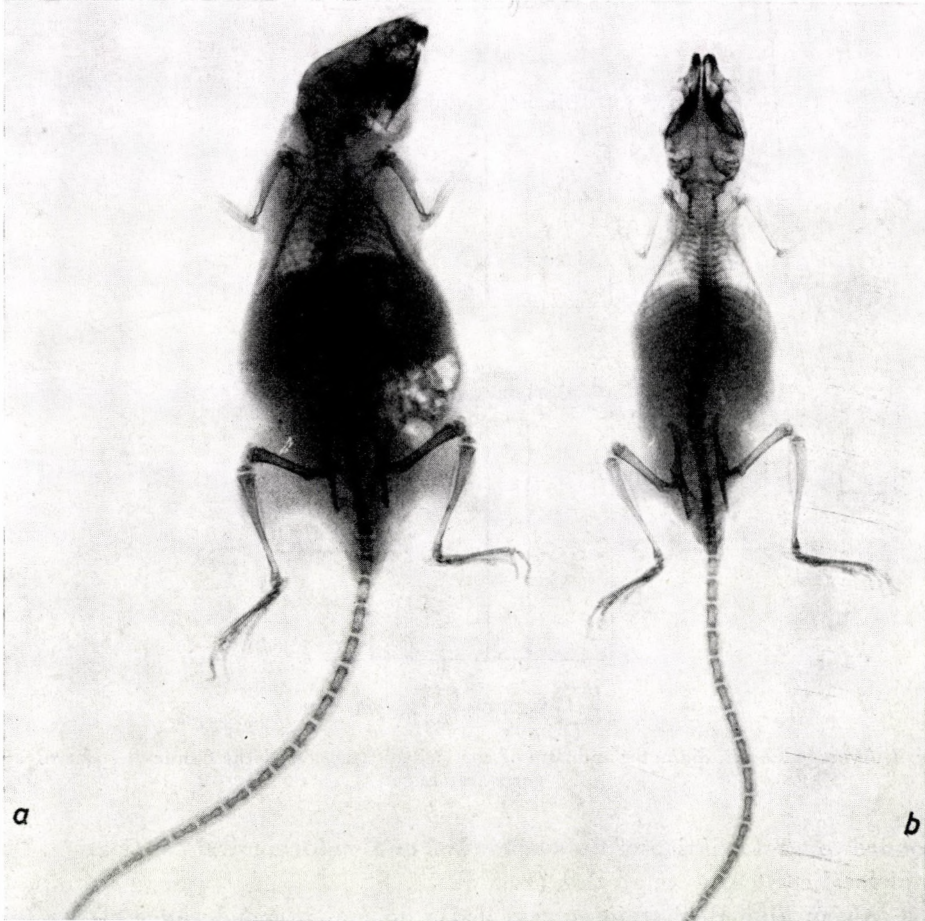


Fig. 3. Radiograms of control (a) and germ-free (b) mice

The distal end of the femur, being the site of most rapid growth, was chosen for histological examination. Control mice displayed normal enchondral ossification (Fig. 5a), while in the germ-free mice the epiphyseal trabecules were thin, slim and rare, with a widening of the intertrabecular spaces. The growth plates were thinner and the number of cells was low in each layer. Hyaline pillars were sparse, displaying little osteoblastic activity. This picture corre-

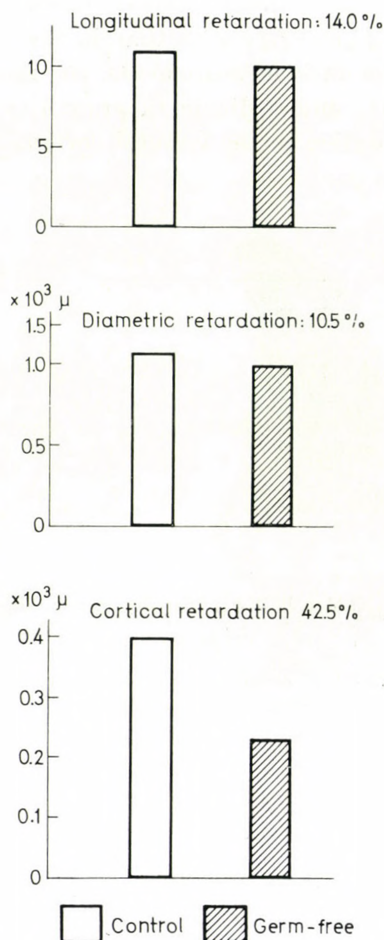


Fig. 4. Average length, diameter and sum of cortical thicknesses of the femur in control and germ-free mice

sponded to a retardation of the enchondral and endochondral ossification. The diaphyseal cortices were all thin (Fig. 5b).

Alcian blue PAS staining revealed a normal preponderance of the acid mucopolysaccharides in the distal femoral epiphyseal plates of the control mice. In germ-free mice this staining was not so uniform in the intercellular matrix of the corresponding bones; pink areas appeared in the dominating blue staining, indicating the presence of neutral mucopolysaccharides in the intercartilaginous matrix.

The histological findings were in agreement with the moderate changes of serum calcium and phosphorus levels as well as with the decrease of alkaline serum phosphatase activity (Fig. 6).

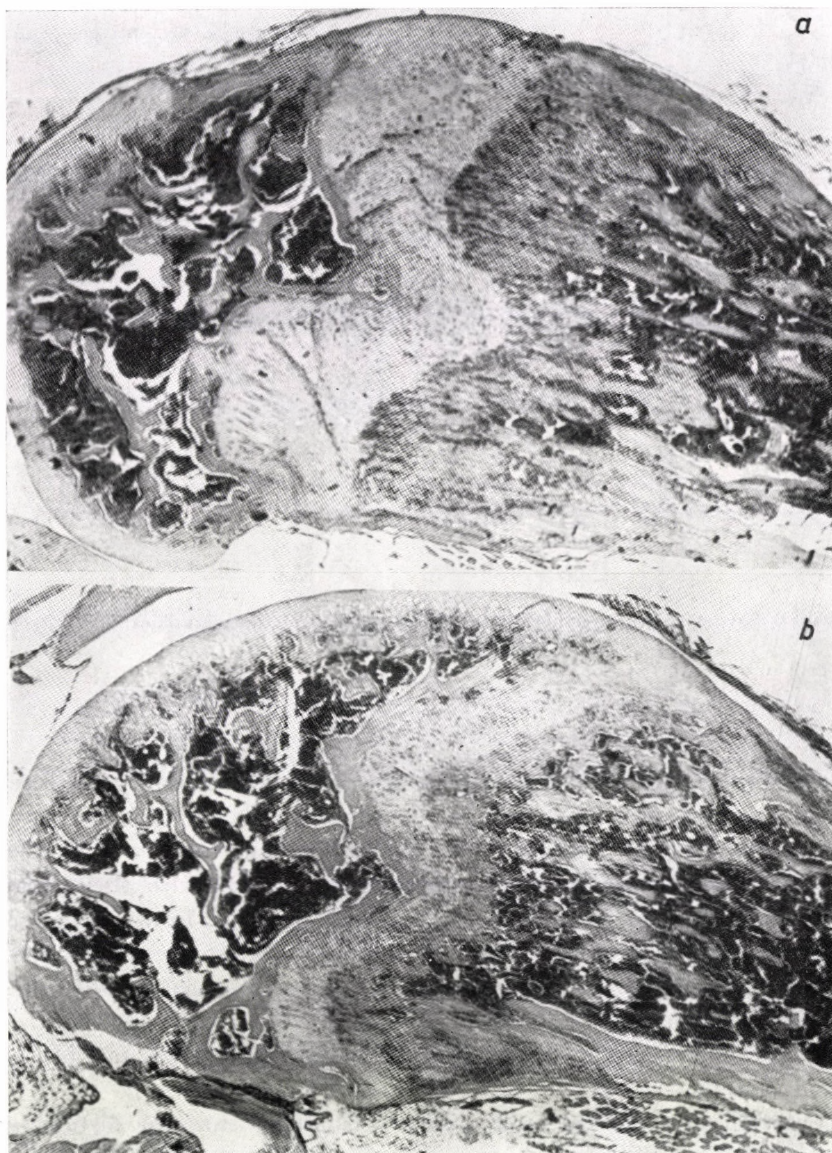


Fig. 5. Microphotograph of distal femoral epiphysis of control (a) and germ-free (b) mice

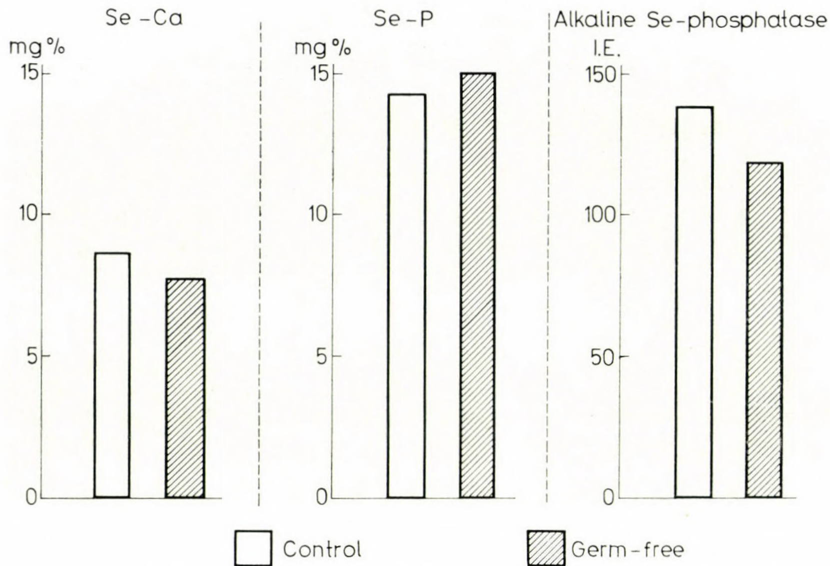


Fig. 6. Serum calcium, phosphorus and alkaline phosphatase values of control and germ-free mice

Discussion

The results indicated that skeletal growth is retarded in germ-free mice As reported earlier, underdevelopment, marked retardation of enchondral ossification and a preponderance of neutral mucopolysaccharides in the growth plate could be observed in young non-germ-free mice with their lymphoid system impaired by neonatal thymectomy or a graft *vs.* host reaction. Functional changes of the immune system, regression or atrophy of lymphoid organs and a decrease of the absolute lymphocyte count were associated with the bone changes in these animals [11—13].

Similar bone changes occurred in germ-free mice with underdeveloped lymphoid system.

It is suggested on the basis of these findings that the disturbance of skeletal growth is primarily due to a hypofunction or lacking function of the thymus dependent lymphoid system.

REFERENCES

1. Glimstedt, G.: Acta path. microbiol. scand. Suppl. **30**, 1 (1936).
2. Gordon, H. A.: Ann. N. Y. Acad. Sci. **78**, 208 (1959).
3. Miyakawa, M.: Ann. N. Y. Acad. Sci. **78**, 221 (1959).
4. Gordon, H. A., Wostman, B. S.: Anat. Rec. **137**, 65 (1960).
5. Wilson, R., Bealmear, M., Sobonya, R.: Proc. Soc. exp. Biol. (N. Y.) **118**, 97 (1965).
6. Bealmear, M., Wilson, R.: Anat. Rec. **154**, 261 (1966).

7. IJAMA, S., YAMANE, T.: The spleen of germ-free animals. In M. MIYAKAWA, T. D. LUCKEY (eds): Advances in Germ Free Research and Gnotobiology. CRC Press, Cleveland 1968, p. 139.
8. MILLER, J. A. F. P., DUKOR, P., GRANT, G., SINCLAIR, N. R. ST. C., SAQUET, E.: Clin. exp. Immunol. **2**, 531 (1967).
9. LONG, C.: Biochemists Handbook. Spon, London 1961, pp. 873—876.
10. BEREK L.: Experientia (Basel) **25**, 633 (1969).
11. BREEK, L., BÁNOS, ZS., SZERI, I., ANDERLIK, P., ASZÓDI, K.: Experientia (Basel) **24**, 721 (1968).
12. BEREK, L., ASZÓDI, K., BÁNOS, ZS., ANDERLIK, P., SZERI, I.: Experientia (Basel) **25**, 982 (1969).
13, BEREK, L., BÁNOS, ZS., ANDERLIK, P., SZERI, I., ASZÓDI, K.: Experientia (Basel) **26**, 92 (1970).

Address of the authors:

LÁSZLÓ BEREK
Department of Orthopaedics, Semmelweis University Medical School
Karolina út 27, Budapest XI, Hungary

ZSUZSANNA BÁNOS
Institute of Microbiology, Semmelweis University Medical School
Hőgyes Endre utca 7—9, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 291—295, 1971

EFFECT OF THE DILUENT ON THE POCK COUNT OF VACCINIA VIRUS DILUTIONS

By

J. SZATHMÁRY and L. HEGEDÜS

Institute for Serobacteriological Production and Research "Human"
(Director: I. VETŐ), *Budapest*

(Received February 25, 1972)

Summary. Raising from 10% to 20% of the skimmed milk content of the McIlvaine buffer used as diluent of vaccinia virus did not influence the pock count obtainable on the chorioallantoic membrane of the chick embryo. Tween 80 (0.1—0.5%) in the plain buffer resulted in pock counts comparable to those obtained in the presence of 10% skimmed milk. Using the plain buffer as diluent, the pock count was 1.4—3.4-fold lower. This difference decreased or disappeared if the virus had been subjected to sonic or trypsin treatment. Supposedly, the dispersity of the vaccinia virions is increased by the milk or the detergent, and this effect is less pronounced if, due to enzymatic or sonic treatment, the dispersity of the virus is nearly optimal.

In a previous report [1] we have shown that an enrichment of the diluent (McIlvaine buffer) of vaccinia virus by addition of 10% skimmed milk resulted in a 2—5.8-fold (on the average, 3.7-fold) increase in the pock count of the virus, as tested on the chorioallantoic membrane (CAM) of the chick embryo. Considering the practical importance of this finding in the infectivity titration of smallpox vaccine, we have continued our investigations. In the present work, the concentration of skimmed milk in the diluent was increased and, in other experiments, Tween 80 [2] was added to the diluent in various concentrations.

Materials and methods

Vaccinia virus suspensions. The "Budapest" vaccine strain was propagated in calf skin. The pulp was treated with phenol and trifluorotrichloroethane (Frigen 113, Farbwerke Hoechst) and centrifuged. The partially purified virus (dermovaccine) thus obtained was resuspended in 5% peptone solution, distributed at 0.2 ml volumes in ampoules and freeze-dried in a Usifroid apparatus [3]. The dry virus was resuspended in McIlvaine buffer before use.

Diluents. To 0.004 *M* McIlvaine buffer, sterilized in an autoclave, 50 μg/ml streptomycin and 500 U/ml penicillin were added. McIlvaine buffer enriched with skimmed milk was prepared as recommended by BOULTER [4]. As Tween 80, a Reanal (Budapest) preparation was used.

Ultrasonic treatment. For each experiment, lyophilized vaccine from three ampoules was resuspended in 3 ml McIlvaine buffer (1 : 5 dilution). This suspension was diluted 1 : 100 in McIlvaine buffer before subjecting to sonic treatment in an MSE 100 W-26 Kc/sec Ultrasonic Disintegrator. Five ml dilution cooled in melting ice was treated for 8 × 15 seconds, with intervals of about 15 seconds. Further dilutions from the sonicated and control solutions were prepared immediately after sonic treatment.

Trypsin treatment. A 0.25% solution was prepared from Trypsin 1 : 250 (Difco Laboratories, Chicago, Ill.) in McIlvaine buffer. Lyophilized vaccine from several ampoules was resuspended in 1 ml McIlvaine buffer/ampoule. From the mixed suspension, 1 ml was transferred into each of two test tubes. Subsequently, 1 ml trypsin solution was added to one of the samples and 1 ml buffer to the other, which served as control. Thus, the vaccine was diluted

1 : 10. The tubes were incubated in a water bath of 37 °C for the period indicated in Table III and cooled in cold water thereafter.

Pock count was determined as described by WESTWOOD *et al.* [5]. The CAMs of 5—8 11—12-day-old chick embryos were inoculated with each mixture. The volume of the inoculum was 0.1 ml. The pocks were counted after an incubation for 48 hours at 37 °C.

Results

Three to 20 parallel tests were carried out with each diluent, and a control test with 10% skimmed milk in McIlvaine buffer was included in each experiment. The pock counts were expressed in per cent of the control value (Table I).

Table I

Relative pock counts formed by vaccinia virus on CAM using diluents of variable composition

Diluent		Relative pock count*		
Skimmed milk	Tween 80			
per cent, McIlvaine buffer		Mean	Minimum	Maximum
10	—	100	77	135
20	—	109	62	140
—	0.01	81	69	94
—	0.1	101	92	120
—	0.2	106	73	130
—	0.5	88	69	110
—	1.0	67	60	71
—	2.0	46	40	51
10	0.01	108	67	142
10	0.1	100	71	115
10	0.2	97	86	114
20	0.1	108	88	134
20	0.2	92	78	124

* The average pock count obtained with McIlvaine buffer enriched with 10% skimmed milk equals 100.

An increase of the skimmed milk component from 10% to 20% did not influence the pock count. The effect of Tween 80 was concentration dependent. It should be noted that the pock count obtained with the plain buffer was, on the average, 27% of the pock count obtained with the control diluent. This implies that the pock count was raised even by such a low Tween 80 concentration as 0.01%. To reach the control value, 0.1—0.5% Tween 80 had to be added to the buffer. In the case of still higher concentrations, the pock count was suppressed by the toxic effect of the detergent.

Combination of 10% or 20% skimmed milk with the effective concentrations of Tween 80 failed to increase further the pock count.

The stability of each virus dilution at 37 °C was examined in 2 or 3 parallel tests. The stability of the virus proved to be lower in the diluents in the presence of which the pock count was relatively low (diluents containing 0.01, 0.5, 1.0 or 2.0% Tween 80 without skimmed milk) than in the control diluent. In the other diluents, the virus was as stable as in the control.

In a previous paper [1] we attributed the pock-count-raising effect of skimmed milk to its protein components which prevent virions from being aggregated. To check this assumption, we disaggregated the clumps by ultrasonic or trypsin treatment before determining the pock count in the presence and in the absence of skimmed milk. We presumed that in this case the difference in pock count would disappear. Results are shown in Table II.

Table II

Effect of milk in the diluent on the pock count of sonicated and untreated vaccinia virus

Experiment No.	Diluent (McIlvaine buffer)	Dilution*	Pock count of		R value for	
			untreated	treated	untreated	treated
1	without milk	$10^{-5.5}$	34	51	1.5	1.1
	with 10% milk		51	60		
2	without milk	$10^{-6.0}$	.	25		1.1
	with 10% milk		22	28		
3	without milk	$10^{-5.5}$	7	13	2.1	1.7
	with 10% milk		15	23		

. = not done.
R = quotient of pock count obtained in the presence and in the absence of milk.
* Volume of inoculum, 0.1 ml.

It is clearly seen that in the case of untreated virus the pock count was increased by the presence of milk. The sonicated virus showed no such difference in experiments 1 and 2. In experiment 3, the R value (see Table II) was higher than expected.

Trypsin treatment led to similar results (Table III).

The R quotients show that, in agreement with literary data [6—8], 15 minutes and 30 minutes pretreatment increased, whereas 60 minutes pretreatment reduced, the pock count as determined in plain buffer. Presumably, incubation at 37 °C for 60 minutes, in itself, contributed to the pock reduction.

The R_1 quotients show the effect of trypsin treatment on the number of pock-forming units as determined in the presence of 10% skimmed milk. In this respect, 15 minutes pretreatment caused hardly any change in, whereas

Table III

Effect of milk in the diluent on the pock count of trypsin-treated and untreated vaccinia virus

Experiment No.	Trypsin treatment, minutes	Diluent McIlvaine buffer		R	R_1	R_2
		without milk	with 10% milk			
1	0	20	63	1.7	1.1	3.1
	15	34	72			2.1
2	0	22	76	1.4	0.4	3.4
	30	31	32			1.0
3	0	24	43	0.6	0.3	1.8
	60	15	13			0.8

Inoculum: 0.1 ml of a 10^{-5} diluted vaccinia virus suspension.
R = quotient of pock count for trypsinized virus and that for the untreated vaccinia virus in the absence of milk.
R_1 = quotient of pock count for trypsinized virus and that for untreated virus in the presence of milk.
R_2 = quotients of pock count obtained in the presence and in the absence of milk.

30 or 60 minutes pretreatment significantly reduced the number of pock-forming units.

The R_2 values are expressing the quotients of the pock counts obtained with McIlvaine buffer containing 10% milk and those obtained with plain buffer. For the untreated virus, the R_2 quotient ranges between 1.8 and 3.4. Trypsin treatment, depending on its duration, tends to reduce the R_2 value.

Discussion

In the pock count obtainable with vaccinia virus, the dispersity of the virus plays an important role. For this reason aggregation of the virions should be minimized before pock titration. Some disaggregation can be achieved by simple mechanical means [9] or by adding 10% skimmed milk to the diluent [1]. The present studies have shown that an elevation of the diluent's milk content to 20% failed to increase the pock count and the milk could be replaced by 0.1—0.5% Tween 80 without any loss of pocks.

