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PLASMA FIBRONECTIN CONCENTRATION IN GERMFREE AND CONVENTIONAL MICE OF VARIOUS AGE

K. Cseh, Piroska Anderlik, Ilona Szeri, Zsuzsanna Bános, L. Jakab and L. Kalabay

Third Department of Medicine, and Institute of Microbiology, Semmelweis University Medical School, Budapest

(Received September 15, 1987)

Lower plasma fibronectin (FN) levels were detected in C3H germfree (Gf) mice as compared to conventional (Cv) ones. FN levels increased with aging both in Gf and Cv mice. Stimulation of the immune system with live Newcastle disease virus (NDV) vaccine increased the FN levels in young germfree mice, but had no effect in conventionals.

Fibronectin (FN) is a high molecular mass adhesive glycoprotein (MV 440 000 d) produced by several cell types. It is found in an insoluble form on the surfaces of various types of cells, associated with basement membranes and connective tissue, and it is present in a soluble form in plasma and in other body fluids [1–7].

One of the most important effect of soluble plasma FN is its opsonic activity. The effective operation of the mononuclear-phagocyte system (MPS) depends on the concentration of plasma FN [1, 2]. The phagocytic activity of MPS is impaired when plasma FN concentration is reduced and improves on restoration of it [5–8]. Phagocytic cells express cell surface receptors for FN, and ingest tissue debris, various blood-borne particles, bacteria and bacterial proteins opsonized by FN. FN plays a role in the control of the motility and soluble mediator production of phagocytic cells [5, 6, 9, 10].

A protein analogous to human FN seems to be present in other vertebrate species. In mice, plasma and certain cell surface have revealed a protein with biological and physicochemical characteristics very similar to that of the human FN [11]. It has been proved that the phagocytic activity of germfree (Gf) mice is decreased compared with that of conventional (Cv) mice [12].

Károly Cseh
Third Department of Medicine, Semmelweis University Medical School
Eötvös út 12, H-1121 Budapest, Hungary

Piroska Anderlik, Ilona Szeri, Zsuzsanna Bános, Lajos Jakab, László Kalabay
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

Data concerning the plasma FN level of Gf mice, however, are not known. The present study is concerned with the plasma FN level of intact Gf and Cv mice with different age and the effect of treatment with live Newcastle disease virus (NDV) containing attenuated NDV vaccine on the plasma FN level in young mice.

Materials and methods

Experimental animals. Germfree and conventional C3H mice (LATI, Gödöllő, Hungary) of both sexes and different age were used. The germfree state was maintained as described earlier [12].

Purification of FN, standard production. Plasma FN was separated from pooled mouse sera (30 ml) as described earlier [10], the homogeneity of isolated FN was greater than 99%.

Preparation of anti-FN-antibodies (aFN). Rabbits were immunized with the purified mouse FN. The monospecifity of aFN was checked by crossed immunoelectrophoresis against normal mouse plasma, mouse plasma depleted for FN and purified mouse FN, similarly as it was described for aFN to human FN [10].

Determination of mouse plasma FN level. Blood was collected from the ophthalmic plexus of mice using sodium citrate as an anticoagulant. Plasma FN concentrations were determined by electroimmunodiffusion [13]. Purified mouse FN was used as a standard for FN measurements.

Determination of protein concentration was performed as described by Lowry et al. [14].

Vaccine. The attenuated mezogene variety (H strain) of the fowl plague virus (NDV) propagated in embryonated chicken egg was used (Phylaxia, Budapest). Five weeks old Gf and Cv mice were treated intraperitoneally with 0.2 ml of NDV vaccine. FN level was determined seven days after the treatment.

Statistical analysis. The Student "*t*" test was used for statistical analysis of data. The accepted level of significance was $p = 0.05$.

Results

Plasma FN and total protein concentrations of Cv and Gf mice with various age are shown in Table I. The plasma FN concentrations varied with age both in Gf and Cv mice. The values were significantly higher in the 12 months old mice than in the 6 weeks old ones. However, in the Gf mice values were

Table I

Plasma FN and total protein concentrations of Cv and Gf mice of various age

Groups	Age of mice	Number	Concentration of total protein $\mu g/ml \times 10^4$ $\bar{X} \pm SE$	Concentration of plasma FN $\mu g/ml$ $\bar{X} \pm SE$	Plasma FN/total protein $\bar{X} \pm SE\%$
Gf	6 weeks	15	n.t.	417±78	n.t.
	12 months	16	5.49±0.10	600± 8	1.1 ±0.02
Cv	6 weeks	13	3.52±0.18	556±42	1.58±0.08
	12 months	16	4.90±0.10	683±14	1.39±0.05

n.t. = not tested

significantly lower than those were found in Cv mice of same age. The plasma FN level of 12 months old Gf mice corresponded to the value of the 6 weeks old Cv mice. The total protein content in 12 months old Gf mice was significantly higher than of the conventionals of same age, however, the ratio of FN/total protein content was lower in Gf mice than in conventionals.

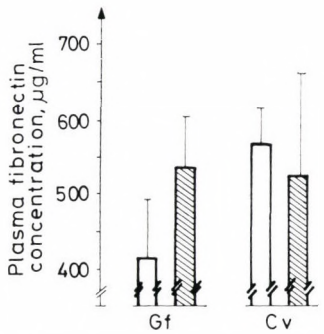

Fig. 1. Effect of atteneuated NDV vaccine on the plasma FN level in young mice. Open columns, untreated mice; shaded columns, NDV-treated mice

The effect of attenuated NDV vaccine on the plasma FN level was investigated in young mice. Plasma FN concentrations determined seven days after the treatment are shown in Fig. 1. In the case of Cv mice the plasma FN level did not differ significantly in vaccinated and untreated groups. However, in Gf mice, the plasma FN level of vaccinated animals was significantly higher compared to that of the untreated ones. The plasma FN level of vaccinated Gf mice was in the range of the values of Cv mice.

Discussion

We have found higher plasma FN levels in all groups of C3H mice as compared to human plasma concentrations (200–400 µg/ml) determined by the same method. However, the increase of FN level with aging of mice is in accordance with our previous observation that the human plasma FN level exhibited a slight gradual increase from age 20 to 60 [15]. The plasma FN concentration of Gf mice has not so far been investigated. Our present results showed that the plasma FN concentrations were lower in Gf than in Cv mice of same age. This result is in accordance with earlier studies which have suggested that macrophage activity of Gf animals differs considerably from that of Cv animals. Adherence and chemotactic activity of macrophages are significantly less intense, lysosomal enzyme activity and phagocytosis are also smaller in the cells of Gf animals than those of conventionals [3, 16–20].

It is also known that the function of the mononuclear phagocyte system greatly depends on the concentration of plasma FN: in case of decreasing FN concentration the phagocyte function is impaired [1, 2, 5–7, 21]. It may be assumed that the altered phagocytic activity is in connection with the lower level of plasma FN found in Gf mice. The reason for this could be a decreased FN production because of the lack of normal microbial flora, which may be also responsible for an unsatisfactory specific immune response and non-specific resistance in Gf mice [22–24]. Our results show that the lower plasma FN level of Gf mice could play a role in the failure of non-specific resistance of Gf mice.

It has been found that the plasma FN concentration of Gf mice was increased to the level of Cv mice of the same age by attenuated the NDV vaccine, however, the FN level of Cv mice was not influenced. This result supports the assumption that the reason for the low level of FN in Gf mice could be the lack of stimulant effect of the normal microbial flora on FN production.

REFERENCES

1. Cseh, K., Török, J., Kalabay, L., Marticsek, J., Szénási, P., Benedek, Sz., Jakab, L.: Ann Immunol Hung **26,** 807 (1986).
2. Cseh, K., Jakab, L., Török, J., Kalabay, L., Pozsonyi, T.: Acta Microbiol Hung **32,** 175 (1985).
3. Cseh, K., Jakab, L., Török, J., Marticsek, J., Benedek, Sz., Kalabay, L., Pozsonyi, T.: Ann Immunol Hung **24,** 299 (1984).
4. Cseh, K., Jakab, L., Török, J., Marticsek, J., Kalabay, L. Jr., Benedek, Sz.: Ann Immunol Hung **24,** 307 (1984).
5. Cseh, K., Jakab, L., Török, J., Marticsek, J., Szénási, P., Kalabay, L. Jr., Pozsonyi, T.: Ann Immunol Hung **24,** 325 (1984).
6. Mosher, D. F.: Ann Rev Med **35,** 561 (1984).
7. Zerlauth, G., Wolf, G.: Am J Med **77,** 685 (1984).
8. Blumenstock, F. A., Weber, P., Saba, T. M.: Adv Exp Med Biol **115,** 613 (1982).
9. Bevilacqua, M. P., Amrani, D., Mosesson, M. W., Bianco, C.: J Exp Med **153,** 42 (1981).
10. Cseh, K., Jakab, L., Török, J., Kalabay, L., Marticsek, J., Pozsonyi, T., Benedek, Sz.: Immunol Lett **9,** 301 (1985).
11. Zetter, B. R., Martin, G. R.: Proc Natl Acad Sci USA **75,** 2324 (1978).
12. Budavári, I., Anderlik, P., Bános, Zs., Fürész, J., Szeri, I.: Acta Microbiol Hung. **32,** 215 (1985).
13. Laurell, C. B.: Scand J Lab Clin Invest Suppl **29,** 21 (1972).
14. Lowry, H. O., Rosenbrough, N. H., Farra, A. L., Randall, R. J.: J Biol Chem **193,** 265 (1951).
15. Cseh, K., Kalabay, L., Jakab, L., Török, J., Marticsek, J., Benedek, Sz., Pozsonyi, T., Szklenárik, Gy.: Orv Hetil **127,** 577 (1986).
16. Bruhn, H. D., Heimbruger, N.: Haemostasis **5,** 189 (1976).
17. Choate, J. J., Mosher, D. F.: Cancer **51,** 1142 (1983).
18. Jungi, T. W., McGregor, D. D.: Infect Immun **19,** 553 (1978).
19. Morland, B., Smievoll, A. P., Midtvedt, T.: Infect Immun **26,** 1129 (1979).
20. Tsahava, O. V.: Gnotobiologiya Medicina, Moscow 1972, p. 136.
21. Kawamura, K., Tanaka, M., Kamiyama, F., Higashino, K., Kishimoto, S.: Clin Chim Acta **131,** 101 (1983).
22. Lev, M., Battisto, J. R.: Immunology **19,** 47 (1970).
23. Ueda, K., Yamasaki, S., Someya, Jpn J Microbiol **17,** 533 (1973).
24. Thorbecke, G. J., Gordon, H. A., Wostmann, B. S., Wagner, M., Reyniers, I. A.: J Infect Dis **101,** 237 (1957).

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RISK INVOLVEMENT WITH *STAPHYLOCOCCUS AUREUS* EXOTOXINS AMONG PYOGENIC SKIN INFECTIONS WITH SPECIAL REFERENCE TO TOXIC SHOCK SYNDROME

SMITA NAIDU, K. RAJYALAKSHMI and A. S. NAIDU

Institute of Preventive Medicine,
Public Health Laboratories and Food (Health) Authority,
A. P., Hyderabad, India

(Received February 19, 1988)

The sera of three patients involved in a non-menstrual toxic shock syndrome outbreak showed TSST-1 at a concentration above 40 ng/ml and no antibodies of IgG class against the toxin. A *Staphylococcus aureus* strain producing TSST-1 was isolated from one of the patients. *S. aureus* strains showing similar phage type and biotype patterns were isolated from 473 out of 876 close contacts with the patients involved in this outbreak. This carriage and spread was traced to pyogenic skin infections (mainly "folliculitis") among this population. Serological studies indicated that during the early phase of the outbreak anti TSST-1 titres were low then rose gradually. In a further related study the frequency of enterotoxin production was found 48.5% among staphylococci isolated from pyogenic skin infections. Our data indicated that pyogenic skin infections are potential source for toxic shock syndrome.

Staphylococcus aureus cause many infections in humans among which pyoderma is one of the most common and important afflictions of the skin. It has been indicated that 5 to 9% of individuals suffer each year from skin infections due to staphylococci of low invasiveness [1].

S. aureus produce a variety of extracellular protein toxins inimical to the tissues of man and animals. Despite extensive research on the role of toxins in staphylococcal illnesses, it was only in toxic shock syndrome (TSS), scalded skin syndrome and staphylococcal food poisoning that the role of toxins has been firmly established [2, 3].

Staphylococcal food poisoning episodes due to food handlers suffering from pyogenic skin infections and healthy carriage is widely known [4]. Nosocomial transmission of *S. aureus* through hospital personnel has been documented in over 200 postoperative TSS cases [5]. Furthermore, we have recently reported that toxic shock syndrome toxin-1 (TSST-1) producing *S. aureus* strains cause

SMITA NAIDU, A. S. NAIDU*
Department of Medical Microbiology, Lund University
Sölvegatan 23, S-223 62 Lund, Sweden

K. RAJYALAKSHMI
Vice Chancellor, S.P.M.
Visvavidyalayam, Tirupati, A. P., India

* Corresponding author

fatal outbreaks of non-menstrual toxic shock syndrome among population through pyogenic skin infections [6].

In this study we have investigated the TSST-1 serology during a non-menstrual TSS outbreak. We have also screened for the incidence of staphylococcal enterotoxin (SE) and TSST-1 production among pyogenic skin infections.

Materials and methods

Bacterial strains. *S. aureus* strains were isolated from TSS patients and healthy persons involved in an outbreak of non-menstrual TSS in a newly raised police battalion in Hyderabad, India. Further, a total of 68. *S. aureus* strains isolated from patients suffering from pyogenic skin infections at the Dermatology Clinic, Osmania General Hospital, Hyderabad, India were tested. These strains were isolated from various clinical types of pyoderma as listed in Table I.

Sera. A total of 847 serum samples collected during a non-menstrual TSS outbreak were tested. These samples included acute phase sera of 3 TSS patients, 29 sera from close contacts of TSS patients, 19 sera from the members of the medical staff, and 799 sera from the remaining members of the battalion. Serum samples were stored at −20 °C until tested.

Chemicals. Pure SEA, SEB, SEC, SED, TSST-1 and corresponding antisera were kindly supplied by Professor M. S. Bergdoll, Food Research Institute, Madison, Wisconsin, USA. Antibodies (goat) for primary coating and horseradish peroxidase conjugated antibodies against SEA, SEB, SEC, SED and TSST-1 were kind gifts from Professor S. H. W. Notermans, National Public Health Laboratories, Bilthoven, Netherlands. Ortho-phenylenediamine substrate and bovine serum albumin were purchased from Sigma Chemicals Co., Mo., USA. Crystal violet stain was from E. Merck, Darmstadt, FRG. Blood agar base No. 2, DNAse agar and brain heart infusion broth were purchased from Hi-Media, Bombay, India. Unless otherwise stated all the chemicals used in the preparation of buffers and solutions were of analytical grade.

TSST-1 and anti TSST-1 assay. *S. aureus* isolates were cultivated according to the dialysis-sac culture technique described by Donnelly et al. [7]. The presence of TSST-1 in the culture supernatants and the anti TSST-1 titres in serum samples were detected by an optimal sensitivity plate method [8], and also by ELISA [9].

Staphylococcal enterotoxin (SE) assay. *S. aureus* strains isolated from pyoderma were grown according to the dialysis-sac culture technique and the culture supernatants were tested by enzyme linked immunosorbent assay (ELISA) developed and described by Notermans et al. [9].

Subspecies typing. *S. aureus* isolated were differentiated into subspecies based on their human, bovine and canine host origin according to the typing procedure described by Meyer [10]

Phage typing. Typing of the *S. aureus* isolates with International basic set of phages was done at the ICMR-Staphylococcal Phage Typing Center, Maulana Azad Medical College, New Delhi, India.

Results

Non-menstrual TSS outbreak. The clinical history of the non-menstrual TSS outbreak was presented elsewhere [6]. During this investigation, the three TSS patients succumbed to illness showed symptoms of high fever, hypotension, vomiting and/or diarrhoea. Necropsy demonstrated a widespread congestion of internal organs with haemorrhage and necrosis. The sera of the three patients showed the presence of TSST-1 ($>$40 ng/ml) but no detectable titres of TSST-1 antibodies of IgG class. From one of the three patients, a *S. aureus* strain was isolated that produced TSST-1 but not any of the SE. It belonged

Table I

Enterotoxin production among S. aureus isolated from various clinical types of pyoderma

	No. tested	No. positive	Staphylococcal enterotoxin type							
			A	B	C	D	E	A&C	B&C	DAC
Folliculitis	19	10	0	2	5	0	0	2	1	0
Furunculosis	10	5	0	0	4	0	0	1	0	0
Impetigo	15	8	2	0	4	1	0	1	0	0
Abscess	3	1	0	0	1	0	0	0	0	0
Sec. infection	19	7	2	1	2	0	0	1	0	1
I.E.D.*	2	2	0	1	0	0	0	1	0	0
Total	68	33	4	4	16	1	0	6	1	1

* Infectious eczematoid dermatitis

to *S. aureus* biovar. *hominis* according to the Meyer classification and showed susceptibility to phage type 75 of group III.

A total of 876 individuals of the battalion were studied for the skin carriage of TSST-1 positive *S. aureus* and were serologically evaluated for the presence of TSST-1 and anti TSST-1. Among this group 473 (54%) individuals harboured TSST-1 positive *S. aureus* strains belonging to a similar biovar and phage type described with the patients. Folliculitis was found to be common and widespread among the battalion. Serological studies with this selected population revealed that during the early phase of the outbreak the anti TSST-1 prevalence was low and rose rapidly by the fifth week (i.e. fifth week after the first diagnosed case of TSS). TSST-1 (< 10 ng/ml of sera) and TSST-1

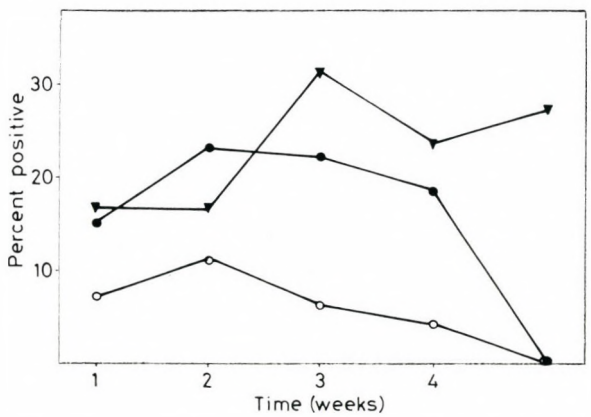

Fig. 1. Serological status of the population for TSST-1 and anti TSST-1 during the non-menstrual toxic shock syndrome outbreak. ○ = TSST-1; ● = TSST-1 + anti TSST-1; ▼ = anti TSST-1

and anti TSST-1 levels were high during the second week and the frequency gradually subsided by the fifth week (Fig. 1).

Incidence of toxin production by S. aureus isolates from pyoderma. Out of 68 *S. aureus* strains isolated from pyoderma patients, 48.5% produced enterotoxins and none produced TSST-1. Different SE types produced by *S. aureus* originating from various clinical types of pyogenic skin infections is shown in Table I. Isolates from IED, impetigo and folliculitis cases showed high degree of enterotoxigenicity. SEC was commonly produced. Mixed combinations with other enterotoxins were expressed only with SEC.

Majority of the SE positive strains were, in majority, untypable by phages (60.6%). Enterotoxin production among the pyoderma strains was found in III, IV and mixed phage groups.

According to the subspecies typing, pyoderma isolates were classified as *S. aureus* biovar. *hominis* (72.1%), *S. aureus* biovar. *bovis* (25.0%) and *S. aureus* biovar. *canis* (2.9%). *S. aureus* biovar. *hominis* (32.3%) and biovar. *bovis* (14.7%) were potent enterotoxin producers.

Discussion

In studies where documented illnesses have been associated with specific enterotoxin or TSST-1 producing strains of *S. aureus*, patients with wound infections and pyogenic skin infections have shown the largest antibody response [11]. These infections were also implicated for TSS when colonized with a potent TSST-1 producing *S. aureus* [6, 12].

During this outbreak we have observed that the population rapidly developed antibodies to TSST-1. This immunity was mainly due to the high degree transmission of the TSS strain in the population through skin carriage and pyoderma. It has been reported that *S. aureus* clinical isolates possess high cell surface hydrophobicity [13, 14] and bind to serum/connective tissue proteins such as fibrinogen, IgG, fibronectin, vitronectin, laminin and collagen [15]. Such interactions probably contribute to the transmission and colonization properties of staphylococci.

The possible risks involved in predisposing TSS and staphylo-enterotoxicosis were evaluated by screening for SE and TSST-1 producing strains among pyogenic skin infections. The frequency of SE positivity was high among strains isolated from pyoderma and with no TSST-1 production. However, none of these patients were complicated any further with enterotoxicosis during the course of this investigation. It was reported that the populations from developing countries have higher antibody titres to at least one enterotoxin, SEB, because of the widespread exposure of these people to staphylococcal infections [11, 16].

The isolation rate of the TSS strains was high (54%) among the persons involved in the TSS outbreak, however, only those lacking anti-TSST-1 titres developed TSS. This finding further supports that TSS occurs only in anti TSST-1 deficient persons. Pyogenic skin infections due to toxin producing *S. aureus* strains (without any apparent clinical toxicosis) probably provide a natural acquired immunity in exposed population. On the other hand, pyogenic skin infections may pose risks in persons lacking protective antibodies against SE and TSST-1. The original report by Todd et al. [17] that TSS as a paediatric illness and the common occurrence of menstrual TSS among young women [18] indicates an age-associated risk involvement in TSS. Age-specific prevalence of antibodies against TSS toxin was observed in a US based sero-surveillance and it was found that antibody titres rising with the increasing age reached a plateau with the population reaching the age of 40 years [19].

Enterotoxigenic *S. aureus* strains of animal origin cause food poisoning episodes [4]. The prevalence of zoonosis among pyogenic skin infections and SE producing *S. aureus* biovar. *bovis* causing human infections has been reported [20, 21]. Olsvik et al. [22] have recently reported that most of the *S. aureus* strains isolated from bovine mastitis produce TSST-1. In this study we have observed SE production among *S. aureus* biovar. *bovis* and biovar. *canis*. Though none of the animal isolates from pyoderma produced TSST-1, the possible role of zoonosis in TSS cannot be excluded.

To summarize, our findings indicate that *S. aureus* causing TSS can spread rapidly in the population through superficial pyogenic skin infections such as folliculitis. In developing countries, tropical climate, profuse sweating and poor hygienic practices are predisposing factors for pyodermas [12, 15]. Since TSS like many other newly emerged diseases is underdiagnosed, there is an immediate necessity to evaluate such risk involvements. A recent report on the widespread zoonosis among pyogenic skin infections further complicates this problem [15].

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REFERENCES

1. Noble, W. C., Somerville, D. A.: Microbiology of the Human Skin. Lloyd Luke, London 1981.
2. Bergdoll, M. S.: In Falmagne, P., Alouf, J. E., Fehrenbach, F. J., Jeljaszewicz, J., Thelestam, M. (eds): Bacterial Protein Toxins. Gustav Fisher Verlag, Stuttgart 1986. p. 321.
3. Freer, J. H., Arbuthnott, J. P.: Rev J Pharmacol Therap **19,** 581 (1986).
4. Naidu, A. S., Rajyalakshmi, K.: Proc 2nd World Congress of Food Borne Infections, Intoxications. Vol. 1. Berlin-West 1986. p. 194.

5. Kreiswirth, B. N., Kravitz, G. R., Schlievert, P. M., Novick, R. P.: Ann Intern Med **105,** 704 (1986).
6. Naidu, A. S., Rathna, K., Nirmala, P., Yojana Devi, D., Rajyalakshmi, K.: Lancet **2,** 1454 (1986).
7. Donnelly, C. B., Leslie, J. E., Black, L. A., Lewis, K. H.: Appl Microbiol **15,** 1382 (1967).
8. Robbins, R., Gould, S., Bergdoll, M. S.: Appl Microbiol **28,** 946 (1974).
9. Notermans, S., van Leeuwen, W. J., Dufrenne, J., Tips, P. D.: J Clin Microbiol **18,** 1055 (1983).
10. Meyer, W.: Int J Syst Bacteriol **17,** 387 (1966).
11. Jozefczyk, Z.: J Infect Dis **130,** 1 (1974).
12. Naidu, A. S., Rathna, K., Smita Naidu, Rao, P. N., Rajyalakshmi, K.: Acta Microbiol Hung **35,** 35 (1988).
13. Ljungh, A., Hjertén, S., Wadström, T.: Infect Immun **47,** 522 (1985).
14. Rozgonyi, F., Szitha, K. R., Hjertén, S., Wadström, T.: J Appl Bacteriol **59,** 451 (1985).
15. Wadström, T.: Zentralbl Bakteriol [A] **266,** 191 (1987).
16. McGann, V. G., Rollins, J. B., Mason, D. W.: J Infect Dis **124,** 206 (1971).
17. Todd, J., Fishaut, M., Kapral, F., Welch, T.: Lancet **2,** 1116 (1978).
18. Shands, K. N., Schmid, G. P., Dan, B. B., Blum, D., Guidotti, R. J., Hargrett, N. T., Anderson, R. L., Hill, D. L., Broome, C. V., Band, J. D., Fraser, D. W.: N Engl J Med **303,** 1436 (1980).
19. Vergeront, J. M., Stolz, S. J., Crass, B. A., Nelson, D. B., Davis, J. P., Bergdoll, M. S.: J Infect Dis **148,** 692 (1983).
20. Naidu, A. S., Reddy, S. V., Rajyalakshmi, K.: Indian J Pathol Microbiol **28,** 333 (1985).
21. Rao, P. N., Naidu, A. S., Ramana Rao, P., Rajyalakshmi, K.: Zentralbl Bakteriol [A] **265,** 218 (1987).
22. Olsvik, O., Adekeye, J., Fossum, K.: Ann Meeting of ASM B-196, 57 (1987).

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HISTAMINE PRODUCTION BY *HAEMOPHILUS*

ZSUZSANNA CSUKÁS and ÁGNES BARÁTH

Institute of Microbiology, Semmelweis University Medical School, and Central Research Institute of Food Industry, Budapest

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Eight *Haemophilus influenzae* and four *Haemophilus parainfluenzae* strains, all freshly isolated from patients, were examined for histamine production. Histamine was produced by all the *H. influenzae* (0.06 μg – 2.6 μg/100 ml) and by two of the *H. parainfluenzae* strains (46.5 – 47.9 μg/100 ml). It is concluded that histamine which plays an important role in the pathogenesis of chronic aspecific respiratory diseases, may derive not only from degranulation of mast cells, but also produced by *Haemophilus*.

Lower respiratory tract secretions taken by transtracheal needle biopsy or fibre bronchoscopy during acute exacerbation of chronic aspecific respiratory disease (CARD) are abundant in bacteria. In lower numbers, bacteria can also be isolated from samples taken in the asymptomatic period [1–3]. *Staphylococcus aureus*, *Haemophilus influenzae*, *Haemophilus parainfluenzae* and *Streptococcus pneumoniae* are the species isolated most commonly [1, 4, 5]. Metabolites and cellular decomposition products of these bacteria come into a continuous tact with the bronchial serosa and the pulmonary tissues.

We [6, 7] have demonstrated the cell-damaging effect of *Haemophilus* enzymes and endotoxin in the pulmonary tissue. In the course of these processes, histamine and mediators are released, which may cause clinical symptoms and histological changes.

Alternatively, histamine may be synthesized by decarboxylation of histidine. Histamine-producing strains are known in certain groups of intestinal bacteria, e.g. *Lactobacillus* species [8]. Sheinman et al. [9] were the first to report in 1987 on *H. influenzae* species exercising a histidine-decarboxylase activity.

Since histamine plays a decisive role in the pathomechanism of CARD, is seemed to be of interest to examine for histamine production *H. influenzae* and *H. parainfluenzae* strains freshly isolated by us from patients.

ZSUZSANNA CSUKÁS
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

ÁGNES BARÁTH
Central Research Institute of Food Industry
Herman Ottó út 15, H-1022 Budapest, Hungary

Materials and methods

Haemophilus strains. Six of the 12 strains used in the experiments had been isolated from the lower respiratory tract of patients with chronic bronchitis, two from ear secretions of children suffering from otitis, two from the CFS of children with meningitis, one from haemoculture and one from prostate secretion.

The following characters were determined: growth factor determination by Sims' method, sucrose and xylose decomposition, ONPG test, haemolysin, indole and nitrate production [10].

Eight of the strains proved to be *H. influenzae* and four *H. parainfluenzae.* The freshly isolated strains were maintained on chocolate agar.

Methods. The strains were subjected to three consecutive passages in a broth medium enriched with 10 μg/ml haematin (NBCO, Cleveland), 10 μg/ml DPN (Reanal, Budapest), 1% glucose and 2% L-histidine (Reanal, Budapest), and 0.5% pyridoxine-HCl (Reanal, Budapest), pH 6.2. The basal broth (pH 7.2) contained 0.3% Difco meat extract, 1% Bacto peptone (Difco), 0.2% yeast extract and 0.5% NaCl.

The cultures were centrifuged at 6 000 rpm after each passage and the deposit was inoculated into fresh medium. The bacteria were tested for histidine decarboxylase activity. The histamine content of the supernatants was examined by thin-layer chromatography and in a Biotronik LC 2000 automatic amino acid analyzer.

Examination of the actual histidine decarboxylase activity of cells. Peptone water enriched with growth factors was used as control medium [10]. For testing *Haemophilus* strains for the enzyme, the same medium was enriched with 2% histidine. The strains were incubated for 24, 48, 72 or 96 h; the change in pH was indicated by a change of colour of bromcresol purple and cresol red [10].

Thin-layer chromatography. Silicagel G layer was used in methanol: ammonium hydroxide: n-butanol (50 : 30 : 70) mixture. Ninhydrin reagent was used as developer [11].

Biogenic amine analysis in amino acid analyser. Histamine was assayed by a cation-exchange chromatographic method in the Biotronik LC 2000 analyzer. Dowex 50 WX/8 resin was used. Potassium citrate was the eluent buffer and ninhydrin the detecting reagent [12].

Results

The *Haemophilus* strains proved to be negative for histidine decarboxylase activity when examined in the nutrient medium and remained negative both after 10 serial passages in the presence of the substrate and when the histidine concentration was raised to 4%.

Table I

Histamine production by Haemophilus strains

Species	Designation of strain	Histamine produced	
		nmol/100 ml	μg/100 ml
H. influenzae	14	5.2	0.6
	24	19.2	2.2
	31	1.3	0.2
	37	0.4	0.06
	38	11.8	1.3
	81	23.1	2.6
	84	8.5	1.0
	T 2	7.3	0.9
H. parainfluenzae	6	—	—
	10	—	—
	32	408.3	46.5
	35	420.3	47.9

Thin-layer chromatography of the medium showed no unequivocally evaluable results, though, in case of several strains uncertain spots were detected at the level of the control histamine spot. These findings led us to introduce a more sensitive technique for histamine detection.

The results of the biogenic amine analysis of the *Haemophilus* cultures that had undergone three consecutive passages are presented in Table I. The basal nutrient medium contained 2.8×10^{-9} mol/100 ml, i.e. 0.3 μg/100 ml, histamine. This control value was subtracted from the result obtained for each strain.

Discussion

Histamine, being fixed in the mast cells of all tissues and tissue fluids may be released during allergic reactions on the effect of endogenous, autoendogenous or heterogeneous allergens [13]. It has been proved by sensitive methods, developed in the last decade for detection of biogenic amines, that microorganisms may contribute to this process as heterogeneous antigens on the one hand, and by producing histamine on the other.

Autolytic processes in the muscular tissue, supported by bacterial proteolytic enzymes, produce free histidine, which may be decarboxylated by bacterial enzymes to histamine [8].

Only few bacterial species are known to possess histidine-decarboxylase activity, and this activity is not characteristic of all strains within one species. Food poisonings called attention to histamine production by *Morganella morganii* [14], *Klebsiella pneumoniae* [15] and *Hafnia alvei* [16]. These bacteria, proliferating in tinned fish, or in cheese products elaborated toxic amounts of histamine. Behling and Taylor [17], comparing the histamine production by these species, distinguished two groups, viz. *M. morganii*, *K. pneumoniae* and *Enterobacter aerogenes* produced large ($>$ 100 mg/100 ml) whereas *H. alvei*, *Citrobacter freundii* and *Escherichia coli* produced little ($<$ 25 mg/100 ml) amounts of histamine. Histamine-producing *Clostridium perfringens* strains, too, have been isolated from tinned fish [18]. Edwards and Sandine [19] *Streptococcus faecium*, *Streptococcus mitis* and *Lactobacillus plantarum* strains that synthesized little amounts of histamine. *Lactobacillus buchneri* was isolated from a cheese causing food poisoning [20]. Strains showing decarboxylase activity occur in the species *Lactobacillus delbruckei* [20]. Ryser et al. [21] estimated the histamine production of various *Pseudomonas* species between 3.2 and 50 mg/100 ml.

Histamine production by *Haemophilus* strains is a little-examined function of this genus, though its existence had already been proposed by clinicians [22, 23]. Sheinman et al. [9] were the first to demonstrate histamine produc-

tion by *Haemophilus* strains in vitro. They found 7 of 10 *H. influenzae* strains to produce histamine.

Our own investigations have confirmed the histamine synthesis by *Haemophilus* strains. All the eight *H. influenzae* strains and even two of the four *H. parainfluenzae* strains proved to be positive.

Detection of histamine production demands a sensitive technique. We failed to detect histamine by the biochemical method, even thin-layer chromatography gave unappreciable results. Our failure is explainable by the very low histamine level (0.06–2.6 μg/100 ml) that was demonstrated in the amino acid analyzer in *H. influenzae* cultures. Strikingly higher histamine concentrations (46.5–47.9 μg/100 ml) were found in two of the *H. parainfluenzae* cultures while the other two proved to be negative. It should be noted that even the highest concentrations were as low as about 1/1000 of the concentrations found in cultures of histamine-synthesizing intestinal bacteria.

Histidine decarboxylase is an adaptive enzyme. We measured its activity after three consecutive passages in a nutrient medium containing the substrate. The negative result did not turn into positive after further passages or when the substrate concentration was raised. Therefore, presence or absence of the enzyme may be regarded as a strain-specific marker.

We found no appreciable relationship between the disease the strain was isolated from and the histamine production by the strain. All the strains isolated from chronic bronchitis proved to be positive. The two *H. parainfluenzae* strains that synthesized the largest amount of histamine, derived from blood and from sperm samples.

Turnbull et al. [22] found an increased histamine level and *H. influenzae* antigens in intrinsic asthmatic clinical pictures in which no allergic aetiological factor could be detected and the serum IgE level was low. Sheinman et al. [9] demonstrating histamine production by some of their *H. influenzae* strains, attributed an importance to *Haemophilus* strains in the development and worsening of CARD cases.

Further experiments should be made to show whether the little amount of histamine produced by the *Haemophilus* strains is enough to induce contraction of vascular and bronchial smooth muscles. It should be considered in this respect that *Haemophilus* may persist on bronchial and alveolar serous membranes for years.

REFERENCES

1. Turk, D. C., May, J. R.: *Haemophilus influenzae* its Clinical importance. English Universities Press, London 1967.
2. Clarke, C. W., Hannant, C. A., Scicchitano, R.: Thorax **36,** 665 (1981).
3. Hans, H., Morris, J. F., Samson, S.: Am Rev Respir Dis **116,** 41 (1977).
4. Smith, C. B., Golden, C. A., Kanner, R. E., Renzetti, A. D.: Lancet **1,** 1253 (1976).
5. Laurenzi, G. A., Potter, R. T., Kass, E. H.: N Engl J Med **265,** 1273 (1961).

6. Csukás, Zs., Horváth, J., Slowik, F., Bertók, L.: Acta Microbiol Hung **30,** 139 (1983).
7. Csukás, Zs., Cserba, I., Ráskay, M.: Egészségtudomány **28,** 243 (1984).
8. Taylor, S. T.: Critical Review in Toxicology **17,** 91 (1986).
9. Sheinman, B. D., Devalia, J. L., Crook, S. J., Tabaqchali, S., Davies, R. J.: J Allergy Clin Immunol **77,** 208 (1986).
10. Lányi, B. (ed.): Public Health and Clinical Bacteriology. Standard Methods (Hung.) National Institute of Hygiene, Budapest (1980).
11. Dévényi, T., Gergely, T.: Aminoacids, Peptides and Proteins (Hung.) Medicina, Budapest (1971).
12. Baráth, Á., Halász, A., Fehér, J., Németh, Zs.: 6th Danube Symposium on Chromatography. Varna 1987. Abstracts p. 281.
13. Knoll, J.: Pharmacology (Hung.). Vol. 2. Medicina, Budapest 1983.
14. Eitenmiller, R. R., Wallis, J. W., Orr, J. H., Phillips, R. D.: J Food Prot **44,** 815 (1981).
15. Taylor, S. L., Guthertz, L. S., Leatherwood, M., Lieber, E. R.: Appl Environ Microbiol **37,** 274 (1979).
16. Havelka, B.: Cesk Hyg **12,** 343 (1967).
17. Behling, A. R., Taylor, S. L.: J Food Sci **47,** 1311 (1982).
18. Recsei, P. A., Moore, W. M., Snell, E. E.: J Biol Chem **258,** 439 (1983).
19. Edwards, S. T., Sandine, W. E.: J Dairy Sci **64,** 2431 (1981).
20. Huynh, Q. K., Snell, E. E.: J Biol Chem **260,** 2794 (1985).
21. Ryser, E. T., Marth, E. H., Taylor, S. L.: J Food Prot **47,** 378 (1984).
22. Turnbull, L. S., Turnbull, L. W., Leitch, A. G., Crofton, J. W.: Lancet **1,** 526 (1977).
23. Bisgaard, H., Mygind, N.: 13th Congress of the European Academy of Allergiology and Clinical Immunology. Budapest 1986. Excerpta Medica, Elsevier, Amsterdam 1986. pp. 1–9.

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TOXIC SHOCK SYNDROME TOXIN-1 (TSST-1) PRODUCTION IN STAPHYLOCOCCAL INFECTIONS AND SEROLOGICAL IMPLICATIONS WITH PATIENTS AND HEALTHY CONTROLS

A. S. Naidu, S. Vörös, G. Lőczi, Zs. Kienle, B. Brasch and L. Emődy

Department of Medical Microbiology, University of Lund, Lund, Sweden, Institute of Microbiology, University Medical School, Pécs, Hungary, and Public Health Station, Pécs, Hungary

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TSST-1 production occurred in 4.6% among *Staphylococcus aureus* strains isolated from divergent clinical sources in South-West Hungary. Patients suffering from staphylococcal infections, whether or not harbouring TSST-1 positive strains, exhibited low anti TSST-1 titres in their acute phase sera compared to the healthy control population. A *S. aureus* strain isolated from toxic shock syndrome (TSS) was a high collagen binder and a low fibronectin and fibrinogen binder suggesting the role of connective tissue adhesion in colonization and low invasive property of TSS strains.

Toxic shock syndrome (TSS) is a staphylococcal illness, recognized early during this decade, affecting persons of any age and of either sex with an estimated case fatality rate of 5.6% [1–3]. The frequency of TSS reported in the United States has significantly increased after the CDC has formulated the criteria of TSS case definition in 1980 [4], but in Europe this illness is less frequently reported probably due to underdiagnosis [5].

In Sweden, Switzerland, Netherlands, France, Federal Republic of Germany and United Kingdom, TSS is found sporadically [5–11] and usually the diagnosis is linked with laboratories performing TSS research. These laboratories have reported a high frequency of toxic shock syndrome toxin-1 (TSST-1) producing *Staphylococcus aureus* in various clinical illnesses without any apparent symptoms of TSS. However, the epidemiological situation of this disease in the European continent is yet unknown. Studies evaluating the risk factors,

A. S. Naidu
Department of Medical Microbiology, University of Lund Sölvegatan 23, S-223 62 Lund, Sweden

Sándor Vörös, Zs. Kienle, B. Brasch, Levente Emődy
Institute of Microbiology, University Medical School
Szigeti út 12, H-7643 Pécs, Hungary

Géza Lőczi
Public Health Station
Szabadság u. 7, H-7601 Pécs, Hungary

have indicated that majority of the population acquire antibodies to TSST-1 and the immunodeficient persons contract the illness [11–13].

In this study based in Hungary, we have investigated the incidence of TSST-1 producers, estimated the anti TSST-1 levels in staphylococcal infections and the TSST-1 sero-status in a selected group of healthy population. We have also studied the binding properties of TSST-1 producing *S. aureus* to serum and connective tissue proteins.

Materials and methods

Bacterial strains. A total of 65 *S. aureus* strains isolated from various clinical specimens, at the routine diagnostic division of the Institute of Microbiology, University Medical School, Pécs, were investigated for the production of TSST-1.

Sera. Sera of patients suffering from staphylococcal illnesses were obtained from the Department of Gynaecology and Dermatology, University Medical School, Pécs. A total of 33 patients suffered from acute staphylococcal infections and one convalescent patient from TSS. The "acute phase serum specimens" were obtained within a week after the onset of illness.

Furthermore, 100 serum samples from healthy individuals randomly selected from either sex were collected from the County Blood Transfusion Centre, Pécs.

Chemicals. IgG anti TSST-1 coat and the anti TSST-1 HRPO conjugate were kindly provided by S. H. W. Notermans, National Public Health Laboratories, Bilthoven, Netherlands. Pure TSST-1 was a gift from M. S. Bergdoll, Food Research Institute, Wisconsin, USA. Protein-A was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Orthophenylene diamine (No. P-1526, Lot. No. 54F-5003) was purchased from Sigma Chemicals Co., St. Louis, MO, USA. Highly purified fibronectin from porcine plasma was a gift from BioInvent International AB, Lund, Sweden. Fibrinogen purified from human plasma (Batch 61857) and human immunoglobulin G were purchased from Kabi, Stockholm, Sweden. Vitrogen 100™ collagen (contains 95% type I and 5% type III collagens, Lot. No. 87H18. 3) was purchased from Collagen Corporation, California, USA. All chemicals used for the preparation of buffer solutions were of analytical grade.

TSST-1 production and assay. Test cultures were grown and TSST-1 was produced according to the dialysis sac culture method [14] and the cell-free culture supernatant was obtained as described earlier [15]. Culture supernatants were tested for the presence of TSST-1 by a direct (sandwich) enzyme linked immunosorbant assay (ELISA) as described by Notermans et al. [12], with modifications. The sensitivity of the ELISA is estimated to 2 ng TSST-1/ml. False positive reactions due to the interference of protein A, during staphylococcal toxin assays have been described [16]. However, in our assay protein A did not interfere.

Detection of IgG antibodies to TSST-1. The anti TSST-1 titres were estimated by an enzyme linked immunosorbant assay. In brief, 0.1 ml of pure TSST-1 (0.2 μg/ml) in 0.02 M PBS, pH 7.2, was added to each well and the microtiter plate (Nunc) was incubated at 37 °C for 1 h and then at 4 °C overnight. The plates were washed thrice with PBS containing 0.05% Tween-20. The uncoated hydrophobic sites of the polystyrene surface were blocked with 0.2 ml of 1% (w/v) bovine serum albumin in PBS. Plates were incubated at 37 °C for 1 h and then at 4 °C overnight. Washing was repeated. A serum dilution of 1 : 100 in PBS-Tween containing 0.02% sodium azide and 0.02% human serum albumin was prepared. Test sera (0.1 ml) were added and incubated at 37 °C for 1 h. After washing, 0.1 ml of antihuman IgG peroxidase conjugate (diluted to 1 : 100 in PBS-Tween) was added and incubated at 37 °C for 30 min. Orthophenylenediamine (OPD) substrate (0.1 ml) prepared in 34 mM citrate buffer, pH 5.4 with 0.005% hydrogen peroxide was added and incubated at 30 °C for 10 min. The enzyme reaction was terminated with 0.02 ml 4 N sulphuric acid. The colour development was measured by light adsoprtion at 450 nm (Titertek-Multiscan).

Binding of ^{125}I labeled serum and connective tissue proteins to TSST-1 producing S. aureus. Fibrinogen, fibronection, IgG and collagen type 1 were labeled with ^{125}I by the chloramine T method [17]. For binding experiment *S. aureus* strains were grown in typticase soy broth (TSB) at 37 °C for 18 h. Cells were washed once and suspended in PBS, to a density of

10^{10} cells/ml. The method used for quantitating the binding of labeled proteins to bacteria was according to Fröman et al. [18].

Phage typing. Sensitivity of *S. aureus* to bacteriophages was investigated with the International Basic set of phages. Phage lytic activity was checked at 1 RTD and at 100 RTD.

Biovar typing. Crytal violet (CV) test is the base for this classification scheme and the typing was done according to Meyer [19].

Results

Three (4.6%) of the 65 *S. aureus* clinical isolates originating from pyogenic infections produced TSST-1. Eighty nine per cent of the strains were *S. aureus* var. *hominis* exhibiting CV-type C/D and the rest 11% comprised *S. aureus* var. *canis* exhibiting CV-type E. None of the strains belonged to *S. aureus* var. *bovis*.

The majority of the *S. aureus* isolates were lyzed by phage sets of the mixed group (37.7%), group II (20.8%) and group III (20.8%). Only 5.7% were associated with group I and 11.3% were non-typable.

The IgG antibody levels to TSST-1 antigen were examined at 1 : 100 serum dilutions. The median extinction value for the patient group was 0.275, whereas the healthy control group showed a significantly higher median value of 0.475 (Fig. 1).

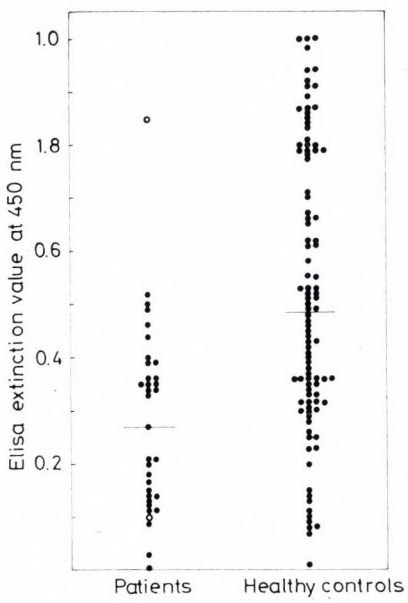

Fig. 1. TSST-l antibody titres in the sera (1 : 100 dilution) of patients with staphylococcal infections and healthy control population. The bisecting line is the median extinction value. The open circles in the patients column represent the acute (bottom) and convalescent (top) phase sera of the toxic shock syndrome patient

Table I

*Binding of ^{125}I labeled serum and connective tissue proteins by TSST-1 producing S. aureus isolates**

^{125}I protein	*S. aureus* strain		
	POTE-49	POTE-52	POTE-64
Fibrinogen	70.6	150.8	107.7
Fibronectin	29.7	97.2	48.7
Immunoglobulin-G	94.5	107.5	98.6
Collagen type 1	245.5	32.9	33.3
TSST-1 production	4.2 μg/ml	1.2 μg/ml	3.1 μg/ml
Phage type	Untypable	I/29	I/29
Biovar	hominis CV-type C/D	hominis CV-type C/D	hominis CV-type C/D
Source	Pus/skin	Vagina	Wound/skin
Clinical diagnosis	TSS	Sec.infection	Pyoderma

* Values are relative percentage to serum protein binding by *S. aureus* strain Cowan 1

One of the patients suffering from infectious eczematoid dermatitis developed typical signs and symptoms related to TSS according to CDC criteria [2]. This patient harboured a TSST-1 producing *S. aureus* strain (POTE-49).

The serum and connective tissue protein binding ability of the three TSST-1 positive strains was studied. *S. aureus* strain POTE-49, the isolate from the TSS case showed significantly higher binding to collagen type 1 than non-TSS clinical isolates and produced a relatively high level of toxin. On the other hand, fibrinogen and fibronectin binding of the TSS isolate was remarkably low compared to the non-TSS isolates (Table I).

Discussion

A high incidence of TSST-1 producing *S. aureus* strains from clinical sources, devoid of apparent clinical symptoms of TSS, has been reported [6–10]. Since staphylococcal infections are common, it was also indicated that the population generally acquires antibodies against TSST-1 with the increase in age and that the immunodeficient persons contract the TSS illness [10].

Phage group I type 29/52 complex and non-typable strains were implicated with TSS, however, strong association has also been indicated with strains from phage group III [1, 17]. In our study the low frequency of these phage groups might explain the low incidence of TSST-1 positivity (4.6%), as compared to the other reports [5–7]. Our three *S. aureus* isolates produced high amounts of TSST-1 similar to the international reference strains.

There was a striking relation between the crystal violet binding pattern an toxigenic staphylococci. All the three TSST-1 isolates were subclassified as *S. aureus* var. *hominis* with CV-type C/D. In a recent study, TSST-1 production was found associated with CV-type C/D and this property was more pronounced than the association with phage types [5].

Our serological data indicated that the patients, whether or not harbouring TSST-1 producing *S. aureus* strains, exhibited low antitoxin levels in their acute phase sera. Conversely, the healthy control population possessed significantly elevated mean level of anti TSST-1. In a US based population surveillance, it was shown that anti TSST-1 antibodies had been acquired rapidly by 20 years of age, 84% of the population having an anti-TSST-1 titre of $\geq 1 : 100$. By age 40, the prevalence had reached a plateau of 97% [13]. Similar studies in Britain showed that healthy individuals (82%) aged 14 to 56 years had elevated antibody titres when compared to only 18% in the acute phase sera of TSS patients [9]. In accordance with these data, our finding extrapolates that the majority of the population generally acquires immunity against TSST-1, through staphylococcal infections, and that the presence of a TSST-1 strain does not necessarily lead to TSS, if the patient is immunologically protected. On the other hand, serological studies have indicated that the acute phase sera from patients with TSS lack antibody to TSST-1 [9, 20, 21]. During our study, the patient with apparent symptoms of TSS, had low anti TSST-1 titre in his acute phase, but significantly elevated titre was detected after two months convalescence.

It is known that *S. aureus* colonize traumatized connective tissues and blood clots in wounded tissues by binding to fibrinogen, fibrin, fibronectin and various tissue collagens [22, 23]. Binding of *S. aureus* to serum spreading factor has also been recently described [24]. Our preliminary studies have indicated that TSST-1 producing staphylococci also bind to vitronectin known as S-protein or serum spreading factor (Paulsson, Naidu, Dahlbäck, Wadström, unpublished data). Our finding that the TSS strain is a high collagen binder and a low fibronectin and fibrinogen binder as compared to TSST-1 producing non TSS strains may indicate that connective tissue protein i.e., collagen adhesion is an important step in its colonization.

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REFERENCES

1. Todd, J., Fishaut, M., Kapral, F., Welch, T.: Lancet **2,** 1116 (1978).
2. Centers for Disease Control: MMWR **29,** 441 (1980).
3. Chesney, P. J., Davis, J. P., Purdy, W. K., Wand, P. J., Chesney, R. W.: JAMA **246,** 741 (1981).
4. Dan, B. B., Sgands, K. N.: Pediatric Annals **10,** 490 (1981).
5. Dickgiesser, N., Brombacher, A.: Zentralbl Bakteriol [A] **265,** 323 (1987).
6. De Nooy, M. P., van Leeuwen, W. J., Notermans, S.: J Hyg Camb **89,** 499 (1982).
7. Naidu, A. S., Kamme, C., Ljungh, A., Wadström, T.: In Press
8. Vischer, W. A., Stadler, H., Faupel, D. M.: Schweiz Med Wochenschr **115,** 1196 (1985).
9. de Saxe, M. J., Hawtin, P., Wieneke, A. A.: Postgrad Med J **61,** 5 (1985).
10. Melconian, A. K., Fleurette, J., Brun, Y.: J Hyg (Lond) **94,** 23 (1985).
11. Christensson, B., Hedström, S. A.: Acta Pathol Microbiol Immunol Scand **93,** 87 (1985).
12. Notermans, S., van Leeuwen, W. J., Dufrenne, J., Tips, P. D.: J Clin Microbiol **18,** 1055 (1983).
13. Vergeront, J. M., Stolz, S. J., Crass, B. A., Nelson, D. B., Davis, J. P., Bergdoll, M. S.: J Infect Dis **148,** 692 (1983).
14. Donnelly, C. B., Leslie, J. E., Black, L. A., Lewis, K. H.: Appl Microbiol **15,** 1382 (1967).
15. Naidu, A. S., Reddy, S. V., Rajyalakshmi, K.: Indian J Path Microbiol **28,** 333 (1985).
16. Rosten, P. A., Bartlett, K. H., Chow, A. W.: J Clin Microbiol **25,** 327 (1987).
17. Greenwood, F. C., Hunter, W. M., Glover, J. S.: Biochem J **89,** 114 (1963).
18. Fröman, G., Switalski, L. M., Faris, A., Wadström, T., Höök, M.: J Biol Chem **259,** 14899 (1984).
19. Meyer, W.: Int J Syst Bacteriol **17,** 387 (1966).
20. Naidu, A. S., Rathna, K., Nirmala, P., Yojana Devi, D., Rajyalakshmi, K.: Lancet **2,** 1454 (1986).
21. Stolz, S. J., Davis, J. P., Vergeront, J. M., Crass, B. A., Chesney, P. J., Wand, P. J., Bergdoll, M. S.: J Infect Dis **151,** 883 (1985).
22. Wadström, T., Speziale, P., Rozgonyi, F., Ljungh, A., Maxe, I., Ryden, C.: Zentralbl Bakteriol Suppl **16,** 83 (1987).
23. Wadström, T.: Zentralbl Bakteriol [A] **266,** 191 (1987).
24. Fuquay, J. I., Loo, D. T., Barnes, D. W.: Infect Immun **52,** 714 (1986).

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COMPARISON OF THE EFFECTS OF PERITONEAL AND SPLEEN CELLS OF SYNGENEIC OR ALLOGENEIC ORIGIN ON THE TAKE OF TRANSPLANTABLE MURINE TUMOURS

ERIKA KARCZAG, J. MINÁROVITS and I. FÖLDES

Microbiological Research Group
of the National Institute of Hygiene, Budapest

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We compared the effects of various potential effector cells of syngeneic or allogeneic origin on the take of a spontaneous adenocarcinoma (SP4) and Lewis lung (LL) carcinoma. As reported earlier, syngeneic resident (non-activated) peritoneal cells (PC) did not inhibit the take of these tumours. On the contrary, transfer of resident PC from allogeneic donors suppressed the tumour take. Syngeneic and allogeneic PC activated by poly I : C or by a combination of indomethacin, poly I : C and Syncumar ("combined treatment") inhibited the tumour take to a similar extent. Syngeneic spleen cells (from untreated mice or from donors underwent "combined treatment") did not inhibit the take of Lewis lung tumour. Transfer of activated allogeneic spleen cells resulted in a stronger inhibition of tumour take than the transfer of resident allogeneic spleen cells.

We demonstrated earlier that syngeneic murine peritoneal cells (PC) activated by poly I : C (a synthetic interferon inducer) or by a combination of indomethacin, poly I : C and Syncumar (referred to as "combined treatment") are capable to inhibit the take of certain transplantable murine tumours in adoptive transfer experiments [1, 2]. As allogeneic cells are capable to stimulate potential antitumoural effector cells of the host without any activation [3], we have tested whether peritoneal cells from allogeneic mice treated with poly I : C or underwent "combined treatment" have an increased tumour inhibitory capacity than syngeneic PC activated the same way. We have found that activated syngeneic and allogeneic PC inhibited the take of SP4 and Lewis lung (LL) tumour to a similar extent.

The spleen also contains potential antitumoural effector cell populations such as natural killer (NK) cells and natural cytotoxic (NC) cells and poly I : C is known to enhance the tumouricidal potential of splenic natural killer cells [4–6]. We compared, therefore, the sensitivity of LL carcinoma to syngeneic PC and spleen cells. Surprisingly, syngeneic spleen cells (resident or from mice underwent "combined treatment") did not inhibit the take of LL tumour, though LL tumour cells are known to be sensitive to NK cells in vitro [4].

ERIKA KARCZAG, JÁNOS MINÁROVITS, ISTVÁN FÖLDES*
National Institute of Hygiene, Microbial Research Group
Pihenő út 1, H-1529 Budapest, Hungary

* Corresponding author

This indicates that activated PC are different from NK and NC cells. On the contrary, transfer of allogeneic spleen cells (resident or activated) suppressed the take of LL tumour. Activation of host effector cells as well as allogeneic lymphocyte cytotoxicity could contribute to this effect [3, 7–10].

Materials and methods

Animals. Seven to eight weeks old Balb/c and C57Black/6 inbred mice (LATI, Gödöllő, Hungary) were used.

Tumours. A spontaneous adenocarcinoma (SP4) of a female Balb/c mouse origin was established as a transplantable tumour in our laboratory. It was maintained in syngeneic female mice by serial transplantation injecting 2×10^5 cells intramuscularly. Lewis lung carcinoma (LL) was maintained in syngeneic female mice by injecting 2×10^5 cells intramuscularly.

Chemicals. Poly I : C sodium salt A grade Lot 702045 (Calbiochem, La Jolla, Ca, USA) was dissolved in saline and kept at 4 °C overnight before use. Indomethacin (Chinoin, Budapest) was dissolved in 96% ethanol to a concentration of 10 mg/ml and further diluted in saline to the appropriate final concentration immediately before administration. It was then injected in a fine suspension form. Syncumar, 3-(α)4-nitrophenyl(-βacetylethyl)-4-hydroxy-coumarin, a gift of Alkaloida (Tiszavasvári, Hungary) was dissolved in sterile saline containing 0.1 N NaOH and neutralized with 0.1 N HCl. Syncumar solutions were prepared freshly before administration.

Treatment of animals. Mice were injected with 100 μg poly I : C intraperitoneally 18 h before cell collecting. Alternatively, they were injected with 100 μg indomethacin, 100 μg poly I : C and 100 μg Syncumar intraperitoneally, 42, 18 and 3 h before cell collecting, respectively ("combined treatment", as described previously [2]).

Peritoneal cells (PC). Cells were obtained by peritoneal lavage using 2.0 ml of medium 199 containing 10 mM HEPES. The collected PC were washed two times with the same medium at 4 °C and then counted. Washed PC were resuspended in sterile saline before transfer.

Spleen cells. Preparation of spleen cell suspensions and lysis of red blood cells was performed as described by Mishell et al. [11]. The cells were washed twice in medium 199 containing 10 mM HEPES and resuspended in sterile saline before transfer.

Separation of adherent and non-adherent cells. Adherent and non-adherent cells were separated by plastic adherence. Briefly, PC or spleen cells were maintained for 2 h in plastic tissue culture dishes in medium 199 containing 10 mM HEPES and 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere. Non-adherent cells detached were removed by careful repeated washings with warm culture medium, and treated with carbonyl iron + magnet [12] to remove the remaining phagocytic cells. After washing two times with medium 199 at 4 °C, nonadherent cells were resuspended in sterile saline.

Experimental procedure. The effect of PC and spleen cells on tumour take was tested by means of the Winn's assay [13]. PC or spleen cells and cell suspensions of the tumour cells were prepared in concentrations indicated and mixed immediately before intramuscular injection into the right thigh of the recipient mice. They were monitored two times weekly for tumour appearance and growth. Tumours could be detected by palpation when they were approximately 3 mm in diameter. The two dimension diameters of growing tumours were measured subsequently. Groups of recipient mice consisted of at least ten animals.

Results

Resident syngeneic PC, as reported earlier [1], stimulated the take of SP4 adenocarcinoma; on the contrary, resident PC from allogeneic mice inhibited the take of the tumour (Fig. 1).

PC from poly I : C stimulated syngeneic and allogeneic mice inhibited the take of SP4 tumour (Fig. 2).

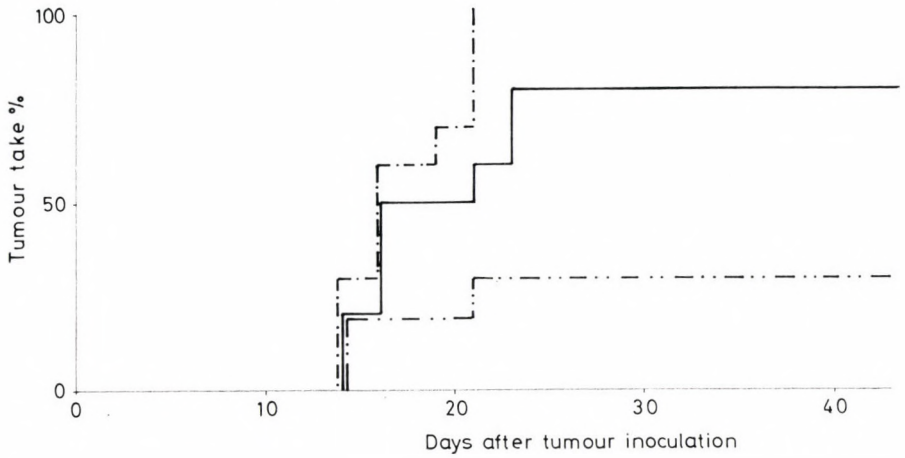

Fig. 1. Effect of resident PC on the take of SP4 adenocarcinoma. Recipient mice were inoculated with 2×10^5 SP4 cells (———) and with 2×10^5 SP4 cells mixed with resident PC from syngeneic Balb/c mice (— . —) or from allogeneic C57B1/6 mice (— . . —). PC : tumour cell ratio, 10 : 1

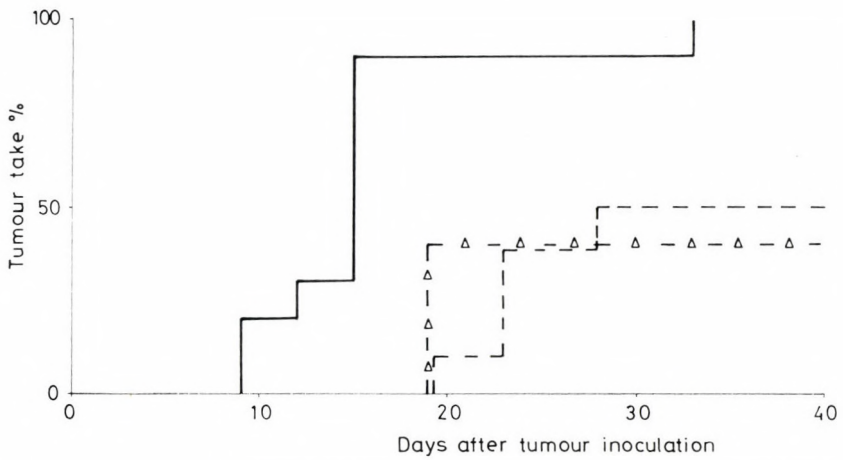

Fig. 2. Effect of poly I : C activated PC on the take of SP4 adenocarcinoma. Recipient mice were inoculated with 2×10^5 SP4 cells (———), with 2×10^5 SP4 cells mixed with poly I : C activated PC from syngeneic Balb/c mice (△ — △) or from allogeneic C57B1 6 mice (— — — —). PC : tumour cell ratio, 25 : 1

Both resident allogeneic PC and PC from allogeneic donors underwent "combined treatment" suppressed the take of LL carcinoma; transferring of activated PC, however, resulted in a more marked inhibition of tumour take (Fig. 3).

Non-adherent PC from syngeneic and allogeneic mice underwent "combined treatment" inhibited the take of LL tumour to a similar extent (Fig. 4).

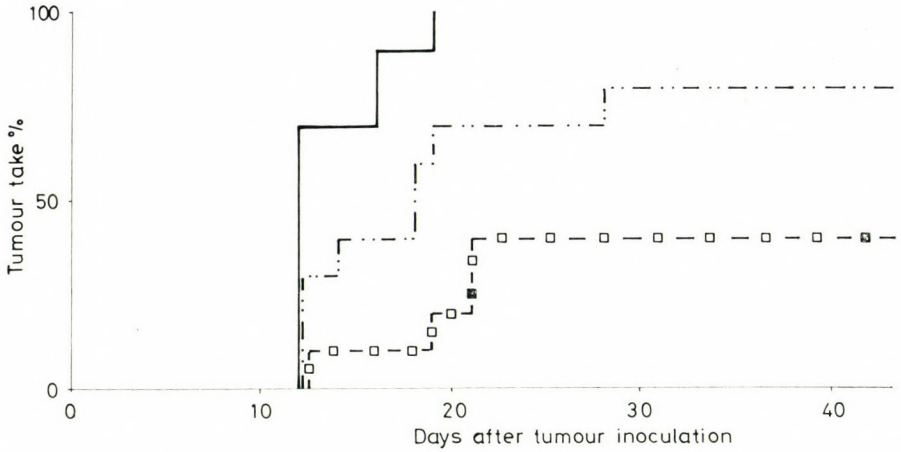

Fig. 3. Effect of allogeneic PC on the take of Lewis lung (LL) carcinoma. Recipient mice were inoculated with 10^5 LL cells (———), with 10^5 LL cells mixed with PC from untreated Balb/c mice (— . . —) or from Balb/c mice underwent "combined treatment" (□ — □). PC : tumour cell ratio, 50 : 1

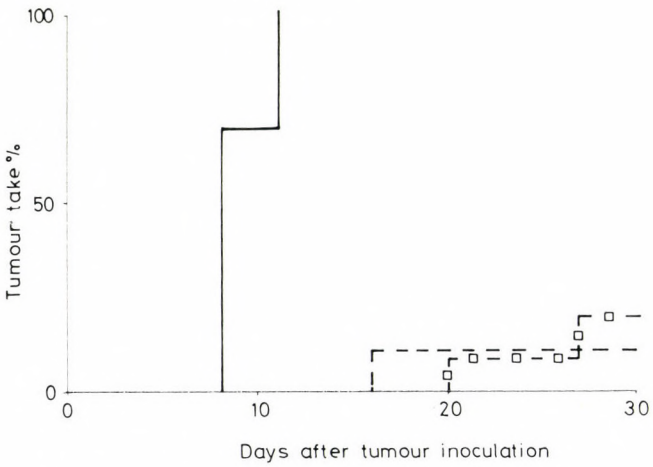

Fig. 4. Effect of activated plastic non-adherent PC on the take of Lewis lung (LL) carcinoma. Recipient mice were inoculated with 10^5 LL cells (———), with 10^5 LL cells mixed with plastic non-adherent PC from syngeneic C57Bl/6 mice underwent "combined treatment" (— — — —) or from allogeneic Balb/c mice underwent "combined treatment" (□ — □). PC : tumour cell ratio, 50 : 1

Syngeneic resident spleen cells or spleen cells from syngeneic donors underwent "combined treatment" did not inhibit the take of LL tumour (Fig. 5).

Plastic non-adherent cells separated from spleens of untreated syngeneic donors or from spleens of syngeneic mice underwent "combined treatment" did not either suppress the take of LL tumour. Plastic non-adherent resident PC

from syngeneic mice were also ineffective, while plastic non-adherent PC from syngeneic mice underwent "combined treatment" proved to be tumour-inhibitory [2] (Fig. 6).

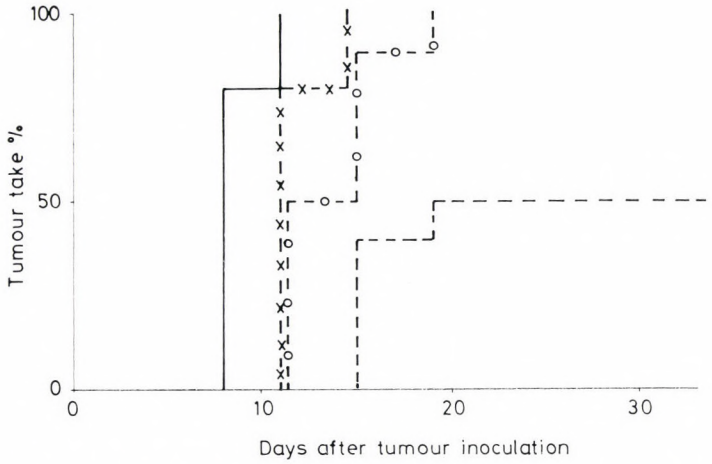

Fig. 5. Effect of syngeneic spleen cells and PC on the take of Lewis lung carcinoma (LL). Recipient mice were inoculated with 10^5 LL cells (———), with 10^5 LL cells mixed with spleen cells from untreated C57B1/6 mice (× — — ×) or from C57B1/6 mice underwent "combined treatment" (○ — — ○). One group of mice inoculated with 10^5 LL cells mixed with PC from C57B1/6 mice underwent "combined treatment" served as positive control (— — — —)

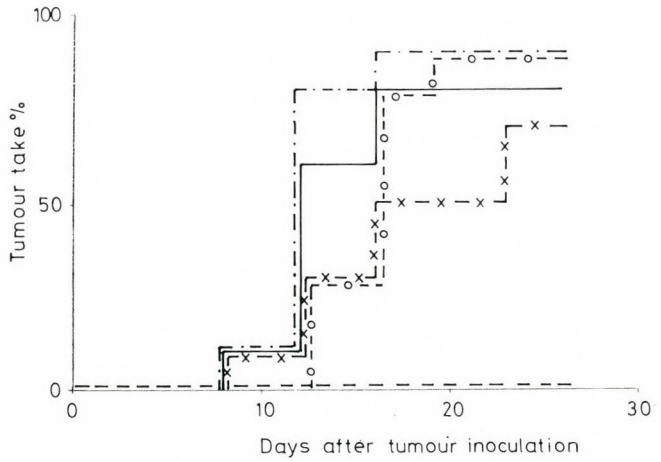

Fig. 6. Effect of syngeneic plastic non-adherent spleen cells and PC on the take of Lewis lung (LL) carcinoma. Recipient mice were inoculated with 10^5 LL cells (———), with 10^5 LL cells mixed with plastic non-adherent PC from untreated C57B1/6 mice (— . —) or from C57B1/6 mice underwent "combined treatment" (— — — —). Other groups of mice were inoculated with 10^5 LL cells mixed with spleen cells from untreated C57B1/6 mice (× — ×) or from C57B1/6 mice underwent "combined treatment" (○ — ○)

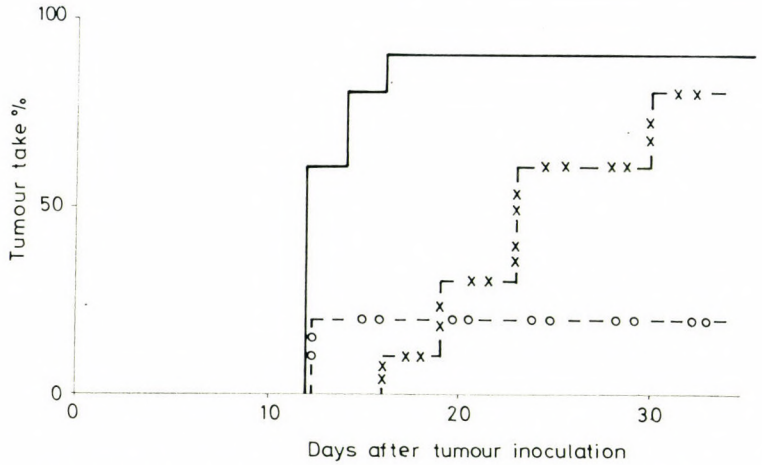

Fig. 7. Effect of allogeneic spleen cells on the take of Lewis lung (LL) carcinoma. Recipient mice were inoculated with 10^5 LL cells (———), with 10^5 LL cells mixed with spleen cells from untreated Balb/c mice (× × — × ×) or from Balb/c mice underwent "combined treatment" (○○ — — ○○)

Resident spleen cells from allogeneic donors delayed the take of LL tumour, transfer of spleen cells from allogeneic mice underwent "combine treatment", however, resulted in a more marked suppression of tumour take (Fig. 7).

Discussion

In a previous study we have shown that PC and non-adherent cells separated from PC of poly I : C treated syngeneic mice markedly inhibited the take of a chemically induced fibrosarcoma and a spontaneous adenocarcinoma in Winn-type experiments [1]. Poly I : C-stimulated syngeneic PC, however, failed to suppress the take of LL carcinoma and P815 mastocytoma; only transfer of PC obtained from mice treated with a combination of indomethacin, poly I : C and Syncumar inhibited the take of LL carcinoma, and even these cells did not influence the growth of the P815 mastocytoma [2].

As transferred non-activated allogeneic cells per se are capable to stimulate potential antitumoural effector cells of the host [3] and to suppress tumour take (Figs 1, 3, 7) we have tested whether PC from allogeneic donors injected with poly I : C or underwent "combined treatment" suppress the take of SP4 tumour and LL carcinoma more efficiently than similar cells of syngeneic mice. We have found no marked differences. We also compared the effect of syngeneic spleen cells and PC on the take of LL carcinoma, a tumour suspectible to the attack of spleen NK cells in vitro [4]. Syngeneic spleen cells from untreated mice or from donors underwent "combined treatment" failed to

inhibit the take of LL tumour. Thus, the effector cells in the PC population mediating the suppression of the take of LL tumour seem to be different from splenic effector cells responsible for natural cell-mediated cytotoxicity [5, 14]. Contrary to syngeneic spleen cells, transfer of allogeneic splenocytes resulted in inhibition of the take of LL tumour. This effect can be explained either by activation of antitumoural effector cells of the host [3], or by cytotoxic activity of the transferred cells [7–10]. Allogeneic spleen cells recognize either the products of the major histocompatibility complex [7] on the surface of tumour cells if present, or other tumour-associated antigens [8, 15] and recognition structures [5].

In conclusion, our data show that activated PC of syngeneic or allogeneic mice are capable to suppress the take of malignant tumours. Their tumour-inhibitory potential is comparable to that of activated allogeneic spleen cells. Non-adherent activated PC seem to be different from NC and NK cells and are potential candidates in immunotherapy of certain malignant tumours.

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REFERENCES

1. Minárovits, J., Karczag, E., Földes, I.: Acta Microbiol Hung **34,** 197 (1987).
2. Karczag, E., Minárovits, J., Földes, I.: Acta Microbiol Hung **34,** 207 (1987).
3. Kawase, I., Komuta, K., Namba, M., Yokota, S., Ogura, T., Kishimoto, S.: Cancer Res **46,** 1047 (1986).
4. Herberman, R. B., Holden, H. T.: Adv Cancer Res **27,** 305 (1987).
5. Stutman, O., Lattime, E. C.: Lymphokines **12,** 107 (1985).
6. Lattime, E. C., Pecoraro, G. A., Cuttito, M., Stutman, O.: In Herberman, R. B. (ed.): NK Cells and Other Natural Effector Cells. Academic Press, New York 1982. p. 179.
7. Bonmassar, E., Goldin, A., Cudkowicz, G.: Transplantation **12,** 314 (1971).
8. Ostrand-Rosenberg, S., Clements, V.: Immunogenetics **26,** 1 (1987).
9. Vidrnová, M., Nouza, K., Nemec, M.: Neoplasma **31,** 369 (1984).
10. Rolstad, B., Fossum, S.: Immunology **60,** 151 (1987).
11. Mishell, B. B., Shiigi, S. M., Henry, C. et al.: In Mishell, B. B., Shiigi, S. M. (eds): Selected Methods in Cellular Immunology. W. H. Freeman and Co., San Francisco 1980. p. 4.
12. Sanderson, C. J., Clark, J. A., Taylor, G. A.: Nature **253,** 376 (1975).
13. Winn, H. J.: J Immunol **86,** 228 (1961).
14. Den Otter, W.: Cancer Immunol Immunother **21,** 85 (1986).
15. Kwak, L. W., Kucuk, O., Melvold, R. W., Williams, R. M.: Science **220,** 959 (1983).

Acta Microbiologica Hungarica 36 (1), pp. 33–39 (1989)

CHARACTERIZATION OF ACTIVATED PERITONEAL CELLS INHIBITING THE TAKE OF TRANSPLANTABLE MURINE TUMOURS

J. MINÁROVITS, ERIKA KARCZAG and I. FÖLDES

Microbiological Research Group of the National Institute of Hygiene, Budapest

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We studied the properties of activated peritoneal cells (PC) inhibiting the take of SP4 spontaneous adenocarcinoma and Lewis lung carcinoma in syngeneic mice. Treatment of the poly I : C activated PC from Balb/c mice suppressing the take of SP4 tumour with anti-asialo GM1 antibody and complement before transfer did not affect their tumour-inhibitory potential. PC from Balb/c nude mice treated with poly I : C also inhibited the take of SP4 tumour. Spleen cells from untreated or poly I : C treated Balb/c and Balb/c nude mice, however, did not inhibit the take of SP4 adenocarcinoma. Treatment of peritoneal cells activated by a combination of poly I : C, indomethacin and Syncumar (referred to as "combined treatment") with anti-asialo GM1 antibody and complement could not, or could only partly abolish their tumour-inhibitory potential. The cells mediating the suppression of the take of Lewis lung tumour proved to be Thy-1,2$^{\pm}$, Lyt-1^{-}, Lyt 2,2^{-} cells. We conclude that the activated peritoneal cells inhibiting the take of SP4 adenocarcinoma and Lewis lung tumour are different from NK cells, NC cells and LAK cells and represent a distinct antitumoural effector cell population.

We reported earlier that peritoneal cells (PC) from poly I : C treated Balb/c mice inhibit the take of a spontaneous adenocarcinoma (SP4) and a benzpyrene induced fibrosarcoma (BaF1) in syngeneic mice [1]. The cells responsible for the tumour-inhibitory effect proved to be plastic non-adherent cells in case of SP4 tumour; both plastic adherent and non-adherent cells contributed, however, to the inhibition of the take of BaF1 fibrosarcoma [1]. The take of Lewis lung carcinoma (LL) could not be inhibited by peritoneal cells from poly I : C treated syngeneic mice; peritoneal cells activated by a combination of indomethacin, poly I : C and Syncumar ("combined treatment"), however, inhibited the take of LL tumour [2]. The cells mediating this effect also proved to be plastic non-adherent cells. Here we report a further characterization of activated peritoneal cells mediating the inhibition of the take of SP4 and LL tumours.

Based on negative selection experiments using various antibodies plus complement and based on the resistance of SP4 and LL tumours to spleen cells (see also our accompanying paper), we conclude that the activated plastic

JÁNOS MINÁROVITS, ERIKA KARCZAG, ISTVÁN FÖLDES*
National Institute of Hygiene, Microbiological Research Group
Pihenő út 1, H-1529 Budapest, Hungary

* Corresponding author

non-adherent PC inhibiting the take of these tumours, are different from natural killer, natural cytotoxic (NC) and lymphokine activated killer (LAK) cells [3–14] and represent a distinct antitumoural effector cell population.

Materials and methods

Animals. Seven to eight weeks old inbred Balb/c and C57B1/6 mice (LATI, Gödöllő, Hungary) were used. Seven weeks old Balb/c nude mice were purchased from the Biological Research Center, Szeged, Hungary.

Tumours and chemicals used were the same as described in the accompanying article [15].

Treatment of animals. Balb/c female mice were injected with 100 μg poly I : C intraperitoneally 18 h before cell collecting. C57B1/6 female mice were injected with 100 μg indomethacin, 100 μg poly I : C and 100 μg Syncumar intraperitoneally 42, 18 and 3 h before cell collecting, respectively, as described previously [2].

Peritoneal cells were collected as described in the accompanied article [15].

Separation of plastic adherent and non-adherent cells. PC were maintained for 1 h in plastic tissue culture dishes in medium 199 containing 10 mM HEPES and 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere. Non-adherent cells were removed by a careful repeated washing with warm culture medium. Non-adherent cells were treated with carbonyl iron + magnet [16] to remove remaining phagocytic cells and washed two times with medium 199 at 4 °C before treatment with antisera + complement, or were passed over a nylon fiber column immediately after removal from the plastic tissue culture dishes.

Passage of plastic non-adherent PC over a nylon fiber column. For the separation of cells by nylon wool adherence, a slightly modified method of Julius et al. [17] was used. A 10 ml syringe was filled with 0.85 g of sterile washed and dried nylon wool. The column was preincubated with medium 199 containing 10 mM HEPES and 10% fetal calf serum and filled with plastic non-adherent PC resuspended in the same culture medium (5 ml cell suspension per column). Then the column was closed and incubated at 37 °C in a 5% CO_2 atmosphere for 1 h. Nylon wool non-adherent cells were collected by washing slowly the column with 20 ml prewarmed culture medium. They were washed two times in medium 199 at 4 °C before treatment with antisera plus complement.

Treatment of PC with antisera plus complement. All treatments were performed under conditions recommended by the suppliers of the antibodies. Plastic non-adherent or plastic and nylon wool non-adherent PC were resuspended in medium 199. Mouse anti-Thy-1,2 monoclonal antibody, mouse anti-Lyt-2,2 monoclonal antibody (Cedarlane Laboratories, Hornby, Ontario, Canada) or normal rabbit serum used as control was added to the cells in a final concentration of 1 : 20 and they were incubated at 4 °C for 60 min. Then the cells were centrifuged and resuspended in medium 199 containing guinea pig complement (Human, Budapest) in a final concentration of 1 : 20. After incubating at 37 °C for 45 min in a 5% CO_2 atmosphere, the cells were washed in medium 199, centrifuged and resuspended in sterile saline.

Treatment of cells with rabbit anti-asialo GM1 antibody (Wako Chemicals, Neuss, FRG) or with control rabbit serum was performed as follows: PC were incubated in medium 199 containing a 1 : 100 or 1 : 50 dilution of the antiserum at room temperature for 60 min, centrifuged, and resuspended in medium 199 containing guinea pig complement in a final concentration of 1 : 5. After incubating at 37 °C for 45 min in a 5% CO_2 atmosphere, the cells were washed in medium 199, centrifuged and resuspended in sterile saline. Treatment of PC with rat anti-Lyt-1 monoclonal antibody (Sera Lab Ltd, Crawley Down, Sussex, England) diluted 1 : 50 and guinea pig complement diluted 1 : 40 was performed in a single step at 37 °C for 37 min. After washing in medium 199 the cells were resuspended in sterile saline.

Spleen cells. Preparation of spleen cell suspensions from spleens of untreated or poly I : C treated Balb/c and Balb/c nude mice and lysis of red blood cells was performed as described by Mishell et al. [18]. The cells were washed twice in medium 199 containing 10 mM HEPES and resuspended in sterile saline before transfer.

Experimental procedure was similar to that described in the accompanying article.

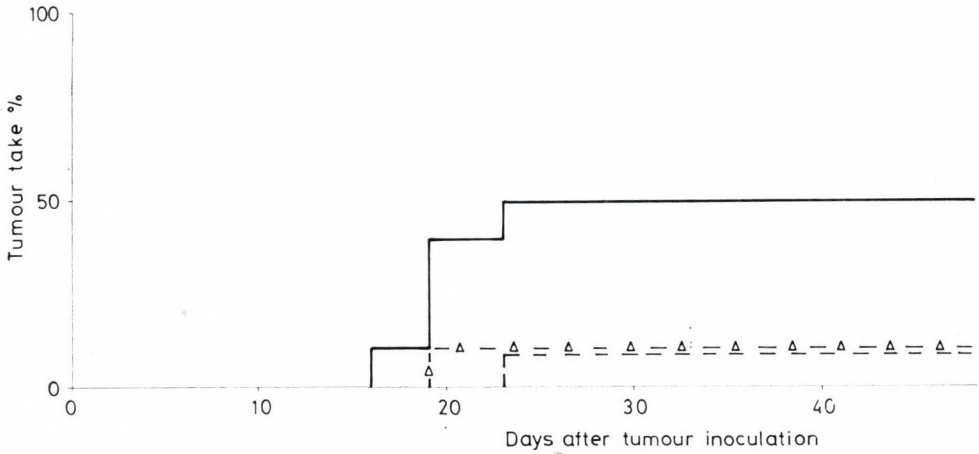

Fig. 1. Effect of anti-asialo GMl antibody plus complement treatment on the tumour-take inhibitory action of plastic non-adherent PC from poly I : C treated mice. Mice were inoculated intramuscularly with 2×10^5 SP4 tumour cells (———) and with 2×10^5 SP4 cells mixed with poly I : C activated plastic nonadherent PC treated with either normal rabbit serum plus complement (△ — △) or rabbit anti-asialo GMl antibody plus complement (— — — —). PC : tumour cell ratio, 18 : 1

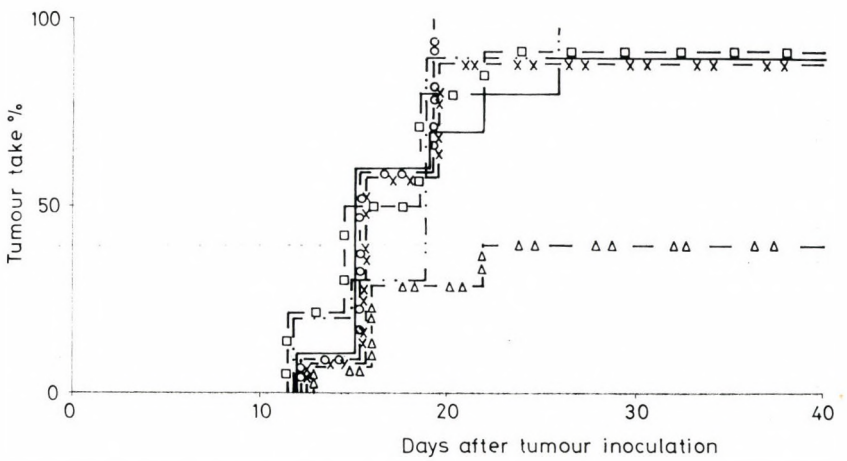

Fig. 2. Comparison of the effects of PC and spleen cells from Balb/c nude mice and spleen cells from Balb/c mice on the take of SP4 adenocarcinoma. Mice were inoculated intramuscularly with 2×10^5 SP4 cells (———) and with 2×10^5 SP4 cells mixed with PC (△△ — △△) or spleen cells (× × — × ×) from poly I : C treated Balb/c nude mice. Other groups of mice were inoculated with 2×10^5 SP4 cells mixed with spleen cells from untreated Balb/c nude mice (○○ — ○○) and from untreated (□ — □) or poly I : C treated (— . . —) Balb/c mice. Effector cell : tumour cell ratio, 50 : 1

Results

Pretreatment of poly I : C activated plastic non-adherent PC with anti-asialo GM1 antibody plus complement did not influence the capacity of these cells to suppress the take of SP4 tumour (Fig. 1). Spleen cells from untreated and poly I : C treated Balb/c or Balb/c nude mice did not inhibit the take of SP4 adenocarcinoma; PC from poly I : C treated Balb/c nude mice, however, inhibited the take of SP4 tumour (Fig. 2).

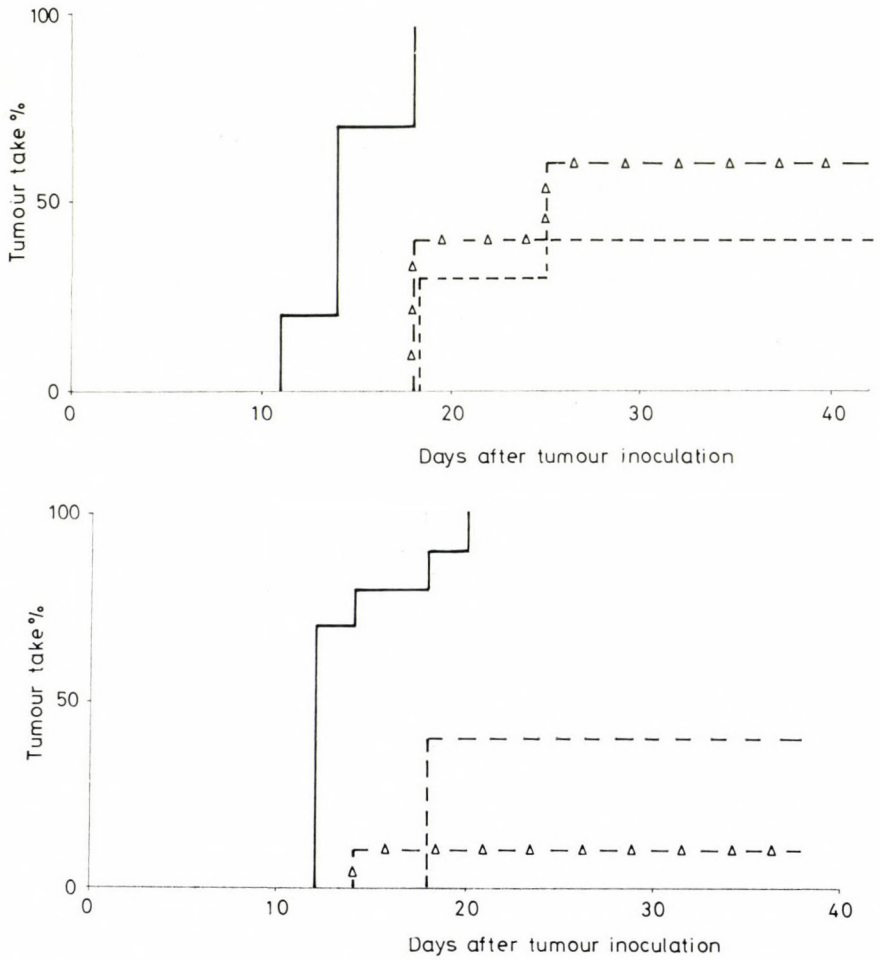

Fig. 3. Effect of anti-asialo GM1 antibody plus complement treatment on the tumour-take inhibitory action of plastic and nylon wool non-adherent PC from mice underwent "combined treatment". Mice were inoculated intramuscularly with 10^5 Lewis lung (LL) carcinoma cells (———) and with 10^5 LL cells mixed with plastic and nylon wool nonadherent activated PC treated with either normal rabbit serum plus complement (△ — △) or rabbit anti-asialo GM1 antibody plus complement (— — — —). PC : tumour cell ratio, 45 : 1. Dilution of sera, 1/50 (upper figure) and 1/100 (lower figure)

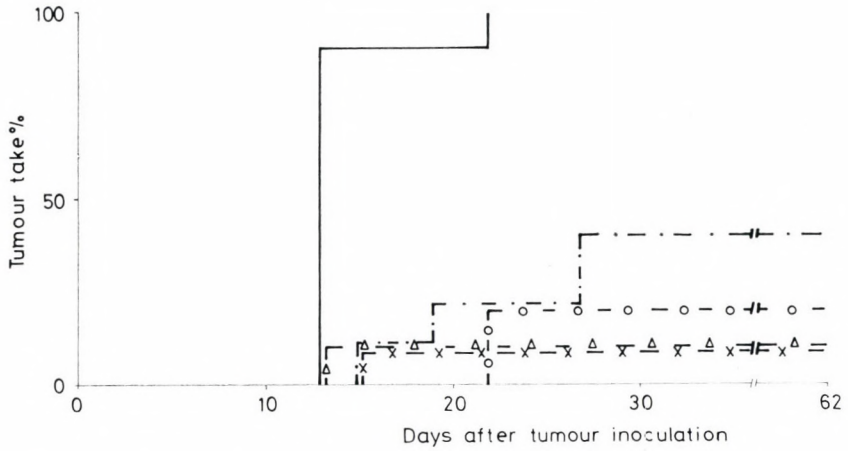

Fig. 4. Effect of anti-Thy-1,2, antiLyt-1 or anti-Lyt-2,2 antibody plus complement treatment on the tumour-take inhibitory effect of plastic non-adherent PC from mice underwent "combined treatment". Mice were inoculated intramuscularly with 10^5 Lewis lung (LL) carcinoma cells (———) and with 10^5 LL cells mixed with activated PC treated with normal rabbit serum plus complement (△ — △), anti-Thy-1,2 antibody plus complement (— . —), anti-Lyt-1 antibody plus complement (× — ×) or anti Lyt-2,2 antibody plus complement (○ — ○)

Treatment of plastic and nylon wool non-adherent PC from C57Bl/6 mice underwent "combined treatment" with anti-asialo GM1 antibody plus complement could not, or could only partly, abolish their capacity to inhibit the take of LL tumour (Fig. 3). Treatment of plastic non-adherent activated PC with anti-Lyt-1 or anti-Lyt-2,2 monoclonal antibody plus complement did not abolish their tumour-inhibitory potential, either; treatment with anti-Thy-1,2 monoclonal antibody plus complement, however, partially abrogated the tumour-suppressing effect of activated PC (Fig. 4).

Discussion

In principle, each cell type capable of selective suppression of the growth of neoplastic cells (without the destruction of normal cells) is a potential candidate for tumour therapy. Specifically sensitized T cells may be effective in the therapy of immunogenic tumours [9, 19]; other cell types (macrophages, NK cells, NC cells and LAK cells) could be used for the treatment of both immunogenic and non-immunogenic neoplasms [3–9, 20–25]. We demonstrated earlier that poly I : C activated PC contain mor ethan one antitumoural effector cell population [1].

In the present study the plastic non-adherent effector cells present in poly I : C activated PC were shown to be resistant to treatment with anti-asialo GM1 antibody plus complement. In addition, we could not detect similar

cells in spleens of untreated or poly I : C treated Balb/c nude mice. Thus, the activated plastic non-adherent PC, inhibiting the take of SP4 tumour are different from NK cells, since NK cells are asialo GM1 positive cells [13] and they are present in high levels in Balb/c nude spleens [3]. The activated PC are most probably different from NC cells and they are not in vivo generated LAK cells, either, since spleens of normal Balb/c mice (a rich source of NC cells and LAK precursors [5, 7]) do not contain effector cells capable to inhibit the take of SP4 tumour (Fig. 2).

Plastic non-adherent, non-phagocytic cells mediate the inhibiton of the take of LL tumour by PC from mice underwent "combined treatment" as well [2]. Passage over a nylon wool column did not abolish their tumour-suppressing effect. Since being Lyt-2$^-$ cells, they are most probably not in vivo generated LAK cells (murine LAK cells are known to be Lyt-2$^+$ cells [9]). They are not typical NC cells, either, since their tumour-inhibitory effect was partly abolished by treatment with anti-Thy-1,2 serum plus complement (NC cells are Thy-1$^-$ cells, [6]). They also do not correspond to typical NK cells, as their tumour-inhibitory capacity could not, or could be only partly, abolished by treatment with anti-asialo BM1 antibody plus complement. In addition, syngeneic spleen cells were unable to inhibit the take of LL tumour [15]. The partial sensitivity of PC activated by "combined treatment" to the effect of anti-Thy-1,2 antibody (or anti-asialo GM1 antibody) and complement could be explained by a low density of target antigens on the cell surface and/or a relative resistance to complement-mediated lysis [11]. In addition, the possibility has to be considered that the tumour-inhibitory effect of PC activated by "combined treatment" is mediated by an heterogenous population of effector cells [10–12].

We conclude that the activated peritoneal cells inhibiting the take of SP4 adenocarcinoma and LL tumour are different from NK cells, NC cells and LAK cells and represent a distinct antitumoural effector cell population, a novel candidate for immunotherapy.

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REFERENCES

1. Minárovits, J., Karczag, E., Földes, I.: Acta Microbiol Hung **34,** 197 (1987).
2. Karczag, E., Minárovits, J., Földes, I.: Acta Microbiol Hung **34,** 207 (1987).
3. Herberman, R. B., Holden, H. T.: Adv Cancer Res **27,** 305 (1978).
4. Riccardi, C., Barlozzari, T., Santoni, A., Herberman, R. B., Cesarini, C.: J Immunol **126,** 1284 (1981).
5. Stutman, O., Paige, C. J., Feo Figarella, E.: J Immunol **121,** 1819 (1978).
6. Paige, C. J., Feo Figarella, E., Cuttito, M. J., Cahan, A., Stutman, O.: J Immunol **121,** 1827 (1978).

7. Shain, B., Holt, C. A., Lilly, F.: J Immunol **129,** 722 (1982).
8. Yang, J. C., Mulé, J. J., Rosenberg, S. A.: J Immunol **137,** 715 (1986).
9. Rosenberg, S.: JNCI **75,** 595 (1985).
10. Minato, N., Reid, L., Bloom, B. R.: J Exp Med **154,** 750 (1981).
11. Bykowsky, M. J., Stutman, O.: J Immunol **137,** 1120 (1986).
12. Stutman, O., Lattime, E. C.: Lymphokines **12,** 107 (1985).
13. Kasai, M., Iwamori, M., Nagai, Y., Okumura, K., Tada, T.: Eur J Immunol **10,** 175 (1980).
14. Lattime, E. C., Ishizaka, S. T., Pecoraro, G. A., Koo, G., Stutman, O.: In Herberman, R. B. (ed.): NK Cells and Other Natural Effector Cells. Academic Press, New York 1982. p. 187.
15. Karczag, E., Minárovits, J., Földes, I.: Acta Microbiol Hung **36,** 25 (1989).
16. Sanderson, C. J., Clark, J. A., Taylor, G. A.: Nature **253,** 376 (1975).
17. Julius, M. H., Simpson E., Herzenberg, L. A.: Eur J Immunol **3,** 645 (1973).
18. Mishell, B. B., Shiigi, S. M., Henry, C. et al.: In Mishell, B. B., Shiigi, S. M. (eds): Selected Methods in Cellular Immunology. W. H. Freeman and Co., San Francisco 1980. p. 4.
19. North, R. J.: Adv Immunol **35,** 89 (1984).
20. Fidler, I. J.: Cancer Res **45,** 4714 (1985).
21. Saito, M., Nanjo, M., Aonuma, E., Noda, T., Nakadate, I., Ebina, T., Ishida, N.: Int J Cancer **33,** 271 (1984).
22. Morales, A., Pang, A. S. D.: Cancer Immunol Immunother **21,** 156 (1986).
23. Lattime, E. C., Pecoraro, G. A., Cuttito, M., Stutman, O.: In Herberman, R. B. (ed.): NK Cells and Other Natural Effector Cells. Academic Press, New York 1982. p. 179.
24. Merluzzi, V. J., Savage, D. M., Souza, L., Boone, T., Mertelsmann, R., Welte, K., Last-Barney, K.: Cancer Res **45,** 203 (1985).
25. Rayner, A., Grimm, E. A., Lotze, M. T., Wilson, D. J., Rosenberg, S. A.: JNCI **75,** 67 (1985).

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BIOFILM MODEL EXPERIMENTS IN VITRO WITH *ESCHERICHIA COLI* AND *SHIGELLA* STRAINS*

I. KÉTYI

Institute of Microbiology, University Medical School, Pécs

(Received May 9, 1988)

Escherichia coli and *Shigella* were cultured together in a glucose-free minimal medium completed only by hog gastric mucin. In mixed cultures inoculated simultaneously one member of the pair more or less overgrew the other. This organism remained predominant when superinfected with its strain pair after 1, 3, 5 or 7 days incubation. Other signs of the quasi biofilm character of such cultures are the higher level of free polysaccharides and enhanced viscosity in the medium and higher streptomycin resistance of the culture. Around the bacteria a capsule-like material can morphologically be demonstrated.

In a previous paper [1] it was shown that the ability of utilizing mucin is a general character of the enteric bacteria, because of their alpha-glycosidase-permease activities. In these experiments a minimal medium completed by hog gastric mucin was used fulfilling the criteria of the so called "limited nutrient condition". According to Costerton et al. [2] it favours the development of a bacterial biofilm. Bacteria in biofilms are attached to some surface, produce an exopolysaccharide matrix in which they are resistant against many external factors.

In this paper we demonstrate the quasi biofilm characters exerted in a very simple cultural environment.

Material and methods

Strains used are listed in Table I.

Media. A minimal medium composed according to Rothman and Corwin [3] was used without glucose. As complete media Luria's broth and LB agar, for selective counting of Lac$^+$ and Lac$^-$ colonies Endo agar was applied.

Mucin. A hog gastric mucin preparation (Granular Mucin, Type 1701-W, Wilson Labs., Chicago, Ill. USA) was used at 5% concentration, sterilized at 100 °C for 1 h.

Mixed cultivation. Minimal media completed mostly by a final concentration of 0.25% mucin were inoculated simultaneously with about 10^5 germs of two strains. Cultures inoculated separately served as control. After 24 h incubation at 37 °C the rates of the colony forming units (c.f.u.) were calculated by plating on Endo agar, one of the strain pairs being Lac$^+$, the other Lac$^-$.

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

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In an other series of experiments only one member of the strain pairs was inoculated into the above medium and incubated at 37 °C for 1, 3, 5 and 7 days. This strain was termed "resident". Thereafter the other member of the strain pair was also inoculated, as a "superinfecting" strain. The initial c.f.u. rates were determined at the time of superinfection. After 24 h incubation the rate of resident and superinfecting strains was estimated by plating. Mixed cultures obtained by simultaneous inoculation served as control.

Examination of antagonistic factors. For the determination of any antagonistic factor which may or may not be involved in the resulting growth rates beside the simple cross streaking test, the following method was applied: 96 h old minimal-mucin cultures of the tested strains were passed through membrane filters (Schleicher-Schüll, BA85, 0.45 nm, Dassel, FRG) and the growing abilities of the strains were tested in the filtrate of their strain pair recompleted by 0.25% of mucin. A filtrate of the same strain served as control.

Determination of free polysaccharide content of 7 days old culture filtrates was done by the phenol-sulfuric acid method of Dubois et al. [4]. The values were expressed in glucose equivalents per 100 μl. The filtrate of a sterile medium and that of a 24 h old culture served as control.

Estimation of viscosity of the above filtrates was performed with a rheometer (Type HEVIMET 40). Data of the minimal velocity gradient (l/S) and the threshold voltage (MPAS) were informative about the viscosity.

Sensitivity of minimal-mucin cultures against the killing effect of streptomycin. The minimum inhibitory concentration (MIC) of streptomycin was determined for bacteria being in "planctonic" phase, i.e. in LB cultures. Graded doses of streptomycin were added to 6 h old and 7 days old minimal-mucin cultures. The simultaneous planctonic control was the 6 h old LB culture. The minimal killing dose of streptomycin was determined by plating after overnight incubation.

Morphological appearance of bacteria living in minimal-mucin culture was studied in an India ink preparation counterstained with fuchsin.

Results

Experiments with mixed cultures obtained by simultaneous inoculation. Five ml aliquots of glucose-free minimal medium completed with mucin (0.5, 0.25, and 0.125%) were inoculated in this series of experiments with six pairs of *E. coli* and *Shigella* strains. The growth rates of the strain pairs were estimated by plating. Strains cultured separately served as control. Because the growth rates with different mucin concentrations did not show substantial differences, their mean values were compared (Table I). The growth rates of cultures inoculated separately did not show marked differences, the maximum

Table I

List of strains

Designation	Marker used	Other data
Escherichia coli O143, No. 2	Lac^+	virulent
O124, No. 34	Lac^-*	virulent
"normal", murine**	Lac^+	avirulent
Shigella sonnei, No. 87	Lac^-*	1st phase is virulent
Shigella flexneri 3, No. 20780	Lac^-	avirulent

* Late lactose fermentation
** Isolated from the faeces of a healthy mouse

difference being only 1 : 3. On the other hand, in mixed cultures one of the strains showed more or less marked predominance: if *E. coli* O143 was cultured together with *E. coli* O124 or *Shigella flexneri* 3, it reached a count 5 and 166

Table II

Rates of growth of some strains of E. coli and Shigella in separated and mixed cultures in minimal medium completed by granular mucin

Strain pairs		Rates of growth (c.f.u.), No. 1: No. 2	
No.1	No. 2	separately cultured	co-cultured
E. coli O143	— *E. coli* O124	1: 1.2	1: 0.21
E. coli O143	— *S. sonnei*	1: 0.89	1: 70.8
E. coli O143	— *S. flexneri* 3	1: 0.41	1: 0.006
E. coli murine	— *E. coli* O124	1: 0.62	1: 0.006
E. coli murine	— *S. sonnei*	1: 0.49	1: 0.07
E. coli murine	— *S. flexneri* 3	1: 0.31	1: 0.14

Minimal media completed by granular mucin in 0.5, 0.25 and 0.125% concentrations were inoculated separately and simultaneously with the above strain pairs. After incubation at 37 °C for 24 h the number of colony forming units (c.f.u.) was determined by plating. The rates of growth were calculated from the mean values obtained for the three concentrations of mucin

Table III

Rates of growth of E. coli and S. sonnei as "resident" and "superinfecting" strains in minimal medium completed by 0.25% granular mucin

	Resident strain grown for			
	1 day	3 days	5 days	7 days
	rates of c.f.u. (resident: superinfecting)			
	Resident strain, E. coli O143 No. 2			
Initial rates	238:1	9.1:1	1.2:1	0.9:1
Rates after mixed cultivation for 24 h	180:1	10:1	41:1	35:1
	Resident strain, S. sonnei No. 87			
Initial rates	1:1	1.7:1	0.8:1	0.7:1
Rates after mixed cultivation for 24 h	20.5:1	29:1	12.5:1	4.2:1
	Control: mixed cultivation after simultaneous inoculation			
Initial rate *(E. coli: S. sonnei)*			4.1:1	
Rate after mixed cultivation for 24 h			1:55.3	

Minimal media completed by 0.25% of granular mucin were first inoculated by one of the cultures ("resident strain"). After incubation for 1, 3, and 7 days the resident cultures were superinfected with the other strain. After a 24 h incubation of the mixed culture, c.f.u. values were determined. The initial rates of the resident and superinfecting strains were also recorded. Simultaneous inoculation of both strains served as control

Table IV

Rates of growth of E. coli and S. flexneri 3 as "resident" and "superinfecting" strains in minimal medium completed by 0.25% granular mucin

	Resident strain grown for			
	1 day	3 days	5 days	7 days
	rates of c.f.u (resident: superinfecting)			
	Resident strain, E. coli "normal", murine			
Initial rates	1.25:1	0.35:1	1.1:1	5:1
Rates after mixed cultivation for 24 h	200:1	100:1	333:1	333:1
	Resident strain, S. flexneri 3 "20780"			
Initial rates	0.55:1	0.55:1	0.03:1	0.008:1
Rates after mixed cultivation for 24 h	0.15:1	0.08:1	0.04:1	0.03 :1
	Control: mixed cultivation after simultaneous inoculation			
Initial rate *(E. coli:S. flexneri)*		3.1:1		
Rate after mixed cultivation for 24 h		1:0.14		

For explanation see Table III

times higher, respectively, than that of the latter. However, *Shigella sonnei* showed a 70-fold increase compared to *E. coli* O143. The *E. coli* strain of murine origin had a marked overgrowing ability over all of its partners: *E. coli* O124, *S. sonnei*, and *S. flexneri* 3 (166, 14 and 7-fold, respectively, Table II).

Experiments with not simultaneously inoculated mixed cultures. In this series of experiments three pairs of strains were chosen and the minimal mucin media were inoculated first with only one member. The superinfection of their

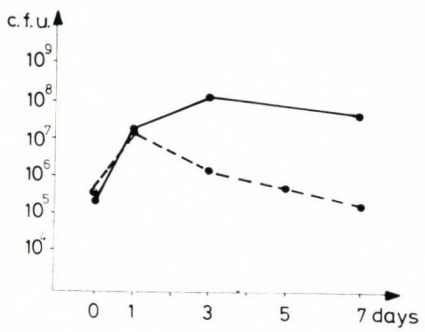

Fig. 1. Colony forming unit values for strains *E. coli* "murine" (● — — — ●) and *S. flexneri* 3 "20780" (● — — — ●) cultured separately for 7 days in minimal medium completed by 0.25% granular mucin

cultures with the other member of the pair was done only after incubations for 1, 3, 5 or 7 days. After 24 h of mixed incubation the c.f.u. rates were estimated by platings. Simultaneously inoculated mixed cultures served as control.

In the case of strains *E. coli* O143 and *S. sonnei* (Table III) the predominance of one of the partners depended on the residence: in 1, 3, 5, or 7 days old *E. coli* cultures superinfected with *S. sonnei*, the former remained predominant, although in the simultaneously incubated mixed culture a massive outgrowth by *S. sonnei* was observed. The same phenomenon appears in the reciprocal experiment (Table III).

The above pattern, namely the overgrowth of the resident strain did not appear for the murine *E. coli* and *S. flexneri* 3 pair. In this case the failure (Table IV) may be due to a stepwise and rapid declination of the c.f.u. values of the "resident" *S. flexneri* culture (Fig. 1), losing more than two log10 exponents at the end of the week.

The first pattern was demonstrated for the third strain pair: murine *E. coli* and O124. In contrast to an overgrowth of O124 by the murine strain in the control (about 200-fold), as a resident, O124 maintained its predominancy (Table V). This is surprising in view of the fact that the culture filtrate of the murine *E. coli* markedly suppressed the growth of O124 (Table VI). No such a filtrable antagonistic factor was detected in cases of the other strain pairs.

Table V

Rates of growth of E. coli murine and O124 as "resident" and "superinfecting" strains in minimal medium completed by 0.25% granular mucin

	Resident strain grown for			
	1 day	3 days	5 days	7 days
	rates of c.f.u. (resident: superinfecting)			
	Resident strain, E. coli murine			
Initial rates	1.2:1	2.2:1	5:1	2:1
Rates after mixed cultivation for 24 h	117:1	173:1	285:1	43.:1
	Resident strain, E. coli O124 No. 34			
Initial rates	0.7:1	14:1	0.14:1	0.2:1
Rates after mixed cultivation for 24 h	7.7:1	6:1	8:1	3.2:1
	Control: mixed cultivation after simultaneous inoculation			
Initial rate (murine: O124)		0.6:1		
Rate after mixed cultivation for 24 h		1:0.005		

For explanation see Table III

Table VI

Effect of the culture filtrate on the growth of strains used as superinfecting agents under limited nutrient condition

"Pairs"	Strains and their designations	Relative growing ability in culture filtrate of the	
		coresponding strain	other strain
1	*S. sonnei* No. 87	1	1
	E. coli O143 No. 2	1.7	1
2	*E. coli* murine	1	1
	S. flexneri 3 No. 20780	1	1.5
3	*E. coli* murine	1	1.1
	E. coli O124 No. 34	1	0.05

Four days old cultures of the above strains in minimal medium completed by 0.25% granular mucin were filtered. Five ml aliquots of each filtrate were recompleted by 0.25% mucin. The strains were inoculated into the filtrate of their own culture (control) and into that of the other member of the pair. After incubation of 37 °C for 24 h, the c.f.u. values were determined and inhibition of growth was estimated by comparing growth in the two kinds of filtrate

Table VII

Changes in the polysaccharide content and viscosity in the supernatants of the minimal mucin cultures of the murine E. coli strain

Age of the cultures and conditions	PS content (100 μl) glc equivalent	Viscosity	
		min. velocity gradient (l/S)	threshold voltage (MPAS)
Control: sterile medium, 7 days at 37 °C	11.4 μg	158.82	0.69
24 h old culture*	11.5 μg	161.19	0.71
7 days old culture	12.7 μg	168.23	0.75

* Before inoculation, the sterile medium was kept at 37 °C for 7 days

Other signs of biofilm, or biofilm-like character of the minimal mucin cultures. First the free polysaccharide content and viscosity of the culture filtrate of a 7 days old murine *E. coli* culture was investigated. Controls included a 24 h culture in minimal mucin medium preincubated for six days, and a sterile medium incubated for 7 days. These were done to exclude errors due to the release of polysaccharides from the mucin during the long incubation. Data presented in Table VII show an elevated concentration of free polysaccharides and an enhanced viscosity in the filtrate of the 7 days old culture.

It is known that bacteria living in the exopolysaccharide matrix of a biofilm exhibit an elevated resistance against antibiotics. After the preliminary determination of MIC values using LB cultures, 1, 2.5, 5.0 and 7.5 MIC

doses of streptomycin were added to the 24 h and 7 days old minimal mucin cultures. The results are summarized in Table VIII. These data suggest that already in the 24 h old cultures in minimal mucin medium all strains, except *S. flexneri* 3 showed an enhanced resistance against streptomycin. A more detailed study was carried out with the murine *E. coli* strain showing the most definite enhanced resistance. The controls were modified so that the minimal are was represented by a 6 h old culture in minimal mucin and the planctonic control was a 6 h old LB culture reaching a c.f.u. value of $5 \times 10^8 - 1.0 \times 10^9$. Streptomycin was added in concentrations of 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μg per ml. The results are shown in Table IX.

It is clear that a somewhat higher dose is needed for the killing of log phase, as well as stationary phase cultures. There was no substantial difference between the log phase LB and minimal mucin cultures (2 and 4 MIC, i.e. 50 and 100 μg/ml, respectively). On the other hand, the 7 days old minimal mucin

Table VIII

Resistance of the minimal mucin cultures against the killing effect of streptomycin

Strains and designation	Resistance against killing by streptomycin							
	24 h old cultures				168 h old cultures			
	1	2.5	5	7.5× ×MIC	1	2.5	5	7.5× ×MIC
E. coli O143 No. 2	R^L	R	R	S	R^L	R	R	S
E. coli O124 No. 34	R^L	R	R	S	R^L	R	R	S
E. coli murine	R^L	R	R	S	R^L	R^L	R	R
S. sonnei No. 87	R^L	R	R	S	R^L	R	R	S
S. flexneri 3 No. 20780	R	S	S	S	R	S	S	S

R^L = bacterial lawn on plates after overnight incubation with the addition of streptomycin; R = resistance (survival); S = sensitivity (killed).
MIC values determined for LB cultures were between 12.5 and 25 μg/ml of streptomycin

Table IX

The killing effect of streptomycin on E. coli murine cultures in glucose-free minimal granular mucin medium

Cultures	Resistance against streptomycin	
	×MIC	μg/ml
Control: 6 h old planctonic in LB medium	2	50
6 h old culture in minimal mucin medium	4	100
168 h old culture in minimal mucin medium	14	350

Six and 168 h old cultures mixed with multiple MIC doses of streptomycin were incubated overnight and plated on LB agar plates. The c.f.u. values before the addition of streptomycin were about $0.8–2.5 \times 10^9$ for LB cultures and about 1×10^8 for minimal mucin cultures

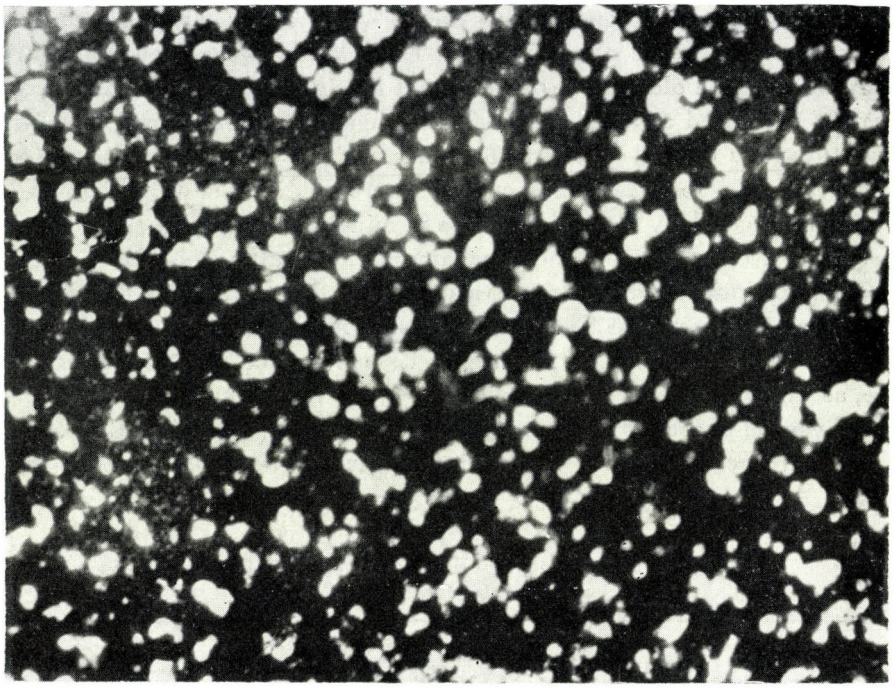

Fig. 2. An India ink preparation of the murine *E. coli* strain from a 7 days old minimal mucin culture (×1000)

culture of the murine *E. coli* showed an marked resistance (350 μg/ml or 14 MIC).

To demonstrate whether in our minimal mucin cultures the bacteria are or are not present in a matrix of exopolysaccharides, the murine *E. coli* strain was incubated for 1 and 7 days in minimal mucin medium. Using the simple method of India ink preparations, no capsule-like material was seen in the 24 h cultures, but the 7 days old cultures showed a very characteristic picture (Fig. 2): bacteria, or bacterial clusters were seen in a wide capsule-like material which was not stained by fuchsin and most of the incorporated bacteria also remained unstained.

Discussion

In a minimal medim completed only with hog gastric mucin, pairs of bacterial cultures showed a characteristic biological pattern: the organism inoculated first maintained a predominancy over the superinfecting agent. This pattern, explained by the residency of the primarily inoculated strain, was evident independently of the ability of one or the other member of the pair to overgrow when inoculated simultaneously, and even if the ability of

the superinfecting strain to produce a filtrable inhibitory factor against the resident strain. This peculiar pattern reminded us to the results of our early mouse experiments [5]. The essence of these in vivo observations was that if mice depleted of their aerobic bowel flora by streptomycin treatment, were orally infected with an (apathogenic) strain of *E. coli*, a monoflora developed resulting in a long-term carriership. Any other strain of *E. coli* introduced orally thereafter disappeared from the bowel within a week. Not knowing its mechanism, this phenomenon was called at that time "inplantation antagonism" which depended only on the priority of the strains.

The minimal mucin medium used throughout our present experiments may be accepted as an environment of "limited nutrient condition". According to the review of Costerton et al. [1] such environments favour the development of a biofilm. The essential difference of bacteria living in planctonic phase or in a biofilm is that in the latter they are present in a matrix composed mainly by exopolysaccharides. This matrix acts not only by "trapping" nutrients, but defends bacteria against phagocytes, antibodies, biocides or antibiotics.

Signs of the above characteristics were demonstrated in minimal mucin medium cultures beside the predominance of the resident strain, the filtrate of such cultures is rich of free polysaccharides, its viscosity is enhanced, the bacteria are relatively resistant against the killing effect of streptomycin and they are seemingly embedded in a wide, capsule-like material. We have to take into consideration that in our model system there is no further supply of nutrients and at least after one week the bacteria are in a declination phase. Therefore this in vitro model may represent only a "quasi biofilm".

According to Freter [6] our assumption about mucosal invasion is oversimplified: "The interaction of a bacterium with the intestinal mucosa is a multifaceted process which cannot be indentified, let alone be quantitated by means of a single test". He assumes that there are many steps of the mucosal association, but not all of these need to function in a pathogenic bacterium, but some of them may have to function in the natural inhabitants of the bowel flora (in vivo synthesis of bacterial chemoreceptors, synthesis of flagella, chemotactic attraction to the surface of mucus gel, penetration of the mucus gel, adhesion to the receptors in the mucus gel, chemotaxis in the deep areas of intervillous space, multiplication of mucosa-associated bacteria, etc.).

From the considerations outlined, it is clear that in the future research should concentrate on the distinct steps of "preepithelial invasion" in cases of different pathogenic and non pathogenic entities. We hope that our test medium more or less modified will be useful for some of these approaches.

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REFERENCES

1. Kétyi, I.: Acta Microbiol Hung **35,** 389 (1988).
2. Costerton, J. W., Cheng, K.-J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., Marrie, T. J.: Ann Rev Microbiol **41,** 435 (1987).
3. Rothman, S. W., Corwin, L. M.: Infect Immun **5,** 103 (1972).
4. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F.: Nature **168,** 167 (1951).
5. Rauss, K., Kétyi, I.: Zentralbl Bakteriol [A] **177,** 161 (1960).
6. Freter, R.: In Ouchterlony, Ö., Holmgren, J. (eds): Cholera and Related Diarrheas. 43rd Nobel Symposium, Stockholm, 1978. S. Karger, Basel 1980. p. 155.

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THE FREQUENCY OF AEROBACTIN PRODUCTION AND ITS EFFECT ON THE PATHOGENICITY OF HUMAN *ESCHERICHIA COLI* STRAINS

I. Gadó, Hedda Milch, Éva Czirók and Mária Herpay

National Institute of Hygiene, Budapest

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A total of 981 human *Escherichia coli* strains (including 632 strains isolated between 1979 and 1983 and 349 strains isolated in 1987) was examined for aerobactin production by biological qualitative test. Aerobactin positivity was found in 55.1% and 47.3%, respectively, in the two groups of strains, while enterochelin was produced nearly by 100% of the strains. Aerobactin production was significantly more frequent than the average among blood and CSF strains samples and serogroup O2 and O6 strains. Aerobactin was more frequent among isolates with K1 or K5 antingens and producing haemolysin and mannose-resistant haemagglutination than among the ones lacking these virulence factors. A strict correlation was found between the pathogenicity in mouse following intraperitoneal infection and the frequency of aerobactin production. The distribution of the LD_{50} values of the aerobactin positive strains was shifted towards the lower values comparing to the aerobactin negative ones, proving statistically the effect of aerobactin in the increase of pathogenicity.

Iron is essential for the majority of microorganisms, but it is practically insoluble under aerobic conditions and at biological pH [1], or it is bound to the proteins of the microorganisms as transferrin and lactoferrin [2]. The iron uptake of bacteria is mediated by the excretion of iron carrying molecules of low molecular weight (siderophores [3]) which molecules posses a high affinity to Fe^{+++} ions. The expression of the siderophore system [4] is produced by the lack of iron.

Two kinds of siderophores are excreted by *E. coli:* a phenolate-type compound: enterochelin [5] and a hydroxamate compound: aerobactin (aer). The latter was found first by Gibson and Magrath [6] in *Enterobacter aerogenes;* in *Escherichia coli* it was demonstrated that the virulence of the host cell was considerably increased by the iron-uptake system localized on the Col V plasmid [7–9]. Later it was observed that the genes of aer-production [10, 11] and Fe^{+++} aer-receptor [12, 13] can also be located on other plasmids [14] or on the chromosome [15].

Though enterochelin shows a higher affinity to Fe^{+++} than aer [16], the in vivo effectiveness of enterochelin is decreased by its linkage to serum-albumin [17] and by the fact that in contrast to aer, the molecule is decomposed

István Gadó, Hedda Milch, Éva Czirók, Mária Herpay
National Institute of Hygiene
H-1966 Budapest, P.O.B. 64, Hungary

after a single use [18, 19]. The effect of aerobactin in increasing pathogenicity was demonstrated by infection experiments [9, 20, 21].

The occurrence of strains carrying aer cluster among the *E. coli* clinical isolates is not of random character. According to Stuart et al. [22] it is fundamental that the more iron deficient is the environment the group of isolates originate from, the more frequent is aer production among them. The published data are not unanimous. Cercenado et al. [23] did not found differences in the frequency of aer production of blood and stool isolates. Besides the selective effect, clonal connections were also observed [14]. Our present work aimed to find further evidence for the effect of aer in the increase of pathogenicity in connection with the presence of other virulence factors. On the other hand, we wanted to establish the non-random character of the occurrence of aer and to examine whether this non-random character can be completely explained by selection due to iron limiting conditions in the past of the strains.

Materials and methods

Bacterial strains. (a) A total of 632 *E. coli* strains isolated from humans between 1979 and 1983 were examined. Properties of the isolates were determined continously during the given period. The detection of siderophore production was carried out in 1986 and 1987, from strains recultured from lyophilized ampoules. (b) Out of the *E. coli* strains isolated from human samples in 1987, 349 were examined.

Serological examination of O antigens was carried out by the agglutination method of Ørskov and Ørskov [24].

Detection of K1 and K5 capsular antigens was performed by testing phage sensitivity to K1 and K5 specific phages [25, 26].

Haemolysin production (Hly) was tested on blood agar [27].

Mannose-resistant haemagglutination (HA) was determined as described by Czirók et al. [28], using human erythrocytes.

LD_{50} *test* was carried out by intraperitoneal infection of mice [29].

Antibiotic resistance was determined by the use of "Resistest" disks (Human Budapest) to streptomycin, neomycin, kanamycin, gentamicin, tetracycline, ampicillin, co-trimoxazole, chloramphenicol and colistin, in case of strains of group (a); for strains of group (b) the above listed antibiotics were supplemented with nitrofurantoin and nalidixic acid and colistin was omitted.

Qualitative biological detection of siderophores was carried out using Simon-Tessman agar, modified by Rabsch and Reissbrodt [30], supplemented with 160 μm α, α-dipyridyl. As indicator bacteria for enterochelin, *Salmonella typhi-murium* LT-2 TA 2700 sidA1, and for aer *E. coli* LG 1522 ara fepA lac leu mtl proC rpsl supE thi tonA trpE xyl (Col V-K30) were used. The indicators produce no siderophore, the former can take up Fe^{+++}-enterochelin only, the latter Fe^{+++}-aer only. The examined strains were inoculated from fresh cultures by tooth-picks.

Statistical analysis. For significance the chi-square test [31] was used. The average frequency of aer production of each groups was taken as reference basis. The difference was considered as significant if $P \leq 0.05$.

Results

The frequency of aer production. The average frequency of aer production was 55.1% among the human *E. coli* strains isolated between 1979 and 1983. Grouping the strains according to different respects resulted in considerable

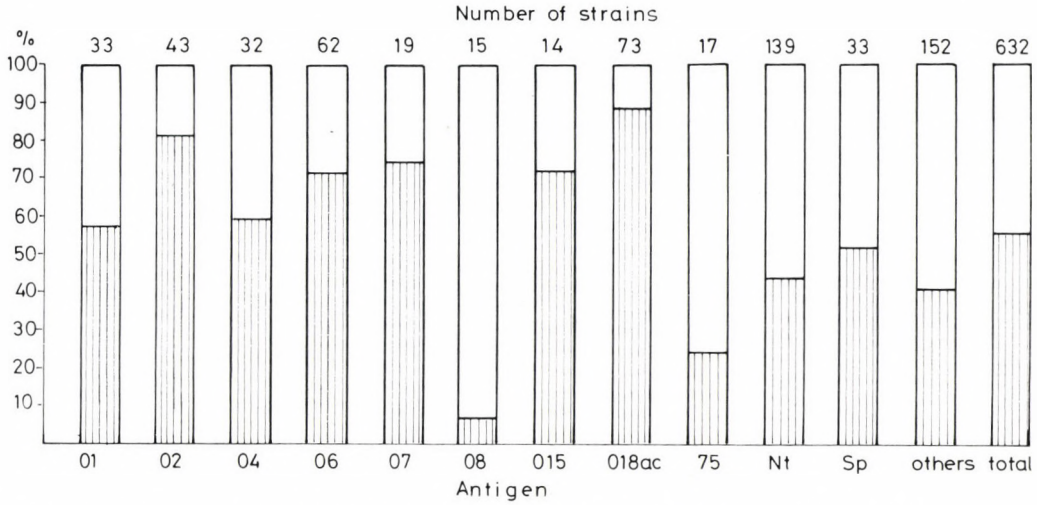


Fig. 1. Incidence of aerobactin production in frequent *E. coli* serogroups. Solid columns: aerobactin produced; open columns: aerobactin not produced. Nt = not typable; Sp = spontaneous agglutination; Others = 68 kinds of O antigen. P values: O1, 0.8–0.7; O2, < 0.001; O4, 0.7–0.5; O6, 0.02–0.01; O7, 0.2–0.1; O8, < 0.001; O15, 0.3–0.2; O18ac, < 0.001; O75, 0.01–0.001; Nt, 0.01–0.001; Sp, 0.7–0.5; others, 0.01–0.001

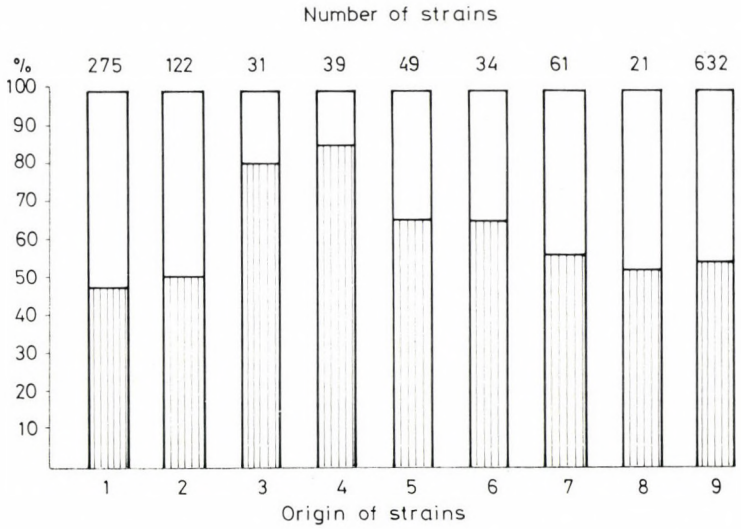


Fig. 2. Frequency of aerobactin production among *E. coli* strains isolated from different specimens. Solid columns: aerobactin produced; open columns: aerobactin not produced. Origin of strains and P values: 1, faeces (0.05–0.02); 2, urine (0.3–0.2); 3, blood (0.01–0.001); 4, CSF (< 0.001); 5, pus and wound swab (0.2–0.1); 6, ear, nose and throat swab (0.3–0.2); 7, genitals and embryo (0.95–0.90); 8, miscellaneous (isolated from different organs, skin, abdominal cavity, bile and source of strain not stated) (0.8); 9, total

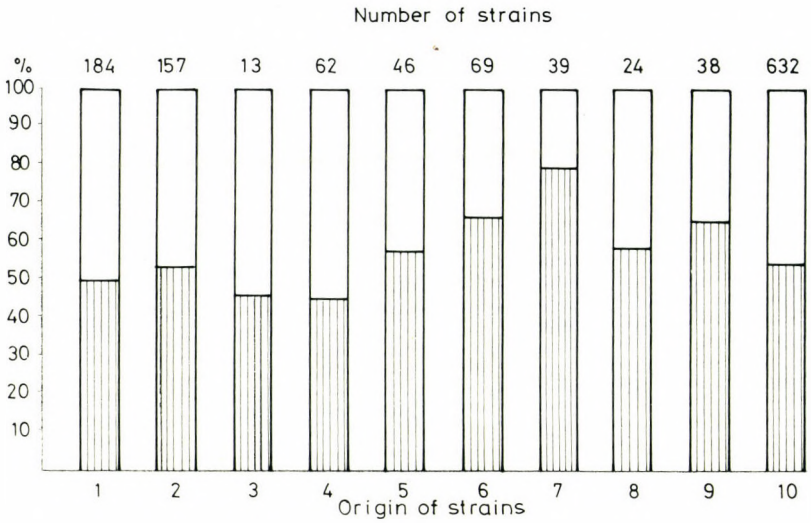


Fig. 3. Frequency of aerobactin production among *E. coli* strains isolated from patients with different diagnosis. Solid columns: aerobactin produced; open columns: aerobactin not produced. Origin of strains and P values: 1, healthy subjects (0.2–0.1); 2, patients with enteritis (0.7–0.5); 3, asymptomatic bacteriuria (0.7–0.5); 4, urinary tract infection (0.2–0.1); 5, pyelonephritis (0.9–0.8); 6, sepsis (0.1–0.05); 7, meningitis (0.01–0.001); 8, miscellaneous: appendicitis, gangrene, abscess, cholecystitis, tonsillitis, peritonitis, leukaemia, prostatitis (0.8–0.7); 9, diagnosis not stated (0.8–0.7); 10, total

differences from the average. The incidence of aer$^+$ strains in serogroups O2, O18 and O6 was 81.4%, 87.7% and 71.0%, respectively. Aer$^+$ strains occurred significantly less frequently in serogroups O8, O75 and among nontypable strains (6.6%, 23.5% and 43.1%, respectively, Fig. 1).

Grouping the strains according to their origin (specimen and diagnosis), it was found that aer$^+$ was markedly higher among strains isolated from CSF and blood than from other sources (Figs 2 and 3). Among faecal strains a negative tendency was observed.

Aer producers were found in 73.8% among the strains carrying four kinds of virulence factors singly or combined and they were found in 36.7% among the strains without virulence factors (Table I). Generally the frequency was higher than the average in case of a single virulence factor (in some categories the difference from the average was not significant because of the few numbers of strains). Aer$^+$ was rather rare among the strains producing haemolysin only, but it was frequent among the strains possessing HA, or HA + K5 antigen beside Hly.

The frequency of aer$^+$ was significantly higher among the multiple resistant strains (resistant to three or more antibiotics) and it was lower than the average among the antibiotic sensitive ones (Table II).

Examining human *E. coli* strains of group (b) it was of interest to find further evidences for some features of the non-random character of aer production. The average of aer$^+$ incidence was 47.3%; the correlation with O

Table I

Frequency of aerobactin production among strains carrying different virulence factors

Virulence factor	(a) 1979—1983				(b) 1987			
	No. of strains	Aerobactin producers			No. of strains	Aerobactin producers		
		No.	%	P		No.	%	P
Nil	319	117	36.7	< 0.001	203	75	36.9	0.02–0.01
Total No. of virulence factor carrying strains	313	231	73.8	<0.001	141	86	61.0	0.01–0.001
Kl*	78	65	83.3	< 0.001	7	5	71.4	0.3 –0.2
K5*	7	5	71.4	0.5 –0.3	3	3	100.0	0.1 –0.05
Hly	35	9	25.7	< 0.001	31	11	35.5	< 0.001
HA	41	28	68.3	0.1 –0.05	33	22	66.7	0.05–0.02
K1 +Hly	3	2	66.7	0.7 –0.5	—	—	—	—
Kl +HA	36	28	77.8	0.01–0.001	9	9	100.0	0.01–0.001
Hly+HA	75	60	80.0	< 0.001	48	28	58.3	0.2 –0.1
K5 +Hly	5	3	60.0	0.9 –0.8	1	1	100.0	0.3 –0.2
K5 +HA	3	3	100.0	0.2 –0.1	2	2	100.0	0.2 –0.1
Kl +Hly+HA	3	2	66.7	0.7 –0.5	3	2	66.7	0.7 –0.5
K5 +Hly+HA	27	26	96.3	< 0.001	4	3	75.0	0.3 –0.2
Not examined					5	4		
Total	632	348	55.1		349	165	47.3	

* Among Kl and K5 antigen-carrying strains isolated between 1979 and 1983, 4 and 7 strains, respectively, were sensitive to phages after heat-treatment at 42 °C only

Table II

Frequency of aerobactin production and antibiotic resistance

Resistance	(a) 1979—1983				(b) 1987			
	No. of strains	aerobactin producers			No. of strains	aerobactin producers		
		No.	%	P		No.	%	P
Sensitive	252	114	45.2	0.05–0.02	195	64	32.8	0.01–0.001
Resistant to 1–2 drugs	165	92	55.7	0.7 –0.5	67	43	64.2	0.02–0.01
Resistant to 3–6 drugs	158	99	62.6	0.05–0.02	87	58	66.7	0.01–0.001
Total	575	305	53.0		349	165	47.2	

Table III

Frequency of aerobactin producer strains isolated in 1987 according to O antigens and source

O antigen	No. of strains	Aerobactin producers		P	Source of strains	No. of strain	Aerobactin producers		P
		No.	%				No.	%	
2*	16	12	75.0	0.05–0.02	faeces*	82	22	26.8	< 0.001
4*	19	9	47.4	0.99	urine*	102	58	56.8	0.1 –0.05
6*	46	31	67.4	0.02–0.01	blood*	33	29	87.8	< 0.001
18**	8	6	75.0	0.2 –0.1	pus and wound swabs*	13	9	69.2	0.2 –0.1
78***	11	8	72.7	0.1 –0.05	ear-, nose-, throat swabs*	55	30	54.2	0.5 –0.3
Nt**	130	56	43.0	0.5 –0.3	genitals**	12	1	8.3	0.01–0.001
Others §	119	43	36.1	0.05–0.02	miscellaneous*§§	26	9	34.6	0.3 –0.2
					not stated	26	7	26.9	0.05–0.02

Total No. of strains: 349; aer$^+$: 165 (47.3%)

* The results are in agreement with those obtained in group (a)
** Qualitative difference from the results in group (a)
*** Included in Fig. 1 as "others"
§ 47 kinds of O antigen
§§ Gastric juice, peritoneal fluid, bile and fomites

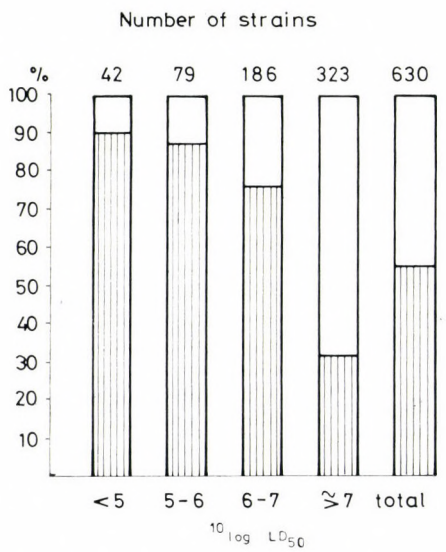


Fig. 4. Frequency of aerobactin production among *E. coli* strains of different pathogenicity. Solid columns: aerobactin produced; open columns: aerobactin not produced. P values are < 0.001 in every category

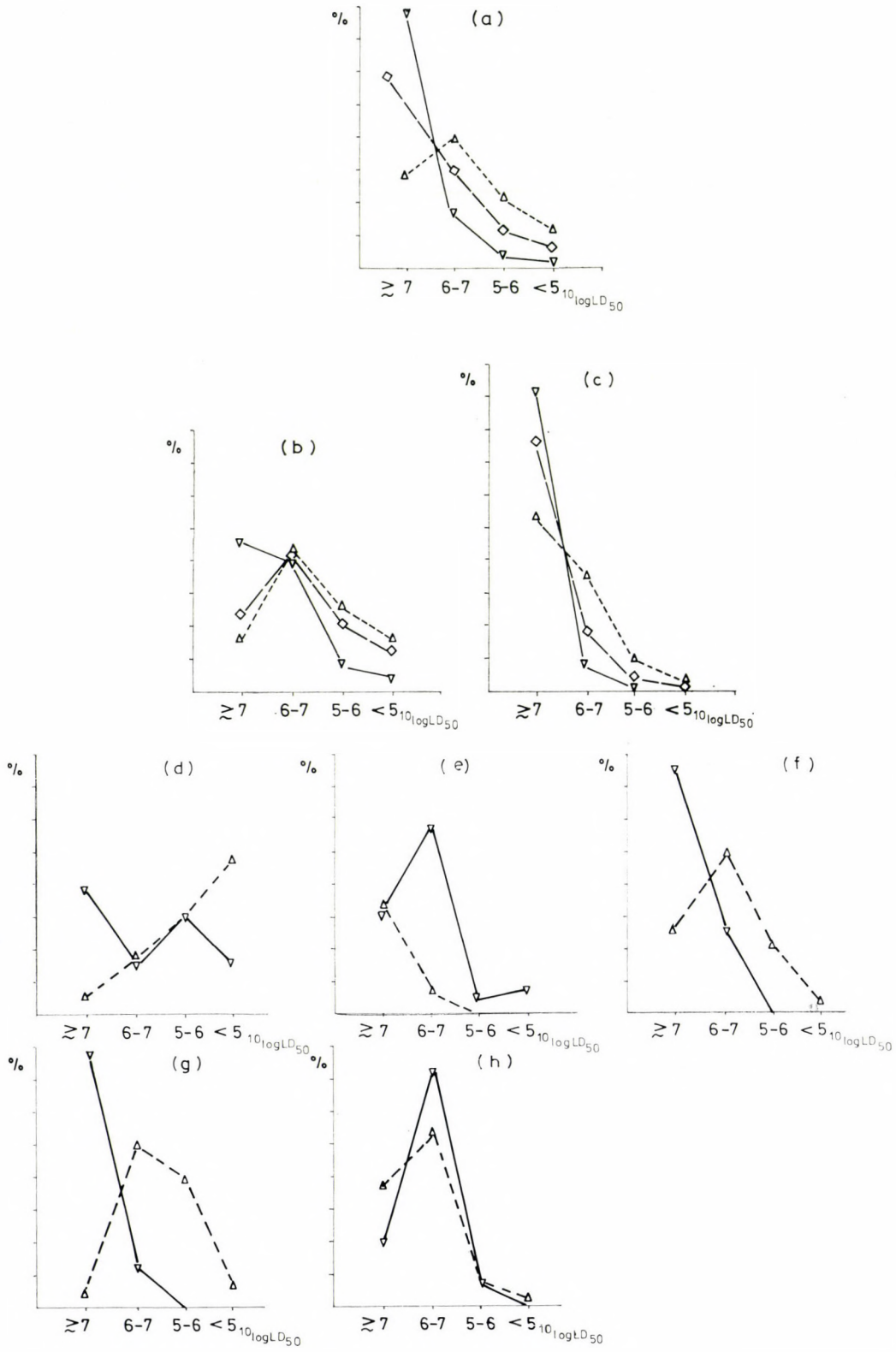
antigen was reproducible, except for serogroup O18 and the nontypable strains. There was a correlation with the former data in the case of high aer$^+$ frequency among strains isolated from blood and with the low frequency among strains originated from stool samples (Table III). The association of aerobactin with other virulence factors was demonstrated in all groups of evaluable samples (Table I).

Considering antibiotic resistance (Table II), the difference between the two groups of strains (a and b) was that not only the multiple resistant strains (as in group a), but also the strains resistant to 1 or 2 antibiotics showed higher aer$^+$ frequency than the average.

Connection between aerobactin production and pathogenicity. Grouping the *E. coli* strains isolated between 1979 and 1983 according to LD_{50} values for mice revealed that a strict correlation existed between pathogenicity and frequency of aer production (Fig. 4). When the LD_{50} was lower than 10^7, the incidence of aer$^+$ was more frequent than the average and it was significantly lower when the LD_{50} was 10^7 or higher. Figure 5 presents this correlation in another respect: the LD_{50} distribution curves of aer$^+$ an aer$^-$ strains are compared. Figure 5a shows, that the distribution of aer$^+$ strains is shifted towards the lower LD_{50} values, whether other virulence factors are carried by them (Fig. 5b), or not (Fig. 5c). This shift was reflected in respect of different virulence factors, except for strains possessing Hly or Hly + HA (Fig. 5d–h).

Discussion

Aerobactin production was observed in about 50% of the strains of both groups of examination, while enterochelin was produced in nearly 100% (only two negative strains were found). The non-random character of incidence of aer was established by our data. The aer producers were predominant among the strains isolated from iron-poor specimens (blood, CSF). In patients with sepsis the result was less convincing, probably because some of the strains were not isolated from blood. Literary data concerning blood samples are in concordance [20, 22, 23] and are also in agreement with our results. In contrast to the publication of Cercenado et al. [23], among faecal strains the aerobactin producers occurred considerably less frequently than the average. Shand et al. [32] published that in *Proteus mirabilis* and *Klebsiella pneumoniae* urinary tract infections the multiplication of bacteria took place under iron restricted conditions and this resulted in the change of OMP pattern. Our results that the frequency of aer$^+$ is not higher than the average among urinary strains indicates that the iron-poor condition is not characteristic of the urinary tract. The results of Carbonetti et al. [33] concerning UTI strains are similar to ours, except that they found a high frequency among strains of pyelonephritis origin. Publications on the aer$^+$ frequency of CSF strains were not found.


%
(a)
≥ 7
6-7
5-6
< 5
10 log LD50
(b)
(c)
(d)
(e)
(f)
(g)
(h)

The significant differences found among the most frequent serogroups can not be explained only by selection. Though, serogroup O4 and O6 strains (isolated between 1979 and 1983) from blood and CSF were represented equally (12 and 13%, respectively), the frequency of aer$^+$ was significantly higher among O6 strains. On the other hand, serogroup O2 strains from blood and CSF represented only 2%, yet the incidence of aer$^+$ was very high. Accordingly, the differences observed, may be attributed to a clonal backround. The results concerning serogroup O18 strains can be explained by the combination of selective and clonal factors: these cultures were of blood and liquor origin in 36 and 25%, respectively, i.e. the aer$^+$ frequency, which was about the average or under the average as a consequence of clonal origin, might be distored to positive direction due to selection.

Aer production was associated with the different virulence factors, except Hly production. The background of this association may be attributed to a selection: the multiplication or the survival of bacteria is promoted by the virulence factors under conditions which are unfavourable in addition to the iron-poor environment, i.e. a higher selective effect of iron restriction may appear. (In case of Hly selection this is not possible because of the iron release from the haemoglobin.) This selection hypothesis is not supported by the high aer$^+$ frequency of Hly + HA and Hly + HA + K5 strains: Hly associated with other virulence factors should also inhibit the development of high aer$^+$ frequency.

The association of antibiotic resistance and aer production can be explained in two ways: if resistance is considered as virulence factor, it can be interpreted in the above mentioned manner, or the aer cluster may be carried by the R plasmid (which is most probably responsible for resistance in case of multiple resistance), similarly as Col V (wich is associated with aer production in 92.0%).

In accordance with literary data the pathogenicity in mouse infection was increased by aer production. This is obvious from the association of aer production and the low LD_{50} values (Fig. 4) and from the shift of distribution curves of LD_{50} values towards the lower values in the presence of aer production (Fig. 5). In addition to the literary data, we demonstrated statistically that pathogenicity was increased by aer production in the presence of other virulence factors, too.

Fig. 5. Distribution of aerobactin producer and aerobactin non-producer strains according to LD_{50} values. (a) independently of virulence factors; (b) virulence factors other than aer are also carried; (c) virulence factors other than aer are not carried; (d) Kl; (e) Hly; (f) HA; (g) Kl + HA; (h) Hly + HA. ◇ total number of strains; △ aer$^+$; ▽ aer$^-$

REFERENCES

1. Weinberg, E. D.: Microbiol Rev **42,** 45 (1978).
2. Aisen, P., Leibman, A.: Biochim Biophys Acta **257,** 314 (1972).
3. Lankford, C. E.: Crit Rev Microbiol **2,** 273 (1973).
4. Neilands, J. B.: Ann Rev Biochem **50,** 715 (1981).
5. O'Brien, I. G., Gibson, F.: Biochim Biophys Acta **215,** 393 (1970).
6. Gibson, F., Magrath, D. I.: Biochim Biophys Acta **192,** 175 (1969).
7. Williams, P. H.: Infect Immun **26,** 925 (1979).
8. Stuart, S. J., Greenwood, K. T., Luke, R. K. J.: J Bacteriol **143,** 35 (1980).
9. Williams, P. H., Warner, P. J.: Infect Immun **29,** 411 (1980).
10. Braun, V.: FEMS Microbiol Lett **11,** 215 (1981).
11. Warner, P. J., Williams, P. H., Bindereif, A., Neilands, J. B.: Infect Immun **33,** 540 (1981).
12. Bindereif, A., Braun, V., Hantke, K.: J Bacteriol **150,** 1472 (1982).
13. Grewal, K. K., Warner, P. J., Williams, P. H.: FEBS Lett **140,** 27 (1982).
14. Valvano, M. A., Silver, R. P., Crosa, J. H.: Infect Immun **52,** 192 (1986).
15. Valvano, M. A., Crosa, J. H.: Infect Immun **42,** 245 (1984).
16. Harris, W. R., Carrano, C. J., Raymond, K. N.: J Am Chem Soc **101,** 2722 (1979).
17. Konopka, K., Bindereif, A., Neilands, J. B.: Biochemistry **21,** 6503 (1982).
18. Rosenberg, H., Young, I. G.: In Neilands, J. B. (ed.): Microbial Iron Metabolism. Academic Press, New York 1974. p. 67.
19. Braun V., Brazel-Faisst, C., Schneider, R.: FEMS Microbiol Lett **21,** 99 (1984).
20. Montgomerie, J. Z., Bindereif, A., Neilands, J. B., Kalmanson, A. M., Guze, L. B.: Infect Immun **46,** 835 (1984).
21. Lafont, J. P., Dho, M., D'Hauteville, H. M., Bree, A., Sansonetti, P. J.: Infect Immun **55,** 193 (1987).
22. Stuart, S. J., Greenwood, K. T., Luke, R. K. J.: Infect Immun **36,** 870 (1982).
23. Cercenado, E., Baquero, F., Delgado-Iribarren, A., Martinez, J. L.: Ann Inst Pasteur (Paris) **137** B, 297 (1986).
24. Ørskov, I., Ørskov, F.: Med Microbiol Immunol **163,** 99 (1977).
25. Gross, R. J., Cheasty, T., Rowe, B.: J Clin Microbiol **6,** 548 (1977).
26. Gupta, D. S., Jann, B., Schmidt, G.: Golecki, J. R., Ørskov, I., Ørskov, F., Jann, K.: FEMS Microbiol Lett **14,** 75 (1982).
27. Czirók, E.: Acta Microbiol Hung **32,** 183 (1985).
28. Czirók, E., Marton, A., Csik, M., Solt, M.: Acta Microbiol Hung **31,** 187 (1984).
29. Czirók, E., Milch, H., Vincze, I.: Egészségtudomány **30,** 217 (1986).
30. Rabsch, W., Reissbrodt, R.: J Basic Microbiol **10,** 663 (1985).
31. Horváth, I.: Kvantitatív mikrobiológiai eljárások. Akadémiai Kiadó, Budapest 1974.
32. Shand, G. H., Anwar, H., Kadurugamuwa, J., Brown, M. R. W., Silverman, S. H., Melling, J.: Infect Immun **48,** 35 (1985).
33. Carbonetti, N. H., Boonchai, S., Parry, S. H., Väisänen-Rhen, V., Korhonen, T. K., Williams, P. H.: Infect Immun **51,** 966 (1986).