In our opinion, the present results support our assumption that the milk in the diluent acts by increasing the dispersity of the virus. If this had been increased by sonic or enzyme treatment before the virus was titrated, the addition of milk to the McIlvaine buffer caused little, if any, increase, in pock count.

Since the heat stability of McIlvaine buffer containing 10% skimmed milk is also favourable [1], we use this fluid as diluent for routine vaccinia virus pock titration.

Acknowledgements. We are indebted to Dr. Z. Csizér for valuable assistance in sonic treatment and to Mrs. Aranka A. Bak for excellent technical assistance.

REFERENCES

1. Szathmáry, J., Hegedüs, L., Koller, M.: Acta microbiol. Acad. Sci. hung. **18**, 61 (1971).
2. Medzon, E. L., Bauer, H.: Virology **40**, 860 (1970).
3. Szathmáry, J., Szentirmay, E., Sikos, K., Hegedüs, L., Koller, M.: Ann. Immunol. hung. **13**, 57 (1969).
4. Boulter, E. A.: J. Hyg. (Lond.) **55**, 502 (1957).
5. Westwood, J. C. N., Phipps, P. H., Boulter, E. A.: J. Hyg. (Lond.) **55**, 123 (1957).
6. Gifford, G. E., Klapper, D. G.: Arch. ges. Virusforsch. **26**, 321 (1969).
7. Nomura, M., Nishimura, Ch., Kitaoka, M.: Jap. J. Med. Sci. Biol. **18**, 249 (1965).
8. Kim, K. S., Dalton, D., Sharp, D. G.: Virology **38**, 503 (1969).
9. Galasso, G. J., Sharp, D. G.: J. Immunol. **88**, 339 (1962).

Address of the authors:

József Szathmáry, László Hegedüs
Institute for Serobacteriological Production and Research "Human"
Szállás u. 5–7, Budapest X, Hungary

Acta microbiol. Acad. Sci. hung. 18, 297—306, 1971

LIPIDS IN STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI CULTURED IN THE PRESENCE OF HUMAN SERUM

By

I. Rédai, A. Réthy, P. Sebessi-Gönczy and L. Váczi

Institute of Microbiology (Director: L. Váczi), *University Medical School, Debrecen*

(Received March 1, 1972)

Summary. Extractable lipids comprised 20% of the dry weight of *Staphylococcus aureus* cultured in the presence of 10% human serum. As compared to cultivation in serum-free medium, there was a 40-fold increase in free fatty acid content and a 7—10-fold increase in mono, di and triglyceride content. Phospholipids showed a twofold increase and amounted to 17.5% of the total lipid fraction. In serum medium, unsaturated fatty acids accumulated in all lipid fractions, but most of them were present in the free fatty acid fraction.

The extractable lipid content of *Escherichia coli* grown in serum medium was 5.9—6.1% of the dry material. The increase in lipid content was due to the accumulation of neutral lipids. In the presence of serum the phosphatidylethanolamine content of *E. coli* decreased and lysophosphatidylethanolamine and other unidentified phospholipids appeared.

Phospholipids are basic constituents of the membrane structure. The distribution of phospholipid constituents is in a dynamic metabolic association with the structure of membranes and is responsible for various processes such as the active transport of cations [1, 2] or mitochondrial functions [3—5].

Cultural conditions including pH, temperature, ingredients of the medium or oxygenation, are of great influence on phospholipid synthesis in Gram-positive and in Gram-negative bacteria [6—8]. An alteration of the membrane phospholipid constituents causes a change in membrane stability [9, 10].

Little is known about the alterations in phospholipid synthesis and membrane structure in bacteria multiplying *in vivo*. Our previous studies have shown that the unsaturated fatty acid content increases if *Staphylococcus aureus* is cultured in the presence of lipoprotein. When the organism is grown in the peritoneal cavity of the guinea pig, its unsaturated fatty acid content increases to 36—42% [11].

In the present paper an account is given of the phospholipids and neutral lipids of *Staph. aureus* and *Escherichia coli* cultured in the presence of human serum.

Materials and methods

Strains. Staph. aureus strains 100 and 80/81 were isolated in our institute. *E. coli* strain 30006 was obtained from the Hungarian National Collection of Medical Bacteria, Budapest.

Cultural methods were as described in [8]. The serum used was a pool obtained from healthy adults.

Dry weight determination. Bacteria grown for 18 hours were centrifuged, washed twice in saline and freeze-dried. The amount of extracted lipids was expressed as per cent of the weight of freeze-dried bacteria.

Lipid extraction. The method of BLIGH and DYER as modified by FRERMAN and WHITE [12] was used. Extraction was performed twice, the fractions were united and dried by evaporation in inert gas. The raw total lipid fraction was purified in Sephadex-G25 fine column as described by WELLS and DITMER [13].

Fractionation of lipids. Purified total lipids were separated on silicic acid column as described by VORBECK and MARINETTI [14], and by thin-layer chromatography.

Thin-layer chromatography was carried out in petroleum ether : ether : acetic acid (82 : 18 : 1 v/v) solvent. *Staph. aureus* phospholipids were fractionated with two-dimensional thin-layer chromatography. For the first dimension chloroform : methanol : acetic acid : water (52 : 20 : 7 : 3 v/v), for the second dimension chloroform : methanol : water (65 : 25 : 4 v/v) solvent was used.

Two-dimensional thin-layer chromatography of *E. coli* phospholipids was performed in chloroform: methanol : concentrated ammonium hydroxide (70 : 30 : 2 v/v), then in chloroform : methanol : acetic acid : water (85 : 20 : 12 : 3 v/v) solvent.

After separation the phospholipids were eluted from the plates and were quantitatively determined by considering their phosphorus content according to the method of BARTLETT [15].

Extraction and determination of fatty acids were performed as described previously [16]. In examining ^{32}P uptake, the culture medium was supplemented with 3000 μC $KH_2{}^{32}PO_4$ per litre. After 18 hours cultivation the phospholipids were extracted, separated on Sephadex G-25 fine and on silicic acid columns, then chromatographed on paper impregnated with silicic acid as described by MARINETTI *et al.* [17]. Radioactivity of the phospholipid components was measured with an end-window Geiger–Müller counter or by autoradiography.

Results

Staph. aureus cultured in the absence of serum yielded 2.8—3% extractable lipids. If the same organism was grown in a medium supplemented with 10% serum, the total lipid yields amounted to 20% of the dry weight of bacteria.

Table I

Distribution of extractable lipids in Staph. aureus

	Serum-free medium*		Serum-medium**	
	Per cent of total lipid	Per cent of dry bacteria	Per cent of total lipid	Per cent of dry bacteria
Phospholipids	73.3 ± 1.5	1.71 ± 0.04	17.5 ± 2.3	3.77 ± 0.43
Free fatty acids	4.5 ± 0.6	0.12 ± 0.01	29.8 ± 0.8	5.83 ± 0.2
Mono and diglycerides	7.7 ± 1.6	0.23 ± 0.04	11.1 ± 0.6	2.38 ± 0.1
Triglycerides	4.6 ± 1.1	0.13 ± 0.03	7.6 ± 1.0	1.6 ± 0.2
Pigment and other substances soluble in organic solvents	12	.	36	.

* Average of 4 parallel cultivations.
** Average of 3 parallel cultivations.

Table I shows the percentage distribution of lipids in the total lipid fraction of *Staph. aureus*. The data indicate that in the presence of 10% human serum the lipid metabolism of *Staph. aureus* shows a considerable change. The main feature of the alteration is a decrease in the relative amount of phospholipids and an increase in the relative and absolute amount of neutral

lipids. In serum-free medium 73% of extractable lipids corresponded to phospholipids, in serum medium this fraction figured in 17.5%. Although the relative amount of phospholipids decreased in the presence of serum, their absolute amount as reckoned per total dry material increased more than 2-fold. The increase in non-esterified free fatty acids was very high (approximately 40-fold). The absolute increase in mono, di and triglycerides was 7—10-fold.

From Fig. 1 it is evident that in serum medium as compared to serum-free medium the ratio of phospholipids shows a considerable change and new phospholipid components appear. In the presence of serum the amount of lysyl-phosphatidylglycerol, cardiolipin and phosphatidic acid increases and the amount of phophatidylglycerol decreases.

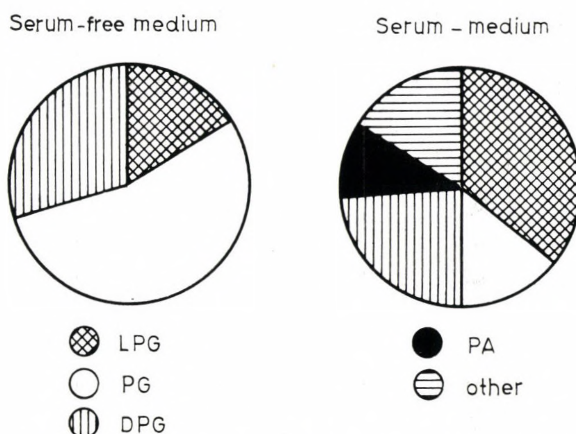


Fig. 1. Distribution of phospholipids in *Staph. aureus*, on the basis of phosphorus determination after two-dimensional thin-layer chromatography. LPG = lysyl-phosphatidylglycerol, PG = phosphatidylglycerol, DPG = cardiolipin, PA = phosphatidic acid, other = unidentified components

Table II shows the percentage distribution of main fatty acids in neutral lipids of *Staph. aureus*. In staphylococci grown with 10% human serum the free fatty acid fraction consists in more than 50% of oleic acid and the fraction contains only straight-chain saturated and unsaturated fatty acids. Branched-chain fatty acids characteristic of staphylococci were detected only in traces. In the mono- and diglyceride fractions, high amounts of short-chain (less than C_{12}) fatty acids were demonstrated; the main components were palmitic acid and stearic acid. The ratio of unsaturated fatty acids was very low, amounting to 4% of the total fatty acids; in this fraction branched-chain fatty acids were present in higher amounts than unsaturated fatty acids. The results indicate that in fatty acid spectrum the mono- and diglycerides are similar to phospholipids.

Table II

*Fatty acid content of neutral lipids in Staph. aureus**

Fatty acids	Free fatty acids		MG + DG		TG	
	Control	10% serum	Control	10% serum	Control	10% serum
$<C_{12}$	+	+	18.3	34.0	—	—
C_{12}	3.2	1.4	1.4	3.7	20.1	5.2
iC_{13}	2.8	+	1.2	2.7	+	+
C_{13}	+	+	+	+	+	+
$C_{14:1}$	2.7	+	1.2	3.1	21.7	2.5
iC_{14}	+	+	—	—	—	—
C_{14}	2.7	+	1.0	3.3	15.5	3.2
aiC_{15}	10.3	+	34.8	6.1	13.5	1.8
C_{15}	+	+	—	—	—	—
C_{16}	13.2	14.3	4.7	32.3	22.0	40.3
C_{17}	—	—	—	—	—	—
iC_{17}	2.5	+	2.8	+	—	—
aiC_{17}	2.2	+	4.2	+	—	—
$C_{18:2}$	2.3	10.9	+	+	—	+
$C_{18:1}$	16.9	63.4	0.4	0.9	—	28.2
C_{18}	2.4	3.4	11.8	13.9	7.2	14.3
iC_{19}	6.2	—	1.1	—	—	—
aiC_{19}	2.6	—	2.6	—	—	—
iC_{20}	3.1	—	—	—	—	—
C_{20}	12.3	—	13.5	—	—	—

* The figures indicate percentages for total fatty acid content. Cultures: control = Casitone (Difco) medium; 10% serum = Casitone (Difco) medium supplemented with 10% human serum.

In the triglyceride fraction the main component was palmitic acid; oleic acid comprised 28%, total unsaturated fatty acids 35%, of the total fatty acid content. In this fraction branched-chain fatty acids occurred in low amounts. The fatty acid distribution in the neutral lipid fraction indicated that the fatty acid content differed considerably in bacteria grown in the presence and in the absence of serum. The high increase in the free fatty acid fraction (in which unsaturated fatty acids were present in 80%) may be assumed to originate from lipoproteins in the medium released probably as an effect of extracellular staphylococcal lipase.

Table III shows the distribution of the main fatty acids of phospholipids. Staphylococci cultured in the presence and in the absence of serum differed in the quantitative distribution of phospholipid fatty acids. New components, however, were not demonstrated. In serum medium the amount of branched-

Table III

*Fatty acid content of phospholipids in Staph. aureus**

Fatty acids	Serum-free medium			Medium with 10% human serum		
	DPG	PG	LPG	DPG	PG	LPG
C_{12}	2.0	0.5	9.3	0.4	0.1	0.5
C_{13}	+	+	+	0.6	1.6	+
iC_{14}	0.7	0.5	4.0	1.4	2.5	0.8
C_{14}	1.3	0.6	+	3.4	3.6	2.9
aiC_{15}	37.8	15.2	42.3	25.0	19.1	13.1
C_{15}	1.5	1.0	5.7	+	+	+
$C_{16:1}$	3.3	0.4	+	2.6	1.5	1.5
C_{16}	7.7	1.2	9.6	15.4	9.0	31.9
aiC_{17}	3.3	+	+	+	+	+
iC_{17}	9.7	6.8	+	+	+	+
C_{17}	+	31.7	+	2.6	2.6	2.6
$C_{18:1}$	+	+	+	13.1	5.3	21.0
C_{18}	17.3	10.4	23.8	14.2	16.5	19.9
aiC_{19}	+	2.4	+	0.4	1.1	+
iC_{19}	+	+	+	0.2	0.8	+
$C_{20.1}$	+	+	+	6.2	7.6	0.2
C_{20}	14.5	8.7	+	13.7	28.0	4.9

* The figures indicate percentages for total fatty acid content.

chain fatty acids decreased and the amount of straight-chain fatty acids increased considerably. At the same time, the ratio of unsaturated fatty acids increased, especially for LPG and cardiolipin. In total fatty acid content LPG and PG may be identical, but in the distribution of individual fatty acids they are different. For example, the ratio of some fatty acids in PG and in LPG was as follows: palmitic acid 9% and 31%, oleic acid 5.3% and 21%, arachic acid 28 and 4.9%, respectively. These data indicate that for the synthesis of PG and LPG the organism utilizes different diglycerides or, perhaps, it may continue to acylate fatty acids in the already synthesized phospholipid molecule.

The difference in fatty acid components of LPG and PG was observed in staphylococci cultured without serum.

The alteration in phospholipid synthesis is demonstrated by the results of ^{32}P uptake experiments. Fig. 2 shows an autoradiogram of staphylococcal phospholipids; autoradiography confirmed the results obtained with analytical methods. Alterations in the radioactivity of phospholipids indicate that in the presence of human serum there is a change in the synthesis of phospholipids: lysyl-phosphatidylglycerol and cardiolipin synthesis increases and phosphatidylglycerol synthesis decreases.

The lipid metabolism in *E. coli* also changed in the presence of serum. The amount of extractable lipids increased to twofold. The ratio of fractions in total lipid considerably differed in bacteria cultured with and without serum. It was characteristic that in the presence of serum more than 50% of extractable lipids corresponded to neutral lipids.

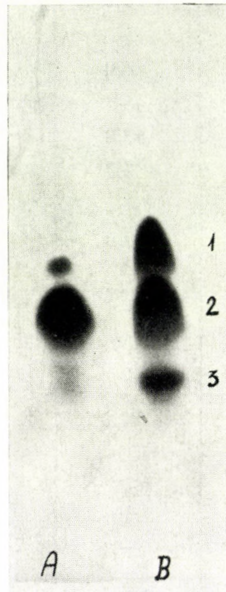


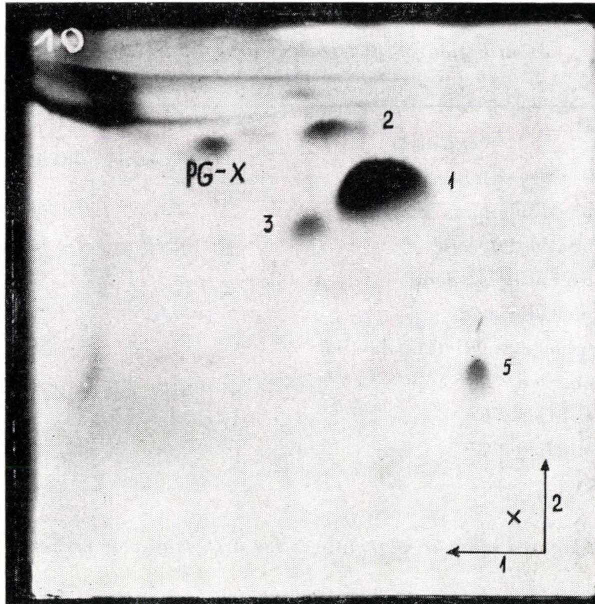
Fig. 2. Autoradiogram of ^{32}P-labelled phospholipids in *Staph. aureus.* A = Casitone (Difco) medium, B = Casitone (Difco) medium + 10% human serum. Phospholipids were chromatographed on paper impregnated with silicic acid in diisobutylketone : acetic acid : water (40 : 25 : 5 v/v) [17]. Identified components: 1 = cardiolipin, 2 = phosphatidylglycerol, 3 = lysyl-phosphatidylglycerol

There was also a change in the phospholipid content and in the ratio of phospholipid components (Fig. 3). Two-dimensional chromatography indicated that *E. coli* synthesizes new phospholipids when grown with serum.

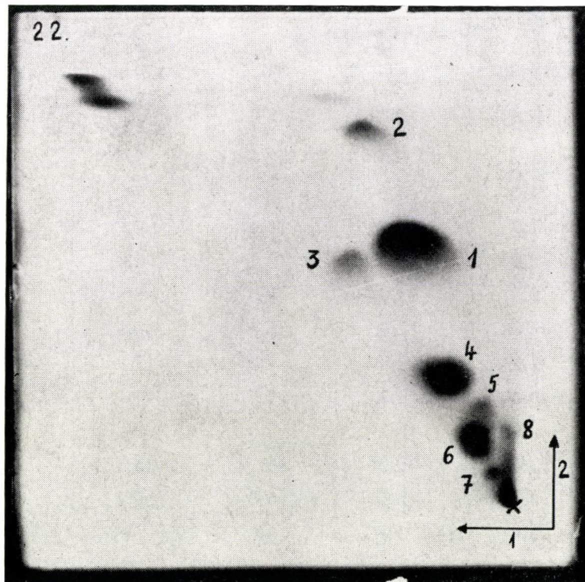
The distribution of *E. coli* phospholipids is shown in Table IV. The main component is phosphatidylethanolamine in both serum-containing and serum-free media but in the former it occurred in lower amounts. The phosphatidylglycerol and cardiolipin content was independent of the mode of cultivation.

In *E. coli* grown with serum, in addition to the above main components, 4 new components appeared. Component designated with "4" in Fig. 3b corresponded to lyso-phosphatidylethanolamine, the remaining 3 components were not identified.

Culturing in serum also influenced neutral and phospholipid fatty acid components in *E. coli*. Fatty acid distribution in neutral lipids is shown in Table V.



a)



b)

Fig. 3. Two-dimensional thin-layer chromatogram of phospholipids in *E. coli* 30006. Dimension 1: chloroform : methanol : concentrated ammonium hydroxide (70 : 30 : 2 v/v). Dimension 2: chloroform : methanol : acetic acid : water (85 : 20 : 12 : 3 v/v). a = cultured in serum-free medium, b = cultured in medium supplemented with 10% serum. 1 = PE, 2 = DPG, 3 = PG, 4 = lyso-PE, 5—8 = unidentified components

Table IV

*Distribution of phospholipids in E. coli cultured in the presence and absence of serum**

Phospholipid	Casitone (Difco) medium	
	without serum	with 10% serum
Phosphatidylethanolamine	1.6	1.06
Phosphatidylglycerol	0.15	0.16
Diphosphatidylglycerol	0.16	0.19
Phosphatidic acid	0.01	0.02
Lyso-phosphatidylethanolamine "4"	—	0.4
Unidentified "5"	—	0.15
Unidentified "6"	—	0.09
Unidentified "7"	—	0.04
PG-X	0.02	—

* The figures indicate percentages for dry weight of bacteria.

Table V

*Fatty acid content of neutral lipids in E. coli**

Fatty acids	Serum-free medium			Medium with 10% human serum		
	DG + MG	FFA	TG	DG + MG	FFA	TG
$C_{12:1}$	0.9	+	7.3	+	6.5	+
C_{12}	1.2	5.3	11.5	2.4	7.6	0.9
$C_{14:1}$	1.3	3.5	+	—	+	+
C_{14}	7.9	3.2	16.6	4.2	5.7	2.3
$C_{15:1}$	7.0	1.0	3.4	0.8	+	0.3
$C_{16:1}$	9.5	12.9	6.5	3.5	5.4	3.4
C_{16}	33.8	12.6	6.5	45.9	15.1	35.4
beta-OH—C_{14}	1.7	2.8	5.2	1.8	10.5	—
ΔC_{17}	8.4	8.3	7.7	—	4.5	1.4
$C_{18:2}$	3.8	10.1	5.9	—	15.9	—
$C_{18:1}$	13.6	22.2	10.1	23.2	12.4	41.2
C_{18}	3.7	2.5	6.0	18.1	7.3	8.5
ΔC_{19}	6.9	3.1	—	—	+	6.4

* The figures indicate percentages for total fatty acid content.