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ISOLATION OF A DNA SEQUENCE STIMULATING RECOMBINATION IN YEAST

M. MINK

Department of Microbiology, József Attila University
Szeged

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A series of DNA sequences was rescued from the yeast *Saccharomyces cerevisiae* transformed by a gene library and selected for the *cdc35ts*$^+$, *TRP1*$^+$ phenotype. These sequences did not complement the *cdc35ts* mutation, and were found in various amounts and orientations in degraded plasmids. A similar phenomenon was demonstrated when the *HIS3* gene was cloned into one of them: a highly deleted plasmid was rescued from complemented homozygous diploid yeast cells, in which the *HIS3*$^+$/*his3*$^-$ character was inherited at a 2 : 2 ratio. These results suggest that the insert sequences rescued from the *cdc35ts* transformants stimulate vigorous non-reciprocal recombination events by the transfer of *HIS3* gene or the *TRP-ARS* fragment. This event was detected in the transformation of *cdc35* or *his3*$^-$ hosts and was followed by the re-isolation of the degraded plasmid molecules.

The plasmid-chromosome interaction is a powerful tool in the study of recombination in yeast. This event occurs independently of whether the communicating region is carried by a replicative [1], or an integrative plasmid [2], or merely by a restriction fragment [3]. The mechanism of the interaction can be interpreted in terms of a single or double crossover, resulting in the complementation, disruption or replacement of the target allele [4]. The changes detected in the chromosome by genetic or biochemical methods are often non-reciprocal, i.e. even deletion of the non-essential regions of the plasmid DNA occurs [5], which might be interpreted through the non-equivalent hierarchy of the plasmid and chromosomal DNAs. This event is usually observed in gene cloning by complementing mutations with a DNA pool derived from a gene library. The rescue of plasmids containing non-complementing sequences may occur too, which happens without apparent selective pressure. The efforts made to clone *CDC35* gene resulted in the isolation of non-complementing insert sequences to which a function could be attributed on the basis of their survival without selective pressure or of their deleted and inverted structure. In order to test these hypotheses, experiments were performed to decide whether such sequence is responsible for its own survival or is involved in the promotion of recombination.

MÁTYÁS MINK
Department of Microbiology, József Attila University
Középfasor 52, H-6720 Szeged, Hungary

Materials and methods

Yeast transformation and rescue. The commercially available haploid yeast *Saccharomyces cerevisiae* temperature sensitive strain *(a, cdc35-lts, adel, arg4, ural, his7, trpl)* was transformed [6] with a wild-type yeast DNA library constructed by Nasmyth and Reed [7] carrying the *TRP-ARS* fragment in the vector, and selected for the *ts*$^+$, *TRP1* phenotype on minimal medium (MM) plates (2% glucose, 0.67% yeast nitrogen base w/o amino acids and auxotrophic requirements, at a 20 mg/l concentration). The cells of those transformant colonies growing at 37 °C were liberated from the top agar and converted into sphaeroplasts [8]. Total DNA was isolated by the lysis of sphaeroplasts in 120 mM Tris-HCl, pH 8.0, 30 mM EDTA, 1% (v/v) Triton X-100 at 65 °C for 10 min, and 20 mg/l RNase treatment at 37 °C for 10 min, followed by phenol extraction and ethanol precipitation. *E. coli* HB101 competent cells were then transformed [9] with this DNA and selected for ampicillin resistance (Ap^r). A homozygous diploid *his3*$^-$ yeast strain was constructed by crossing AH202 *(a, leu2–3, 2–112, his3, trp1)* with GRF18 *ura2* $\triangle/\alpha$, *leu2–3,2–112, his3–11,3–15, ura2)*, resulting in the *leu2*$^-$ *his3*$^-$ phenotype. Transformants were selected for *HIS3*$^+$ on MM + leucine plates. Sporulation was achieved by plating diploids onto sporulation medium (0.1% yeast extract, 0.25% glucose, 2% potassium acetate). Non-selective YEPD medium (2% glucose, 2% peptone, 1% yeast extract) was used for mitotic stability test. All solid media contained 2% agar.

Results and discussion

Rescue of non-complementing sequences. The structures of plasmids of the Ap^r *Escherichia coli* colonies were analysed by means of miniscreens [9]. The majority of the clones consisted of the vector without any insert of yeast DNA (Fig. 1). In a few instances, insert-containing plasmids were isolated (Fig. 1, pMM3, 4 and 5). Their structures indicate that they can hardly be interpreted as artefacts produced in the construction of the gene library. These plasmids contain different amounts of the same insert in the two orientations; in pMM4 and 5, some of the non-functioning parts of the pBR322 sequences were lost (Fig. 1). This insert DNA hybridizes to total yeast DNA under stringent conditions (10, not shown), and do not complement the *cdc35ts* mutation [11, 12]. These findings suggested that this insert somehow promotes recombination. The rescue of this insert DNA in PMM4 without the *TRP-ARS* fragment, however, hinted at the ability for its own maintenance, which can be explained as a function of an autonomously replicating sequence *(ARS*, [13]), or a centromere *(CEN*, [14]). To test these ideas, the *HIS3* gene [15] was cloned into pMM4, where the putative function can be studied, as follows. Deletion of the *Sal*I-*Pvu*II fragment of the pBR322 sequences of pMM4 was acieved by partial digestions and re-isolations of DNA fragments of the correct size from agarose gels. The *Bam*HI digested, Klenow repaired *Xho*I cut fragment of the *HIS3*$^+$ gene [15] was ligated by fusing the *Sal*I-*Xho*I sites and the blunt ends of the vector and the HIS3 gene, reconstructing the *Bam*HI site. The resulting plasmid, pNW13 (Fig. 1), having a single *Sal*I site in the cryptic region, was used for targeted transformation into the homologous region [16] of the *his3*$^-$, *leu2*$^-$ homozygous diploid strain.

Genetic and biochemical analysis of the HIS3$^+$ *yeast transformants.* Diploid transformants were selected on MM + leu plates, and then transferred onto

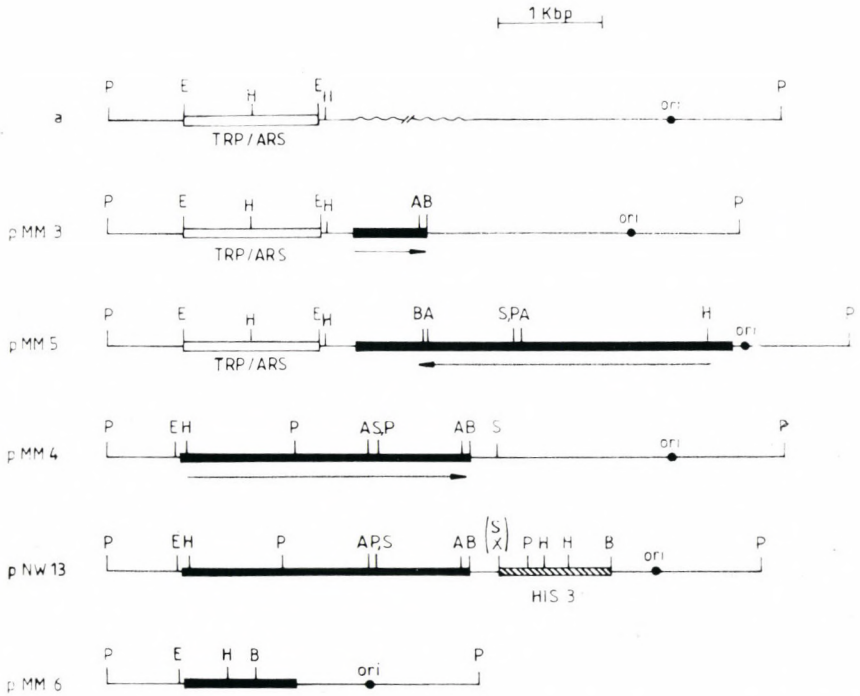

Fig. 1. Physical maps of plasmids rescued from *cdc35ts* yeast transformants growing at 37 °C (pMM3, 4 and 5) and from *HIS3*$^+$ complemented cells (pMM6); pNW13, carrying the *HIS3* gene. a: the structure of the library DNA; wavy line: the inserted yeast DNA. E: *Eco*RI, P: *Pst*I, B: *Bam*HI, H: *Hind*III, S: *Sal*I, A: *Ava*I

sporulation medium. Asci were dissected and a random spore analysis was made by plating a liquots of spore suspensions onto MM + leu + ura + + trp+/-his plates. A 2 : 2 segregation of the *HIS3*$^+$/*his3*$^-$ markers was observed, showing a nuclear inheritance, i.e. the integrated state of the *HIS3*$^+$ gene. The *HIS3*$^+$ character was stably inherited, as was judged via several replica platings of the haploid cells on YEPO plates, then by a selection on MM + leu + trp + ura+/-his plates: all of the clones were grown without histidine. These results suggested that the insert DNA was not an *ARS* or a *CEN* element.

The total DNA was isolated from the diploid transformants, digested with *Hind*III and separated in agarose gels. Undigested DNA was loaded, too. pBR322 DNA was nick-translated with ^{32}P and was used as a probe for hybridization under stringent conditions ([10], Fig. 2). Undigested DNA gave three bands corresponding to an integrated plasmid, and two, non-stoichiometric bands, which may correspond to OC and CCC forms of plasmid DNA circles (from top to bottom). When this DNA was digested with *Hind*III, two bands appeared in the autoradiogram (Fig. 2) with sizes of 4.2 and 3.6 kilobasepairs

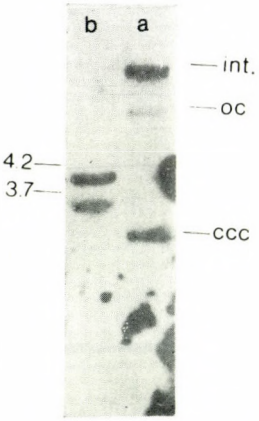

Fig. 2. Hybridization of undigested (a) and *Hin*dIII digested (b) total yeast DNA from the pNW13 transformants to a pBR322 probe

(kbp). The same total yeast DNA was used to transform *E. coli* to Ap^r. Analysis of the clones revealed the rescue of a highly deleted 3.7 kbp plasmid, a derivative of pNW13 (Fig. 1, pMM6), apparently missing the *HIS3*$^+$ region and the bulk of the pMM4 insert.

Conclusions

A 2 : 2 segregation of the *HIS3*$^+$/*his3*$^-$ loci was observed when pNW13 transformed diploids were dissected, showing a nuclear inheritance of the transformed marker. The hybridization pattern of the undigested DNA suggested the presence of the pBR322 sequences in an integrated and in a free circular form. *Hin*dIII digestion converted these three bands into two fragments, corresponding to cutting-out of the integrated sequence and formation of a linear band from the circular DNA. The pMM6 plasmid DNA, a derivative of pNW13, was rescued from transformants having the same size and a single *Hin*dIII site (pMM6), corresponding to the circular DNA in Fig. 2. These results can be explained as follows.

(i) The transforming pNW13 DNA, if integrated in diploid cells, had lost the *HIS3*$^+$ sequences, since no 4.2 kbp fragment can be expected by integration into any of the homologous regions, when digested with *Hin*dIII. Formation of such a fragment can only be expected from partial deletion of the pMM4 insert too.

(ii) Further degradation of this insert took place, when pMM6 plasmid DNA was formed in the transformant diploid cells (Fig. 1).

(iii) Half of the cells became stable *HIS3*$^+$ as a result of a non-reciprocal exchange between the pNW13 plasmid sequences and the homologous regions of the chromosomes.

(iv) A similar phenomenon was detected on the deletion/inversion of DNA sequences *(E. coli* and yeast) in pMM3, 4 and 5 plasmids.

The results suggest that the non-complementing region rescued from transformed *cdc35ts* cells growing at 37 °C has a strong directive recombinogenic effect, provoking conversion of the *HIS3* gene from plasmid to chromosome in diploid cells.

REFERENCES

1. Falco, S. C., Rose, M., Botstein, D.: Genetics **105,** 843 (1983).
2. Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J.: Cell **33,** 25 (1983).
3. Struhl, K.: Gene **26,** 231 (1983).
4. Struhl, K.: Nature **305,** 391 (1983).
5. Clancy, S., Mann, C., Davis, R. W., Calos, M. P.: J Bacteriol **159,** 1065 (1984).
6. Hinnen, A., Hicks, J. B., Fink, G. R.: Proc Natl Acad Sci USA **75,** 1929 (1978).
7. Nasmyth, K. A., Reed, S. I.: Proc Natl Acad Sci USA **77,** 2119 (1980).
8. Ferenczy, L., Pesti, M.: Curr Microbiol **7,** 157 (1982).
9. Maniatis, T., Fritsch, E. F., Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, 1982.
10. Southern, E. M.: J Mol Biol **98,** 503 (1975).
11. Casperson, G. F., Walker, N., Bourne, H. R.: Proc Natl Acad Sci USA **82,** 5060 (1985).
12. Kataoka, T., Broek, D., Wigler, M.: Cell **43,** 493 (1985).
13. Kearsey, S.: EMBO J **2,** 1571 (1983).
14. Tschumper, G., Carbon, J.: Gene **23,** 221 (1983).
15. Struhl, K., Stinchcomb, D. T., Scherer, S., Davis, R. W.: Proc Natl Acad Sci USA **76,** 1035 (1979).
16. Orr-Weaver, T. L., Szostak, J. W., Rothstein, R. J.: Proc Natl Acad Sci USA **78,** 6354 (1981).

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MOLECULAR CLONING AND PHYSICAL MAPPING OF THE DNA OF HUMAN ADENOVIRUS TYPE 35

W. Gy. Kang, Gy. Berencsi, Mária Takács, Z. Ascher, G. Fejér and I. Nász

Institute of Microbiology, Semmelweis University Medical School, Budapest

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The prototype strain of the human adenovirus type 35 (AV35) was examined. *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, and *Sal*I restriction endonucleases were used for the mapping of DNA fragments. Three original maps were constructed, and previously published maps were somewhat modified. A *Pst*I-specific fragment library was also prepared and characterized using the pBR322/*E. coli* system. Some of the recombinants seem to be applicable for rapid DNA diagnostics, and for the comparative mapping of type- and subgroup-specific DNA sequences. The comparative presentation of physical maps of subgroup B human adenoviruses might improve the efficiency of genotyping of adenoviruses using restriction endonucleases.

The isolation of human adenovirus type 35 (AV35) has been reported first in 1977 [1]. It has been characterized as the member of subgroup B adenoviruses and could be classified together with types AV 11, AV 14 and AV 34 as DNA homology cluster II of subgroup B adenoviruses [2]. These viruses possess affinity for the organs of the urogenital tract, and are frequently isolated from immuno-compromized patients i.e. from patients suffering from acquired immune deficiency syndrome (AIDS) [3].

Relatedness of DNA sequences of subgroup B human adenoviruses to other subgenera has been compared by DNA heteroduplex studies [4] and by DNA-DNA liquid hybridization [5]. Nevertheless, detailed examination of AV35 DNA including restriction endonuclease mapping and cloning has been published only by Valderrama-Leon et al. [6].

Since natural isolates of AV14/35 have been obtained recently also in Hungary (G. Fejér, M. Uj, G. Szűcs and Gy. Berencsi, unpublished data), the quick identification of the members of DNA homology cluster II of human subgroup B viruses became practical requirement. In this paper the construc-

W. Gy. Kang
Institute of Experimental Biology of the Academy of Sciences, Democratic People's Republic Korea Pyongyang, Korea

György Berencsi*, Mária Takács, Zoltán Ascher, György Fejér, István Nász
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

* Corresponding author

tion of new restriction endonuclease maps of the AV35 DNA, and the preparation of an overlapping fragment library of internal *Pst*I fragments are described. The cloning system used allowed refinment of restriction endonuclease maps of AV35 DNA published earlier by others [6]

Materials and methods

Viruses and tissue cultures. The prototype strain of human AV35 was obtained from Dr. M. N. Oxman, San Diego, California, USA, [1]. Inocula for the suspension cultures were regularly prepared on the human cell line "293" transformed by the DNA of human AV5 [7]. Not more than two passages were sufficient to increase virus titre for the efficient infection of HEp2 suspension cultures grown in Ca^{++}-free Eagle's MEM supplemented with 2 mM arginine-HCl, 0.09 parts of newborn calf serum and alternating pairs of conventional antibiotics (streptomycin, 100 μg/ml; penicillin 200–500 μg/ml; kanamycin 50 μg/ml; ampicillin 100 μg/ml; erythromycin 50 μg/ml; cephalexin 100 μg/ml). The cultivation and infection of the "Cincinati line" of HEp2 cells in suspension cultures was described in detail [8, 9].

Purification of virion and viral DNA. Virus-infected cells were pelleted 7 or 11 days after infection by low speed centrifugation. Fresh medium was added to the infected cells on the fourth and seventh day following infection. The cell pellet was extracted twice with 0.4 M NaCl, 5 mg/ml Triton X 100 and 10 mM Tris–HCl, pH 7.5 for 10 min at room temperature, and centrifuged at 5000 *g* at +4 °C for 20 min. The supernates were united, and the virus particles were purified by equilibrium gradient centrifugation using preformed CsCl gradients (1.1 to 1.4 g/ml) in 10 mM Tris–HCl, pH 7.5 [10, 11].

The viral DNA was purified from dialyzed fractions of the CsCl gradients containing virus particles. Samples were treated with predigested pronase (1 mg/ml) in the presence of 10 mg/ml SDS and 20 mM Tris–HCl, pH 7.5 at 37 °C for 60 min. Deproteinization was done with water-saturated phenol and chloroform : isoamylalcohol according to standard protocols [10–12].

Separation of restriction endonuclease fragments of DNA. Restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I and *Pst*I were purchased from Reanal (Budapest) and used as described earlier [11]. Horizontal agarose slab gels were prepared from low EEO agarose (No. A-6013; Sigma Chemical Co., St. Louis, Missouri, or HGT/P agarose; SeaKem, Marine Colloids Rockland, Maine, USA) and run at 1 to 2 V/cm [13]. The gels (8 to 12 mg/ml) were stained with ethidium bromide, and photographed on Fortepan 400 films (Forte, Vác, Hungary).

*Hin*dIII fragments of the human AV type 1 DNA were used as size markers [3, 11]. The physical maps of human AV2 DNA were generated by computer processing of sequence data kindly put to our disposal by R. Roberts (Cold Spring Harbor Labs., Cold Spring Harbor, N. Y., USA).

Partial heat-denaturation of restriction fragments was preformed in formamide : SDS mixture (10 mg/ml SDS in 100 mM Tris–HCl, pH 7.2; ratio 1 : 1) at different temperatures [14]. DNA fragments of identical electrophoretic mobility were identified by blot-hybridization [13]. Synpore (Prague, Czechoslovakia) or Schleicher-Schüll (Dassel, FRG) nitrocellulose filters were used as DNA carrier. The DNA samples were labeled using the nick translation kit of Bethesda Research Labs. (Gaithersburgh, Maryland, USA), according to the protocol of the manufacturer. The alpha-^{32}P-ATP was supplied by Izinta (Budapest) in ethanol : water (1 : 1). The specific activities were between 13 and 110 TBq/mM. Sephadex G-50 columns were used for determination of the specific activity of the labeled DNA [13].

Blot hybridization was performed in 0.5 parts, or 0.55 parts of formamide at 37 °C in the presence of 6×SSC. The filters were pretreated with non-fat dry milk dissolved in the appropriate concentration of formamide and 10 mg/ml SDS [13, 15, 16]. The filters were washed twice with prewarmed (60 °C) 1×SSC and 1 mg/ml SDS in order to obtain intermediate stringency of hybridization. The autoradiography was performed at room temperature for several days if required. Duplicate films HS11 (Orwo, Wolfen, GDR) or Medifort (Forte, Vác, Hungary) were usually exposed.

Cloning of PstI DNA fragments of AV35. The pBR322 and *Escherichia coli* HB101 host/vector system was used [13, 17]. The AV35 DNA was partially digested with *Pst*I endonuclease under controlled conditions, and linear pBR322 DNA was ligated with the prepara-

tion, when most of the DNA was partially cut, but all final DNA fragments could be already recognized in the gels [18]. Ligation, transfection, and reisolation of the recombinants were exactly performed as suggested by Maniatis et al. [13]. Recombinants pAd12RIC and pAd5XhoC carrying left terminal fragments of AV12 and AV5 DNA, respectively [19], were included into some of the experiments.

Identification of recombinants. The clones were isolated and cultivated in YTB. The alkaline lysis procedure was chosen following chloramphenicol amplification (170 μg/ml) for plasmid purification. The endonuclease digestion, and fragment identification was identical with procedures used for fragments isolated from viral DNA [13].

Results

The restriction endonuclease fragment patterns are shown in Fig. 1. The patterns were obtained in gels of 10 mg/ml with *Kpn*I (lane 1), *Pst*I (lane 2), *Bam*HI (lane 4) *Hin*dIII (lane 5), *Sal*I (lane 6) and *Eco*RI (lane 8) enzymes. The patterns of *Hin*dIII, *Bam*HI, and *Pst*I were found to be corresponding to the data published earlier [6, 20]. The lengths of the fragments were determined in comparative experiments to the *Hin*dIII fragments of AV1 [11] and the data are given in Table I.

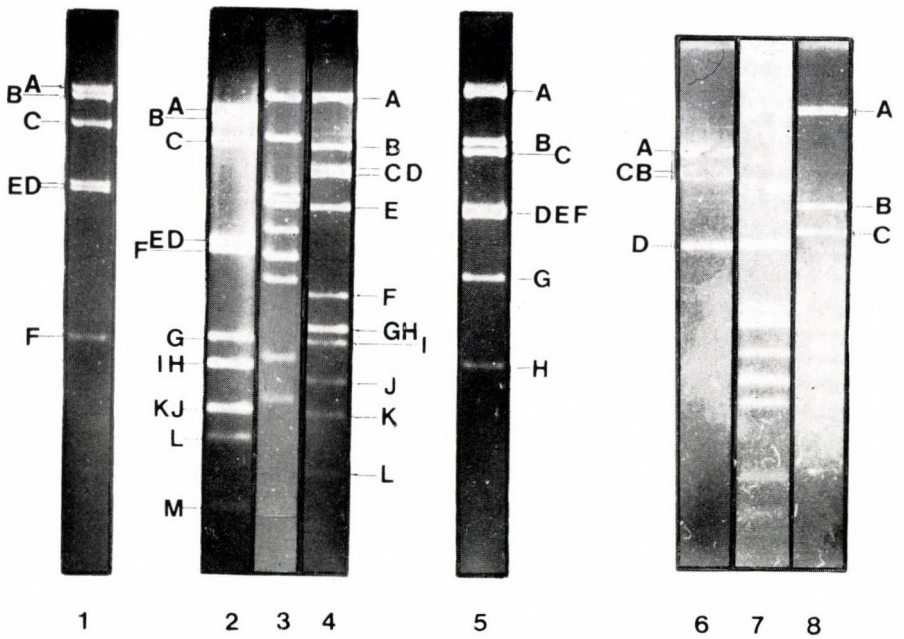

Fig. 1. Electrophoretic patterns of restriction endonuclease fragments of AV35 DNA. The electrophoretic separation was performed in an agarose slab gel of 10 mg/ml, at room temperature and 2 V/cm for 16 h. Controls (lanes 3 and 7 for two different slab gels) were *Hin*dIII cut DNA of human AV-1 [11]. Enzymes used for the digestion of AV35: *Kpn*I (lane 1), *Pst*I (lane 2), *Bam*HI (lane 4), *Hin*dIII (lane 5), *Sal*I (lane 6), and *Eco*RI (lane 8). Capital letters indicate fragments of decreasing length M_r, listed in the columns of Table I

Table I

Estimated number of basepairs of restriction endonuclease fragments of AV35 DNA

Fragments	Result obtained with restriction endonucleases in kilobase pairs					
	*Eco*RI	*Sal*I	*Kpn*I	*Hind*III	*Bam*HI	*Pst*I
A	22.3	11.2	10.35	10.65	9.05	7.75
B	7.3	9.4	9.5	5.8	5.25	6.9
C	6.0	9.3	6.6	5.05	4.3	5.6
D	—	5.4	3.7	3.5	4.2	2.65
E	—	—	3.6	3.45	3.3	2.65
F	—	—	1.6	3.4	1.98	2.5
G	—	—	—	2.3	1.67	1.5
H	—	—	—	1.35	1.67	1.3
I	—	—	—	—	1.47	1.3
J	—	—	—	—	1.15	1.05
K	—	—	—	—	0.95	1.05
L	—	—	—	—	0.6	0.8
M	—	—	—	—	—	0.5
Total	35.6	35.3	35.35	35.5	35.59	35.55

The viral DNA was digested with pairs of restriction endonucleases as described in previous papers [10, 11, 21]. The tentative physical maps constructed are shown in Fig. 2. In order to make later discussion easier, all the data available on human subgroup B restriction endonuclease maps have been summarized in Fig. 2. Three of the maps are published first in this paper, and some fragments of *Bam*HI and *Pst*I maps were identified first by us.

Molecular cloning of the DNA of AV35. The most complex physical map was obtained with the *Pst*I enzyme (Fig. 2 and reference [6]). Therefore the viral DNA was partially digested with *Pst*I, and a fragment library was cloned into pBR322/*E. coli* system. The total AV35 genome except the two terminal DNA fragments could be recovered from the clones. The viral DNA fragments recovered from the recombinants are summarized in Fig. 3. The black horizontal bars above the physical map of the viral DNA indicate the DNA fragments present in individual recombinants. The recombinants were digested with different restriction endonucleases, and the results are shown by vertical arrows (restriction endonuclease sites indentified in the cloned AV35 DNA fragments) and by open horizontal bars (*Bam*HI, *Hind*III and *Eco*RI fragments recovered from the recombinants shown below) in Fig. 3.

The DNA composition of some of the recombinants from the total of 26 clones characterized in detail are shown in Fig. 4. The recombinants have been recut with *Pst*I enzyme. The control was *Pst*I-fragments of viral DNA (lane 1). The linear pBR322 DNA is visualized as a series of bands present in all but the control lane. The fragment compositions in the different recombinants

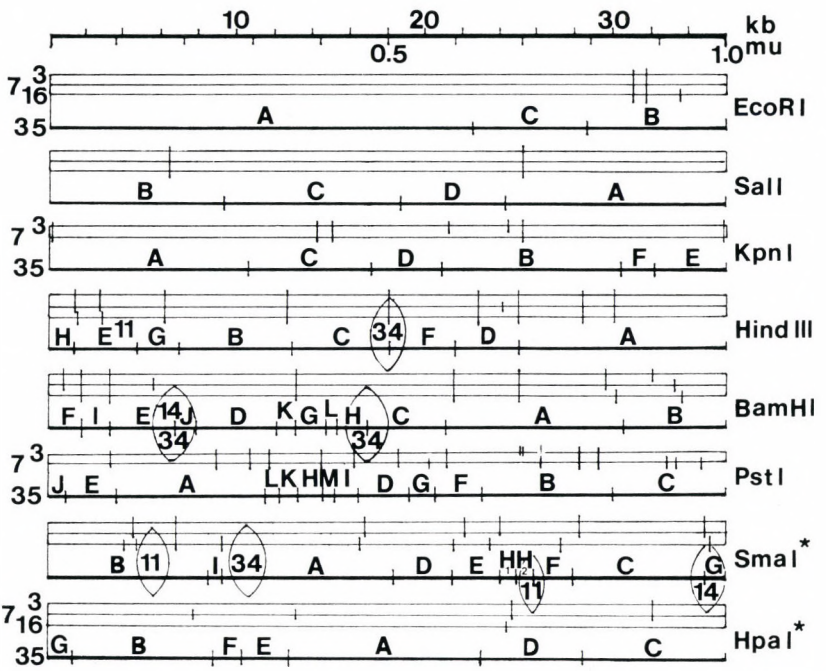

Fig. 2. Comparison of restriction endonuclease maps of the DNA of human subgroup B adenoviruses. The scale is given in both kilobase pairs (kb) and map units (mu). Capital letters indicate only the fragments of AV35 DNA. Asterisks indicate that the *Sma*I and *Hpa*I maps (not examined in this work) have been redrawn from the paper of Valderrama-Leon et al. [6] without modification. The maps of types 3, 7, and 16 which belong to the DNA cluster I of subgroup B human adenoviruses were taken from numerous references [22, 25–29], except for the *Pst*I maps. The latter has been measured by us, and the left half was determined by Engler and Kilpatrick [23] in 1981. Numbers indicate possible differences in AV11, 14 and 34 genomes on the basis of published fragment patterns [2, 20, 24, 33]

shown are the following: D + G + *F* (lane 2), *D* + *G* (lane 3), *D* + *G* + *I* + + *M* + *H* (lane 4; note that the band *H* + *I* is more intensively stained than fragment *G*), *K* (lane 5; note that the identical fragment *J* is the terminal one, therefore it cannot be cloned in the system used), *E* and *D* (lane 6 and 8), *H* and *I* (lanes 7 and 11), *M* (lane 9), *F* + *B* (lane 10), and *B* (lane 12).

Blot hybridization using cloned AV35 fragments. In order to construct DNA probes for DNA diagnostics the authentic sequences within the recombinants were controlled. The recombinants were labeled by nick translation in vitro and the blotted DNA from viruses and other recombinants were identified with these ^{32}P-samples. The control of the two largest recombinants, and the separation of recombinants carrying inserts of identical lengths are shown in Fig. 5.

The largest recombinant, carrying *Pst*I-fragments of AV35 *A* + *L* + + *K* + *H* was labeled with ^{32}P isotope. These sequences can be indentified

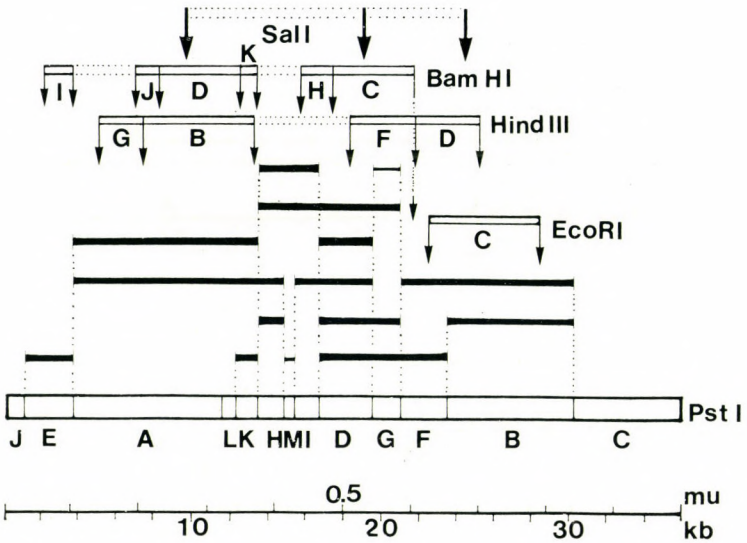

Fig. 3. *Pst*I-fragment combinations present in the fragment library prepared in pBR322/*E. coli* system. The *Pst*I map of AV35 DNA is shown below, together with the scale (map units and kilobase pairs). The horizontal solid bars indicate the DNA regions present in different recombinants. The vertical dotted lines indicate *Pst*I-sites which had been attached to the single *Pst*I-site of the pBR322 in different recombinants. Open horizontal bars indicate different restriction endonuclease fragments, which had been recovered from recombinants using the enzymes inserted into the corresponding level of the figure. Vertical arrows indicate restriction endonuclease sites detected in the specific inserts

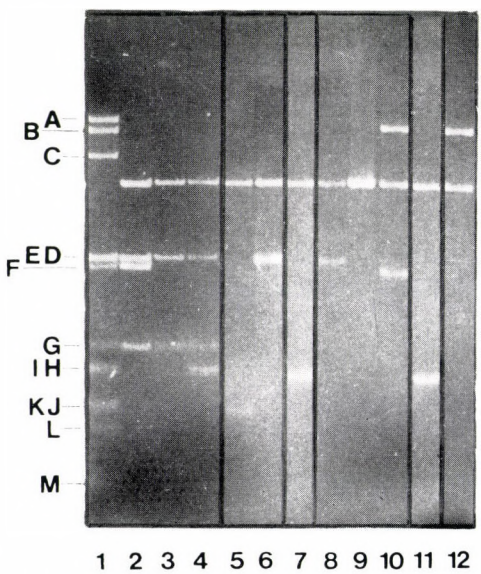

Fig. 4. *Pst*I-fragments of AV35 DNA reisolated from the pBR322 recombinants. Separation in agarose gel (10 mg/ml) was done as described in the legend of Fig. 1. The control was *Pst*I--digested AV35 DNA. Lanes 2 to 12 were used for the separation of *Pst*I-digested recombinants. The band present in all lanes represents the unit length pBR322 molecule

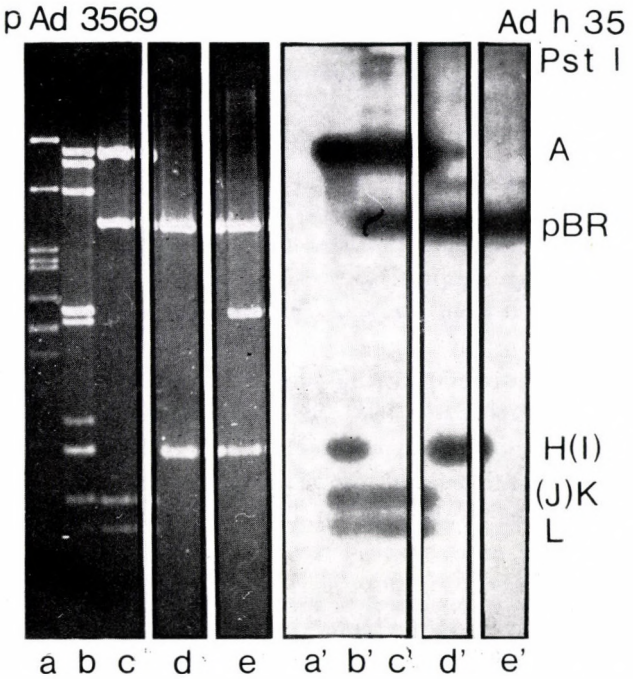

Fig. 5. Blot hybridization of AV1, AV35 and recombinant DNA fragments with the largest recombinant found in the *Pst*I fragment library. pAd 3569 was the protocol number of the clone, labeled by nick translation. Only fragments detected by autoradiography are denoted by capital letters

in lanes *b* and *b'* which contained only AV35 DNA. Only fragments $A + H + K + L$ give autoradiographic signals in lane *b'* with labeled recombinant pAd35PstI-69. Lane *c* contained a blotted recombinant preparation, without the presence of fragment *H*. The autoradiographic pattern shows complete homology of fragments $A + K + L$ in addition to the signal given with pBR322 sequences (lane *c'*). The recombinants in lanes *d* and *e* may carry either *Pst*I-*H* or *PstI-I* inserts of the same electrophoretic mobility. The autoradiographic analysis was necessary to prove, that only the plasmid in lane *d* is carrying *Pst*I-*H*, and the sample of lane *e*/*e'* possesses no homology to the probe. All other recombinants shown in Fig. 4 have been characterized by one of the procedures described above.

The nick-translated DNA probe in the experiment shown in Fig. 5 was representing 10.9 kb of the AV35 genome. It is an important result of this experiment that under the hybridization conditions used (0.55 parts of formamide) the recombinant revealed only very small homology to the DNA fragments of AV1 DNA (lane *a*/*a'*). Very weak signal was detected above the *Hin*dIII-*B* fragment of the AV type 1 genome (not labeled in Fig. 5).

Discussion

The DNA of subgroup B human adenoviruses has been examined by many authors with different procedures [2, 4–6, 20, 22–31]. Most of the work have been done with the first DNA cluster: physical mapping [22, 25–29]; cloning [23]; heteroduplex mapping [4]; sequence homology [5, 31, 32]. Data on DNA cluster II became available only recently [2, 4–6, 20, 24, 33]. The present work, however, described the first summarizing restriction endonuclease maps on a comparative manner. Three maps of AV35 DNA are original, and minor corrections have been done in the *Bam*HI, and *Pst*I maps published earlier [6].

DNA diagnostics is of growing importance in the case of adenoviruses [2, 20, 24]. As mentioned earlier [14], *Sal*I, *Kpn*I and *Pst*I patterns also can be used to differentiate subgenera from each other, and the two DNA clusters of subgroup B may be differentiated, too. Applying restriction endonuclease maps in connection with the evaluation of diagnostic examination of restriction endonuclease patterns, more specific results may be obtained. Some type specific differences are drawn (numbers 11, 14, and 34 in Fig. 2) using the patterns published by Adrian et al. [20, 24].

The fragment library cloned into the pBR322/*E. coli* system is of practical importance. Using the recombinants as reagents for DNA hybridization, type-specific and subgroup-specific sequences can be mapped, and subgroup-specific probes may be obtained for rapid diagnosis. The experiment presented in Fig. 5 indicates that subgroup-specific differences may be obtained even with probes of 10.9 kb. The DNA of AV1 possesses *Hin*dIII-fragments $D + B + I + J$ between map units 0.11 to 0.4 overlapping with the isotope-labeled AV35 fragment. In spite of this only *Hin*dIII-B gave very weak homology (lane *a*/*a*' in Fig. 5) under the conditions of hybridization used.

The polarity of the physical maps presented has been measured by DNA fragment denaturation ([14]; experiments not presented). The colinearity of all maps available proves the correct polarity of the maps.

Acknowledgements. The skilled technical assistance of Miss MÁRIA VÉGH and KATALIN BEDECS is very much appreciated. We thank DR. P. MEDVECZKY for the gift of *Kpn*I restriction endonuclease, and Dr. TH. ADRIAN for preprints of Ref. 20. We are indebted to Dr. MÁRIA TAKÁCS-HASZPRA for the computer programme, which enabled us to search for restriction sites in published adenovirus DNA nucleotide sequences.

REFERENCES

1. Stalder, H., Hierholzer, J. C., Oxman, M. N.: New J Clin Microbiol **6,** 257 (1977).
2. Wadell, G.: Curr Topics Microbiol Immunol **110,** 181 (1984).
3. Horowitz, M. S., Valderrama, G., Korn, R., Spigland, I.: UCLA Symp Mol Cell Biol **16,** 187 (1984).
4. Garon, C. F., Berry, K. W., Hierholzer, J. C., Rose, J. A.: Virology **54,** 414 (1973).

5. Green, M., Mackey, J. K., Wold, W. S. M., Ridgen, P.: Virology **93,** 481 (1979).
6. Valderrama-Leon, G., Flomenberg, P., Horowitz, M. S.: J Virol **56,** 647 (1985).
7. Graham, F. L., Smiley, J., Russell, W. C., Nairu, R.: Virology **30,** 59 (1977).
8. Berencsi, Gy., Dyachenko, N. S., Tarassishin, L. A., Vantsak, N. P., Kovalishin, G. G., Kiseleva, E. K., Zhovnovataya, V. Z., Ádám, É., Nász, I.: Acta Microbiol Hung **33,** 233 (1986).
9. Belák, S., Virtanen, A., Zabielski, J., Rusvai, M., Berencsi, Gy., Petterrsson, U.: Virology **150,** 3 (1986).
10. Belák, S., Berencsi, Gy., Rusvai, M., Lukács, K., Nász, I.: Arch Virol **77,** 181 (1983).
11. Medveczky, P., Zvavizion, B. A., Berencsi, Gy., Chaplygina, N. M., Szomolányi, É., Naroditsky, B. S.: Nász, I.: Arch Virol **67,** 85 (1981).
12. Palmiter, R. D.: Biochemistry **13,** 3606 (1974).
13. Maniatis, T., Fritsch, E. F., Sambrook, J.: Molecular Cloning — A Laboratory Manual. Cold Spring Harbor, New York 1982.
14. Takács, M., Berencsi, Gy., Lengyel, A., Nász, I.: Acta Virol **27,** 289 (1983).
15. Smith, G. E., Summers, M. D.: Anal Biochem **109,** 123 (1980).
16. Johnson, D. A., Gautsch, J. W., Sportsman, J. R., Elder, J. H.: Gene Anal Techniques **1,** 3 (1984).
17. Peden K. W. C.: Gene **22,** 277 (1983).
18. Palkonyai, L., Berencsi, Gy., Geck, P., Nász, I.: Acta Microbiol Hung **30,** 179 (1983).
19. Bos, J. L., Polder, L. J., Bernards, R., Schrier, P. I., Van der Elsen, P. J., Van Der Eb, A. J., Van Ormondt, H.: Cell **27,** 121 (1981).
20. Adrian, Th., Wadell, G., Hierholzer, J. C., Wigand, R.: Arch Virol **91,** 277 (1986).
21. Berencsi, Gy., Medveczky, P., Naroditsky, B. S., Chaplygina, N. M., Zavizion, B. A., Tikhonenko, T. I., Nász, I.: Acta Microbiol Acad Sci Hung **25,** 97 (1978).
22. Varsányi, T., Winberg, G., Wadell, G.: FEBS Lett **76,** 151 (1977).
23. Engler, J. A., Kilpatrick, B. A.: Gene **13,** 125 (1981).
24. Adrian, Th., Wigand, R., Hierholzer, J. C.: Arch Virol **84,** 79 (1985).
25. Buescher, M. A., Roninson, I., Padmanabhan, R.: Gene **18,** 123 (1982).
26. Daniell, E.: J Virol **19,** 685 (1976).
27. Sekigawa, K., Fujinaga, K.: Virology **82,** 509 (1977).
28. Tibbetts, C.: J Virol **24,** 564 (1977).
29. Tibbetts, C.: Cell **12,** 243 (1977).
30. Van Ormondt, H., Galibert, F.: Curr Topics Microbiol Immunol **110,** 191 (1984).
31. Fujinaga, K., Yoshida, K., Yamashita, T., Shimizu, Y.: Curr Topics Microbiol Immunol **110,** 53 (1984).
32. Kang, W. G.: Ann Immunol Hung **26,** 1003 (1986).
33. Kang, W. G.: Thesis. Budapest 1986.

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TENTH INTERNATIONAL SYMPOSIUM ON LISTERIOSIS

TENTH INTERNATIONAL SYMPOSIUM ON LISTERIOSIS

and

ROUND TABLE DISCUSSION

on

METHODS OF ISOLATION AND CHARACTERIZATION OF LISTERIA

PÉCS, HUNGARY, AUGUST 21–26, 1988

Acta Microbiologica Hungarica 36 (2—3), pp. 79—85 (1989)

Opening addresses

J. TIGYI

Head of the Biological Section of the Hungarian Academy of Sciences; University Medical School, Pécs, Hungary

On behalf of the Biological Section of the Hungarian Academy of Sciences and its Local Committee in Pécs, I greet you warmly on the occasion of the Tenth International Symposium on Listeriosis.

It is a great honour for us, that you have choosen Pécs second time to hold this meeting here. The value of this meeting is elevated by the support of WHO. The newest international epidemiological data illustrate well the importance of the world-wide listeriosis problem. The role of food-stuffs in the spreading of pathogens involved in these researches and discussions food producers and controllers besides medical doctors and veterinarians. It is urgent to solve the uncleared problems of listeriosis because of the high mortality rate of pregnant women, their fetuses and new born babies as well as the immune-compromised patients. Different problems of methodology, epidemiological tracing and surveillance will be discussed also and we hope on the basis of these consultations that there will be possibility to prepare a document which will promote a better organized, more unified and more effective work in the world.

Our town and its surroundings are very rich in historical monuments and events and I wish all of you to be acquainted with our history and present life, enjoy this town and have a very successful conference.

Á. VASS

Head of Department for Hygiene and Epidemiology, Hungarian Ministry of Social Affairs and Health, Budapest, Hungary

It is a particular pleasure for me to welcome the participants at the Tenth International Listeriosis Symposium and round table conference on behalf of Dr. Judit Csehák, Minister of Social Affairs and Health and of the Ministry's senior staff. We are really pleased that after 1972, Hungary can host this important scientific meeting for the second time. The selection of my country to host this meeting for a second time is of particular importance to us, since it expresses the recognition of scientific research work of Hungarian experts in connection with this pathogen whose importance has been

growing in recent years. My thanks are due to the city of Pécs, which, after having been the host of several international and national medical symposia of outstanding importance, will make it possible for experts of the World Health Organization's Regional Office of Europe, of the International Organization of Veterinary Health as well as for scientists from 25 countries, among them Hungary, too, to have worthwhile discussions. The importance of this meeting is also stressed by the fact that WHO's Health for All By the Year 2000 strategy includes a target on combating and controlling communicable diseases and hazards.

A disease with high case fatality rate, listeriosis in humans occurs in the newborn and in pregnant women in about half of the cases. A cause of concern is that much remains to be answered about the disease despite intensive research. In the eighties, epidemics developed in the United States, Canada and Switzerland and spread through contaminated foodstuffs, causing much concern and fear. This also urges the scientific community to make serious efforts to clear scientific issues which have remained unanswered so far and to step up international cooperation. This meeting in Pécs may be a valuable step towards attaining this goal. I wish every success to its work.

K. Bögel

Chief, Veterinary Public Health, Division of Communicable Diseases, WHO, Geneva, Switzerland

It is a great pleasure for me to represent the WHO Regional Office for Europe and to convey the greetings of Dr. Aswall, the Regional Director. He requested me to wish you a successful conference and, in particular, to thank all those who have for many years faithfully cooperated with the World Health Organization.

Listeriosis is a dramatic though sporadic disease which in recent years has moved into the foreground of zoonotic diseases in Europe as well as in North America. The epidemiology of the majority of human cases is not yet known and we face the problem of widespread occurrence of the infectious agent, the distinct conditions for its accumulation and amplification in animals and the environment, the susceptibility of certain individuals and the various pathways of infection. Much research is still required to elucidate all the critical paths and the conditions of this disease. We are glad to see that efforts continue in the search for methods of prevention and control, not only at microbiological, environmental and technological level, but also by applying newest methodologies of molecular pathology and epidemiology. The Congress in Pécs may bring us a step further in the understanding of the infection and the orientation of further research in all its complexity.

The WHO Collaborating Centre at the Institute of Veterinary Medicine in Berlin (West) issued in 1987 a voluminous book on the Joint WHO/ROI Consultation on Prevention and Control of Listeriosis, which still serves as a basis for discussions and decisions. The conclusions of this joint meeting were summarized in a special WHO document and reconfirmed in its essence by a WHO Informal Working Group held in February this year in Geneva.

Of course, meetings of this kind organized by WHO aim more at the practical issues of research coordination and management of surveillance, epidemiological conditions and cases of disease. In view of the great gap of knowledge concerning decisive factors leading to spread and disease, WHO welcomes the opportunity to co-sponsor this Conference which will concentrate on the scientific roots and approaches in listeriosis epidemiology and prevention. Personally, I am very happy to meet with so many friends here in Pécs and to be able to work with you on the emerging problem of listeriosis.

N. SKOVGAARD

Secretary and Treasurer of the International Committee on Food Microbiology and Hygiene; Royal Veterinary and Agricultural University, Frederiksberg, Denmark

I have the honour on behalf of the "International Committee on Food Microbiology and Hygiene" of the "International Union of Microbiological Societies" to address this opening session of the Tenth International Symposium on Listeriosis.

For those not quite familiar with the committee I can inform you that it was founded during the "International Congress on Microbiology", held in Rome, 1953. Since then the committee has convened 13 international symposia covering many different fields of food microbiology and is in good progress to plan its fourteenth symposium in Oslo, Norway, 1990. Working parties and round table conferences have been held in between. In 1984 we launched the International Journal of Food Microbiology in collaboration with the "International Union of Microbiological Societies" and the journal has turned out to be quite successful.

It is now well established that food plays a major role in the epidemiology of listeriosis and the committee has been very happy to support and cooperate in the planning of the symposium. One person deserves to be mentioned in this connection, the secretary Dr. Béla Ralovich, who, I can assure you, has succeeded in overcoming many local and also cross boundary problems.

We look forward to a successful and enjoyable symposium and we are confident that its deliberations will improve our knowledge of *Listeria* which has challenged mankind so severely the latest years.

H. SEELIGER

International Committee of Systematic Bacteriology, Taxonomic Subcommittee of Listeria, Erysipelothrix and related organisms; Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, FRG

Since the previous speakers have already covered most of what I intended to say, I take the liberty of reminding you that this is the second time that workers interested in *Listeria* and listeriosis have convened for a symposium in Pécs. Only very few of today's participants were among those who attended the meeting in the late fall of 1972. This meeting in 1972 became a mile-stone in the history of listeriosis research. It was the International Subcommittee of *Listeria* of ICSB within IUMS that accepted the invitation of the organizers to this second meeting in the hope that again Pécs would serve the function of bringing together scientists from the eastern and western hemisphere to exchange new knowledge and recent developments in the field. As a matter of fact nobody would have dared to anticipitate not only a second Pécs meeting 16 years ago but also the role *Listeria monocytogenes* has taken during the past years in human epidemiology as cause of food-borne infections, primarily in those parts of the world which are rich and progressive. At the same time new methods are offered to cope quicker and more efficiently with the problems of diagnosis, monitoring of food production and hopefully, with the prevention of the illness. With thousands of human cases of listeriosis every year and hundreds with fatal outcome the time has come to initiate a world-wide campaign to reduce the recognized new danger to avoidable proportions. With this in mind, we extend to all of you our best wishes for a full success of this Tenth International Symposium on the Problems of Listeriosis.

B. LÁNYI

Chairman of the Bacteriology Section of the Hungarian Society of Microbiology; National Institute of Hygiene, Budapest, Hungary

On behalf of the Hungarian Society of Microbiology I take pleasure to extend a very warm welcome to all participants in the Tenth International Symposium on the Problems of Listeriosis. In view of the world-wide listeria--hysteria in association with foodborne infections, it is not at all unusual to hear of a new meeting on listeriosis, and yet, the Executive Committee and members of our Society were delighted to receive the news that the International Listeria Meeting held in Hungary in 1972, would be followed this year by another symposium in our country. I hope that this meeting is going to provide ample opportunities to tighten the links among the numerous listeria workers, microbiologists specialized in other fields as well as epidemiologists, food hygienists and practitioners.

In Hungary, we have well-organized, efficient networks for medical and veterinary microbiology. Laboratories devoted to medical, food and environmental bacteriology have been established in the Public Health Stations during the past sixty years, and recently hospital bacteriology laboratories have been joined to this service. We have state regulations governing the operation of bacteriology laboratories and there is a newly formed Microbiology Advisory Council to the National Institute of Hygiene, which is in charge of elaborating guidelines, performing quality control supervisions and evaluating statistical data.

In cooperation with epidemiologists, we have made a good progress in surveying salmonellosis and other enteric diseases, hospital infections, resistance to antibiotics, etc. As to listeriosis, owing mainly to the enthusiastic contribution of my friend, Dr. Béla Ralovich, a way was opened up for a good progress, too. However, as to the prevention of this disease, there are still many unanswered questions in our country and elsewhere, and I hope, the present meeting will furnish clue to some of these problems.

L. VÁRNAGY

Chairman, Veterinary Association of the Hungarian Society of Agricultural Sciences, Budapest, Hungary

It is my privilege to convey the greetings and good wishes of the Hungarian veterinarians to this conference for a successful and memorable meeting. One of the main tasks of the Hungarian Veterinary Service has always been the continuous preventive and regulatory work on the area of zoonoses and of foodborne diseases. In this area we have been working in close collaboration with the public health service of our country. These harmonized joint efforts could be traced back to almost a century when excellent young professors (like Professor Hutyra, Professor Preisz, and Professor Aujeszky) became founders of the Hungarian veterinary epidemiology, microbiology and state veterinary medicine. As a result, we were successful in eradication of several animal diseases that represented a constant threat to human health. In the last decade, for instance, a good collaboration between the two services helped us to eradicate bovine tuberculosis, bovine and porcine brucellosis.

Foodborne diseases are also continuously monitored, checked and fought with joint efforts of the public health service and of the veterinary service of this country. New developments in diagnostic and laboratory techniques, and on the area of new or newly recognized diseases (like immunodefficiencies) are bringing new tasks for both human and veterinary scientists. One such infection — characterized by almost exponentially growing awareness — is listeriosis.

It will be the task of this conference (having almost all the distinguished scientists and specialists of this field together) to help us to get an up-to-date and realistic picture of this disease in both human and veterinary medicine, and to define areas where the most urgent actions should be taken in order to protect human health and/or animal losses. I am convinced that this conference will accomplish these main tasks and will be regarded in retrospect as one of the key events in the row of listeria research meetings. I am sure that the symposium will establish and renew collegial acquaintanceships among specialists of listeriosis from different nations.

Based on the forementioned, traditionally good collaboration between human and veterinary medicine and on our professional interest, the Hungarian veterinarians are looking very much forward to learning about the major outcomes of this meeting. Therefore we are prepared to report these briefly in the Hungarian Veterinary Journal.

Finally, in the name of the Veterinary Association of the Hungarian Society of Agricultural Sciences I have the pleasure to welcome our gests at this important symposium. We wish you all a very good professional meeting, with good, constructive and friendly discussions and a very pleasant stay in Hungary.

Z. MOLNÁR

Vice-President of the City Council of Pécs, Hungary

On behalf of the Town Council of Pécs as a host allow me to greet warmly all the international and national lecturers as well as all the participants and guests of the Tenth International Symposium on Listeriosis. It is a great honour for us that this Conference has been organized in our town.

Pécs is an industrial town with a lot of workers employed by the mine-industry. Our 2000 years old town is well known of the preservation of the traditions, she has been considered as an important center of science and culture of the country. We try to promote the values of the earlier centuries with an increasing number of museums and with renewing the historical centre of the town.

We are glad to notice that Pécs has become the host of many conferences and these scientific meetings can fulfill two tasks: on the one hand they make possible for our experts to improve their knowledge and on the other hand we hope to make new friends for the people living in this region.

Pécs is not only the basic centre of the Hungarian industry and mining but she has been the scientific centre of our country for many centuries, too. The first University of Hungary was founded in Pécs, in 1367 as the fifth one in Europe, following the foundation of the universities in Paris, Prague, Krakow and Vienna. Presently Pécs is the scientific centre of the South Hun-

garian region. The university staff and that of the different research institutions help us to solve the everyday life problems of society, economics and health creating the common interest and cooperation among the representatives of science and practice.

In this way science has become part of our life, it helps to improve our development and future existence. We believe that the common work will promote the peaceful coexistence of people. I hope that your Symposium will result in multilateral cooperation between the theoretical and practical specialists and that it will improve the further researches in this field. I know that the successful meetings have always very strict time-tables, but in spite of this fact I believe that you will have the possibility to visit our town and see lot of the nice historical monuments. I hope that you will enjoy them and you will be impressed not only by the culture of past centuries but you can make consequences on the everyday life of the inhabitants, too. You will see how we try to fulfill the economic, cultural and civilization requirements of people of our days based on the rich heritage of the former centuries. I wish you to get acquainted with this town of 2000 years old history and feel yourselves well here. I hope that you will visit us officially or privately in the near future also and I can promise you that you will be always our distinguished guests and will be welcomed to Pécs. Once more I wish you a successful conference.

M. Bauer

Rector of the University Medical School, Pécs Hungary

It is my privilege to greet you on behalf of the Medical University of Pécs. Our University willingly offers home and facilities for outstanding scientific activities. It is a great honour that you have chosen Pécs as a site of the Congress and I dare to hope that this choice is indicative of certain recognization and appreciation of the work done here in this field. I wish you much success and a good time here.

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Overview lectures

MULTISECTORAL APPROACH IN THE PREVENTION OF HUMAN LISTERIOSIS

K. Bögel

Veterinary Public Health, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland

A new dimension has been added to our understanding of human listeriosis through the study of large-scale or common source outbreaks of the disease related to food of animal origin or to the episode due to coleslaw prepared from cabbage which may have been contaminated shortly prior to harvesting by an infected sheep flock. In Switzerland and the Federal Republic of Germany individual and relatively isolated cases were related to soft cheese. In the literature of the first half of this century we find epidemiological observations suggestive for the foodborne transmission of the infection in addition to diaplacentar, aerosol and contact infection. *Listeria monocytogenes* appears to be mainly amplified in spread and growth by mammalian species and, in particular, some of the food producing domestic animals. Whether this is associated with animal disease seems to be of no great human health significance except in situations where people purposely handle such sick animals and are therefore at particular risk of infection.

All the aspects of the epidemiological pattern such as cycles of animal infection and disease, the role of the environment, cross-contamination along the food chain, and man as victim and excretor permit to look at the overall epidemiological process from different angles with a risk of overestimating the one or the other factor and conditions leading to the human disease. Gaps in knowledge can easily lead to a biased description of the epidemiology of a disease particularly if this affects regulatory issues or product markets.

It may therefore be wise to subject the scientific analysis and suggested practical approaches to a neutral and well established managerial methods which we may call systems research. The complexity of approaches which we think of as reasonable in listeriosis prevention would entail wide intersectoral cooperation.

Konrad Bögel
Veterinary Public Health, Division of Communicable Diseases, World Health Organization,
1211 Geneva, 27-Switzerland

The first question we should ask is in which sectors do we find resources for the surveillance and prevention of the disease? Of course, the reply must relate to a distinct national set-up but may be generalized to some extent. Resources including laboratory services could be located in the following sectors.

Public health, including individual health care;
Animal health, including farming practice;
Environment, including waste disposal and rendering;
Municipalities, slaughter hygiene and food inspection;
Trade, e.g. export/import requirements;
Interior, e.g. for standardization of tests;
Defense, e.g. food microbiology;
Private industries:
— animal feed;
— milk and milk products;
— meat and meat products;
— drugs and vaccine development.

A systematic analysis must show for each of these sectors:

(a) where and how *L. monocytogenes* infection and contamination detected — contribution to surveillance;
(b) which general measures of prevention are being taken, and
(c) which interventions are foreseen in case of a positive finding.

Following such an approach we may recognize that *L. monocytogenes* infection and contamination is not ubiquitous in certain ecosystems, e.g. Alpine or pre-Alpine hilly areas in Europe or different facilities in the dairy industries. The occurrence in the environment may have its own pattern, possibly in relation to the rates of infection in certain animal species. Another epidemiological pattern appears in foodstuffs since cross-contamination during present slaughter practices appears to be the overwhelming determining factor. The pattern may further change during food processing and storage whereby the often uncontrolled storage conditions in households, particularly in refrigerators, could result in an epidemiological pattern which shows very little relation to the original distribution of the agent in domestic animals, man and the polluted environment.

Yet, there is no better solution than to provide food and environment originally free of the infectious agent. This requires multisectoral cooperation reaching into the environment, the entire food chain and the international trade. What could be done by the individual sectors to reduce the load of *L. monocytogenes* infection and contamination?

Animal production and health

(a) Control (including surveillance) of infection in animals;
(b) Establishment and management of *L. monocytogenes*-free herds;
(c) Disinfection of production and slaughter premises;
(d) Development of slaughter technologies avoiding cross contamination;
(e) Reduction of pollution by organic matters, phosphates and nitrates in the environment.

Trade

(a) Monitoring of exports and imports for *L. monocytogenes;*
(b) Harmonization of regulatory procedures;
(c) Assessment and establishment of bactericidal methods.

Municipalities and rural communities

(a) Environmental monitoring of food processing and storage;
(b) Identification and monitoring of critical control points;
(c) Research on decontamination procedures;
(d) Establishment, education and surveillance in GMP;
(e) Research and measures concerning survival and growth of *L. monocytogenes* in households.

Education and research

(a) Establishment of guidelines for education — consumers, farmers, food processes, veterinary public health and environmental health;
(b) Coordinated research by university institutes on: virulence, epidemiology, preventive measures, rapid diagnosis and treatment;
(c) Teaching of intersectoral approaches in *L. monocytogenes* prevention.

Health

(a) Establishment of services for rapid diagnosis and treatment of human cases of listeriosis;
(b) Research on zoonoses and routes of human infection;
(c) Development of methods for the protection of high risk groups of people: e.g. decontamination of vehicles of infection and of environment;
(d) Assessment of the role of human carriers and control measures;
(e) Improvement of regulatory issues;
(f) Cooperation with industries on prevention and control;
(g) Professional and public education.

Food industry

(a) Promotion of HACCP approaches;
(b) Research on control points;
(c) Development and application of procedure for growth inhibition;

(d) Operational research on sanitation measures;

(e) Cooperation in surveillance, regulatory issues and education.

The managerial approach to resource mobilization in research, education and operations is, in its basic functions, neutral and can, therefore, be applied irrespective of the local epidemiological pattern or policy of prevention and control. Surely, such an approach will predominantly help to add information and to fill gaps in our knowledge. We may then be in a better position to circumscribe the problems of ubiquitous environmental contamination and distinct foci of growth of *L. monocytogenes* and contamination, as well as the problem of levels of infection and concentrations of *L. monocytogenes* in the environment, the production animals, and the food chain.

Intersectoral cooperation requires a structural element to coordinate the cooperation of the whole institutional framework involved. Not all countries possess an intersectoral committee which joins aspects on environmental health, veterinary public health and food safety. For the question of *L. monocytogenes* infection, zoonoses committees or food hygiene committees are generally insufficient in their composition. It should, however, not be difficult to enlarge existing coordinating bodies in order to elaborate a comprehensive programme.

International organization are presently working in three major directions; firstly, towards new efforts in animal production hygiene including promotion of good husbandry practice (major problem: community participation) and good slaughterhouse practice (major problem: research and development of hygiene to prevent cross-contamination). Secondly, special efforts are made to improve surveillance and hygiene in the food processing sector including regulatory issues and consumer education. Thirdly, research and development of bactericidal and growth preventing procedures continues, including food irradiation.

Obviously the solution can be seen in a system of general hygiene, monitoring and intervention. We may, however, also think of actions oriented towards some of the most critical epidemiological foci and paths only, e.g. by reducing rates of infection and contamination in some selected farms and their environment or by restricting particularly hygienic measures at slaughter to selected groups of animals for production of distinct food items which are eaten raw or not subject to sufficient bactericidal treatment, and finally by selection of food items meeting distinct criteria for the high risk groups of consumers.

However, this range of possibilities shows clearly that we can improve safety considerably if we work together intersectorally with a joint responsibility for animal production and food safety. This will not only have a positive impact on the control of listeriosis, but generally on animal production and product hygiene.

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INTERNATIONAL ASPECTS OF THE CONTROL OF ANIMAL LISTERIOSIS

J. L. Bind

Veterinary Laboratory, Tours, France

In response to a proposal by the International Animal Health Code Commission, the Office International des Epizooties (O. I. E.) has drawn up two lists of animal diseases, for the purpose of notifications to be submitted by Member Countries, and has recommended measures to be applied by Member Countries with regard to international trade.

List "A" includes the most serious and highly contagious diseases. List "B" contains transmissible diseases having important socio-economic and/or health implications for Member Countries, and having a significant effect on international trade in animals and animal products.

It is clear that animal listeriosis does not fall into category "A". The question is whether the time has now come to propose its inclusion in category "B". To answer this question it is necessary to consider the various implications of the disease, concerning the field of animal health, the relationship between the animal form of the disease and human health, and problems related to the contamination of animal products.

1. *Animal health and Listeria*

Listeriae can be isolated from a large number of animal species, notably from cattle, goats, sheep, carnivores, rodents, birds, fish and wild animals. Firstly, it is important to draw a distinction between the carrier state and the disease itself.

1.1. *Carrier state*

Listeria is an organism present in the environment, and as such is found worldwide. Listeriae can easily be isolated in most animal species without there necessarily being any clinical manifestation of the disease.

Jean-Louis Bind
Veterinary Laboratory
46 Avenue Gustave Eiffel, 37023 Tours Cedex, France

1.2. *The disease*

1.2.1. *General remarks*

Different factors are involved in the clinical expression of the disease in carrier animals.

— Increased susceptibility of certain species. Goats, sheep and cattle are more susceptible to the disease than other species. This may be related to a particular method of feeding. For example, the correlation between the introduction of silage and the appearance of the disease has been demonstrated by Nicolas et al. [1].

— A particular physiological state. Immunodepression, dietary imbalance, intercurrent diseases, vaccinal stress, gestation or the neo-natal period are all factors inducing the clinical expression of listeriosis.

— Heightened virulence of certain strains. Epidemiological studies, particularly those carried out at the time of outbreaks, have demonstrated the existence of "epidemic" phage-types which are more virulent than others.

1.2.2. *Clinical expression*

The disease exists in two basic forms: abortive and neo-natal form or meningeal form. In both cases the prognosis is serious and the mortality rate very high. However, though the mortality rate is high, the morbidity rate remains very low. Within a single herd, the disease occurs in only a limited number of animals (approximately 5%) even though up to 80% of the herd are carriers.

1.2.3. *Incidence of the diseae*

Although no systematic screening network exists, it is evident that clinical listeriosis is present worldwide. However, taking into account the low morbidity of listeriosis, the economic effects of the meningeal form or the abortive form are slight.

A survey carried out by Martel [2] clearly showed that infectious aetiology accounted for a third of all cases of abortion, and *Listeria* for 1.8% of bacteria-related abortions.

2. *Animal listeriosis and human health*

2.1. *Direct transmission*

Given our present level of knowledge, listeriosis can no longer be considered a zoonosis in the strict sense of the term. Cases of direct contamination are exceptional. Moreover, a comparison of strains isolated in humans with

those in animals shows that, in most cases, different serotypes or phage-types are involved. Statistics prove that in France the urban population is affected more than the rural population, though the latter is in more frequent contact with animals. Thus, Goulet et al. [3] have shown that the incidence of listeriosis is highest in Ile de France, the host highly urbanised region. These points were debated at the February 1988 meeting of a working group on foodborne listeriosis. The majority of experts attending the meeting in Geneva were of the opinion that listeriosis in man is essentially of food or environmental origin.

2.2. *Contamination of animal products*

2.2.1. *Dairy products*

— Milk. Mammary excretion of *Listeria* is, in fact, very rare in the natural state. Nevertheless, mastitis due to *Listeria* does exist. Contamination of milk is principally caused by pollution from the environment during milking, possibly of faecal origin. However, since milk from different establishments is pooled, a process of dilution takes place. It can be considered that correctly conducted pasteurization results in a product devoid of risk to human health.

— Dairy products. Contamination occurs chiefly during manufacturing processes and commercialization. Statistics published by Bind[4] show that the percentage of contamination in cheeses made from pasteurized milk is equal to that of cheeses made from raw milk.

2.2.2. *Meat products*

Similar reasoning can be applied to meat products. The presence of *Listeria* in the faeces of many mammals and birds seems to play a large part.

2.2.3. *Other food products of animal origin*

Fish and other seafood, such as crab meat and prawns, may be contaminated. The widespread presence of *Listeria* in numerous plant species should also be borne in mind.

3. *Effect of animal listeriosis on international trade in animals*

List "B" designates the category of transmissible diseases having important socio-economic and/or health implications for Member Countries, and having a significant effect on international trade in animals and animal products. Is the inclusion of animal listeriosis in list "B" justified at the present time?

With regard to list "B", the following criteria must be considered.

— Animal listeriosis, due to its low morbidity, cannot be considered

a transmissible disease having important socio-economic and/or health implications for Member Countries.

— However, the carrier state can have a significant effect on animal products.

The following point must also be discussed. *Listeria* is a bacterium present in the environment, and its distribution is wordwide. In this context, international trade procedures cannot significantly alter the epidemiological situation in the countries affected. Furthermore, it is quite unthinkable, given the existing state of our knowledge of the epidemiology of listeriosis, to seek to eliminate the pathogen carriage by any one species.

It would therefore seem premature to propose the inclusion of animal listeriosis in list "B". However, the introduction of the following measures is to be strongly recommended.

— The organization of a screening programme for animal listeriosis.

— An in-depth study of strains isolated in animals (serotypes, phage types).

— A comparison of such strains with those from animal products and patients.

— A study of the epidemiology of animal listeriosis and its evolution.

— A study of the relationship between animal feeding and the carrier state.

— An evaluation of the minimal infectious dose in animals.

— A study of the prevention and control of the disease.

Were the implementation of these recommendations to bring to light significant variations concerning the health status of animal listeriosis in different countries, it would then be appropriate to refer the case of animal listeriosis to the International Animal Health Code Commission of the O. I. E.

In summary, the O. I. E. has drawn up two lists of animal diseases, List "A" and List "B", according to their seriousness and their effect on international trade in animals and animal products.

Animal listeriosis should automatically be excluded from List "A". However, certain criteria for List "B" might, a priori, justify its inclusion.

After a review of the implications of animal listeriosis in the fields of animal and human health, and also in problems of contamination of animal products, the author concludes that it is still too early to include animal listeriosis in List "B".

REFERENCES

1. Nicolas, J. A., Espaze, E. P., Rocourt, J., Videau, N., Cornejals, M. J., Catinal, B., Courtieu A. L.: Rec Med Vet (In press).
2. Martel, J. L.: Avortements infectieux non brucelliques des Bovins. Ass. Et. de la Reproduction Animale. 1982. 15–19.
3. Goulet, V., Leonard, J. L., Celers, J.: Rev Epid Santé Publ **34,** 191 (1986).
4. Bind, J. L.: Review of latest information concerning data about repartition of *Listeria* in France. WHO Working Group on Foodborne Listeriosis. Geneva, February 1988.

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LISTERIOSIS — A WORLDWIDE ISSUE

B. RALOVICH

Institute of Public Health and Epidemiology, University Medical School, Pécs, Hungary

The Fifth International Symposium on Problems of Listeriosis was organized here at Pécs in 1972. Participants of this meeting emphasized the importance of the newest selective and enrichment media containing trypaflavine and nalixidic acid, the results of surveillance on the ecology of *Listeria* and the observations in relation to the virulence markers. Necessity of an effective active immunization was also mentioned [1].

Since the Fifth meeting Sutton Bonington [2], Varna [3], Madrid [4] and Nantes [5] were the hosts of consecutive symposia.

It is a honour for us that we have been authorized to organize this Tenth Symposium. For this kind gesture I should like to express my thanks to Professor Seeliger and Dr. Jones the Chairman and the Secretary of IUMS-ICSB Subcommittee on *Listeria* and Related Bacteria, for the fruitful cooperation to Dr. Bögel, the representative of WHO, and for the continuous important support of the organization to Professor Mossel and Professor Skovgaard the President and the Secretary of IUMS-ICFHM, to Dr. Medve, the Secretary of State for Health, to Professor Tigyi, the Head of the Biological Section of the Hungarian Academy of Sciences, to Professor Mészáros, the Chairman of the Hungarian Society of Microbiology, to Professor Várnagy, the Chairman of the Veterinary Association of the Hungarian Society of Agricultural Sciences, to Professor Kovács, the Rector of the University of Veterinary Science, to Professor Bauer, the Rector of the University Medical School of Pécs, to Mr Molnár, the Deputy-president of the City Council of Pécs, to other colleagues and, last but not least, to a number of commercial firms. Without their moral and financial helps we would not have been able to organize this Symposium.

During the last 16 years a lot of efforts have been done to clear different unknown problems of listeriosis. It is impossible to cover all studies and

BÉLA RALOVICH
Hungarian Meat Research Institute
Gubacsi út 6/b, H-1453 Budapest, Hungary

results in this short lecture. Therefore, I shall only focus our attention on some questions of theoretical and practical exposing those for further discussions.

In view of taxonomy, the use of haemolytic and pothogenic properties for classification of *Listeria* has not proved to be a lucky idea. The extensive studies of Rocourt and her colleagues yielded important results. On the basis of DNA relatedness and some few phenotypical properties they have come to the conclusion that the genus *Listeria* contains seven species: *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. grayi* and *L. murrayi* [6]. *L. seeligeri* strains generally do not cause typical beta-haemolysis on blood agar and its haemolysin differs from those of *L. monocytogenes* strains [7]. Cell wall constituents and serotypes of *L. monocytogenes* and *L. seeligeri* strains are identical [8–10]. On the basis of the results of gene probes both strains have similar and different DNA fragments [11–13]. The haemolytic property of *L. seeligeri* strains seems to me somewhat similar to that of *L. innocua* and *L. welshimeri* strains.

Now it is clear, as it was assumed in my book, that pathogenic property of *Listeria* strains is a multifactorial phenomenon. Besides haemolysin, lipolytic effect, different enzyme and metabolic activities (catalase, superoxide dismutase, iron transport system and so on), surface antigens and monocytosis-producing activity of *Listeria* strains have to be studied for an understanding of their virulence and mode of their pathogenicity at the molecular level. It is a problem that we know little about the stability of phenotypical and genetical characteristics of listeriae and I expect some more interesting observations on this field [14]. Pine et al. [15] published from CDC in 1987 about spontaneous loss of the beta-haemolytic character and virulence of *L. monocytogenes* reference strains.

On the basis of our present experience it can be stated that human and animal pathogenic and non-pathogenic species belong to the genus *Listeria*. Single members of a pathogenic species may be virulent or avirulent. To determine virulence of listeriae we published our observations on the markers of virulence first in 1970 and in the following years [16–18]. The newest experience has been presented on this Symposium [19]. Our opinion is generally unchanged. The newest results of genetical and pathogenetical works have verified the pathogenic importance of beta-haemolysin and the correctness of our conclusions [20–25]. In my opinion beta-type haemolysis on blood agar containing sheep or horse blood usually means that the given *Listeria* strain is more or less virulent. It is natural that the exact characterization of a strain requires the determination of the other markers of virulence, too, because results of cultivation on blood agar, Anton's test, chick-embryo pathogenicity, cell culture infection and mouse test represent somewhat different sides of virulence of a single *Listeria* strain. In contrast to my opinion, some of the authors believe that "the capacity of a *Listeria* isolate alone to damage erythro-

cytes does not, in any case, attest to its virulence" [26]. On the basis of my investigations performed during the last 20 years the non-haemolytic listeriae are non-virulent or avirulent.

Beta-haemolytic *L. monocytogenes* and *L. ivanovii* strains have been considered as virulent for humans and animals. On the basis of some preliminary results it was declared that *L. seeligeri* strains are practically apathogenic [27, 28]. The truth is that both virulent and avirulent strains belong to this species [29]. Neither beta-haemolytic property nor virulence seem to be species linked characteristics of listeriae.