The same fatty acids were present in serum-containing and in serum-free medium cultures, but they differed in quantitative distribution. In Table VI the fatty acid components of the main phospholipids are presented.

Table VI

*Fatty acid content of phospholipids in E. coli**

Fatty acids	Serum-free medium			Medium with 10% human serum			
	DPG	PG	PE	DPG	PG	PE	Component "4"
C_{12}	11.6	4.9	3.0	3.2	3.5	4.4	4.9
$C_{14:1}$	10.8	6.8	5.3	5.4	7.1	6.3	5.1
C_{14}	7.2	7.0	3.0	4.5	4.7	3.8	4.1
aiC_{15}	+	+	+	+	+	+	1.9
C_{15}	5.7	+	—	1.1	+	—	1.3
$C_{16:1}$	6.3	5.6	6.2	1.8	3.2	3.1	5.9
C_{16}	26.6	31.7	42.6	33.6	40.8	42.4	28.1
aiC_{17}	4.5	+	—	0.8	+	—	10.4
ΔC_{17}	8.2	18.4	17.8	18.6	16.2	18.5	5.8
C_{17}	6.2	—	—	6.3	—	—	1.0
$C_{18:2}$	+	+	+	+	+	+	3.8
$C_{18:1}$	5.1	8.1	7.7	3.4	5.2	7.3	13.3
C_{18}	7.8	1.0	+	3.4	3.4	+	8.9
ΔC_{19}	+	16.5	14.4	17.9	15.9	14.2	5.5

* The figures indicate percentage for total fatty acid content.

The fatty acid distribution in phospholipids also indicated that the mode of cultivation influenced only the percentage distribution of fatty acids. Accumulation of unsaturated fatty acids characteristic of *Staph. aureus* was not observed in *E. coli* phospholipids, where the ratio of these substances in the total fatty acid content showed a decrease. In *E. coli* phospholipids the ratio of short-chain fatty acids decreased and the ratio of straight-chain saturated and cyclopropane fatty acids increased. The increase in CP-ring-containing fatty acids was observed mainly within cardiolipin.

Discussion

The results presented have confirmed our previous findings and are in agreement with data indicating changes in lipid synthesis after environmental alterations [6—11]. Fatty acid analysis of the staphylococcal lipid fractions showed that the accumulation of unsaturated fatty acids observed earlier for total lipids affects mainly the neutral lipid fraction.

In the presence of serum there is an alteration also in the unsaturated fatty acid content of phospholipids but, as compared to the control culture, the increase is not higher than twofold. In the presence of human serum sta-

phylococci accumulate lipids corresponding to 20% of the weight of dry bacteria. The overwhelming majority of the accumulated lipid originates from the environment. The examinations indicate that environmental polar lipids (serum-phospholipids) cannot be utilized directly, namely, no traces of them were demonstrated in staphylococci.

Our findings confirm the data of WHITE and FRERMAN [18] that lysyl-phosphatidylglycerol and phosphatidylglycerol fatty acids occur in significantly different amounts in *Staph. aureus*. These results failed to confirm the assumption of HOUTSMUELLER and VAN DEENEN [7] that phosphatidylglycerol is a precursor of lysyl-phosphatidylglycerol.

Our results indicating a change in phospholipid synthesis in *E. coli* cultured with serum agree with the observation of BARBU and LUX [19].

Recently lysyl-phosphatidylglycerol and cardiolipin have been regarded as structural components [20]. As these phospholipids show a qualitative and quantitative alteration after culturing in serum medium as compared to cultures grown without serum, it may be concluded that in the presence of serum the membrane structure of bacteria undergoes changes. On the basis of the finding that unsaturated and cyclopropane ring-containing fatty acids show an increased ratio, it may be assumed that in serum medium the functional state of the membrane also suffers alterations and becomes looser in structure.

REFERENCES

1. LESLIE, R. B.: Membranes and bioenergetics. In CHAPMAN, D. (ed.): Biological membranes. Academic Press, New York 1968, p. 289.
2. AMES, G. F.: J. Bact. **95,** 833 (1968).
3. ANSELL, G. D.: In HAWTHORNE, J. N.: The role of phospholipids. Vol. 3. Elsevier, Amsterdam 1964, p. 256.
4. AZZI, A., FLEISCHER, S., CHANCE, B.: Biochem. biophys. Res. Commun. **36,** 322 (1969).
5. GELLMAN, N. S., LUKAYANOVA, M. A., OSTROVSKI, D. N.: Respiration and phosphorylation of bacteria. Plenum Press, New York 1967.
6. MARR, A. S., INGRAHAM, J. L.: J. Bact. **84,** 1260 (1962).
7. HOUTSMUELLER, I. M. T., VAN DEENEN, L. L. M.: Biochim. biophys. Acta (Amst.) **106,** 564 (1964).
8. VÁCZI, L., RÉDAI, I., RÉTHY, A.: Acta microbiol. Acad. Sci. hung. **14,** 293 (1967).
9. ITERSON, W., OP DEN KAMP, J. A. F.: J. Bact. **99,** 304 (1969).
10. OP DEN KAMP, J. A. F., RÉDAI, I., VAN DEENEN, L. L. M.: J. Bact. **99,** 298 (1969).
11. RÉDAI, I., RÉTHY, A., ROZGONYI, F.: Acta microbiol. Acad. Sci. hung. **16,** 219 (1969).
12. FRERMAN, F. F., WHITE, D. C.: J. Bact. **94,** 1869 (1967).
13. WELLS, A. M., DITMER, J. C.: Biochemistry **2,** 1259 (1963).
14. VORBECK, M. L., MARINETTI, G. V.: J. Lipid Res. **6,** 3 (1965).
15. BARTLETT, G. R.: J. biol. Chem. **234,** 466 (1959).
16. VÁCZI, L., RÉTHY, A., RÉDAI, I.: Acta microbiol. Acad. Sci. hung. **11,** 375 (1964/65).
17. MARINETTI, G. V., ERBLAND, J., KOCHEN, I.: Fed. Proc. **16,** 837 (1957).
18. WHITE, D. C., FRERMAN, F. F.: J. Bact. **95,** 2198 (1968).
19. BARBU, E., LUX, M.: C. R. Acad. Sci. (Paris) Ser. D **268,** 449 (1969).
20. GOULD, R. M., LENARTZ, W. L.: J. Bact. **104,** 1135 (1970).

Address of the authors:

IMRE RÉDAI, ANTAL RÉTHY, PÉTER SEBESSI-GÖNCZY, LAJOS VÁCZI
Institute of Microbiology, University Medical School
Debrecen 12, Hungary

Acta microbiol. Acad. Sci. hung. 18, 307—317, 1971

SEROLOGICAL STUDIES ON PSEUDOMONAS AERUGINOSA O GROUP LIPOPOLYSACCHARIDES

By

Maria M. Ádám, T. Kontrohr and Edith Horváth

National Institute of Public Health (Director: T. Bakács), *Budapest, Institute of Microbiology (Director:* K. Rauss), *University Medical School, Pécs and Institute of Experimental Medicine (Director:* E. Stark), *Hungarian Academy of Sciences, Budapest*

(Received March 2, 1972)

Summary. Purified lipopolysaccharides prepared from 23 type strains of Lányi's antigenic schema by the phenol-water method and ultracentrifugation were examined with direct precipitation and passive haemagglutination test in O immune sera. Precipitation in unabsorbed sera yielded specific reactions at moderate dilutions of the LPS extracts. The agglutination pattern for LPS-coated erythrocytes was similar to the agglutination pattern for heated bacteria in unabsorbed and in highly specific absorbed subgroup sera. The close correlation in serological behaviour between phenol-water extracts and bacteria indicated that LPS was responsible for O antigen specificity.

The chemistry of endotoxic substances in the cell wall of *Pseudomonas aeruginosa* has been studied by several authors [1 – 10]. It has been shown that lipopolysaccharides extracted from *Ps. aeruginosa* strains are serologically specific and can be used for the detection of antibodies in immune sera [11]. Much of the work has been done with only a few strains of the species and no systematic investigations have been performed into the chemical structure of every *Ps. aeruginosa* antigen. The elucidation in recent years of the antigenic structure of the organism and classification by O group and subgroup antigens by the use of a sufficient number of isolates has yielded a basis for such studies.

In undertaking the present examinations we have been inspired by systematic immunochemical studies [12, 13] which yielded important data for the structural features of O-specific chains in *Enterobacteriaceae*. We prepared lipopolysaccharides (LPS) from type strains of Lányi's *Ps. aeruginosa* antigenic schema [14]. The purpose of the experiments was to prove that LPS extracts contain the substances responsible for serological specificity. These investigations are considered a first step of studies of the chemical structure of *Ps. aeruginosa* O antigens.

Materials and methods

Bacterial cultures. Twenty-three strains representing Lányi's O antigenic groups and subgroups [14] were used. The cultures were maintained by freeze-drying. The strains and extracts prepared from them were designated according to Lányi's new numbers [15].

Cultivation was performed in a layer of liquid medium distributed into Roux flasks or in shaken Erlenmeyer flasks at 37 °C for 22 hours. The medium contained the following ingre-

dients: beef extract (Central Slaughterhouse, Budapest), 30 g; peptone (Richter, Budapest), 20 g; yeast extract Cellamin (Human, Budapest), 10 g; glucose, 5 g; NaCl, 3 g; $Na_2HPO_4 \cdot 12H_2O$, 4 g; tap water, 1000 ml. The medium was adjusted to pH 7.4 and sterilized at 115 °C for 30 minutes. The bacteria were killed with 0.5% formalin and centrifuged. The deposit was weighed either after drying in acetone or in the wet state.

Extraction of LPS was performed with WESTPHAL and JANN's phenol-water method [16]. After dialysis and concentration by evaporation, the extracts were freeze-dried. In order to purify the preparation, it was rehydrated to give a 3% aqueous solution and sedimented by ultracentrifugation at 105 000 *g*. After 3 subsequent ultracentrifugations at 105 000 *g* each lasting for 4 hours the purified extract was re-lyophilized and stored.

Purity of LPS preparations was checked by determining their UV absorption spectra [17]. The extracts were dissolved approximately at 0.01% concentration in 0.1 *N* NaOH [16] so as to give absorbance in the range of 0—1.0. The measurements were performed in an Opton or in a Perkin-Elmer registrating spectrophotometer.

Immune sera were prepared in rabbits with bacteria heated at 75 °C for 1 hour [14]. Heterologous agglutinins were absorbed with cultures heated at 100 °C for 2 hours. Absorbed sera for the determination of O subgroup antigens were prepared as described by LÁNYI [14].

Bacterial agglutination was performed with bacteria heated at 100 °C for 2½ hours in saline, then at 130 °C in glycerol. The tubes were incubated in water bath at 50 °C overnight [14].

Precipitation test was carried out in tubes 4 mm in diameter. Immune serum diluted 1 : 2 was overlayered with LPS basic solution (0.5 mg LPS per ml saline) and its twofold serial dilutions. A definite precipitation ring appearing at the point of contact between the two liquids after incubation for 10 minutes at room temperature was recorded as a positive reaction.

Passive haemagglutination test was based on the method of NETER *et al.* [18]. Titrations were performed by the use of TAKÁTSY's microtitrator [19]. Sheep erthrocytes preserved in Alsever solution were washed in saline and the deposit was resuspended in an equal volume of 0.5% LPS dissolved in saline and heated previously at 100 °C for 1 hour. Then 8 volumes of saline were added and the mixture was incubated at 37 °C for 2 hours. After treatment the erythrocytes were washed 3 times then resuspended in 50 volumes of saline. To 0.025 ml aliquots of twofold serum dilutions 0.025 ml aliquots of erythrocyte suspension were added. The results were read after 60 minutes incubation at 37 °C temperature.

Results

Purity of LPS. Acetone-dried bacteria yielded 0.4—3.0% purified LPS. After ultracentrifugation the majority of extracts showed no absorption maximum at 260 mμ, indicating that the preparations were free from nucleic acid contamination. Absorption curves showed usually a course similar to that presented in Fig. 1. The curve for the supernatant after the first centrifugation was characterized by a high peak (curve 1), while the curve obtained for the deposit after 3 subsequent ultracentrifugations indicated the absence of contaminating nucleic acids (curve 2). Seven out of the 23 extracts gave a definite peak at 260 mμ even after the third ultracentrifugation (Fig. 2). After a fourth ultracentrifugation the contaminant disappeared (Fig. 3) from all extracts except 170 016 and 170 021, in which the peak persisted after further ultracentrifugation (Fig. 4). The latter two preparations were then centrifuged at 12 000 *g* for 30 minutes. The UV spectrum of the deposit obtained in this manner was characteristic of pure LPS and the substance responsible for the peak at 260 mμ remained in the supernatant (Fig. 5).

Precipitation test with LPS. Direct precipitation tests with unabsorbed immune sera are shown in Table I. LPS preparations representing all O anti-

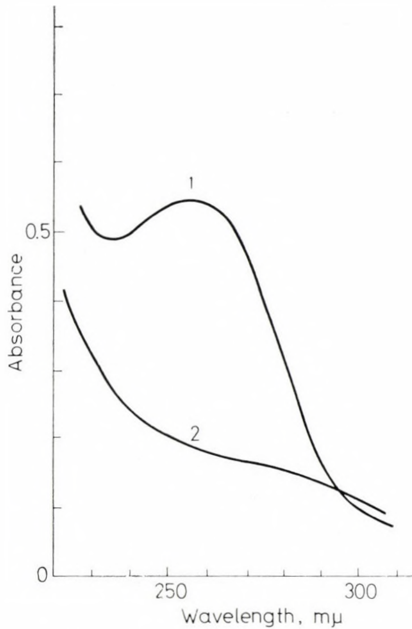


Fig. 1. UV absorption spectrum of phenol-water extracts obtained with the majority of *Ps. aeruginosa* strains; 1 = supernatant after 4 hours ultracentrifugation at 105 000 *g*, 2 = deposit after 3×4 hours ultracentrifugation at 105 000 *g*

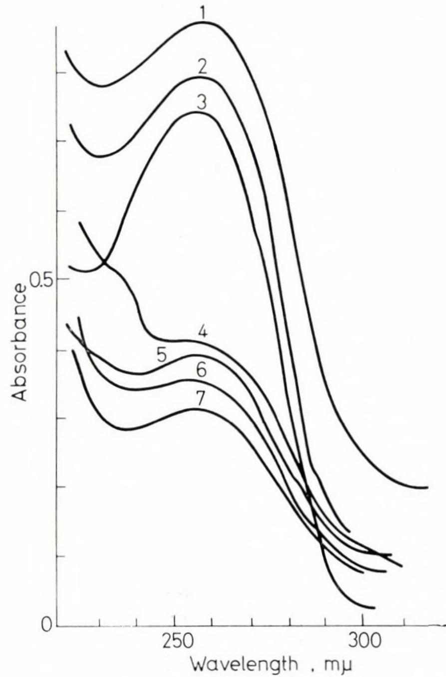


Fig. 2. UV absorption spectrum of phenol-water extracts of certain *Ps. aeruginosa* strains after 3×4 hours ultracentrifugation at 105 000 *g*; 1 = 170 021, 2 = 170 016, 3 = 170 017, 4 = 170 013, 5 = 170 015, 6 = 170 005, 7 = 170 012

genic groups and subgroups reacted with homologous sera in the range of LPS dilutions of 1 : 8—1 : 128. The extract prepared from strain 170 017 (group O8) was the only exception: it failed to react in repeated experiments including undiluted and diluted serum. As with other extracts the most definite reaction appeared if the sera were diluted 1 : 2 and the LPS basic solution to 1 : 4, cross precipitation tests were performed under these conditions. Table I clearly demonstrates that precipitation with LPS preparations was highly group and subgroup specific: cross reactions were observed only between O antigens sharing common group specific factors. As no heterologous precipitation was noted, there was no need to absorb the sera. Some experiments performed with absorbed sera gave no readable results.

Passive haemagglutination. Cross-agglutination of erythrocytes coated with *P. aeruginosa* LPS antigens is shown in Table II. The homologous reaction was characterized, as a rule, by a high titre haemagglutination. It is evident that certain sera were highly specific without absorption. With the

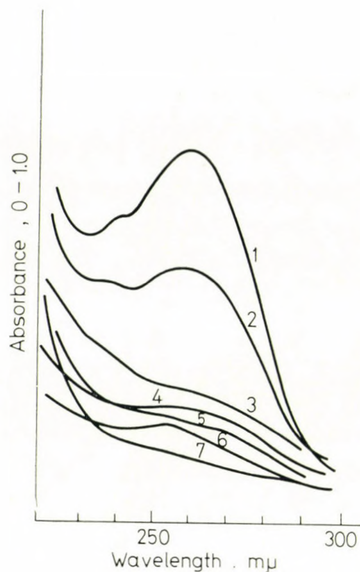


Fig. 3. UV absorption spectrum of phenol-water extracts of the strains shown in Fig. 2 after 4×4 hours ultracentrifugation at 105 000 *g*; 1 = 170 021, 2 = 170 016, 3 = 170 015, 4 = 170 017, 5 = 170 005, 6 = 170 012, 7 = 170 013

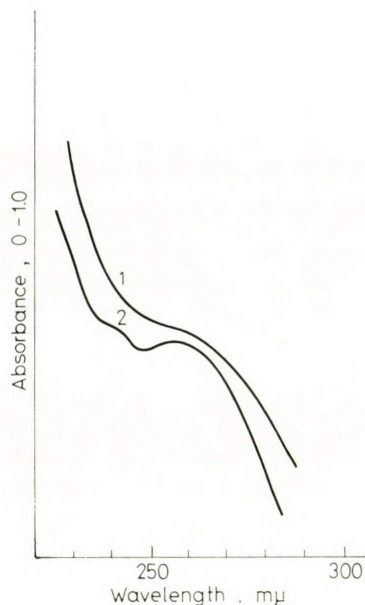


Fig. 4. UV absorption spectrum of phenol-water extracts of strains 170 021 (1) and 170 016 (2), after 5×4 hours ultracentrifugation at 105 000 *g*

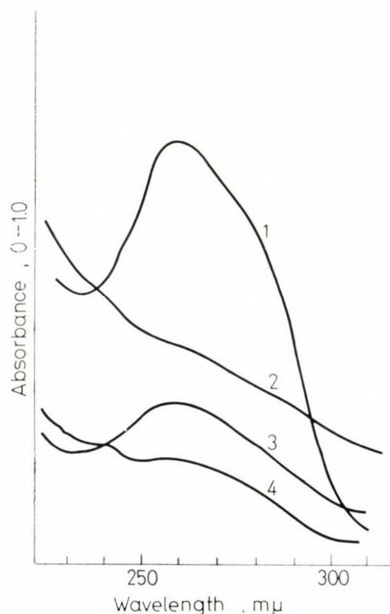


Fig. 5. UV absorption spectrum of phenol-water extract of strains shown in Fig. 4 after 5×4 hours ultracentrifugation at 105 000 *g* and 30 minutes centrifugation at 12 000 *g*; 1 = 170 016 supernatant, 2 = 170 016 deposit, 3 = 170 021 supernatant, 4 = 170 021 deposit

Table I

Tube precipitation test with Ps. aeruginosa lipopolysaccharides

Lipopolysaccharide		Homologous titre*	Precipitation with other sera**
O antigen	Type strain		
1	170 001	16	—
2	170 002	32	—
3a, b	170 003	32	3c; 3a, d; 3a, d, e; 3d, f
3c	170 004	16	3a, b; 3a, d; 3a, d, e; 3d, f
3a, d	170 005	64	3a, b; 3c; 3a, d, e; 3d, f
3a, d, e	170 006	32	3a, b; 3c; 3a, d; 3d, f
3d, f	170 007	16	3a, b; 3c; 3a, d; 3a, d, e
4a, b	170 008	64	4a, c; 4a, d
4a, c	170 009	32	4a, b; 4a, d
4a, d	170 010	16	4a, b; 4a, c
5a, b, c	170 011	32	5a, b, d; 5a, d
5a, b, d	170 012	16	5a, b, c; 5a, d
5a, d	170 013	128	5a, b, c; 5a. b, d
6	170 014	32	—
7a, b	170 015	8	7a, c
7a, c	170 016	16	7a, b
8	170 017	—	—
9	170 018	8	—
10a	170 019	64	10a, b
10a, b	170 020	16	10a
11	170 021	16	—
12	170 022	32	—
13	170 023	8	—

* Highest dilution of LPS basic solution (0.12 mg LPS/ml) giving precipitation with the homologous serum diluted 1 : 2.

** Immune sera reacting at 1 : 2 dilution with LPS antigens shown in left hand column (LPS basic solutions were used at 1 : 4 dilution).

majority of sera, however, several reactions not interpretable on the basis of the O antigenic schema were recorded (non-italicized figures in Table II). Antibodies responsible for these reactions could be removed by absorption with one of the heterologous antigens. After absorption with such cultures the serum retained its capacity to agglutinate erythrocytes coated with homologous and with related group-specific antigens (Table II, figures printed in italics). A 1—3 exponent decrease in the titre for homologous and with related group-specific antigens was frequently observed after heterologous absorption.