Listeriosis is studied extensively mainly in Europe, USA, Japan and in some other countries. Data originating from these countries are rather insufficient for a final evaluation. Our epidemiological knowledge has been summarized several times, here only the newest developments will be mentioned [17, 30–36].

Human listeriosis can be characterized by two epidemiological patterns: "endemic" sporadic cases and "epidemic" outbreaks. Surveillance systems for human listeriosis in different countries are different and, in general, they are not sufficiently effective. The laboratory system for this purpose may be considered adequate for example in the UK, Hungary, GDR, Denmark, Sweden, Japan, France, the USA, FRG, the Netherlands, Canada, Switzerland, Czechoslovakia, Belgium. More or less active surveillance has been performed in the USA, France, UK, Denmark, Canada and Switzerland. The reported incidences range from less than 1 to 11.3 per million from Hungary, Scandinavian countries, France, the UK and the USA. In a study performed in the USA it was found that about 120 sporadic listeriosis cases occurred per million births. In view of the differences in reporting systems and activity of surveillance nobody knows whether the aforementioned data reflect different diagnostic routines, food hygienic practices, dietary habits, surveillance methods or epidemiological situations. Apparent increases in the incidence of human listeriosis have been noted in different countries. A verified increase was observed in the UK, Switzerland, Denmark, Belgium, Sweden and France.

Pregnant women and their fetuses or newborn babies as well as those persons whose immune system is compromised or incompetent are at high risk. However, such cases have also been diagnosed in which predisposing factor(s) could not be found yet. It would be very good to known exactly why does listeria infection manifest in illness in some persons but not in others. As the proportions of some risk-groups in the population are rising increase of number of listeriosis cases may be counted with.

The infectious dose for humans is not known but its value may considerably be influenced by the actual susceptibility or, in other words, by the momentary physiological condition of the host. On the basis of American and

Swiss observations, it seems that ingestion of 10^3–10^4 germs is dangerous. It is also a fact that consumption of home made sausages containting a few cells of virulent listeriae per gram failed to cause disease in Hungary [37].

The incubation period of listeriosis is generally 1 day after cutaneous or ocular infection and from 1 to 10 weeks after oral ingestion. Therefore, importance of the carriage of listeriae by healthy persons and importance of the effects which can influence the health condition of humans (viral or bacterial infections, hormonal changes at birth, alteration of iron metabolism and so on) helping manifestation of listeriosis cannot be emphasized too much. In persons with impaired condition, consumption of a low number of virulent listeriae may have serious consequence.

The source of listeriae and the convey of infection are very important questions. Numerous examinations of faeces verify that cattle, pig, sheep and poultry may harbour and excrete listeriae. These microorganisms can be found in milk, nasal, throat and vaginal discharges of animals. Also part of healthy persons carry listeriae. Besides these, *Listeria* strains can be found in waste water, manure, surface water, vegetation, soil and in any place contaminated with unsatisfactorily treated human and animal excreta. This fact is no surprise, since a similar situation exists, for example, in case of salmonellae.

On the basis of the last FAO/WHO report of 1985 listeriosis belongs to zoonoses. A small proportion of human cases is acquired by direct contact with infected animals or humans. Diseases of fetuses and newborn babies which represent about the half of all human cases in different countries originate very frequently from their mothers, but the source and the time of infection of the mothers are rarely known. This is true for other cases, too.

During the last seven years three major and important verified outbreaks of listeriosis were reported. In 1981 in Nova Scotia coleslaw was the transmitter, in 1985 in Los Angeles a Mexican style soft cheese and in 1983–87 in Canton of Vaud Vacherin soft cheese. In the Boston outbreak in 1983, the epidemiological evidence suggested a similar role of milk but there was no microbiological confirmation [38–41]. Besides these, some other outbreaks have been observed in different countries without verification of their sources, in Slovakia, 1981 [42]; FRG, 1983 [43]; GDR, 1985 and 1986 [44]; Denmark, 1986 [45]; Austria, 1986 [46]; USA, 1987 [47]. It can be supposed that foodstuffs might have played some role in these outbreaks, too. Now, on the basis of the evidences, one can state that foodborne transmission of *L. monocytogenes* may be one of the most probable explanation for many cases of epidemic human listeriosis and this explanation may also be valid for some of the sporadic cases, too. However, in the verified epidemics, the pathogenic agent was transmitted by food, the source of contamination (animals, employees or environment) is not known.

It cannot be accepted that human listeriosis is a soil-borne disease [36]. I am reluctant to accept such a definition that "*L. monocytogenes* is perhaps best considered as an environmental contaminant". Also it seems unadvisable to me to divide listeric infections into two nosological units. This idea was proposed at the Meeting of the WHO Informal Working Group on Foodborn Listeriosis in 1988, but some of the attendees were against it [48].

Virulent listeriae are human and animal pathogenic microorganisms which originate primarily from healthy and ill animals as well as humans. Listeriae can survive and sometimes also multiply in the environment. Foods produced or processed in a contaminated non-hygienic environment may really be contaminated but I think that the most important primary sources of listeriae contaminating raw foods of animal origin (milk, raw milk products, raw meats, fresh raw meat products) are the animals themselves. Hands of the employees and instruments can convey the agent, especially when the hygienic standards are low.

Further studies are necessary to elucidate the realities of epidemiology of listeriosis and the importance of food production and food industry in the spreading of listeriae. For this purpose existence of an effective surveillance system and a reference laboratory in each country is necessary.

A careful consideration is justified, as the predominant serotypes and phage types of *Listeria* strains isolated from foodstuffs and humans have been found somewhat different in France and in the UK, and in my book I wrote about similar differences in cases of those strains which had been isolated from animals and humans in different countries. However, this general observation does not exclude that special single epidemiological connections may exist.

As to the epidemiology of animal listeriosis in different countries we have only a few data and, therefore, it is very difficult to speak about this problem. Animal listeriosis has at least two important aspects. One of them is the direct economic loss in consequence of the disease. The other is the danger of contamination in food production and food industry. The less the number of animal carriers, the lower the probability of production of contaminated raw foods. To prevent listeria infection in animals, there are two not only theoretical possibilities. The first is to elevate the hygienic level of keeping conditions and to feed animals with good quality feeds. I think that SPF animals may have importance in this respect in future. The second may be active immunization and effective bacteriological control. These questions are also open for discussions.

At present it is impossible to eliminate completely all listeriae from all foods. I think that milk and dairy products do not transmit listeriae if they are adequately pasteurized before marketing or processing. In our country milk is pasteurized at 76 °C for 37 s [49]. After this treatment milk will

not contain living listeriae unless of course post-pasteurization recontamination occurs [50]. The problem is more difficult with raw meats and non-heat-treated transformed meat products. Fermentation process may result in decrease or elimination of listeriae in sausages. These questions require more attention and studies, so far no evidence about human listeriosis occurred after consumption of meat products. It is important to emphasize that listeriae can multiply at 4 °C, that is, refrigeration is not a way to protect the quality of foods. They can also survive freezing.

There was a general agreement on the following problems at the meeting of the WHO Informal Working Group on Foodborne Listeriosis [36].

(i) Processed foods, after application of any listericidal process, in intact packages should be free of listeriae.

(ii) Any processed food contaminated with virulent *L. monocytogenes*, should be removed from the market.

(iii) Any category of foods which has been verified to be associated with human listeriosis, should be confiscated.

In our country the Ministry of Health regulates food-hygienic microbiological examinations and qualification of food-stuffs for the Public Health Service and other laboratories involved in this type of work. On the basis of this order food suitable for human consumption may not contain any human pathogenic germ [17].

I hope we shall hear some more about national and international aspects of food and animal trade in connection with listeriosis. I known of restrictions in relation to cheeses in some countries — for example in Switzerland —, to icecream — in the USA —, as well as the cheeses between Common Market countries and the USA, furthermore to seafoods between the USA and Japan as well as Korea.

In the present situation education of medical and catering employees and consumers is very necessary. They should know about the danger of potential presence of listeriae in raw foods, transformed foods and processed foods subsequently handled. Those persons who belong to the high risk groups should consume such foods only after sufficient heat treatment. Also, an important aspect of prevention is the appropriate kitchen hygiene, in order to limit contamination not only with *Listeria* but with *Salmonella* and other microorganisms.

As the methodological questions will be discussed in detail on the Round Table Discussion and several papers deal with genetical problems of listeriae, clinical aspects of listeriosis and the question of treatment, it remains for me to express my thanks for your attention and let me to wish you a successful and useful meeting.

REFERENCES

1. Proceedings of the International Scientific Meeting on the Problems of Listeriosis. Pécs, October, 6–7, 1972. Acta Microbiol Acad Sci Hung **19,** 269 (1972).
2. Woodbine, M. (ed.): Problems of Listeriosis. Proceedings of the Sixth International Symposium. Nottingham, September, 1974. Leicester University Press, Leicester 1975.
3. Ivanov, I. (ed.): Proceedings of the Seventh International Symposium. Varna, September, 1977. National Agroindustrial Union Center for Scientific Information, Sofia 1979.
4. Baquero, F. (ed.): Abstracts Book of VIIIth International Symposium on the Problems of Listeriosis. Madrid, September, 22–27, 1981. ISOPOL, Madrid 1981.
5. Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): Listeriose, Listeria, Listeriosis — 1985–1986. Proceedings of the 9th International Symposium on the Problems of Listeriosis. Nantes, September 17–20, 1985. University of Nantes, Nantes 1986.
6. Rocourt, J.: Thesis. Université de Paris-Sud, Centre D'Orsay, 1987.
7. Goebel, W., Kathariou, S., Kuhn, M., Sokolovic, Z., Kreft, J., Köhler, S., Funke, D., Chakraborty, T., Leimeister-Wächter, M.: Infection **16,** S 143 (1988).
8. Fiedler, F., Seger, J., Schrettenbrunner, A., Seeliger, H. P. R.: System Appl Microbiol **5,** 360 (1984).
9. Lamont, R. J., Petrie, D. T., Melvin, W. T., Postlewaite, R.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): Listeriose, Listeria, Listeriosis — 1985–1986. Proceedings of the 9th International Symposium on the Problems of Listeriosis. Nantes, September 17–20, 1985. University of Nantes, Nantes 1986. p. 41.
10. Ruhland, G. J., Fiedler, F.: System Appl Microbiol **9,** 40 (1987).
11. Leimeister-Wächter, M., Chakraborty, T.: Acta Microbiol Hung **36,** 211 (1989).
12. Mengaud, J., Vicente, M. F., Chenevert, J., Pereira, J. M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J. C., Cossart, P.: Infect Immun **56,** 766 (1988).
13. Datta, A. R., Wentz, B. A., Hill, W. E.: Appl Environ Microbiol **53,** 2256 (1987).
14. Ralovich, B., Ewan, E. P., Emődy, L.: Acta Microbiol Hung **33,** 19 (1986).
15. Pine, L., Weaver, R. E., Carlone, G. M., Pienta, P. A., Rocourt, J., Goebel, W., Kathariou, S., Bibb, W. F., Malcolm, G. B.: J Clin Microbiol **25,** 2247 (1987).
16. Ralovich, B., Forray, A., Mérő, E., Málovics, I.: Zentralbl Bakteriol (A) **214,** 231 (1970).
17. Ralovich, B.: Listeriosis Research; Present Situation and Perspective. Akadémiai Kiadó, Budapest 1984.
18. Ralovich, B. S., Kokryakov, V. N., Mazing, Yu. A., Rotova, G. M., Pigarevskii, V. E.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): Listeriose, Listeria, Listeriosis — 1985–1986. Proceedings of the International Symposium on the Problems of Listeriosis. Nantes, September 17–20, 1985. University of Nantes, Nantes 1986. p. 177.
19. Ralovich, B.: Int J Food Microbiol **8,** 269, (1989).
20. Gaillard, J. L., Berche, P., Sansonetti, P.: Infect Immun **52,** 50 (1986).
21. Leimeister-Wächter, M., Chakraborty, T., Goebel, W.: Ann Microbiol (Paris) A **138,** 252 (1987).
22. Vicente, M., Perez-Diaz, J. C., Baquero, F., Chenevert, J., Groisman, E., Mengaud, J., Moniz Pereira, J., Rauzier, J., Gicquel-Sanzey, B., Cossart, P.: Ann Microbiol (Paris) A **138,** 250 (1987).
23. Gaillard, J. L., Berche, P., Mounier, J., Richard, S., Sansonetti, P.: Infect Immun **55,** 2822 (1987).
24. Farber, J. M., Speirs, J. I.: J Clin Microbiol **25,** 1463 (1987).
25. Stelma, G. N., Reyes, A. L., Peeler, J. T., Francis, D. W., Hunt, J. M., Spaulding, P. L., Johnson, C. H., Lovett, J.: J Clin Microbiol **25,** 2085 (1987).
26. Rocourt, J., Seeliger, H. P. R.: Ann Microbiol (Paris) A **138,** 277 (1987).
27. Rocourt, J., Alonso, J. M., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 359 (1983).
28. Zinn, U., Hof, H., Rocourt, J.: In Ryc, M., Franek, J. (eds): Proceedings of the International Symposium on "Bacteria and the Host", May 14–17, 1985, Prague, Czechoslovakia. Avicenum, Czechoslovak Medical Press, Prague 1986. p. 253.
29. Rocourt, J., Hof, H., Schrettenbrunner, A., Malinverni, R., Bille, J.: Schweiz Med Wochenschr **116,** 248 (1986).
30. McLauchlin, J.: J Appl Bacteriol **63,** 1 (1987).
31. McLauchlin, J., Audurier, A., Taylor, A. G.: J Med Microbiol **22,** 367 (1986).
32. Ralovich, B.: In Kiss, I., Deák, T., Incze, K. (eds): Microbial Associations and Interactions in Food. Akadémiai Kiadó, Budapest 1984. p. 99.
33. Ralovich, B. S.: In Schönberg, A. (ed.): Listeriosis; Joint WHO/ROI Consultation on Prevention and Control. Vet Med Hefte No. **5,** 21 (1987).

34. Schmidt-Wolf, G., Seeliger, H. P. R., Schrettenbrunner, A.: Zentralbl Bakteriol (A) **265,** 472 (1987).
35. WHO, 1987. Report of the WHO Consultation of Prevention and Control of Listeriosis. Berlin (West), December 10–12, 1986. WHO/CDS/VPH/ 87. 69.
36. WHO, 1988. Foodborne Listeriosis. Report of a WHO Informal Working Group, Geneva, February 15–19, 1988. WHO/EHE/FOS/.
37. Ralovich, B., Proksza, Á.: Abstracts of 10th International Symposium on Listeriosis, Pécs, 22–26 August 1988, p. 58.
38. Schlech, III. W. F., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H.; Nicholls, E. S., Broome, C. V.: N Engl J Med **308,** 203 (1983).
39. James, S. M., Fannin, S. L., Agee, B. A., Hall, B., Parker, E., Vogt, J., Run, G., Williams, J., Lieb, L., Salminen, C., Prendergast, T., Werner, S. B., Chin, J.: MMWR **34,** 357 (1985).
40. Bille, J., Glauser, M. P.: Bull Bundesamt Gesundheitswesen **3,** 28 (1988).
41. Fleming, D. W., Cochi, S. L., McDonald, K. L., Brondum, J., Hayes, P. S., Plikaytis, B. D., Holmes, M. B., Audurier, A., Broome, C. V., Reingold, A. L.: N Engl J Med **312,** 404 (1985).
42. Elischerová, K.: Personal communication.
43. Nicolai-Scholten, M. E., Potel, J., Natzschka, J., Pekker, St.: Immun Infekt **13,** 76 (1985).
44. Ortel, S.: Personal communication.
45. Frederiksen, W.: Personal communication.
46. Allerberger, F.: Personal communication.
47. Broome, C. V.: Personal communication.
48. Rothschild, L.: Food Chemical News **30,** 3 (1988).
49. Farkas, Gy., Szakály, S., Ralovich, B.: Abstracts of 10th International Symposium on Listeriosis. Pécs, 22–26 August 1988, p. 59.
50. Mossel, D. A. A.: Personal communication.

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MONITORING FOR *LISTERIA MONOCYTOGENES* IN THE FOOD SUPPLY

R. E. ENGEL

International Scientific Liaison, USDA, FSIS Washington, D. C., USA

During the 1980's, listeriosis emerged as a significant foodborne illness. USDA's Food Safety and Inspection Service (FSIS) began monitoring both cooked and raw products for *Listeria* in 1987. To date, meat and poultry have not been associated with any cases of listeriosis. Outbreaks have been traced to cabbage, soft cheese and other dairy products. Listeriosis is usually manifested as meningitis, meningo-encephalitis, or septicaemia. Although information is scant, the Centers for Disease Control estimate that there are at least, 1600 human cases each year and 415 deaths.

Listeria monocytogenes is more difficult to control than many other foodborne pathogens because it can grow slowly under refrigeration and is somewhat resistant to heat and salt. This is cause for concern because of the growing number of refrigerated ready-to-eat meat and poultry products on the market.

The National Academy of Science study on poultry inspection is one of our basic guides for microbial control. The report noted a complex association between microorganisms on or in poultry at slaughter and associated disease in humans. The report noted that more information about the health risks associated with many microorganisms is needed, but that microbial contamination appears to be the greatest risk area associated with poultry consumption. The report recommended:

— Focus on microbial control where this will reduce human illness;

— More research and epidemiological studies to determine health risks associated with many microorganisms on poultry;

— Identification and monitoring of the critical control points at which known pathogenic organism such as *Salmonella* and *Campylobacter* are introduced into the poultry production system;

RONALD E. ENGEL
International Scientific Liaison, Food Safety and Inspection Service
Washington, D. C. 20250 USA

— Educational programs for producers, food preparers, and persons in all other sectors of the poultry system, including retail labelling on proper handling to prevent illness associated with microbial contamination.

The WHO Informal Working Group report stated that the primary means of transmission to humans of *L. monocytogenes* is through contamination of foodstuffs at any point in the food-chain from source to kitchen. In September 1987, FSIS began to monitor and test for *Listeria* in cooked and roast beef, and cooked corned beef. As of mid July, 1988 of the 421 monitoring samples tested, seven have been positive for *Listeria*. The first sample to test positive was taken on October 14, 1987 from cooked corned beef. Verification samples also tested positive. However, a recall was not necessary because none of the product had reached the public. The second sample to test positive was taken on November 30, 1987 from cooked corned beef. Verification samples for *Listeria* tested negative, indicating that no further action was required.

In January 1988, FSIS began monitoring for *Listeria* in canned, sliced luncheon meats. As of mid July, 120 samples had been tested and all were negative. In April 1988, monitoring for *Listeria* in prosciutto was initiated. On May 6, 1988 a monitoring sample of prosciutto tested positive for *Listeria*. The testing procedure used to detect *Listeria* in cooked, ready-to-eat meat and poultry is as follows. Monitoring samples are collected from a plant's current production, but the product is not withheld from the market. The samples are analyzed in an FSIS laboratory. If the monitoring sample tests positive, the firm is notified so it can rectify the situation immediately. Then, six intact verification samples are collected, frozen, and sent to the nearest FSIS laboratory. Processors are encouraged, but not required, to hold the product until the test results are available. If none of the six verification samples tests positive, no further action is taken. If one or more of the six verification samples test positive, FSIS requests a voluntary recall of the lot represented by the six intact verification samples, if any of the product has entered commerce. In addition, the agency places the plant under an intensive sampling program for five months, testing a variety of products, to assure that the problem has been brought under control.

FSIS began monitoring domestic raw beef for *Listeria* in January 1987 for informational purposes only. As of mid July, 36 of 544 samples had tested positive for *Listeria*. Consumers are told that cooking meat and poultry to the recommended internal temperature should be sufficient to kill any *Listeria* that may be present on the raw product. Standard food safety practices should always be followed.

FSIS has contracted for research to learn more about dry and steam-heat destruction of *Listeria* in meat and poultry, and will join with other US agencies and other nations in providing information to help food handlers prevent the survival and growth of the bacteria. In addition, meat and poultry

organizations are working to develop good manufacturing practices to control *Listeria* in the plant environment.

A major strategy for controlling foodborne listeriosis includes all measures for physical intervention in the transmission of the pathogen. Monitoring and control of processing environmental hazards (HACCP) should become an accepted regimen for all processors intent on controlling foodborne listeriosis.

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LISTERIOSIS, MAN-MADE?

H. P. R. SEELIGER

Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, FRG

Listeriosis has been considered for decades as zoonosis and listed by WHO terminology as such with the understanding that it is a naturally occurring disease with primary spread among animals. This view does, however, not take into account that in many instances animal listeriosis is a food-borne infection, originating from *Listeria monocytogenes* organisms living in soil on decaying vegetables or elsewhere in the environment polluted with faecal matter from which the organism gets access to silage. There it can multiply, when acidification is insufficient. Silage is a product developed by man and has become an indispensable animal feed during the stabling period. Silage-borne infections are frequent in winter and early spring. There may subsequently be epizootic spread among animals and occasionally transmission to man, either directly or via meats, etc. In man the disease would then be classified as zoo-anthroponosis with eventual transmission from man to man in obstetric wards and quite obviously during pregnancy when the fetus is affected. The past few years have, however, made evident that human listeriosis in sporadic cases and in epidemic form is also a foodborne infection, the true dimensions of which require further study. Although listeriosis has been placed into the category of a geonosis, i.e. a soilborne environmental infection, as primary source of human and animal disease, it may secondarily behave like a genuine zoonosis. Obviously the present categories of classification are somewhat insufficient to place listeriosis in one single entity only.

The disease has been observed in man with slowly increasing frequency since the twenties, the first proved case of meningitis having been described in France (1921). Except a few outbreaks of epidemic proportions in the German Democratic Republic and in Northern part of Federal Republic of Germany, practically all reported cases have been sporadic. Major outbreaks in Nova Scotia, Canada, as well as in Massachusetts and in California, USA, during the past few years and recent waves of infections observed in certain areas of Switzerland, Austria and the USA, caused a great deal of concern,

HEINZ P. R. SEELIGER
Institute of Hygiene and Microbiology, University of Würzburg,
Josef-Schneider-Str. 2. Bldg. 17. D-8700 Würzburg, FRG

as they mainly involved milk, dairy products or raw vegetables consumed as salad.

So far the medical literature covers around 10 000 bacteriologically proved cases, but extremely little has become known as to its epidemiology and the pathogenetic processes leading to overt disease.

Obviously, human listeriosis does not differ clinically or histologically from the same disease as it was observed in a wide range of animal species, particularly in domestic animals, but also among free living ruminants such as deer. The similarity of tissue lesions provoked in all susceptible species indicates similar pathways of the infection, which is caused by an obviously non-host-adapted soil bacterium with a surprising pathogenic potential.

When it was discovered that listeriosis in domestic animals predominates during the stabling period with silage as main food, interest was focused on its quality. This resulted in the noteworthy findings that in numerous instances it contained virulent *L. monocytogenes* due to faulty methods of production. As already stated listeriosis in dairy cattle still is a disease often resulting from man-produced food.

Although a similar mode of transmission from listeria-containing food, such as milk, has been suspected since the early fifties in Germany, no proof for this was obtained until only a few years ago, when an outbreak in Minnesota (USA) alerted the public health authorities. Summarizing the accumulated knowledge since then, there is no more any doubt that *L. monocytogenes* reaches man primarily by food via three different routes: (i) milk and dairy products; (ii) meats and raw meat products; (iii) vegetables and salads soiled with contaminated sewage, dirt and faecal matter.

This does not exclude a few other modes of transmission, such as direct contact with listeria-infected animals and their excretions as well as inhalation of dust from contaminated stables — as has been observed among veterinaries and agricultural workers.

Of great importance for both, animals and man, is the transmission during pregnancy from the maternal body to the fetus with resulting fetal wastage, abortion or still-birth. There again a contact infection with infected body fluids is easily possible and may lead to an occasional carrier state in the tonsillar region and in the intestinal tract as well as to cross-infection in obstetric wards.

Obviously the great majority of individuals ingesting the organisms will not come down with apparent clinical infection, regardless whether or not prevention is tried. This, of course, does not exclude for instance enteritic episodes, which so far have never been investigated as to possible listeric infections. The hypothesis of clinically inapparent infection does not necessarily indicate that the ingested *L. monocytogenes* cells are little or not virulent or only present in numbers too small for causing infection. It may be more

likely that the individuals in contact with them, are quite resistant to infection or even immune due to previous exposure with resulting formation of T cells reactive with other Gram-positive bacteria, as recently reported by Prof. Kaufmann from Ulm (FRG).

The alarming human outbreaks and findings in various countries seem to indicate that the main entry of *L. monocytogenes* into man is the oral route by means of listeria-containing food. It may well be assumed that a normal production of gastric acid as essential part of digestion would kill most listeria-cells due to their sensitivity to pH-levels lower than 3.5. That this will not prevent periods of carrier-state with faecal excretion of the organisms, has been proved beyond doubt by reports from the Netherlands on laboratory personnel and during a prospective study of pregnant women in the Federal Republic of Germany, recently also in surveys among workers in French slaughter-houses.

For more or less unknown reasons, *L. monocytogenes* will, however, invade the human body, leading occasionally to severe clinical syndromes, these including meningitis, septicaemia and abortion. Nevertheless, listeric infection will usually pass rather unnoticed. It is unknown and a matter of conjecture whether the uptake of live listeria-cells will lead to local inflammation of the oro-pharyngeal region or in the intestine with resulting syndromes which may be sore throat, flu-like or characterized by enteritis. It has to be admitted that presently no clear view exists as to what happens along the mucous membranes at the primary site of infection. One may speculate that certain structures on the surface of listeria-cells seem to favour the adherence or penetration into epithelial cells or defence cells with resulting propagation. In this context white blood cells and primarily monocytes appear on the local scene, being attracted by lipids of the listeria-cells which have been analyzed and called monocytosis-producing agent (MPA).

This is why immunologists for their studies make use of *L. monocytogenes* cells if they want to obtain monocytes in the peritoneal washings of listeria-infected rodents. According to recent observations from France, this attraction of monocytes may perhaps be the decisive step for the further development of severe forms of listeriosis, because such reticulo-endothelia-cells will sometimes fail to kill the phagocytosed organisms (as they should), but rather carry them to other parts of the body. It is known that listerial-cells are not susceptible to natural killer agents as contained in the blood, nor to administered antibiotics whilst they are contained in such cells. This holds true also for immunosuppressed individuals, be it on account of natural disease, of application of corticosteroid therapy or of progressed age or combinations of them.

The presentation of *Listeria* organisms to T cells in normal individuals would start a process which will finally lead to resistance or even immunity

to *Listeria* and serologically related organisms, and vice versa. Thus only under special circumstances *L. monocytogenes* will cause severe or fatal clinical syndromes after it has crossed the local defence barriers during an endogenous or exogenous process, leading to septicaemia or meningoencephalitis.

The question arises whether and to what extent *L. monocytogenes* will give rise to the development of antibodies of various nature to cope with or to indicate present or past listeria-infection. Many individuals, beginning in the earliest childhood, give evidence of IgM- and IgG-serum-antibodies reactive with *Listeria* somatic (O) and flagellar (H) antigens. Due to cross-reactions between the O antigens of *Listeria* and Gram-positive pathogenic and non-pathogenic cocci, it is, however, questionable whether such *Listeria* O antibodies are really specific for and indicative of this species — even if they can be demonstrated after appropriate absorption procedures with staphylococci and enterococci. On the other hand, H antibodies against *Listeria* seem to be much more specific and thus pointing to previous or past infection.

Clinical experience has shown that even in bacteriologically proved cases, O or H antibodies may not appear in the serum of the patients, perhaps as consequence of early chemotherapy preventing antibody formation. It remains, therefore, open whether the respective antibodies as found among 30 to 40% of the examined individuals, are really a result of previous listeric infection. If this were true, one would have perhaps a parameter to indicate a state of immunity or at least increased resistance, as observed with listeria-reactive T cells.

At present, all this remains essentially a matter of conjecture. Extended scientific studies are indispensable, in order to evaluate the state of susceptibility of human beings against listeric infection and to provide a reliable diagnostic test.

Moreover, it appears that the haemolysin of *L. monocytogenes* — which is different from the haemolysin of *Listeria ivanovii* and probably also of *Listeria seeligeri* — plays an important role in the process of invasion and subsequent virulent action. This haemolysin is very closely related to streptolysin-O, again making evaluation of antibody-titers extremely difficult.

The possibility of cross protection originating from streptococcal infections can thus not be denied.

Only very limited experience indicates that killed listeria-cells will lead to cutaneous reactions, the interpretation of which is entirely open and hypothetical at this time. The present state of ignorance concerning both, the genesis and development of lesions in the body due to *L. monocytogenes* and the absence of reliable and recognized tests to determine the state of immunity or susceptibility, are the principal reasons of the uncertainty among the medical profession to what extent human health and human life is endangered

by *L. monocytogenes*, although there can be no doubt from available scientific evidence that, regardless the origin, all organisms belonging to *L. monocytogenes* have to be considered potential pathogens.

A last entirely unsolved problem is the infectious dose, i.e. the minimal amount of pathogenic *L. monocytogenes* cells to cause infection in the various age groups as well as in the healthy or predisposed individuals.

Returning to the question, posed in the title of this presentation "Listeriosis, man-made?" experience with animal feed and new knowledge accumulated only in the eighties leave little doubt that only a small fraction of the clinical cases among animals and men are the result of what may be called a natural and almost unavoidable infection resulting from ingestion of raw feed or food contaminated with soil-borne bacteria. The greater part of the observed clinical cases seems likely to be the result of alimentation with man-made food stuff. So far the production of all sorts of food took place without any consideration of *Listeria* as a possible contaminant of milk-borne products, of uncooked meat and meat-products, of certain types of fresh vegetables and salads, but also of seafood (such as shrimps) and of many convenience foods with a long shelf-life. The unique properties of *L. monocytogenes* to grow at low temperatures used to keep food fresh, to tolerate well elevated salt concentrations and the failure to produce any offensive odours, are extremely well suited to escape even highly critical monitoring of such food. It thus appears that progress in civilized and highly developed communities has actually greatly contributed to make listeriosis a human and veterinary public health problem. It cannot be just a coincidence that red-smear cheeses as produced in wide parts of Europe, have been found more frequently to contain *L. monocytogenes* on their surfaces than cheeses from sheep milk in the Near East with only about 1% listeric contamination. The almost exclusive contamination of the surfaces (crust, rind) of such cheeses indicates spread of the organisms in the later stages of manufacturing. The extremely frequent contamination by *L. monocytogenes* of deep-frozen poultry-meat and so-called tatar pork likewise points to serious hygienic faults in the handling and manufacture of such products. The modern methods of refrigeration with the prolonged shelf-life of convenience — and perishable food favour in a unique fashion not only the survival, but also the growth of *L. monocytogenes*, eventually to levels endangering human and animal health.

With all this in mind, one can dare to say that listeriosis in its greater part is a man-made disease. This has been clearly recognized by the World Health Organization which in one of its most recent reports calls for combined efforts of all concerned to minimize wherever possible the present risk by appropriate methods at all levels, although it must be assumed that the naturally occurring listeria-infections will never be eradicated.

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TAXONOMIC POSITION OF *LISTERIA* STRAINS

DOROTHY JONES

Department of Microbiology, University of Leicester, Leicester, UK

Bacterial taxonomy is the process of classifying bacteria into orderly groups or taxa on the basis of as much information as possible. These groups are then named by an internationally agreed method [1] for purposes of communication. Good, stable classification and nomenclature are essential for bacteriological work of all kinds. All biological classifications are subject to change as new information becomes available, but bacterial classifications have been particularly unstable because of problems peculiar to these organisms; paucity of morphological detail, the virtual absence of useful fossil records and the relatively recent development of methods to study the bacterial genome. Thus, bacterial classifications are, in the main, phenetic (based on the phenotypic properties of organisms living today), not phylogenetic (based on evolutionary evidence).

The marked improvements that have taken place in the construction of bacterial classifications in the last twenty years are due mainly to the development of new techniques for investigating the properties of the organisms, especially macromolecular composition and structure, and to the use of computers for the handling and analysis of the plethora of information derived from comprehensive taxonomic studies. These studies are leading to the production of stable phenetic classifications [2] and are also providing the basis for a phylogenetic classification [3]. Not surprisingly, there is very frequently a lack of congruence between the older and newer phenetic classfi-. cations and between these and the emerging phylogenetic classifications. This is especially so in the case of the older phenetic classifications. This discrepancy is now being addressed and active efforts are being made to reconcile phenetic and phylogenetic classifications of bacteria [4]. The aim is to produce a good, scientific classification with a stable nomenclature that reflects the evolutionary processes and pathways that have resulted in the emergence of contemporary bacterial forms.

In the case of the listeriae the results of numerical taxonomic, chemical

DOROTHY JONES
Department of Microbiology, University of Leicester
P. O. Box 138, University Road, LEI 9HN Leicester, UK

and nucleic acid hybridization and sequencing studies in particular have led both to changes in the composition of the genus *Listeria* and in our perception of the relatedness of the genus to other groups of Gram-positive, rod-shaped bacteria. However, not all the problems relating to a good, stable, scientific classification of the listeriae have been resolved. In this contribution the current classification and nomenclature of the bacteria will be reviewed.

Taxonomy of the genus Listeria

The genus *Listeria* [5] was created for the Gram-positive, catalase positive, rod-shaped bacteria isolated, described and named "*Bacterium monocytogenes*" by Murray et al. [6]. In the past twenty years the genus has been expanded from containing only the type species, *Listeria monocytogenes*, to contain, in addition, *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria ivanovii*, *Listeria grayi*, *Listeria murrayi* and *Listeria denitrificans* [7]. Although all eight species were validly named, these authors chose to treat the last three as species incertae sedis. *L. denitrificans* was so designated because while it had been recognized for many years that these bacteria were not members of the genus *Listeria*, they could not be placed with confidence in any other described taxon (see [7], for further discussion and references). Recently the results of 16S rRNA cataloguing studies have provided unequivocal evidence for the reclassification of *L. denitrificans* in a new genus *Jonesia* as *Jonesia denitrificans* [8].

L. grayi and *L. murrayi* were treated as incertae sedis by Seeliger and Jones [7] because of the conflict between the interpretation of the results of numerical taxonomic [9—13] and chemical [13—16] studies on the one hand, and DNA-DNA hybridization studies [10, 17, 18] on the other. The results of the numerical taxonomic and chemical studies all indicated that *L. grayi* and *L. murrayi* were phenotypically members of the genus *Listeria* but the results of the DNA-DNA hybridization studies supported the suggestion of Stuart and Welshimer [10] that these bacteria be transferred to a new genus for which they proposed the name "Murraya" (see [7] for fuller discussion). This problem has been resolved by the recent results of 16S rRNA cataloguing studies [19] which indicated that *L. murrayi* is closely related on this criterion (similarity coefficient 0.73) to *L. monocytogenes*. Consideration of their results together with the close phenotypic similarity, referred to above, between *L. murrayi* and *L. grayi* and the other species of the genus *Listeria* led these workers to propose that *L. murrayi* and *L. grayi* should be retained in the genus *Listeria* [19]. More recent chemical and numerical taxonomic studies [20, 21] also support this view. Thus, currently the genus *Listeria* comprises seven validly named species, *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi* and *L. murrayi*.

Intrageneric composition of the genus Listeria

The genus is a well circumscribed taxon (see Seeliger and Jones [7], for most references) containing mesophilic, Gram-positive, non-sporing, non-mycelial forming, motile rods that grow aerobically and anaerobically and produce mainly L(+) lactic acid from the fermentative metabolism of glucose. All are catalase-positive and produce cytochromes [21, 22]. Exogenous citrate is not utilized. The cell wall contains a directly cross-linked peptidoglycan based upon mesodiaminopimelic acid (variation Aly of Schleifer and Kandler [23]), associated with teichoic acids; lipoteichoic acids are present [24]. Mycolic acids are not present. The long chain fatty acids consist predominantly of straight chain saturated, *anteiso-* and *iso*-methyl-branched chain types. The major fatty acids are 14-methylhexadecanoic (anteiso-$C_{17:0}$) and 12 methyl-tetradecanoic (anteiso-$C_{15:0}$). Menaquinones as the major respiratory quinones; the major menaquinone contains seven isoprene units (MK-7). The mol % G + C content of the DNA is 36–42 (T_m).

A detailed account of the intrageneric classification of the genus and the differentiation of the seven species is given by Rocourt (this symposium) and will not be dealt with here. There are, however, two areas that justify some comment. The current classification of strains, formerly all included in the one species *L. monocytogenes sensu lato*, as *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri and L. ivanovii* is based mainly, but not entirely, on the results of DNA-DNA hybridization studies [18]. Phenotypically, all the taxa except possibly *L. ivanovii*, are very similar [7, 21, 24–28]. Indeed, as noted by Feresu and Jones [21], although five clusters were recovered among the listeriae in their numerical taxonomic study *(L. seeligeri* and *L. welshimeri* were not included) all exhibited 87% similarity and in the absence of the genomic data of Rocourt et al. [18] it would have been difficult to regard the taxa as separate species; rather, the results would have indicated subspecies. However, it has been proposed recently [4] that — "The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less ΔT_m. Both values must be considered". On these criteria the strains studied by Rocourt et al. [18] represent at least five species. A recent electrophoretic study of strains of *L. monocytogenes*, *L. innocua*, *L. seeligeri* and *L. ivanovii* [29] indicated that all the species were genetically dissimilar and also indicated that the population of *L. monocytogenes* studied could be separated into two groups. Of interest in this context is that two distinct groups of *L. monocytogenes* strains were recovered in the study of Feresu and Jones [21] but, as noted previously, these authors would not, on the basis of their results alone, have considered them as distinct species but as subspecies.

More work must be done to search for additional phenotypic characters

studies [9, 39, 40] all indicated that the genus *Listeria* was most closely related to distinguish the five taxa *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*. Until this is done it would be unwise to name further species. When only a few phenotypic characters are available for differentiation errors can ensue for a variety of reasons and there is always the problem of plasmid encoded characters that can be lost or gained. Haemolysis is a particularly important character for differentiation and it is known that non-haemolytic variants of *L. monocytogenes* occur, if rarely [30].

The other area of uncertainty is the relationship of *L. grayi* and *L. murrayi* to each other. As noted earlier, Rocourt et al. [19] have proposed that both taxa be retained in the genus *Listeria*, but made no comment on whether they should be regarded as two distinct species or subspecies. The results of numerical taxonomic [12, 21], chemical [20, 27] and DNA-DNA hybridization studies [10, 17] indicate that *L. grayi* and *L. murrayi* are sufficiently closely related to be considered as subspecies of one species [10, 12, 21, 31]. In this case the name *L. grayi* [32] would take precedence and the subspecies would be designated *L. grayi* subspecies *grayi* and *L. grayi* subspecies *murrayi*.

Intergeneric placement of the genus Listeria

In the seventh edition of Bergey's Manual [33] the genus *Listeria* was placed together with *Erysipelothrix* in the family *Corynebacteriaceae* the type pecies of which was the genus *Corynebacterium*. This placement was undoubtedly one of convenience, made on the basis that the listeriae were Gram-spositive, non-sporing, catalase-positive rods. The placement of *Erysipelothrix*, a catalase-negative genus, close to *Listeria* is not surprising because several early workers had argued a close relatedness between the two genera on the basis of information available at that time and Wilson and Miles [34] stated that they treated *Erysipelothrix* and *Listeria* as separate genera very reluctantly because, in their opinion the differences between the two taxa did not warrant separate generic status.

Murray [35] thought that *Listeria* might be placed in a family *Brevibacteriaceae*. Jones et al. [22] noted a similarity between *Listeria* and the description given by Thornley and Sharpe [36] for some Gram-positive rods isolated from chicken meat. It is of interest that some of the bacteria described by Thornley and Sharpe [36] have now been classified as *Brochothrix thermosphacta* [21] and others as a new genus, *Carnobacterium* [37].

The earliest attempt to quantify the relatedness of *Listeria* to other Gram-positive bacteria was the numerical taxonomic study of Sneath and Cowan [38]. This study indicated that *Listeria* was more closely related to the streptococci and staphylococci than to *Corynebacterium diphtheriae*. However, since the study was based on old laboratory data these authors felt unable to recommend any taxonomic changes. Later numerical taxonomic

to the genera *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Kurthia* and especially to *Brochothrix* (at that time named *Microbacterium thermosphactum)*. These results together with the results of cell wall analyses [23, 41] led to the removal of *Listeria* from the family *Corynebacteriaceae*. In the eighth edition of Bergey's Manual [42] *Listeria* was listed together with *Erysipelothrix* and *Caryophanon* as "genera of uncertain affiliation" associated with the family *Lactobacillaceae*. *Brochothrix* and *Kurthia* were, however, treated with the Coryneform Group of bacteria.

Since that time all the evidence from further numerical taxonomic [11, 12, 21] and cell wall [15, 16, 20, 24, 43, 44] studies, together with the results of other chemical analyses [13, 14, 21, 45–48] has not only reinforced the distinctness of the genus *Listeria* but also confirmed the results of earlier studies that indicated that the genus is related to the lactic acid bacteria, the genera *Bacillus* and *Kurthia* and closely related to the genus *Brochothrix* [7, 49]. All these genera contain DNA with a mol % G + C content of less than 55 and are members of the *Clostridium—Lactobacillus—Bacillus* branch of the Gram-positive bacterial phylogeny constructed by Stackebrandt and Woese [50] on the basis of 16S rRNA cataloguing studies. Analysis of the 16 S rRNA of *L. monocytogenes* and *Brochothrix thermosphacta* [51] indicated that both species were specifically, though moderately related, and formed one of several sublines within the *Bacillus—Lactobacillus—Streptococcus* cluster of the "Clostridium" sub-branch of the Gram-positive bacteria. The later, similar studies of Rocourt et al. [19] also showed that both *L. monocytogenes* and *L. murrayi* were closely related to *Brochothrix thermosphacta*. Although the genera *Listeria* and *Brochothrix* are closely related they are sufficiently distinct [7, 8, 21, 49, 51] to be regarded as separate genera for the present. There is as yet, no good scientific definition of a genus [4].

REFERENCES

1. Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R., Clark, W. A. (eds): International Code of Nomenclature of Bacteria, 1976 Revision. American Society for Microbiology, Washington D. C. 1975.
2. Staley, J. T., Krieg, N. R.: In Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holt, J. G. (eds): Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins, Baltimore 1986. pp. 965–968.
3. Woese, C. R.: Microbiol Rev **51,** 221 (1987).
4. Wayne, L. G., Brenner, D. J., Colwell, R. R. et al.: Int J System Bacteriol **37,** 463 (1987).
5. Pirie, J. H. H.: Nature **145,** 264 (1940).
6. Murray, E. G. D., Webb, R. A., Swann, M. B. R.: J Path Bacteriol **29,** 407 (1926).
7. Seeliger, H. P. R., Jones, D.: In Sneath, P. H. A., Mair, N. J., Sharpe, M. E., Holt, J. G. (eds): Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins, Baltimore 1986. pp. 1235–1245.
8. Rocourt, J., Wehmeyer, U., Stackebrandt, E.: Int J System Bacteriol **37,** 266 (1987).
9. Stuart, M. R., Pease, P. E.: J Gen Microbiol **73,** 551 (1972).
10. Stuart, S. E., Welshimer, H. J.: Int J System Bacteriol **24,** 177 (1974).
11. Jones, D.: J Gen Microbiol **87,** 52 (1975).
12. Wilkinson, B. J., Jones, D.: J Gen Microbiol **98,** 399 (1977).

13. Jones, D., Feresu, S. B., Collins, M. D.: In Courtieu, A-L., Espaze, E. P., Reynaud, A. (eds): Listeriose—Listeria—Listeriosis. Proceedings of the International Symposium on the Problems of Listeriosis. Université de Nantes, Nantes 1985–86. pp. 29–34.
14. Collins, M. D., Jones, D., Goodfellow, M., Minnikin, D. E.: J Gen Microbiol **111,** 453 (1979).
15. Fiedler, F., Seger, J.: Syst Appl Microbiol **4,** 444 (1983).
16. Fiedler, F., Seger, J., Schrettenbrunner, A., Seeliger, H. P. R.: Syst Appl Microbiol **5,** 360 (1984).
17. Stuart, S. E., Welshimer, H. J.: Int J System Bacteriol **23,** 8 (1973).
18. Rocourt, J., Grimont, F., Grimont, P. A. D., Seeliger, H. P. R.: Curr Microbiol **7,** 383 (1982).
19. Rocourt, J., Weymeyer, U., Cossart, P., Stackebrandt, E.: Int J System Bacteriol **37,** 298 (1987).
20. Ruhland, G. J., Fiedler, F.: System Appl Microbiol **9,** 40 (1987).
21. Feresu, S. B., Jones, D.: J Gen Microbiol **134,** 1165 (1988).
22. Jones, D., Sneath, P. H. A., Woodbine, M.: Proceedings of the 3rd International Symposium on Listeriosis. Bilthoven, The Netherlands 1966. pp. 161–164.
23. Schleifer, K. H., Kandler, O.: Bacteriol Rev **36,** 407 (1972).
24. Fiedler, F.: Infection **16,** Suppl. 2. 592 (1988).
25. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 65 (1983).
26. Rocourt, J., Catimel, B.: Zentralbl Bakteriol (A) **259,** 489 (1985).
27. Lamont, R. J., Petrie, D. T., Melvin, W. T., Postlethwaite, R.: In Courtieu, A-L., Espaze, Reynaud, A. E. (eds): Listeriose—Listeria—Listeriosis. Proceedings of the International Symposium on the Problems of Listeriosis. Université de Nantes, Nantes 1985–86. pp. 41–46.
28. McLauchlin, J.: J Appl Bacteriol **63,** 1 (1987).
29. Kressebuch, H., Aeschbacher, M., Bille, J., Bannermann, E., Rocourt, J., Piffaretti, J. C.: Abstracts of the Annaul Meeting of the American Society for Microbiology. 1988. D-65. p. 82.
30. Pine, L., Weaver, R. E., Carlone, G. M., Pienta, P. A., Rocourt, J., Goebel, W., Kathariou, S., Bibb, W. F., Makolm, G. B.: J Clin Microbiol **25,** 2247 (1987).
31. Seeliger, H. P. R., Schrettenbrunner, A., Pongratz, G., Hof, H.: Zentralbl Bakteriol (A) **252,** 176 (1982).
32. Errebo-Larsen, H., Seeliger, H. P. R.: Proceedings of the Third International Symposium in Listeriosis. Bilthoven, The Netherlands 1966. pp. 35–39.
33. Breed, R. S., Murray, E. G. D., Smith, N. T.: Bergey s Manual of Determinative Bacteriology. (7th ed.) Williams and Wilkins, Baltimore 1957.
34. Wilson, G. S., Miles, A. A.: Topley and Wilson's Principles of Bacteriology and Immunity. (5th ed.) Vol. 1. Arnold, London 1964. pp. 528–535.
35. Murray, E. G. D.: Second Symposium of Listeric Infection. Artcraft Printers, Bozeman 1962. p. 72.
36. Thornley, M. J., Sharpe, M. E.: J Appl Bacteriol **22,** 368 (1959).
37. Collins, M. D., Farrow, J. A. E., Phillips, B. A., Feresu, S., Jones, D.: Int J System Bacteriol **37,** 310 (1987).
38. Sneath, P. H. A., Cowan, S. T.: J Gen Microbiol **19,** 551 (1958).
39. Davis, G. H. G., Newton, K. G.: J Gen Microbiol **56,** 195 (1969).
40. Davis, G. H. G., Fomin, L., Wilson, E., Newton, K. G.: J Gen Microbiol **57,** 333 (1969).
41. Cummins, C. S., Harris, H.: J Gen Microbiol **14,** (1956).
42. Seeliger, H. P. R., Welshimer, H.J.: In Buchanan, R. E., Gibbons, N. E. (eds): Bergey's Manual Determinative Bacteriology. Williams and Wilkins, Baltimore 1974. pp. 593–596.
43. Srivastava, K. K., Siddique, I. H.: Infect Immun **7,** 700 (1973).
44. Kamisango, K., Saiki, I., Tanio, Y., Okamura, H., Araki, Y., Sekikawa, I., Asuma, I., Yamamura, Y.: J Biochem **92,** 23 (1982).
45. Kosaric, N., Carroll, K. K.: Biochim Biophys Acta **239,** 428 (1971).
46. Tadyon, R. A., Carroll, K. K.: Lipids **6,** 820 (1971).
47. Shaw, N.: Adv Appl Microbiol **17,** 63 (1974).
48. Collins, M. D., Jones, D.: Microbiol Rev **45,** 316 (1981).
49. Kandler, O., Weiss, N.: In Sneath, P. H. A., Mair, N. J., Sharpe, M. E., Holt, J. G. (eds): Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore 1986. pp. 1209–1234.
50. Stackebrandt, E., Woese, C. R.: In Carlile, M. J., Collins, J. F., Moseley, B. E. B. (eds): Molecular and Cellular Aspects of Microbial Evolution. Cambridge University Press, Cambridge 1981. pp. 1–31.
51. Ludwig, W., Schleifer, K. H., Stackebrandt, E.: FEMS Microbiol Letts **25,** 199 (1984).

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Special lectures

1. EXPERIMENTAL INFECTION

BIOLOGICAL AND IMMUNOLOGICAL PROPERTIES OF AVIRULENT STRAIN OF *LISTERIA INNOCUA*

EVA MENČÍKOVÁ, M. MÁRA, Z. MIKOVÁ and J. PRŮCHOVÁ

Institute of Medical Microbiology and Immunology, Charles University School of Medicine, and SUKL, Prague, Czechoslovakia

The strain isolated by Dr. J. H. Welshimer from plants has antigenic formula V (VI) IX; XV; XI; AB, C — serovar 6a, is non-haemolytic, produces lipase, and toxic factor Ei, is avirulent for adult mice, but causes encephalitis in sucklings. In organs of intravenously injected mice the strain persists and multiplies for 1–3 weeks. The protective effect against listerial infections in mice of this strain administered 2–14 days before challenge is dose depending. After 3 weeks induces resistance of guinea pigs to infection with *Mycobacterium tuberculosis* H37Rv measured by spleen weight and Feldman index. The hypersensitivity induced in animals is detectable by factor Ei and PPD or OT tuberculins using MIF method. A suspension of living cells of this strain injected intraperitoneally causes resistance *to Mycobacterium kansasii* in mice, measured by inhibition of loss of weight and decrease of the number of bacillus in their lungs.

The immunization of animals against listeriosis is of practical significance. For this treatment is available the use of viable suspensions of avirulent strains of *Listeria*. Hasenclever and Karakawa [1] and independently Osebold and Sawyer [2] have described the possibility of inducing the immunity against listeriosis by intradermal or subcutan administration of very low doses of virulent *Listeria* strains. This immunity is increased by repetition of immunization. The applicaton of the strain Welshimer, which is, as we have demonstrated, practically without any pathogenic effects, is possible in relatively high doses.

Materials and methods

For studies on biological and immunological properties of avirulent listeriae we used a strain originally isolated by Dr. J. W. Welshimer from plants supplied to us by Dr. Elischerová. The antigenic structure was established by H. P. R. Seeliger as follows: O antigens V, (VI), IX, XV, XI; H antigens AB, and C. The strain belongs to serovar 6a of the species *Listeria innocua*. Strain Welshimer is non-haemolytic. Production of lipase was demonstrable on nutrient agar supplemented with Tween 80. The strain is not virulent for adult mice even in doses of 10^9 c.f.u. administered intraperitoneally or intravenously.

EVA MENČÍKOVÁ, M. MÁRA, Z. MIKOVÁ
Institute of Medical Microbiology and Immunology, Charles University School of Medicine
Studničkova 7, 128 00 Prague 2, Czechoslovakia

J. PRŮCHOVÁ
SÚKL, Prague, Czechoslovakia

Results and discussion

After intravenous administration of an LD_{50} analogous to that of the virulent strain of *Listeria monocytogenes* Bratislava 1, after 4 h strain Welshimer accumulated in the spleen, and to a lesser degree in the liver and persisted in both organs for 7 days (Fig. 1). Exceptionally it was detectable in some animals in the liver or brain after 21 days using the method of enrichment of homogenized aliquots of tissue at 4 °C. In organs of animals infected with *L. monocytogenes* histological examination revealed the usual development of characteristic listerioms in liver with focuses of necrosis in spleen (after 4 days) healing in 7–11 days. Strain Welshimer induces the formation of mixed nodes (leucocytes and round cells) from 1–2 days after infection in the liver and listeriae were present in the Kuppfer cells. The neurotropic activity of strain Welshimer was proved in suckling mice [3]. Clinical symptoms of encephalitis were apparent usually from 5 days after i.c. innoculation of 3×10^4–-3×10^6 c.f.u. Histological examination revealed a non-purulent predominantly periventricular situated encephalitis with destruction of the ependyma of lateral ventricles in advanced cases. Multiplication of listeriae was demonstrated even in the cytoplasm of macrophages of the stroma of plexus chorioideus [4]. Antilisterial effect of the suspension of this strain administered intraperitoneally is demonstrated in Fig. 2 showing the time dependence. After the administration 14 to 2 days before challenge the resistance of mice was significant, the administration one day after infection caused an increased lethality. The immunity remained unchanged for three weeks [5].

The infection induced by sublethal doses of *Mycobacterium kansasii* in mice is characteristic by decreased weight of animals between 5 to 25 days

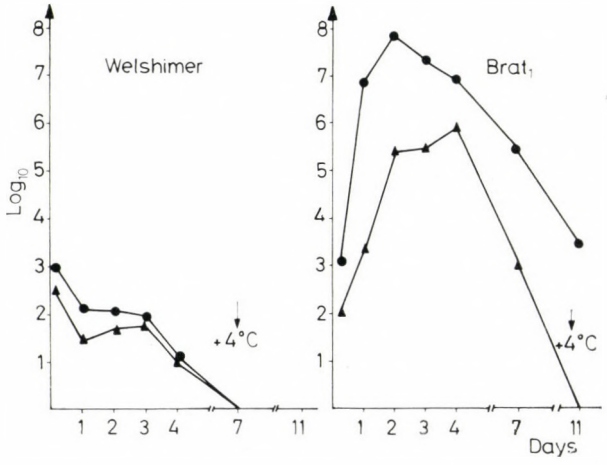

Fig. 1. Number of c.f.u. of *L. monocytogenes* (Brat. 1) and *L. innocua* (Welshimer) in 100 mg of tissue. ▲ liver, ● spleen

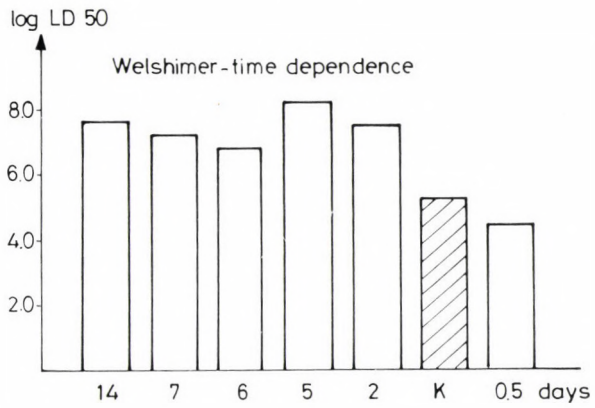

Fig. 2. Antilisteric effect of *L. innocua* (Welshimer) on mice. Time dependence — days of administration

after challenge. The animals were infected with 0.25 mg per mouse intravenously. After intraperitoneal administration of 10^6 c.f.u. of *L. innocua* strain Welshimer per mouse 5 days before challenge with *M. kansasii* significantly inhibited the weight decrease between 12 to 19 days, but the number of mycobacteria in the lungs was not influenced (Fig. 3). After repeated low doses of *L. innocua*, this inhibition was demonstrable 26 to 33 days after infection and between 19 to 33 days after challenge the number of mycobacteria in the lungs significantly diminished (Fig. 4).

Strain Welshimer caused in the experimental tuberculosis model a significant resistance measured by decreased values of spleen weight and Feldman

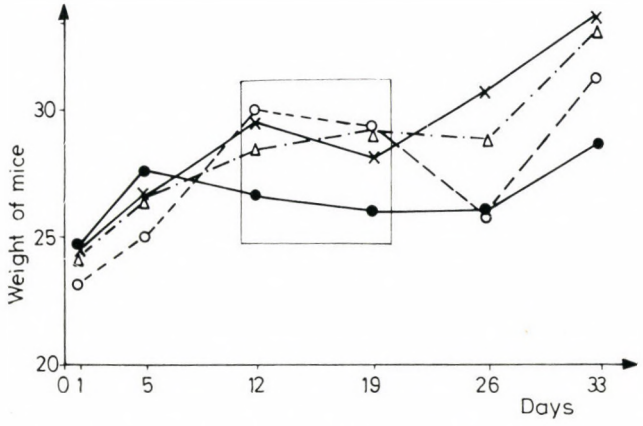

Fig. 3. Weight of mice during the infection of *M. kansasii* after immunization with *L. innocua* (Welshimer). ×——× control; △–·–△ strain Welshimer; ●—● *M. kansasii;* ○---○ strain Welshimer + *M. kansasii*

index (Fig. 5). Strain Welshimer was administered intraperitoneally in doses 10^8 or 10^9 c.f.u. per mouse. The Feldman index represents tuberculous changes in the lungs, liver, spleen and lymph nodes [6]. Maximal infestation was expressed as 100 units. The level of resistance depended on dose and time of application. Repeating high doses of *Listeria* had only minimal effect [7].

Administration of the strain Welshimer (3×10^8 c.f.u.) three weeks before transplantation of sarcoma 180 to the mice induced a protection measured by lethality. The same effect was exerted by strain Welshimer on Krebs 2 carcinoma. The effect is presented in Fig. 6 as the survival time [8].

The immunization of guinea pigs or rabbits caused a delayed hypersensitivity. This phenomenon was detectable by the method of MIF using listerial

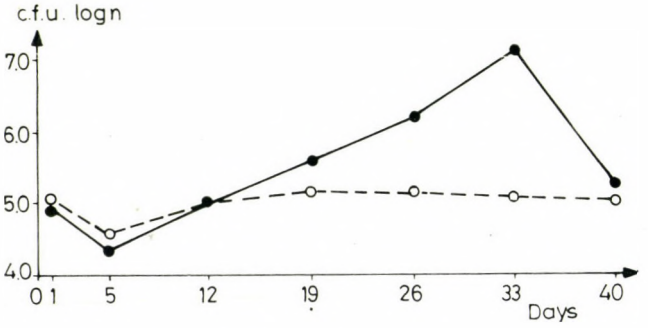

Fig. 4. Number of c.f.u. of *M. kansasii* in lung of mice. ●—● *M. kansasii;* ○---○ strain Welshimer + *M. kansasii*

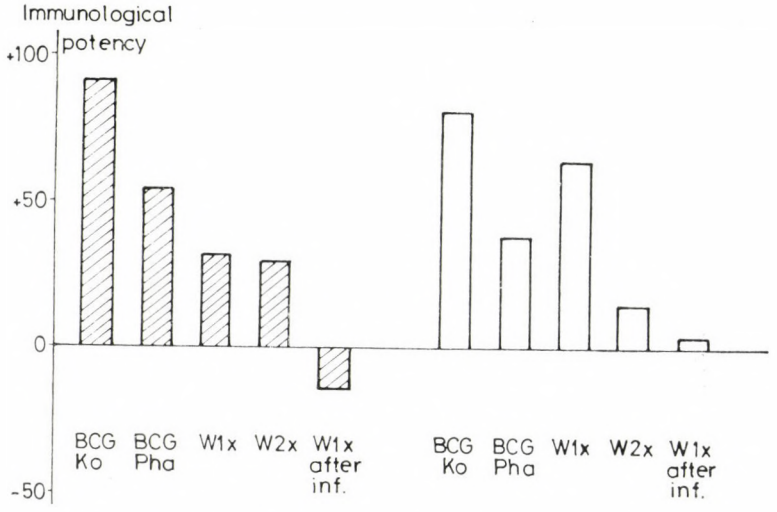

Fig. 5. Immunological potency of BCG strains and *L. innocua* (Welshimer) in the model of experimental tuberculosis on guinea pigs. Left part, weight of spleens; right part, Feldman index

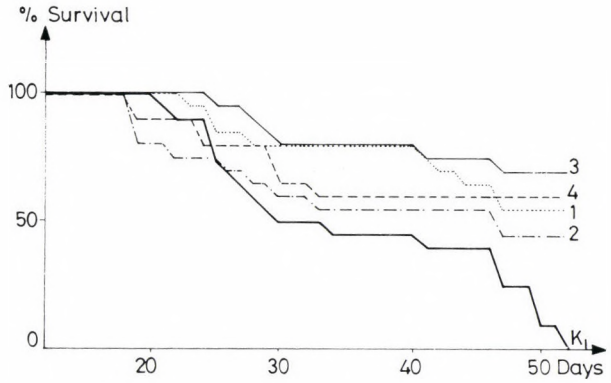

Fig. 6. Immunostimulation of mice against Krebs 2 carcinoma expressed as percentage of survival time. K_1 control (Krebs 2-carcinoma only); 1, BCG Prague 725; 2, *L. innocua* (Welshimer); 3, *L. innocua* (Welshimer) and BCG lipids; 4, BCG lipids. Immunostimulation was made 8 weeks (BCG) or 3 weeks (listeriae) before transplantation of tumour

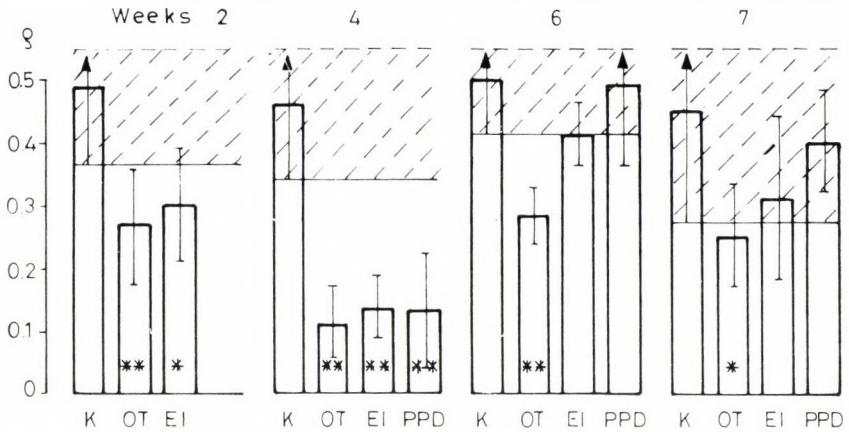

Fig. 7. Determination of delayed hypersensitivity of guinea pigs induced by *L. innocua* (Welshimer) and detected with listerial factor Ei, PPD or old tuberculin (OT)

factor Ei, PPD or old tuberculin (OT) as antigens. The hypersensitivity persists from the second to seventh week. This in vitro method is not so specific as the skin test (Fig. 7) [9]. The precipitation in Ochterlony's procedure is specific for two different strains. After hydrolysis of the suspension of the strains for 1 h with 0.1 N acetic acid, this strain specificity has been lost as in the case of Gram-negative bacteria.

All the immunizing and immunomodulating qualities of the avirulent strain Welshimer and lack of the pathogenic properties presume this strain for using as a good viable vaccine for immunization of sheep.

REFERENCES

1. Hasenclever, H. F., Karakawa, W. W.: J Bacteriol **74,** 584 (1957).
2. Osebold, J. W., Sawyer, M. T.: J Immunol **78,** 262 (1957).
3. Patočka, F., Menčíková, E., Seeliger, H. P. R., Jirásek, A.: Zentralbl Bakteriol (A) **243,** 490 (1979).
4. Menčíková, E., Patočka, F., Jirásek, A., Mára, M.: Folia Microbiol **21,** 237 (1976).
5. Mára, M., Miková, Z., Menčíková, E., John, C.: Čs Epidemiol **27,** 15 (1978).
6. Feldman, W. H.: Rev Tuberc **48,** 248 (1943).
7. Mára, M., Průchová, J., John, C., Šmerák, P., Šir, Z.: Folia Microbiol **26,** 52 (1981).
8. Mára, M., Nedvědová, V., Miková, Z., Hynčica, V.: Neoplasma **26,** 703 (1979).
9. Mára, M., Průchová, J., Šmerák, P., John, C., Miková, Z., Nedvědová, V., Šir, Z.: International Symposium on BCG Vaccines and Tuberculins, Budapest Hungary, 1983. Develop Biol Standard **58,** 379. (S. Karger, Basel 1986).

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OBSERVATION ON THE PRESENCE OF *LISTERIA MONOCYTOGENES* IN AXONS

A. Otter and W. F. Blakemore

Department of Clinical Veterinary Medicine, University of Cambridge, England

The brains from 16 sheep diagnosed as listerial encephalitis on the basis of clinical signs and histopathology were examined to determine the types of inflammatory cells present and for bacteria. *Listeria monocytogenes* were detected by immunocytochemistry in the brains of the 16 sheep and were also demonstrated in the trigeminal ganglion from the same side as the most severely affected side of the medulla. Bacteria were observed within myelinated axons in white matter tracts of the brain and in peripheral nerve axons of the trigeminal nerve. Their location in axoplasm was confirmed by electron microscopy. In order to investigate whether axonal presence can be equated with axonal transport, a model of infection has been established in mice in which bacteria are injected into the sciatic nerve. Paralysis of the injected leg occurs 7–12 days later and examination of the spinal cord reveals lesions very similar to those found in the brains of affected sheep.

It is generally accepted that the lesions of encephalitis in sheep due to infection by *Listeria monocytogenes* result from a focal infection of the brain-stem with the trigeminal nerve being the favoured route of entry of the organism [1–3]. However, the means by which bacteria travel within the nerve is not known. This report describes the appearance of *L. monocytogenes* within axons of the peripheral and central nervous system of field cases in sheep and following experimental infections in mice, observations which support the concept that *L. monocytogenes* can be transported within axons.

Materials and methods

Sheep tissue. The brains and heads from 16 cases of listerial encephalitis in sheep were obtained from two Veterinary Investigation Centres. The tissue had been fixed by immersion in 10% formal saline; coronal sections at the level of the thalamus, superior colliculus, pons and medulla oblongata were embedded in paraffin wax. The Gasserian ganglia, trigeminal, facial and hypoglossal nerves were removed from the heads and transverse sections were embedded in paraffin wax. Five micron sections were stained with heamatoxylin and eosin and also by Gram's method. *Listeria* organisms were identified in sections using rabbit anti-*Listeria* antiserum (Difco) and peroxidase-conjugated goat anti-rabbit (ICN Biomedicals Ltd.), 1 : 800 and 1 : 100 respectively. The reaction was developed using 3–3' diaminobenzidine as chromagen in the presence of hydrogen peroxide. Brain-stem and Gasserian ganglion material from the most heavily infected sheep was post-fixed in osmium tetroxide for

Arthur Otter, W. F. Blakemore
Department of Clinical Veterinary Medicine, University of Cambridge
Madingley Rd, CB3 OES Cambridge, England

electron microscopy, before routinely processing and embedding in Taab resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

Mouse model. Six to eight week old ICI mice were anaesthetised using equal volumes of a 50% solution of Fentanyl/Fluanisone ("Hypnorm" Janssen) and a 50% solution of Midazolam ("Hypnovel" Roche), at a dose of 7–10 ml per kilogram given intraperitoneally. The skin and subcutaneous tissue over the lateral aspect of the left thigh were incised and the sciatic nerve exposed. Using a 30 guage needle 10 μl of a suspension containing 10^8 *L. monocytogenes* type 1/2a (NCTC 10887) per ml was injected into the sciatic nerve at a point about 1 cm above the stifle joint. Any bacterial suspension that leaked at the site of inoculation was removed with a sterile swab. The wound was closed and a metal staple applied to the skin. The mice were allowed to recover and observed daily for signs of paralysis. At the onset of bilateral hindleg paralysis they were anaesthetised and perfused with 4% paraformaldehyde via an intracardiac catheter. The spinal cord and sciatic nerves were removed and blocks from lumbar spinal cord (L2–L3) were embedded in paraffin wax. Five micron sections were stained with haematoxylin and eosin and adjacent sections were labelled by the indirect immunoperoxidase method as above. Sections of sciatic nerve were post fixed in osmium tetroxide and routinely processed for electron microscopy.

Results

Light microscopy. Lesions characteristic of listeric encephalitis were found in the CNS of all 16 sheep examined. They were most numerous in the brain-stem, though evidence of infection was detected in cerebrum and cerebellum in some sheep. The focal lesions or "microabscesses" consisted of 3 cell patterns. In most sheep the majority of cells were macrophages; in some sheep the predominant cell type was the neutrophil, and in others there was a mixture of both cell types with the neutrophils undergoing degeneration. Large areas of malacia were present in some sheep. Perivascular cuffing of mononuclear cells was also seen. It was not possible to detect bacteria in sections stained with haematoxylin and eosin but they were visible in sections stained by Gram's method. Bacteria were present in microabscesses in those sheep in which the predominant inflammatory cell type was the neutrophil and in these sheep organisms were also present in adjacent areas of parenchyma free of inflammatory cells. A few bacteria were detected in fibre tracts where they were present within inflammatory cells associated with degenerating fibres and in linear arrangements in intact nerve fibres (Fig. 1).

Inflammatory cells were present in perineural and epineural connective tissue of the proximal parts of cranial nerves and in the Gasserian ganglia. In general the changes were restricted to the same side as the most severely affected side of the brain, but occasionally the contralateral trigeminal ganglion was also affected. The most severely affected ganglia were those from the two sheep in which the greatest number of bacteria were observed in the brain and in these sheep mononuclear cell infiltrates were present in perineural and epineural connective tissue and there were foci of predominantly polymorphonuclear leucocytes within the nerve fascicles.

Using immunocytochemistry *L. monocytogenes* were demonstrated in the brains and Gasserian ganglia of the 4 sheep in which bacteria had been identified using Gram's method.