Table II

Agglutination of LPS-coated erythrocytes

Lipopolysaccharide		Immune									
O antigen	Type strain	1	2	3a, b	3c	3a, d	3a,d,e	3d, f	4a, b	4a, c	4a, d
1	170 001	*5120*	—	—	40	—	160	40	—	—	—
2	170 002	—	10 240	—	—	—	—	—	—	—	—
3a, b	170 003	—	—	*1280*	*1280*	*1280*	*640*	*160*	—	—	—
3c	170 004	—	—	*160*	*10 240*	*320*	*640*	*160*	—	—	—
3a, d	170 005	—	—	*160*	*320*	*2560*	*2560*	*1280*	—	—	—
3a, d, e	170 006	—	—	*80*	*1280*	*640*	*2560*	*160*	—	—	—
3d, f	170 007	—	—	*80*	*1280*	*320*	*640*	*20 480*	—	—	—
4a, b	170 008	—	—	—	160	—	160	80	*2560*	*2560*	*640*
4a, c	170 009	—	—	—	—	—	—	—	*1280*	*2560*	*640*
4a, d	170 010	—	—	—	40	—	—	40	*40*	*80*	*2560*
5a, b, c	170 011	—	—	—	—	—	—	—	—	—	—
5a, b, d	170 012	—	—	—	160	—	80	160	80	—	80
5a, d	170 013	—	—	—	—	—	—	—	—	—	—
6	170 014	—	—	—	160	—	40	160	80	—	160
7a, b	170 015	—	—	—	40	—	40	160	160	—	160
7a, c	170 016	40	—	—	640	—	80	320	160	—	320
8	170 017	—	—	—	—	—	—	—	—	—	—
9	170 018	—	—	—	—	—	—	—	—	—	—
10a	170 019	—	—	—	320	—	40	40	80	—	160
10a, b	170 020	—	—	—	160	—	40	40	80	—	80
11	170 021	—	—	—	160	—	—	—	160	—	80
12	170 022	—	—	—	—	—	—	—	—	—	—
13	170 023	—	40	—	—	—	—	—	—	—	—

* Reciprocals of serum dilutions; figures printed in italics designate titres demon-

The parallelism between bacterial agglutination with heated suspensions and passive haemagglutination with LPS-coated erythrocytes was evident from the fact that the two methods gave very similar cross-agglutination patterns. Examinations in sera absorbed for subgroup determination yielded especially convincing data, since, in order to ensure high subgroup-specificity, these sera were absorbed with great amounts of bacteria. Reactions obtained in unabsorbed group sera and in absorbed subgroup sera are presented in Table III for O3, in Table IV for O4, in Table V for O5, in Table VI for O7 and in Table VII for O10 antigens. Low titre weak reactions, due to a decrease in antibody content after absorption with great amounts of culture, could be improved by using 3 times concentrated LPS for sensitizing erythrocytes.

in PS. aeruginosa O sera

sera*												
5a, b, c	5a, b, d	5a, d	6	7a, b	7a, c	8	9	10a	10b	11	12	13
640	—	—	—	—	—	—	—	640	—	—	—	—
—	—	—	—	—	—	—	40	*80*	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
80	—	—	—	—	—	—	—	—	—	—	—	—
80	—	—	—	—	—	—	—	640	—	—	—	—
640	80	—	40	—	160	160	160	10 240	80	160	—	80
—	—	—	—	—	—	80	—	—	—	—	—	—
—	40	80	40	—	80	—	40	5120	80	160	—	40
10 240	*320*	*5120*	—	—	*80*	80	160	—	—	—	—	—
5120	*1280*	*5120*	40	—	160	80	160	2560	80	320	—	—
5120	*1280*	*5120*	—	—	80	80	80	320	—	—	—	—
80	80	—	*1280*	—	160	—	80	40	—	160	—	80
640	160	—	—	*1280*	*640*	—	160	640	—	160	—	80
640	160	—	40	*160*	*2560*	40	40	5120	160	320	—	—
—	—	—	—	—	—	*1280*	320	—	—	—	—	—
—	—	—	—	—	—	—	*320*	—	—	—	—	—
320	80	—	40	—	320	—	40	*5120*	*320*	320	—	40
1240	320	—	40	—	640	—	160	*2560*	*640*	320	—	80
640	40	—	—	—	160	—	320	—	40	*640*	—	40
—	—	—	—	—	—	—	80	—	—	—	*5120*	—
—	—	—	—	—	—	—	—	1280	—	—	—	*2560*

strable after the absorption of the serum with heterologous antigens.

Discussion

Absorption spectra of *Ps. aeruginosa* extracts showed that the usual phenol-water method and ultracentrifugation yielded preparations free from nucleic acids. Extracts of type strains representing groups O7a, 7c and O11 showed the presence of contamination (possibly a nucleic acid of unusually high molecular weight), until they had been subjected to a subsequent centrifugation at low speed. As to whether this behaviour is characteristic of the corresponding serogroups or only of certain strains, needs further examination.

The main purpose of the present study was to investigate whether purified LPS preparations represented O antigens determined by bacterial agglutination in LÁNYI's antigenic schema [14, 15].

Table III

Agglutination of heated bacteria and of

O antigen and type strain			Immune		
			3a, b 170 003 unabsorbed	3b 170 003 absorbed by 170 005	3c 170 004 unabsorbed
3a, b	170 003	Bacteria	1280	80	640
		LPS	1280	40	1280
3c	170 004	Bacteria	160	—	5120
		LPS	160	—	10 240
3a, d	170 005	Bacteria	160	—	80
		LPS	160	—	320
3a, d, e	170 006	Bacteria	160	—	640
		LPS	80	—	1280
3d, f	170 007	Bacteria	160	—	20
		LPS	80	—	1280

Precipitation test with LPS preparations in unabsorbed sera yielded specific reactions, but only at moderate dilutions of the extracts. The precipitation pattern showed a striking similarity to the agglutination pattern of living cells in unabsorbed O sera: LÁNYI described that living bacteria, either in tube or in slide agglutination, seldom gave heterologous reactions and in unabsorbed sera the agglutination pattern reflected the group specificity more definitely if living bacteria were used instead of heated ones [14].

Table IV

Agglutination of heated bacteria and of LPS-coated erythrocytes in Ps. aeruginosa O4 sera

O antigen and type strain			Immune sera					
			4a, b 170 008 unabsorbed	4b 170 008 absorbed by 170 009	4a, c 170 009 unabsorbed	4c 170 009 absorbed by 170 008	4a, d 170 010 unabsorbed	4d 170 010 absorbed by 170 009 + 170 024
4a, b	170 008	Bacteria	5120	80	2560	—	640	—
		LPS	2560	40	2560	—	640	—
4a, c	170 009	Bacteria	1280	—	5120	80	640	—
		LPS	1280	—	2560	40	640	—
4a, d	170 010	Bacteria	160	—	320	—	5120	160
		LPS	40	—	80	—	2560	80

LPS-coated erythrocytes in Ps. aeruginosa O3 sera

sera						
3c 170 004 absorbed by 170 003 + 170 006	3a, d 170 005 unabsorbed	3d 170 005 absorbed by 170 003	3a, d, e 170 006 unabsorbed	3e 170 006 absorbed by 170 004 + 170 005	3d, f 170 007 unabsorbed	3f 170 007 absorbed by 170 005
—	1280	—	160	—	160	—
—	1280	—	640	—	160	—
640	160	—	320	—	40	—
320	320	—	640	—	160	—
—	1280	80	640	—	640	—
—	2560	80	2560	—	1280	—
—	320	40	1280	160	1280	—
—	640	20	2560	80	160	—
—	640	—	320	—	2560	640
—	320	—	640	—	20480	320

In contrast, the agglutination patterns for LPS-coated erythrocytes showed a similarity to the patterns obtained with bacteria heated at high temperature. It was shown that in certain unabsorbed sera considerable titres appeared for strains having entirely different O antigens [14]. These heterologous reactions were frequently not reproducible as their appearance varied with the batch of the serum. After absorption with strains showing the non-group-specific reaction, these sera yielded agglutination patterns identical for heated and for living cells [14]. In the present experiments LPS-coated erythro-

Table V

Agglutination of heated bacteria and of LPS-coated erythrocytes in Ps. aeruginosa O5 sera

O antigen and type strain		Immune sera					
		5a, b, c 170 011 unabsorbed	5c 170 011 absorbed by 170 012	5a, b, d 170 012 unabsorbed	5b 170 012 absorbed by 170 013	5a, d 170 013 unabsorbed	5d 170 013 absorbed by 170 011
5a, b, c 170 011	Bacteria	5120	160	640	160	320	—
	LPS	10240	20	320	40	5120	—
5a, b, d 170 012	Bacteria	1280	—	5120	160	2560	160
	LPS	5120	—	1280	80	5120	80
5a, d 170 013	Bacteria	2560	—	1280	—	2560	80
	LPS	5120	—	1280	—	5120	80

Table VI

Agglutination of heated bacteria and of LPS-coated erythrocytes in Ps. aeruginosa O7 sera

O antigen and type strain			Immune sera			
			7a, b 170 015 unabsorbed	7b 170 015 absorbed by 170 016	7a, c 170 016 unabsorbed	7c 170 016 absorbed by 170 015
7a, b	170 015	Bacteria	5120	320	640	—
		LPS	1280	80	640	20
7a, c	170 016	Bacteria	1280	—	5120	160
		LPS	160	—	2560	80

Table VII

Agglutination of heated bacteria and of LPS-coated erythrocytes in Ps. aeruginosa O10 sera

O antigen and type strain			Immune sera		
			10a 170 019 unabsorbed	10a, b 170 020 unabsorbed	10b 170 020 absorbed by 170 019
10a	170 019	Bacteria	2560	2560	—
		LPS	5120	320	—
10a, b	170 020	Bacteria	1280	5120	320
		LPS	2560	640	320

cytes gave very similar results: non-group-specific haemagglutinations were eliminated by absorption with a small amount of heterologous bacteria.

Table I and Table II demonstrate that precipitation and haemagglutination tests with LPS were in close correlation with bacterial O agglutination. Tables III—VII prove that bacterial and LPS antigens give similar agglutination patterns in highly specific subgroup sera. The validity of subgroup classification could not be demonstrated by direct LPS precipitation, since subgroup sera had, in the course of their absorption, been diluted above the level yielding readable precipitation.

Some differences from LÁNYI's schema can be summarized as follows. One serum (O9) showed a low group-specific titre for LPS-coated erythrocytes after absorption with heterologous culture. One serum (O8) failed in repeated experiments to precipitate the homologous LPS. These findings may indicate that the strains used for the preparation of the corresponding sera were defective in O antigens. The correctness of this assumption seems to be confirmed by the fact that recently it has been found that type strains for groups O8 and O9 tend to agglutinate spontaneously after autoclaving [20].

From the results it may be concluded that in *Ps. aeruginosa*, as in other Gram-negative bacteria, the LPS extractable with phenol-water is responsible for O antigen specificity. Extracts prepared in the manner described are suitable for the detection of O specific antibodies by the use of precipitation or passive haemagglutination.

REFERENCES

1. Egami, F., Shimomura, F., Ishihara, H., Homma, J. Y., Sagehashi, K., Hosoya, S.: Bull. Soc. Chim. biol. **36**, 779 (1954).
2. Homma, J. Y., Hamamura, N., Naoi, M., Egami, F.: Bull. Soc. Chim. biol. **40**, 647 (1958).
3. Naoi, M., Egami, F., Hamamura, N., Homma, J. Y.: Biochem. Z. **330**, 421 (1958).
4. Gray, G. W., Wilkinson, S. G.: J. gen. Microbiol. **39**, 385 (1965).
5. Clarke, K., Gray, G. W., Reaveley, D. A.: Nature (Lond.) **208**, 586 (1965).
6. Roberts, N. A., Gray, G. W., Wilkinson, S. G.: Biochim. biophys. Acta (Amst.) **135**, 1068 (1967).
7. Fensom, A. H., Gray, G. W.: Biochem. J. **114**, 185 (1969).
8. Michaels, G. B., Eagon, R. G.: Proc. Soc. exp. biol. (N. Y.) **131**, 1346 (1969).
9. Wilkinson, S. G.: J. Bact. **104**, 1035 (1970).
10. Fensom, A. H., Meadow, P. M.: FEBS Letters **9**, 81 (1970).
11. Gaines, S., Landy, M.: J. Bact. **69**, 628 (1955).
12. Westphal, O., Lüderitz, O.: Path. Microbiol. **24**, 870 (1961).
13. Lüderitz, O., Staub, A. M., Westphal, O.: Bact. Rev. **30**, 192 (1966).
14. Lányi, B.: Acta microbiol. Acad. Sci. hung. **13**, 295 (1966/67).
15. Lányi, B. : Acta microbiol. Acad. Sci. hung. **17**, 35 (1970).
16. Westphal, O., Jann, K.: In Whistler, R. H. (ed.): Methods in Carbohydrate Chemistry. Vol. 5. Academic Press, New York 1965.
17. Westphal, O., Lüderitz, O., Eichenberger, E., Keiderling, W.: Z. Naturforsch. **7b**, 536 (1952).
18. Neter, E., Bertram, L. F., Zak, D. A., Murdock, M. R., Arbesman, C. E.: J. exp. Med. **96**, 1 (1952).
19. Takátsy, G.: Acta microbiol. Acad. Sci. hung. **3**, 191 (1955).
20. Lányi, B.: Personal communication.

Address of the authors:

Maria M. Ádám
National Institute of Public Health
Gyáli út 2–6, Budapest IX, Hungary

Tivadar Kontrohr
Institute of Microbiology, University Medical School
Szigeti út 12, Pécs, Hungary

Edit Horvath
Institute of Experimental Medicine, Hungarian Academy of Sciences
Szigony u. 43, Budapest VIII, Hungary

Acta microbiol. Acad. Sci. hung. 18, 319—326, 1971

INCIDENCE AND HYGIENIC IMPORTANCE OF PSEUDOMONAS AERUGINOSA IN WATER

By

L. Némedi and B. Lányi

Public Health Station (Director: F Gács) *and National Institute of Public Health (Director:*. T. Bakács), *Budapest*

(Received March 20, 1972)

Summary. Routine sanitary qualification of water samples has been completed with the detection of *Pseudomonas aeruginosa* in addition to determination of coliform and enterococcal counts. As compared to the usual membrane filter technique, enrichment in selenite–brilliant green broth was highly efficient for the isolation of *Ps. aeruginosa*, especially when examining polluted waters. Membrane filtration of 37,153 samples yielded *Ps. aeruginosa* in 9.5%, while in enrichment medium 1372 specimens were positive in 38.2%. As shown with the enrichment method, the incidence of *Ps. aeruginosa* was 3.4—22.3% in various kinds of drinking water samples, 37.2% in industrial water circuits, 23.4—84.4% in swimming pools, 86.2% in surface waters, and 95.7% in sewage. Coliform bacteria were absent from 117 out of 227 drinking water specimens positive for *Ps. aeruginosa*: in samples containing both organisms, coliforms occurred usually in smaller numbers than *Ps. aeruginosa*. Serological typing according to Lányi's schema showed a predominance of *Ps. aeruginosa* O4 strains. Serogroups O1, O3 and O7 were also frequently encountered.

The sanitary importance of *Pseudomonas aeruginosa* has been pointed out by several authors [1, 2, 5, 7, 9, 10, 12—14, 19, 20, 22]. The studies of Hoadley and McCoy [6], Ringen and Drake [16] and Lányi, Gregács and Ádám [9] indicate that the presence of *Ps. aeruginosa* in water is associated with direct or indirect faecal pollution. As the organism is a potentially pathogenic agent, it may be considered an indicator of the sanitary quality of the water.

In the Laboratory of Water Biology of the Budapest Public Health Station since 1965 routine bacteriological examinations have been performed for the detection of *Ps. aeruginosa*. From data obtained for various water and sewage samples, conclusions have been drawn as to the degree of potential danger due to the appearance of the organism. The degree of association between the incidence of coliform bacteria and *Ps. aeruginosa* has also been studied in respect to the hygienic value of *Ps. aeruginosa* isolations.

Materials and methods

Water samples were collected in the area of Budapest. Bacteriological examinations were performed as described in the Hungarian Standard for the Examination of Water [11].

Isolation of Ps. aeruginosa. Two methods were used. The membrane filter technique was the same as that used for the detection of coliforms, but membranes placed on modified Endo medium were examined also for pseudomonad colonies. For the enrichment method, brilliant green–selenite broth [15] originally described by Stokes and Osborne [18] was used. Each

of 5 tubes with 5 ml broth was inoculated with a 5 ml portion of the sample. Specimens expected to contain large numbers of bacteria were diluted previously 1 : 10 or 1 : 100, so that 5×0.5 ml or 5×0.05 ml aliquots of the original sample were examined. The inoculated tubes were incubated at 37 °C for 48 hours, then the cultures were streaked on brilliant green agar [15] and the plates were incubated at 42 °C for 24 hours.

Identification of Ps. aeruginosa. Pigment production, gelatin liquefaction, oxidase reaction [15] and growth on agar slants and brilliant green agar at 42 °C [10] were examined.

Serological typing. Determination of the O group of *Ps. aeruginosa* strains was performed according to the schema of LÁNYI [10] by slide agglutination from blood agar plates.

Ps. aeruginosa counts were determined directly from the membranes or by the most probable number method in the case of enrichment cultures.

Results

1. *Selective cultivation of Ps. aeruginosa.* The use of the enrichment medium for the detection of *Ps. aeruginosa* occurred to us when observing that selenite–brilliant green agar, originally designed for salmonellae, did not inhibit the growth of pseudomonads which, in fact, even tended to overgrow the former organisms. If the medium was diluted with an equal volume of the specimen examined, its selectivity for pseudomonads increased.

Investigations into the selectivity of the medium are summarized in Fig. 1. *Ps. aeruginosa* grew well after 24 hours in undiluted or 1 : 2-diluted selenite–brilliant green broth, even if the inoculum had been as small as 1–10 colony-forming units per 5 ml medium. The cultures were transferred to brilliant green agar at 18, 24, 48, 72 and 96 hour intervals. The plates incubated at 42 °C showed abundant growth of *Ps. aeruginosa* in all transfers but the 18-hour one. In contrast, *E. coli* grew only if an inoculum corresponding to 10^7–10^9 colony-forming units had been added to 5 ml medium, but only in the enrichment broth, as brilliant green agar subcultures showed no evidence of growth. In mixed cultures *Ps. aeruginosa* rapidly suppressed *E. coli*. In a mixed culture of *Salmonella* and *Ps. aeruginosa* the former appeared in large numbers, but later it was overgrown by *Ps. aeruginosa*. As *Ps. aeruginosa* multiplied somewhat better in enrichment broth diluted 1 : 2, in subsequent examinations an equal volume of the specimen or its appropriate dilution was added to the medium and incubation lasted for 48 hours.

Table I shows that, as to the detection of *Ps. aeruginosa*, the enrichment method was superior to membrane filtration in all kinds of specimens. It is also evident that the more polluted the sample, the more efficient the enrichment method. This finding is explained by the fact that *Ps. aeruginosa* colonies growing on the membrane filter can easily be distinguished if the coliform count is low, but great numbers of coliforms mask the pseudomonad colonies. Dilution of highly polluted samples is not of much aid, since in these the relative number of *Ps. aeruginosa* is much smaller than that of coliform bacteria.