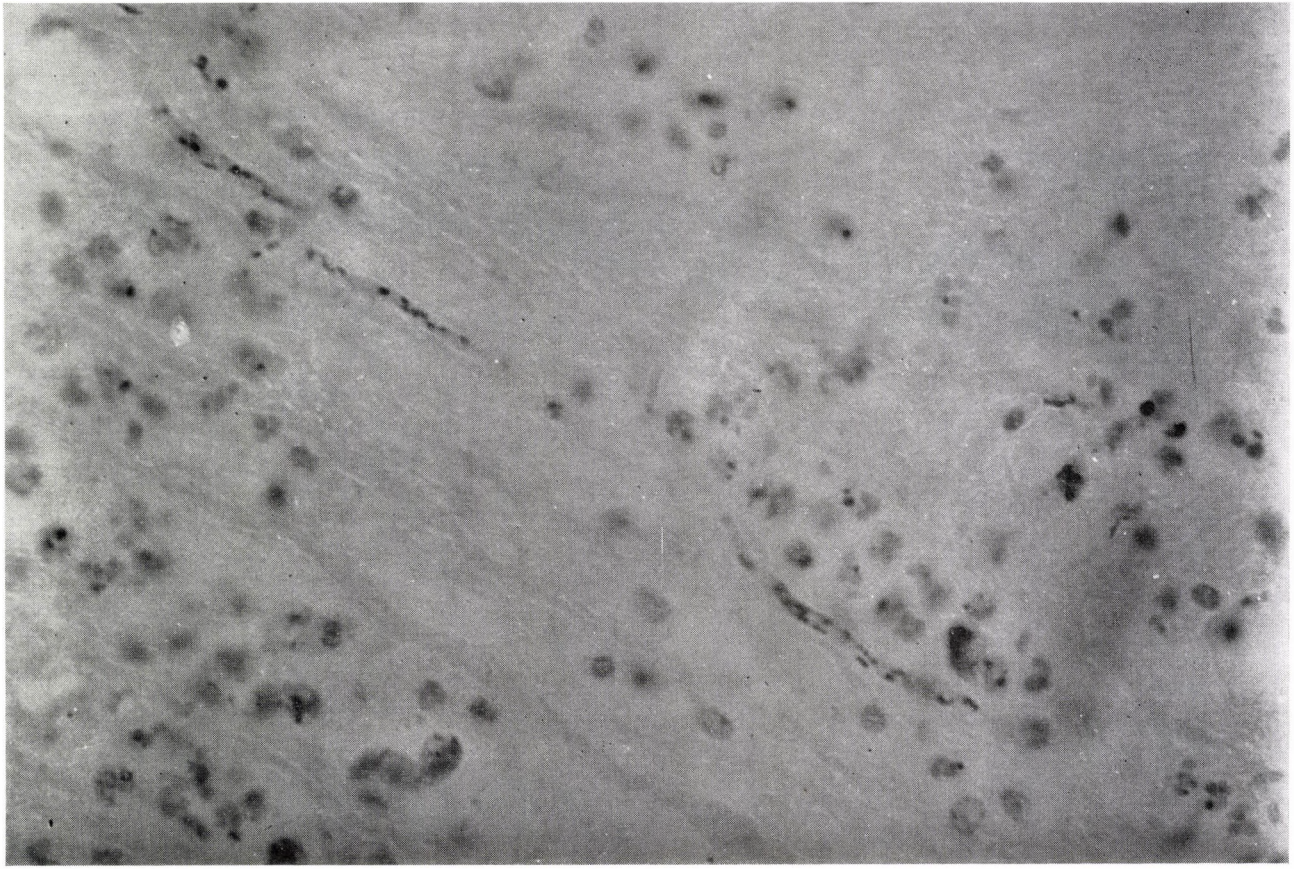

Fig. 1. Medulla (sheep). Immunoperoxidase labelled *L. monocytogenes* in intact nerve fibres and in neutrophils associated with degenerated fibres ×1000

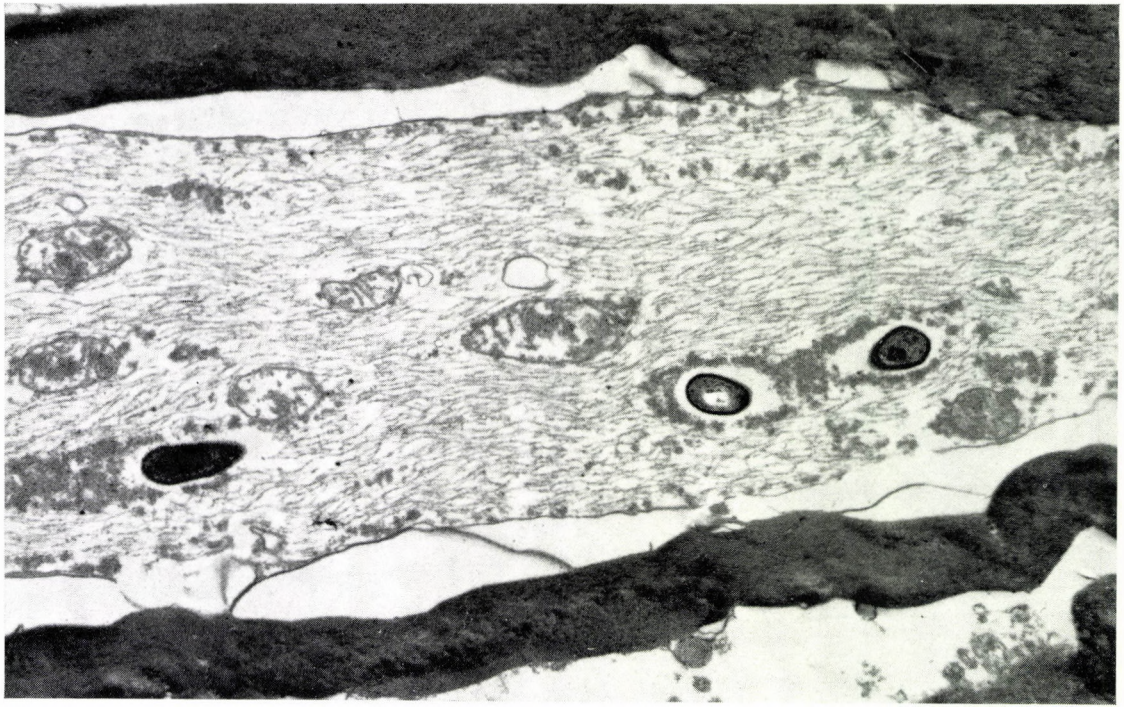

Fig. 2. Medulla (sheep). Longitudinal section of a myelinated axon showing 3 bacteria located within axoplasm. ×24 300

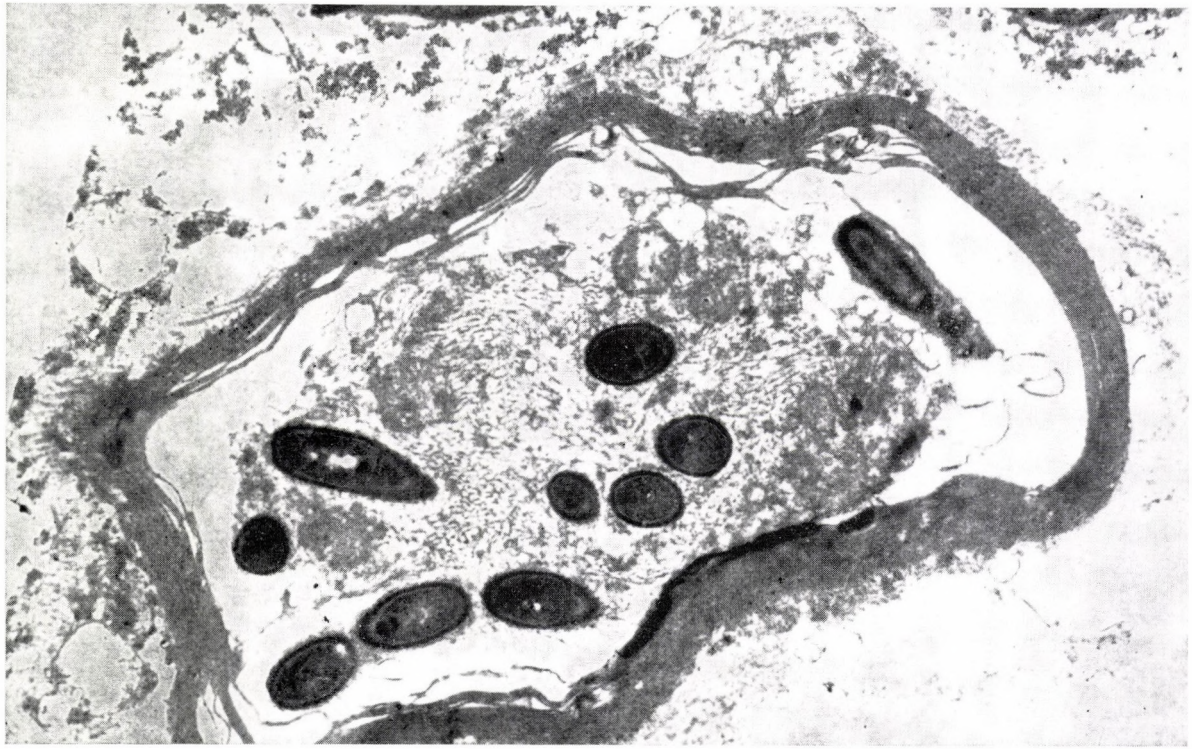

Fig. 3. Gasserian ganglion (sheep). Transverse section of a myelinated axon with several bacteria. ×17 700

Transmission electron microscopy. In brain-stem material from the most heavily infected sheep bacteria were located extracellularly, singly or in small groups, and were often associated with the cytoplasm of degenerating cells. They were also seen in membrane-bound phagocytic vacuoles of neutrophils. None were seen in macrophages. The bacteria in neutrophils appeared capable of dividing as evidenced by the development of transsecting cell walls. Less commonly the bacteria were detected in the axoplasm of myelinated axons (Fig. 2). In such sites they appeared to have normal morphological features with an intact cell wall; no flagellae were present. There was no disruption of the normal structure of the axon, the bacteria lying free within axoplasm surrounded by an electron transparent zone with a collection of electron-dense material present around the halo. They were not present in membrane-bound vacuoles. In several areas more than one bacteria was present in a single intact axon.

In the Gasserian ganglion from the same side as the most severely affected side of the brain-stem, similar changes were present as seen in the brain parenchyma. Bacteria were seen in neutrophils and were present within axons (Fig. 3).

Mouse model. Following anaesthesia the mice made an uneventful recovery and were eating and drinking normally by the next day. Between 7–14 days after inoculation the mice started knuckling the toes of the inoculated leg and within 2 days complete flaccid paralysis of the leg developed. The mice seemed otherwise unaffected and continued to eat and drink and if left a further 1–2 days developed bilateral hindleg paralysis. Examination of lumbar spinal cord of these mice by light microscopy showed lesions similar to those observed in the brains of sheep with focal accumulations of inflammatory cells and perivascular cuffs. *L. monocytogenes* were identified in lesions by immunocytochemistry. Bacteria were more commonly seen in animals which developed paralysis within 7–8 days after inoculation than in mice which developed paralysis after 13–14 days. Bacteria were seen in neutrophils and occasionally in axons. In the sciatic nerve there was extensive loss of axons and proliferation of fibrous connective tissue; changes were most marked at the site of inoculation. In the dorsal root ganglion there was no significant loss of neuronal cell bodies and there was no inflammation.

Discussion

Bacteria were only observed in the brains of 4 of the 16 sheep examined. In these animals the predominant inflammatory cell type was the neutrophil A similar pattern was seen in the experimental mice — bacteria were found in those animals which developed paralysis within 7–8 days which showed

a neutrophilic cellular infiltrate but were difficult or impossible to find in mice which became paralysed at longer times post inoculation in which lesions were characterized by infiltration of macrophages. In clinical cases one has no knowledge of the length of incubation period. Our observations from the mouse experiments suggest that those sheep in which neutrophils were the predominant inflammatory cell may represent animals which developed clinical signs earlier after inoculation than the animals which had a predominantly macrophage infiltration.

L. monocytogenes was demonstrated within axons of the peripheral and central nervous system in field cases of encephalitis in sheep and in central axons in experimental mice. The axons appeared morphologically normal which indicates that the bacteria were not just present as an end stage phenomenon in degenerating axons, an observation similarly made by others investigating infection with *Mycobacterium leprae* in man [4]. Our finding shows that *L. monocytogenes* can be transported within axons, a finding which supports, but does not prove that *L. monocytogenes* could reach the brain by retrograde axonal transport as postulated by Charlton and Garcia [3]. However, further studies are therefore needed to establish if *L. monocytogenes* is transported in the axonal compartment of the trigeminal nerve and if so, by what mechanism the organism enters the axon.

REFERENCES

1. Asahi, O., Hosoda, T., Akiyama, Y.: Am J Vet Res **18,** 147 (1957).
2. Barlow, R. M., McGorum, B.: Vet Rec **116,** 233 (1985).
3. Charlton, K. M., Garcia, M. M.: Vet Pathol **14,** 297 (1977).
4. Yoshizumi, M. O., Asbury, A. K.: Acta Neuropath (Berl.) **27,** 1 (1974).

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COMPARATIVE STUDIES ON SUSCEPTIBILITY OF THE MOUSE *(MUS MUSCULUS)* AND THE VOLE *(MICROTUS ARVALIS)* TO *LISTERIA MONOCYTOGENES* AND *ERYSIPELOTHRIX RHUSIOPATHIAE*

F. KEMENES, J. DURST and F. VETÉSI

Phylaxia Veterinary Biologicals Co., Budapest, Public Health Station, Szolnok, and Department of Pathology, University of Veterinary Sciences, Budapest, Hungary

The susceptibility of mice and voles to intraperitoneal and subcutan injection of virulent strains has been tested comparatively on 261 mice and 120 voles. Using various germ-doses, 50 per cent lethality has been established. *Listeria monocytogenes* serovar 1/2a killed mice in doses of 120 000 and 1.5×10^6 germs when injected intraperitoneally and subcutaneously and voles in doses of 1800 and 12 000 germs, respectively. Thus voles proved 100 to 120 times more susceptible than mice. *Erysipelothrix rhusiopathiae* killed mice injected both intraperitoneally or subcutaneously in minimal doses, such as 25 germs, but voles survived the infection with 1 to 5 million germs. This indicates that voles are resistant to *E. rhusiopathiae*, but more susceptible to *L. monocytogenes* than mice. In contrast, both these rodent species proved susceptible to the obligate pathogenic *Francisella tularensis* and invariably succumbed to septicaemia when injected with a few of these bacteria.

The Swiss mouse — as experimental animal — is generally held susceptible to *Listeria monocytogenes* and to *Erysipelothrix rhusiopathiae*. On the other hand it is less known that for experimental murine listeriosis at least 100 000 listeriae are needed to get 50% mortality after intraperitoneal injection. Therefore, investigations were made by many authors to find a more susceptible animal for experimental listeriosis. Recently we have undertaken a comparative study on the susceptibility of the mouse and the vole to listeriae and swine erysipelas bacteria to find out differences in their dose-dependent experimental infections.

Materials and methods

A total of 261 white mice weighing 22 to 25 g and 120 common voles weighing 22–38 g were used. The mice were purchased from the State Laboratory Animal Breeding Stock (LATI, Gödöllő, Hungary), the voles were obtained from our Vole Breeding Stock (Phylaxia, Budapest). The haemolytic (virulent) *Listeria monocytogenes* strain of type 1/2a was isolated

FERENC KEMENES
Phylaxia Veterinary Biologicals Co.
H-1486 Budapest, P. O. B. 23, Hungary

JÁNOS DURST
Public Health Station
Ady Endre út 35, H-5001 Szolnok, Hungary

FERENC VETÉSI
Department of Pathology, University of Veterinary Sciences
Rottenbiller u. 50, H-1077 Budapest, Hungary

from an ovine brain with fatal listeriosis. The *Erysipelothrix rhusiopathiae* strain of type Ia was cultivated from the heart blood of a swine died from acute erysipelas. Both of the strains were maintained on blood agar plates and in stabbed agar. Every second month these strains were passaged in mice in order to secure their virulences. The reisolated strains in Tarozzi's liver broth were incubated at 38 °C and the 20 h growth was diluted with 1% peptone water from 1 : 10 to 1 : 10×10^6. Thereafter these dilutions were injected at 0.2–0.2 ml quantities subcutaneously or intraperitoneally to four mice grouped according to different dilutions. The mice were observed for 10 days, meanwhile the dead animals were dissected and bacteriological cultures were made. The 50% mortality per group means that two out of 4 mice died in listeriosis or in swine erysipelas.

Results and discussion

The lethality-rate of mice and voles infected intraperitoneally or subcutaneously with different doses of *L. monocytogenes* can be seen in Tables I and II, respectively. The tables show that mice and voles infected intraperitoneally were more susceptible to *L. monocytogenes* than those infected subcutaneously. Furthermore, it is also shown that voles are 100–120 times more susceptible than mice.

Irrespective of the two modes of experimental infection the mortality-rate of both rodent species to *E. rhusiopathiae* varied to a great extent (see

Table I

Mortality of mice and voles infected intraperitoneally with L. monocytogenes

Bacterial count in 0.2 ml	Mice	Voles
1 200 000*	3/4**	4/4
120 000	2/4	4/4
12 000	0/4	3/4
1 200	0/4	3/4
120	0/4	0/4

* The basic dilution of Tarozzi's broth culture contained cc 6 million bacterial/ml
** Denominator: number of animals infected, numerator: number of animals died of listeriosis

Table II

Mortality of mice and voles infected subcutaneously with L. monocytogenes

Bacterial count in 0.2 ml	Mice	Voles
1 500 000*	3/4**	4/4
150 000	1/4	4/4
15 000	0/4	3/4
1 500	0/4	3/4
150	0/4	0/4

* The basic dilution of broth culture contained cc 7.5 million bacteria/ml
** See Table I

Table III

Mortality of mice and voles infected subcutaneously or intraperitoneally with E. rhusiopathiae

Bacterial count in 0.2 ml	Mice	Voles
5 000 000*	8/8**	5/8
500 000	8/8	2/8
50 000	8/8	0/8
5 000	8/8	0/8
500	8/8	0/8
50	7/8	0/8
5	5/8	0/8

* The 20 hour's liver broth culture contained cc 25 million bacteria/ml
** See Table I

Table III). The mice were killed by a few (3–25) erysipelas bacteria, whereas at least 500 000 bacteria were needed parenterally to kill some of the voles In resistant voles infected subcutaneously into the knee-joint region with 200 000 erysipelas bacteria no chronic arthritis developed. In contrast to resistant rats infected in the same way, severe arthritis developed in the

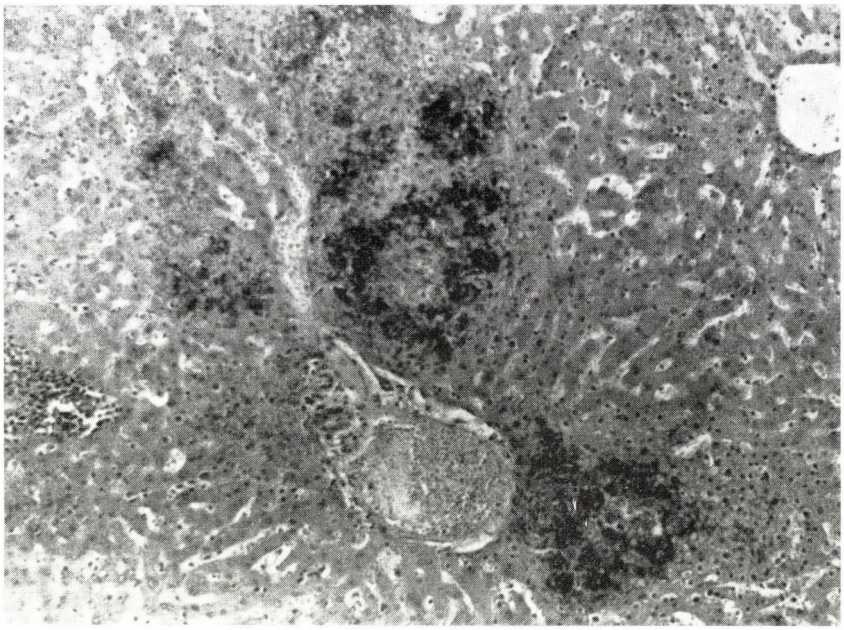

Fig. 1. Two necrotic inflammatory foci are seen around an enlarged blood vessel in hepatic section of the vole died in listeriosis. Haemalaun-eosin staining. ×320

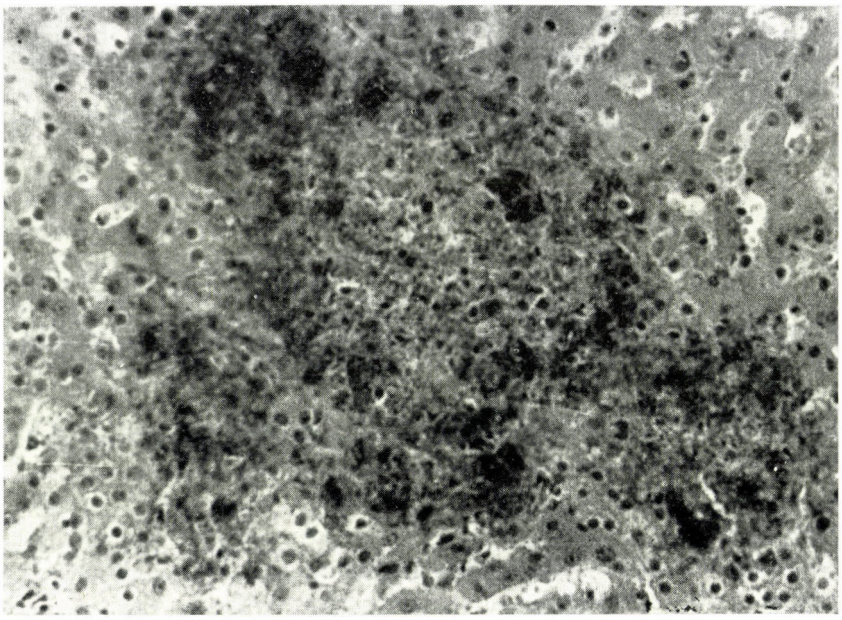

Fig. 2. A somewhat areactive necrotic area is shown in hepatic section of the vole died in listeriosis. Haemalaun-eosin staining. ×670

vicinity of the infected site. Mice infected intraarticularly with *E. rhusiopathiae* succumbed 4–6 days in fatal septicaemia.

Pathological and histopathological lesions of voles died in listeriosis were investigated in 28 cases. Enlarged and degenerated parenchymal organs were found in the cadavers of them. Smaller or greater necrotic inflammatory foci were present in the hepatic tissue chiefly around the blood vessel (Fig. 1). In other hepatic sections larger necrotic areas with a few cell-infiltration only were shown (Fig. 2).

From these comparative investigations it is clear that the vole is more susceptible to *L. monocytogenes* than the mouse. On the other hand the mouse is more susceptible to *E. rhusiopathiae* than the resistant vole. In contrast to this, both of these rodent species proved more susceptible to the obligate pathogenic *F. tularensis* and invariably succumbed to septicaemia when injected parenterally with a few of this bacterial-species. Consequently, the systematically related, close to each other enlisted *L. monocytogenes* and *E. rhusiopathiae* differ considerably in pathogenicity, and accordingly this biological property can be employed only as a limited marker in their bacterial classification.

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RESEARCH ON LISTERIOSIS IN MILK COWS WITH INTRAMAMMARY INOCULATION OF *LISTERIA MONOCYTOGENES*

J. BRYNER, I. WESLEY and M. VAN DER MAATEN

USDA-ARS, National Animal Disease Center, Ames, Iowa, USA

The purpose of our work was to produce 600 litre quantities of milk containing (per ml) 5000 viable *Listeria monocytogenes*, for certification of standard pasteurization technique. Preliminary research indicates deep (10 cm) inoculation via teat canal with 3 injections, (10^3, 10^5, 10^7) per 10-day interval, was better than intraocular or per os route for establishing chronic *L. monocytogenes* mastitis. Although milk yield diminished 25%, chronic *L. monocytogenes* shedding persisted for 8 months. Sheeding in individual cows varied from 10^3 to 10^5 listeriae/ml. Dexamethasone injections increased *L. monocytogenes* shedding in milk ca. 1000-fold. Three of 30 cows were pregnant during the experiment and produced normal calves, indicating that intramammary *L. monocytogenes* infection was localized and did not migrate to the pregnant uterus. Four of 30 cows developed CNS symptoms of listeriosis, and one died. From 11 cows, *L. monocytogenes* was isolated at necropsy, from mammary tissues (9/11), supramammary lymph nodes (9/11), liver (6/11), spleen (5/11), and brain (6/11) by cold enrichment culture. Serum antibody titres of infected cows increased from 1 : 160 to 1 : 10 280 in most cows. Large scale pasteurization trials led to the conclusion that the present pasteurization guidelines of the FDA are adequate to inactivate listeriae in whole milk.

Listeria monocytogenes is a facultative intracellular, Gram-positive rod which produces bacteriaemia, neurologic disturbances, abortion, and death in animals and humans. As a free-living bacterium it survives and grows in vegetation, dairy and meat products, waste materials, and in water with minimal nutrients. Growth occurs at temperatures near freezing up to 43 °C, replicating rapidly in refrigerated milk to 10^8 cells/ml, surviving for months in cold without apparent spoilage. *L. monocytogenes* establishes inapparent, chronic, intramammary infection in lactating cows, going undetected for months, with a potential to contaminate large batches of milk in dairy processing plants. Recent Public Health concerns [1, 2] that *L. monocytogenes* might survive heat pasteurization prompted US Food and Drug Administration (FDA) to reevaluate milk pasteurization standards (71.7 °C; 15 s). To this end, FDA and USDA cooperated to establish an *L. monocytogenes*-infected herd of cows to supply bulk quantities (600 litres) of milk with ca. 10^3 *L. monocytogenes*/ml. National Animal Disease Center (NADC) scientists at Ames, Iowa infected 34 lactating cows, collected milk, and sent 13 shipments (7500 litres) of cold (5 °C) milk via airplane to Cincinnati (1500 km) for pasteuriza-

JOHN BRYNER, I. WESLEY, M. VAN DER MAATEN
National Animal Disease Center
P.O. Box 70, Dayton Road 2300, Ames, Iowa, 50010 USA

tion trials. This paper reports methods used to establish chronic intramammary *L. monocytogenes*-infection in cows, cultural enumeration of *L. monocytogenes* in milk, and the immune response in cattle.

Materials and methods

Bacteria. L. monocytogenes, strain Scott A, serotype 4b was used as the infecting bacterium. Cultures were maintained on blood agar plates (5% bovine blood in Brain Heart Infusion Agar) at 20 °C to 30 °C, subcultured twice, weekly, and incubated microaerophilically.

Cows. Thirty four Holstein cows 3 to 7 years of age, in early lactation were divided into 4 groups. Groups I, II, III (27 cows) were injected with 10^6 to 10^7 c.f.u. of *L. monocytogenes* deep (10 cm) into mammary teat canal, repeatedly 3 to 9 times, at 7 to 14 day. Group IV (7 cows) were injected twice with 10^2 and twice with 10^3 c.f.u. weekly.

Cultural isolation. Morning milk collections were cultured (100 μl) to blood agar plates (McBrides, 1 plate; BHIA 2 plates), incubated at 30 °C or ambient (20 °C). During the early phases of this study, 10 ml samples of milk were cold-enriched in trypticase soy-yeast extract TSYE) broth (100 ml) at 5 °C for up to 3 months, and plated twice monthly. One per month milk samples were treated with ultrasound to release intracellular and clumped *L. monocytogenes*. After 2 days incubation *L. monocytogenes* colonies were enumerated by 10× microscope, and reflected light. Phase contrast microscopic examination showed motile, tumbling rods typical of *L. monocytogenes;* haemolysis on 5% blood agar, serology, and biochemical test reactions confirmed *L. monocytogenes*.

Serology. Pre- and post-inoculation serum samples from all cows were tested for *L. monocytogenes* agglutinins with tube and microtiter methods. Serum samples were obtained from 55 humans to survey for naturally occurring anti-*L. monocytogenes* titer.

Dexamethasone. To increase *L. monocytogenes* shedding in milk 5 cows were injected daily, 3 days, with dexamethasone. The effects on milk yield, *L. monocytogenes* numbers, and leukocyte count in blood and milk were evaluated [3].

Results and discussion

Because serotype 4b *L. monocytogenes* was the most commonly isolated type in outbreaks of human listeriosis, we tested serum from 4 herds of cattle (n = 82) for naturally occurring 4b-agglutinins. Results (Table I) show that 70% of cattle had titres to *L. monocytogenes* (1 : 20 to 1 : 1280). However, there was no detectable correlation between anti-listeria titre and susceptibility to infection following injection of the same *L. monocytogenes* serotype. In a similar survey, we tested serum from 55 persons who worked at the

Table I

Serum titres to L. monocytogenes serotype 4b

Titre	Bovine serum	Human serum
Negative to 1 : 20	35	55
1 : 40 to 1 : 80	31	0
1 : 160 to 1 : 640	15	0
1 : 1280	1	0

Table II

Schedule of intramammary injections of L. monocytogenes

Cows	Dates	Injections	Dose injected
3	March—April	8	10^3, 10^6, 10^7, 10^9
15	May—August	7	10^2, 10^4, 10^6, 10^9
9	September—December	5	10^7, 10^8
6	December—January	4	10^2, 10^3, 10^4

NADC to determine previous experience with *L. monocytogenes* serotype 4b. Among individuals tested were animal caretakers, veterinarians, microbiologists, and office personnel; 2/55 had titres (1 : 20). Table II shows injection schedule for experimental cows. Although the dose varied from 10^2 to 10^9 and the number of injections varied (2 to 8), there was no apparent correlation between chronic shedding of *L. monocytogenes* in milk vs serum antibody titre.

Table III gives *L. monocytogenes* shedding of 3 cows used to determine optimum methods for infecting and assaying parameters of the experiment. Although these are typical of the cows (n = 34) a few cows resisted infection and rarely shed more than 10 *L. monocytogenes*/ml of milk, whereas, most cows were chronic shedders of 500 to 50 000 *L. monocytogenes*/ml. Daily culture yielded highly variable *L. monocytogenes* plate counts in individual cows; milk pooled in bulk refrigeration tank contained 3×10^3 *L. monocytogenes*/ml. Cows that developed chronic intramammary infection have remained infected throughout their lactation period (9 to 12 months), and three that produced normal calves retained infection into their next lactation. Dexamethasone administration caused a rise in body temperature, increase in blood leukocytes and milk somatic phagocytes, decline in eosinophils, 25% loss of milk yield, and a transient, 1000-fold increase in *L. monocytogenes* shedding in milk. Several infected cows developed pneumonia, CNS symptoms, etc., possibly due to underlying stress of listeriosis, and one died. Systemic antibiotic therapy was used to treat lameness, non-specific mastitis, fevers, and

Table III

L. monocytogenes colonies/ml cultured from infected milk

Week	Cow 1033	Cow 1177	Cow Chancy
1	0	0	0
4	100	200	990
6	1270	1440	2000
9	1290	1580	5310

Table IV

L. monocytogenes isolated at necropsy by cold enrichment

Cow	Mammary	Lymph node	Liver	Spleen	Brain	Blood
1	+	+	−	+	+	−
2	+	+	+	+	−	−
3	+	−	+	+	+	−
4	+	+	+	−	+	−
5	−	−	−	−	+	−
6	+	+	−	−	−	−
7	+	+	−	−	+	−
8	+	+	+	+	−	−
9	+	+	+	+	−	−
10	−	+	+	−	+	−
11	+	+	−	−	−	−
Total	9	9	6	5	6	0

other conditions. Culture of eleven infected cows at necropsy yielded *L. monocytogenes* isolations from mammary glands, supramammary lymph nodes, liver, spleen, and brain (Table IV). Although *L. monocytogenes* bacteriaemia occurs in infected humans, *L. monocytogenes* was not isolated from blood of infected cows by direct plating nor by cold enrichment.

Conclusions

Chronic mastitis can be induced experimentally with intramammary injection of *L. monocytogenes*. Susceptibility to *L. monocytogenes*-mastitis appears to be unrelated to serum antibody titre to the organism. Intramammary inoculation of *L. monocytogenes* and infection of mammary gland does not result in detectable bacteriaemia in cows. The average shedding rate in-cows with chronic *L. monocytogenes*-mastitis is approximately 3×10^3 *L. monocytogenes*/ml of milk. Once chronic *L. monocytogenes*-mastitis is established, the cow may remain infected for life and shed *L. monocytogenes* in milk indefinitely, or until treated. Data derived from pasteurization trials led to the conclusion that the present pasteurization guidelines of the FDA are adequate to inactivate *L. monocytogenes* in whole milk.

REFERENCES

1. Bunning, V. K., Crawford, R. G., Bradshaw, J. G., Peeler, J. T., Tierney, J. T., Twedt, R. M.: Appl Environ Microbiol **52,** 1398 (1986).
2. Doyle, M. P., Glass, K. A., Berry, J. T., Garcia, G. A., Pollard, D. J., Schultz, R. D.: Appl Environ Microbiol **53,** 1433 (1987).
3. Wesley, I. V., Bryner, J. H., Van Der Maaten, M. J.: Am J Vet Res (In press).

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2. EPIDEMIOLOGY AND THERAPY

OVERVIEW OF LISTERIOSIS IN COUNTRIES OF THE MEDITERRANEAN ZOONOSES CONTROL PROGRAMME

(A REVIEW)

G. Papadopoulos E. Simos

Mediterranean Zoonoses Control Centre, Athens, Greece

Listeriosis is a bacterial zoonosis affecting domestic and wild animals as well as man. Its geographical distribution is described as ubiquitous but the disease is more common in temperate climates than in the tropics or subtropics [1].

Though an uncommon cause of human disease, the case fatality rate is high; approximately one third of cases have resulted in deaths or stillbirths, a fact that has caused concern among health professionals, governments, food industries and the public.

Listeriosis is recognized and studied mostly in industrialized countries while in developing countries of Africa, Asia, South America and in those of the Mediterranean area, reported prevalence is non existent or low. This may be due to the lack of adequate surveillance system.

Of all *Listeria* species, only *Listeria monocytogenes* has been regularly implicated as being pathogenic to man and animals. For many years after the first isolation of *L. monocytogenes*, in the nearly twenties, all further isolates came from animals suffering from or having had contacts with the disease. It has been isolated most often from sheep, cattle and man and occassionally from wild animals, birds, reptiles, fish, turtles, snails etc., but now is considered more as an environmental contaminant [2, 3].

The disease in man and animals. Cases of listeriosis in man are reported infrequently, although they have been described in all parts of the world. Because of its diphtheroid appearance, variable staining properties and beta-haemolysis, *L. monocytogenes* may be confused with some other bacteria. The most important clinical manifestations are: meningitis or meningo-encephalitis, septicaemia, intra-uterine infections of the foetus, and newborn infections.

George Papadopoulos, Evangelos Simos
Mediterranean Zoonoses Control Centre
P.O. Box 3904, 10210 Athens, Greece

Almost all mammals and birds are susceptible to the disease. Among domestic animals sheep and cattle are most often affected followed by goat, pig and fowl. The most common clinical manifestations of animal listeriosis are encephalitis, septicaemia and abortion. In the cow the predominant form

Table I

Situation of listeriosis in countries of the Mediterranean zoonoses control programme

Country	1984	1985	1986
Afghanistan	(+)	+	(+)
Bulgaria	+V	( ) PnTV	Ov+CaV*
Cyprus	—	—	Ov(+)T
Democratic Yemen	...	...	
Egypt	—Qf	— 0000	...
F. R. G.	Bov(+)Ov(+)	BovOv+)(T	BovOv+
France	+ ..	+	+
Greece	+ tev	OvCap+)(tvV	OvCap+)(tvV
Italy	(+)	...	—
Jordan	—		
Lebanon	—	—	—
Libya	...	—	—
Portugal	+	+	+
Saudi Arabia			—
Somalia	—	—	—
Spain	Bov+( )	Bov+	Bov+
Sudan	...	...	...
Syria	—	—	—
Tunisia	?	?	—
Turkey	—	...	...
Yemen	—	Ov 1981	

Code

Animal group

Bov	bovine (including buffalo)
Cap	caprine
Ov	ovine

Disease occurrence

0000	Never recorded
—	Not recorded
year	Year of last occurrence
?	Suspected but not confirmed
(+)	Exceptional occurrence
+	Low sporadic occurrence
+..	Disease exists; distribution and occurrence entirely unknown
( )	Confined to certain regions
( )	Ubiquitous
...	No information available

Disease control

Pa	Control programme for only some areas of the country or certain types of breeding
Pn	Control programme for the whole country
Qf	Quarantine and other precautions at frontier
T	Treatment
te	Testing
tv	Voluntary testing
V	Vaccination
*	Notifiable disease

is abortion while in sheep encephalitis and abortion is also frequent. Listeriosis in farm animals is classified as follows [4]: (a) Infection of the pregnant uterus with subsequent abortion, stillbirth or neonatal; death (b) visceral or septicaemic infections; (c) encephalitis of ruminants; (d) infectious bovine keratitis; (e) bovine mastitis; (f) mixed infections.

Epidemiology—transmission. The epidemiology of *L. monocytogenes* is an enigma. This bacterium is perhaps best considered, although not exclusively, as an environmental contaminant whose primary means of transmission to humans is through foodstuffs contaminated during production and processing. Human listeriosis is more prevalent in the summer and early fall, and the disease has been observed most frequently in temperate zones. In the animals, movement of replacement cattle or sheep into a feedlot has often resulted in outbreaks. Mastitis in cows often results in contamination of the milk that may harbour large numbers of the organism. The modes of transmission are not well understood although there is evidence for direct and indirect transmission.

The lack of elucidation in the mode of spread mainly due to the fact that (a) listeriosis is a disease of low incidence; (b) there is no surveillance and data collection system in most countries; (c) there is no satisfactory typing system for isolates, and (d) *L. monocytogenes* is common in the environment and is frequently carried in the gut without causing disease.

The presence of L. monocytogenes in foods. Of all foods cheeses have been found to be frequently contaminated with listeriae and associated with human disease. Variation in manufacturing practices results in opportunities for post-process contamination. In raw ready to eat meat products up to 30% have been reported to contain *L. monocytogenes.* In sausages, post-processing manipulation appears to be responsible for contamination. Cooked fish and other seafoods as well as salad vegetables may also be contaminated with *L. monocytogenes.*

Listeriosis in countries of the MZCP. Table I shows the situation of listeriosis in countries of the Mediterranean Zoonoses Control Programme (WHO/FAO/OIE Animal Health Yearbook). It is quite clear from this table that in most of the Northern Africa and the Arab Peninsula countries which participate in the MZCP there are no available data. In other countries like Bulgaria, Cyprus, France and Portugal there are available data and some control measures are taken. Finally, in countries where data on human or animal listeriosis are collected there is clear evidence of increased reported cases in recent years. The disease became notifiable lately in F. R. G. and according to our information, in some other countries (e.g. Greece).

Prevention and control. Prevention of listeriosis is an extremely difficult task. There are no vaccines killed, or living attenuated of proven value. Lack of epidemiologic information hampers prevention and control. What is needed

is a systematic collection of epidemiological data, and development of adequate monitoring systems. Public education and awareness of professional and risk groups are also important.

Preventive measures should be directed towards animal husbandry, food production, food processing, and good manufacturing practices in connection with implementation of the principles of hazard analysis of control critical points. General and specific recommendations to public health authorities on how to ensure safeguarding the consumer as well as recommendations to the food industry are given in detail in the report of the WHO Informal Working Group 1988 on foodborne listeriosis [5].

International cooperation and the role of the MZCC/MZCP. International cooperation and intersectoral collaboration is fundamental for the improvement of surveillance, prevention and control of listeriosis. The MZCC/MZCP could facilitate this cooperation by coordinating collection and dissemination from and to the region of available information in collaboration with other international organizations, like OIE and WHO Collaborating Centres which are willing to participate to this effort.

REFERENCES

1. Seeliger, H. P. R.: Listeriosis. Hafner Press, New York 1961.
2. Steele, J. H.: Handbook series in Zoonoses. CEC Press, Inc., Florida 1979.
3. World Health Organization: Report of the WHO Consultation on Prevention and Control of Listeriosis. W. Berlin 1986.
4. Gitter, M.: In Collins, C. H. (ed.): Isolation and Identification of Microorganisms of Medical and Veterinary Importance. Society for Applied Bacteriology, London 1985. pp. 191–200.
5. World Health Organization: Foodborne listeriosis. Report of a WHO Informal Working Group, Geneva 1988.

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LISTERIOSIS OF ANIMALS: PROBLEMS AND RESEARCH

I. A. Bakulov

All-Union Research Institute of Veterinary Virology and Microbiology, Pokrov, USSR

In a pig farm virulent *Listeria* were found in 25% of animal tissues and environmental object and 7% in human tonsil samples from patients with tonsillitis. The isolates were characterized by serological and phage-typing. Purified cytoplasm and cell wall fractions and subfractions protected laboratory animals in 40—58%.

In the USSR listeriosis of animals has been recorded in all kinds of farm and domestic animals and also in wild animals in different regions of the country. The intensity of manifesting of the epizootic process according years, areas and kinds of animals varied. In 1977–1987 cattle were frequently affected in Kazakhstan, sheep in the Ukraine and swine in the RSFSR. In general, country sheep were the most affected. The years 1962—1966, 1970–1972, 1974, 1979 were the most unfavourable. In 1982–1985 the occurrence decreased. The prevalence of the disease as an average was in cattle 0.03, sheep 1.33, swine 1.13. The lethality decreased. Probably this reduction is associated with the increasing effectiveness of medical preventive measures. The disease occurred again as sporadic cases and was characterized by seasonal prevalence in sheep.

Materials and methods

We have developed methods and means used for serological investigations of listeriosis:

— a diagnostic set for agglutination test (AT) to detect listeric agglutinating macroglobulin (19 S) and microglobulin (7 S) antibody. AT is used for diagnosis of listeriosis together with other assays and also for detection in inapparently ill and survived animals;

— dry cytoplasmic antigen for CFT;

— a diagnostic set for a passive haemagglutination test (PHT) for veterinary and medical laboratories.

Results and discussion

Together with medical specialists we examined animals and humans for listeriosis in a big pig complex and also determined the contamination of

I. A. Bakulov
All-Union Research Institute of Veterinary Virology and Microbiology
Vladimirskaja oblast. Pokrov 601120, USSR

the surroundings. Virulent strains of *Listeria* were found in 25% of animal tissues and environmental objects and 7% in human tonsil samples from patients with chronic tonsillitis. Listeriae were also found in 22% of women having different urogenital and obstetric symptoms. In 58.4% of positive cases serogroup I listeriae were found, serogroup II occurred in 30.9% and both groups were shown in 10.7%.

Serological studies confirmed a wide distribution of *Listeria* in humans and animals in the area of the pig complex. The average titre for personnel of this complex was 15.6 times higher than in persons of a control group (industrial workers), and 2.1 times higher than in people living near this farm, but not working there.

Based on usual methods we studied 420 strains of *Listeria*, isolated in the USSR from various sources (man, 21; cattle, 28; sheep and goats, 201; pigs, 45; fowl, 10, environment, 24). As a reference we used the strain kindly provided by H. Seeliger, D. Jones and I. Ivanov. All tested strains had typical morphology, grew well on usual media, formed typical colonies on solid media and produced turbidity of a meat-peptone broth, Serogroup I was represented by 363 strains, serogroup II by 57 strains.

Diagnostic *Listeria* bacteriophages L 2 A and L 4 A were isolated to identify the isolated strains and establish their serogroup affiliation. Bacteriophages are also used for bacteriophage titre growth test which promotes to determine the presence of *Listeria* in the examined pathological material without isolation of a pure culture of the agent. With the help of 7 types of *Listeria* phages the tested *Listeria* strains were divided into 9 phage types.

Using DNA-DNA hybridization we established the relationship of *Listeria monocytogenes* strains and their distinction from *Listeria grayi* and *Listeria murrayi* strains. The homology level between the two latter (88–100%) suggests their belonging to the same species.

Listeria denitrificans had an average homology level of 7%, and therefore it was excluded from the genus *Listeria*.

Investigation of energetic metabolism of *Listeria* cells showed that they had a definite ability to split glucose via the hexosemonophosphate pathway under conditions of an almost total absence of glycolytic splitting. There were also found cytochromes "a" and "b" in whole cells of low virulent and virulent strains in different quantitative ratio in these groups of strains.

A work was performed to determine the role of L-forms in the pathogenesis of the disease. The virulence of experimentally induced L-forms was proved. Radioactive isotope-labeled *Listeria* predominantly accumulated in the brain and also in the liver and spleen.

"Methodical recommendations in diagnostics of listeriosis of animals and man" and "Instruction in prophylaxis and control of animal listeriosis" have been developed.

For developing a vaccine against listeriosis we disintegrated listeriae and prepared purified cytoplasm and cell wall fractions, subfractions of membrane ribosomes and glycoproteins. These preparations protected laboratory animals in 40–58% of cases, though the cell wall fraction appeared less immunogenic. The addition of adjuvants failed to increase the immunogenicity of these preparations. In experiments with farm animals (sheep), negative results and even a sensibilizing effect of the above mentioned preparations were observed.

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LISTERIOSIS IN AUSTRIA — REPORT OF AN OUTBREAK IN 1986

F. ALLERBERGER and J. P. GUGGENBICHLER

Federal Public Health Laboratory Innsbruck, Austria

In 1986 *Listeria monocytogenes* was isolated from various specimens of 28 patients in Austria: 24 were newborn infants, 4 patients were adults (25–73 years). All four adult patients and five of 20 mothers had consumed unpasteurized milk. Nine of 20 mothers and two of four adult patients had received vegetables from biologic agriculture. The consumption of raw milk and biologically grown vegetables as possible source of infection cannot be dismissed. The fatality rate (5 of 28) was remarkably low; in our experience there was no difference in the therapeutic outcome between the use of a combination of ampicillin or penicillin with an aminoglycoside or of amplicillin alone; all were effective.

In the last years an increasing number of patients with listeria infections has been reported [1–3]. There is still some controversy regarding epidemiology and treatment of this serious disorder. We are reporting an epidemic outbreak of *Listeria monocytogenes* infections in 28 patients which occurred in Austria in 1986.

Materials and methods

In 1986 a total number of 39 cases of human listeriosis came to knowledge of the Austrian Federal Ministry of Health. Twenty-eight culture-proven cases of bacterial infection with verification by the Listeria Reference Laboratory Würzburg (H. P. R. Seeliger) were included in our study. Determination of lysotypes was performed at the Institute Pasteur Paris (J. Rocourt).

Results

In 1986 *Listeria monocytogenes* was isolated from various specimens (Table I) of 28 patients in two laboratories in Austria (Serovar 1/2a, 24 strains; serovar 4b, 4 strains). Patients originated from the four western districts of Austria namely Vorarlberg, Tirol, Salzburg and Upper Austria with a total population of 2.4 million people. The last epidemic occurrence of listeriosis was registered in the eastern part of Austria five years earlier — 10 cases in the years 1980/81 [4]. From Austria three isolates of human origin were

FRANZ ALLERBERGER, J. P. GUGGENBICHLER
Federal Public Health Laboratory
Schopfstrasse 41, A-6020 Innsbruck, Austria

Table I

Sources of specimens — 28 cases of listeriosis / Austria 1986

Patient	Specimen
Neonatal infants:	
B.	meconium, throat swab, ear swab
K.	ventricular aspirate
L.	blood
H.	blood, ear swab
R.	cerebrospinal fluid
P.	cerebrospinal fluid
G.	blood
M.	blood
R.	blood
H.	blood
B.	blood
M.	blood, cerebrospinal fluid
N.	blood, ear swab
A.	blood
P.	ear swab
H.	endotracheal tube
H.	endotracheal tube
S.	cerebrospinal fluid
M.	endotracheal tube
K.	ventricular aspirate
S.	blood
W.	blood, cerebrospinal fluid
S.	blood, meconium
S.	cerebrospinal fluid
Adults:	
K. M.	cerebrospinal fluid
L. J.	cerebrospinal fluid
K. A.	blood
E. R.	blood

typed in 1982 at the Listeria Reference Laboratory Würzburg, one strain in 1983, none in 1984 and 1985 and 17 strains in 1986 and 1987, respectively. In 1988 (including March) two Austrian strains of human origin were typed at the German Reference Laboratory.

Out of the 28 patients in 1986, 24 were newborn infants; 23 infants had early onset disease with signs and symptoms of septicaemia and respiratory distress either immediately or within 12 h after delivery. Further evaluation of these patients disclosed a bacterial meningitis in 10, septicaemia in 13 patients. One patient had late onset disease on day six with signs and symptoms of bacterial meningitis. The adult patients were in an age range between 25 and 73 years. Only one of these four patients showed compromised host defence mechanisms (blastic crisis, CML). Two patients suffered from meningitis (a 73-year-old man fell ill five days after incision of a perianal abscess), two from septicaemia.

A careful history with emphasis on epidemiologic data and environmental factors was obtained in 20 of the 24 mothers of newborn patients and in all adults. Pregnancy was uneventful in all cases until the end of pregnancy; 16 mothers had a short course of fever — most frequently with symptoms of an acute upper respiratory tract infection prior to delivery. Four mothers had a subclinical unrecognized course. Five of 20 mothers were consuming raw unpasteurized milk and nine of 20 mothers were eating vegetables from biologic agriculture during pregnancy. Ten of 20 mothers lived on or visited farms during pregnancy. Contact with sick animals was denied in all cases. All adult patients had consumed raw uncooked milk; two of four had received vegetables from biologic agriculture. Three of four were living on or visiting farms; none has had contact with sick animals.

During this epidemic *L. monocytogenes* was also isolated in several instances from contaminated cheese. Determination of lysotypes of four listeria strains (serovar 1/2a) isolated from cheese yielded the lysotype 44/77/12029/10/43. Lysotype 10/43 was found seven times in eight strains (serovar 1/2a) isolated from human sources; an identical lysotype as in cheese was only isolated once.

Five of 28 patients died. Two infants died prior to diagnosis and administration of antimicrobial agents. A late sequela was observed in one infant (hydrocephalus). Two other infants who died and the one who suffered from hydrocephalus had received cephalosporines (cefuroxime, cefoxitin, cephalothin). An adult with listeria-septicaemia and compromised host defense was treated with chloramphenicol. Despite of rapid defrevescence, no improvement in the clinical condition was observed and the patient died on day five of the therapy. Ampicillin alone (three patients), a combination of ampicillin with an aminoglycoside (14 patients), ampicillin with penicillin (two patients) and penicillin in combination with an aminoglycoside (three patients) were used successfully in the treatment of our patients; no difference was observed in the immediate clinical response and the ultimate outcome of patients.

Discussion

The consumption of raw milk and biologically grown vegetables as possible sources of infection in our patients cannot be dismissed. Usually in Tirol 92% of the consumed milk is pasteurized and processed in dairy farms; only 8% is directly sold by farmers. Seeliger reported in 1951 the consumption of raw uncooked milk as a suspected cause of a listeria epidemic in Halle, GDR [5]. Also cream, sour milk and cheese were reported as a potential source of infection [2]. Although *L. monocytogenes* was repeatedly isolated from cheese — in our outbreak — this seems to be of lesser importance; only in one child was an identical lysotype found. Vegetables have been incrimi-

nated as an important source of infections [6–8]. It seems that vegetables grown under conditions of biological agriculture pose an increasing hazard over products grown with artificial fertilization.

While *L. monocytogenes* infections seem to be primarily a food-borne disease listeriae can be a resident of the faecal flora [9]. It is likely that the source of infection in our 73-year-old patient, who fell ill after incision of a perianal abscess, originated from the bowel flora. Perianal operations could present a potential risk of introducing these bacteria into the bloodstream.

The contact with sick animals as a source of human infection seems to be negligibly low. This correlates well with results obtained by other authors [9].

Various antimicrobial agents have been used in the treatment of listeria infections. However, there is still a debate about the most effective therapy of this serious illness. From previous reports in the literature and from our own data it is quite clear that a listeria-infection should not be treated with cephalosporines alone or in combination. Our data also suggest that therapy with chloramphenicol — despite of adequate in vitro sensitivity and an excellent CSF penetration — is unsatisfactory. Many in vitro investigations on combinations of ampicillin with aminoglycosides have shown a synergistic mode of action [10]. In our experience there was no difference in the therapeutic outcome between the use of a combination of ampicillin or penicillin with an aminoglycoside or of ampicillin alone; all were efficacious. Hof and Gückel [11] reported the lack of synergism of ampicillin and gentamicin in experimental listeriosis using mice; using a microcalorimetric apparatus we recently demonstrated a missing synergism between ampicillin and gentamicin in vitro [12]. The use of a single effective antimicrobial agent with relatively little toxic effect, such as ampicillin, could be preferred to the use of a combination of ampicillin with an aminoglycoside with potentially serious toxic effects; we believe the ultimate test of superiority of a therapeutic regimen must come from further clinical studies.

REFERENCES

1. Bille, J., Glauser, M. P., Mean, F., Aubort, J. D., Nicolet, J., Breer, C., Schällibaum, M., Gutzwiller, F., Rickenbach, M.: Bulletin de l'Office fédéral de la santé publique **50,** 413 (1986).
2. James, S. M., Fannin, S. L., Agee, B. A.: Morbid Mort Weekly Rep **34,** 357 (1985).
3. Schuster, G., Borkhardt, H. L.: Z Gesamte Hyg **33,** 261 (1987).
4. Hirschl, A., Rotter, M., Koller, W., Stanek, G.: Wien Klin Wschr **92,** 44 (1980).
5. Seeliger, H. P. R.: Listeriosis. Hafner, New York 1961.
6. Bryan, F. L.: In Riemann, H. (ed.): Food-borne Infections and Intoxications. Academic Press, New York—London 1969. pp. 272–273.
7. Rocourt, J., Seeliger, H. P. R.: Médecine et Maladies Infectieuses **15,** 721 (1985).
8. Schlech, W. F., Lavigne, P. M., Bortolussi, R. A.: N Engl J Med **308,** 203 (1983).
9. Emmerling, P., Schröter, G., Hof, H.: Med Welt **36,** 1517 (1985).
10. Azimi, H., Korányi, K., Lindsey, D.: Amer J Clin Pathol **72,** 974 (1979).
11. Hof, H., Gückel, H.: Infection **15,** 40 (1987).
12. Allerberger, F.: Thermochimica Acta **119,** 113 (1987).

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LISTERIA-MENINGITIS AND -SEPTICAEMIA IN IMMUNOCOMPROMISED PATIENTS

S. ORTEL

Institute of Medical Microbiology and Epidemiology,
Reference Laboratory for Listeriosis, Martin Luther University, Halle/S., GDR

In the period from 1980–86 we obtained 51 strains of *Listeria* from meningitis in adults for serotyping and phage-typing. Ten strains were associated with meningitis and 3 with septicaemia of immunocompromised patients. They suffered from leukaemia, diabetes, Hodgkin's disease, alcoholism, lupus erythematodes. The lethality rate in these patients was 70%, in other patients with meningitis 30%. Phage typing has shown that 4b strains were often determined by the phage-code 00010 and similar codes. This phage-pattern might be specific for meningitis strains. The immunocomprised patient is especially endangered in taking up listeriae from the environment, but it must also be in consideration that listeriae may easy gain access from the gut into the vessels.

In the past years we observed more frequently *Listeria monocytogenes* meningitis in adults. The illness becomes often fatal, in spite of an adequate therapy with ampicillin and gentamicin. Meningitis and listeria-septicaemia were especially observed in immunocompromised older patients in which the immundefence was injured by diabetes, leukaemia, meagerness, alcoholism, lupus erythematodes and other diseases.

Materials and methods

The strains for serotyping and phage-typing were obtained from bacteriological laboratories of the GDR. Phage typing was done in a multi-centre study [1], serotyping with factor-sera prepared by Impfstoffwerk Dessau (GDR). Clinical data, as far as available, were collected from the histories of patients treated in different hospitals.

Results

The cases which we have observed can be distributed into 4 groups. Table I shows this classification. In group I we summarize 41 cases of meningitis in patients with normal defence without co-existing diseases. The number of male and female patients was nearly the same, the death rate 30%. In

SIEGFRIED ORTEL
Institute of Medical Microbiology and Epidemiology, Martin Luther University
Leninallee 6, 4020 Halle/S. GDR

Table I

Listeria-meningitis in adults with normal defence and in immunocompromised patients including septicaemia and local listeria-infections

Patient-group	Number	m	f	Died			Died %
				m	f	Together	
I. Listeria-meningitis in adults with normal defence	41	20	21	6	6	12	30
II. Listeria-meningitis in immunocompromised patients	10	3	7	2	5	7	(70)
III. Listeria-septicaemia in immunocompromised patients	3	1	2	1	1	2	(66)
IV. Local listeria-infections (abscesses) in immunocompromised patients	2	0	2	0	0	0	—
Total	56	24	32	9	12	21	37.5

group II we present 10 cases of meningitis in immunocompromised patients. The male and female ratio is 3 : 7. The death rate is very high, 70%. Also in group III, in which we collected 3 cases of listeria-septicaemia in immunocompromised patients, the death rate is about 66%. In group IV we list 2 cases of abscesses in patients with diabetes. It must be mentioned in connection with the death rates that the listeria-meningitis has often contributed to, but it was not always the cause of the death.

Table II

Age distribution in adult patients with listeria-meningitis

Year	m	f	Total		
20—25	3	3	6		
26—30	1	1	2		
31—35	1	2	3		
36—40	1	1	2		
41—45	2	1	3		
46—50	2	2	4		
51—55	3	2	5		
56—60	4	3	7		
61—65	2	4	6		
66—70	3	6	9	36 = 64.3%	
71—75	2	4	6		24 = 43%
76—80	—	2	2		
81—85	—	1	1		
Total	24	32	56		

The age distribution (Table II) shows that 36 patients (~64%) were older than 50 years and 24 (~43%) were older than 60 years. The oldest patient with listeria-meningitis was a female of 85 years.

Tables III, IV and V give a representation about the cases of meningitis, septicaemia and local listeria-infections in immunocompromised patients. The basic illnesses, which were responsible for the damage of the immunsystem were: diabetes, (6); myeloid leukaemia, (3); alcoholism, (2) and meagerness (hypophysical), Hodgkin's disease and lupus erythematodes (one of each).

Table III

Listeria-meningitis in immunocompromised patients

Patient	Sex	Age years	Occupation	Meningitis	Illness	Serovar	Phagovar	Outcome
1	f	23	—	1980	leukaemia	4b	n. typ.	died
2	m	30	invalid rentier	1984	Hodgkin's disease	4b	n. typ.	died
3	f	68	country woman	1985	diabetes	1/2a	n. typ.	died
4	m	60	invalid rentier	1985	diabetes	4b	00010	died
5	m	32	employee	1985	leukaemia	4b	00010	healed
6	f	20	nurse	1985	meagerness	4b	00010	healed
7	f	65	rentier	1985	diabetes	4b	n. typ.	died
8	f	60	rentier	1985	alcoholism	1/2a	n. typ.	died
9	f	46	—	1986	alcoholism	1/2a	n. typ.	died
10	f	65	farmer	1986	diabetes	4b	51604	died

Table IV

Listeria-septicaemiae in immunocompromised patients

Patient	Sex	Age years	Occupation	Year of septicaemiae	Illnes	Serovar	Phagovar	Outcome
11	f	63	rentier	1978	leukaemia	1/2a	n. typ.	died
12	m	70	rentier	1984	cerebrocardial insufficiency	1/2a	21100	died
13	f	50	invalid rentier	1986	lupus erythematodes	1/2a	04200	healed

Table V

Local listeria-infections (abscesses) in immunocompromisedd patients

Patient	Sex	Age years	Occupation	Year of infection	Illness	Serovar	Phagovar	Outcome
14	f	78	rentier	1977	diabetes	4b	n. typ.	healed
15	f	79	rentier	1985	diabetes	1/2a	n. typ.	healed

The clinical symptoms were typical for meningitis: high temperatures up to 39.8 °C, shivering, headache, stiffness of the neck, unconsciousness and coma. In the CSF high numbers of cells (5000–10 000) were shown.

The results of phage typing of the 15 *Listeria* strains which were isolated from CSF, blood and abscesses of the immunocompromised patients show that none of the 1/2a serovar strains from meningitis cases was typeable, whereas 2 strains isolated from blood were determined by Codes 21100 and 04200. Out of serovar 4b strains from immunocompromised patients 3 belonged to Code 00010 and one to Code 51064. The typing-results of the 36 strains from meningitis (Table VI) show that from the 10 1/2a strains only two were typeable. They were determined by Codes 20024 and 22100. But it might be of interest, that in 4b Code 00010 and similar strains as for instance 00110, 00100 and 00001 were frequent. We raise the question, if these phage-types might be specific for meningitis strains, similarly as in *Staphylococcus aureus*, where the phage-pattern "71" is supposed to be specific for impetigo.

The antibiotic-susceptibility of the isolated *Listeria* strains showed a good susceptibility to ampicillin, co-trimoxazole, erythromycin, carbenicillin, azlocillin and mezlocillin. The known resistance to cefuroxim, cefotaxim and cefotiam could be confirmed. Therefore, the last-mentioned antibiotics should not be used in the treatment of listeriosis in any case, especially not in meningitis. Based on our own investigations with 100 strains of *L. monocytogenes* of the serovars 1/2a and 4b, ciprofloxacin had a very good effect on listeriae [2]. The MIC's ranged between 0.5 to 2.0 mg/l and were lower than the MIC's of norfloxacin and ofloxacin [3, 4].

Considering the above findings, in the therapy of meningitis and septicaemia a correct treatment was prescribed in most cases. Ampicillin and gentamicin or kanamycin combinations were often given. Oxytetracycline, high

Table VI

Phagovars of 36 Listeria strains isolated from meningitis in adults

Serovar 1/2a (10 strains)		Serovar 4b (26 strains)	
Code	20024 (1+)	Code	00010 (7×)
	22100 (1×)	Code	00110 (4×)
		Code	00100 (1×)
Untypeable	8 strains	Code	00001 (1×)
		Code	00210 (1×)
		Code	00400 (1×)
		Code	01651 (1+)
		Code	01401 (1×)
		Code	51604 (1×)
		Untypeable	8 strains

doses of penicillin (40 million IU/day) as an initial therapy, followed by ampicillin were effective. In one case continuous infusion of ampicillin and intramuscular gentamicin resulted in a recovery. Azlocillin was also effective. In the treatment of listeria-meningitis the application of ciprofloxacin and its combination with ampicillin are of especial significance. This therapy might be important in severe cases of listeria-meningitis and -septicaemia.

Discussion

Our investigations about listeria-meningitis and -septicaemia, especially in immunocompromised patients, have shown that meningitis cases in adults and older persons are frequently connected with a high lethality rate. Patients with immune deficiency are especially endangered by infection with listeriae from the environment. Also the possibility that listeriae immigrate from the gut into the vessels must be taken into consideration.

It should also be added that the elderly patient often exhibits an unhygienic behaviour and therefore the uptake of listeria from his environment is favoured. It might be otherwise of great interest that the patient with an acquired immune deficiency syndrome (AIDS) is not especially endangered by *L. monocytogenes*, which is not a common pathogen in patients with AIDS. Jacobs and Murray [5] observed that listeriae rarely infect AIDS-patients. This is as yet curious and unexplained. The reasons for the unexpectedly low incidence if listeria-infections in patients with AIDS might be based on genetic factors or epidemiological influences, for instance an alteration of the intestinal flora in these patients, (most homosexuals having a history of gastrointestinal tract infections), and multiple courses of antibiotic therapy which perhaps alter their flora and thus decrease exposure to *L. monocytogenes*. But also immune and non-immune defence mechanism may be part of this problem which remains as yet unsolved.

REFERENCES

1. Rocourt, J., Audurier, A., Courtieu, A. L., Durst, J., Ortel, S., Schrettenbrunner, A., Taylor, A. G.: Zentralbl Bakteriol (A) **259,** 489 (1985).
2. Brettschneider, W., Wilhelms, D., Sandow, D., Ortel, S.: Zschr Klin Med **44,** 793 (1989)
3. Reynaud, A. E., Espaze, E. P., Papin, S. A., Courtieu A. L.: Proceedings of the 9th International Symposium on the Problems of Listeriosis, Nantes 17–20, Septembre, 1985. Université de Nantes, Nantes 1986. pp. 449–455.
4. Schrettenbrunner, A., Schröter, G., Rocourt, J.: Proceedings of the 9th International Symposium on the Problems of Listeriosis, Nantes 17–20 September, 1985. Université de Nantes, Nantes 1986. pp. 443–448.
5. Jacobs, J. L., Murray, H. W.: Arch Int Med **146,** 1299 (1986).

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THE TREATMENT OF *LISTERIA MONOCYTOGENES* INFECTIONS IN THE CENTRAL NERVOUS SYSTEM

ANA CARVAJAL, SUSANNE SAMUELSSON, N. P. ROTHGARDT and W. FREDERIKSEN

State Serum Institute, Copenhagen, Denmark

Fifty-four cases of *Listeria monocytogenes* meningitis and meningoencephalitis occurred in Denmark between 1981 and 1986. The aim of the present study was to consider the antibiotic treatment of these patients, 33 males and 21 females aged from 16 months to 91 years: average (AV) 54 years. One or more predisposing factors (PF) were found in 30 patients (55%): steroid treatment (15), cancer (12), alcoholism and/or cirrhosis (10), diabetes mellitus (5), and renal allograft (2). Follow-up varied from 3 months to 5 years. Ampicillin (AMP) was used as the only antibiotic in 14 patients, and with an aminoglycoside (AMI) in 38 but AMI was withdrawn within 24–48 h in 14. High doses of AMP were used (AV 16 g/day) generally for two weeks (AV 11 days), but AMI was usually withdrawn in the first week (AV 5 days). The mortality rate (MR) was 39% and varied greatly between previously healthy patients (PHP) and those with one or more PF: only 2 out of 24 PHP died, both admitted to hospital with respiratory insufficiency and shock. No significant differences between survivors and non-survivors were observed either in the doses, duration and administration of AMP, or use of AMI, although a higher number of PF was found in the non-survivors. Relapse occurred in at least 3 immunocompromised patients. Sequelae were reported in 9 patients. Death occurred in 6 cases within 24–48 h of admission. Delayed diagnosis and treatment together with the underlying disease seemed responsible for the high MR. More effective treatments in immunocompromised patients should be tried.

The recommended antibiotic for the treatment of *Listeria monocytogenes* infections in the central nervous system (CNS) is penicillin G or ampicillin (AMP) [1–3]. Combination with an aminoglycoside (AMI) has been widely suggested, because of the synergistic effect [1–4]. However, this combination is not generally accepted [4–6] and the optimal therapy of listerial CNS infections remains to be determined [1, 4]. The use of erythromycin, chloramphenicol, and vancomycin for patients allergic to penicillin has produced controversial results [3, 4, 7–9] and co-trimoxazole is increasingly being used because of its good penetration into the CNS and the possibility of extended oral administration after clinical recovery [2, 7, 10].

We reviewed the treatment of *L. monocytogenes* CNS infections in a homogeneous population with similar medical care, in order to see whether there were any differences in the treatment used, antibiotic combinations, dosage, and duration.

ANNA CARVAJAL
32, Artemidos Str., 166 73 Ano Voula Athens, Greece

SUSANNE SAMUELSSON, N. P. ROTHGARDT, WILHELM FREDERIKSEN
State Serum Institute
80, Amager Boulevard, DK-3400 Copenhagen S., Denmark

Materials and methods

The materials and methods have been described in other reports presented at this Symposium. Briefly, the study comprised 54 confirmed *L. monocytogenes* CNS infections, reported in patients other than neonates during a six-year period, 1981–87. Case histories were retrospectively reviewed and a questionnaire was filled in for each patient. The data on the antibiotic treatment and other details considered relevant for the prognosis of the patients are presented here. Follow-up varied from three months to five years. Thirty-three (61%) of the patients were male and the age range was 16 months to 91 years, with a mean of 54 years. There were four children under 10 years (Table I). Thirty out of 54 patients (55%) had at least one pre-existing condition known to favour listerial infections, and a higher number of these underlying conditions (UC) (42) was found in patients who died than in survivors (16) (Table II).

Table I

L. monocytogenes CNS infections: age and sex distribution

Age (years)	Male	Female	Total
1—10	1	3	4
11—20	0	0	0
21—30	4	2	6
31—40	1	1	2
41—50	6	3	9
51—60	7	3	10
61—70	8	1	9
71—80	5	5	10
>81	1	3	4
Total	33	21	54

Male/female ratio: 1.5

Table II

CNS infection: underlying conditions

	Survivors (10)	Non-survivors (20)
Malignancy	2	10
Steroid treatment	3	12
Other immunosuppressives	2	7
Alcoholism	4	3
Cirrhosis	2	1
Renal transplant	—	2
Diabetes mellitus	2	3
Miscellaneous	1	4
No. patients with more than one	3	12

Results

Initial antibiotic treatment. Six patients, namely two previously healthy patients (PHP) and four with UC received oral penicillin or oral AMP before admission. Pre-diagnostic antibiotics were given in hospital to 19 patients, only 4 of whom were PHP, for 1 to 3 days: oral penicillin (1), intravenous penicillin G alone (5) or with other antibiotics (3), AMP alone (1) or with an AMI (5), cefotaxim alone (1), and cephalosporin plus AMI (3).

Treatment of L. monocytogenes infection. When listerial infection was suspected AMP was the only antibiotic given in 14 cases (10 PHP) and in combination with an AMI in 38 cases, but the AMI was withdrawn in 14 patients within 24–48 h. High doses of intravenous AMP were used in all cases (average 16 g/day) generally for two weeks (average 11 days), but AMI was usually given for a shorter period than AMP (average 5 days). During treatment the AMP dosage was reduced in 14 cases and in two patients a small intramuscular dose of 2 g/day and 1 g/day followed the initial high dose of intravenous AMP. After the conventional intravenous treatment, oral AMP was given to 6 patients and 3 others received oral co-trimoxazole after intravenous treatment. Another patient received intravenously erythromycin 2 g/day plus intravenously AMP for 7 days after withdrawal of AMI. Five patients died during the first 24–48 h of treatment, one on AMP alone and 4 on AMP plus AMI. Another received cefuroxime plus tobramycin plus metronidazole for the 48 h before death, and one received no antibiotics. Relapse occurred in at least three patients and one of them had two microbiologically confirmed relapses before discharge to another hospital where the clinical history was not available. Unsuccessful treatment with chloramphenicol was given in two cases, and low-dose intramuscularly AMP in the third. Sequelae were reported in 9 patients: hemiparesis, aphasia, disphasia and facial paralysis.

Other treatment measures. Artificial ventilation was given to 16 patients and peritoneal dialysis to one.

Complications. Pleuropulmonary disease was reported in 21 cases, shock in 12, renal impairement in 11, and disseminated intravascular coagulation in one.

Outcome. The delayed mortality rate was 39%, but in 3 cases the cause of death could not be directly related to *L. monocytogenes* infection. The mortality rate of PHP and patients with underlying conditions varied widely: only two out of 24 PHP died, an 88-year-old woman and a 51 year-old-man, both admitted to hospital with respiratory insufficiency and shock.

Necropsy reports were available in 12 cases, but postmortem examination of the CNS was done in only 8, and in none of them were microbiological cultures done. Pulmonary involvement was reported in 9, encephalitis in 5 and pericarditis in one case.

Discussion

No differences were found in the antibiotic treatment of survivors and that of non-survivors, but the mortality rate depended on underlying diseases and predisposing factors, as reported in other studies [1, 4, 11]. Old age is considered a predisposing factor in some studies [11]. Pulmonary involvement and shock worsened the prognosis in this series. Death occurred in six cases within 24–48 h of admission and late diagnosis could be responsible for the outcome. AMP alone was as effective as AMP combined with AMI, as used in this study. Reduction of the AMP dose and withdrawal of AMI after a few days of treatment, as seen in this series, has no pharmacological support: the AMP level in the CNS fell during convalescence and the synergistic effect of AMP plus AMI should be tried in order to achieve a bactericidal effect at the site of the infection to prevent relapse [1, 2, 8]. The use of oral AMP after intravenous treatment given in 6 cases in this series does not seem to be justified, because it fails to penetrate the CNS [2]. Other drugs able to reach the site of infection by the oral route have been used successfully in the treatment of *L. monocytogenes* CNS infections [2, 7, 10].

High doses of penicillin or AMP are generally preferred in the treatment of listerial infections [3–5, 9]. Although the duration of treatment is not well defined, the high mortality rate in immunocompromised patients and the relapse rate would suggest a period of 3–6 weeks [1, 3, 4, 7, 9, 10].

From the findings it may be concluded that (i) the mortality rate in *L. monocytogenes* CNS infections depends on the underlying condition of the patients; (ii) AMP alone was as useful as the combination of AMP and AMI used in this study; (iii) the initial dose of the antibiotics, which permits clinical recovery, should not be reduced during the treatment unless there are other reasons; (iv) when a synergistic combination of AMP plus AMI is chosen for the treatment of listerial infections, AMI should not be withdrawn unless there are other reasons; (v) more effective antibiotic regimens should be tried in immunocompromised patients; longer treatment and the use of oral drugs able to reach the CNS are advisable.

REFERENCES

1. Armstrong, D.: In Mandell, G. L., Douglas, R. G. Jr., Bennett, J. E. (eds): Principles and Practice of Infectious Diseases. 2nd ed. John Wiley, New York 1985. pp. 1177–1182.
2. Kucers, A., Bennett, N. Mck (eds): The use of antibiotics. A comprehensive review with clinical emphasis. 4th ed. William Heinemann, London 1987.
3. Hooper, D. C., Pruitt, A. A., Rubin, R. H.: Medicine (Baltimore) **61,** 166 (1982).
4. Bouvet, E., Suter, F., Gibert, C., Witchitz, J. L., Bazin, C., Vachon, F.: Scand J Infect Dis **14,** 267 (1982).
5. Hansen, P. B., Jensen, T. H., Lykkegaard, S., Kristensen, H. S.: Scand J Infect Dis **19,** 55 (1987).

6. Hof, H., Gückel, H.: Infection **15,** 40 (1987).
7. Günther, G., Philipson, A.: Rev Infect Dis **10,** 53 (1988).
8. Richards, J., Swann, R. A., Ponton, A. W. G.: J Infect **16,** 65 (1988).
9. Watson, G. W., Fuller, T. J., Elms, J., Kluge, R. M.: Arch Intern Med **138,** 83 (1978).
10. Spitzer, P. G., Hammer, S. M., Karchmer, A. W.: Rev Infect Dis **8,** 427 (1986).
11. Pollock, S. S., Pollock, T. M., Harrison, M. J. G.: Quarterly J Med **53,** 331 (1984).

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THE TREATMENT OF *LISTERIA MONOCYTOGENES* SEPTICAEMIA

ANA CARVAJAL, SUSANNE SAMUELSSON, N. P. ROTHGARDT and W. FREDERIKSEN

State Serum Institute, Copenhagen, Denmark

Thirty cases of *Listeria monocytogenes* septicaemia occurred in Denmark between 1981 and 1986. The aim of this study was to consider the treatment of these patients, 18 males and 12 females aged from 20 to 87 years: average (AV) 65 years. One or more predisposing factors (PF) were found in 90% of the patients, mainly cancer (16), steroid treatment (12), cirrhosis and/or alcoholism (8), and diabetes mellitus (3). Follow-up varied from 3 months to 5 years. Ampicillin (AMP) alone or with an aminoglycoside (AMI) was the treatment in 9 and 16 cases, respectively. One patient was successfully treated with penicillin G and another received oral co-trimoxazol after recovered with carbenicillin plus AMI. AMP doses were lower than used in listerial meningitis (AV 5 g/day vs. 16 g/day), and the duration was variable: from one to 21 days (AV 8 days). The mortality rate was 50%. No significant differences between survivors and non-survivors were observed either in the antibiotic treatment (doses, duration, administration, and use of AMI), or the number and kind of PF found. The cause of septicaemia could not be established in most cases but 3 endocarditis, 2 perianal abscesses and one pericarditis were found in the non-survivors. Pulmonary involvement was present in 13 patients and CNS infection suspected in 10. Early diagnosis, adequate doses and duration of antibiotic treatment, and the use of drugs capable to penetrate purulent collections (microabscess and abscess formations) should improve the prognosis of *L. monocytogenes* septicaemia.

Listeria monocytogenes septicaemia in adults other than pregnant women and neonates is not well-characterized clinically; it will present as an acute septicaemia, unsuspected and of unknown origin until a blood culture reveils the microorganism. "Primary" bacteraemia due to *L. monocytogenes* is reported with increasing frequency and it is the second commonest clinical form of listeriosis after infections of the central nervous system (CNS) [1–6]. Although penicillin G and ampicillin (AMP) are the most used antibiotics in many studies, the prognosis of *L. monocytogenes* septicaemia varied greatly [2–6]. We reviewed the treatment of *L. monocytogenes* septicaemia in Denmark during a six-year period, 1981–1987, in order to assess the prognosis of the infection and establish whether there were any differences in the antibiotic treatments given.

ANA CARVAJAL
32, Artemidos Str., 166 73 Ano Voula Athens, Greece

SUSANNE SAMUELSSON, N. P. ROTHGARDT, WILHELM FREDERIKSEN
State Serum Institute
80, Amager Boulevard, DK-3400 Copenhagen S., Denmark

Materials and methods

The materials and methods have been described in other reports presented at this Symposium. Briefly, the study comprised 30 septicaemic patients whose blood cultures grew *L. monocytogenes*. Neonates and pregnant women were not included; patients with established meningitis were also not considered. Case histories were retrospectively reviewed and a questionnaire was filled in for each patient. The data on the antibiotic treatment and other details considered relevant for the prognosis of the patients are presented here. Follow-up varied from 3 months to 5 years. Nineteen (63%) of the patients were male and the age range was 20 to 87 years, with a mean of 65 years. Twenty-seven out of 30 patients (90%) had at least one pre-existing underlying condition (UC) and the only three previously healthy patients were aged 77, 86 and 87 years. The miscellaneous group had ulcerative colitis (2), were receiving treatment with antacid (2), had uraemia and haemodialysis (1), posttransfusional AIDS (1), splenectomy (1), granulocytopenia (1), myxoedema (1), pancreatitis (1), and asthma (1).

Results

Initial antibiotic treatment. None of the patients were reported to have received antibiotic treatment before admission. Pre-diagnostic antibiotics were given to 22 patients, 14 survivors and 8 non-survivors, for one to 10 days: oral AMP (1), intravenous AMP alone (3), or with an aminoglycoside (AMI) (11), AMP with other antibiotics (3), intravenous erythromycin (2), piperacillin with AMI (1), and cephalothin (1).