2. *Incidence of Ps. aeruginosa in water and sewage.* Table I demonstrates that, if examined with the comparable enrichment technique, the incidence

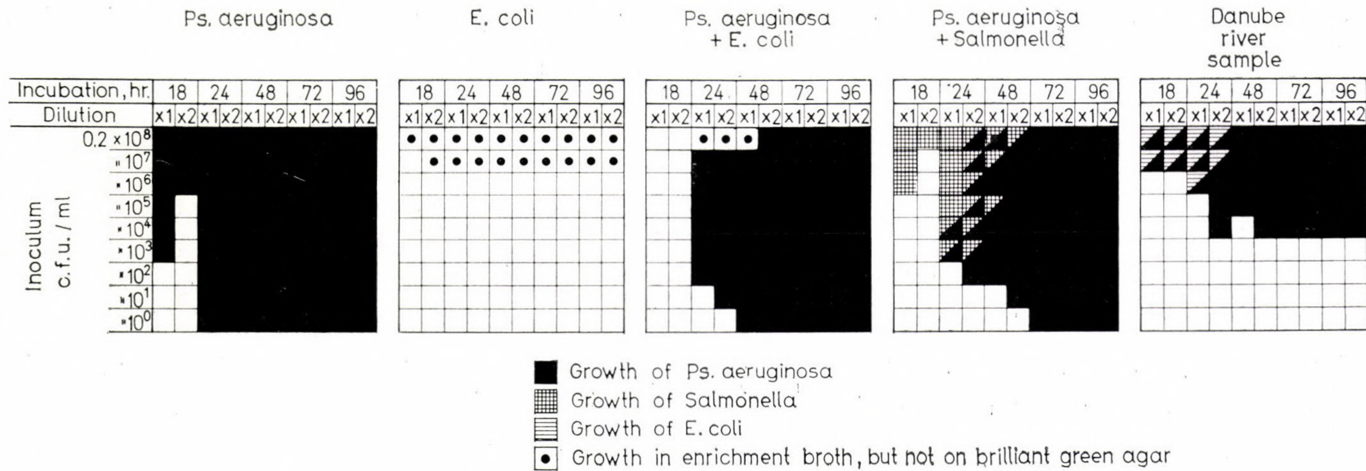


Fig. 1. **Selective cultivation of *Ps. aeruginosa*. Bacteria ranging from 0.2 to 0.2×10^8 c.f.u./ml were seeded into undiluted ($\times 1$) and 1 : 2 diluted ($\times 2$) selenite–brilliant green broth, then after 18, 24, 48, 72 and 96 hours incubation streaked onto brilliant green agar plates**

Table I

Comparison of membrane filter and enrichment technique for the detection of Ps. aeruginosa in water and sewage

Specimens	Method*	No. of specimens	Specimens positive for *Ps. aeruginosa*		Average *Ps. aeruginosa* counts per 100 ml
			No.	%	
Municipal water supply	MFT	34 429	88	0.2	5.5
	SBG	353	12	3.4	3.8
Springs	MFT	79	5	6.3	5.8
	SBG	48	4	8.3	16.0
Mineral and soda water	MFT	210	41	19.0	24.0
	SBG	228	51	22.3	20.0
Wells	MFT	1 047	9	0.8	270.0
	SBG	109	17	15.6	49.0
Industrial circuits and	MFT	497	31	6.2	196.0
containers	SBG	118	44	37.2	452.0
Inlets of swimming pools	MFT	170	11	6.4	8.0
	SBG	81	19	23.4	100.0
Swimming pools	MFT	235	8	3.4	.
	SBG	161	136	84.4	701.0
Surface waters	MFT	406	2	0.5	.
	SBG	204	176	86.2	690.0
Sewage	MFT	80	—	—	—
	SBG	70	67	95.7	2500.0
Total	MFT	37 153	195	0.5	
	SBG	1 372	526	38.3	

* MFT = membrane filter technique, SBG selenite–brilliant green enrichment technique.
. = *Ps. aeruginosa* count not determinable.

of *Ps. aeruginosa* shows a wide variation according to the kind of specimen examined. The right hand column of Table I shows the average of *Ps. aeruginosa* counts per 100 ml volume of different samples. In membrane filter examinations these values were determined by direct colony counting, in enrichment cultures the data were obtained by the most probable number method.

Table II shows the number of *Ps. aeruginosa* positive samples not containing coliform bacteria. A surprisingly great number of drinking water specimens fell into this category (118 out of 227). That is, more than half of the *Ps. aeruginosa* positive samples would have been qualified satisfactory on the basis of a sole coliform test. In industrial water circuits the situation was even worse: 70—80% of the specimens positive for *Ps. aeruginosa* failed to contain coliform bacteria.

Table II

Incidence of coliform bacteria in specimens positive for Ps. aeruginosa

Specimens	Method*	Specimens positive for *Ps. aeruginosa*		No. of specimens showing higher *Ps. aeruginosa* counts than coliform counts
		Total No.	Coliform negative	
Municipal water supply	MFT	88	60	80
	SBG	12	5	5
Springs	MFT	5	5	5
	SBG	4	2	2
Mineral and soda water	MFT	41	11	38
	SBG	51	20	47
Wells	MFT	9	6	6
	SBG	17	8	11
Industrial circuits and containers	MFT	31	26	31
	SBG	44	33	40
Inlets of swimming pools	MFT	11	7	7
	SBG	19	10	17
Swimming pools	MFT	8	—	—
	SBG	136	5	45
Surface waters	MFT	2	—	—
	SBG	176	—	1
Sewage	MFT	—	—	—
	SBG	67	—	—
Total	MFT	195	115	167
	SBG	526	83	168

* MFT = membrane filter technique, SBG = selenite–brilliant green enrichment technique.

The right hand column of Table II shows the number of *Ps. aeruginosa* positive specimens in which this organism was present in greater numbers than were the coliforms. In drinking water samples *Ps. aeruginosa* usually exceeded the coliforms, but in swimming pools, surface water and sewage the latter predominated.

Table III presents the incidence of *Ps. aeruginosa* serogroups in various specimens. The commonest serogroup was O4 (18.5%). Serogroups O1, O7 and O3 were next in order (14.1, 11.2 and 11.0%, respectively). These four serogroups comprised, accordingly, 54.8% of all isolates. The appearance of serogroup O4 was usually associated with more frequent isolations of coliform bacteria. The relative frequency of O1 cultures was associated with a mass *Ps. aeruginosa* pollution in the Municipal Mineral Water Plant.

Discussion

Members of the order *Pseudomonadales* are widely distributed in nature and most of them are normal inhabitants of surface waters. *Ps. aeruginosa* is the only wide-spread pseudomonad that has become a potential pathogen for higher animals and man, and it appears probably in water as a result of faecal pollution. In order to survey the incidence of this organism in a variety of water specimens, we have introduced the selenite–brilliant green broth–brilliant green agar technique, which allows a sensitive detection of *Ps. aeruginosa* especially in highly polluted specimens.

Our data indicated that neither the incidence of *Ps. aeruginosa* positive drinking water samples nor the number of the organism in them was negligible. This is well illustrated by the fact that chlorinated municipal water, mineral water and well water specimens contained *Ps. aeruginosa* in 3.4, 22.3 and 16.5%, respectively; at the same time the incidence of the corresponding specimens qualified unsatisfactory in view of their coliform content was 9.0, 37.4 and 39.3%, respectively.

Ps. aeruginosa was isolated remarkably frequently from industrial water circuits. Coliform bacteria, in contrast, seldom occurred in such specimens. This finding deserves special interest, as pipelines carrying industrial water run usually parallel with drinking water pipes and thus there is a hazard of communication.

We have observed that from water supply equipments polluted with *Ps. aeruginosa* the organism is difficult to eliminate and disinfecting procedures

Table III

Distribution of Ps. aeruginosa

Specimens	No. of strains	*Ps. aeruginosa* serogroups				
		1	2	3	4	5
Municipal water supply	12	1	—	—	6	1
Springs	4	1	2	—	—	1
Mineral and soda water	62	36*	—	3	4	7
Wells	18	3	—	1	5	2
Industrial circuits and containers	47	8	—	3	24	—
Inlets of swimming pools	21	1	—	7	—	6
Swimming pools	285	24	18	45	29	27
Surface waters	337	46	14	33	81	23
Sewage	147	12	14	11	25	23
Total	933	132	48	103	174	90

* All *Ps. aeruginosa* O1 strains were isolated from the Municipal Mineral Water Plant.

effective against coliforms fail to kill pseudomonads. For example, from a private piped water supply of a holiday-house, *Ps. aeruginosa* strains of the same serogroup were isolated for years despite all efforts of cleaning. In a mineral water plant the equipment used for cooling the water was a continuous source of *Ps. aeruginosa.* Serological determination of the isolates was especially useful in such examinations. In our experience, polluted piped water systems remain positive for *Ps. aeruginosa* longer than for coliforms, that is, repeated examinations show the presence of *Ps. aeruginosa* rather than of coliform bacteria. HURST and SUTTER [8] have also found that in water pseudomonads survive longer and are more resistant to chlorination than coliform bacteria. Accordingly, there is good reason to assume that *Ps. aeruginosa* is of special importance as to the sanitary condition of drinking water and that it is desirable to consider *Ps. aeruginosa* counts in addition to coliform and enterococcal counts as an indicator of pollution.

Our specimens from swimming pools contained *Ps. aeruginosa* in 84.4%, and the average count for this organism was almost as high as in surface waters or sewage (701 bacteria per 100 ml sample). That the presence of this organism should not be neglected in qualifying swimming pool samples is evident from the work of FAVERO *et al.* [5] who described the predominance of *Ps. aeruginosa* in insufficiently disinfected swimming pools and pointed out that the organism is one of the principal causative agents of otitis externa among swimmers.

The fact that the incidence of *Ps. aeruginosa* in Budapest surface waters (86.2%) is almost as high as in sewage (95.7%) is indicative of a great degree of sewage-load on our natural waters. Specimens originating from the river Danube at sampling points situated above the city were less frequently pol-

serogroups in various specimens

(LÁNYI's antigenic schema)									
6	7	8	9	10	11	12	13	P	ND
—	4	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
2	2	1	—	3	—	—	—	—	4
2	—	1	—	2	—	—	—	—	2
5	1	4	—	1	1	—	—	—	—
1	3	—	—	1	—	—	—	—	2
31	39	17	1	27	2	—	—	7	18
36	44	8	4	8	3	1	1	13	22
18	12	15	—	3	5	—	—	3	6
95	105	46	5	45	11	1	1	23	54

P = polyagglutinable strains, ND = serogroup not determined.

luted with *Ps. aeruginosa* than specimens taken under the city. Similar observations were described by HOADLEY and McCOY [7]; they showed that *Ps. aeruginosa* occurred with greater frequency in the neighbourhood of sewage inflows than in other parts of surface waters.

Serological typing indicated that there was no significant difference between specimens in the distribution of *Ps. aeruginosa* serogroups. The predominating serogroups were the same as those isolated by LÁNYI *et al.* [9] in 1966 from water, sewage and faecal specimens collected in the country area surrounding Budapest city.

REFERENCES

1. BULLOCK, G. L.: Symposium Fish Microbiology. Developments in Industrial Microbiology **5**, 101 (1964).
2. BULLOCK, G. L.: Appl. Microbiol. **13**, 89 (1965).
3. DRAKE, C. H.: Hlth Lab. Sci. **3**, 10 (1966).
4. EWING, W. H., JOHNSON, J. G.: Int. Bull. bact. Nomencl. **10**, 223 (1960).
5. FAVERO, M. S., DRAKE, C. H., RANDALL, G. B.: Publ. Hlth Rep. (Wash.) **79**, 61 (1964).
6. HOADLEY, A. W., McCOY, E.: Hlth Lab. Sci. **3**, 20 (1966).
7. HOADLEY, A. W., McCOY, E.: Appl. Microbiol. **13**, 575 (1965).
8. HURST, V., SUTTER, V.: J. infect. Dis. **116**, 151 (1966).
9. LÁNYI, B., GREGÁCS, M., ÁDÁM, M.: Acta microbiol. Acad. Sci. hung. **13**, 319 (1966/67).
10. LÁNYI, B.: Acta microbiol. Acad. Sci. hung. **13**, 295 (1966/67).
11. MSZ Szabványgyűjtemények 35. Vízvizsgálati szabványok. Budapest 1963.
12. NÉMEDI, L.: Acta microbiol. Acad. Sci. hung. **12**, 181 (1965).
13. NILSSON, P. O., THÖRNE, H.: Nord. vet. Med. **15**, 239 (1963).
14. NILSSON, P. O., THÖRNE, H.: Nord. vet. Med. **14**, 538 (1963).
15. Módszertani Útmutató. Országos Közegészségügyi Intézet, Budapest 1969.
16. RINGEN, L. F., DRAKE, C. H.: J. Bact. **64**, 841 (1952).
17. REITLER, F.: J. appl. Bacteriol. **20**, 145 (1957).
18. STOKES, J. L., OSBORNE, W. W.: Appl. Microbiol. **3**, 217 (1955).
19. SUTTER, V., HURST, V., GROSSMAN, M., CALONJE, R.: J. Amer. med. Ass. **197**, 854 (1966).
20. SUTTER, V., HURST, V.: Ann. Surg. **163**, 597 (1966).
21. THÖRNE, H., KYRKJEBÖ, A.: Acta vet. scand. **7**, 289 (1966).
22. WOKATSCH, R.: Zbl. Bakt. I. Abt. Orig. **192**, 468 (1964).

Address of the authors:

LÁSZLÓ NÉMEDI
Public Health Station
Váci út 174, Budapest XIII, Hungary

BÉLA LÁNYI
National Institute of Public Health
Gyáli út 2—6, Budapest IX, Hungary

Acta microbiol. Acad. Sci. hung. 18, 327—331, 1971

EFFECT OF TILORONE HYDROCHLORIDE ON THE EXPERIMENTAL RABIES OF MICE

By

F. FORNOSI, MARGARET TÁLAS and G. WEISZFEILER

National Institute of Public Health (Director: T. BAKÁCS) *and Microbiological Research Group (Director:* G. WEISZFEILER) *of the Hungarian Academy of Sciences, Budapest*

(Received March 24, 1972)

Summary. A single intragastric administration of tilorone hydrochloride completely protected Swiss mice against an intracerebral challenge with 6 intracerebral, or subcutaneous challenge with 6 subcutaneous, LD_{50} of the CVS strain of rabies virus. The challenge was applied 19 hours after treatment. The same pretreatment prolonged the incubation period if the challenge dose was larger (100 or 1000 LD_{50}). Interferon (IF) was demonstrable 48 hours after challenge even in untreated mice: in the serum after subcutaneous challenge and in both the brain tissue and the serum after intracerebral challenge. The IF level of the mice pretreated with tilorone increased in the brain after intracerebral challenge and in the blood serum after subcutaneous challenge.

Utilization of interferon (IF) and IF inducers in the prophylaxis of rabies has been considered by several authors. However, neither poly I : C nor NDV protected Swiss mice against the CVS strain of rabies virus [1, 2]. In the present work, the effect of a single intragastric administration of tilorone hydrochloride on experimental rabies induced by the CVS strain has been studied.

Materials and methods

Mice. Swiss mice bred at the National Institute of Public Health, Budapest, weighing 14—16 g, were used.

Virus. The CVS strain of rabies virus was kindly supplied by the Research Institute for Poliomyelitis and Viral Encephalitides of the Soviet Academy of Medical Sciences, Moscow. In our Laboratory, the strain had undergone two intracerebral passages in three-week-old Swiss mice. Brains from the second passage were suspended in four volumes of distilled water containing 2% horse serum. The stock suspension thus obtained was stored at −60 °C until used. Virus dilutions were made in a phosphate buffered saline (PBS), pH 7.2, containing 2% horse serum. The virus dilutions were kept in melting ice until inoculated into mice.

Tilorone hydrochloride (WM. S. Merrel Co., Cincinnati, Ohio, U.S.A.), 12.5 mg/ml was dissolved in PBS. Of this solution 0.2 ml (160—170 mg/kg) was introduced into the stomach of each animal through a gastric tube adjusted to a tuberculin syringe.

Challenge. Mice were infected subcutaneously or intracerebrally 19 hours after tilorone treatment.

IF titration. Serum dilutions prepared in PBS and 20% brain emulsions dialysed for 48 hours at pH 2.0, and subsequently for 24 hours at pH 7.6, were incubated with washed L-cell tube cultures for 4 hours. Subsequently, the medium was poured off and the cultures were infected with 50 CPD_{50} of Semliki Forest virus. Parker's 199 enriched with 2% calf serum served as maintenance medium. Results were read after an incubation for 72 hours. IF titre is expressed in the highest dilution by which the cytopathic effect of the challenge virus was completely prevented.

Sampling. Three mice were killed in each group, 48 hours and 5 days after intracerebral, and 48 hours and 6 days after subcutaneous, challenge with rabies virus. The serum samples as well as the brains from the three mice were pooled. From each brain pool a 20% homogenate was prepared.

Results

Table I shows the effect of tilorone hydrochloride on mice intracerebrally infected with rabies virus. For the mice pretreated with the antiviral agent, the LD_{50} was approximately 6-fold the control value (0.8 log units). If the inoculum was larger than 10 LD_{50}, the mortality rate was not influenced but the average incubation period was prolonged by the drug.

Table I

Effect of tilorone hydrochloride administered intragastrically on intracerebral infection with the CVS strain of rabies virus

Treatment		Virus dilution					Neg log LD_{50}/0.03 ml
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Tilorone** + CVS	1*	—	8/8	9/10	2/8	—	
	2*	—	6.6	6.9	8.5	—	5.6
	3*	—	2.1	1.9	1.0	—	
CVS (control)	1*	9/9	9/10	10/10	8/9	1/10	
	2*	—	6.0	6.4	6.8	—	6.4
	3*	1.7	2.0	2.1	2.2	—	

** Tilorone HCl 2.5 mg/14—16 g Swiss mouse 19 hours before virus challenge
1* Numerator = number of died, denominator = number of treated animals
2* Arithmetical mean of incubation period in days
3* Arithmetical mean of clinical illness in days

There was no difference in clinical symptoms between pretreated and untreated mice. Nevertheless, for the tilorone-treated mice the average duration of clinical illness tended to increase on increasing the challenge dose. A similar tendency was not notable in the control group.

In Table II, the effect of tilorone pretreatment on the subcutaneous infection of mice is shown. In this case, too, the LD_{50} for the pretreated mice was approximately 6-fold the corresponding control value. In the case of inocula larger than 10 LD_{50}, mortality was not reduced but the incubation period was prolonged by tilorone treatment. The average duration of illness seemed to be in direct relation to the challenge dose.

The serum IF titre was 1 : 10, 1 : 20, 1 : 160 and 1 : 40 at 2, 6, 18 and 48 hours, respectively, after the intragastric administration of 2.5 mg tilorone hydrochloride. The challenge virus was given at the expected peak of the serum IF curve, *i.e.*, 19 hours after treatment. Table III shows the IF levels

Table II

Effect of tilorone hydrochloride administered intragastrically on subcutaneous infection with the CVS strain of rabies virus

Treatment		Virus dilution				Neg log LD_{50}/0.1 ml
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	
Tilorone** + CVS	1*	7/9	2/8	1/9	—	
	2*	7.6	11.0	—	—	1.59
	3*	2.4	< 1.0	—	—	
CVS (control)	1*	9/9	7/10	1/9	0/10	
	2*	6.8	9.6	—	—	2.35
	3*	2.0	1.0	—	—	

For explanation see Table I

in the pooled serum and pooled brain of the mice killed 48 hours after challenge. It is clearly seen that IF was induced even by the challenge virus itself. In mice challenged subcutaneously, the serum IF titre was low (1 : 10 — 1 : 20) and no IF could be demonstrated in the brain. After intracerebral challenge, the titre reached 1 : 64 — 1 : 128 in both the serum and the brain of the control mice. In subcutaneously challenged mice the serum IF titre, in intracerebrally challenged ones the brain IF titre, increased more intensely. In the brains of the mice challenged subcutaneously, IF could not be detected even if the mice

Table III

Interferon levels in serum and brain of mice 48 hours after infection with CVS strain of rabies virus*

Pre-treatment with tilorone	Virus dilution	Way of infection	Interferon titres** in 0.5 ml	
			serum	brain
yes	10^{-4}	icer	160	512
yes	10^{-6}	icer	80	256
no	10^{-3}	icer	80	128
no	10^{-6}	icer	80	64
yes	10^{-1}	scut	640	< 20
yes	10^{-3}	scut	320	< 20
no	10^{-1}	scut	20	< 20
no	10^{-3}	scut	20	< 20

* In each test pooled samples from 3 mice
** Reciprocals of dilutions

had been pretreated with tilorone. An increase in the challenge dose was in general accompanied by an increased IF production.

Five and 6 days after subcutaneous challenge of mice, low IF levels (1 : 20) were demonstrated in the serum, but no IF could be detected in the brain of the mice pretreated with tilorone. In the other groups, IF could be detected neither in the serum nor in the brain.

Discussion

Tilorone hydrochloride, 2,7-bis (2-diethyl-aminoethoxy-fluoren-9-one dihydrochloride), is a broad-scale antiviral agent and a good IF inducer [3, 4]. Its IF-inducing effect was clearly demonstrated in the present experiments.

Nemes *et al.* [1] examined the protective effect of poly I : C on the CVS-26-7 strain of rabies virus. They administered the challenge into the plantar pad or intracerebrally. Average survival time of the pretreated mice as compared to that of the controls was prolonged, whereas protection was poor: the survival rate was increased by 30% if 10 LD_{50} was administered as challenge.

Fenje and Postič [5, 6] administered poly I : C to rabbits intravenously or intramuscularly within 24 hours after a challenge with rabies street virus. They demonstrated a significant protection.

Atanasiu *et al.* [2] called attention to the fact that the IF sensitivity of the rabies virus depends on the host. Induction of IF by NDV was followed by no protection, in spite of the high serum IF level.

In our own investigations, after a single intragastric administration of tilorone hydrochloride, IF appeared in the serum, but not in the brain. Forty-eight hours after intracerebral challenge with rabies virus, IF was demonstrable in the brain tissue as well. The IF level of the brain was higher in the mice pretreated with tilorone than in the control mice. IF produced in the brain tissue was also demonstrable in the serum, whereas no detectable amounts of the serum IF diffused into the brain. This phenomenon points to the probable role of the blood-CSF barrier. An interpretation of the practically identical protection against subcutaneous and intracerebral infection, involving differences in the pathomechanism, needs more differentiated investigations.

Acknowledgement. The authors are indebted to the WM. S. Merrel Co., Cincinnati, Ohio, U.S.A. for the sample of tilorone hydrochloride.

REFERENCES

1. Nemes, M. M., Tytell, A. A., Lampson, G. P., Field, A. K., Hilleman, M. R.: Proc. Soc. exp. Biol. (N.Y.) **132,** 775 (1969).
2. Atanasiu, P., Barroeta, M., Tsiang, H., Favre, S.: Ann. Inst. Pasteur **119,** 767 (1970).

3. Krueger, R. F., Mayer: G. D.: Science **169**, 1213 (1970).
4. Mayer, G. D., Krueger,R., F.: Science **169**, 1214 (1970).
5. Fenje, P., Postić, B.: Nature (Lond.) **226**, 171 (1970).
6. Fenje, P., Postić, B.: J. infect. Dis. **123**, 426 (1971).