Treatment of L. monocytogenes septicaemia. When listerial infection was suspected AMP was the only antibiotic given in 7 cases and in combination with an AMI in 15 cases (seven survivors and eight non-survivors). One patient recovered with intravenous penicillin G, 15 million IU/day for 7 days in combination with methicillin, 6 g/day and methicillin alone in the same dose for a further 3 days (*Staphylococcus aureus* was isolated from the sputum) Another patient was successfully treated with carbenicillin, 30 g/day plus tobramycin for 16 and 27 days, respectively (*Pseudomonas aeruginosa* was solated from the sputum; he was given artificial ventilation and had lung abscess). After carbenicillin was withdrawn, oral co-trimoxazole, 800/160 mg was administered every 12 h for 11 more days, and finally oral erythromycin for a further 3 weeks completed the antibiotic treatment for this patient. Penicillin G, 3 million IU/day for 6 days, followed by oral AMP (1.4 g/day for 14 days) was given to one patient with rectal carcinoma, who died during the oral treatment. No autopsy was done. Another patient received intravenously erythromycin, because of penicillin allergy, 1.0 g/day for 2 days, followed by oral erythromycin in the same dose of 1.0 g/day for 14 days. Two weeks after this treatment the patient died in another hospital with pulmonary emboli and septicaemia. No autopsy report was available.

The intravenous AMP dosage, the most used antibiotic given to 25 patients varied from 2–15 g/day, but the majority of the patients received 4–6 g/day. The duration of the treatment also varied: from 1 to 21 days

(average 8 days). Two patients died during the first 24–48 h of AMP plus AMI treatment and another patient received no antibiotics.

Oral treatment after intravenous treatment. Oral AMP was given in 7 cases, 4 survivors and 3 patients who died, and another 4 patients received oral erythromycin (3) and co-trimoxazole (1). Other antibiotics were metronidazole given to one patient with ulcerative colitis for 21 days; seven patients received short treatments for 24–48 h: fucidic acid, cephalothin, cephalexin, cefotaxim and metronidazole.

Other treatment measures. Artificial ventilation was given to one patient.

Complications. Pulmonary involvement was reported in 13 cases (5 survivors), shock in three, and renal impairment in two. CNS infection was suspected in 10 cases, one of them with focal neurological symptoms (facial paralysis), endocarditis was suspected in two, perianal abscess occurred in two and lung abscess in one patient.

Outcome. Fourteen patients died, 14 recovered and no follow-up was possible in two cases. Death could not be directly related to *L. monocytogenes* infection in two cases.

Autopsy reports were available in 8 cases. Pulmonary involvement was reported in all autopsies, endocaridits in 3 and pericarditis in one case. Neither post-mortem examination of the CNS nor microbiological cultures were done in any patient.

Discussion

L. monocytogenes bacteraemia was considered in this study when the microorganism was isolated from the blood cultures of the patients and samples from other sites, if done, were negative. Endocarditis, pericarditis, and abscesses were therefore also included. It was not possible to establish as to whether these conditions were the source of the infection or a secondary site of *L. monocytogenes* infection. "Uncomplicated" bacteraemia, as reported in other studies [3, 6] or as observed in pregnant women, was not present in this series. The mean age was higher than that observed in patients with established CNS infections, as was the number of the UC and patients with pulmonary involvement.

Patients with symptoms suggesting CNS infection were found in this and other series [3, 6]. Half of the patients died. The reported mortality rate in *L. monocytogenes* septicaemia varied highly in the different studies: from 11 to 88% [2–6]. This might be because the term bacteraemia is used for transitory bacteraemia, urinary tract infection, or for such severe infections as cerebritis, hepatitis, abscesses, endocarditis, and pericarditis [3, 6–11].

A special comment is called for when pulmonary involvement occurs in the presence of *L. monocytogenes* bacteraemia. It has been recognized that

pulmonary involvement is a feature of disseminated infection and it should be considered as a possible primary or secondary site of *L. monocytogenes* infections in immunocompromised patients [1], as was indeed seen in this study. Pulmonary involvement, the diversity in the presentation of *L. monocytogenes* CNS infections, and the fact that their presence may be unclear in some of the patients in this and other studies [1—3, 12] makes it advisable to treat "primary" bacteraemia in the same way as meningitis [6].

The AMP doses used in this series were lower than those used in CNS infections (averaging 5 g/day versus 16 g/day) and the duration of treatment was shorter (averaging 8 days versus 11 days). The small number of patients receiving treatments other than AMP does not permit any conclusion to be drawn in this study, although various other treatments have proved useful in other studies [3, 9, 12]. Post-mortem examinations of the CNS and microbiological cultures of patients who died of *L. monocytogenes* bacteraemia would help to define the source and the possible secondary sites of the infection, which could not be determined in this and other studies [1–3, 5, 6].

It may be concluded that (i) all possible diagnostic methods should be used to establish "primary" *L. monocytogenes* bacteraemia, as a part from cases with local sites of infection (e.g. CNS); (ii) pulmonary involvement as a secondary infection should be considered; (iii) high doses of AMP or penicillin G should be used, specially in immunocompromised patients with symptoms suggestive of CNS or endocarditis and/or pulmonary infection; (iv) microbiological studies and CNS examination should be done at autopsy of patients who died from *L. monocytogenes* bacteraemia in order to improve our knowledge of this severe condition.

REFERENCES

1. Ananthraman, A., Israel, R. H., Magnussen, C. R.: Respiration **44,** 153 (1983).
2. Larsson, S., Cronberg, S., Winblad, S.: Acta Med Scand **204,** 503 (1978).
3. Nieman, R. E., Lorber, B.: Rev Infect Dis **2,** 207 (1980).
4. Samra, Y., Hertz, M., Altmann, G.: Postgrad Med J **60,** 267 (1984).
5. Winslow, D. L., Holloway, W. J., Scott, E. G.: Infect Dis Rev **6,** 187 (1981).
6. Stamm, A. M., Dismukes, W. E., Simmons, B. P., Cobbs, C. G., Elliott, A., Budrich, P., Harmon, J.: Rev Infect Dis **4,** 665 (1982).
7. Al-Dajani, O., Khatib, R.: J Infect Dis **147,** 961 (1983).
8. Carvajal, A., Frederiksen, W.: Rev Infect Dis **10,** 616 (1988).
9. Holoshitz, J., Schneider, M., Yaretzky, A., Bernheim, J., Klajman, A.: Am J Med Sci **288,** 34 (1984).
10. Hardie, R., Roberts, W.: J Infect **8,** 256 (1984).
11. Yu, V. L., Miller, W. P., Wing, E. J., Romano, J. M., Ruiz, C. A., Bruns, F. J.: Am J Med **73,** 773 (1982).
12. Scheer, M. S., Hirschman, S. Z.: Mt Sinai J Med **49,** 411 (1982).

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LATENT LISTERIOSIS — ITS DIAGNOSIS BY BACTERIOLOGY AND SEROLOGY IN FIVE CASES

C. ROMAÑA and MONIQUE SAGE

Sanitation Department, Barcelona, Spain

The failure of agglutination tests in the diagnosis of latent listeriosis (chronic and asymptomatic) are partly due to the fact that deficient antigens in general use have discredited the serology in this infection. However, using good live antigens or antigens devitalized at moderate temperature and conserved by lyophilization, excellent diagnostic results may be obtained by indirect immunofluorescence (IIF). We could confirm this again in five women with a history of pregnancy disorders. All had a positive or strongly positive IIF. *Listeria monocytogenes* could be isolated by cultivation of the cervico-vaginal exudate, cerebrospinal fluid and from the placenta. After one year and more, these mothers continued to have positive IIF, proving that *L. monocytogenes* does not disappear from infected persons, even after a long-lasting treatment.

Under the term of "latent listeriosis" we understand a state of chronic infection by *Listeria monocytogenes* of apparently healthy men and women. The infection is evidenced by indirect immunofluorescence (IIF) of anti-listeric antibodies in the blood at levels above 300, or, in women also by cultures of cervical secretions.

The diagnosis of latent listeriosis by IIF requires the use of antigens that are totally Gram-positive (100%). These could either be live listeria or devitalized listeria, extinguished by moderate heat and then lyophilized. Up to this day we consider the indirect immunofluorescent technique the only adequate serological method for a reliable diagnosis of latent listeriosis. This work wishes to surmount the opposition against the IIF serological diagnostic of the latent listeriosis, if *L. monocytogenes* cannot be isolated.

Case reports

Case 1. Mrs L., 36 years

1978. First pregnancy with a normal delivery.
1980. Second pregnancy. Miscarriage at month two.
1981. Third pregnancy. Surgical delivery at month seven. Isolation of *L. monocytogenes* from the meconium. Child presents with hydrocephalus and tetraplegia. It outlived under antibiotics.

CECILIO ROMANA, MONIQUE SAGE
Department de Sanitat, Generalitat de Catalunya
Avenida Tibidabo, 10, 08022 Barcelona, Spain

1988. Fourth pregnancy: IIF positive at level 1200. Antibiotic therapy to the mother throughout the entire pregnancy. Birth of a normal child. IIF negative.

Case 2. Mrs M., 25 years

1980. First pregnancy. Premature delivery at month six. Child dies after 40 days; no malformation.
1983. *L. monocytogenes* found in cervical secretion. By IIF both parents positive at level 1200. Antibiotic therapy and IIF blood level control prior to a new pregnancy.
1984. Second pregnancy with antibiotics until delivery. Normal delivery of a healthy child. IIF negative.
1988. IIF again positive at level 1200. Initiation of antibiotic therapy prior to a 3rd pregnancy.

Case 3. Mrs MC., 40 years

1986. First pregnancy: Surgical delivery at month seven (fetal distress). *L. monocytogenes* in amniotic fluid and placenta.
1987. IIF control positive at 2400. Antibiotic therapy prior to a new pregnancy.

Case 4. Mrs A., 30 years

1971. First pregnancy: premature delivery at month 6. Child dies two weeks later. Autopsy reveals *L. monocytogenes* in brain, liver and lungs.
1973. Second pregnancy: IIF (+) 800; antibiotic therapy. Normal delivery of a child, which due to insufficient antibiotic treatment of the mother suffers, at day 3, acute listeriosis (IIF + 1600). Complete recovery after ampicillin.
1977. Third pregnancy: IIF (+) 1600. Adequate antibiotic treatment. Normal delivery. Child IIF (−).

Case 5. Mrs C., 23 years

Four pregnancies with miscarriages at months 3, 5, 2 and 4. In the latest abortion *L. monocytogenes* was isolated from the chorionic villi and proved by fetal histopathology. At this time the mother's IIF was positive at 600.

Conclusions

(i) In the above described five cases of latent listeriosis, the isolation of *L. monocytogenes* coincided with a positive serologic IIF finding. (ii) A positive IIF titre, evidence of a latent listeriosis, usually persists even through a long lasting treatment, so that each new pregnancy may pose a new problem.

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LATENT LISTERIOSIS MAY CAUSE HABITUAL ABORTION INTRAUTERIN DEATHS, FETAL MALFORMATIONS WHEN DIAGNOSED AND TREATED ADEQUATELY NORMAL CHILDREN WILL BE BORN

C. Romaña, L. Salleras, and Monique Sage

Sanitation Department, Barcelona, Spain

We endeavoured to prove latent listeriosis (LL), a chronic and asymptomatic infection, in women who suffered from repeated accidents in their pregnancies. By the reduction of immune defences in the pregnant woman, the latent infection may break out again, affecting thus the fetus and causing abortion, stillbirths or malformations. In such women the diagnosis of LL is urgent right from the start of pregnancy, and so is the antilisteria treatment to protect the fetus. Antilisteric antibodies are readily detected by indirect immunofluorescence (IIF), using live antigen or antigen not inactivated by high temperatures of inadequate conservation, as is frequent in currently used agglutination test reactives. Out of the 309 women, 207 had a total of 334 abortions, 67 prematures, 75 stillbirths, 43 malformed living or dead offsprings. High positivity of IIF was the only aetiologic date to explain these accidents. Treatment of new pregnancies of a part of these IIF-positive women led to birth of 152 normal children, negative by IIF for listeriosis. The search for LL at the onset of any pregnancy, same as done in various infections (lues, toxoplasmosis, etc.) is therefore warmly recommended.

In this study, still in progress, we propose to point out the leading part of chronic asymptomatic latent listeriosis in women with a history of repeated abortions, premature newborns and congenital malformations.

Listeria monocytogenes is a widely spread bacterium that occurs in humans and in animals, both domestic and silvatic. Men and women carry it in their digestive and genito-urinary system. During pregnancy, with lowered resistance, the infection may affect the fetus and cause intrauterine death or malformations. Diagnosis of latent listeriosis is easy and reliable, when done by indirect immunofluorescence, provided that an antigen is used, that has not been degraded by procedures involving the inactivation or conservation of the bacteria. Unfortunately the commercially available antigens at present do not fulfil this requirement: due to their inadequate reactivity they have masked up to now the true existence of this infection. We wish to recall, that already in 1960 Rappaport published in Lancet a paper on "Genital Listeriosis as a Cause of Repeated Abortions". This paper has practically been forgotten although the authors could isolate *L. monocytogenes* in 25 habitually aborting women. There were two reasons for this oblivion: (i) a difficulty to

Cecilio Romaña, L. Salleras, Monique Sage
Department de Sanitat, Generalitat de Catalunya
Avenida Tibidabo, 10, 08022 Barcelona, Spain

discover the bacteria in the vaginal exudate; (ii) the usual negativity of serology, generally based on laboratory tests of agglutination.

As a consequence specialists of listeriosis discarded the existence of latent listeriosis giving as an argument the failure of isolating the bacteria and the apparent absence of antilisteric antibodies in the blood.

Clinical histories

The 268 women examined by us had the following clinical histories: 334 abortions, 43 dead prematures, 22 alive prematures, 75 intrauterine deaths, 43 alive or dead malformed newborns.

The most striking malformations were in the antecedent 53 children: cardiopathies 10, hydrocephalus 6, anenchephaly 4, polycystic kidneys 4, bifid spine 3, microcephalia 2, multiples 5, talipes equinus 2, achondroplasty 2, mental retardation 2, paralysis 3, epilepsy 1, cleft lip 1, blindness 1, genito-urinary 2, others 5.

Experimental and conclusions

One of us (C. R.), having a long-time experience in the indirect immunofluorescent diagnosis of toxoplasmosis decided to apply the same method to the diagnosis of listeriosis, using live bacteria of *L. monocytogenes* as an antigen. The results were excellent and from here it was only a short step to discover latent listeriosis. Later, by improving the antigen, we performed the reaction with inactivated and lyophilized bacteria whose antigenic locations had not been destroyed. The results that we present are based on serological tests. We wish to stress, however, that the coincidence between positive IIF reactions and the isolation of *L. monocytogenes* in various cases did confirm to us the value of this immunofluorescent procedure.

The only aetiologic finding in the 268 women examined that could explain the accidents of their pregnancies was the elevated positivity of antilisteric antibodies. On the other hand, once an antilisteric treatment was applied to a great number of these women, the birth of 152 perfectly normal children was attained, most of whom, if not all, were negative for *Listeria*.

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HUMAN LISTERIOSIS IN FRANCE IN 1987

VÉRONIQUE GOULET, E. ESPAZE, I. BASTIDE and I. REBIERE

National Health Laboratory, Paris, and National Listeria Reference Centre, Nantes, France

The surveillance of human listeriosis is based in France on laboratories' reports. From 1983 to 1986 the trend showed a steady increase of the number of cases reported. The highest annual incidence rate was observed in 1986 with 14.7 cases/million population. In 1987 less cases have been reported; about half of the human strains were typed by the National Reference Centre of Listeriosis showing that 62% were 4b, 22% were 1/2a and 13% were 1/2b. Since 1988, the surveillance of listeriosis is computerized using a system of minicomputer terminals (called Minitel) linked via a national telephone network to a large computer. The 70 microbiologists involved in this surveillance must send, on a 24 h basis, data as a soon as they have a new isolate of *Listeria monocytogenes* in their laboratories. An investigation (typing of the strains, case-control study) is made for each cluster detected.

Between 1983 to 1986, according to the notification of isolates in cerebrospinal fluid, human listeriosis seems to have increased in France [1]. The aim of this survey is to evaluate the situation of human listeriosis in France in 1987.

Materials and methods

Cases were identified by the notification of isolated strains of *Listeria monocytogenes* from microbiology laboratories. A questionnaire was send to the 285 microbiologists who had participated at a similar survey in 1984 [2].

Patients were categorized either as maternal/neonatal or adult. A maternal/neonatal case was defined as a case in which *L. monocytogenes* was isolated from a culture of either a pregnant woman or her fetus or a neonate. A mother and neonate pair were counted as one case. An adult case was defined as a case in which *L. monocytogenes* was isolated from a culture of someone other than a pregnant woman or neonate.

Results and discussion

A total of 642 cases were reported by 260 laboratories which represent 62% microbiological hospital laboratories and 67% of beds of acute-care hospitals in France. The incidence rate is 11.6 cases/million population. This

VERONIQE GOULET, I. BASTIDE, I. REBIERE
Laboratoire National de la Santé
25 Boulevard Saint-Jacques, 75014 Paris, France

E. ESPAZE
Centre National de Référence des Listeria
Nantes, France

rate is lower than the real one because not all the French hospitals reported their cases. Thus the incidence can be estimated as around 17 case/million population. Comparing to similar studies performed in 1984 and 1986, the incidence rate is lower than in 1986 and seems similar to that in 1984 [2].

The cases are spread all over the country (Fig. 1). To be able to compare a region to another, an estimated incidence was calculated for each area (Fig. 2). The incidence was estimated taking into account the participation rate of the laboratories in this study. Region "Ile de France" including Paris and its suburbs has the highest incidence rate (28 cases/million population). The west of France which was known to have a lot of human cases of listeriosis in the 970–75 [3] does not have, in this survey, a higher incidence rate than the other regions.

The number of cases registered as materno-neonatal was 332. Isolations were recorded from pregnant woman in 32%, from mothers and her babies in 22%, and from neonates only in 46% of the cases. In pregnant women, isolations were made from blood culture in 55%, from placenta in 45%, and from abortion products in 9% of the cases. In neonates, the most important sites of positive cultures were blood (41%) and cerebrospinal fluid (9%).

Pregnancies resulted in fetal death (26%) and in infected neonate (40%). The case fatality rate of the neonates infected with listeriosis was 26%.

Adults were represented by 310 cases. In 167 cases the microbiologist reviewed the clinical charts to look for a predisposing illness. In 25% of those cases, no predisposing factor for possible immunodeficiency was found. In the 126 other cases, some patients had more than one predisposing illness but only the factor that seemed the most important for immunodeficiency was taken into account: cancer 67, liver diseases 27, diabetes 9, organ transplants 7,

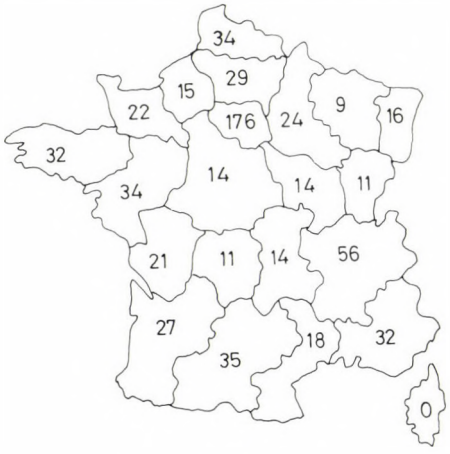

Fig. 1. Human listeriosis in France (1987); geographical distribution of reported cases

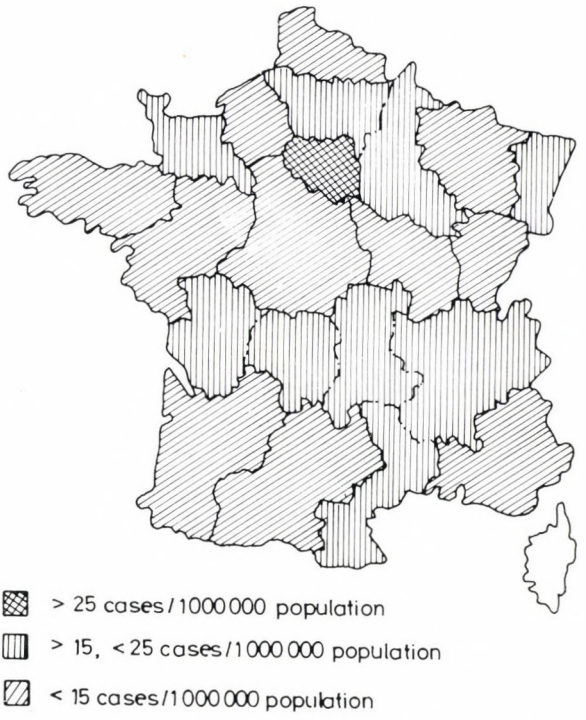

Fig. 2. Human listeriosis in France (1987); geographical distribution of estimated incidence rate

gastrointestinal diseases 6, heart diseases 6, seropositivity to H. I. V. virus 4. In 40% of all adult cases isolates came from cerebrospinal fluid and in 53% only from blood cultures.

The overall mortality rate was 35%. This rate is lower in the group of patients having no predisposing illness (22% as compared to 46% in the other group; $p < 0.02$).

The monthly distribution shows two small peaks of listeriosis in May and October (Fig. 3). The materno-neonatal curve is similar to the total cases curve. It seems that each peak is mainly due to the increase of the materno-neonatal cases. The adults cases are less variable from one month to another. This fact was shown in previous studies made in France [2].

The group of 384 strains of *L. monocytogenes* was reported: 63% group 4; 37% group 1; 269 strains of this study were send to the National Centre of Reference (Professor Courtieu, Nantes) to be typed. The most frequent serovar is 4b (66%), followed by 1/2a (20%) and 1/2b (12%); 3 strains belonged to group 3. The distribution of the serovars is the same for the adults and for the materno-neonatal cases.

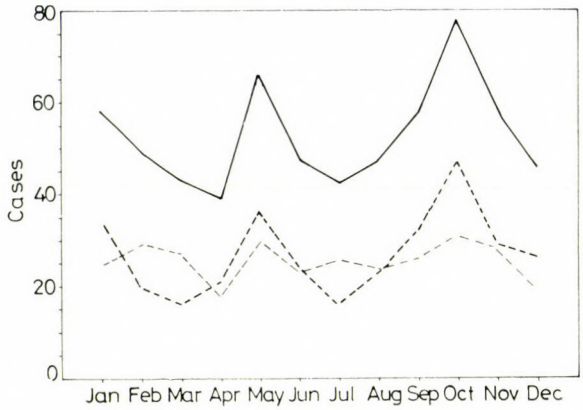

Fig. 3. Human listeriosis in France; monthly distribution in 1987. ——— total cases; - - - - - adult cases; — — — — — maternal-neonatal cases

Summarizing, 642 cases were reported by French hospital laboratories in 1987. No epidemic seems to have occurred during the year. Since food has been incriminated in few outbreaks recently, it seems important to be able to investigate each outbreak. In France the surveillance of listeriosis is made mainly by the National Centre of Listeriosis, and in some cases, reports are made by physicians to the District Health Office when they have an unusual number of cases. Since January 1988, a system based on laboratories reports through a national computer network have started to detect listeriosis clusters as soon as possible. If a cluster is detected by the surveillance, an epidemiological investigation is performed. The patients are questionned on their habits and usual food purchases. The strain of *Listeria* are sent to be phage-typed. If the strains cannot be distinguished by phage-typing, a case-control study is made with another questionnaire. If the same food is incriminated in several cases, investigation is performed by the veterinary authorities.

REFERENCES

1. Goulet, V.: Bull Epid Hebd **29,** 114 (1988).
2. Goulet, V., Leonard, J. L., Celers, J.: Rev Epidém et Santé Publ **34,** 191 (1986).
3. Humbert, G., Duval, C., Fessard, Cl., Meunier, M., Ledoux, A.: Med Mal Infect **6,** 60 (1976).

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3. GENETICS

A GENETIC APPROACH TO DEMONSTRATE THE ROLE OF LISTERIOLYSIN O IN THE VIRULENCE OF *LISTERIA MONOCYTOGENES*

J. Mengaud, Maria Franciska Vicente, J. Chenevert, C. Geoffroy, F. Baquero, J. C. Perez-Diaz and P. Cossart

Unit of Microbial Engineering and Unit of Bacterial Antigens, Institute Pasteur, Paris, France, and Microbiology Service, Ramon Y Cajal Hospital, Madrid, Spain

The locus of insertion of a transposon previously used to obtain a non-haemolytic avirulent mutant was identified: it is the structural gene encoding lisreriolysin O, the thiol-dependent haemolysin, now called *hly*A. The gene was completely sequenced. The preliminary structural and functional study of the chromosomal region containing the gene indicates that *hly*A belongs to a monocistronic transcriptional unit. If it is the case, the transposon insertion would have no major polar effect on downstream genes and would only affect *hly*A expression. These results emphasize the importance of the haemolysin in the virulence of *Listeria monocytogenes*.

Listeria monocytogenes is a Gram-positive bacterium which belongs to the non sporulating clostridia group [1]. It is a facultative intracellular parasite that multiplies in human and animal macrophages and a variety of cell lines [2–5]. Since the pioneering work of Mackaness [6], murine infection by *L. monocytogenes* has been an excellent model for studies of cell-mediated immunity. The purpose of this first part of the introduction is to emphasize that *L. monocytogenes* has also become a model for genetic studies of bacterial virulence determinants of intracellular pathogens.

First, *L. monocytogenes* is not too pathogenic: it is an opportunistic bacterium which essentially affects immunocompromised people and pregnant women. Therefore, it can be manipulated in the laboratory without extreme security conditions, which is not the case, for example, of pathogenic mycobacteria.

The second interesting property of *Listeria* is that it is a fast growing

Jerome M. Mengaud, J. Chenevert, P. Cossart
Unité de Génie Microbiologique, Institut Pasteur
Rue du Dr. Roux, 75724 Paris Cedex 15, France

C. Geoffroy
Unité des Antigenes Bacteriens, Institut Pasteur
Rue du Dr. Roux, 75724 Paris Cedex 15, France

M. F. Vicente, F. Baquero, J. C. Perez-Diaz
Hospital Ramon y Cajal, Servicio de Microbiologia
C. Colmenar KM 9, 600, 28034 Madrid, Spain

bacterium as colonies grow on agar plates in less than two days. This implies that genetics is as easily performed than in *Escherichia coli*.

The third advantage is that genetic tools are now available to study the *Listeria* genome. Conjugative transposons such as Tn*1545* [7] or Tn*916* [8], originally identified in streptococci, can be used to target genes in the *Listeria* chromosome. Conjugation implies close contact, on a filter, between cells carrying the transposon and recipient cells. By a so far unidentified mechanism, the transposon excises from the donor and transfers itself to the recipient where it gets inserted anywhere in the genome. Then a good identification system is needed to detect the mutants of interest. An other genetic tool available is transformation [9]. After protoplast formation, cells are transformed with plasmid DNA in presence of polyethyleneglycol. Alternatively, it is now possible to electroporate the protoplasts. Plasmids able to replicate in *Listeria*, and therefore stably maintained, are those able to replicate in *Bacillus* for example, pMK4, a shuttle vector able to replicate in *E. coli* and *Bacillus subtilis*, as it is a hybrid of pUC8 and pC194 [10]. Other plasmids can be used which present different advantages, for example, pTV1 which carries transposon Tn*917* [11]. This plasmid can be introduced in *Listeria* in a first step by transformation. Since this plasmid has a temperature sensitive replication origin, after raising the temperature, it is possible to select for insertion of the transposon in the chromosome by taking advantage of the drug resistance marker carried by the transposon. This plasmid is thus another tool to target genes in the chromosome. Conjugation can also be performed between *E. coli* and *Listeria* to introduce plasmids in *Listeria*, by use of the recently developed conjugative plasmid pAT187 [12].

Finally, *Listeria* appears as a good model to study the virulence factors of intracellular pathogens as the intravenous or intraperitoneal murine infection is a good animal model: indeed, lesions in humans and mice are very similar. Another model, the "Sprague-Dawley rat" is also used.

The first virulence factor which has been suspected is the haemolysin. The starting observation was that all clinical isolates were haemolytic and that all non-haemolytic *Listeria* were avirulent in the animal model. The first genetic step to evaluate the role of the haemolysin was performed by Gaillard et al. [13]: it was shown that a single copy of transposon Tn*1545* inserted in the genome could abolish both haemolysin production and virulence. A revertant obtained by excision of the transposon recovered haemolytic phenotype and virulence. These results suggested that the transposon had inserted within the structural gene of listeriolysin O or within a gene affecting expression of haemolysin, either by a polar (insertion in an adjacent gene) or a regulatory effect (insertion in a regulatory gene).

In this paper, we present the identification of the gene coding for the SH-activated haemolysin as the locus of insertion of the transposon [14],

the complete sequence of this gene called *hly*A and the characteristic feature of the encoded protein [15], and a preliminary structural and functional description of the chromosomal region which argues in favour of a crucial role of listeriolysin O in virulence.

Results

In culture supernatants of the Tn*1545*-induced non-haemolytic mutant of *L. monocytogenes*, by immunoblotting with an anti-serum raised against purified listeriolysin O, we have detected the presence of a truncated protein of 52 kDa (the secreted listeriolysin O is 60 kDa). This was a first indication that the transposon was inserted in the structural gene. In addition, by taking advantage of the kanamycin resistance gene carried by the transposon, we cloned and sequenced the DNA region corresponding to the locus of insertion. The transposon had inserted in an open reading frame. The homologies detected between the amino acid sequence encoded by this ORF, streptolysin O and pneumolysin demonstrated that the transposon had indeed interrupted the listeriolysin O gene [14].

We then used the cloned fragment to identify in a cosmid previously isolated [16], the DNA fragment containing the complete gene. As shown by immunoblotting of the cellular extracts of *E. coli* carrying the cosmid, the gene is indeed expressed in *E. coli* under its own promoter. The gene was sequenced. It codes for a protein of 529 amino-acids which share strong homologies with streptolysin O and pneumolysin [17] (Fig. 1). This confirms previous observations that all SH-activated haemolysins cross-react. These cytolysins are characterised by the fact that they bind to cholesterol and are inactivated by thiol alkylating agents [18, 19]. It is interesting to note that the three proteins share the strongest homology in an eleven amino-acid peptide which contains the unique cysteine of each of the three proteins and that the transposon, in the mutant, was inserted just before the region coding for this probably essential peptide.

Does the transposon have any effect on other genes or is the inactivation of the listeriolysin O gene by itself responsible for the avirulence of the mutant? To answer this question we first examined the structure of the region containing the gene. As the original cosmid contained some rearrangements, a 3.7 kilobases *Bam*HI fragment was recloned in pUC8 and identified by colony hybridization. The total sequence of the insert was determined (data not shown): *hly*A is followed by an other gene located 340 basepairs downstream from it. There is no known operon having such a long intergenic region. In addition, downstream from the *hly*A gene at a distance of 50 basepairs, we could detect a structure which has all the features of known terminators of transcription. Upstream from *hlyA*, we detected an open reading frame

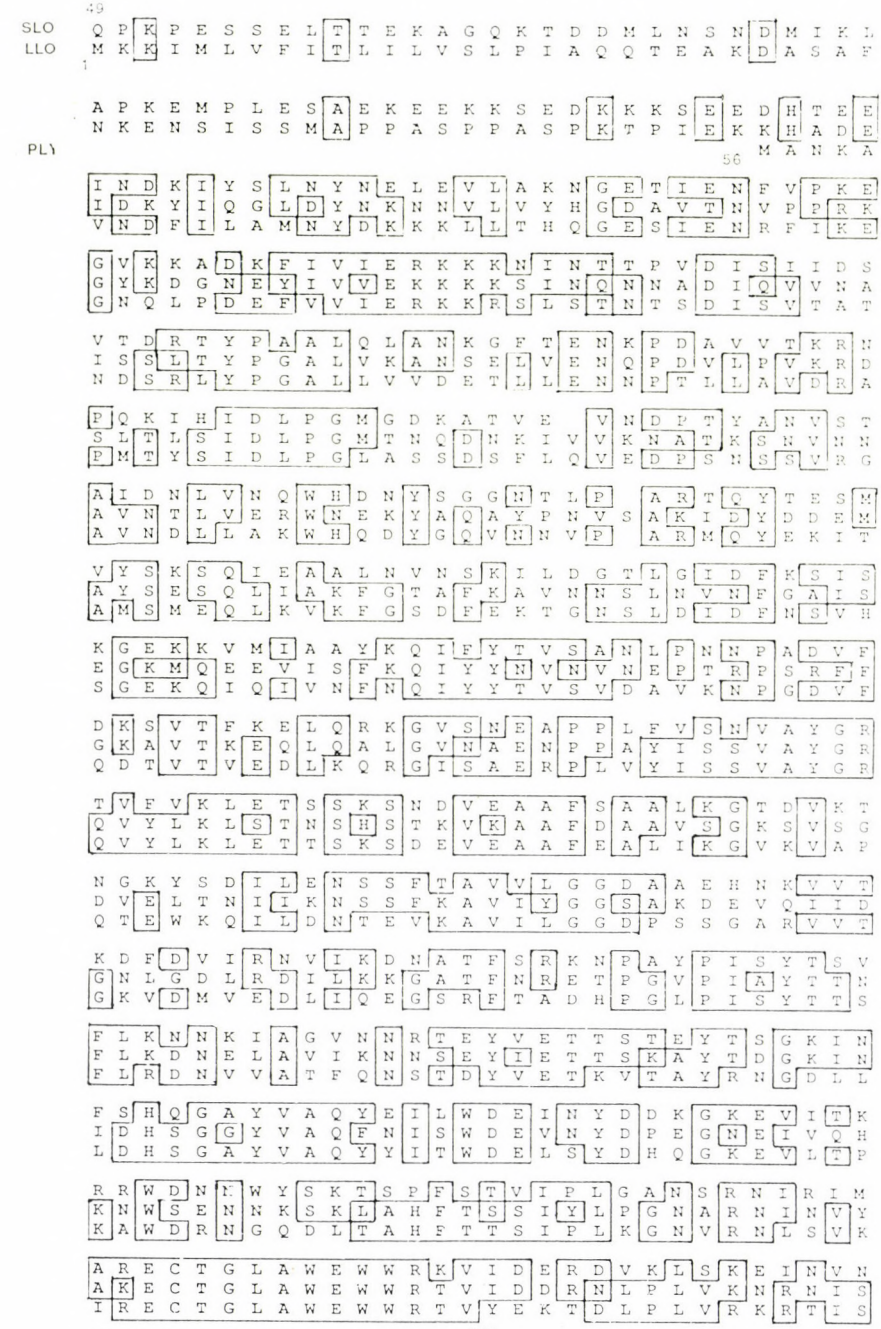

Fig. 1. Alignment of listeriolysin O (LLO), streptolysin O (SLO), and pneumolysin (PLY). Identical amino-acids present in two or three sequences have been boxed. Numbers refer to amino acid coordinates in the sequence

transcribed in the other direction. The distance of *hyl*A to the putative translation initiation codon of the upstream gene is 240 basepairs. These data indicate that *hly*A probably belongs to a monocistronic transcription unit and is the only gene transcribed from its own promoter.

To demonstrate this hypothesis, the promoter region of *hly*A was localized by classical techniques of Sl nuclease mapping and primer extension. These techniques involve preparation of messenger RNAs: in preliminary experiments, RNAs were prepared from exponential cultures (to be reported elsewhere). Under those conditions, the unique promoter region is located 150 basepairs upstream from the ATG initiation codon of *hly*A. By northern blot analysis, we could show that the size of the RNA transcript corresponds exactly to the size of the gene: the RNA starting at the *hly*A promoter is terminating just after the gene. These preliminary results indicate that the haemolysin gene belongs to a monocistronic unit.

Conclusions

We have shown that a transposon affecting the virulence of *L. monocytogenes* had interrupted *hly*A, the structural gene coding for the SH-activated haemolysin, now called listeriolysin O [14]. The gene was then completely sequenced [15]. This is the first gene to be sequenced in *Listeria*. Its GC content was in complete agreement with the calculated values [20]. The preliminary results of the structural and functional study of the chromosomic region containing *hly*A argue that the Tn*1545* insertion had probably no major polar effect on adjacent genes as the listeriolysin O gene seems to belong to a monocistronic unit. Experiments are in progress to examine this aspect more carefully. If these results are confirmed, the transposon insertion would only have affected the expression of listeriolysin O, therefore demonstrating its crucial role in virulence.

REFERENCES

1. Seeliger, H. P. R., Jones, D.: In Sneath, P. H. A., Mair, N. S., Sharpe, N. E., Holt, J. G. (eds): Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore 1986. p. 1235.
2. Farber, J. M., Speirs, J. I.: J Clin Microbiol **25,** 1463 (1987).
3. Gaillard, J. L., Berche, P., Mounier, J., Richard, S., Sansonnetti, P.: Infect Immun **55,** 2822 (1987).
4. Havell, E. A.: Infect Immun **54,** 787 (1986).
5. Portnoi, D. A., Jacks, P. S., Hinrichs, D. J.: J Exp Med **167,** 14 (1988).
6. Mackaness, G. B.: J Exp Med **116,** 381 (1962)
7. Courvalin, P., Carlier, C.: Mol Gen Genet **205,** 291 (1986).
8. Franke, A., Clewell, D.: Cold Spring Harbor Symp Quant Biol **45,** 77 (1980).
9. Vicente, M. F., Baquero, F., Perez-Diaz, J. C.: Plasmid **18,** 89 (1987).

10. Sullivan, M. A., Yasbin, R. E., Young, F. E.: Gene **29,** 21 (1984).
11. Youngman, P., Zuber, P., Perkins, J. B., Sandman, K., Igo, M., Losick, R.: Science **228,** 285 (1985).
12. Trieu-cuot, P., Carlier, C., Martin, P., Courvalin, P.: FEMS Microbiol Lett **48,** 289 (1987).
13. Gaillard, J. L., Berche, P., Sansonnetti, P.: Infect Immun **55,** 2822 (1986).
14. Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J. L., Cossart, P.: Infect Immun **55,** 3225 (1987).
15. Mengaud, J., Vicente, M. F., Chenevert, J., Moniz Pereira, J., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J. C., Cossart, P.: Infect Immun **56,** 766 (1988).
16. Vicente, M. F., Baquero, F., Perez-Diaz, J. C.: FEMS Microbiol Lett **30,** 77 (1985).
17. Kehoe, M. A., Miller, L., Walker, J. A., Boulnois, G. J.: Infect Immun **55,** 3228 (1987).
18. Geoffroy, C., Gaillard, J. L., Alouf, J. E., Berche, P.: Infect Immun **55,** 1641 (1987).
19. Smyth, C. J., Duncan, J. L.: In Jeljaszewicz, J., Wadström, T. (eds): Bacterial Toxins and Cell Membranes. Academic Press, Inc., New York 1978. p. 129.
20. Stuart, S. E., Welshimer, H. J.: Int J Bacteriol **23,** 8 (1973).

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ISOLATION AND CHARACTERIZATION OF GENES CODING FOR PROTEINS INVOLVED IN THE CYTOLYSIS BY *LISTERIA IVANOVII*

A. Haas, J. Kreft and W. Goebel

Institute of Genetics and Microbiology, University of Würzburg, Würzburg, FRG

We established a library of chromosomal DNA of *Listeria ivanovii* in the pTZ19R plasmid system, using *Escherichia coli* DH5alpha as the host. One recombinant clone reacted strongly with a polyclonal antiserum raised against the listeriolysin O and a second exoprotein (24kDa) of *L. ivanovii*, which is most probably also involved in cytolytic processes. The recombinant *E. coli* clone may contain part of the listeriolysin O gene of *L. ivanovii*.

Analysis of bacterial cytolysins by genetic manipulation is of special interest because the genes encoding cytolytic factors can be removed from their natural genetic background and transferred to a different one, e.g., an *Escherichia coli* cell. Thus, detailed biochemical analysis becomes possible. Here we present a study using methods of molecular biology to isolate genes coding for cytolytic factors of the strongly haemolytic *Listeria ivanovii*.

Materials and methods

Bacteria. L. ivanovii strain SLCC 2379 and *E. coli* strain DH5alpha were used.

Isolation of chromosomal DNA of L. ivanovii has been carried out according to [1] with some minor modifications.

Isolation of plasmid-DNA. Large scale plasmid-preparation and cesium chloride density-gradient centrifugation was performed as described in [2], with an additional proteinase and RNase treatment after alkaline lysis. Small scale plasmid isolation was performed according to [3].

DNA-ligations, digestions with restrictions enzymes, DNA/DNA-hybridizations, formation of completent cells were performed as described in [2]. For DNA/DNA-hybridization assays the nitrocellulose was incubated in hybridization buffer for 2 h at 50 °C, the denatured DNA probe was added, and the nitrocellulose incubated overnight at 50 °C. The filter was washed twice with 5×SSC/0.1% SDS at 40 °C for 20 min each. Following autoradiography, the nitrocellulose was washed again with 1×SSC/0.1% SDS at 40 °C for twice 20 min and again used for autoradiography. DNA was labelled using the Boehringer Mannheim random primed labelling kit.

Screening of the library has been performed according to [4] using a polyclonal antiserum raised in rabbits against a mixture of 58kDa (LLO) and 24kDa proteins of *L. ivanovii* (dilution of the antiserum 1 : 1000). The 24kDa protein co-purified by thiodisulfide exchange chromatography of culture supernatants of *L. ivanovii* and could not be separated from the LLO under native conditions.

Albert Haas, Jürgen Kreft, Werner Goebel
Institute of Genetics and Microbiology, University of Würzburg
Röntgenring 11, D-8700 Würzburg, FRG

Immuno (Western-) Blots were performed as described in [5] using an anti-SLO-serum (generous gift of Dr. J. Alouf, Paris: Charge 94) or anti-LLO-serum (as described above), at a dilution of 1 : 1000 and 1 : 100, respectively. To reduce non-specific binding, all sera were preadsorbed to cellular lysates of *E. coli* DH5 alpha harbouring the pTZ19R plasmid without a DNA insert.

Haemolytic assays. Cellular lysates of *E. coli* were first treated with 10 mM dithiothreitol during 15 min at 37 °C. Then they were incubated with 1% erythrocytes in phosphate-buffered saline (pH 6.0) at 37 °C for 30 min and centrifuged. In the supernatant, haemolysis was measured spectrophotometrically at 543 nm.

Results

A total of 4500 recombinant *E. coli* clones were obtained by ligation of Sau3A partially digested *L. ivanovii* chromosomal DNA into the BamHI digested vector pTZ19R and subsequent transformation into *E. coli* DH5alpha. Ampicillin-resistant *E. coli* transformants were first phenotypically screened for a *Listeria* DNA insert by colour reaction of LB agar containing 0.2 mM IPTG and 40 mg/l X-Gal (recombinant *E. coli*-clones appear colourless [2]). Fifty-one of these clones were randomly chosen for insert DNA size analysis. The molecular weight of *Listeria* insert DNA varied from 0.3 kb to 12.0 kb, with an average of 3.9 kb.

If one presupposes that the chromosome of *L. ivanovii* is of about the same size as the *E. coli* chromosome, and that all DNA fragments are ligated

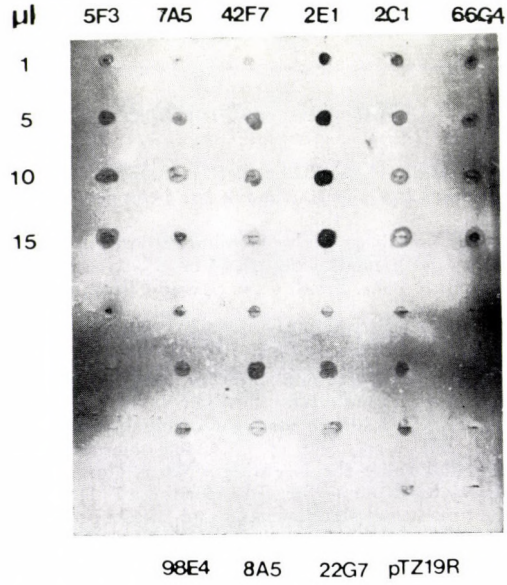

Fig. 1. Dot-blot immunoassay: cellular lysates of those recombinant *E. coli*-clones which reacted strongly in the colony-immunoblot were dotted onto nitrocellulose. The antigenic proteins were detected with anti-LLO-serum (1 : 1000), followed by visualization of primary antibody binding by a second, peroxidase conjugated swine-anti-rabbit-antibody (1 : 1000). "pTZ19R" marks the negative control

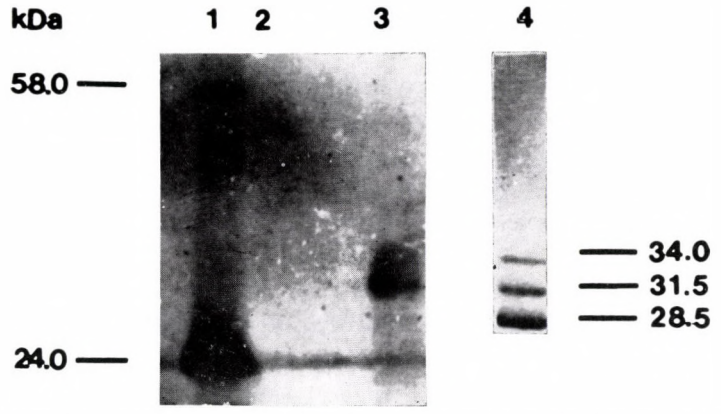

Fig. 2. Western-blot immunoassay using anti-LLO-serum, visualization as in Fig. 1. Lane 1: culture supernatant of *L. ivanovii;* lane 2: cellular lysate of *E. coli* DH5alpha (pTZ19R); lanes 3 and 4: cellular lysate of *E. coli* 2El. The proteins were probed with an anti-LLO-serum (lanes 1, 2 and 3 at a dilution of 1 : 1000, lane 4 at 1 : 100)

and stably transformed with the same frequency, then the probability that any gene is included in the library is 98% [6].

Out of the 4500 recombinant *E. coli* clones 9 were strongly positive in the colony-blot immunoassay with anti-LLO-serum. Using a dot-blot immunoassay of cellular lysates of these 9 recombinants, one clone remained strongly positive (Fig. 1). This *E. coli* clone was called #2E1.

In a Western-blot assay using the anti-LLO-serum (described in the methods section), 3 proteins were detected in cellular lysates of #2E1 which reacted immunologically, having molecular weights of 34.0, 31.5 and 28.5 kDa, respectively (Fig. 2, lane 4). Using the anti-LLO-serum at a dilution 1 : 1000, only one of these proteins (having a molecular weight of 31.5 kDa) was detected (Fig. 1, lane 3). The 34.0 kDa protein seems to be the primary gene product, and both other proteins may be degradation products, as proteolysis of recombinant proteins occurs rather often in *E. coli* [7]. Surprisingly, none of these proteins reacted immunologically in a Western-blot analysis with anti-streptolysin O (SLO-) serum, but there was a reaction with another protein having a molecular weight of 24. OmkDa.

The *Listeria*-DNA insert of #2E1 is 4.5 kb and hybridizes with three distinct fragments of *L. ivanovii* chromosomal DNA digested with EcoRI (1.3 kb, 5.4 kb and 7.0 kb molecular weight, respectively). It also hybridizes with chromosomal DNA from *L. monocytogenes* 1/2a EGD, digested with EcoRI and HindIII (molecular weights of 2.4 kb and 2.65 kb, respectively [8].

Cellular lysates of #2E1 do not show enhanced haemolytic activity compared with a vector-containing strain of *E. coli* DH5alpha. This result was also found after preincubation of the lysates in 10 mM dithiothreitol, which elevates the haemolytic potential of listeriolysin O [9].

Discussion

It has been shown in this work that it is possible to construct a gene library of *L. ivanovii* chromosomal DNA in the pTZ19R plasmid system. We used this vector because there is evidence that in such a system a higher stability of *Listeria* DNA inserts is achieved compared with lambda phage and cosmid cloning systems. Furthermore, characterization of the cloned DNA and its gene product(s) is much simplified.

The *E. coli*-clone #2E1 harbours a 4.5 kb *Listeria* DNA insert in the pTZ19R vector. This *E. coli* clone reacts strongly with the anti-LLO-serum, which has been prepared by immunizing rabbits with listeriolysin O and a 24 kDa-protein of *L. ivanovii*. We suggest that the 24 kDa-protein is most probably involved in the regulation of cytolytic processes or is a cytolysin itself. The 24 kDa-protein binds to erythrocyte membranes and studies with transposon mutants of *L. ivanovii* revealed that it may be regulated in a coordinated fashion with the sphingomyelinase and/or lecithinase genes. Apparently, a primary gene product, having a molecular weight of 34.0 kDa, is expressed in #2E1 from the inserted *Listeria* DNA. The gene for the 24.0 kDa-protein cannot encode a protein of this molecular weight, neither as a protein having a signal sequence for transport, nor as a fusion protein with the vector's beta-galactosidase.

Therefore, we suggest that the recombinant *L. ivanovii* DNA in #2E1 consists at least partially of the genetic information corresponding to the amino terminal part of listeriolysin O of *L. ivanovii*. This *Listeria* DNA fragment apparently bears its own transcription and translation signals, because the partial gene is expressed in *E. coli* without induction of transcription. Of course, we cannot exclude the possibility that there exists a protein in *L. ivanovii* which cross-reacts with the anti-LLO-serum used in our studies and which is only slightly expressed in *L. ivanovii*, but which, upon cloning into a multi-copy-plasmid, such as pTZ19R, is expressed at high levels in *E. coli*. Such a gene product would then be detected by the colony-immunoassay. One can speculate that the epitope(s) of listeriolysin O reacting with the anti-SLO-serum lie(s) within the carboxyterminal part of the protein. This suggestion is supported by the fact that amino acid sequence comparisons between the SH-activated cytolysins reveal significant homologies only in the carboxyterminal part of these proteins [10]. This region is, according to our hypothesis, not expressed in *E. coli* #2E1 and therefore no cross-reaction of the recombinant protein from this partial gene with anti-SLO-serum would be expected.

The recombinant 24 kDa-protein which is expressed in #2E1 and which cross-reacts immunologically with the anti-SLO-serum used in our experiments is most probably the product of another gene lying within the *Listeria*

DNA insert of this recombinant plasmid. Further characterization will reveal if the proteins reacting immunologically with anti-LLO-serum are related to the listeriolysin O of *L. ivanovii*.

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REFERENCES

1. Flamm, R. K., Hinrichs, D. J., Thomashow, M. F.: Infect Immun **44,** 157 (1984).
2. Maniatis, T., Frotsch, E. F., Sambrook, J.: Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, New York 1982.
3. Birnboim, H. C., Doly, J.: Nucl Acids Res **7,** 1513 (1979).
4. Lo, R. Y., Cameron, L. A.: Biochem Cell Biol **64,** 73 (1986).
5. Kathariou, S., Metz, P., Hof, H., Goebel, W.: J Bacteriol **169,** 1291 (1987).
6. Clarke, L., Carbon, L.: Cell **9,** 91 (1976).
7. Miller, C. G.: In Neidhardt, F. C. (ed.): *Escherichia coli* and *Salmonella typhimurium.* ASM, Washington 1987. p. 680.
8. Leimeister-Wächter, M., Chakraborty, T., Goebel, W.: Ann Microbiol (Paris) A **138,** 252 (1987).
9. Geoffroy, C., Gaillard, J.-L., Alouf, J. E., Berche, P.: Infect Immun **55,** 1641 (1987).
10. Mengaud, J., Vicente, M.-F., Chenevert, J., Pereira, J. M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.-C., Cossart, P.: Infect Immun **56,** 766 (1988).

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PURIFICATION AND CHARACTERIZATION OF CYTOLYSINS FROM *LISTERIA MONOCYTOGENES* SEROVAR 4b AND *LISTERIA IVANOVII*

J. KREFT, D. FUNKE, R. SCHLESINGER, F. LOTTSPEICH and W. GOEBEL

Institute of Genetics and Microbiology, University of Würzburg, and Max-Planck-Institute for Biochemistry, Martinsried, FRG

Several exoproteins from *Listeria monocytogenes* serovar 4b (NCTC 10527) and *Listeria ivanovii* (ATCC) 19119, SLCC 2379), respectively, have been purified to homogeneity by thiol-disulfide exchange chromatography and gel filtration. Both strains produce a haemolytic/cytolytic protein of Mr 58 kDa, which has all the properties of a SH-activated cytolysin, the prototype of which is streptolysin O (SLO), and this protein has therefore been termed listeriolysin O (LLO). In addition a protein of Mr 24 kDa from culture supernatants of *L. ivanovii* co-purified with LLO. The N-terminal aminoacid sequences of both proteins from *L. ivanovii* have been determined. By mutagenesis with transposons of Gram-positive origin (Tn916 and Tn1545), which have been introduced via conjugation into *L. ivanovii*, several phenotypic mutants (altered haemolysis on sheep blood agar or lecithinase-negative) were obtained. Results on the properties of these muntants will be presented.

It is generally accepted that listeriolysin O, a SH-activated exotoxin produced by several pathogenic *Listeria*, plays an important role in the pathogenesis of these facultative intracellular bacteria [1, 2]. In order to elucidate if *Listeria* strains other than *Listeria monocytogenes* serovar 1/2a produce such toxins, we undertook a search for the detection and subsequent purification/characterization of such exoproteins from different *Listeria* strains and species.

The use of conjugative transposons from Gram-positive bacteria (transposons Tn916 and Tn1545) has proven to be helpful in the identification of virulence factors from *Listeria* [2, 3], therefore we tried to adapt this method to *Listeria ivanovii*.

Materials and methods

Bacteria. L. ivanovii (ATCC 1919, SLCC 2379), *L. monocytogenes* serovar 4b (NCTC 10527) nad *Rhodococcus equi* (NCTC 1621) were from the strain collection of the Institute for Hygiene and Microbiology, University of Würzburg. *Streptococcus faecalis* CG110 (with Tn916) was donated by D. B. Clewell (Ann Arbor, USA), *L. monocytogenes* BM4140 (with Tn1545) and *Escherichia coli* BM2962 (with pAT93) by P. Courvalin (Paris). Bacteria were grown on brain heart infusion broth (BHI), Gibco/ or blood agar base (BAB No. 2, Oxoid), supplemented as described below, at 37 °C.

JÜRGEN KREFT, D. FUNKE, R. SCHLESINGER, W. GOEBEL
Institute of Genetics and Microbiology, University of Würzburg
Röntgenring 11, D-8700 Würzburg, FRG

F. LOTTSPEICH
Max Planck Institute for Biochemistry
Martinsried, FRG

Purification of listeriolysin was done by thiol-disulfide exchange chromatography on thiopropyl-Sepharose 6B (Pharmacia) and gel filtration of Biogel P-100 (Biorad) as decribed [4].

SDS polyacrylamide gel electrophoresis of proteins was performed with TCA-precipitated culture supernatants of 12.5% slab gels [5].

Chromosomal and plasmid DNAs were isolated according to published procedures [6, 7] with minor modifications.

Transposon mutagenesis of L. ivanovii was performed essentially as described [2] by filter matings of *Streptococcus* (with Tn916) or *L. monocytogenes* (with Tn1545) and a streptomycin-resistant (SmR) mutant of *L. ivanovii*.

Procedures for DNA-(Southern-) hybridization and immunoblotting have been previously described [2].

Haemolysin assays were performed as decribed in the adjacent paper [8].

In enzymatic tests for sphingomyelinase (Smase) and phospholipase (Plase) TNPAL--sphingomyelin or p-nitrophenyl-phosphorylcholine, respectively, were used as substrates [9, 10].

Results

Purification and characterization of listeriolysin O and the 24 kDa protein. By thiodisulfide exchange chromatography and gel filtration [4] we could purify from BHI-culture supernatants (concentrated about 50-fold by ultrafiltration) of both *L. ivanovii* and *L. monocytogenes* serovar 4b a single protein of Mr 58 kDa, which fulfilled all the requirements for a SH-activated cytolysin of the streptolysin O (SLO) type [11], namely haemolytic activity, inactivation by oxygen or micromolar amounts of cholesterol, activation by SH-reagents (dithiothreitol, DTE) and immunological cross reaction with anti-SLO (unpublished [12]). These cytolysins were therefore identified as listeriolysin O (LLO). In the case of *L. ivanovii* a second major supernatant protein of Mr 24 kDa copurified with LLO. The possible role of this protein has still to be elucidated. The N-terminal aminoacid sequences of LLO and the 24 kDa protein from *L. ivanovii* have been determined (Fig. 1).

Transposon mutagenesis. L. ivanovii was mutagenized by the conjugative transposon Tn916 [2] and Tn1545 [13], respectively. Tn916 proved to be

	1								10					
LLO L. ivanovii	Asp	Ala	Ser	Val	Tyr	Ser	Tyr	Gln	Gly	Ile	Ile	X (Ser)	His	Met	Ala
LLO L. monocyt.	Asp	Ala	Ser	Ala	Phe	Asn	Lys	Glu	Asn	Ser	Ile	Ser	Ser	Met	Ala
									20					
LLO L. ivanovii	Pro	Pro	Ala	X (Ser)	Pro	Pro	Ala	Ser						
LLO L. monocyt.	Pro	Pro	Ala	Ser	Pro	Pro	Ala	Lys						
24 KDa	Ala	Asp	Ile	Leu	X	Pro	Met	Pro	Ile	Asn	Gln	Ile	Phe	Pro	Asp
	Pro/Leu	Asp	Leu	Ala											

Fig. 1. N-terminal aminoacid sequence of LLO from *L. ivanovii*, compared to LLO from *L. monocytogenes* serovar 1/2 [14] and of the 24 kDa protein from *L. ivanovii*. X means an amino acid which could not be determined precisely (most presumably serine). Residues homologous to LLO from *L. monocytogenes* are underlined

rather inefficient when looking for altered haemolytic/phospholipolytic phenotypes of the transconjugants. Tn1545 was much more successful, yielding five mutants of a relevant phenotype among 3000 recipients. The screening of all transconjugants was done on BAB/blood agar plates +/— DTE or with

Table I

Relevant properties of Tn1545 mutants

Strain	WT	20/24	8/6	44/2
58 KDa	+	—	—	+
24 KDa	+	+	—	—
Smase	+	+	+	—
Plase	+	+	—	+
CAMP	+	+	+	—

Smase: Sphingomyelinase C activity
Plase: Phospholipase C (lecithinase) activity

erythrocytes pretreated with *R. equi* culture supernatant or on egg yolk agar plates.

L. ivanovii mutants with relevant phenotypes were analyzed by Southern hybridization of their chromosomal DNAs to probes specific for Tn916 or Tn1545, thus demonstrating the presence of the respective transposon in the chromosome of the mutant transconjugants (data not shown). Furthermore the exoprotein pattern, the enzymatic (Smase and Plase) activities in concentrated culture supernatants and the behaviour in the classical CAMP-test of the mutants were compared with the wildtype strain. Smase and Plase could be differentiated by the use of specific substrates (see above). The results from the most interesting mutants are shown in Table I.

Discussion

From the results described above several conclusions can be drawn: (i) *L. ivanovii* (ATCC 19119, SLCC 2379) and *L. monocytogenes* serovar 4b (NCTC 10527) both produce and secrete a haemolysin/cytolysin comparable to streptolysin O (SLO), therefore termed listeriolysin O (LLO), with a Mr of 58 kDa. The N-terminal aminoacid sequence of LLO from *L. ivanovii* shows significant homology to LLO from *L. monocytogenes* serovar 1/2a [14]. (ii) Transposon mutagenesis of *L. ivanovii* proved to be possible and useful, in particular with Tn1545. Several mutant types were obtained, where one or several exoproteins were no longer secreted, pointing to a possible coor-

dinate regulation of the genes responsible for these proteins. Comparison of the phenotypes, protein patterns and enzymatic activities showed that most presumably the sphingomyelinase present in the wild type *L. ivanovii* is the factor which interacts with the *R. equi* exosubstance in the CAMP-reaction.

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REFERENCES

1. Gaillard, J. L., Berche, P., Sansonetti, P.: Infect Immun **52,** 50 (1986).
2. Kathariou, S., Metz, P., Hof, F., Goebel, W.: J Bacteriol **169,** 1291 (1987).
3. Berche, P., Gaillard, J. L., Sansonetti, P. S.: J Immunol **138,** 2266 (1987).
4. Geoffroy, C., Alouf, J. E.: J Biol Chem **258,** 9968 (1983).
5. Laemmli, U. K.: Nature **227,** 680 (1970).
6. Birnboim, H. C., Doly, J.: Nucl Acids Res **7,** 1513 (1979).
7. Flamm, R. K., Hinrichs, D. J., Thomashow, M. F.: Infect Immun **44,** 157 (1984).
8. Haas, A., Kreft, J., Goebel, W.: Acta Microbiol Hung **36,** (1989).
9. Gatt, S., Dinur, T., Barenholz, Y.: Biochem Biophys Acta **530,** 503 (1978).
10. Kurioka, S., Matsuda, M.: Anal Biochem **75,** 281 (1976).
11. Smyth, C. J., Duncan, J. L.: In Jeljaszewicz, J., Wadström, T. (eds): Bacterial Toxins and Cell Membranes. Academic Press, New York 1978. p. 129.
12. Goebel, W., Kathariou, S., Kuhn, M., Sokolovic, Z., Kreft, J., Köhler, S., Funke, D., Chakraborty, T., Leimeister-Wächter, M.: Infection **16,** 149 (1988).
13. Collatz, E., Carlier, C., Courvalin, P.: J Bacteriol **156,** 1373 (1983).
14. Mengaud, J., Vicente, M. F., Chenevert, J., Pereira, J. M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J. C., Cossart, P.: Infect Immun **56,** 766 (1988).

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TOWARDS A PHYSICAL MAP OF THE *LISTERIA* CHROMOSOME: THE PULSED FIELD ELECTROPHORESIS APPROACH

M. F. VICENTE, J. C. PÉREZ-DIAZ, R. AMILS, F. BAQUERO and I. MARIN

Microbiology Service, H. Ramon y Cajal Centre, Madrid, Spain

The possibility of the electrophoretic study of very large fragments of the *Listeria* chromosome (DNAs up to 1000 Kb) may help to the understanding of the physical organization and functional mapping of the entire genome of this organism. Several experiences were done to apply this technology to *Listeria*. Agarose inserts were prepared with intact cells and protoplasts, and lysis was induced in situ. Inserts with a convenient amount of DNA were cleaved in situ again by diffusing restriction enzymes into the agarose and submitted to one dimensional electric field that was periodically inverted, to cause changes of direction of the DNA fragments. We obtained a good band resolution of bands with *Eco* R1 and *Hind*III. Experiments are in progress to select other restriction enzymes leading to larger fragments. This technique must be combined with the use of blotting with known DNA sequences, such as the corresponding to the haemolysin, to arrive to a comprehensive map of the *Listeria* chromosome. Cloning of *Listeria* genes involved in several biochemical characteristics of *Listeria*, or at least a good collection of mutants will be quite necessary for the progress of such approach.

The technique of pulsed field gel (PFG) electrophoresis [1] has many applications, some of which are just beginning to be explored [2, 3]. It allows investigation of an entire genome in one gel by resolving DNA fragments from 20 000 to 2000 Kilobase pairs. A directional switching of the electric field causes molecules to change direction in the gel. Separations are possible apparently because the time each fragment takes to alter its shape and migrate in a new direction, varies as a function of its length. We have used this technique to help understand the physical organization and functional mapping of the genome of *Listeria monocytogenes* LO28.

Materials and methods

Agarose inserts were prepared with intact cells and protoplasts [4] and lysis of cells was induced in situ (in the agarose inserts).

The quantity of DNA in the agarose inserts was tested in a one dimensional electric field, that was periodically inverted, to change the direction of the DNA fragments.

Agarose inserts with the convenient amount (100 μg) of DNA were cleaved in situ by diffusing restriction enzymes into the agarose and again submitting to a pulsed field gel.

M. F. VICENTE, J. C. PÉREZ-DIAZ, R. AMILS, F. BAQUERO, I. MARIN
Servicio de Microbiologia, H. Ramon y Cajal
Madrid, Spain

Results

Good resolution of DNA chromosomal fragments from *L. monocytogenes* LO28 was obtained (it was impossible to detect any plasmid of high molecular weight).

Digestion of the DNA with several different restriction enzymes (Table I) produced many bands (Fig. 1). However, with NotI restriction enzyme, six fragments were resolved (Figs 2 and 3). Three of these are smaller than the

Table I

Restriction enzymes used in this study

BamHI	BanII	BclI	ClaI	DraI	*Eco*RI	*Hind*III
KpnI	NarI	NcoI	NheI	NotI	NruI	PvuI
SacI	SacII	SalI	ScaI	SfiI	SpHI	StuI
XbaI	XhoI					

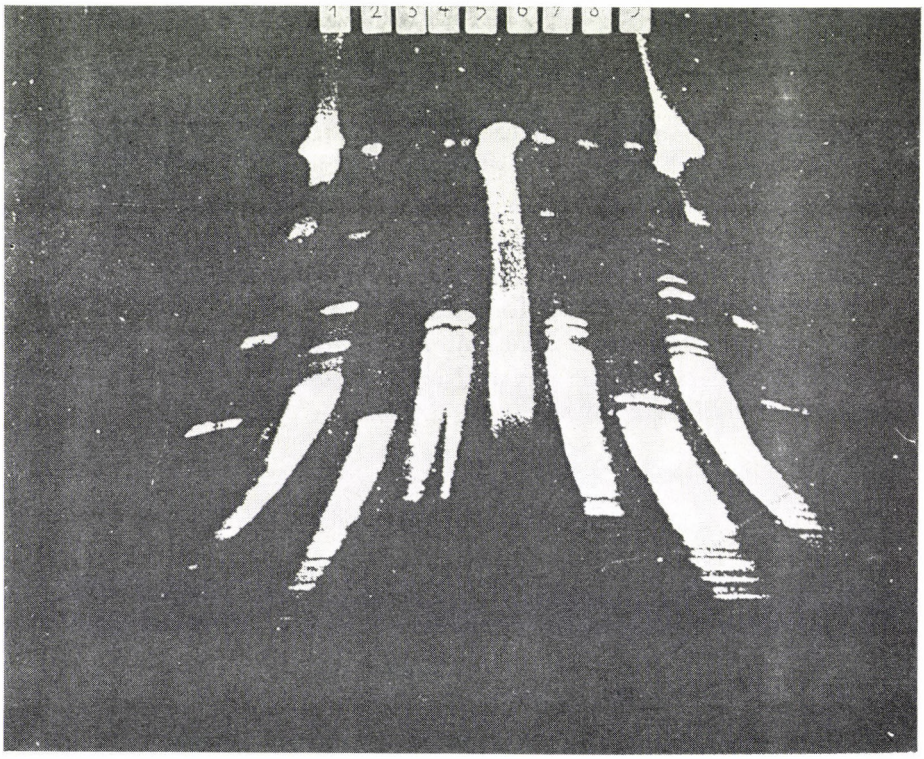

Fig. 1. Pulsed field gel (A) with DNA agarose inserts of *L. monocytogenes* LO28 cleaved with NcoI (2), NruI (3), ScaI (4), SpHI (5), StuI (6), ClaI (7), ScaII (8) and size standard fragments (1, 9)

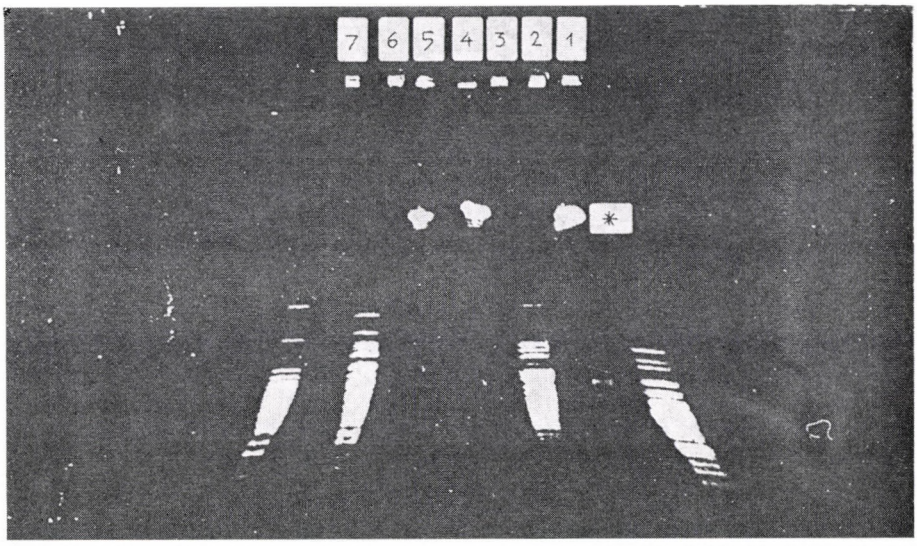

Fig. 2. Digestion of the agarose inserts of *L. monocytogenes* by NheI (1), NotI (2), NarI (3), SfiI (5), SaII (6), ScaI (7) and size standard fragments (4); pulsed field gel (B)

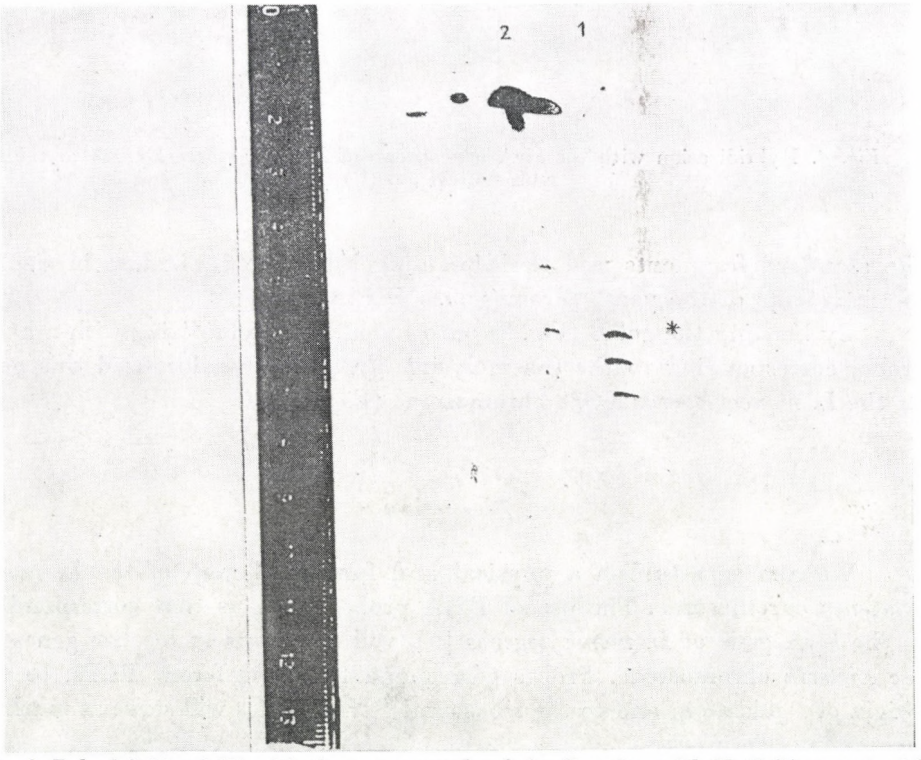

Fig. 3. Pulsed field gel (C) with the same sample of the digestion with NotI (1) except with extra pulses. It is possible to resolve three extra fragments; the control is the fragments of the yeast chromosomes (2)

Fig. 4. Hybridization with the *hly*A gene probe of *L. monocytogenes* LO28 to the pulsed field gel (B)

size standard fragments and the remaining three are equivalent in size to the fragments of the yeast chromosomes DNAs [5].

By blotting the gel B (Fig. 2) onto nylon, and hybridizing with a DNA probe, corresponding to the (haemolysin) *hly*A gene, we localized this gene on the *L. monocytogenes* LO28 chromosome (Fig. 4).

Discussion

We aim to establish a physical and functional map of the *L. monocytogenes* chromosome. The use of DNA probes, such as that corresponding to the *hly*A gene of *L. monocytogenes* [6], will enable us to localize genes on the *Listeria* chromosome. Probes correspond to the different PBPs, to the oirigin of replication, and to the ribosomal RNAs, genes will serve as markers per preliminary work.

In conclusion the methodology of pulse field electrophoresis seems to be promising for the understanding of the genetic arrangement of the *Listeria*

chromosome. It will be necessary in the near future to prepare DNA probes corresponding to the different functional or phenotypic traits of *Listeria* in order to localize these genes in the chromosomal DNA.

REFERENCES

1. Carle, G. F., Frank, M., Olson, M. V.: Science **232,** 65 (1986).
2. Garvey, E. P., Santi, D. V.: Science **233,** 535 (1986).
3. Kohara, Y., Akiyama, K., Isono, K.: Cell **50,** 495 (1987).
4. Vicente, M. F., Baquero, F., Pérez-Diaz, J. C.: Plasmid **18,** 89 (1987).
5. Schwartz, D. C., Cantor, C. R.: Cell **37,** 67 (1984).
6. Mengaud, J., Vicente, M. F., Chenevert, J., Moniz Pereira, J., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Pérez-Diaz, J. C., Cossart, P.: Infect Immun **56,** 766 (1988).

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REACQUISITION OF VIRULENCE OF HAEMOLYSIN-NEGATIVE *LISTERIA MONOCYTOGENES* MUTANTS BY COMPLEMENTATION WITH A PLASMID CARRYING THE *hly*A GENE

M. F. VICENTE, J. MENGAUD, J. CHENEVERT, J. C. PÉREZ-DIAZ, C. GEOFFROY, F. BAQUERO, P. COSSART and P. BERCHE

Microbiology Service, H. Ramon y Cajal Centre, Madrid, Spain, Unit of Bacterial Genetics, and Unit of Bacterial Antigens, Institute Pasteur, Paris, and Microbiology Laboratory, H. Necker Infants' Hospital, Paris, France

An haemolysin negative mutant of *Listeria monocytogenes* LO28 produced by insertion of Tn917 resulted in an avirulent derivative. The *hly*A gene of the same strain was previously cloned in *Escherichia coli* and retransfered by transformation to the *hly*$^-$ derivative, after subcloning in the shuttle vector pMK4. The transformant strain (L828) reacquired an haemolytic activity at a similar level than the wild strain. The virulence of this *hly*$^+$ transformant was estimated by determining the LD_{50} in Swiss mice infected intravenously. With increasing doses of bacteria a *hly*$^-$ control strain (transformant with only pMK4) appeared to be totally avirulent; however, no significant difference in virulence was found between the *hly*$^+$ transformants and the wild strain. Seriol viable counts in the liver and spleen of infected mice demonstrated an increase in number of L828 *hly*$^+$ transformants at 48 h, but the *hly*$^-$ control transformants were rapidly eliminated. These results confirm that the production of haemolysin is a major factor in the pathogenic capabilities of *L. monocytogenes*.

Listeria monocytogenes can be considered as a peribiont as defined by Isenberg: an organism capable on tolerating plant, animal and human biospheres [1]. These organisms tend to progress to the pathogenic level essentially if the host susceptibility or general immune status permits the expression of microbial harmfulness. Pathogenicity is always the result of a vast continuum of interactions that requires the participation of the individual host and the microorganism. Therefore a single biological property cannot explain pathogenicity. Nevertheless, in the absence of certain bacterial factors, there is almost no possibility for disease production. This paper presents in

M. F. VICENTE, J. C. PÉREZ-DIAZ, F. BAQUERO
Servicio de Microbiologia, H. Ramon y Cajal
Madrid, Spain

JEROME MENGAUD, J. CHENEVERT, P. COSSART
Unité de Génie Microbiologique, Institut Pasteur
Rue du Dr. Roux, 75724 Paris Cedex 15, France

C. GEOFFROY
Unité des Antigenes Bactériens, Institut Pasteur
Rue du Dr. Roux, 75724 Paris Cedex 15, France

vivo evidence of the key-role of the listeriolysin O as a factor enabling the production of lethal infection in mice.

The first genetic indications of the importance of the haemolysin in the pathogenesis of *Listeria* infections were obtained through the comparison of the pathogenic activity of wild haemolytic strains versus non-haemolytic isogenic mutants in experimental murine infections. These mutants were obtained by transposon mutagenesis, with Tn1545 [2] or Tn916 [3]. Insertion of a single copy of these genetic elements in the gene encoding the haemolysin resulted in an Hly$^-$ mutant, producing a truncated haemolysin as detected by immunoblot [3, 4]. Hly$^-$ mutants were unable to provoke disease in mice, being rapidly eliminated from the blood and organs whatever the level of the infecting dose. Moreover, Hly$^-$ mutants did not generate any delayed sensitivity, probably because of the lack of bacterial replication into the host cells [5]. The confirmation of these results required a positive evidence of the role of the haemolysin as a factor enabling the pathogenic activity of *L. monocytogenes*.

Materials and methods

The *Listeria* haemolysin gene further designed as *hly*A was originally cloned from the pathogenic and haemolytic strain *L. monocytogenes* LO28 in a cosmid vector pHC79 of *Escherichia coli* in 1985 [6]. A Hly$^-$ mutant of this strain was obtained by transposon mutagenesis, but using Tn917 which encodes erythromycin resistance. In order to reintroduce the haemolysin gene into this *Listeria* derivative, the haemolysin gene containing region (about 8.5 Kb) was subcloned into a bifunctional shuttle vector (pMK4, containing a determinant for chloramphenicol resistance) able to replicate both in *E. coli* and in *Listeria*, and used to transform *Listeria* by the DNA transformation procedures recently developed in this genus [7]. The introduction of this genetical construction pCL103 into Hly$^-$ *Listeria* mutants obtained both with Tn1545 in strain EGD [2] or Tn917 (see above) resulted in a recovery of the haemolysin production. As genetic rearrangements were detected in pCL103, a new clone from the *Listeria* chromosome containing the haemolysin gene in a much shorter (3 Kb) piece of DNA was then obtained in the vector pUC8 and then subcloned in pMK4. The resulting plasmid, pLIS4, was also sufficient to restore the haemolysin production when it was introduced in the above mentioned Hly$^-$ mutants.

Results and discussion

The structural gene encoding the haemolysin determinant (*hly*A) was sequenced from the region firstly cloned in the cosmid pHC79 and reconfirmed by sequencing of pLIS4. The deduced protein sequence revealed the presence of a putative 25-aminoacid signal sequence; the secreted form of the haemolysin would have 504 amino acids, corresponding to the molecular weight of the purified protein, 58 kilodaltons [8]. Homologies of the sequence of this haemolysin with streptolysin O and pneumolysin was found: an 11 amino acid peptide is conserved in the three proteins containing the unique cysteine known to be essential for the lytic activity. The name listeriolysin O had been given to

the thiol-activated haemolysin encoded by the sequenced gene. Interestingly listeriolysin O is the only SH-dependent haemolysin active at pH 5 [9], which may argue for the role of this toxin in the phagosome disruption and intracellular bacterial replication.

In order to obtain positive evidence of the role of listeriolysin O in *Listeria* pathogenesis, specific pathogen-free ICR female swiss mice were inoculated by intravenous route with various concentrations of the different *Listeria* derivatives obtained in the previous experiments. A list of these strains is presented in Table I. Virulence was determined by (i) determining the 50% lethal dose (LD_{50}); and (ii) following the viable bacteria in organs: liver and spleen were aseptically removed and serial dilutions of whole organ homogenates were plated on tryptic soy agar. The LD_{50} for the wild haemolytic strain LO28 was $2.1 \times 10^{5.5}$; the hly$^-$ mutants obtained by Tn917 insertion proved to be totally avirulent, since mice survived to challenge as high as $>10^{9.5}$ organisms and did not exhibit abscesses in organs. The presence of the shuttle plasmid pMK4 did not influence the expression of virulence, since the *hly*$^-$ strain harbouring this plasmid has the same LD_{50} as the hly$^-$ mutant. As it is shown in Table II, the presence of *hly*A, in pLis4, restores the patho-

Table I

Bacterial strains used in experimental infection

Strains	Antibiotic resistance	Haemolytic activity	Cells/ml*
LO28 (wild)	—	900 HU/ml	2.7×10^8
LO28: :Tn*917*	erythromycin	< 10 HU/lml	5.0×10^8
LO28: :Tn*917*.pMK4	erythromycin chloramphenicol	< 10 HU/ml	6.0×10^8
LO28: :Tn*917*. pLis4	erythromycin chloramphenicol	1.200 HU/ml	4.0×10^8

* Culture in charcoal-treated broth (10 h at 37 °C)

Table II

Listeria LD_{50} determination in Swiss mice

Strain	Haemolytic phenotype	LD_{50}*
LO28 (wild)	+	$10^{5.2}$
LO28: :Tn*917*	—	$>10^{9.5}$
LO28: :Tn*917*.pMK4	—	$>10^{9.5}$
LO28: :Tn*917*.pLIS4	+	$10^{6.7}$

* The LD_{50} was determined on groups of 5 female Swiss mice by the probit method, after intravenous infection

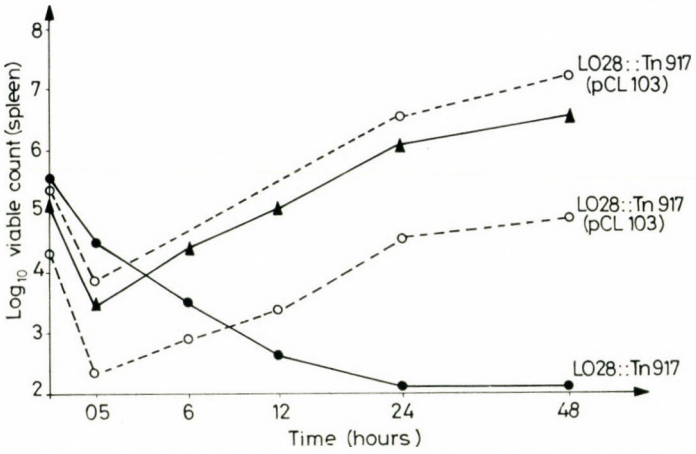

Fig. 1. Bacterial survival was followed in mice infected intravenously (2×10^6) by wild-type *L. monocytogenes* LO28 (▲ — ▲), *L. monocytogenes* Hly⁻ mutant with the Tn917 (● — ●). Results are expressed as the mean log bacteria per organ

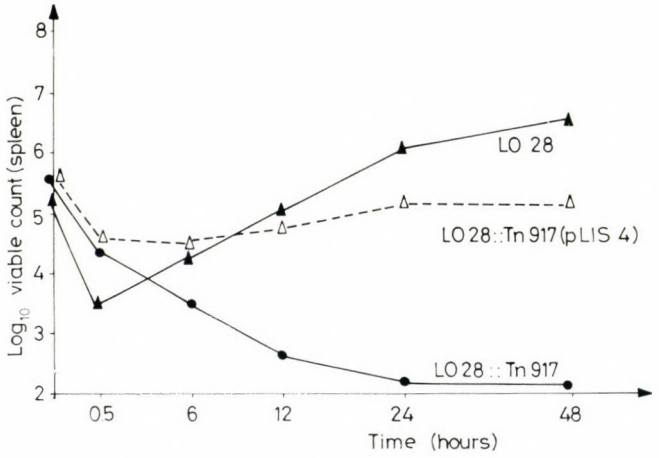

Fig. 2. Bacterial survival was followed in mice infected intravenously (2×10^6) by wild-type *L. monocytogenes* LO28 (▲ — ▲), strains pLIS4 (△ — △) and pCL103 (○ — ○). Results are expressed as the mean log bacteria per organ

genic power of the mutant, indicating the reacquisition of virulence by complementation with the plasmid carrying only the listeriolysin O gene. In Figs 1 and 2 are shown the results concerning the viable count of *Listeria* cells in organs. After an initial reduction in bacterial counts in liver or spleen, as with the wild type strain, the complemented mutants carrying the cloned *hly*A gene increased its number, which demonstrate bacterial multiplication into the animal tissues. On the contrary, the non-complemented Hly⁻ mutants were rapidly eliminated. The secretion of listeriolysin O seems to be essential

to bacterial growth in host tissues where bacteria are trapped by professional phagocytic cells. This toxin appears to be a key-factor in the pathogenesis of *L. monocytogenes* infections.

REFERENCES

1. Isenberg, H. D.: Clin Microbiol Review **1,** 40 (1988).
2. Gaillard, J. L., Berche, P., Sansonnetti, P.: Infect Immun **52,** 50 (1986).
3. Kathariou, S., Kohler, S., Kuhn, M., Goebel, W.: Ann Microbiol (Paris) A **138,** 256 (1987).
4. Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J. L., Cossart, P.: Infect Immun **55,** 3225 (1987).
5. Berche, P., Gaillard, J. L., Sansonnetti, P. J.: J Immunol **138,** 2266 (1987).
6. Vicente, M. F., Baquero, F., Pérez-Lopez, J. C.: FEMS Microbiol Lett **30,** 77 (1985).
7. Vicente, M. F., Baquero, F., Pérez-Diaz, J. C.: Plasmid **18,** 89 (1987).
8. Mengaud, J., Vicente, M. F., Chenevert, J., Moniz Pereira, J., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Pérez-Diaz, J. C., Cossart, P.: Infect Immun **56,** 766 (1988).
9. Geoffroy, C., Gaillard, J. L., Alouf, J. E., Berche, P.: Infect Immun **55,** 1641 (1987).

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4. EPIDEMIOLOGICAL TRACING

TYPING OF *LISTERIA MONOCYTOGENES* FOR EPIDEMIOLOGICAL STUDIES USING DNA PROBES

N. A. SAUNDERS, A. M. RIDLEY and A. G. TAYLOR

Central Public Health Laboratory, Colindale, London, UK

We have investigated the possibility of using restriction fragment length polymorphisms (RFLPs) to distinguish different strains of *Listeria monocytogenes*. Cloned DNA fragments (probes) were selected from a bacteriophage lambda gene library of *L. monocytogenes* (L1428). DNAs from two lambda clones consisting of a total of approximately 20kb of probe DNA were labelled with biotinylated dUTP for use in these experiments. The criteria for probe selection were the ability both to hybridise with all strains of *L. monocytogenes* and to reveal RFLPs. Southern blots of restriction endonuclease (Nci I) digested DNAs from test strains of *L. monocytogenes* were hybridized to the probe. Following washing, biotinylated probe remaining bound to the filters was detected using the Blugene reagents (Gibco/BRL). Under these experimental conditions strain-specific restriction fragments were revealed. We have examined 64 strains of *L. monocytogenes* belonging to serogroup 1/2 which were not apparently epidemiologically associated and 19 patterns were observed. Epidemiologically related strains gave identical patterns of restriction fragments.

Serotyping of *Listeria monocytogenes* [1] can subdivide the species into 13 serovars. However, most strains causing disease belong to the three serovars 1/2a, 1/2b and 4b [2] and so serotyping is of restricted value in epidemiological studies.

Phage typing has also been employed [3] and an evaluation of this method [4] showed it to be reproducible and to give excellent discrimination between strains. One disadvantage of phage-typing is that the overall typeability of strains (reaction with at least one phage preparation) was found to be only 64% for British isolates, with 82% of serogroup 4 and 37% of serogroup 1/2 strains phage typeable. We have shown previously [5] that cloned, biotin-labelled DNA sequences derived from *Legionella pneumophila* can be employed to probe Southern blots of restriction endonuclease treated genomic DNA and that the inter-strain restriction fragment length polymorphisms (RFLPs) can be used as the basis of a typing method for this species. Here we show that a similar method can be employed for typing *L. monocytogenes* strains. The method is particularly useful in the case of the 1/2 strains.

NICHOLAS A. SAUNDERS, A. M. RIDLEY, A. G. TAYLOR
Division of Microbiological Reagents and Quality Control,
Central Public Health Laboratory
61 Colindale Avenue NW9 5HT London, United Kingdom

Materials and methods

Bacterial strains and preparation of chromosomal DNA. Isolates of *L. monocytogenes* identified on the basis of biochemical and serological reactions were cultured on Marmite agar for 18 h harvested in saline containing 10 mg/ml lysozyme incubated for at least 10 min at room temperature and pelleted by centrifugation. The cells from 1 plate were resuspended in 200 μl of 5 M GuSCN containing 0.1 M EDTA pH 7.0 and incubated for 30 min at room temperature. The lysate was diluted to 500 μl with buffer containing 150 mM NaCl, 15 mM EDTA and 50 mM Tris-HCl, pH 8.0 and then emulsified with an equal volume of chloroform : isoamyl alcohol 24 : 1 (v/v). The phases were separated by centrifugation for 2 min in an Eppendorf centrifuge. To 400 μl of the aqueous phase was added 200 μl of 7.5 M ammonum acetate and 324 μl isopropanol. The fibrous DNA precipitate formed was collected by centrifugation and washed twice with 100% ethanol. The DNA was finally dried in vacuo and resuspended in 100 μl TE buffer containing 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA.

Restriction endonuclease digestion and agarose-gel electrophoresis. Bacterial DNA (5 μg) was digested for 1–2 h with 8 units Nci 1 (NBL Ltd) in 50 μl of the buffer supplied by the manufacturer. Following incubation dye solution (25 μl) containing 10% Ficoll and 0.001% bromophenol blue was added and the digest was stored at −40 °C until required. The restriction fragments were separated by agarose gel electrophoresis on 0.8% agarose at 25 volts (1.25 V/cm) for 18 h in Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) containing 0.5 μg/ml ethidium bromide. A mixture of restriction fragments (2 ng) comprising Pst 1 and EcoR1 digests of λ phage DNA was run as a molecular weight standard. Following electrophoresis the gel was soaked twice for 15 min in 0.25 M HCl, twice for 15 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and then in neutralizing solution (1 M Tris-HCl, pH 0.8, 1.5 M NaCl) for between 15 min and 2 h.

Southern transfer of DNA was accomplished by vacuum blotting on a Vacublot apparatus (Applied Bionetics) using 10× SSC as transfer buffer and nylon filters (Hybond-N, Amersham International plc). The filters were air-dried, covered in Mobilrapper II and exposed to long-wave ultra-violet light (3 min) from a transilluminator (UV Products Inc).

Preparation of probe DNA. λ NS32 and λ NS35 were selected from a library of *L. monocytogenes* (wild-type strain L1428, serovar 4b) EcoR1 fragments in the λ vector λ gtWES. λB. which was constructed as described previously [6]. Bacteriophage particles were purified and the DNA extracted from them also as described previously [6].

Labelling and hybridization of probes. Probes λ NS32 and λ NS35 were labelled by primer extension using biotin-11-dUTP (Gibco Ltd) and hybridized to Southern blots of Nci 1 digested *L. monocytogenes* DNA as detailed previously [6]. Following incubation the filters were washed at low-stringency in 2×SSC, 0.1% SDS at 50 °C (2×15 min and 1×30 min). The filters were air-dried and treated with the Blu-gene biotin detection reagents (Gibco Ltd).

Results and discussion

Selection of probe sequences and other conditions. The probe used in these studies consists of two sequences selected from a group of eight clones picked at random from a bacteriophage lambda genomic library of *L.monocytogenes* DNA. The probes were selected on the basis that (i) they had broad specificity and were able to hybridize, under conditions of low stringency, with strains of different serotypes of *L.monocytogenes* and (ii) that when the target *L.monocytogenes* DNA was treated with Nci 1 the probes hybridized to restriction fragments having a broad range of molecular sizes. Nci 1, which cuts target DNA at the sequence 5'-CCGGG-3', was selected because the restriction fragments generated gives rise to a relatively uncomplicated pattern of bands, which are of sufficient number to provide useful discrimination between strains. Nylon membranes to which the DNA fragments are coupled cova-

lently by UV illumination are used as they are easy to handle and because efficient binding of small DNA species (400–1000 base pairs) is achieved.

Examination of strains. We have examined 143 *L.monocytogenes* strains and have observed 28 different patterns of bands. The 79 isolates of serogroup 4 were distributed between 11 pattern types and the 64 serogroup 1/2 strains were sub-divided into 19 types. Analysis of the numbers of strains in each pattern type (Table I) reveals that 81% of the serogroup 4 strains were indistinguishable using the RFLP method.

This result indicates that the serogroup 4 isolates and particularly those of serovar 4b are closely related genotypically. The greater genetic diversity among the 1/2 strains, particularly those of serovars 1/2a and 1/2c, is shown by their relatively even distribution between the 19 relevant pattern types. A correlation may be seen between the degree of phage-typeability of each

Table I

RFLP types of 143 L. monocytogenes strains

RFLP type	No. of strains							total
	serogroup 4			serogroup 1				
	4*	4b	4 not b	1/2*	1/2a	1/2b	1/2c	
1	12	34	17					63
2	2			1	3	14		20
3				1				1
4					1			1
5		1	1					2
6			3					3
7			1					1
8		1			3	1	4	9
9					6		1	7
10				1	2			3
11						3	1	4
12			2					2
13			1					1
14					2			2
15					2	1		3
16					1		1	2
17					1		1	2
18					1			1
19					1			1
20			1					1
21					2			2
22				1	1			2
23					1			1
24	2							2
25	1							1
26				1				1
27					1			1
28				3	1			4
Total	17	36	26	8	29	19	8	143

* The serovar of some strains was not determined

λ 1 2 λ 3 4 λ 5 6 λ 7 8 λ 9 10 λ 11 12 λ 13 14 λ

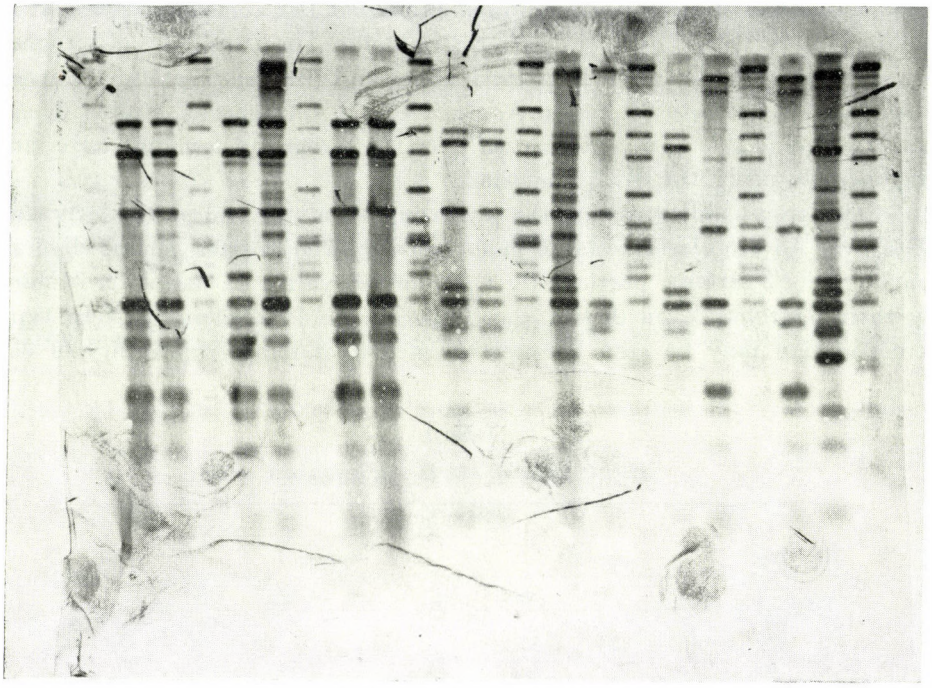

Fig. 1. DNAs isolated from λ NS32 and λ NS35 were labelled with biotin-11-dUTP by primer-extension. The labelled DNAs were mixed in equal proportion of biotinylated residues and used to probe a Southern blot, of Nci 1 digested DNAs from *L. monocytogenes* isolates (1 μg/ /strain) run on an agarose gel. The blot was washed under non-stringent conditions. Tracks marked λ show a mixture of Eco R1 and Pst 1 digest of λ DNA. The strains examined were run in the remaining tracks as follows: 1. 85449, serogroup 4b; 2, 85450, serogroup 4b; 3, 86303, serogroup 4b; 4, 86304, serogroup 4b; 5, 86305, serogroup 4b; 6, 86306, serogroup 4b; 7, 87179, serogroup 1/2; 8, 87183, serogroup 1/2; 9, 87193, serogroup 1/2; 10, 87198, serogroup 1/2; 11, 87236,serogroup 1/2; 12, 87423, serogroup 1/2; 13, 87426, serogroup 1/2; 14, 86776, serogroup 1/2

serogroup and its genotypic homogeneity. The more diverse serogroup 1/2 strains being less susceptible to phage infection and lytic growth.

Analysis of the restriction patterns obtained from 28 strains for which phage typing and epidemiological data was available revealed 8 RFLP patterns. The results show that all epidemiologically-linked strains gave indistinguishable types by both phage and RFLP methods. However, in one case, a pair of strains (serogroup 4) indistinguishable by phage-typing were distinguished by the RFLP method and in several instances strains could only be distinguished by phage-typing.

Figure 1 shows a blot, probed with λ32 and λ35, of the restriction fragment patterns of 14 of the 28 phage-typed strains. Indistinguishable patterns,

obtained from 5 isolates of serogroup 4, are shown in tracks 1, 2, 4, 5 and 6. Pairs of strains with an epidemiological relationship which gave indistinguishable restriction fragments are in the following tracks; 1 and 2 (serovar 4b strains from an outbreak of listeriosis in California, USA); 7 and 8 (serogroup 1/2 strains from a case of cross-infection); 12 and 13 (serovar 1/2a strains isolated from different clinical specimens obtained from the same individual). Other distinct patterns can be seen in tracks 3, 9, 10 and 14.

Further work will be required to assess fully the reproducibility of the RFLP typing method. However, preliminary data from the examination of different cultures of the same strain and of epidemiologically linked strains is encouraging. To date the patterns obtained in these cases have been indistinguishable. Evaluation of a similar method for typing *L.pneumophila* strains has shown that RFLPs, which are the basis of the method, are very stable [7].

It is anticipated that the method described here will be of value in epidemiological studies of *L.monocytogenes* particularly for serogroup 1/2 strains.

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REFERENCES

1. Seeliger, H. P. R., Hohne, K.: In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Vol. 13. Academic Press, New York 1979. pp. 31–49.
2. McLauchlin, J.: J Appl Bacteriol **63,** 1 (1987).
3. Audurier, A., Chatelain, R., Chalons, F., Piechaud, M.: Ann Microbiol (Paris) B **130,** 179 (1979).
4. McLauchlin, J., Audurier, A., Taylor, A. G.: J Med Microbiol **22,** 367 (1986).
5. Saunders, N. A., Kachwalla, N., Harrison, T. G., Taylor, A. G.: In Fleurette, J., Bornstein, N., Marmer, D., Surgot, M. (eds): "Colloque Legionella". Collection Fondation Marcel Merieux, 1987. pp. 197–203.
6. Saunders, N. A., Harrison, T. G., Kachwalla, N., Taylor, A. G.: J Gen Microbiol **134,** 2363 (1988).
7. Saunders, N. A., Harrison, T. G., Haththotuwa, A., Kachwalla, N., Taylor, A. G.: J Med Microbiol. In press.

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GENE PROBES FOR THE DETECTION OF *LISTERIA* SPP.

MICHAELA LEIMEISTER-WÄCHTER and T. CHAKRABORTY

Institute of Genetics and Microbiology, University of Würzburg, Würzburg, FRG

Four cloned genes of *Listeria monocytogenes* coding for listeriolysin O, beta-haemolysin, CAMP-factor and a DTH-inducing protein (DTH-18) were used as gene probes in DNA-DNA hybridization with different listerial references strains. The fragment encoding for DTH-18 is the only probe reacting specifically with pathogenic strains of *L. monocytogenes* and *Listeria ivanovii.*

Several recent outbreaks of listeriosis transmitted by contaminated food, like soft cheese [1], milk [2], or coleslaw [3] have emphasized the need for a rapid method in detection and identification of the bacterium. An alternative to the time-consuming standard cultural method is the use of specific DNA probes in DNA hybridization [4]. Specific probes for pathogenic *Listeria* should ideally be genes encoding virulence factors. Putative factors contributing to the virulence of *Listeria* include haemolysins [5, 6], a CAMP-factor [7, 8] and a protein causing delayed type hypersensitivity (DTH) [9]. The genes coding these factors have been isolated from a recombinant listerial gene library and were used as probes in southern hybridization.

Materials and methods

A gene library of Listeria monocytogenes EGD serovar 1/2a (obtained from S. H. E. Kaufmann) has been constructed in *Escherichia coli* DH5alpha with plasmid pUC18 using standard cloning procedures [10]. Recombinants were isolated via immuno-colony blots with hyperimmune antisera against streptolysin O (a gift of J. Alouf), listeriolysin O and a soluble fraction of strain EGD capable of eliciting DTH-reaction in immune mice and inducing proliferative responses of T-lymphocytes from *Listeria*-immune mice [11].

Gene probes were obtained by digesting the plasmids with appropriate restriction endonucleases to obtain internal fragments of the genes. The fragments were eluted from agarose gels and radioactively labelled with α^{32}PdATP.

For detection of homologous sequences to the different probes used, different chromosomal DNAs were cut with restriction endonucleases and DNA hybridization was performed by prehybridizing nitrocellulose filters for 4 h at 65 °C in 6× SSC (1× SSC —0.15 M NaCl, 0.015 M Na_3 citrate), 0.1% SDS, 5× Denhardt's (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumine), 100 μg/ml denatured salmon sperm DNA. The radiolabelled probe was then added and the blot incubated over night at 65 °C. The filters were washed twice for 30 min, each at 55 °C in 0.2× SSC, 0.1% SDS.

MICHAELA LEIMEISTER-WÄCHTER, T. CHAKRABORTY
Institut für Genetik und Mikrobiologie, Universität Würzburg
Röntgenring 11, D-8700 Würzburg, FRG

Results

Results of DNA hybridization with the different probes are summarized in Table I.

Table I

Hybridization reactions of different Listeria type strains with DNA probes enconding listeriolysin O (internal fragment or oligonucleotides M27 and M34), β-haemolysin, CAMP-factor and DTH 18

Ref. No.		Biotype	Serotype	Hybridization reaction					
				listeriolysin O			β-hly	CAMP	DTH 18
				LisO	M27	M34			
EGD		*L. monocytogenes*	1/2a	+	+	+	+	+	+
SLCC	5764	*L. monocytogenes*	1/2a	+	+	+	+	+	+
NCTC	7973	*L. monocytogenes*	1/2a	+	+	+	+	+	+
SLCC	53	*L. monocytogenes*	1/2a	+	+	+	+	+	+
SLCC	4013	*L. monocytogenes*	4b	+	+	+	+	+	+
SLCC	5489	*L. monocytogenes*	4b	+	+	+	+	+	+
NCTC	5105	*L. monocytogenes*	3a	+	+	+	+	+	+
ATCC	19119	*L. ivanovii*	5a	+	−	+	+	+	+
NCTC	11288	*L. innocua*	6a	−	−	+	+	+	−
SLCC	5334	*L. welshimeri*	6a	−	−	+	+	+	−
SLCC	3954	*L. seeligeri*	1/2b	+	−	+	+	+	−
		L. murrayi		−	+	−	−	−	−
		L. grayi		−	+	−	−	−	−

Listeriolysin O

All strains of *L. monocytogenes* tested (1/2a, 4b, 3a) hybridized with the 0.9 kb internal fragment of listeriolysin O. *Listeria seeligeri* and *Listeria ivanovii* gave positive signals albeit with a different set of restriction fragments. Using different oligonucleotide probes from the C-terminus of listeriolysin O positive reactions were even obtained with *Listeria welshimeri*, *Listeria murrayi* and *Listeria grayi*.

Beta-haemolysin

The second haemolytic principle isolated encodes a putative β-haemolysin present on a 0.8 kb EcoRI fragment. This DNA probe reacted with all *Listeria* strains excepting *L. murrayi* and *L. grayi*.

CAMP-factor

Similar results were obtained with a 0.4 kb Ndel-Hpal fragment of pLMl encoding the CAMP-factor. All *Listeria* strains except *L. murrayi* and *L. grayi* hybridized with this probe.

dth-18

The 1.1 kb gene probe coding for the 18 kD DTH-inducing protein showed specific signals with all *L. monocytogenes* strains and *L. ivanovii*. No hybridization reaction could be detected with all other *Listeria* species even when less stringent hybridization and washing conditions were used. The efficacy of the probe has recently been studied (see also the contribution of S. Notermans) testing a large number of different *Listeria* strains and confirms our results.

Discussion

We have used four cloned putative virulence factors as probes for *Listeria* species in southern hybridization assays. Two of them, namely β-haemolysin and CAMP-factor, were shown to be specific for the genus *Listeria* excepting *L. murrayi* and *L. grayi* and can therefore be used to detect *Listeria* species other than *L. murrayi* and *L. grayi*

Although the listeriolysin O is to date the only factor shown to be involved in the pathogenesis of listerial infections in a mouse model [5, 6] the gene probes tested so far are not specific for pathogenic *Listeria*. Other fragments from the listeriolysin O gene or synthetic oligonucleotides have to be tested to obtain a more specific gene probe.

The gene probe encoding the DTH-18 protein is the only probe specific for pathogenic *Listeria*. We believe it to be the probe of choice in the use in identifying pathogenic *Listeria*.

REFERENCES

1. Bannister, B. A.: Infect Immun **48,** 165 (1987).
2. Beckers, H. J., Soentoro, P. S. S., Delfgon-van Asch, E. H. M.: Int J Food Microbiol **4,** 249 (1987).
3. Schlech, W. F., Lavigue, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nicholls, E. S., Broome, C. V.: N Engl J Med **308,** 203 (1983).
4. Kingsbury, D. T.: Tibtech **5,** 107 (1985).
5. Gaillard, J. L., Berche, P., Sansonnetti, P.: Infect Immun **52,** 50 (1986).
6. Kathariou, S., Metz, P., Hof, H., Goebel, W.: J Bacteriol **169,** 1291 (1987).
7. Groves, R. D., Welshimer, H. J.: J Clin Microbiol **5,** 559 (1977).
8. Skalka, B., Smola, J., Elischerova, K.: J Clin Microbiol **15,** 503 (1982).
9. Antonissen, A. C. J. M., Lemmens, P. J. M. R., van den Bosch, J. F., van Boven, C. P. A.: FEMS Microbiol Lett **34,** 91 (1986).
10. Maniatis, T., Fritsch, E. F., Sambrook, J.: Molecular cloning, a laboratory manual. Cold Spring Harbour, New York 1982.
11. Kaufmann, S. H. E.: Infect Immun **45,** 234 (1984).

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THE USE OF THE *LISTERIA MONOCYTOGENES* DTH GENE FOR THE DETECTION OF PATHOGENIC BIOVARS IN FOOD

S. NOTERMANS, K. WERNARS and T. CHAKRABORTY

Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, Institute of Genetics and Microbiology, University of Würzburg, Würzburg, FRG

A gene probe encoding a *Listeria monocytogenes* delayed type hypersensitivity factor (DTH) was used for the detection of pathogenic biovars/serovars of the genus *Listeria* isolated from soft cheese. A total of 187 strains, isolated by plating techniques, were screened. The probe reacted with all isolated serovars of *L. monocytogenes* (except one 4b strain) and did not react with any of the *Listeria innocua* strains. Using the mouse bio-assay, as described by Kaufmann, a significant correlation was found between probe reaction and pathogenicity.

Outbreaks of listeriosis caused by indirect transmission by food, have been traced to dairy products [1, 2] and to vegetables [3]. Current methods for isolation and determination of *Listeria monocytogenes* are laborious and time-consuming. The procedure includes enrichment in (selective) liquid media, subsequent plating of the enrichment fluid on isolation media and testing of suspected colonies by a number of biochemical and serological tests. Alternative methods for rapid and reliable detection of *L. monocytogenes* in food samples are required. A promising technique is the use of DNA hybridization and a DNA probe specific for *L. monocytogenes*. Recently, a probe encoding a delayed type hypersensitivity factor (DTH) has been described by us [4]. This DNA-probe reacted with all reference strains of *L. monocytogenes* and *Listeria ivanovii* except for *L. monocytogenes* of serogroup 4a. Hybridization was found to be absent in *Listeria seeligeri*, *Listeria grayi*, *Listeria murrayi*, *Listeria innocua* and *Listeria welshimeri*. In this study we describe the screening of 187 *Listeria* strains isolated from soft cheeses for the presence of the gene. Furthermore a number of strains were tested for pathogenicity using the mouse bio-assay as described by Kaufmann [5].

SERVE NOTERMANS, K. WERNARS
National Institute of Public Health and Environmental Protection
P.O. Box 1, 3720 BA Bilthoven, The Netherlands

T. CHAKRABORTY
Institut für Genetik und Mikrobiologie, Universität Würzburg
Röntgenring 11, D-8700 Würzburg, FRG

Materials and methods

Cultures. Strains were isolated from soft cheeses and from other food products by conventional techniques. Additionally, *L. monocytogenes* (strain EGD), *L. grayi* (strain L499) and *L. murrayi* (strain L497) were used. All strains were serotyped according to the antigenic scheme of Seeliger and Höhne [6]. Biotyping was according to Rocourt et al. [7].

DNA probe. Plasmid pLM10 [4] is a pUC18-clone containing an 1.1 kb *L. monocytogenes* DNA insert. This insert, encoding a delayed type hypersensitivity factor (DTH) was excized and purified by two successive runs of agarose gel electrophoresis and electroelution. To serve as a probe, 200 ng of this DNA was ^{32}P labelled by the method of Feinberg and Vogelstein [8].

Colony hybridization. The colony hybridization procedure used was described previously [9].

Mouse bio-assay. Pathogenicity of *Listeria* strains was tested in a mouse bio-assay as described by Kaufmann [5]. In this assay 10^4–10^5 bacteria are injected intravenously into mice. After 2 or 4 days the animals are sacrificed and the number of *Listeria* are determined present in the spleen. Strains are considered pathogenic if the number of bacteria found, exceeds the number that was injected previously.

Results and discussion

An ideal probe for detection of *L. monocytogenes* would be a probe encoding a well understood virulence factor. However, the mechanism of pathogenic action of *L. monocytogenes* is poorly understood. Among the factors that appear to contribute to the virulence of *L. monocytogenes* are haemolysins [10–13], invasive factors [14], factors that cause delayed hypersensitivity [5, 15]. In this study we have used a DNA probe encoding a delayed type hypersensitivity factor. *Listeria* strains isolated from soft cheeses were tested for hybridization reaction with this probe. Serotypes and biotypes of *Listeria* strains isolated from soft cheeses are summarized in Table I. Of *L. monocytogenes* only strains of serovars 1/2a, 1/2b and 4b were found. All these strains, except one 4b strain, reacted with the DNA probe used. All other *Listeria* strains isolated were of biovar *L. innocua* and belonged to serogroups 6a and 6b. None of these *L. innocua* strains reacted with the DNA probe. These results are in accordance with those found earlier [9].

Table I

DNA hybridization reactions of serotypes and biotypes of Listeria strains isolated from soft cheeses by plating techniques using DNA probe encoding a delayed type hypersensitivity factor

No. of strains	Serovar	Biotype (frequency)	Number (and per cent) with hybridization signals
77	1/2a	*L. monocytogenes*	77 (100%)
10	1/2b	*L. monocytogenes*	10 (100%)
7	4b	*L. monocytogenes*	6 (86%)
62	6a	*L. innocua*	0 (0%)
31	6b	*L. innocua*	0 (0%)

Table II

*Persistence of Listeria strains in spleens of intravenously infected mice**
Pathogenicity test described by Kaufmann [5]

Strain	Biovar	Serovar	Isolation source	Hybridization reaction**	^{10}log count/g of spleen***	
					day 2	day 4
EGD	*L. monocytogenes*	1/2a	ref. strain	+	7.4	8.3 +
L44	*L. monocytogenes*	1/2b	soft cheese	+	6.2	7.1 +
L83	*L. monocytogenes*	1/2c	food	+	5.4	++
L19	*L. monocytogenes*	3a	food	+	6.4	6.5
L12	*L. monocytogenes*	3b	food	+	8.4	++
L98	*L. monocytogenes*	3c	food	+	8.2	++
L99	*L. monocytogenes*	4a	faeces	—	6.0	5.9 +
L49	*L. monocytogenes*	4b	soft cheese	+	7.0	++
L148	*L. monocytogenes*	4b	soft cheese	+	6.5	++
L311	*L. monocytogenes*	4b	soft cheese	+	8.3	++
L312	*L. monocytogenes*	4b	soft cheese	—	5.2	++
L578	*L. seeligeri*	4c	food	—	<2.0	<2.0
L73	*L. innocua*	6a	soft cheese	—	2.0	3.7
L106	*L. innocua*	6b	soft cheese	—	<2.0	<2.0
L498	*L. grayi*		ref. strain	—	<2.0	<2.0
L457	*L. murrayi*		ref. strain	—	<2.0	<2.0

* 10^4–10^5 of organism were injected respectively in 4 mice
** Hybridization was carried out using the DTH gene as probe
*** Average of two mice; + mouse died before sampling

Seven strains isolated from soft cheeses and 9 controls were tested for pathogenicity in the mouse bio-assay. With this test pathogenicity was observed for all *L. monocytogenes* strains tested (Table II). Also a strain of serovar 4a and the strain of serovar 4b isolated from soft cheese, which did not react with the DNA probe, were pathogenic. With all other biovars negative results were obtained and in most cases the organisms had been cleaned from the spleen of the test animals. These findings demonstrates a strong correlation between pathogenicity and the presence of the DTH-gene *Listeria* strains. Previously Datta et al. [16] tested a haemolysin gene probe for detection of *Listeria*. However, by a lack of data relating to biotype, serovar and haemolytic activity it is not possible to interprete their results in relation to pathogenicity. Since strong homology exists between listeriolysin, streptolysin O and pneumolysin-genes [17, 18] it may be expected that a DNA-probe encoding listeriolysin will cross react with these related toxin-genes when testing samples for the presence of *L. monocytogenes*. Furthermore it has been demonstrated already that the listeriolysin gene is present in *L. grayi* and *L. murrayi*, strains which are regarded as non-pathogenic.

The results obtained with the DNA probe encoding a DTH factor prove that is suited for detection of pathogenic *Listeria* strains present in food.

The colony hybridization procedure can be used for confirming the presence of *L. monocytogenes* on agar plates. Also enrichment cultures can be tested directly for the presence of *L. monocytogenes* by using a filtration technique. In the near future, however, it might be possible to detect small numbers of *L. monocytogenes* in food by the so called polymerase chain reaction method as described by Verlaan-de Vries et al. [19]. With this technique target DNA is reproduced using an in vitro technique.

REFERENCES

1. Fleming, D. W., Cochi, S. L., MacConald, K. L., Brondum, J., Hayes, P. S., Plikaytis, B. D., Holmes, M. B., Audurier, A., Broome, C. V., Reingold, R. L.: N Engl J Med **312,** 404 (1985).
2. James, S. M., Fannin, S. L., Agee, B. A., Hall, B., Parker, E., Vogt, J., Run, G., Williams J., Lieb, L., Salminen, C., Pendergast, T., Wernar, S. B., Chin, J.: MMWR **34,** 357 (1985).
3. Schlech, W. F., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nicholls, E. S., Broome, C. V.: N Engl J Med **308,** 203 (1983).
4. Chakraborty, T., Leimeister-Wächter, M., Goebel, W., Wernars, K., Notermans, S.: Letter to Lancet (To be published). 1988.
5. Kaufmann, S. H. E.: Infect Immun **45,** 234 (1984).
6. Seeliger, H. P. R., Höhne, K.: In Bergen, T., Norris, J. R. (eds): Methods in Microbiology. Vol. 13. Academic Press, New York, Toronto, Sydney, San Francisco 1979. pp. 31–49.
7. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 56 (1983).
8. Feinberg, A. P., Vogelstein, P.: Anal Biochem **132,** 6 (1983).
9. Notermans, S., Chakraborty, T., Dufrenne, J., Heuvelman, C. J., Maas, H., Jansen, W., Wenars, K., Guinee, P.: Appl Environ Microbiol (In press).
10. Gaillard, J. L., Berche, P., Sansonnetti, P.: Infect Immun **52,** 50 (1987).
11. Hof, H.: Med Microbiol Immunol **173,** 207 (1984).
12. Kathariou, S., Metz, P., Hof, H., Goebel, W.: J Bacteriol **169,** 1291 (1987).
13. Kathariou, S., Köhler, S., Kuhn, M., Goebel, W.: Ann Bacteriol (Paris) A **138,** 256 (1987).
14. Gaillard, J. L., Berche, P., Mounier, J., Richard, S., Sansonnetti, P.: Ann Microbiol (Paris) A **138,** 255 (1987).
15. Antonissen, A. C. J. M., Lemmens, P. J. M. R., van den Bosch, J. F., van Boven, C. P. A.: FEMS Microbiol Lett **34,** 91 (1986).
16. Datta, A. T., Wentz, B. A., Hill, W. A.: Appl Environ Microbiol **53,** 2256 (1987).
17. Leimeister-Wächter, M., Chakraborty, T., Goebel, W.: Ann Microbiol (Paris) A **138,** 252 (1987).
18. Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J. L., Cossart, P.: Infect Immun **55,** 3225 (1987).
19. Verlaan-de Vries, M., Bogaard, M. E., van den Elst, M., van Boom, J. H., van der Eb, A. J., Bos, J. L.: Gene **50,** 313 (1986).

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FURTHER RESULTS AND EXPERIENCES WITH PHAGE-TYPING OF *LISTERIA*

S. ORTEL

Institute of Medical Microbiology and Epidemiology, Reference Laboratory for Listeriosis, Martin Luther University, Halle/S, GDR

Results of phage-typing of 22 strains from 16 listeriosis cases, which were isolated during an epidemic outbreak in the district of Magdeburg, showed that most strains were determined by the octal codes 73611, 73615, 73655, 73645. We suppose that this was the epidemic strain, although also other phage-patterns (40000, 00600, 03200) were found. The source of the epidemic outbreak could not cleared up. The purpose of further investigations was to establish how far phage-patterns of *Listeria* from the same patients from different materials might be identified as identical, similar or different. We give examples of typing results in 1/2a and 4b strains and compare the results concerning an significant or non-significant alteration.

From May until October 1985, in the district of Magdeburg an accumulation of listeriosis was observed [1]; 16 premature babies and infants and one adult became ill, 9 of the former died or were born dead.

Materials and methods

The Listeria strains and clinical data from this outbreak were obtained from the Institute of Medical Microbiology of the Medical Academy of Magdeburg. The strains, including those from our laboratory were serotyped with factor-sera and phage-typed with the international phage-set [2]. For interpretation of the results the Octal-Code system was used [3].

Results

Figure 1 shows the distribution of the cases. It demonstrates that the first case was observed in May, further cases followed in June, July and August; the epidemic lasted until October. The highest incidence-rate was observed in August with 6 cases. The slow ascent, peak and falling off, is usually typical for epidemic outbreaks.

In Table I the results of serotyping and phage-typing and some clinical data of the cases are summarized. All the 25 isolated strains from 17 patients belonged to serovar 4b. Concerning the phage-patterns, the first isolated

SIEGFRIED ORTEL
Institute of Medical Microbiology and Epidemiology, Martin Luther University
Leninallee 6, 4020 Halle/S. GDR

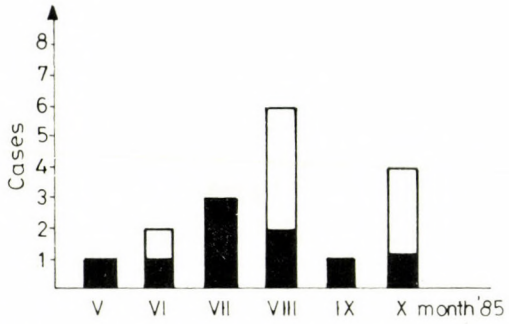

Fig. 1. Distribution of listeria cases during the epidemic. Closed columns, stillborn or fatal cases

Table I

Serovars and phagovars of the isolated L. monocytogenes strains

Patient	Sex	Born	Ill date	Source	Serovar	Phagovar (Code)	Clinical
1	f	23. 5.85	28. 5.85	liver	4b	71615●	respiratory
				lung	4b	73615●	distress syndrome, stillborn died
2	m	25. 6.85	26. 6.85	CSF	4b	n.typ.	meningitis dead
3	m	3. 6.85	27. 6.85	CSF	4b	40000	meningitis
4	m	2. 7.85	8. 7.85	gut	4b	n.typ.	abortus stillborn
5	f	8. 7.85	12. 7.85	brain	4b	73611●	abortus stillborn
6	f	19. 7.85	24. 7.85	small gut	4b	73605●	premature birth, dead
7	m	6. 8.85	11. 8.85	blood	4b	00600	premature birth, septicaemiae
8	m	13. 8.85	14. 8.85	meconium	4b	01640	premature birth
9	f	21. 8.85	24. 8.85	stomach	4b	01601	meningitis
				auditory passage	4b	73615●	died
10	m	21. 8.85	31. 8.85	CSF	4b	03200	meningitis
				eye	4b	43200	
11	m	23. 8.85	23. 8.85	blood	4b	41601	premature birth, dead
12	f	28. 8.85	28. 8.85	stomach	4b	73655●	meningitis
				blood	4b	73645●	
13	m	19. 9.85	29. 9.85	small gut	4b	strains	
				gut	4b	no more	abortus
				brain	4b	available	dead
				liver	4b	for typing	
14	f	6.10.85	17.10.85	CSF	4b	51645	meningitis
15	f	17.10.85	17.10.85	stomach	4b	43655○	respiratory
				blood	4b	43645○	distress syndrome died
16	f	20.10.85	23.10.85	CSF	4b	73645●	meningitis
17	f	6. 5.63	24.10.85	urine	4b	43651○	nephritis

● non-significant alteration
○ questionable alteration

strains (in May) corresponded to codes 71615 and 73615 and most strains, which were isolated later on fell into similar or identical codes, as for instance 71615, 73615, 73611, 73605, 73615, 73655, 73645. The alteration is not significant, i.e., these types could have originated from the epidemic strain. Also listeriae with codes 43655, 43645, 43651 might be similar to one another. However, strains with codes 40000, 00600, 01640, 01601, 03200, 43200, 41601 and two non-typeable strains. were quite different from the proposed epidemic strains and had no association with the epidemic.

The geographical distribution of the cases and their phage-patterns are shown in Fig. 2. It demonstrates that the listeriosis-cases distributed over the whole district of Magdeburg and were observed in the South, in the Middle and in the North. The phage-patterns 73615, 71615, 73655, 73605 were preponderant in little villages, whereas in the city different phage patterns of non-epidemic strains were more often observed, also non-epidemic-strains. Serotyping, phage-typing and epidemiological inquiries allowed no conclusion as to the source of the outbreak. The results of phage typing show, that during the outbreak in the environment strains of different phage-patterns may have occurred and that the number of infected mothers and other persons might have been higher than that of the manifest listeriosis cases. Our further experiences

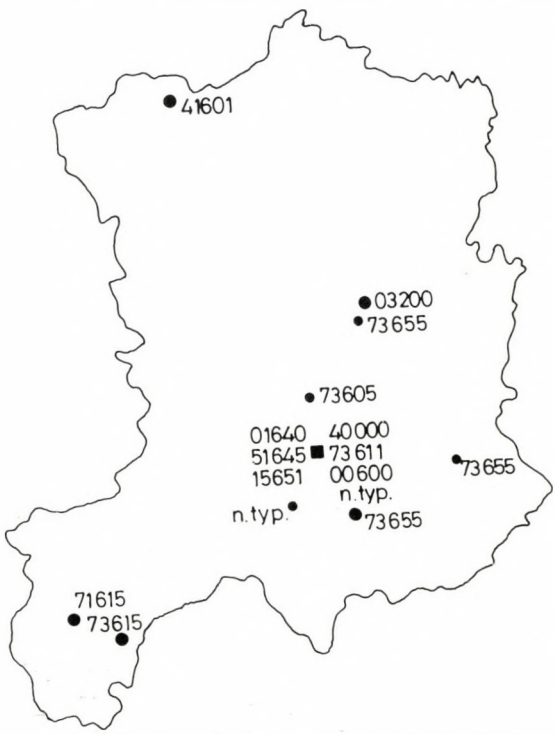

Fig. 2. Geographical distribution of phage patterns in the district of Magdeburg

Table II

Phage patterns of L. monocytogenes 4b strains isolated from different sites of the same patients

Case	Source	Serovar	Phagovar (Code)
1 premature baby	blood	4b	04610
	throat	4b	11601
	stomach	4b	01601
	ear	4b	11601
	eye	4b	01611
	nose I	4b	01601
	meconium	4b	00601
	nose II	4b	33601
2 premature baby	meconium	4b	41611
	throat	4b	01600
	stomach	4b	23600
	eye	4b	03600
3 premature baby	stomach	4b	13601
	ear	4b	00201
	throat	4b	41615
	blood	4b	01601
	meconium	4b	41605
mother	lochia	4b	43605
	faeces	4b	41605
4 premature baby	meconium	4b	51601
	throat	4b	53601
	stomach	4b	53615
mother	lochia	4b	73615
5 premature baby	throat	4b	01240
	ear	4b	01240
	CSF	4b	01240
	meconium	4b	01240

with phage-typing deal with results we obtained for strains isolated from the same patients at different sites (Table II).

Case 1 demonstrates serovars and phage-patterns from 8 different specimens of the same patient. The typing of the different strains show no significant alteration, because the three end-digits of the code were nearly identical.

Also in Case 2 there seems to be no significant alteration of the strains in the four different sites, because the end-digits of the code were identical.

In the third case we show a comparison between strains of the baby and his mother. Also in this example we have a non-significant alteration, except one strain isolated from the ear. This strain belonged to code 00201.

Table III

Phage patterns of L. monocytogenes isolated from different sites of the same patients; non-significant alteration

Case	Source	Serovar	Phagovar
4	stomach	1/2a	00020
	meconium	1/2a	00020
	ear	1/2a	00020
	eye	1/2a	00020
12	blood	4b	01600
	stomach	4b	01601
	meconium	4b	00601
	ear	4b	11601
	eye	4b	01611
	nose	4b	01600
	throat	4b	11601
15	meconium	4b	41611
	stomach	4b	23600
	eye	4b	03600
	throat	4b	01600

Similar results are demonstrated in the fourth example, as the strain of the mother, isolated from lochia, is identical with the strains isolated from the baby.

In Case 5, all strains from different materials also show identical phage-patterns. Table III shows similar results in three other patients.

Discussion

This synopsis and our experiences in this field demonstrates that, as also shown by other authors [4–11] phage-typing gives reliable results in epidemiological problems. But the interpretation of phage-typing results by using each 15 phages for typing serovar 1/2a and serovar 4b giving 5 code digits shows that three identical digits should be necessary to determine a strain to be of the same kind. Further investigations may show, whether we can conclude common directions of these results. The Octal Code, which is also used by other authors [4, 5, 9–12] with success in epidemiological investigation and in confirmation, reporting and comparing results involves the definition of characteristic reactions, especially if the number of phages used is larger than 20 or 30.

Ralovich and co-workers [11] have also shown alterations of phage-patterns and biotypes in different materials of the same patients; in such cases alterations were possible and phage-typing should be used with great caution in epidemiological work and conclusions.

REFERENCES

1. Schuster, G., Borkhardt, H.-L.: Z Ges Hyg **33,** 261 (1987).
2. Rocourt, J., Audurier, A., Courtieu, A. L., Durst, J., Ortel, S., Schrettenbrunner, A., Taylor, A. G.: Zentralbl Bakteriol (A) **259,** 489 (1985).
3. Audurier, A., Chalons, F., Toucas, M.: Ann Microbiol (Paris) A **130,** 345 (1979).
4. Audurier, A., Chatelain, R., Chalons, F., Piéchaud, M.: Ann Microbiol (Paris) B **130,** 179 (1979).
5. Audurier, A., Taylor, A. G., Carbonelle, B., McLauchlin, J.: Clin Invest Med **7,** 229 (1984).
6. McLauchlin, J., Audurier, A., Taylor, A. G.: J Med Microbiol **22,** 367 (1986).
7. McLauchlin, J., Audurier, A., Taylor, A. G.: J Med Microbiol **22,** 357 (1986).
8. Ortel, S.: Z Ges Hyg **27,** 837 (1981).
9. Ralovich, B., Audurier, A., Ortel, S., Angyal, T., Proksza, A.: Acta Microbiol Hung **30,** 103 (1983).
10. Ralovich, B., Audurier, A., Hajtós, I., Berkessy, E., Pitron-Szemerdi, M.: Acta Microbiol Hung **33,** 9 (1986).
11. Ralovich, B., Ewan, E. P., Emődy, L.: Acta Microbiol Hung **33,** 19 (1986).
12. Taylor, A. G., McLauchlin, J., Green, H. T., Macaulay, M. B., Audurier, A.: Lancet **2,** 1106 (1981).

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INTERNATIONAL PHAGE TYPING CENTER FOR LISTERIA: REPORT FOR 1987

JOCELYNE ROCOURT and BÉNÉDICTE CATIMEL

Unit of Bacterial Ecology, Institute Pasteur, Paris, France

A total of 3400 *Listeria* strains were sent for identification and/or phage typing during 1987. These strains mainly originated from Europe. They were isolated from humans, animals and mostly from foodstuffs, thus reflecting the increasing interest concerning the view that listeriosis is a foodborne disease. Phage typing proved to be a useful tool for epidemiological survey. That was especially evidenced during studies of the outbreak of human listeriosis in Switzerland, for which a contaminated cheese was incriminated as the source of contamination.

Among the methods so far described for typing *Listeria* isolates, serovar determination and phage typing remain the most commonly used because of the fine characterization of the strains they provide. Their usefulness has been pointed out several times, especially during hospital cross-infections as well as during the recent outbreaks of human listeriosis [1–5]. Phage typing used for studying some of these epidemics demonstrated that strains isolated from human pathological samples, and in some cases from foodstuffs, belong to the same phage type, thus strongly suggesting that a particular food was implicated as the source of human contamination.

More than 10 000 strains were sent to the International Phage Typing Centre for phage typing and/or identification since 1984. These strains originated from 31 various countries of the world (Fig. 1). The present report concerns strains received in 1987.

Materials and methods

Strains were identified according to previously described methods [6]. The serogroup of *Listeria monocytogenes* isolates (when the serovar was not conveyed by the correspondent) was determined using unabsorbed sera anti-serogroup 1/2 and anti-serogroup 4. The procedure used for phage typing was those previously standardized during a multicenter study, with the *L. monocytogenes* phages selected at this occasion and experimental phages for the other species of the genus *Listeria* [7–9]. Results were analyzed using and IBM PC computer with Filling Assistant data file.

JOCELYNE ROCOURT, BÉNÉDICTE CATIMEL
Institut Pasteur, Unité d'Ecologie Bactérienne
25 rue Dr. Roux, 75724 Paris Cedex 15, France

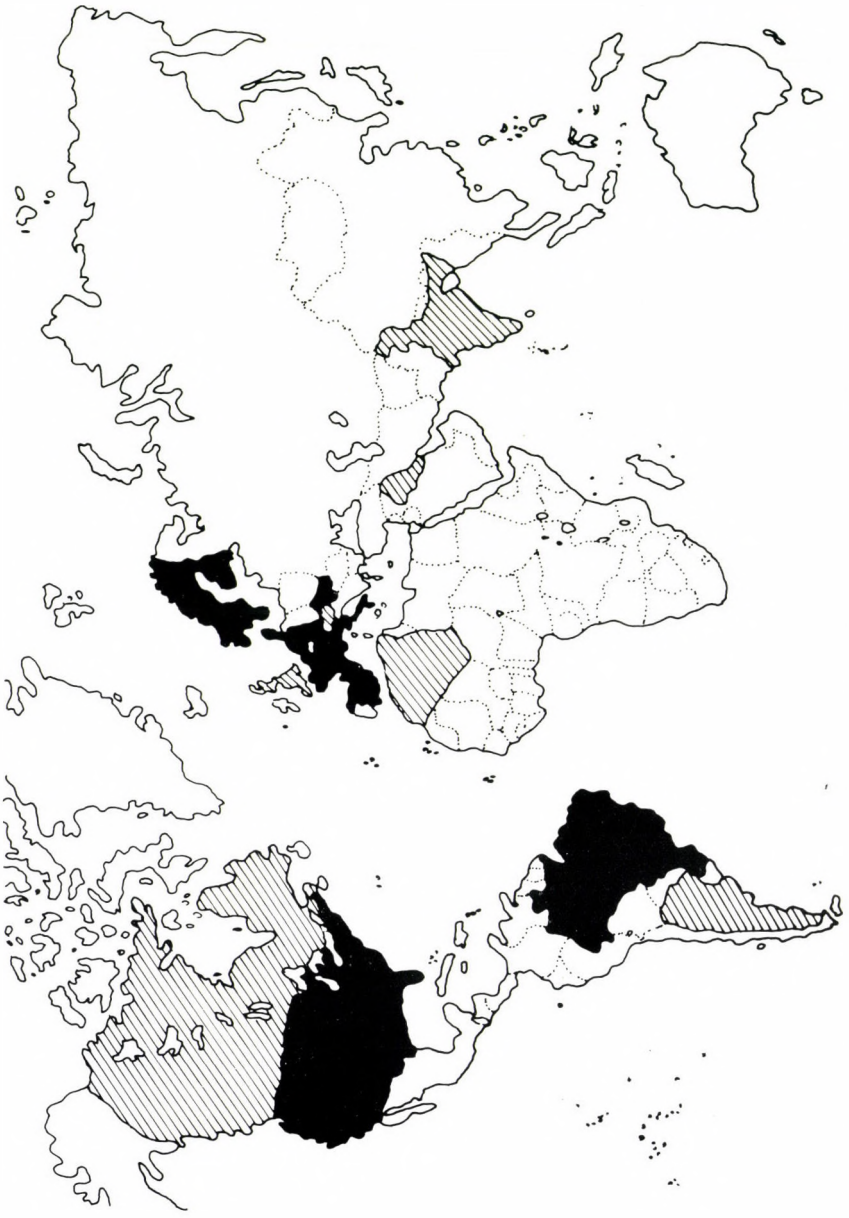

Fig. 1. Geographical distribution of strains. Shaded areas, 1–49 strains; closed areas, 50 or more strains

Results and discussion

Characterization of the strains. A total of 3394 strains were received during 1987, from 13 different countries. Identification, serogroup determination and phage typing results are shown in Tables I and II.

Epidemiological aspects

France. See Espaze et al. [10] in this number.

Switzerland. Since the epidemic of human listeriosis which occurred in 1984 in the district of Vaud [3], this infection was persisting at an endemic

Table I

Geographical origin of the strains and percentage of typeable strains according to species

Country	*L. monocytogenes*		*L. ivanovii*	*L. innocua*	*L. welshimeri*	*L. seeligeri*
	1/2*	4*				
France	907 (55%)	459 (67%)	2	325 (57%)	6	75 (42%)
Austria	14	1	0	0	0	0
Belgium	13	48 (87%)	0	0	0	0
Canada	4	2	0	0	0	0
Denmark	81 (45%)	63 (82%)	0	1	1	0
Spain	1	19 (95%)	0	0	0	4
Finland	66 (74%)	31 (75%)	0	14	0	0
Italy	37 (75%)	39 (61%)	0	46 (60%)	0	3
N. Zealand	6	29 (97%)	0	0	0	0
FRG	188 (43%)	52 (67%)	0	99 (49%)	15	10
Sweden	4	8	0	0	0	0
Switzerl.	165 (54%)	346 (91%)	1	104 (50%)	0	8
USA	16 (25%)	12	0	0	0	0
Total	1502 (53%)	1109 (76%)	3 (66%)	619 (54%)	22 (57%)	100 (43%)

* Serogroups

Table II

Origin of the strains

Species	Human	Animal	Food*	Environment
L. monocytogenes	602	195	1711	103
L. ivanovii	0	0	3	0
L. innocua	17	35	475	42
L. welshimeri	0	0	22	0
L. seeligeri	0	0	95	0
L. grayi	1	0	0	0
Total	621	231	2306	145

* Food and food industry

level. Strains belonging to two phage types (including the epidemic one) were regularly recovered from patients. Careful epidemiological investigations during four years led to isolate strains of the same phage types from a particular cheese, providing strong evidence that this food was responsible for the human infection [2].

Belgium. Human listeriosis is particularly well studied in this country, this infectious disease being included with some others in a surveillance program. We receive each year all the strains collected during the past year. According to the phage types, the evolution of human listeriosis seems to be very similar to that observed in France: a great majority of sporadic cases, sometimes amplified in small clusters.

Denmark. Phage typing of strains recovered from patients between 1981 and 1987 showed the emergence of a cluster of strains characterized by a single phage type, thus suggesting the occurrence of a recent outbreak in this country. No relation, using phage typing, between these strains and those isolated from foodstuffs in 1987 could be established.

Austria. Strains from six patients involved in an outbreak during July 1986 were indistinguishable by phage typing [5].

FRG. Among 95 strains recovered from chicken, only 25% were phage typeable. This low percentage remains unexplained (we faced similar results four years ago with Belgian strains of the same origin). Two suspicions of cross-infections in nursery wards were reinforced by phage typing (strains with same phage type).

Italy. In the Ferrara area, human listeriosis was never reported until 1987 when the first two cases were diagnosed. No relationship between the phage type of these strains and those of the strains isolated from foodstuffs and environment [11] in this area for three years could be detected.

Finland. A total of 96 strains of veterinary origin were studied. The predominance of serogroup 1/2 strains among *Listeria monocytogenes* isolates (80 %) as well as the phage types observed resembled those previously observed for strains from similar origin in Norway [12].

New Zealand. A retrospective study of 35 strains collected from patients between 1982 and 1986 suggested that cross-infections occurred at least two times in the same hospital.

It may be concluded that phage typing undoubtely participated a great deal to explain some aspects of the epidemiology of human listeriosis, especially in contributing to demonstrate that this infection may be considered in some cases as a foodborne disease. Nevertheless, the percentage of non typeable strains which is sometimes relatively high (Table II), as well as eventual difficulties in reproducibility of some results, emphasize the necessity to undertake typing using new methodologies based on molecular markers as well.

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REFERENCES

1. Audurier, A., Taylor, A., Carbonnelle, B., McLauchlin, J.: Clin Invest Med **7,** 229 (1984).
2. Bille, J., Mean, F., Rocourt, J.; Glauser, M. P., et al.: Épidemie de listériose humaine dans le canton de Vaud. II. Épidemiologie. 56. Jahresversammlung der Schweizerischen Gesellschaft.
3. Malinverni, R., Bille, J., Perret, Cl., Regli, F., Tanner, F., Glauser, M. P.: Schweiz Med Wschr **115,** 2 (1985).
4. McLauchlin, J., Audurier, A., Taylor, A. G.: J Med Microbiol **22,** 367 (1986).
5. Tulzer, G., Bauer, R., Daubek, Puza, W. D., Eitelberger, F., Graber, C., Heinrich, E., Hohenauer, L., Stojakovic, M., Wilk, F.: Klin Pädiatr **199,** 325 (1987).
6. Rocourt, J., Grimont, F., Grimont, P. A. D., Seeliger, H. P. R.: Curr Microbiol **7,** 383 (1982).
7. Rocourt, J., Audurier, A., Courtieu, A. L., Durst, J., Ortel, S., Schrettenbrunner, A., Taylor, A. G.: Zentralbl Bakteriol (A) **259,** 489 (1985).
8. Rocourt, J., Catimel, B., Schrettenbrunner, A.: Zentralbl Bakteriol (A) **259,** 341 (1985).
9. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Zentralbl Bakteriol (A) **251,** 505 (1982).
10. Espaze, E. P., Gautreau, D., Catimel, B., Miegeville, A. F., Rocourt, J., Courtieu, A. L.: Acta Microbiol Hung **36,** 231 (1989).
11. Luppi, A., Rocourt, J., Bucci, G., Maini, P.: Boll Ist Sieroter Milan **65,** 108 (1986).
12. Gronstol, H., Rocourt, J., Catimel, B.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): Listeriose, Listeria, Listeriosis. Proc. 9th International Symposium on the Problems of Listeriosis. Université de Nantes, Nantes 1986.

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AN EPIDEMIOLOGICAL SURVEY OF HUMAN LISTERIOSIS IN FRANCE DURING 1987, USING SEROTYPING AND PHAGE TYPING

E. P. Espaze, D. Gautreau, Bénédicte Catimel, A. F. Miegeville, Jocelyne Rocourt and A. L. Courtieu

National Listeria Reference Center, Nantes, and Unit of Bacterial Ecology Institute Pasteur, Paris, France

During 1987 the French National Reference Center for Listeria received, from this country, 384 *Listeria monocytogenes* strains isolated from human listeriosis cases. A constant exchange of information and strains between the Reference Center and the Bacterial Ecology Unit of the Institute Pasteur of Paris allowed complete characterization of these isolates, using serotyping and phage typing. Among the strains studied 22%, 13% and 63%, respectively belonged to serovars 1/2a, 1/2b, and 4b, but this distribution can vary according to place and time of isolation. Only 58% of strains were phage-typeable (1/2a: 29%), (1/2b: 66%) and (4b: 40%). Phage typing allows to consider that French human cases, in 1987, were mainly sporadic. However, a small number of cases corresponding to similar phage types could be clustered according to place and/or time.

Three hundred and eighty four *Listeria monocytogenes* strains, isolated from 366 human listeriosis cases in France during 1987, were serotyped in the French Reference Center for Listeria (Nantes) and phage typed at the Bacterial Ecology Unit of the Pasteur Institute (Paris). Through a permanent collaboration and information exchanges, most of the strains were precisely characterized. The chronological and geographical serovar and lysovar distribution was studied, showing the complexity of the French listeriosis epidemiology.

Materials and methods

Identifications were performed as previously described by Seeliger et al. [1] and Rocourt et al. [2]. Serovar determinations were carried out as described in [1]. Phage typing determinations were performed according to the Audurier et al. [3] using the *Listeria* phage set selected by an international multicentre study [4].

Results

The results are summarized as follows:

(i) Serovars were determined for 366 *L. monocytogenes* strains isolated from human listeriosis cases in France in 1987. Six serovars were found and

E. P. Espaze, D. Gautreau, A. F. Miegeville, A. L. Courtieu
Centre National de Référence des Listeria
Nantes, France

Jocelyne Rocourt, Bénédicte Catimel
Institut Pasteur, Unité d'Ecologie Bactérienne
25 rue du Dr. Roux, 75724 Paris Cedex 15, France

there were three main serovars: serovar 1/2a, 22%; serovar 1/2b, 13% and serovar 4b, 63%. Serovars 1/2c, 3b and 3c were infrequently found. Since 1984 the annual percentage range was 63–70%. However, in 1988 the 4b serovar percentage could be lower than 50%.

(ii) The serovar distribution varied according to the months of the isolation as shown in Fig. 1. The monthly distribution according to serovars can also vary according to years of isolation.

(iii) The serovar distribution varied according to the geographical region of isolation. Serovar 4b was predominant in most of the regions, except Nor-

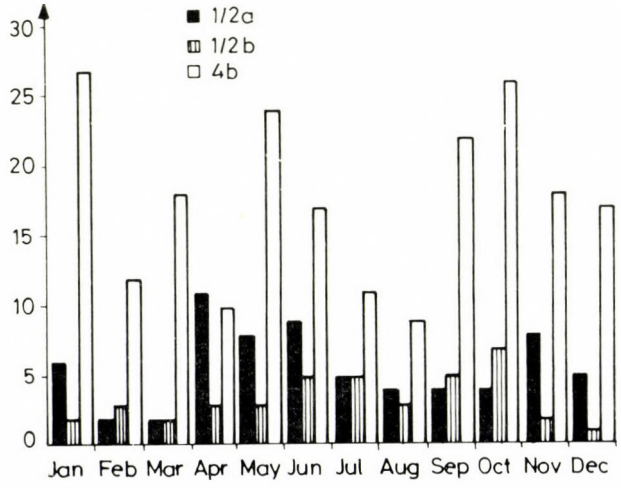

Fig. 1. Monthly distribution of *L. monocytogenes* serovars 1/2a, 1/2b and 4b isolated from 321 human cases in France, 1987

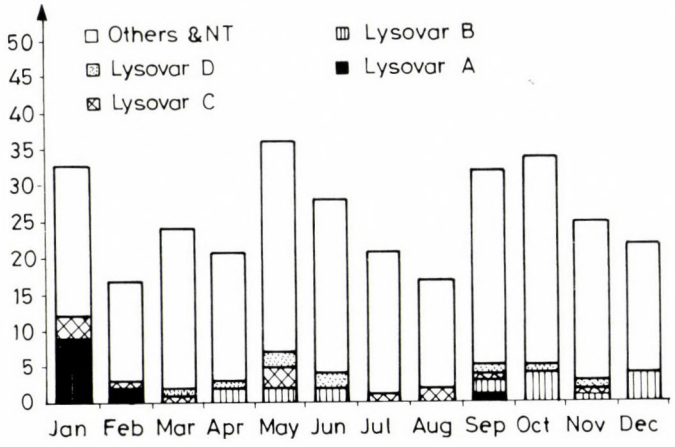

Fig. 2. Monthly distribution of lysovars of *L. monocytogenes* isolated from 320 human listeriosis cases in France, 1987

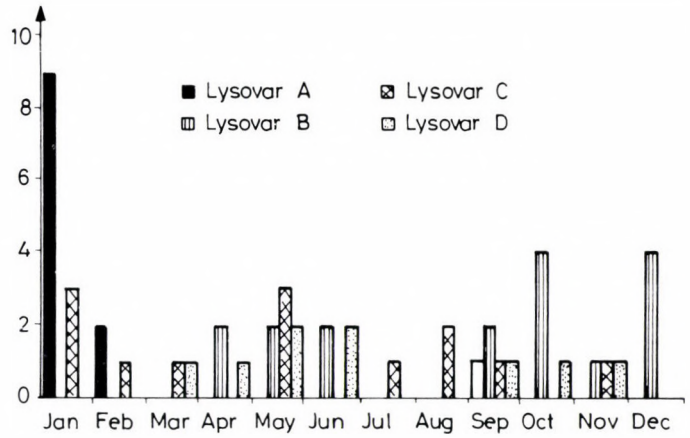

Fig. 3. Monthly distribution of the four main lysovars of *L. monocytogenes* serovar 4b, isolated from human listeriosis cases in France, 1987

mandie, Aquitaine and Ile de France, where its percentages were respectively 30%, 38% and 46% of the *L. monocytogenes* strains isolated.

(iv) Out of the 320 lysovars of determined strains, only 58% were phage-typeable. The percentages of non phage-typeable (NT) strains were respectively: 29% for serovar 1/2a, 66% for serovar 1/2b and 40% for 4b.

(v) There was a wide range of lysovars amongst the *L. monocytogenes* strains studied as shown in Fig. 2. However, four lysovars were more frequently found (lysovars A, B, C, D). Several epidemic bursts of two to five cases, with similar lysovars have been pointed out. These cases are connected with time and/or geographical localization.

(vi) A more important epidemic pike occurred in January (9 cases) and ended in February (2 cases). These cases appeared simultaneously (Fig. 3) in geographical areas which were not close, although, some small clusters could be localy pointed out. These strains belonged to 4b serovar and to 2671 lysovar (lysovar A).

(vii) Three other 4b main lysovars: 2389/47/101/340 (lysovar B), 2425 (lysovar C) and 2389/2425/3274/2671/47/108/340 (lysovar D) were recognized but this time without geographical and/or time connection (Fig. 3).

(viii) The four main lysovars A, B, C and D represent only 16% of the studied strains.

(ix) Amongst the serovar 4b, only 8% of the human investigated strains of *L. monocytogenes* showed some lysovars similar to those determined for food strains during 1987.

Discussion

Human listeriosis epidemiology in France is complex. The number of isolates and the serovar distribution vary according to months and geographical area and could be different one year from another. There is a wide range of lysovars and the main ones represent a small fraction of the studied strains. Their distribution through time and area is not preferential but from time to time some more or less important bursts of few cases can occur. The distribution of human *L. monocytogenes* serovars and lysovars suggests that besides geographically and time connected cases there are some which are serovar and lysovar connected but not geographically related. Sometimes several unrelated cases, with different or same serovars and various lysovars appear during a very short period of time in the same place. In conclusion, the serotyping and phage typing characterization of human origin *L. monocytogenes* strains suggest that in France human listeriosis is represented mainly by sporadic cases and from time to time by little epidemic bursts.

REFERENCES

1. Seeliger, H. P. R., Höhne, K. In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Vol. 13. Academic Press, London—New York 1979. pp. 31–49.
2. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 65 (1983)
3. Audurier, A., Rocourt, J., Courtieu, A. L.: Ann Microbiol (Paris) A **128,** 185 (1977).
4. Rocourt, J., Audurier, A., Courtieu, A. L., Durst, J., Ortel, S., Schrettenbrunner, A., Taylor, A. G.: Zentralbl Bakteriol (A) **259,** 489 (1985).