Address of the authors:

Ferenc Fornosi
National Institute of Public Health
Gyáli út 2–6, Budapest IX, Hungary

Margaret Tálas, Gyula Weiszfeiler
Microbiological Research Group of the Hungarian Academy of Sciences
Pihenő út 1, Budapest XII, Hungary

BOOKS RECEIVED

Juhasz, S. E., Plummer, G. (eds): *Host–Virus Relationship in Mycobacterium, Nocardia and Actinomyces*. Proceedings of a Symposium. Thomas, Springfield, Ill., 1970. 270 pp. Price: US $ 13.75.

Regamey, R. H., Stanic, M., Ungar, J.: *Symposia Series in Immunobiological Standardization*, Vol. 15. *International Symposium on Enterobacterial Vaccines*. Proceedings of the 27th Symposium Organized by the Permanent Section of Microbiological Standardization. Berne, October 9—11, 1969. S. Karger, Basel/München/Paris/London/New York/Sydney, 1971. VIII + 296 pp., 63 figs, 110 tables. Price: SFr 60.—, US $14.40, DM 60.—, £ 6.30.

Regamey, R. H., Bach, J. F., Dormont, J., Eyquem, A., Raynaud, M. (eds): *Symposia Series in Immunobiological Standardization*. Vol. 16. *International Symposium on Antilymphocyte Serum*. Proceedings of the 26th Symposium Organized by the Permanent Section of Microbiological Standardization, Versailles, March 23—25, 1970. S. Karger, Basel/München/London/Paris/New York/ Sydney, 1970. X + 404 pp., 127 figs, 28 pls, 85 tables. Price: SFr 60.—, US $ 14.40, DM 60.—, 120 s.

Hockenhull, D. J. D.: *Progress in Industrial Microbiology*. Vol. 9. J. and A. Churchill, London 1971. Price: £ 4.00.

Madoff, S.: *Mycoplasma and the L Forms of Bacteria*. Gordon and Breach, New York/London/Paris, 1971. 116 pp., 44 figs. Price: £ 5.25, US $ 12.60.

Hobby, G. L. (ed.): *Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Bethesda, Maryland, 1970. XI + 545 pp.

Regamey, R. H., Hennessen, W., Perkins, F. T. (eds): *Symposia Series in Immunobiological Standardization*. Vol. 17. *International Symposium on BCG Vaccine*. Proceedings of the 32nd Symposium Organized by the Permanent Section of Microbiological Standardiza-, tion, Frankfurt (Main), December 1—3, 1970. S. Karger, Basel/München/New York 1971. X + 326 pp. Price: SFr. 60.—, DM 60.—, US $ 14.40, £ 6.60.

Isler, O. (ed.): *Carotenoids*. Birkhäuser Verlag, Basel/Stuttgart, 1971. 932 pp. Price: SFr. 118.—

INDEX

Tenner, C., Rácz, P., Mérő, E.: Significance of "Intracellular Parasitism" in Experimental Salmonella Cystitis 219

Pálfi, A. B.: Virus Content of Sewage in Different Seasons in Hungary 231

Serény, B., Tenner, C., Rácz, P.: Immunogenicity of Living Attenuated Shigellae 239

Weiszfeiler, G., Karasseva, V., Karczag, E.: A New *Mycobacterium* Species: *Mycobacterium asiaticum* n. sp. 247

Pellecuer, M., Galzy, P., Pasero, J.: Use of Mutations in Marking Industrial Yeast Strains 253

Losonczy, G., Tóth, L., Petrás, G., Bognár, S., Lányi, B., Csekes, J.: *Pseudomonas aeruginosa* Infections in an Artificial Respiratory Ward 261

D. Tóth, F., Karsai, T., Váczi, L.: Effects of Rauscher Leukaemia Virus on the Immune System in Susceptible and Resistant Mouse Strains 271

Szeri, H., Anderlik, P., Bános, S., Radnai, B.: Studies on Young Germ-free Mice. I. Stress Reaction of Germ-free Mice 279

Berek, L., Bános, S.: Studies on Young Germ-free Mice. II. Bone Changes in Germ-free Mice 283

Szathmáry, J., Hegedüs, L.: Effect of the Diluent on the Pock Count of Vaccinia Virus Dilutions 291

Rédai, I., Réthy, A., Sebessi-Gönczy, P., Váczi, L.: Lipids in *Staphylococcus aureus* and *Escherichia coli* Cultured in the Presence cf Human Serum 297

Ádám, M. M., Kontrohr, T., Horváth, E.: Serologieal Studies on *Pseudomonas aeruginosa* O Group Lipopolysaccharides 307

Némedi, L., Lányi, B.: Incidence and Hygienic Importance of *Pseudomonas aeruginosa* in Water 319

Fornosi, F., Tálas, M., Weiszfeiler, G.: Effect of Tilorone Hydrochloride on the Experimental Rabies of Mice 327

Books Received 333

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Zacsik Annamária
A kézirat nyomdába érkezett: 1972. V. 16. — Terjedelem: 10,50 (A/5) ív, 36 ábra

72.73856 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

THE JOURNAL OF GENERAL MICROBIOLOGY

Partial contents of Volume 71, Part 3. August 1972

C. A. VERMEULEN and C. VENEMA The Effect of Competence Regime on Competence, DNA Absorption and Integration of DNA in Cultures of *Bacillus subtilis*.

K. K. NIXDORFF and S. SCHLECHT Heterogeneity of the Haemagglutinin Responses to *Salmonella minnesota* R-Antigens in Rabbits.

I. J. BOUSFIELD A Taxonomic Study of Some Coryneform Bacteria.

M. M. TAYLOR *Eubacterium fissicatena* Sp. Nov. Isolated from the Alimentary Tract of the Goat.

Y. OKON, I. CHET and Y. RENIS Lactose-induced Synchronous Sclerotium Formation in *Sclerotium rolfsii* and Its Inhibition by Ethanol.

J. C. H. WESSELS and Y. KOLTIN R-Glucanase Activity and Susceptibility of Hyphal Walls to Degradation in Mutants of *Schizophyllum* with Disrupted Nuclear Migration.

M. R. EVANS and J. D. OWENS Factors Affecting the Concentration of Faecal Bacteria in Land-drainage Water.

P. N. PANDHI and C. PANOS Biosynthesis of D-Alanyl-D-Alanine from L-Alanine by Extracts of a Stabilized L-Form from *Streptococcus pyogenes*.

W. S. NG, J. E. SMITH and J. G. ANDERSON Changes in Carbon Catabolic Pathways During Synchronous Development of Conidiophores of *Aspergillus niger*.

P. J. WHITE The Nutrition of *Bacillus megaterium* and *Bacillus cereus*.

W. DE VRIES, W. M. C. VAN WIJCK-KAPTEIJN and A. H. STOUTHAMER Influence of Oxygen on Growth, Cytochrome Synthesis and Fermentation Pattern in Propionic Acid Bacteria.

A. MCVITTIE Flagellum Mutants of *Chlamydomonas reinhardii*.

M. ECCLESTON and D. P. KELLY Assimilation and Toxicity of Exogenous Amino Acids in the Methane-oxidizing Bacterium *Methylococcus capsulatus*.

F. Y. KAZAMA Ultrastructure and Phototaxis of the Zoospores of *Phlyctochytrium* Sp., an Estuarine Chytrid.

P. A. SWENSON, R. L. SCHENLEY and J. M. BOYLE Effects of 5-Fluorouracil and Pantoyl Lactone on Viability and Division of Ultraviolet-irradiated *Escherichia coli* FIL^{+} and FIL^{-}.

T. J. FOSTER and T. G. B. HOWE Chloramphenicol Acetyl Transferase Formed by Wild-type and Complementing R Factors in *Escherichia coli* k 12.

M. J. CARLILE The Lethal Interaction Following Plasmodial Fusion between Two Strains of the Myxomycete *Physarum polycephalum*.

Price £4.00 net ($12.00 in U.S.A.). Annual subscription £32 net ($106.00 in U.S.A.)

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London NWI 2DB
American Branch: 32 East 57th Street, New York, N.Y. 10022

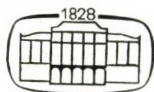
Proceedings of the Microbiological Research Group of the Hungarian Academy of Sciences

Vol. IV

edited by *J. G. Weiszfeiler*

The volumes of this series, published yearly or every other year, summarize the results of the Microbiological Research Group and publish the lectures of international conferences organized by the group. The present volume contains reports on interferon, on the improvement of antituberculous vaccine, on enzyme-inhibiting antibodies, etc. read at the Congress by French, English, Czechoslovakian, Danish, Soviet, Bulgarian and Hungarian research workers. These reports are, without exception, published in this volume for the first time.

In English · 146 pages · 42 figures · 53 tables
Paperback



AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
Budapest

ACTA MICROBIOLOGICA

ТОМ XVIII

РЕЗЮМЕ

MEASLES IMMUNITY IN INFANTS

Z. PALLINGER, G. PALLINGER-SZEKERES

ИММУНИТЕТ К КОРИ У ГРУДНЫХ ДЕТЕЙ

З. ПАЛЛИНГЕР, Г. ПАЛЛИНГЕР-СЕКЕРЕШ

Методом РПГА авторы исследовали 218 сывороток от детей младше 1 года на наличие антител к кори. На основе полученных результатов было установлено:

1. Уровень антител к кори до 5-месячного возраста быстро понижается, до 9-месячного возраста остается на низком уровне, затем опять повышается. Кривая возрастного распределения заболеваемости корью и результаты исследований полностью совпадают: число случаев заболеваемости корью в 5—8-месячном возрасте скачкообразно повышается.

2. Заслуживает внимания помесячное изменение среднего геометрического титра в РПГА у сывороток, полученных от отдельных возрастных групп в различные месяцы года.

SOME PROPERTIES OF THE MEMBRANE-BOUND PENICILLINASE OF BACILLUS CEREUS 569

V. CSÁNYI, I. MILE, I. FERENCZ, É. SZABÓ

НЕКОТОРЫЕ СВОЙСТВА МЕМБРАН-СВЯЗАННОЙ ПЕНИЦИЛЛИНАЗЫ

В. ЧАНЬИ, И. МИЛЕ, ИЛОНА ФЕРЕНЦ, ЕВА САБО

С помощью различных методик авторам удалось выделить из клеток 569/H штамма *B. cereus* три мембран-связанной пенициллиназы, отличающихся друг от друга по некоторым свойствам. Свойства одного из вариантов соответствовали таковым — пенициллиназы, выделенной *Pollock*. На основе экспериментальных данных излагаются выводы относительно механизма образования трех видов связанной пенициллиназы.

EFFECT OF UREA ON THE TWO IODINE INACTIVATION REACTIONS OF BACILLUS CEREUS PENICILLINASE

J. HAJDÚ, I. FERENCZ, I. MILE, V. CSÁNYI

ДЕЙСТВИЕ МОЧЕВИНЫ НА ДВЕ РЕАКЦИИ ЙОДНОЙ ИНАКТИВАЦИИ ПЕНИЦИЛЛИНАЗЫ B. CEREUS

Й. ХАЙДУ, ИЛОНА ФЕРЕНЦ, И. МИЛЕ, В. ЧАНЬИ

Мочевина стимулировала обе ступени реакции йодной инактивации пенициллиназы *B. cereus*, и степень стимуляции была пропорциональна концентрации мочевины. Предварительная инкубация пенициллиназы с 7,5 M мочевины в течение 30 минут изменяла условия йодной инактивации, так как обе реакции протекали при более низком значении pH, чем обычно. Комбинируя мочевину с метициллином — каждое из этих соединений действовало на одну из двух ступеней реакции — и обеспечивая их одновременное действие, можно было достичь практически полной инактивации при обеих изученных степенях pH.

INTERFERON AND ANTIBODY PRODUCTION IN INBRED MICE INFECTED WITH THE RAUSCHER VIRUS

F. D. TÓTH, L. VÁCZI, CL. BERENCSI

ПРОДУКЦИЯ ИНТЕРФЕРОНА И АНТИТЕЛ У INBRED-ШТАММА МЫШЕЙ, ЗАРАЖЕННЫХ ВИРУСОМ RAUSCHER-A

Ф. Д. ТОТ, Л. ВАЦИ, КЛАРА БЕРЕНЧИ

Авторы изучали спленомегалию и продукцию туморспецифических антител и интерферона у inbred-мышей Balb/c, DBA/I и C57BI/IO Sn, зараженных вирусом Rauscher-a. Была выявлена прямолинейная зависимость между резистентностью и степенью продукции антител. В случае умеренно резистентных мышей DBA/I вирус Rauscher-a оказался хорошим индуктором интерферона; у двух других штаммов продукция интерферона была незначительной.

RELATIONSHIP OF LYSOGENY AND MEGACINOGENY IN BACILLUS MEGATERIUM

V. GAÁL, J. MOLNÁR, G. IVÁNOVICS

ВЗАИМОСВЯЗЬ МЕЖДУ ЛИЗОГЕНИЕЙ И МЕГАЦИБОГЕНИЕЙ У BACILLUS MEGATERIUM

ВЕРА ГААЛ, Й. МОЛНАР, ДЬ. ИВАНОВИЧ

Клетки мегационогенного 216 штамма *Bacillus megaterium* были заражены host range фаговым мутантом, полученном из лизогенного штамма 899 I. На агарах, привитых этим материалом, возникшие клоны спор отличались друг от друга с точки зрения профага и мегацин-фактора. Под действием заражения фагом мегацин-фактор мог оказаться лабильным, и в результате этого он мог быть потерян. Такие немегациогенные сегреганты в то же время могли стать лизогенными, но профаг часто не стабилизировался в клетках, и таким способом некоторые изоляты с потерей мегацин-фактора не становились лизогенными. Если после заражения мегацин-фактор оставался, из него образовались лабильные или стабильные лизогенные дериваты. В стабильных изолятах, несущих оба плагмида, преобладает одна из фракций. Культуры этих дериватов растворялись под воздействием митомицина, и кинетика лизиса, а также количественная связь между возникшими продуктами, фагом и мегацином, была различной, соответственно преобладанию факторов. В отдельных изолятах мегацин-фактор полностью репрессировал индукцию профага. Таким образом, оба плазмида по-разному могут препятствовать репликации один-другого, а также индукции.

EFFECT OF PYRAZOCILLIN ON THE INDUCED PENICILLINASE SYNTHESIS OF BACILLUS CEREUS

I. MILE, V. CSÁNYI, I. KOCZKA, É. BADÁR, I. HORVÁTH

ДЕЙСТВИЕ ПИРАЗОЦИЛЛИНА НА СИНТЕЗ ИНДУЦИРОВАННОЙ ПЕНИЦИЛЛИНАЗЫ B. CEREUS

И. МИЛЕ, В. ЧАНЬИ, И. КОЦКА, ЕВА БАДАР, И. ХОРВАТ

Пиразоциллин (1—)2,6 — дихлорофенил (—4-метил-5-пиразолиновый пенициллин) в низкой степени индуцировал синтез пенициллиназы в клетках *B. cereus* 569. Его связь с пенициллинсвязывающим фактором клеток была низкой в противоположность таковой пенициллина, однако — как и в случае пенициллина — связь оказалась необратимой.

LYSOGENIC CONVERSION IN THE SHIGELLA FLEXNERI GROUP

I. KÉTYI, A. VERTÉNYI, I. FINANCSEK

ЛИЗОГЕННАЯ КОНВЕРСИЯ У ГРУППЫ SHIGELLA FLEXNERI

И. КЕТЬИ, АДЕЛ ВЕРТЕНЬИ, И. ФИНАНЧЕК

Из штамма 4в серотипа *Shigella flexneri* авторы выделили температурный фаг с антиген-конвертирующей способностью. Этот фаг, обозначенный P90, — за исключением серотипных репрезентантов, несущих IV антигенный комплекс, — во всех исследуемых штаммах, принадлежавших к различным серотипам, обусловливал антигенную конверсию, сопровождающуюся появлением специфичности IV_1. Антиген, фигурирующий в конвертантах, не идентичен с антигеном IV_1, а находится с ним в антигенной связи а, в — а, с.

В пробах нейтрализации фаг P90 оказался серологически идентичным с фагом Ø IV_1, располагающим подобной конвертирующей способностью. Несмотря на это, между этими двумя фагами нет перекрестной иммунной реакции, и может быть индуцирован двойной лизогенный дериват.

THE EFFECT OF THEOPHYLLINE UPON INDUCED β-GALACTOSIDASE SYNTHESIS IN ESCHERICHIA COLI

J. SCHLAMMADINGER, G. SZABÓ

ДЕЙСТВИЕ ТЕОФИЛЛИНА НА ИНДУЦИРОВАННЫЙ СИНТЕЗ ГАЛЛАКТОЗИДАЗЫ В ESCHERICHIA COLI

Й. ШЛАММАДИНГЕР, Г. САБО

Авторы установили, что теофиллин в концентрации 3×10^{-3} M и выше подавляет синтез β-галактозидазы в полностью индуцированных клетках ***Escherichia coli*** К 12 в зависимости от используемой концентрации. Предполагается «конкуренция» между теофиллином и циклическим АМФ.

EFFECT OF VARIOUS DILUENTS ON STABILITY OF VACCINIA VIRUS DILUTIONS

J. SZATHMÁRY, L. HEGEDÜS, M. KOLLER

ВЛИЯНИЕ РАЗЛИЧНЫХ РАСТВОРОВ НА СТАБИЛЬНОСТЬ РАЗВЕДЕНИЙ ВИРУСА ОСПЕННОЙ ВАКЦИНЫ

Й. САТМАРИ, Л. ХЕГЕДЮШ, М. КОЛЛЕР

В 10^{-5}—10^{-7} разведениях вируса вакцины, проведенных с различными растворами и содержашихся при 37°С, наблюдалось понижение разной степени в числе оспин образующих единиц (ООЕ). Наиболее стабильными оказались разведения, содержавшие сывороточный белок и, особенно, обезжиренное молоко.

Не удалось обнаружить связь между содержанием в вирусных суспензиях бактерий, тканевых детритов и стабильностью различных разведений.

Многоступенчатое разведение вируса вакцины в буферном растворе, в составе которого находилось обезжиренное молоко, давало более высокие ООЕ, чем таковые при разведениях только в одном буфере.

WATER LIMITED REACTION OF BACILLUS CEREUS PENICILLINASE IN NONAQUEOUS SOLVENT

V. CSÁNYI, I. MILE, É. SZABÓ, GY. HARMATH, I. FERENCZ

РЕАКЦИЯ ПЕНИЦИЛЛИНАЗЫ B. CEREUS, ОГРАНИЧЕННАЯ ВОДОЙ. В БЕЗВОДНОМ РАСТВОРЕ

В. ЧАНЬИ, И. МИЛЕ, ЕВА САБО, ДЬ. ХАРМАТ, ИЛОНА ФЕРЕНЦ

Было установлено, что пенициллиназа *B. cereus* может храниться в глицероле без денатурации. Авторы изучали некоторые характерные свойства реакции, катализируемой пенициллиназой, в глицероле с низким содержанием воды. Реакция на уровне насыщения субстрата зависела от содержания воды и энзима.

CORRELATION BETWEEN THE MORPHOLOGICAL VARIABILITY AND RIBOFLAVIN PRODUCING CAPACITY OF EREMOTHECIUM ASHBYII

T. VÁGHY

ВЗАИМОСВЯЗЬ МЕЖДУ МОРФОЛОГИЧЕСКОЙ ИЗМЕНЧИВОСТЬЮ EREMOTHECIUM ASHBYII И СПОСОБНОСТЬЮ ПРОДУЦИРОВАТЬ РИБОФЛАВИН

Т. ВАГИ

Eremothecium ashbyii является генетически лабильным, склонным к спонтанным мутациям грибом. Для установления взаимосвязи между внешним проявлением (с особым вниманием на интенсивность роста, форму, цвет и салтацию) и продуцирующей способностью было проведено изучение 3484 колоний, каждой по отдельности. На основе полученных результатов удалось выбрать идеальный тип колонии (со средней интенсивностью роста, выраженной хромогенностью; не дает изменений в секторе, гладкий, с поверхностью, покрытой воздушным мицелием), который располагает наиболее высокой синтетизирующей способностью.

PHAGE INFECTION OF VITAMIN B_{12} PRODUCING RHIZOBIUM CULTURES

J. SZABÓ SZÜCS

ФАГОВАЯ ИНФЕКЦИЯ КУЛЬТУР RHIZOBIUM, ПРОДУЦИРУЮЩИХ ВИТАМИН B_{12}

Й. САБО С ЮЧ

В процессе выращивания одного старого лабораторного штамма *Rhizobium melilotii* с B_{12}-витаминной активностью автор выделил два фаговых штамма из ферментов с пониженным содержанием действующего вещества. Они отличались между собой по размерам бляшек, бактериальному содержанию и антигенному действию и могли быть успешно использованы для отбора фагорезистентных мутантов Rhizobium высокой B_{12}-витаминной активности. У отобранных бактериальных мутантов была выявлена связь между B_{12}-витаминной активностью и способностью продуцировать капсулу.

BACTERIOLOGIC DIAGNOSIS OF YERSINIA PSEUDOTUBERCULOSIS

J. SZITA, A. SVIDRÓ

БАКТЕРИАЛЬНАЯ ДИАГНОСТИКА YERSINIA PSEUDOTUBERCULOSIS

Й. СИТА, АННА ШВИДРО

1. Было исследовано 6 штаммов *Yersinia pseudotuberculosis:* 5 выделены из лимфатических узлов страдающих болезнью Masshoff-а и один — из удаленного аппендикса.

2. Устойчивость штаммов к нагреванию и действию дезинфицирующих средств была подобна таковой кишечных бактерий.

3. 5 штаммов принадлежали к серотипу IA, 1 штамм — к серотипу IB. Все штаммы проявляли единые биохимические свойства и соответствовали описанным в литературе характеристикам.

4. С диагностической целью методом агглютинации были исследованы 1214 присланных сывороток и 400 сывороток от здоровых лиц (контроль). Титр 1 : 100 считается сомнительным, 1 : 200 и выше нужно рассматривать как положительный.

PHAGE TYPING OF D-GROUP STREPTOCOCCI. I. TYPING OF ENTEROCOCCI WITH ROUMANIAN PHAGES

V. HOCH, G. HÉRMÁN

ЗНАЧЕНИЕ В ГИГИЕНЕ ПИТАНИЯ ФАГ-ТИПИРОВАНИЯ СТРЕПТОКОККОВ ГРУППЫ Д

1. ТИПИРОВАНИЕ ЭНТЕРОКОККОВЫХ ШТАММОВ РУМЫНСКИМ НАБОРОМ ФАГОВ

ВИОЛА ХОХ, Г. ХЕРМАН

Опираясь на результаты исследований заграничных авторов при изучении пищевых отравлений, вызванных энтерококками, с целью эпидемиологического определения условий возникновения отравлений было введено фаг-типирование. Из материала при пищевых отравлениях и проб пищи всего было выделено 557 штаммов, относящихся в серогруппу Д; из них 53% удалось типировать румынскими фагами. С помощью собственного дополнительного набора фагов авторы стремятся увеличить число типируемых штаммов.

PHAGE TYPING OF D-GROUP STREPTOCOCCI. II. ISOLATION OF SUPPLEMENTARY PHAGES FOR CLASSIFICATION OF ENTEROCOCCI UNTYPABLE WITH ROUMANIAN PHAGES

G. HÉRMÁN, V. HOCH

ЗНАЧЕНИЕ В ГИГИЕНЕ ПИТАНИЯ ФАГ-ТИПИРОВАНИЯ СТРЕПТОКОККОВ ГРУППЫ Д

II. ДОПОЛНИТЕЛЬНОЕ ТИПИРОВАНИЕ СОБСТВЕННЫМИ ФАГАМИ СТРЕПТОКОККОВЫХ ШТАММОВ ГРУППЫ Д, НЕ ТИПИРУЕМЫХ РУМЫНСКИМ НАБОРОМ ФАГОВ

Г. ХЕРМАН, ВИОЛА ХОХ

Набором фагов собственного происхождения в 42,9% случаев авторам удалось типировать штаммы, не типируемые румынским набором фагов. Эффективность типирования нетипируемых штаммов была повышена — одновременно с фаг-типированием — температурной обработкой. Собственный набор фагов был приготовлен путем высвобождения фагов с помощью УФ индукции из штаммов, выделенных авторами, и вирулентных штаммов, выдегенных из сточных вод. Лабильность типирующих фагов была понижена добавлением 0,01 М сульфатной магнезии, 10% человеческой сыворотки и 1 М сахарозы, а также изменением pH в кислом направлении. В модельном опыте была изучена возможность повышения температурной чувствительности и резистентности одного из типирующихся фагов.

ATTEMPTS TO DETECT THE PRESENCE OF TEICHOIC ACID IN BACILLUS ANTHRACIS

J. MOLNÁR, B. PRÁGAI

ПОПЫТКА ОПРЕДЕЛЕНИЯ НАЛИЧИЯ TEICHOIC КИСЛОТЫ В BACILLUS ANTHRACIS

Й. МОЛНАР, Б. ПРАГАИ

Применяя различные методы, не удалось выявить наличия teichoic кислоты в клеточных стенках двух штаммов *Bacillus anthracis* и аденин-дефицитного мутанта одного из штаммов. Другими авторами ранее было установлено отсутствие teichoic кислоты в случае *Bacillus cereus*. О дальнейшем филогенетическом родстве между двумя похожими видами *B. anthracis* и *B. cereus*, свидетельствует отсутствие полифосфатов в клеточных стенках.

EFFECT OF INTERFERON TREATMENT ON THE TUMOUR-SPECIFIC ANTIBODY RESPONSE OF BALB/c MICE INFECTED WITH RAUSCHER VIRUS

F. D. TÓTH, L. VÁCZI, CL. BERENCSI

ЭФФЕКТ ИНТЕРФЕРОННОЙ ОБРАБОТКИ НА ПОЯВЛЕНИЕ ТУМОР-СПЕЦИФИЧЕСКИХ АНТИТЕЛ У МЫШЕЙ BALB/c, ЗАРАЖЕННЫХ ВИРУСОМ RAUSCHER-A

Ф. Д. ТОТ, Л. ВАЦИ, КЛАРА БЕРЕНЧИ

Введением экзогенного интерферона можно было замедлить развитие спленомегалии у мышей BALB/c, зараженных вирусом Rauscher-а. У животных, получивших интерферон, можно наблюдать значительное усиление тумор-специфического иммунного ответа, который по времени опережает замедление прогрессии лейкемии. Защитное действие интерферона можно объяснить тем, что он подавляет заражение и злокачественную трансформацию иммункомпетентных прекурзоров-клеток.

SEROLOGICAL DIAGNOSIS OF YERSINIA ENTEROCOLITICA

J. SZITA, M. KÁLI, B. RÉDEY

СЕРОЛОГИЧЕСКАЯ ДИАГНОСТИКА YERSINIA ENTEROCOLITICA

Й. СИТА, МАРГИТ КАЛИ, Б. РЕДЕИ

1. В целях определения распространения в Венгрии заболеваний, вызванных *Yersinia enterocolitica*, по схеме Winblad-а были приготовлены фактор-сыворотки.

2. На основе серотипизации 41 присланного штамма и штаммов, выделенных авторами, делается предположение, что 03 тип *Yersinia enterocolitica* встречается чаще, чем 09 тип. Первый тип был выделен из фекалий 15 зараженных лиц, другой — от 3 лиц.

3. Исходя из средних титров реакции агглютинации типа Widal-а, полученных с сыворотками, присланными с диагностической целью от 249 больных и от 1500 здоровых лиц (контроль), было установлено, что — в соответствии с литературными данными — в Венгрии титр 1 : 40 может рассматриваться как сомнительный, а титр 1 : 80 — положительный.

4. Принимая во внимание серологическую связь между 09 типом *Y. enterocolitica* и *Brucella abortus*, в случае положительной реакции агглютинации с 09 антигеном важно поставить реакцию агглютинации типа Widal-а и с антигеном *Brucella abortus*. При положительном результате с обеими антигенами сыворотку надо истощить, и одновременно с серологическими процедурами необходимо пытаться выявить и сам патогенный агент.

TWENTY YEARS OBSERVATION ON THE STABILITY OF SALMONELLA TYPHI PHAGE TYPES

G. HÉRMÁN, H. MILCH

РЕЗУЛЬТАТЫ 20-ЛЕТНЕГО НАБЛЮДЕНИЯ ОТНОСИТЕЛЬНО СТАБИЛЬНОСТИ ФАГОВЫХ ТИПОВ ШТАММОВ SALMONELLA TYPHI, ВЫДЕЛЕННЫХ ОТ БАКТЕРИОНОСИТЕЛЕЙ И БАКТЕРИОВЫДЕЛИТЕЛЕЙ В ВЕНГРИИ

Г. ХЕРМАН, ХЕДДА МИЛХ

Авторы исследовали штаммы *Salmonella typhi*, выделенные от 3776 бактерионосителей и бактериовыделителей при однократном и повторных выделениях. Была установлена частота встречаемости фаговых типов в Венгрии. У большинства фаговых типов часто можно было наблюдать потерю поверхностного антигена Vi, однако в отношении более часто встречающихся фаговых типов — среди выделителей штаммов фагового типа А, $Д_1$, E_{1a} чаще обнаруживалось появление Vi — отрицательного штамма, чем у выделителей штаммов фаговых типов C_1 или F_1. У 15,9% больных встречался другой фаговый тип или наблюдалось появление изменений в антигенной структуре, влияющих на типирование. Появление штаммов нового фагового типа у одного и того же больного в большинстве случаев может быть объяснено заражением штаммами различных фаговых типов, а в меньшем — изменением фагового типа.

A NEW SALMONELLA SEROTYPE: SALMONELLA ISASZEG (48 : z_{10} : *e*, *n*, *x*)

M. M. ÁDÁM, S. DEÁK

НОВЫЙ СЕРОТИП САЛМОНЕЛЛ: SALMONELLA ISASZEG (48 : z_{10} : e, n, x)

МАРИЯ М. АДАМ, ЖУЖАННА ДЕАК

Описывается новый субродовой 1 серотип Salmonellae: S. isaszeg (48 : z_{10} : e, n, x) выделенный из поверхностной воды.

IRREVERSIBLE ADSORPTION OF EXOPENICILLINASE ON BACILLUS CEREUS 569 CELLS

V. CSÁNYI, I. MILE, T. ROMHÁNYI, P. HORTOBÁGYI, F. HATFALUDI

НЕОБРАТИМАЯ АДСОРБЦИЯ ЭКЗОПЕНИЦИЛЛИНАЗЫ НА КЛЕТКИ B. CEREUS 569

В. ЧАНЬИ, И. МИЛЕ, Т. РОМХАНЬИ, П. ХОРТОБАДЬИ, Ф. ХАТФАЛУДИ

При соответствующих экспериментальных условиях небольшая часть экзопенициллиназы способна необратимо адсорбироваться на поверхность клеток *B. cereus* и других адсорбентов. Авторы изучали наиболее благоприятные экспериментальные условия, необходимые для возникновения необратимой адсорбции. Было найдено, что под действием средств, сильно влияющих на конформацию, во фракции пенициллиназы, не проявляющей необратимой адсорбции, может появиться форма, способная к адсорбции.

Необратимо адсорбированная пенициллиназа при инкубации в сильной щелочи может перейти в раствор. Иммунологические исследования γ-пенициллиназы, возникшей в течение индукции, а также необратимо адсорбированной экзопенициллиназы показали, что между обоими энзимами имеется сходство большой степени. На основе полученных данных авторы далее развили ранее выработанную ими рабочую гипотезу об образовании связанного энзима.

SOME EPIDEMIOLOGIC PROBLEMS OF HUMAN LISTERIOSIS IN HUNGARY

E. MÉRŐ, B. RALOVICH

НЕКОТОРЫЕ ЭПИДЕМИОЛОГИЧЕСКИЕ ПРОБЛЕМЫ ЧЕЛОВЕЧЕСКОГО ЛИСТЕРИОЗА

Э. МЕРЁ, Б. РАЛОВИЧ

С целью определения частоты встречаемости *Listeria monocytogenes* в Венгрии авторы провели исследования фекальных проб от здоровых работников бойни, здоровых лиц других профессий, здоровых беременных жинщин и родильниц и, наконец, от больных гастроэнтеритом.

Выращивание проводилось при $+4°C$ в питательной среде Holman-а и на агаре селективного характера, содержащем трипафлавин, налидиксовую кислоту и бычью сыворотку.

Из 3900 исследованных проб в 3,5% удалось выделить ***Listeria monocytogenes***, штаммы принадлежали к серотипам 1,3 и 4, а часть из них оказывалась не типирующейся. Полученные результаты показывают, что в Венгрии может быть выявлено большое число носителей человеческой ***Listeria monocytogenes*** и с ними, как источниками заражения, безусловно нужно считаться.

PHAGE TYPING OF MYCOBACTERIUM TUBERCULOSIS STRAINS ISOLATED IN HUNGARY

E. VANDRA, T. FODOR

ФАГОТИПИРОВАНИЕ ШТАММОВ MYCOBACTERIUM TUBERCULOSIS, ВЫДЕЛЕННЫХ В ВЕНГРИИ

Э. ВАНДРА, Т. ФОДОР

Авторы изучали распределение по фаговым типам 152 штаммов ***M. tuberculosis*** используя фаги DS6A, GS4E, BGI, D—34 и BKI. Встречаемость штаммов — соответственно группированию по Bates и сотр. — была следующей: A, 8,5; B, 16,5; C, 0 и промежуточные — 75%.

BACTERIAL CARDIOLIPINS AND THEIR SEROLOGICAL ACTIVITY

L. VÁCZI, M. SURJÁN, M. J. KISS, G. FÜST, I. RÉDAI

БАКТЕРИАЛЬНЫЕ КАРДИОЛИПИНЫ И ИХ СЕРОЛОГИЧЕСКАЯ АКТИВНОСТЬ

Л. ВАЦИ, МАРГАРИТА ШУРЬЯН, Й. М. КИШШ, ДЬ. ФЮШТ, И. РЕДАИ

Было проведено определение содержания общих липидов и кардиолипинов у штаммов ***Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Clostridium novyi*** и ***Clostridium tetani.*** Содержание кардиолипинов, отнесенное к сухому материалу упомянутых бактерий, колебалось между 0,2 и 0,7%, общие липиды были представлены 3,1—9,5%. При изменении условий выращивания изменяется общелипидное и общефосфатидное содержание, но количество кардиолипинов остается неизмененным.

Кардиолипины различного вида бактерий не только количественно, но и качественно отличаются между собой: расхождения, наблюдаемые в пропорции глицерин-фосфор, указывают и на структуральную разницу.

Серологическая активность препаратов бактериальных кардиолипинов по чувствительности и специфичности не уступает международным стандартным препаратам. Повторенные через год исследования показали, что препараты стабильны.

SIGNIFICANCE OF THE "EPITHELIAL PHASE" IN EXPERIMENTAL SALMONELLA CONJUNCTIVITIS

C. TENNER, P. RÁCZ, B. RÉDEY

ЗНАЧЕНИЕ «ЭПИТЕЛИАЛЬНОЙ ФАЗЫ» В ЭКСПЕРИМЕНТАЛЬНОМ САЛМОНЕЛЛЁЗНОМ КОНЪЮНКТИВИТЕ

КЛАРА ТЕННЕР, П. РАЦ, Б. РЕДЕИ

При введении в количестве 10^9 различных штаммов *S. typhi murium* и *S. enteridis* в конъюнктивальный мешок морских свинок у последних возникал конъюнктивит с характерным течением. Формирование инфекционного процесса было прослежено гистологически до 28 дня опыта. Авторы нашли, что проникновению штаммов салмонелл в макрофагного типа клетки, находящиеся в более глубоких частях организма, предшествует «эпителиальная фаза». Во время этой фазы часть бактерий не только просто проходит между эпителиальными клетками конъюнктивы, но и внедряется в них и там размножается.

Сравнив морфогенез салмонеллёзного конъюнктивита и шигеллёзного кератоконъюнктивита, авторы установили, что при салмонеллёзном конъюнктивите за короткой «эпителиальной фазой» следует продолжительная «макрофагная фаза» — когда центр инфекционного процесса из эпителиальных областей переходит в клетки типа макрофагов,— тогда как по ходу шигеллёзного кератоконъюнктивита шигеллы в течение всего времени болезни находятся в эпителальных клетках конъюнктивы и роговицы; только в исключительных случаях их можно найти в клетках типа макрофагов, но и тогда их пребывание там кратковременно. Эта разница связана с основными биологическими особенностями обоих патогенных агентов.

SIGNIFICANCE OF "INTRACELLULAR PARASITISM" IN EXPERIMENTAL SHIGELLA CYSTITIS

C. TENNER, P. RÁCZ, B. SERÉNY

ЗНАЧЕНИЕ «ВНУТРИКЛЕТОЧНОГО ПАРАЗИТИЗМА» В ЭКСПЕРИМЕНТАЛЬНОМ ШИГЕЛЛЁЗНОМ ЦИСТИТЕ

КЛАРА ТЕННЕР, Р. РАЦ, Б. ШЕРЕНЬ

Введением в мочевой пузырь *Shigella flexneri* 3a в количестве 2×10^8, а в другой серии опыта — такого же количества *Shigella sonnei* авторы вызывали цистит с обычным течением.

Подробное гистологическое изучение шигеллёз-модели **Bingel**-а в отношении биологических особенностей шигелл привело к тем же самым результатам, как и при гистологической обработке шигеллёзного кератоконъюнктивита: шигеллы могут проникнуть в эпителиальные клетки и размножаться в них, не оказывая сильного цитотоксического действия, в течение всей инфекции шигеллы остаются в пределах эпителиального барьера, полиморфноядерные лейкоциты уничтожают их — при этом высвобождаются токсические вещества, которые повреждают эпителиальные клетки, — только в редких случаях обнаруживаются в цитоплазме клеток типа макрофагов.

По ходу процесса морфогенез язв, образующихся на поверхности мочевого пузыря, напоминает явления в эпителии конъюнктивы при шигеллёзном кератоконъюнктивите: изъявление носит очаговый характер, возникая в нескольких эпителиальных клетках; очаги постепенно увеличиваются и могут слиться. На месте инфекционного процесса образуются общирные эпителиальные дефекты.

Сходство явлений, полученных на модели в двух различных опытах указывает на то, что речь идет об основных биологических свойствах шигелл.

INDIA-INK IMMUNO-REACTION FOR THE RAPID DETECTION OF ENTERIC PATHOGENS

P. GECK

ИММУННАЯ ТУШЬ—РЕАКЦИЯ ДЛЯ БЫСТРОГО ОПРЕДЕЛЕНИЯ ЭНТЕРАЛЬНЫХ ПАТОГЕННЫХ АГЕНТОВ

П. ГЕК

Автор разработал новую серодиагностическую методику для быстрой идентификации патогенных микробов. Новый метод, иммунная тушь—реакция, в опытах на модели был сравнен с классическим методом выращивания для быстрой идентификации *E. coli dyspepsiae*, *Shigella* и *Salmonella*. Сравнительные исследования, проведенные параллельно, показали, что чувствительность иммунной тушь—реакции практически совпадает с таковой выращивания, а её специфичность — со специфичностью иммунфлуоресцентной техники. Цель разработки новой методики — замена иммунфлуоресцентной техники в бактериологической диагностике.

Преимущество иммунной тушь-реакции заключается в том, что для окраски мазков можно применять жидкую китайскую тушь, а для оценки результатов — обычный световой микроскоп. Окрашивание продолжается 5 минут и объединяет в себе примущества прямой, непрямой и модифицированной процедуры окрашивания.

LA RECHERCHE DES SHIGELLA DANS LES EAUX PAR LE TEST DES ANTICORPS FLUORESCENTS ET PAR LA RÉACTION DE GECK

N. D. DUC

ВЫЯВЛЕНИЕ ШИГЕЛЛ В ВОДНЫХ ПРОБАХ МЕТОДОМ ФЛЮОРЕСЦЕНТНЫХ АНТИТЕЛ И РЕАКЦИЕЙ ГЕКК-А

НГУЙЕН ДАНГ ДЮК

Автор провел в Венгрии сравнительное изучение относительно применения флюоресцентных антител и реакции Гекк-а для выявления шигелл из проб воды различного происхождения. Были исследованы осадки, фильтраты и обогащенные культуры 60 водных проб. Методом флюоресцентных антител удалось выявить четыре серотипа шигелл у 38,3% исследованных проб воды, реакция Гекк-а оказалась положительной в 41,1%, а метод выращивания показал положительный результат в 3,3%. Положительные результаты, полученные тремя методами, полностью совпадали. Реакция Гекк-а по сравнению с методом флюоресцентных антител и с выращиванием, является более чувствительным специфическим и быстрым процессом, дешёвым и — особенно с эпидемиологической точки зрения — важным методом, который очень быстро может быть введен в любой бактериологической лаборатории.

SEROLOGICAL GROUPING OF PSEUDOMONAS TYPING PHAGES

J. LANTOS

СЕРОЛОГИЧЕСКАЯ ГРУППИРОВКА ТИПИРУЮЩИХСЯ ФАГОВ PSEUDOMONAS

ЮДИТ ЛАНТОШ

Была проведена серологическая группировка набора стандартных фагов, используемых для типирования штаммов **Pseudomonas**. К 17 фагам была приготовлена антифаговая сыворотка, и фаги были распределены в 10 групп.

Серологическое родство удалось подтвердить и искусственной лизогенией модельных штаммов. Фаговая картина штаммов, полученная таким образом, изменилась, и возник иммунитет к фагам, относящимся в одну и ту же серогруппу (за исключением группы Б).

HOST-SPECIFICITY TYPES IN SHIGELLA FLEXNERI

I. KÉTYI, A. VERTÉNYI

ХОЗЯИННАЯ СПЕЦИФИЧНОСТЬ SHIGELLA FLEXNERI

И. КЕТЬИ, А. ВЕРТЕНЬИ

С помощью модифицированного фагового лизата T5 авторы исследовали 179 штаммов ***Shigella flexneri***, относящихся к серотипам 1a, 1в, 2a, 3 и 6, с точки зрения специфичности клетки-хозяина. Были выявлены два «типа специфичности клетки-хозяина», которые оказались независимыми от антигенной структуры штаммов.

Обсуждается возможная ценность этого явления в таксономии.

SIGNIFICANCE OF "INTRACELLULAR PARASITISM" IN EXPERIMENTAL SALMONELLA-CYSTITIS

C. TENNER, P. RÁCZ, E. MÉRŐ

ЗНАЧЕНИЕ «ВНУТРИКЛЕТОЧНОГО ПАРАЗИТИЗМА» В ЭКСПЕРИМЕНТАЛЬНОМ САЛМОНЕЛЛЕЗНОМ ЦИСТИТЕ

КЛАРА ТЕННЕР, П. РАЦ, Е. МЕРЁ

Авторы провели подробное гистологическое изучение салмонеллёзного чистита, описанного **Bingel**. Было установлено, что в начальный период процесса («эпителиальная фаза») микробы проникают в эпителиальные клетки мочевого пузыря и там проделывают весь цикл развития. Вслед за этим и отчасти параллельно этому салмонеллы проникают в подэпителиальный слой, где размножаются в цитоплазме клеток типа макрофагов. Дегенеративные бактериальные формы можно было наблюдать в основном в цитоплазме полиморфноядерных лейкоцитов, из чего авторы делают вывод, что по ходу воспаления в основном полиморфноядерные лейкоциты уничтожают салмонеллы. Часто в процессе воспаления образовывалась флегмона. Исследование других органов подтвердило генерализацию процесса.

VIRUS CONTENT OF SEWAGE IN DIFFERENT SEASONS IN HUNGARY

A. B. PÁLFI

ВСТРЕЧАЕМОСТЬ ЭНТЕРОВИРУСОВ В СТОЧНЫХ ВОДАХ ПО СЕЗОНАМ

АГНЕШ Б. ПАЛФИ

С целью получения данных относительно циркуляции в популяции вирусных типов по сезонам в 1969 году было проведено вирусологическое исследование 336 проб сточных вод. Из 317 выделенных вирусных штаммов 62 оказались полиовирусом вакционного происхождения. Остальные 255 штаммов были представлены реовирусом 1 типа в 43%, эховирусом 7 типа в 21%, эховирусом 11 типа в 18%; 18% составляли вирусы коксаки-Б 1,3,4 типов, а также эховирусы, принадлежавшие к 1,6,12,14,19 и 20 типам. Наибольшей постоянностью в циркуляции обладал реовирус 1 типа, а также эховирусы 7 и 11 типов. Большую часть штаммов выделили в августе, за этим следовали октябрь, ноябрь и сентябрь. Самая низкая разновидность штаммов наблюдалась в феврале-марте, что совпадает с периодом массивного рассеивания, следующего за полиовакцинацией. Сравнив выделенные типы с таковыми соответствующего периода 1968 года, можно установить, что реовирус 1 типа, вирусы коксаки-Б 1,3 типов, а также эховирусы типов 6 и 7 в течение обоих лет встречались в один и тот же сезон.

IMMUNOGENICITY OF LIVING ATTENUATED SHIGELLAE

B. SERÉNY, C. TENNER, P. RÁCZ

ИММУНОГЕННОСТЬ ЖИВЫХ АТТЕНУИРОВАННЫХ ШИГЕЛЛ

Б. ШЕРЕНЬ, КЛАРА ТЕННЕР, П. РАЦ

Штаммы вирулентных шигелл выращивались на питательных средах, содержавших краску; морфология колоний субкультур была изучена при косом освещении под различным углом. Экспериментальное введение в конъюнктиву морских свинок полученных таким образом вариантов делало возможным отбор большого количества авирулентных вариантов. Эти варианты, как показывают опыты на модели шигеллёзного кератоконъюнктивита, обладают различной иммуногенностью. Предварительное введение вариантов с наибольшей иммуногенной способностью защищало 50% животных при последующем заражении их культурой *Sh. flexneri* гомологичного или гетерологичного типа.

A NEW MYCOBACTERIUM SPECIES: MYCOBACTERIUM ASIATICUM n. sp.

G. WEISZFEILER, V. KARASSEVA, E. KARCZAG

НОВЫЙ ВИД МИКОБАКТЕРИИ: M. ASIATICUM

ДЬ. ВЕЙСФЕЙЛЕР, ВАЛЕНТИНА КАРАСЁВА, ЭРИКА КАРЦАГ

Из 18 штаммов, выделенных от обезьян как *Mycobacterium simiae*, 4 медленнорастущих штамма оказались новым фотохромогенным видом, названным *Mycobacterium asiaticum*, который факультативно патогенен, является вирулентным для мышей и как по своим биохимическим так и по антигенной структуре и типу сензитина отличается от *M. simiae*.

USE OF MUTATIONS IN MARKING INDUSTRIAL YEAST STRAINS

M. PELLECUER, P. GALZY, J. PASERO

ПРИМЕНЕНИЕ МУТАНТОВ ПРОМЫШЛЕННЫХ ДРОЖЖЕВЫХ ШТАММОВ-МАРКЕРОВ

МОНИКА ПЕЛЛЕКЮЭР, П. ГАЛЗИ, Й. ПАСЕРО

Авторы исследовали два штамма дрожжей, *Candida tropicalis*, способных расти на гидрокарбонах, и *Saccharomyces cerevisiae*, культивированный на глюкозе, на их резистентность к некоторым антибиотикам и ионам, считающимся токсичными для дрожжей. Была сделана попытка проверить актидион и канаванин, но самые лучшие результаты были получены с кадмиумом, кобальтом, медью и арсенатом. Представилось возможным выделить ион-резистентные мутанты, которые затем могли быть использованы как штаммы-маркеры, чтобы проследить за поведением данного организма в относительно смешанной популяции промышленной культуры. Предварительные результаты показали, что в условиях эксперимента эта мутация не нарушала выживаемости организмов.

PSEUDOMONAS AERUGINOSA INFECTIONS IN AN ARTIFICIAL RESPIRATORY WARD

G. LOSONCZY, L. TÓTH, G. PETRÁS, S. BOGNÁR, B. LÁNYI, J. CSEKES

ИНФЕКЦИЯ PSEUDOMONAS AERUGINOSA В ПАЛАТЕ ИСКУССТВЕННОГО ДЫХАНИЯ

ДЬ. ЛОШОНЦИ, Л. ТОТ, ДЬ. ПЕТРАШ, Ш. БОГНАР, Б. ЛАНЬИ, ЮДИТ ЧЕКЕШ

Авторы в течение двухлетнего периода провели изучение в отношении 156 больных, показавшее, что в палате искусственного дыхания единичные и множественные ятрогенные инфекции были обычным явлением (255 случаев у 101 больного) и менее часто встречались

среди больных, у которых искусственное дыхание не применялось (26 случаев у 55 больных). Смертельные случаи наблюдались только среди больных с искусственным дыханием (37 умерло от первоначальной болезни, 18 — от ятрогенной инфекции). Особенно часто поражались больные со столбняком.

Из большинства случаев инфекций среди больных, у которых применялось искусственное дыхание, удалось выделить ***Pseudomonas aeruginosa*** в 40 случаях из 65 моноинфекций и в 46 случаях из 55 смешанных инфекций). В остальную часть инфеккций были вовлечены ***Klebsiella, Escherichia coli, Staphylococcus aureus, Proteus.*** Серотипизация ***Ps. aeruginosa*** показала, что большинство инфекций было вызвано штаммами, принадлежащими к трем различным серогруппам (04*a*, 4*d*, 07*a*, 7в и 03*a*, 3*d*). Те же самые серогруппы были выделены из окружения и от сотрудников. В статье обсуждаются пути заражения и профилактические мероприятия.

EFFECTS OF RAUSCHER LEUKAEMIA VIRUS ON THE IMMUNE SYSTEM IN SUSCEPTIBLE AND RESISTANT MOUSE STRAINS

F. D. TÓTH, T. KARSAI, L. VÁCZI

ДЕЙСТВИЕ ВИРУСА ЛЕЙКЕМИИ RAUSCHER-А НА ИММУНСИСТЕМУ ЧУВСТВИТЕЛЬНЫХ И РЕЗИСТЕНТНЫХ ШТАММОВ МЫШЕЙ

Ф. Д. ТОТ, Т. КАРШАИ, Л. ВАЦИ

Заражение мышиных штаммов **BALB/c, DBA/I** и **C57BI/IO Sn** вирусом **Rauscher**-а вызывало увеличение количества α_1, α_2, и β_2 глобулинов пропорционально степени чувствительности данного мышиного штамма. Уровень гаммаглобулина у мышей **BALB/c** и **DBA/I** падает параллельно с развитием лейкемии, в то время как у мышиного штамма **C57BI/I0 Sn** он повышается и остается на уровне, превышающем нормальные величины. Эти изменения могут быть объяснены отчасти действием вируса **Rauscher**-а на иммунсистему, отчасти появлением новых белков, продуцируемых опухолевыми клетками. Степень размножения вируса **Rauscher**-а в лимфатических узлах параллельна чувствительности штаммов мышей и вызывает пропорциональное понижение в продукции иммунспецифического глобулина.

STUDIES ON YOUNG GERM-FREE MICE. I. STRESS REACTION OF GERM-FREE MICE

H. SZERI, P. ANDERLIK, S. BÁNOS, B. RADNAI

ОПЫТЫ НА МОЛОДЫХ GERM-FREE МЫШАХ I. ШТРЕСС-РЕАКЦИЯ GERM-FRE МЫШЕЙ

ХЕЛЕН СЕРИ, ПИРОШКА АНДЕРЛИК, ЖУЖАННА БАНОШ, Б. РАДНАИ

Ответ **germ-free** мышей на холодный штресс отличался от такового обычных мышей в контроле. У **germ-free** мышей наблюдалась повышенная чувствительность к холоду, и вслед за штрессом отсутствовали лимфопеническая реакция и уменьшение веса лимфатических органов. Эти нарушения подобны тем, которые были ранее описаны в отношении обычных мышей после воздействий, повреждающих их лимфоидную систему.

STUDIES ON YOUNG GERM-FREE MICE. II. BONE CHANGES IN GERM-FREE MICE

L. BEREK, S. BÁNOS

ОПЫТЫ НА МОЛОДЫХ GERM-FREE МЫШАХ II. ИЗМЕНЕНИЯ В КОСТЯХ У GERM-FREE МЫШЕЙ

Л. БЕРЕК, ЖУЖАННА БАНОШ

Авторы провели сравнительное изучение костной системы у **germ-free** и обычных мышей. Было установлено, что у **germ-free** мышей с гиполастической лимфоидной системой наблюдается ретардация костной системы.

Предположительно, что причиной нарушения в развитии костной ткани является пониженная функция (или её отсутствие) лимфоидной системы, зависящей от щитовидной железы

EFFECT OF THE DILUENT ON THE POCK COUNT OF VACCINIA VIRUS DILUTIONS

J. SZATHMÁRY, L. HEGEDÜS

ВЛИЯНИЕ РАСТВОРИТЕЛЯ НА ИНФЕКЦИОЗНОСТЬ РАЗВЕДЕНИЙ ВИРУСА ОСПЕННОЙ ВАКЦИНЫ

Й. САТМАРИ, Л. ХЕГЕДЮШ

Повышение содержания обезжиренного молока до 20% вместо 10% в буферном растворе **McIlvaine**, используемом для разведений вируса вакцины, не увеличивало числа оспин на хорионаллантоисной мембране. При добавлении к буферному раствору Tween-а 80 в концентрации 0,1—0,5% число оспин совпадало с таковым при использовании разбавителя с 10% содержанием молока. Более низкое или более высокое содержание Tween-а 80 сопровождалось меньшим числом оспин. После обработки ультразвуком и трипсином разницы в числе оспин, полученных в разбавителе без молока и в разбавителе с 10% содержание молока, не наблюдалось. В противоположность этому в необработанном контроле число оспин было в 1,4—3,4 раза меньше, когда для разведений использовали буферный раствор без молока, чем в случае разбавителя, содержащего 10% молока. Причина разниц, по всей вероятности, заключается в том, что наличие молока препятствует образованию из вирионов аггрегатов или диспергирует уже имеющиеся аггрегаты.

LIPIDS IN STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI CULTURED IN THE PRESENCE OF HUMAN SERUM

I. RÉDAI, A. RÉTHY, P. SEBESSY-GÖNCZY, L. VÁCZI

ИЗУЧЕНИЕ ЛИПИДОВ STAPHYLOCOCCUS AUREUS И ESCHERICHIA COLI, ВЫРАЩЕННЫХ В ПРИСУТСТВИИ ЧЕЛОВЕЧЕСКОЙ СЫВОРОТКИ

И. РЕДАИ, А. РЕТИ, Р. ШЕБЕШШИ-ГЁНЦИ, Л. ВАЦИ

Экстрагируемые липиды ***Staphylococcus aureus*** — при выращиванни в питательной среде с 10% человеческой сыворотки — составляют 20% сухого материала. В процессе выращивания в среде, содержащей липопротеин, содержание свободных жирных кислот повышается в 40 раз, а моно-, ди- и триглицеридов — в 7—10 раз. На фосфолипиды приходится 17,5% в обще-липидном составе, при расчете на сухой материал их количество увеличивается в 2 раза. Накопление ненасыщенных жирных кислот можно наблюдать в каждой липидной фракции, однако в первую очередь это обнаруживается во фракции свободной жирной кислоты.

Экстрагируемые липиды ***Escherichia coli*** — выращивание в присутствии человеческой сыворотки — составляют 5,9—6,1% сухого материала. Повышение содержания-липидов является следствием накопления нейтральных липидов. Под действием сыворотки содержание фосфатидил-этаноламина ***Escherichia coli*** понижается, появляются лизофосфатидил-этаноламин и другие, подробно не идентифицированные фосфолипиды.

SEROLOGICAL STUDIES ON PSEUDOMONAS AERUGINOSA O GROUP LIPOPOLYSACCHARIDES

M. M. ÁDÁM, T. KONTROHR, E. HORVÁTH

СЕРОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ О ГРУППЫ ЛИПОПОЛИСАХАРИДОВ PSEUDOMONAS AERUGINOSA

МАРИЯ М. АДАМ, Т. КОНТРОР, ЭДИТ ХОРВАТ

Очищенные липополисахариды, полученные из 23 типовых штаммов (определенных по антигенной схеме Лани) путем фенольно-водного метода и ультрацентрифугирования, были исследованы методом прямой преципитации и пассивной гемагглютинации в 0 им-

мунной сыворотке. Преципитация в неадсорбированной сыворотке давала специфические реакции при умеренных разведениях липополисахаридных экстрактов. Гемагглютинация, проведенная с эритроцитами, покрытыми липополисахаридом, была подобна таковой прогретых бактерий в неадсорбированной и высоко специфичной подгрупповой сыворотке. Тесная взаимосвязь в серологическом поведении между фенольно-водными экспрактами и бактериями указывает на то, что липополисахариды обусловливают специфичность 0 антигена.

INCIDENCE AND HYGIENIC IMPORTANCE OF PSEUDOMONAS AERUGINOSA IN WATER

L. NÉMEDI, B. LÁNYI

ВСТРЕЧАЕМОСТЬ И ГИГИЕНИЧЕСКОЕ ЗАЧЕНИЕ PSEUDOMONAS AERUGINOSA В ВОДЕ

Л. НЕМЕДИ, Б. ЛАНЬИ

В обычное санитарное исследование качества водных проб, помимо определения числа колиформных и антерококковых бактерий, авторы ввели также выявление **Pseudomonas aeruginosa**. По сравнению с мембранным фильтрованием более эффективным для выделения *Ps. aeruginosa* оказалось обогащение питательной среды селенит-бриллиантовым зеленым бульоном особенно при исследовании загрязненных водных проб. При мембранном фильтровании 37 153 проб только в 05% случаев удалось выделить *Ps. aeruginosa*, тогда как в обогащенной среде 1372 пробы оказались положительными в 38,2%. Методом обогащения встречаемость *Ps. aeruginosa* в пробах питьевой воды различного вида была показана в 3,4—22,3%, 37,2% достигала в промышленных водах, 23,4—84,4% — бассейнах, 86,2% — в поверхностных водах, 95,7% — в канализационных водах. Из 227 проб питьевой воды, положительных на *Ps. aeruginosa* в 117 колиформные бактерии отсутствовали; в пробах, содержащих оба организма, колиформные встречались обычно в меньшем числе, чем *Ps. aeruginosa*. Большая часть выделенных штаммов *Ps. aeruginosa* принадлежала к серогруппе 04 (по схеме Лани). Довольно часто встречались и серогруппы 01, 03, 07.

EFFECT OF TILORONE HYDROCHLORIDE ON THE EXPERIMENTAL RABIES OF MICE

F. FORNOSI, M. TÁLAS, G. WEISZFEILER

ДЕЙСТВИЕ ГИДРОХЛОРИСТОГО ТИЛОРОНА ПРИ ЭКСПЕРИМЕНТАЛЬНОМ БЕЩЕНСТВЕ У БЕЛЫХ МЫШЕЙ

Ф. ФОРНОШИ, МАРГАРИТА ТАЛАШ, ДЬ. ВЕЙСФЕЙЛЕР

Одноразовое внутрижелудочное введение гидрохлористого тилорона почти полностью защищало швейцарских мышей от последующего (спустя 19 часов) внутримозгового или подкожного заражения 10 $ЛД_{50}$ вируса бешенства, штамм CVS. После предварительной дачи тилорона увеличился и инкубационный период при заражении мышей большими вирусными дозами (100, 1000 $ЛД_{50}$). Интерферон можно было обнаружить через 48 часов после заражения и у мышей, не получивших тилорон: при подкожном заражении только в сыворотке крови, в случае внутримозгового введения — как в тканях мозга, так и в сыворотке крови. Уровень интерферона у мышей, получивших тилорон, повысился: после внутримозгового заражения — в тканях мозга, после подкожного введения — в сыворотке крови.

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, Budapest, Keleti Postahivatal, Postafiók 64.

An die gleiche Anschrift ist auch jede für die Redaktion bestimmte Korrespondenz zu richten. Abonnementspreis pro Band: $ 16.00.

Bestellbar bei dem Buch- und Zeitungs-Außenhandels-Unternehmen »*Kultúra*« (Budapest I., Fő utca 32. Bankkonto Nr. 43-790-057-181) 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 de fascicules qui seront réunis en volumes.

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

Acta Microbiologica, Budapest, Keleti Postahivatal, Postafiók 64.

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

Le prix de l'abonnement est de $ 16.00 par volume.

On peur s'abonner à l'Entreprise pour le Commerce Extérieur de Livres et Journaux «*Kultúra*» (Budapest I., Fő utca 32. Compte courant No. 43-790-057-181) ou à l'étranger chez tous les représentants ou dépositaires.

«*Acta Microbiologica*» публикуют грактаты 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, Budapest, Keleti Postahivatal, Postafiók 64.

По этому же адресу направлять всякую корреспонденцию для редакции.

Подписания цена — $ 16.00 за том.

Заказы принимает предприятие по внешней торговле книг и газет «*Kultúra*» (Budapest I., Fő utca 32. Текущий счет № 43-790-057-181), или его заграничные представительства и уполномоченные.

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

ALBANIA

Drejtorija Qändrone e Pärhapjes
dhe Propagandimit tä Libri
Kruga Konferenca e Päzes
Tirana

AUSTRALIA

A. Keesing
Box 4886, GPO
Sydney

AUSTRIA

GLOBUS
Höchstädtplatz 3
A-1200 Wien XX

BELGIUM

Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
162, rue du vidi 5, Place St. Jean
100 Bruxelles

BULGARIA

HEMUS
11 pl Slaveikov
Sofia

CANADA

Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA

Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA

Artia
Ve Smečkách 30
Praha 2
Poštovní Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha 1
SLOVART A. G.
Gorkého
Bratislava

DENMARK

Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND

Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE

Office International de Documentation
et Librairie
48, rue Gay-Lussac
Paris 5e

GERMAN DEMOCRATIC REPUBLIC

Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701
Zeitungsvertriebsamt
Fruchtstraße 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC

Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN

Blackwell's Periodicals
Oxenford House
Magdalen Street
Oxford
Collet's Subscription Import
Department
Dennington Estate
Wellingsborough. Northants.
Robert Maxwell and Co. Ltd.
4—5 Fitzroy Square
London W. 1

HOLLAND

Swetz & Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA

Hind Book House
66 Babar Road
New Delhi 1

ITALY

Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze
Techna
Via Cesi 16
40135 Bologna

JAPAN

Kinokuniya Book-Store Co. Ltd.
826 Tsunohazu 1-chome
Shinjuku-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central

KOREA

Chulpanmul
Phenjan

NORWAY

Tanum-Cammermeyer
Karl Johansgt 41—43
Oslo 1

POLAND

RUCH
ul. Wronia 23
Warszawa

ROUMANIA

Cartimex
Str. Aristide Briand 14—18
București

SOVIET UNION

Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN

Almquist & Wiksell
Gamla Brogatan 26
S—101 20 Stockholm

USA

F. W. Faxon Co. Inc.
15 Southwest Park
Westwood, Mass. 02090
Stechert Hafner Inc.
31 East 10th Street
New York, N. Y. 10003

VIETNAM

Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA

Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

10. XI. 1972

Index: 26.016