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PRELIMINARY STUDIES ON MONOCINE TYPING OF *LISTERIA MONOCYTOGENES* STRAINS

D. WILHELMS and D. SANDOW

Institute of Medical Microbiology, Section of Medicine, Martin Luther University, Halle-Wittenberg, Halle (Saale), GDR

About 58% of *Listeria monocytogenes* strains produced monocines. The titres of 1/2a monocines were higher than those of 4b strains.

The phage typing of serovars of *Listeria monocytogenes* and its epidemiological evaluation are limited. For this reasons it is useful to look for further possibilities of typing *Listeria* serovars. One direction could be the monocine typing [1–3]. Up to now, in the literature only a few data exist. It is not clear, if the monocines can be used for a typing system.

Therefore, a few own preliminary results should be contributed to monocine production of the most important serovars 1/2a and 4b, to their properties and the possible monocine grouping of *L. monocytogenes* strains.

Materials and methods

We tested 88 *L. monocytogenes* strains of serovars 1/2a and 4b from patients with listeriosis. The monocines were produced by means of mitomycin C induction, as described by Ortel [4]. The effective monocine production was proved on indicator strains of serovar 1/2a, 4b and 5. Properties of the strains were determined as indicated in "Results and discussion".

Results and discussion

Characterization of monocines. Table I and Fig. 1 show the frequency of monocine production by *L. monocytogenes* strains as the titres of monocines. The geometric mean titre of monocines produced by serovar 1/2a amounted to 17.4. The mean value of those from serovar 4b was 7.7.

The stability of monocines was tested after storage at 4 °C. The titres tested i mmediately after preparation and monthly over one year showed only a decrease of one step.

D. WILHELMS, D. SANDOW
Institut für Medizinische Mikrobiologie und Epidemiologie der Martin-Luther-Universität
Leninallee, Halle-Wittenberg, 4020 Halle, GDR

Table I

Frequency of monocine producing L. monocytogenes strains

Serovar	Total number	Producers of monocines	Strains not producing monocines
1/2a	56	33 (59%)	23 (41%)
4b	32	18 (56%)	14 (44%)
Total	88	51 (58%)	37 (42%)

Various monocines of serovars 1/2a and 4b were incubated in a series of buffers with pH ranging from 2 to 11 at 37 °C for 24 h. The activity was completely stable in the pH range from 6 to 11. Below pH 6 and above 11 the monocines lost their activity.

The activity of our monocines was destroyed at 60 °C in 1 min.

Like those of Hamon and Péron, our monocines were also resistant to trypsin.

Monocines centrifuged through a gradient of 15 to 45% sucrose showed a peak at about 20% sucrose. Figure 2 shows the result of sucrose gradient centrifugation. The gradient fractions containing monocines were studied by electron microscopy, but no phage like particles could be found after potassium phosphotungstate staining. Therefore, it cannot be decided whether our monocine belong to defect phages as described by Bradley [1].

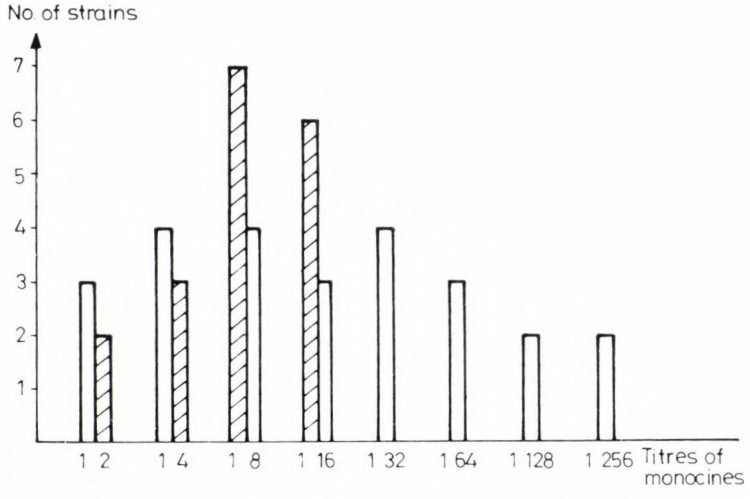

Fig. 1. Titres of monocine producing *L. monocytogenes* strains. Shaded columns, serovar 4b; open columns serovar 1/2a

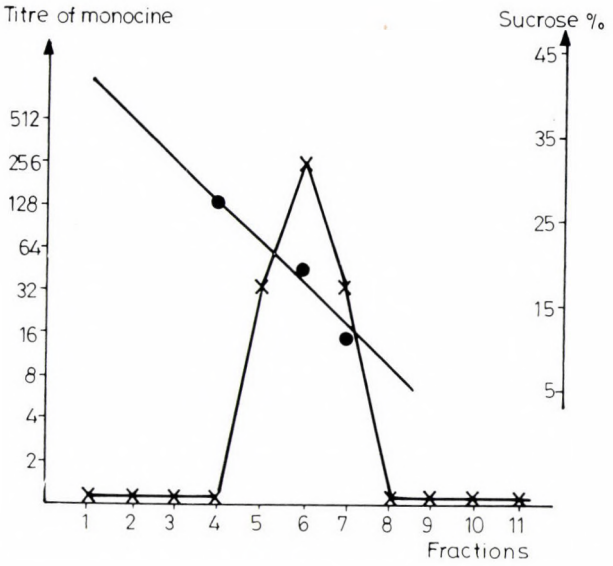

Fig. 2. Ultracentrifugation of monocine preparation through a 15–45% linear sucrose gradient. *L. monocytogenes* strain 1/2a, No. 2459

Monocine typing of L. monocytogenes serovars. We tested 56 monocines of serovar 1/2a and 32 monocines of serovar 4b against 32 patient strains of each serovar. Monocines produced by serovar 4b showed no reactions against strains of the same serovar and only in some cases against strains of serovar 1/2a.

The monocines originating from serovar 1/2a failed to react with strains of serovar 1/2a, but inhibited the growth of serovar 4b in various patterns.

According to the various sensitivity patterns we tried to differentiate these strains in various groups with identical reactions (Table II). No identical monocine susceptibility was shown by 37 strains, and they could not be classified in any of above mentioned groups.

Table II

Group of serovar 4b strains with identical monocine susceptibility

Group of strains	No. of monocine reactions		No. of strains
	positive	negative	
1	0	32	27
2	30	2	6
3	31	1	6
4	1	31	12
			Total 51

Table III

Example of typing of L. monocytogenes

Strains isolated from	Phage type octal code	Monocine pattern*
Mother (vagina)	n. t.	30+/2∅
Newborn (meconium)	n. t.	30+/2∅
Mother (urine)	04200	1+/31∅
Newborn (6 various swabs)	04200	1+/31∅

*Number of reacting monocines

For epidemiological evaluation, we tried to differentiate 4b strains which belonged to the same phage type or could not be phage-typed.

Table III shows an example of typing of *L. monocytogenes* strains isolated from mothers and their newborns. In the first example the strains could not be phage-typed, but had a corresponding monocine pattern. The other example shows strains which had the same phage type and also corresponding monocine pattern.

Summarizing our preliminary results, it may be assumed that about 58% of the tested *L. monocytogenes* strains produced monocines. The titres of 1/2a monocines were higher than those of 4b strains. Furthermore, it seems hopeful to select monocines for typing of *L. monocytogenes* strains belonging to the serovar 4b.

REFERENCES

1. Bradley, D. E.: Bacteriol Rev **31,** 230 (1967).
2. Brandis, H., Smarda, J.: Bacteriocine und bacteriocinähnliche Substanzen, Infektionskrankheiten und ihre Erreger. Vol. 11. Fischer, Jena 1971.
3. Reeves, P.: The Bacteriocins, Molecular Biology, Biochemistry and Biophysics. Vol. 11. Springer, Berlin—Heidelberg—New York 1972.
4. Ortel, S.: Zentralbl Bakteriol (A) **242**, 72 (1978).

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5. LISTERIA IN FOODS AND ENVIRONMENT

LISTERIA: ECOLOGY IN THE FOOD CHAIN

(A REVIEW)

N. SKOVGAARD

Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark

In recent years we have seen an increase in epidemic human listeriosis as well as in sporadic "non-epidemic" cases. Undoubtedly, this reflects the real situation and is not merely the result of greater interest in the organism and improved recognition of it.

The increase in human listeriosis has initiated a major break-through in the detection of the organism in all kinds of samples besides specimens from human beings and diseased animals.

The high prevalence of *Listeria* demonstrated in environmental samples, feeds, domestic and wild animals, and in raw and processed food, etc. during the latest years, is rather the result of the improved isolation technique and does not indicate that *Listeria* has suddenly taken the lead in the microbial world. It has always been there, and when it comes to food, most probably in much higher number and with higher prevalence in former times than to-day. There are, however, certain interesting exceptions from this, as will be explained further

Listeria monocytogenes was only occasionally found in food in the past and it is only in the latest years that this organism has become fully established as a food-borne pathogen which from its many niches in the environment may pass on to man via the food chain. As it is widespread in nature, humans may, however, be exposed to the organism in many other ways such as direct contact with diseased animals or contaminated faeces from diseased or healthy animals. Air-borne infection has thus led to a fatal case of the pneumonic form of listeriosis in a Norwegian farmer who became infected while cleaning his sheep-stable. Food-borne transmission, however, remains the most important route to man.

At a first look it seems contradictory to state on one hand that the recent increase in human listeriosis is caused by food and on the other hand that

NIELS SKOVGAARD
Royal Veterinary and Agricultural University,
Institute of Hygiene and Microbiology
13, Bülowsvej, DK-1870 Frederiksberg C., Denmark

the prevalence in food was higher in former times. However, let us make a hazard analysis of the critical control points in the food chain and try to disclose the secrets of the organism.

Primary production

The farmer is a very important link in the whole food chain. The higher the prevalence of *Listeria* in feeds, the higher the prevalence of animal excretors and the higher also the number of *Listeria* excreted. The importance of animal excretors of bacteria pathogenic to man cannot be over-emphasized.

Listeriosis in animals has been named the "silage disease", as silage of inferior quality, that is, with too high pH, is well-documented to be able to cause listeriosis in susceptible animals. The importance of silage is, however, overstressed. All ruminants feed on vegetables of various kinds besides silage; and all vegetables harbour *Listeria* which readily multiplies in decaying vegetable material and much better than in silage where the intended pH drop tends to arrest the growth of *Listeria*. In Denmark 67% of the cattle population have been shown to excrete *Listeria* species and 53% to excrete *L. monocytogenes*. The figures for *Listeria* species and *L. monocytogenes* in cattle feed were 82% and 62%, respectively. The faecal excretors must be considered to be by far the predmonant reason why 67% on minced beef contain *Listeria* species and 28% also *L. monocytogenes* [1].

When it comes to the pig population, faecal excretors of *Listeria* are down at the low level of only a few percent, with similarly low figures in minced pork [2]. The pigs are raised on dried concentrated feeds and kept indoors, and are thus not exposed to *L. monocytogenes* such as is the case with cattle.

Faecal excretors among the cattle population are also of great importance for the presence of *Listeria* in listerigenic types of cheeses. All raw milk in bulk can be considered to harbour *L. monocytogenes* of faecal origin, not in a high number; in most cases probably far below 1 per ml [3].

Any step taken at farm level to reduce the number of *Listeria* in feed and in domestic animals will diminish the risk to man. General guidelines of good hygienic practice on farms are needed, similar to those elaborated for the later steps in the food chain. Some hygienic rules do exist e.g. for the operation of SPF farms. But these rules are oriented towards prevention of animal diseases and are not necessarily sufficient to prevent human foodborne diseases.

Food manufacturing

Before outlining some critical control points in the production of foods it is essential to remember the following growth characteristics which explain

the ability of the bacteria to establish itself under the most adverse conditions of life:

(i) It grows at refrigeration temperature.

(ii) Growth is enhanced under decreased oxygen concentration and with supplementation of carbon dioxide.

(iii) It is quite tolerant to salt, growing in 10% NaCl or at correspondingly low water activity.

(iv) It does not survive pasteurization of milk, but it is probably able to survive much higher time/temperature combinations in most other foods with lower water activity.

Milk and cheese

The two most critical control points in the production are correct pasteurization and contamination from the environment, especially in the manufacturing of certain types of soft cheese.

Pasteurization. D-values for listeriae in milk have been determined to be in the range of 0.9 s at 71.1 °C [4], the legal limit in the USA for high-temperature/short-time (HT/ST) pasteurization being 71.7 °C for 15 s. This heat treatment should be sufficient to inactive 15 $\log_{10}$ *L. monocytogenes*/ml, far beyond the number present in any batch of raw milk.

The heat resistance of *L. monocytogenes* within bovine polymorphonuclear leucocytes (PMNL), a principal location of phagocytosis in the udder of cows suffering from listeria-mastitis, has under experimental conditions been shown to be in the order of 72.2 °C for 16.4 s [5]. Listeria-mastitis is, however, rather rare, and the number of *Listeria* engulfed in PMNL in batches of milk for pasteurization must be considered to be very low and of no practical importance in comparison with other possibilities for contamination.

Foremost failures in the pasteurization process. HTST pasteurization is not a very sophisticated process, but if not properly controlled it may rather easily lead to disastrous shortcomings.

There are no recorded cases of food poisoning from microorganisms surviving a pasteurization process provided the process has been properly carried out according to the minimum time/temperature conditions recommended by the IDF, i.e. 72 °C for 15 s. The product must give a negative phosphatase test immediately after heat treatment; that is, less than 10 μg of paranitrophenol must be liberated by 1 ml of sample. Figures exceeding this level, or large fluctuations, will indicate that the process is not under control.

Contamination in the pasteurizer is possible, for example through pinholes in heat-exchanger plates in the heat regeneration section. Pasteurizers should always be designed so that processed milk is under a higher pressure than unprocessed milk in the regenerator sections.

Anybody who is familiar with the possibilities of microbial contamination of milk during pasteurization will recognize the importance of proper control during operation. Hazard analysis of the critical control points of the pasteurization is a combination of automatic recording, sampling, testing, and specific inspection procedures, details of which are outside the scope of this paper. It suffices to mention that recording of the pasteurization and cooling temperatures with quick-reacting well-positioned sensors with a thermometric lag of less than 0.2 °C is an essential part of the supervision [6].

Environmental contamination

The pasteurization process has been dealt with to some length because of the controversial nature of the misunderstanding of the presence of *Listeria* in heat treated milk. Improperly pasteurized milk will be a constant source of *Listeria* in the subsequent steps of processing, foremost in soft cheese production.

Whether originating from the raw milk or from environmental sources, *Listeria* species have the ability to establish themselves in any niche where the water activity is sufficiently high, i.e. approximately 0.93, which is pretty low for apathogenic bacteria. This calls for a re-evalutation of the monitoring program established to assure the safety of the final product. It is not enough to look for coliforms and/or *L. innocua*, one has to investigate specifically for *L. monocytogenes*.

Meat and poultry

By far the predominant hygienic problem of public health concern during the slaughtering of animals is the faecal contamination of carcasses and organs with bacteria, e.g. *Salmonella*, *Campylobacter*, *Yersinia*, *Staphylococcus*, etc. The importance of this for human disease is well documented. Faecal contamination is, as mentioned above, also responsible for high prevalence of findings of *L. monocytogenes* especially in raw minced beef and raw poultry. Although an epidemiological link has not been established between human listeriosis and meat and poultry, there can be no doubt that it does play a role.

The long incubation period of listeriosis makes it difficult to find out which food is responsible, unless the food in question has a long keeping time such as cheese. Meat products spoil easily, while cheese kept for a longer time just matures and is still acceptable to the consumer.

Listeria species and *L. monocytogenes* are present with high prevalence in raw meat and poultry and also in many other raw foods such as vegetables, milk, etc. This has to be accepted. But when it comes to meat, the risk can be substantially reduced by improved hygiene such as reducing the risk of faecal contamination.

The *L. monocytogenes* contamination pressure from the raw material as well as from environmental sources constitutes in meat production plants a constant risk for post-processing contamination of products to which a listericidal process has been applied. Data from the American Meat Institute on the *Listeria* spp. incidence in 41 meat plants have shown that 29% of the floors, 37% of the drains, 20% of food contact surfaces, and 5% of the walls and ceilings in post heat-processing areas were contaminated. It is very obvious that it is necessary to re-evaluate the HACCP procedures to reduce wherever technologically feasible the *L. monocytogenes* burden in ready-to-eat meat products, especially those which undergo subsequent handling after the bactericidal process. Contamination of such products with *L. monocytogenes* is unacceptable.

Vacuum packed meats

There seem to be some inexplicable differences in the growth characteristics of *L. monocytogenes* in different types of refrigerated fresh red meat packed in either oxygen permeable or oxygen impermeable bags and kept. At present it seems difficult to predict whether *L. monocytogenes* in vacuum packed raw beef and pork kept for longer periods of time e.g. for maturing purposes, will increase or decrease.

L. monocytogenes, however, in processed meat products packed in oxygen impermeable bags, will be able to propagate during prolonged periods of storage, provided the temperature, the water activity, and other growth limiting factors so permit. The reduced oxygen tension and build-up of CO_2 in the bags will tend to stimulate the growth. Prolonged storage of meat products at low temperature is assumed to be an important factor in the increase of human yersiniosis. It is likely that the same may apply to listeriosis.

REFERENCES

1. Skovgaard, N., Morgen, C.-Aa.: Int J Food Microbiol **6**, 229 (1988).
2. Skovgaard, N., Nørrung, B.: Int J Food Microbiol **8**, 59 (1989).
3. Lovett, J., Francis, D. W., Hunt, J. M.: J Food Protect **50**, 188 (1987).
4. Bradshaw, J. G., Peeler, J. T., Corwin, J. J., Hunt, J. M., Tierney, J. T., Larkin, E. P., Twedt, R. M.: Appl Environ Microbiol **52**, 1398 (1985).
5. Doyle, M. P., Schoeni, J. L.: J Food Protect **50**, 4 (1987).
6. Van den Berg, M. G.: IDF Bulletin No. **200**, 35 (1980)

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PHYSICAL AND CHEMICAL EFFECTS DAMAGING LISTERIAE

(A SHORT REVIEW)

J. M. FARBER

Health Protection Branch, Bureau of Microbial Hazards, Health and Welfare, Ottawa, Canada

This very brief summary attempts to review some of the more recent information on the physical and chemical conditions affecting survival and/or growth of listeriae in foods.

Physical effects

Temperature. Recent studies [1] have demonstrated that *Listeria monocytogenes* is able to grow at temperatures as low as —0.4 to —0.1 °C. The upper temperature limit for growth appears to be between 45 and 50 °C, although there are a number of strains which can grow at 50 °C [2].

pH. Even though the latest Bergey's Manual [3] lists the pH range for growth of listeriae at around 5.6–9.6, recent work has shown that under optimum conditions *L. monocytogenes* can grow at pH values as low as 4.4 at 30 °C, and 5.2 at 4 °C in an enriched trypticase soy broth acidified with HCl [4]. Cabbage juice at pH values ≤ 4.8 proved lethal to *L. monocytogenes*, with rates of inactivation being lower at 5 °C than 30 °C [5]. In addition, most *L. monocytogenes* strains tested failed to grow at or below pH 5.4 in cultured or uncultured whey stored at 6 °C [6].

Water activity. There is little or no information on the a_w limits of growth for *L. monocytogenes*. It is known that under optimum conditions the organism can grow in the presence of 10% NaCl [2], which corresponds to an a_w value of 0.935.

Freezing. Freezing cells in tryptose phosphate broth at —18 °C for 7 or 14 days resulted in decreases in the viable populations of two strains of *L. monocytogenes* by about 0.1 to 0.5 logs. The presence of injured cells ranged from 62–71% and 72–80% for freeze treatments of 7 and 14 days, respectively [7]. Freezing of either poultry [8] or raw milk [9] appears to have no detrimental effect on the organism.

Heating. There has been a lot of work on the heat resistance of *L. monocytogenes* in dairy products, with results varying depending on the heating

J. M. FARBER
Microbiology Research Division, Health Protection Branch, Bureau of Microbial Hazards, Health and Welfare
Ottawa, Ontario, K1A OL2 Canada

menstruum, serotype, initial count, heat apparatus and methodology for recovering heat-stressed cells [10]. Based on current information the WHO Working Group has concluded that "pasteurization is a safe process which reduces the numbers of *L. monocytogenes* occurring in raw milk to levels that do not pose an appreciable risk to human health" [8]. Standard pasteurization of meats also appears to be an effective control measure [10, 11].

Radiation. L. monocytogenes was found to be sensitive to short wave UV energy (100 μW/cm^2) with D-values of around 16–18 s for moist cells and 43 s for dry cells. Long-wave UV energy was not effective against the organism [12].

Gamma irradiation. It appears that *L. monocytogenes* is as sensitive or less sensitive than Gram-negative bacteria to the effects of gamma irradiation. Stegeman [13] obtained D-values for *L. monocytogenes* of 0.2 kGy and 0.38 kGy for *L. monocytogenes* in buffer and minced meat (both at 4 °C), respectively. Irradiation of *L. monocytogenes* with 2 kGy in culture media or pork meat paste resulted in a 7 log reduction in viable counts, although one strain out of three survived a dose as high as 4 kGy [14].

Chemical effects

Some *L. monocytogenes* strains can tolerate up to 20% (w/v) NaCl [3], while others were found to decrease in numbers during 4 °C storage in trypticase soy broth containing 25.5% NaCl, but still remained viable after 132 days [15]. In contrast in unclarified cabbage juice stored at 30 °C, NaCl concentrations greater than 2.5% were lethal for *L. monocytogenes*, while storage of the juice at 4 °C required higher concentrations of NaCl to obtain similar lethality [5].

Although *L. monocytogenes* was able to grow at 4 °C in a 10% glycol solution (containing 0.1% non-fat dry milk), no growth was observed in a 20% glycol solution and a 30% solution proved lethal to the organism [16].

A calf rennet extract containing 5% propylene glycol, 2% sodium propionate and 0.1% sodium benzoate was inhibitory for *L. monocytogenes* in that no viable cells were observed after 42 days at 7 °C with a starting inoculum of 10^5 to 10^7 cells/ml [17].

Other compounds which have been found to be effective in controlling *L. monocytogenes* include sodium benzoate [18], chlorine [19, 20], lysozyme [21], iodine, acid anionic and quaternary ammonia-based sanitizers [22] and liquid smoke [23].

Biological inhibition

A bacteriocin (PA-1) produced by *Pediococcus acidilactici* was found to be both inhibitory and bactericidal for *L. monocytogenes* over a wide pH and temperature range [24].

Work done by Schaack and Marth [25] on the effect of thermophilic starter cultures on the survival of *L. monocytogenes*, demonstrated that although the growth of the organism was inhibited by the thermophilic lactic starter bacteria, it still managed to survive for at least 9 h during all milk fermentations and throughout the yogurt fermentation. Similarly *L. monocytogenes* was able to survive and grow to some extent in a skim milk fermentation (21 or 30 °C) which contained either 0.1, 0.5, 1.0 or 5.0% of a milk culture of *Streptococcus cremoris* or *Streptococcus lactis*, although growth of *L. monocytogenes* was always considerably inhibited when compared to that of the control [26].

REFERENCES

1. Walker, S. J., Stringer, M. F.: J Appl Bacteriol **63,** R20 (1987).
2. Feresu, S. B., Jones, D.: J Gen Microbiol **134,** 1165 (1988).
3. Seeliger, H. P. R., Jones, D.: In Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holt, J. G. (eds): Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore—London 1986. p. 1235.
4. Susan, S. M., Lund, B. M., Brocklehurst, T. F.: Letters in Appl Microbiol **6,** 153 (1988).
5. Conner, D. E., Brackett, R. E., Beuchat, L. R.: Appl. Environ Microbiol **52,** 59 (1986).
6. Ryser, E. T., Marth, E. H.: Can J Microbiol **34,** 730 (1988).
7. Golden, D. A., Beuchat, L. R., Brackett, R. E.: Food Microbiol **5,** 17 (1988).
8. WHO Working Group: Bull of the WHO **66,** 421 (1988).
9. Farber, J. M.: Unpublished data.
10. Farber, J. M.: Int J Food Microbiol. (In Press)
11. Stegeman, H., Hartog, B. J., Stekelenburg, J. P. M., Hartog, D.: Abstracts of the 10th International Symposium on Listeriosis. Pécs, Hungary, 1988. p. 103.
12. Yousef, A. E., Marth, E. H.: J Food Sci **53,** 571 (1988).
13. Stegeman, H.: Abstracts of the 10th International Symposium on Listeriosis. Pécs, Hungary 1988. p. 104.
14. Tarján, V.: Abstracts of the 10th International Symposium on Listeriosis. Pécs, Hungary, 1988. p. 105.
15. Shahamat, M., Seaman, A., Woodbine, M.: Zentralbl Bakteriol (A) **246,** 506 (1980).
16. Petran, R., Zottola, E. A.: J Food Protect **51,** 172 (1988).
17. El-Gazzar, F. E., Marth, E. H.: J Food Protect **51,** 16 (1988).
18. E.-Shenawy, M. A., Marth, E. H.: J Food Protect **51,** 525 (1988).
19. El-Kest, S. E., Marth, E. H.: J Food Protect **51,** 520 (1988).
20. El-Kest, S. E., Marth, E. H.: J Food Protect **51,** 622 (1988).
21. Hughey, V. L., Johnson, E. A.: Appl Environ Microbiol **53,** 2165 (1987).
22. Lopes, J. A., J Daity Sci **69,** 2791 (1986).
23. Messina, M. C., Ahmad, H. A., Marchello, J. A., Gerba, C. P., Paquette, M. W.: J Food Protect **51,** 629 (1988).
24. Pucci, M. J., Vedamuthu, E. R., Kunka, B. S., Vandenbergh, P. A.: Appl Environ Microbiol **54,** 2349 (1988).
25. Schaack, M. M., Marth, E. H.: J Food Protect **51,** 607 (1988).
26. Schaack, M. M., Marth, E. H.: J Food Protect **51,** 600 (1988).

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SEROVARS OF *LISTERIA MONOCYTOGENES* AND *LISTERIA INNOCUA* FROM FOOD

A. Schönberg, P. Teufel and E. Weise

Institute for Veterinary Medicine, Federal Health Office, Berlin, FRG

The increase of listeriosis outbreaks in recent years has mainly been due to the consumption of contaminated dairy products. This prompted a revision of prevention and control measures. Cheese and other foods (meat, poultry meat) were investigated. For isolation, samples were prepared and investigated according to the recommendations of the FDA with some modifications including, in particular, sub-enrichment. Suspicious colonies were tested biochemically followed by serological identification with factor sera. Out of 100 chicken (95 broilers, 5 layers) *Listeria monocytogenes* was isolated from 85 carcases. The contamination rate for minced beef and pork (n = 76) was approximately 30%. That for selected ripened soft cheeses (n = 89) was 9%. The counts of *Listeria* in red meat were usually very low (approximately 10/g). Serovar 1/2a was predominant (78 out of 102 strains). Further serovars detected were: 1/2b, 1/2c, 3a, 4a, 4b, 4d. For comparison, isolated strains of *L. monocytogenes* and *Listeria innocua* were inoculated into chicken embryos. In contrast to the former, *L. innocua* did not kill the embryos.

The increase of listeriosis outbreaks in recent years mainly has been due to the consumption of contaminated dairy products. This prompted a revision of prevention and control measures. In contrast to several other foodborne diseases listeriosis often shows a high case-fatality rate. Foodborne outbreaks with death-rates above 30% occurred in Canada (1981) and Los Angeles (1985) [1]. Thus a systematic investigation of outbreaks was indispensable to identify possible risks in food consumption and to implement appropriate control measures. On the basis of intensified investigations it was possible to demonstrate that the outbreak in Switzerland at the end of 1987 had been caused by soft cheese [2].

In order to collect epidemiological data for the prevention and control of listeriosis, cheese and other foods (meat, poultry meat) were investigated.

Materials and methods

Sixty-six German cheeses and 23 cheeses manufactured in different European countries, 76 samples of minced beef and pork, and 100 chicken (95 broilers, 5 layers) were investigated (Table I). For isolation, samples were prepared and treated according to the recommendations

Arno Schönberg, F. Teufel, E. Weise
Bundesgesundheitsamt, Institut für Veterinärmedizin
Thielallee 88/92, D-1000 Berlin 33, FRG

of the US Food and Drug Administration (FDA) with some modifications including, in particular, sub-enrichment procedures.

Suspicious colonies were tested biochemically followed by serological identification with factorspecific antisera. For control of pathogenicity, 54 isolated strains of *Listeria monocytogenes and Listeria innocua* were inoculated into 10-day-old chicken embryos. Route of inoculation: allantoic sac, approximately 100 cells in 0.1 ml suspension [3].

Results and discussion

Out of 89 cheeses 8 samples (9%) contained *L. monocytogenes*, 17 (18%) *L. innocua*. *L. monocytogenes* strains belonged to the serovars 1/2a (5 strains), and 1/2b (4 strains). One samples of French cheese was positive with both serovars. Additionally, two samples of German cheese contained *L. innocua* serovar 6b (Table II).

Out of 76 samples of minced beef and pork, 29 (38%) contained *L. monocytogenes*, 31 (40%) *L. innocua*. Serotyping of *L. monocytogenes* showed sero-

Table I

Investigated Foods

Specimen	No. of samples
1. *Cheese*	
FRG	66
other European countries	23
2. *Minced Meat*	
beef	38
pork	38
3. *Poultry Meat*	
broilers	95
layers	5
Total number of samples	265

Table II

Listeria in cheese
Total number of samples 89

No. of positive samples	*Listeria* spp.
8 (9%)*	*L. monocytogenes* serovars 1/2a (5 strains) 1/2b (4 strains)
17 (18%)	*L. innocua*

* One sample contained both serovars, two samples contained additionally *L. innocua*

Table III

Listeria in minced meat
Total number of samples 76

No. of positive samples	*Listeria* spp.	
29 (38%)*	*L. monocytogenes* serovars	
	1/2a	(15 strains)
	1/2b	(4 strains)
	1/2c	(8 strains)
	4ab	(1 strain)
	4b	(1 strain)
	4e	(1 strain)
31 (40%)	*L. innocua*	

* One sample contained two serovars, 1/2a and 1/2c. Eight samples contained additionally *L. innocua*

var 1/2a (15 strains), serovar 1/2b (4 strains), serovar 1/2c (8 strains) and one strain each of serovars 4ab, 4b and 4e. Additionally, eight samples were contaminated with *L. innocua* serovar 6a. One sample contained two serovars of *L. monocytogenes* 1/2a and 1/2c (Table III).

Out of 100 chickens, 85 carcasses (85%) contained *L. monocytogenes* of which 31 were additionally contaminated with *L. innocua*, 8 carcasses (8%) contained *L. innocua* and 1 *Listeria welshimeri*. The serological identification of *L. monocytogenes* showed the following results: serovar 1/2a (66 strains), serovar 1/2b (3 strains), serovar 1/2c (7 strains), serovar 3a (3 strains), serovar

Table IV

Listeria in poultry meat
Total number of samples 100

No. of positive samples	*Listeria* spp.	
85 (85%)*	*L. monocytogenes* serovars	
	1/2a	(66 strains)
	1/2b	(3 strains)
	1/2c	(7 strains)
	3a	(3 strains)
	4a	(1 strain)
	4b	(3 strains)
	4d	(4 strains)
8 (8%)	*L. innocua*	
1 (1%)	*L. welshimeri*	

* Two samples contained two serovars, 1/2c and 1/2a, 4a and 4d; additionally 31 out of 85 samples contained *L. innocua*

4a (1 strain), serovar 4b (3 strains) and serovar 4d (4 strains). One sample containing serovar 1/2c was additionally contaminated with serovar 1/2a and one sample containing serovar 4d was additionally contaminated with serrovar 4a (Table IV).

All chicken embryos infected with the isolated strains of *L. monocytogenes* died within 3 days, most of them after 2 days, while *L. innocua* and *L. welshimeri* did not kill the embryos.

Table V

Serovars of L. monocytogenes isolated from food of animal origin

Serovar	Number
1/2a	86
1/2b	11
1/2c	15
3	3
4a	1
4ab	1
4b	4
4d	4
4e	1
Total	126

Out of 265 samples, a total of 126 strains of *L. monocytogenes* were isolated. Serovar 1/2a was predominant with 86 strains followed by serovars 1/2b and 1/2c with 11 and 15 strains, respectively. Other serovars including serovar 4b were rare (Table V). Therefore, it is interesting to note that serovar 4b, which causes 2/3 of the human infections, was only detected in a few samples. It may be assumed that serovar 4b does not play an important role in food contamination. However, it must be taken into account that serovar 4b may be less resistant to inhibitory substances contained in the selective media used for isolation.

While Terplan [4, 5] detected *L. monocytogenes* serovars 1/2a and 1/2c in cheese but not 4b, Breer [6, 7] isolated *L. monocytogenes* from 9 (13%)out of 70 cheese samples from Switzerland. Seven strains belonged to serovar 4b, the others were serovars 1/2a and 3b. Pini et al. [8] isolated 26 strains of *L. monocytogenes* from 222 cheese samples. Sixteen strains belonged to serovars 1/2a–c and nine to serovar 4b.

It should be borne in mind that during the outbreak of listeriosis at the end of 1987 in Switzerland, which had been conveyed by soft cheese, *L. monocytogenes* serovar 4b was isolated from cheese as well as from human pathological material. Both isolates belonged to the same lysotype.

Investigations of meat and meat products in France in 1984 [9] showed contamination rates of *L. monocytogenes* between 10% to 26%. The highest rate was found in frozen beef-steaks. Out of 47 strains of *L. monocytogenes*, 16 belonged to serovar 1/2a, 30 to serovar 1/2c and only one to serovar 4b.

The rate of infections of minced meat with *L. monocytogenes* was 36% in Austria [10]. Investigations of minced beef in Denmark demonstrated *L. monocytogenes* in 28% of the samples [11].

Investigations of poultry meat in Great Britain showed that out of 100 carcasses 60 samples contained *L. monocytogenes*. Serovars 1/2a–c were predominant with 50 strains out of 76 however, 10 strains belonged to serovar 4b [8].

It may be concluded that specific milk products and other raw foods of animal origin may be a potential reservoir for *L. monocytogenes*. The risk of infection associated with foods of animal origin is poorly appreciated by the general public. Therefore, people in medical professions must be familiar with the hazards of certain foods, for high-risk groups [12, 13]. It is implicit that a high level of hygiene-consciousness should exist amongst personel involved in the production and handling of foods liable to contamination. It should be borne in mind that *L. monocytogenes* is particularly common in humid environments in food manufacturing facilities and maintenance of a dry environment is one of the best ways of limiting growth of this organism. General recommendations were made by the WHO Consultation on Prevention and Control of Listeriosis, Berlin (West) 1986 [14], and by the WHO Informal Working Group on Foodborne Listeriosis, Geneva, February 1988 [15].

REFERENCES

1. Broome, C. V.: Vet Med Hefte No. 5, 1987. pp. 5–8.
2. Billo, N.: Bericht während des Sachverständigengespräches über Listeriose in Bundesgesundheitsamt Berlin, 03. 12. 1987.
3. Kampelmacher, E. H., van Noorle-Jansen, L. M.: Ztrbl Bakteriol (A) **221,** 70 (1972).
4. Terplan, G.: Bericht während des Sachverständigengespräches über Listeriose im Bundesgesundheitsamt Berlin, 12. 06. 1986.
5. Terplan, G., Schoen, R., Springmeyer, W., Degle, I., Becker, H.: Vet Med Hefte No. 5, 1987. pp. 98–105.
6. Breer, C.: Proceedings of the Second World Congress Foodborne Infections and Intoxications, Berlin (West), Vol. I. 1986. pp. 230–233.
7. Breer, C.: Vet Med Hefte No. 5, 1987. pp. 101–109.
8. Pini, P. N., Gilbert, R. J.: Int J Food Microbiol **6,** 317 (1988).
9. Nicolas, J., Vidaud, N.: Proceedings of the 9th International Symposium on the Problems of Listeriosis, Nantes 1985. University of Nantes, Nantes 1986. pp. 330–338.
10. Breuer, J., Prändl, O.: Archiv für Lebensmittelhygiene **39,** 28 (1988).
11. Skovgaard, N., Morgen, C. A.: Int J Food Microbiol **6,** 229 (1988).
12. Bulling, E., Schönberg, A., Seeliger, H. P. R.: Dt Ärztebl **85,** 1373 (1988).
13. Seeliger, H. P. R.: Dtsch Med Wschr **112,** 359 (1987).
14. Proceedings on Listeriosis 1986. Vet Med Hefte **5,** (1987).
15. Foodborne Listeriosis. WHO Report, Geneva, 1988. WHO/EHE/FOS/ 88. 5.

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THE INCIDENCE OF *LISTERIA MONOCYTOGENES* IN FARM BULK MILKS IN NE SCOTLAND

D. R. FENLON and J. WILSON

The North of Scotland College of Agriculture, Aberdeen, Scotland, UK

Raw milk samples (c. 20 ml) were taken from approximately 180 farm bulk tanks in the NE of Scotland on 3 occasions over a period from August 1987 to February 1988 and tested for the presence of *Listeria monocytogenes*. The maximum incidence of *L. monocytogenes* (3.8%) was found in the August sampling, with incidences of 1.02% and 2.7% in October and February. Direct enrichment of 0.1 ml was negative and only 2 of the total of 14 positives were obtained by direct enrichment using 1.0 ml of milk, the remainder requiring cold enrichment over a period up to 8 weeks. These findings are similar to two recent USA surveys which suggest that in positive milks the number of *L. monocytogenes* per ml is in the order of 1 organism per ml.

The incidence of listeriosis in humans is increasing. A number of recent outbreaks have been linked to milk or milk products [1]. The consumption of unpasteurized milk or its use for dairy products in many of the reported incidents suggests that the origin of the causative organism, *Listeria monocytogenes*, may be contamination on the farm, either from milking equipment or by the cow itself.

This study describes a comprehensive survey of the incidence of *L. monocytogenes* in farm bulk tanks in North East Scotland. The period of the investigation included seasons when the cows were grazing and when confined inside fed on silage, which when subjected to aerobic spoilage is a known reservoir of large numbers of *L. monocytogenes* [2]. The effect of direct and long term cold enrichment on isolation rates of *Listeria* was also determined.

Materials and methods

Milk samples, ca 20 ml, were obtained from over 180 farm bulk milk tanks on the same day and tested for *Listeria* as described by Fenlon [2] except that the direct enrichment incubation period was extended to 48 h. The first part of the survey (August 1987) used 1.0 ml and 0.1 ml of milk for direct enrichment, and for cold enrichment at 4 °C 20 ml of milk was added to 180 ml of Nutrient Broth No. 2 (Oxoid Ltd.), and tested after 1, 2, 4, 6 and 8 weeks. In the second (October 1987) and third (February 1988) sampling periods, the 0.1 ml direct enrichment was omitted. Cold enrichment was modified as follows, 10 ml of milk were added to two lots of 90 ml Nutrient Broth No. 2, one of which contained 25 gl^{-1} calcium carbonate for pH stabilization. During the third cold enrichment period six paired chalk and non-chalk cold enrichment broths were tested for changes in pH.

DAVID R. FENLON, J. WILSON
The North of Scotland College of Agriculture
581 King Street, Aberdeen AB9 1UD, Scotland, UK

Results

The first sampling period (August 1987) gave no positive isolates by direct enrichment of 0.1 ml of milk and only 2 from 1.0 ml. A further 5 positives were picked up by cold enrichment of the 20 ml samples. Maximum recovery was obtained after 4 weeks incubation. Some of the positive cold enrichment samples were negative after further incubation, this may have been

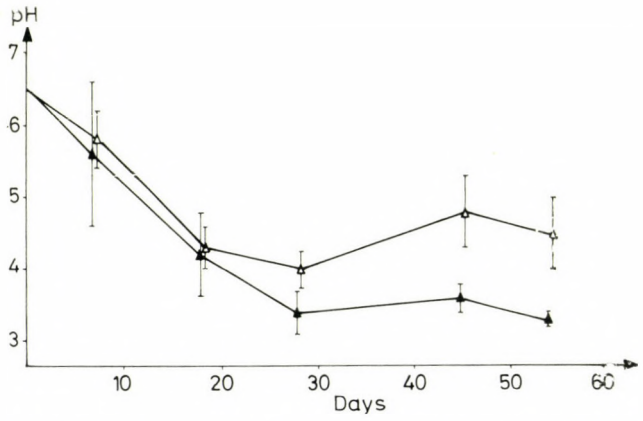

Fig. 1. The effect of 25 gl^{-1} calcium carbonate on the pH of cold enrichment broth containing milk. △ pH in $CaCO_3$ broth, ▲ pH in control broth. Each pH is the mean and SD of six measurements

caused by the pH falling as low as 4.2. Overall an incidence of 3.8% for *L. monocytogenes* was found. The second sampling period gave no isolations of *L. monocytogenes* by direct enrichment, and only two isolations from cold enrichment, one from each type of broth, an overall incidence of 1.02%. The third sampling period gave 5 positive *L. monocytogenes* isolates, 4 from the chalk cold enrichment and 2 from the non-chalk, one isolate was common to both. The effect of the addition of chalk on the pH in the cold enrichment broths is shown in Fig. 1. After 4 weeks without chalk the mean pHs ranged from 4.3–4.6, inclusion of chalk raised these to pH 5.0–5.8.

The relationship between the incidence of *L. monocytogenes* isolation and the total bacterial count (tbc) is shown in Table I. Only one farm bulk tank consistently contained *L. monocytogenes* and its tbc was well below average. The highest incidence of *L. monocytogenes* was found in summer and these farms had a higher mean tbc than the average for all farms tested. The converse was true for the other sampling periods.

Discussion

The findings of this survey indicate that the incidence and level of *L. monocytogenes* contamination of raw bulk milk supplies in Scotland is low. The infrequency of isolation from direct enrichment suggests levels of contamination similar to those found by Lovett et al. [3] of less than one *Listeria* per ml. This is in accord with the results of Hayes et al. [4] who obtained no positive isolations by direct plating, yet 12% of samples yielded *L. monocytogenes* after cold enrichment of 50 ml milk. The higher incidence found in their survey may have been due to the greater volume of milk used, 50 ml compared to 20 ml in this survey and 25 ml in that of Lovett et al. [3].

Hayes et al. [4] reported problems in stabilizing the pH of the cold enrichment broth. The studies reported in this paper showed that the incorporarion of 25 gl^{-1} calcium carbonate maintained the pH above 5.0. This inclusion of chalk in the cold enrichment during the third part of the survey did give a higher rate of isolation, but the low overall incidence of *L. monocytogenes* makes it impossible to arrive at a statistically significant result.

The presence of *L. monocytogenes* was found to be sporadic, only one farm gave positive isolates on all three sampling occasions. Milk from this farm had a consistently low tbc, showing the standard of hygiene to be extremely high. One other farm gave positive isolates on 2 occasions, all other isolates originated from different farms. The seasonal bias towards the colder months found by Lovett et al. [3] was not apparent in this survey. The highest incidence was

Table I

Total bacterial count of bulk tank milks in the week L. monocytogenes isolated

Farm	Total bacterial count per ml milk		
	Summer 87	Autumn 87	Winter 87/88
a	5000	4000	6000
b	86000	—	18000
c	39000	—	—
d	17000	—	—
e	16000	—	—
f	—	—	9000
g	—	6000	—
h	—	—	6000
i	—	—	6000
j	5000	—	—
k	4000	—	—
Mean of listeria positives	25000	5000	9000
Mean of all producers sampled	11000	17000	12000

found in the summer months, when 4 out of the 7 bulk samples found positive were from farms with higher than average tbc's, though all but one were below a level which would incur a penalty from the local milk marketing board (46 000 bacteria per ml). The incidence of *L. monocytogenes* was lower in the two sampling periods during which the cows were housed and fed on silage. The total bacterial count (tbc) of milk found to be positive was lower than average in all but one case. This suggests that hygiene standards and silage feeding are not significant factors affecting the incidence of *Listeria* contamination.

These levels of *Listeria* contamination are unlikely to survive pasteurization but may be sufficient to cause problems with soft cheeses, especially the mould ripened variety manufactured from unpasteurized milk. Ryser and Marth [5] have demonstrated that *L. monocytogenes* can multiply during the ripening of Camembert cheese. The extremely low numbers of the organism found in these surveys may be able to form a sufficient inoculum for subsequent multiplication in the ripening cheese. An analogous situation exists in the fermentation of silage followed by aerobic deterioration reported by Fenlon [6]. In the original grass the listeriae were too few to be detected by direct enrichment, yet when ensiled in conditions where the original fermentation was followed by aerobic deterioration and mould growth numbers often reached 10^6/g. *L. monocytogenes* can survive long periods in acid conditions, when the restriction of low pH is lifted, due to mould growth either utilising or neutralizing the lactic acid, numbers can increase to levels dangerous to humans or animals consuming the product. Consequently all milk should be pasteurized whether intended for the liquid market or for use in the manufacture of dairy produce.

REFERENCES

1. McLauchlin, J.: J Appl Bacteriol **63,** 1 (1987).
2. Fenlon, D. R.: Veterinary Record **118,** 240 (1986).
3. Lovett, J., Francis, D. W., Hunt, J. M.: J Food Protection **50,** 188 (1987).
4. Hayes, P. S., Feeley, J. C., Graves, L. M., Ajello, G. W., Fleming, D. W.: Appl Environmental Microbiology **51,** 438 (1986).
5. Ryser, E. T., Marth, E. H.: J Food Production **49,** 848 (1986).
6. Fenlon, D. R.: Grass and Forage Science **41,** 375 (1986).

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EXAMINATION OF *LISTERIA MONOCYTOGENES* IN MILK PRODUCTS

M. RODLER and W. KÖRBLER

National Institute of Food Hygiene and Nutrition, Budapest, Hungary

The aim of our examinations was to make clear whether listeriae occur in Hungarian milk products, first of all, in cheese. In the course of model-examinations, the most suitable enrichment- and isolation media were selected. An examination method was elaborated involving cold-enrichment and KOH treatment. Milk samples from different parts of the country and cheeses produced by different technologies were examined. To the present, 80 raw milk samples and 100 cheese samples were examined. *Listeria monocytogenes* could be detected in 3 raw milk and 2 cheese samples.

Raw milk may be supposed to contain pathogenic microbes and may be consumed without risk only after adequate heat treatment. According to literary data, milk products play often a considerable role in the spreading of listeriosis. It is sufficient to mention that 49 persons were taken ill with listeriosis in the USA in 1983, 14 of them died. The diseases were associated with milk. In California, 86 illnesses were caused by *Listeria monocytogenes* in the first half of 1985, with fatal outcome in 29 cases; 58 mothers and infants were infected and 21 perinatal deaths occurred. The disease was conveyed by fresh cheese, produced according to Mexican art in the same plant and in the case of which, the heat treatment of the milk was not sufficient. In the UK a 36 years old woman fell ill with meningitis in 1985, due to *L. monocytogenes* infection. The patient consumed imported soft cheese, from which the identical serovar (4b) of the organism could be isolated.

The aim of our examination was to make clear whether *L. monocytogenes* occurs in Hungarian milk products, first of all, in cheese, produced by using different technologies. In the course of model-examinations, the most suitable enrichment and isolation media were selected.

Materials and methods

Media. 1. *Cold enrichment (4 °C) soy broth:* Bacto Tryptone, 17 g; glucose, 0.5 g; soybean peptone, 3 g; NaCl, 5 g; K_2HPO_4, 2.5 g; water, 1000 ml; pH 7.3.

2. *Enrichment (30 °C) broth:* soy broth, 30 g; yeast extract (Oxoid), 6 g; trypaflavine, 0.015 g; nalidixic acid, 0.04 g; water to 1000 ml; pH 7.3.

MIKLÓS RODLER, WALTER KÖRBLER
National Institute of Food Hygiene and Nutrition
Gyáli út 3, H-1476 Budapest, Hungary

3. *Isolation medium:* meat extract (Lab Lemco or Corbovin), 5 g; tryptose (Oxoid), 5 g; peptone (Richter), 5 g; yeast extract (Oxoid), 5 g; NaCl, 5 g; agar (Reanal), 10 g; nalidixic acid, 0.04 g; trypaflavine 0.02 g; water, 1000 ml; pH 7.3.

Cultures. In the model-examinations, the applied *L. monocytogenes* strains, both of human and of animal origin, were from the strain collection of the National Institute of Hygiene, Budapest.

Model examinations. A specimen of 25 g was homogenized in 225 ml enrichment medium and after having distributed the homogenizate onto the isolation medium, both media were incubated at 30 °C for 24 h. After spreading, 0.1 ml from the enrichment medium was inoculated into 10 ml enrichment medium and incubated as described above. After spreading, the medium was left to stand at room temperature for a week and was spread again. At the same time, cold enrichment and, in the case of spreading onto isolation medium, KOH treatment was applied in all cases. Cold enrichment was performed at 4 °C with weekly spreadings, for several weeks. Identification of the isolates was performed by the usual methods.

Results and discussion

In connection with a stillbirth due to *L. monocytogenes* infection, we examined 76 collected raw milk samples of the Creamery of Gyöngyös, Hungary and 4 raw milk samples imported from Chechoslovakia. Furthermore, samples of cheese, produced by different technologies and coming from different parts of the country, were examined.

The results are presented in Table I. Two positive raw milk samples originated from the region where the stillbirth occurred, and one came from Chechoslovakia. *L. monocytogenes* could be detected in two samples of Hajdu cheese (Nagykőrös). This cheese, belonging to the mould cheeses, was processed by the minimum heat treatment prescribed. We recommended the increase of the temperature of pasteurization and called attention to the rigorous keeping of technological regulations.

The importance of employing veterinary measures for milk production should be emphasized. The milk of cows suffering from garget, must not be

Table I

Results of L. monocytogenes examinations

Sample	Number	Positive*	Negative
Raw milk	80	3	77
Soft cheese	25	—	25
Moulded cheese	10	2	8
Cheese ripened by molds	10	—	10
Semi-soft cheese	25	—	25
Hard cheese	10	—	10
Processed cheese	20	—	20
Total	180	5	175

* Serovar 1/2a

mixed into the collected milk. The illness may be due also to *L. monocytogenes* infection. According to literary data, the survival of the pathogen, after the pasteurizing heat treatment, may be due to a high bacterial count and to a protective effect of white blood cells.

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PERSISTENCE OF *LISTERIA MONOCYTOGENES* IN THREE SORTS OF SOIL

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

University Institute of Technology, and University Regional Centre Hospital, Angers, France

Life time of *Listeria monocytogenes* (strain PS 10401, serovar 4b) was studied in three sorts of soil: a chalky soil, poor in organic matters (pH 8.3) a peaty soil rich in organic matters (pH 5.5) a mixture of a chalky and peaty soil (pH 7.9). About 10^5 colony forming units (c.f.u.) per g of dry material were inoculated to each sort of soil which was incubated at 4 °C or at 20 °C. Periodically samples were taken and a numeration of germs was made on Tryptose Soy Agar. When direct numeration was negative, an enrichment was made at low temperature. In peaty soil, *L. monocytogenes* disappeared after incubation of 162 days at 4 °C and 156 days at 20 °C. In chalky soil, *L. monocytogenes* disappeared after 394 days of incubation at 20 °C, whereas at 4 °C, 10^4 c.f.u./g of dry matter were found 1500 days later. In mixture of chalky and peaty soil, 10^4 c.f.u. were found 1500 days after inoculation at 4 °C whereas 10^3 c.f.u. were found at 20 °C. Acid soils do not allow the persistence of *L. monocytogenes* for more than 160 days. Low temperature are significantly favourable to the persistence of *L. monocytogenes*.

The influence of soil on the behaviour of numerous microorganisms has been often shown [1]. The different climatic and environmental components play a role on the presence of some germs, on their concentration and perhaps on their pathogenic nature.

Listeria monocytogenes is a telluric bacterium, often found in environment, in cultivated or fallow lands, in decaying plants [2, 3] or in liquid waste [4]. Thus, evaluating the life time of *L. monocytogenes* in soil and its possible multiplication, seemed of interest to us.

Materials and methods

Organism. L. monocytogenes strain PS 10401, beta haemolytic, serovar 4b, sensitive to numerous phages [5], was employed.

Preparation of soil samples. There different types of soils were used: a chalky soil (pH 8.3, low organic matters content 15.3‰), a peaty soil (pH 5.5, high organic matters content 313.3‰), a mixture of peaty and chalky soil (pH 7.9, average organic matters content 74.9‰).

FRANCOISE PICARD-BONNAUD
Institut Universitaire de Technologie
4, Boulevard Lavoisier, 49045, Angers Cedex, France

J. COTTIN, B. CARBONNELLE
Laboratoire de Bactériologie — Virologie, Centre Hospitalier Régional et Universitaire
4 rue Larrey, 49040, Angers Cedex, France

The dry matter content of each sample was determined after incubation at 105 °C for 15 h with a weighing operation before and after the drying [6]. The samples used were autoclaved 3 times for 20 min at 120 °C for 3 days running. For each sort of soil, aliquots were distributed in tubes all under sterile conditions (Table I). The number of samples is important: it allows us to take two of them at regular intervals, so that the experiments can be continued over several months.

Preservation of samples. Tubes were kept in darkness; the first half of each lot was incubated at 4 °C, the other one at ambient temperature of the laboratory.

Taking and counting of samples. Over 100 days every week, then at longer intervals, two tubes per experiment were taken. The whole content of a tube is used again with a sterile dispersing solution (Bacto peptone, 1 g/litre; sodium pyrophosphate, 5 g/litre) at the rate of 45 ml of dispersing solution for 5 g of soil. After dilution in sterile water, counting was made on "Tryptose Soy Agar" (Biomérieux) Fifty microliters of the suspension were placed on the surface of the agar, then spread with a "buttoned" pipette to obtain a start-shaped isolation. Three Petri dishes, inoculated for each dilution, were incubated for 48 h at 37 °C. Counting was made either by direct reading or by reading on the colony automatic counter.

Cold enrichment. In direct counting, after three samplings when no *L. monocytogenes* was isolated, a cold enrichment was made [7]. Ten ml of enrichment broth (Tryptose Phosphate Broth, Merck) were distributed in each tube. The enrichment samples were kept at 4 °C. Every 3 weeks, 1 ml of the broth was transferred into 10 ml of enrichment broth *(Listeria* enrichment broth base, Merck) enriched by trypaflavine (0.01 g/l), and potassium thiocyanate (37.5 g/l). After two days of incubation at 22 °C if growth was evident, a loopful of the culture was spread on a selective agar for *Listeria* (Merck) enriched by nalidixic acid (0.04 g/l) and trypaflavine (0.01 g/l). Suspect colonies were identified.

Results and discussion

The results are presented in Table II. In chalky soil preserved at 4 °C, bacteria multiplied over a period of 30 days following the culture, growing from 6.8×10^5 c. f. u. to 2×10^7 c. f. u. per g of dry matter of soil. Then the bacterial population decreased to stabilize at 10^4 c. f. u. per g of dry matter. After 1500 days of culture, their number became stabilized at 10^4 c. f. u. per g of dry matter (Fig. 1).

In chalky soil preserved at ambient temperature, the number of c. f. u. increased from $6.8 \times 10.^5$ at the time of culture to 10^7 c. f. u. 23 days after culture. Then the c. f. u. decreased gradually and disappeared 394 days after the beginning of experiment. Enrichment by cold showed the presence of *L. monocytogenes* in 5 samples out of 51 (Fig. 1).

Table I

Number, composition and degree of contamination of the samples studied in the three sorts of soil

Nature of soil	Number of samples	Quantity of dry matter per sample	Inoculum c.f.u. per g of dry matter
Chalky soil: pH 8.3	309	4.87 g	6.8×10^5
Peaty soil: pH 5.5	180	2.31 g	4.4×10^5
Mixture of chalky and peaty soil pH 7.9	312	4.66 g	8×10^5

Table II

Life time and number of c.f.u. of L. monocytogenes strain PS 10401 observed in the three sorts of soil

Nature of soil	Inoculum: c.f.u. per g of dry matter in soil	Temperature of preservation	Maximum number of c.f.u per g of dry matter in soil	Number of c.f.u. per g of dry matter and life time
Chalky soil	6.8×10^5	4 °C	2×10^7, 31 days later	10^4, on 1500th day
		20 °C	10^7, 23 days later	disappearance on 394th day
Peaty soil	4.4×10^5	4 °C	maximal number in the inoculum	disappearance on 162nd day
		20 °C	8.7×10^5 14 days later	disappearance on 156th day
Mixture of chalky and peaty soil	8×10^5	4 °C	10^7 45 days later	10^4 on 1500th day
		20 °C	2×10^7 20 days later	10^3 on 1500th day

In samples of peaty soil preserved at 4 °C, *L. monocytogenes* was found for 162 days. In the same medium, at ambient temperature, its presence was shown for 156 days (Fig. 2).

During the whole period of these two experiments, countings revealed a progressive decrease in the number of bacteria. The cold enrichment made after three successive and negative samplings showed the presence of *L. monocytogenes* in one sample out of 8 preserved at 4 °C, whereas samples preserved at ambient temperature presented no bacterial development after enrichment by cold for three months.

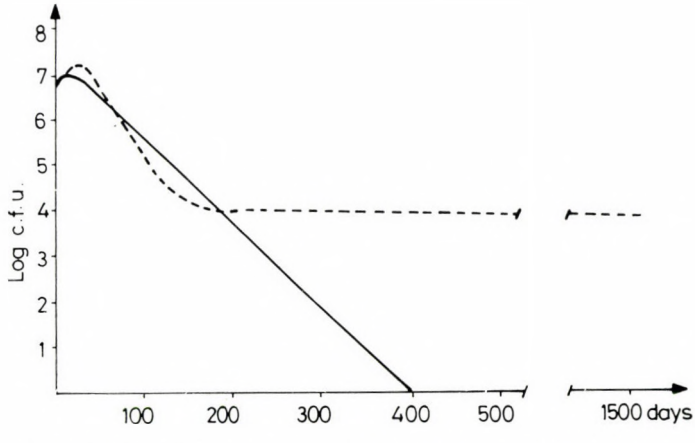

Fig. 1. Logarithm of c.f.u. of *L. monocytogenes* PS 10401 serovar 4b per g of dry matter of chalky soil ——— samples at 20 °C; — — — — samples at 4 °C

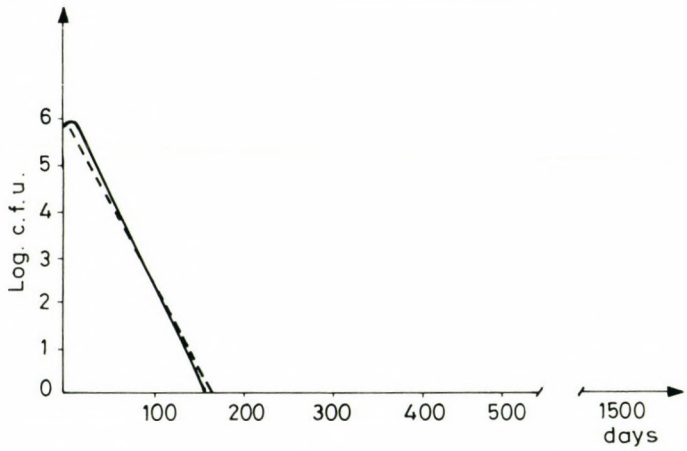

Fig. 2. Logarithm of c.f.u. of *L. monocytogenes* PS 10401 serovar 4b per g of dry matter of peaty soil ——— samples at 20 °C; — — — — samples at 4 °C

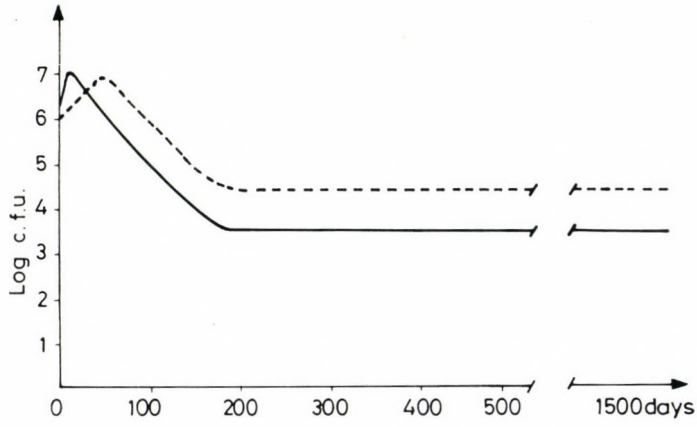

Fig. 3. Logarithm of c.f.u. of *L. monocytogenes* PS 10401 serovar 4b per g of dry matter of mixture of a chalky and peaty soils. ——— samples at 20 °C; — — — samples at 4 °C

In the mixture of peaty and chalky soil preserved at 4 °C, the number c f. u. increased from 8×10^5 at the time of culture to 10^7 c. f. u. per g of dry matter, 40 days after inoculation. Then this number had decreased by the 200th day and became stabilized at 10^4 c. f. u. per g of dry matter. This remained as long as 1500 days after culture (Fig. 3).

In the mixture of peaty and chalky soil preserved at ambient temperature, the number of c. f. u. increased from 8×10^5 at the time of culture to 10^7 four weeks after culture. This number had decreased by the 150th day and stabilized at 10^3 c. f. u. per g of dry matter. This was found 1500 days after culture (Fig. 3).

Discussion

The life possibilities of *L. monocytogenes* in different soils and precise environment conditions have been confirmed. The results are in accordance with the literature: Welshimer [8] kept alive *L. monocytogenes* in a sterile garden soil and at 24–26 °C, the number of viable microorganisms being 3×10^4 bacteria per g of soil after 295 days of incubation. Soil is a better medium than water for *L. monocytogenes* to live [9].

The two environmental factors, often mentioned, intervening in *L. monocytogenes* conservation, are the degree of acidity of the medium and the incubation temperature. In fact, in a peaty soil, the medium acidity cannot allow a preservation superior to 5 months whereas the high organic matter content can ensure a good life for *L. monocytogenes*. In the two other media in which the organic matters content is lower and the pH less acid, *L. monocytogenes* is present after 1500 days in great numbers.

The results were observed in nature where takings made in forests and in damp soils, rich in decaying plants (rich in humus) [10, 11], show that the presence of *L. monocytogenes* declines when the pH becomes acid and the organic matters content increases.

The preservation temperature is an important factor which intervenes in the number of surviving germs. Whatever the medium acidity was, the life times and the countings were superior when samples were preserved at 4 °C. Botzler [9] showed that the bacteria multiplied when the exterior temperature varied between −6 °C and +9 °C. Upsurges in human listeriosis were observed during winter and spring [12].

Thus soil is a medium extremely favourable to the life of *L. monocytogenes*; optimum conditions being a slighty acid medium (superior to 5.5) and low exterior temperature (4 °C).

REFERENCES

1. Weinberg, E. D.: Experientia **43,** 81 (1987).
2. Watkins, J., Sleath, K. P.: J Appl Bacteriol **50,** 1 (1981).
3. Weis, J., Seeliger, H. P. R.: Appl Microbiol **30,** 29 (1975).
4. Cottin, J., Babin, F., Carbonnelle, B.: In Courtieu, A. L. (ed.): Listériose—Listeria—Listeriosis. Proc. 9th Int. Symp. on the Problems of Listeriosis. Université de Nantes, Nantes (France) 1986. p. 227.
5. Audurier, A., Chatelain, R., Chalon, F., Piechaud, M.: Ann Microbiol (Paris) B **130,** 179 (1979).
6. Chandler, D. S., Craven, J. A.: Aust J Res **31,** 547 (1980).
7. Gray, M. L.: J Am Vet Med Assoc **135,** 205 (1960).
8. Welshimer, H. J.: J Bacteriol **80,** 316 (1960).
9. Botzler, R. G.: J Wildlife Dis **10,** 204 (1974).
10. Lehnert, C.: Zentralbl Bakteriol (A) **180,** 350 (1960).
11. Weis, J.: In Woodbine, M. (ed.): Problems of Listeriosis. Leicester University Press, Leicester 1975. pp. 61–65.
12. Goulet, V., Leonard, J. L., Celers, J.: In Courtieu, A. L. (ed.): Listériose—Listeria—Listeriosis. Proc 9th Int Symp on the Problems of Listeriosis. Université de Nantes, Nantes (France) 1986. pp. 372–379.

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PRESERVATION OF THE VIRULENCE OF *LISERIA MONOCYTOGENES* IN DIFFERENT SORTS OF SOIL

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

University Institute of Technology, and University Regional Centre Hospital, Angers, France

Listeria monocytogenes (strain PS 10401, serovar 4b) whose virulence was well known, has been placed for 600 days in a sterile chalky soil (pH 8.3) and in a mixture of chalky and peaty soil (pH 7.9) and incubated at 4 °C and 20 °C. Groups of six OFl mice were challenged by subcutaneous injection in the rear left hind foodpad with each sort of germs. Three days after inoculation, mice were killed by cervical disruption and numeration of *L. monocytogenes* was made in the spleen. Chemical composition of soil does not change virulence of germs. But low temperatures significantly increase virulence.

Listeria monocytogenes is a telluric germ that can contaminate human and animal food. Suspected since 1975 [1], a real food contamination has been shown clearly in human beings [2–4].

It seemed interesting for us to know if the virulence of *L. monocytogenes* could be affected by a lenghty incubation in soil samples. Thus a strain of known virulence has been placed for 600 days in two different sorts of soil incubated at 4 °C and 20 °C. In each case the virulence of the strain has been tested on lots of holoxenic mice under well-defined conditions [5] which allowed a good reproducibility of experiments and the comparison of results.

Materials and methods

Organism. L. monocytogenes strain PS 10401, serover 4b, beta-haemolytic, was used [6]. This strain is sensitive to numerous phages [7]. Its virulence has been determined at the beginning of the experiments. The bacterium was kept in two different sorts of soil and at different temperatures for 600 days.

Soil. Two sorts of soil were used: a chalky soil (pH 8.3, low organic matters content 15.3‰), a mixture of peaty and chalky soil (pH 7.9, average organic matters content 74.9‰). The soils were autoclaved for 20 min at 105 °C, for 3 days running.

Culture of soil samples, preservation and isolation of bacterial strains. The soil samples were cultured by flooding and preserved at 4 °C or at 20 °C in darkness (Table I). After 600 days of incubation, the strains were isolated from each sample and identified [6].

FRANCOISE PICARD-BONNAUD
Institut Universitaire de Technologie
4, Boulevard Lavoisier, 49045, Angers Cedex, France

J. COTTIN, B. CARBONNELLE
Laboratoire de Bactériologie—Virologie, Centre Hospitalier Régional et Universitaire
4 rue Larrey, 49040, Angers Cedex, France

Mice. Six weeks old female holoxenic mice Swiss OF 1 (IFFA CREDO Saint Germain sur Arbresles 69210 France), weighing 25 to 30 g at the beginning of the experiments were used. They were bred under sterile conditions and in a controlled atmosphere. They were fed sterile food and water, ad libitum.

Culture and inoculation. After isolation from different soils, the bacteria have been cultured on brain-heart agar (Biomérieux), for 20 h at 37 °C, then suspended in a buffered solution (pH 6.9). Then the suspension was diluted and the inoculum was standardized by turbidimetry in order to obtain a final concentration of 2×10^6 bacteria per ml [5]. Two lots of 6 mice at the beginning of the experiments (control at day 1) then six lots of 6 mice (day 600) have received 0.05 ml of suspension (10^5 bacteria) per subcutaneous injection in the rear left hind foodpad.

Spleen counts. Three days after inoculation, the mice were killed by cervical dislocation. The spleen was taken off under sterile conditions. It was weighed, submerged in a sterile physiological solution, then ground. After dilution, culture was made in two Petri dishes containing Tryptose Soy Agar (Biomérieux). The counting was made after 48 h of incubation at 30 °C.

Table I

Characters of preservation media for L. monocytogenes PS 10401

Nature of soil	pH of soil	Inoculum: c.f.u. per g of dry matter in soil	Preservation temperature	Preservation time
Chalky soil	8.3	7.5×10^5	4 °C	600 days
Mixture of peaty and chalky soil	7.9	6×10^6	4 °C and 20 °C	600 days

Table II

Virulence of the control strain before its addition to the soil

Lot of mice	Number of animals	Inoculum per animal: subcutaneous injection in the rear left foodpad	Average and standard interval of the logarithm of c.f.u. per spleen
1	6	1.6×10^5	4.52 ± 0.36
2	6	1.8×10^5	4.39 ± 0.31

Table III

Virulence of strain kept for 600 days in different soils and at different temperatures

Nature of soil	Preservation temperature	Number of animals	Inoculum per animal	Average and standard interval of the logarithm of c.f.u. per spleen
Chalky soil	4 °C	6	1.1×10^5	4.84 ± 0.27
		6	1.1×10^5	4.57 ± 0.17
Mixture of peaty and chalky soil	4 °C	6	2.1×10^5	5.02 ± 0.35
		6	1.5×10^5	4.80 ± 0.12
	20 °C	6	1.6×10^5	4.54 ± 0.23
		6	1.8×10^5	4.31 ± 0.16

Results

In Tables II and III the number of viable bacteria are expressed by the logarithm of the c. f. u. (colony forming units) per spleen, and the results of the virulence test are expressed in average by group of 6 mice, by the logarithm of the c. f. u. per spleen. The average, the standard interval and the variance have been calculated according the logarithm values.

The 6 strains preserved in soil, generally had the same virulence as that of the control strain at the beginning of the experiment. Controversely, when different preservation temperatures were compared, the virulences showed significant differences. Bacteria preserved in the mixture of chalky and peaty soil at 4 °C show significant differences from the virulence of the control strain ($t = 2.31$ at 95% — ddl = 22). The difference between the virulence of the strains preserved in the mixture at 4 °C and those preserved at 20 °C ($t = 2.16$ at 95% — ddl = 22) is significant, too.

Discussion

The virulence of *L. monocytogenes* varies with the way and size of inoculation, choice of the dissection day after inoculation, choice of the tested organ [5, 8]. A subcutaneous injection in the foodpad needs a lower dose than the oral route and can be reproduced. The latter way does not take into account the passing of the intestinal mucous membrane. According to Audurier [5] the recovery of *L. monocytogenes* from the spleen is the most adapted method to evaluate the virulence and to measure precisely the lethal activity. Moreover, *L. monocytogenes* serovar 4b constantly colonized the spleen and the lymphatic ganglions draining the point of inoculation [5, 8]. Whatever the inoculation method is, the infection develops during the three first days and decreases from the 4th day following the appearance of immunity phenomena [9]. Thus the evaluation of the virulence in the spleen after inoculation by a subcutaneous foodpad injection seems to be the most reliable method to study the action of the environmental factors on virulence.

The role of the temperature on the pathogenicity of *L. monocytogenes* has been already studied. *L. monocytogenes* injections into the air sac of chick embryos show variations in the expression of virulence of strains incubated at 4 °C, 20 °C and 37 °C [10]. The same variations have been observed with some strains of *L. monocytogenes* kept for 6 months on agar medium at 4 °C and 6 °C [11]. These results would seem to be in close correlation with the seasonal peaks of animal and human epidemics which have been observed in autumn and winter [12].

REFERENCES

1. Carbonnelle, B., Cottin, J., Parvery, F., Chambreuil, G., Kouyoumdjian, S., Lelirzin, M., Cordier, G., Vincent, F.: Rev Epidémiol Santé Publ **26,** 451 (1978).
2. Beuchat, L. R., Brackett, R. E., Hao, D. Y. Y., Conner, D. E.: Can J Microbiol **32,** 791 (1986).
3. Linnan, M. J., Mascola, L., Xiao, D. L., Goulet. V., Salminen, C., Fannin, S., Broome, C. V.: In Courtieu, A. L. (ed.): Listériose—Listeria—Listeriosis. Proceedings of the 9th International Symposium on the Problems of Listeriosis. Université de Nantes, Nantes (France) 1986. p. 227.
4. Schlech, W. E., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V. et al.: N Engl J Med **308,** 203 (1983).
5. Audurier, A., Pardon, P., Marly, J., Lantier, F., Loulergue, J.: Ann Immunol (Paris) B **132,** 191 (1981).
6. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 65 (1983).
7. Audurier, A., Chatelain, R., Chalon, F., Piechaud, M.: Ann Microbiol (Paris) B **130,** 179 (1979).
8. Rocourt, J., Alonso, J. M., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 359 (1983).
9. Berche, L. A.: Immunol **56,** 707 (1985).
10. Wood, L. W., Woodbine, M.: Zentralbl Bakteriol (A) **243,** 74 (1979).
11. Durst, J., Zentralbl Bakteriol (A) **233,** 72 (1975).
12. Goulet, V., Leonard, J. L., Celers, J.: In Courtieu, A. L. (ed.): Listériose—Listeria—Listeriosis. Proceedings of the 9th International Symposium on the Problems of Listeriosis. Université de Nantes, Nantes (France) 1986. pp. 372–379.

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THERMAL RESISTANCE OF *LISTERIA MONOCYTOGENES* IN SAUSAGE MEAT

J. M. FARBER, A. HUGHES, R. HOLLEY and B. BROWN

Health and Welfare Canada Health Protection Branch, Microbiology Research Division, Ottawa, Canada and John Labatt Brewing Company Limited, London, Canada

The heat resistance of a mixture of 10 different strains of *Listeria monocytogenes* inoculated into ground meat and ground meat plus cure was examined. D-values for ground meat ranged from 1.01 min at 62 °C to 13.18 min at 56 °C. The D-values obtained for ground meat plus cure were approximately 5–8 fold times higher than those for ground meat alone. These results imply that rare meats and possibly some cooked fermented meats may not be heated adequately to inactivate *Listeria*.

Ever since the outbreak of listeriosis in Massachusetts in 1983 linked epidemiologically to the consumption of pasteurized milk [1], the thermal resistance of *Listeria monocytogenes* has been intensively studied.

Most of these studies have been performed using milk (either sterile or raw) or broth as the heating menstruum and have demonstrated an inability of *L. monocytogenes* to survive a pasteurizing heat treatment [2–6].

However, results from a recent study by Doyle et al. [7] using milk from cows artificially infected with *L. monocytogenes* strain Scott A, suggested that *L. monocytogenes* may be afforded extra heat protection when it resides within bovine leukocytes, and may actually survive a minimum HTST treatment (71.7 °C for 16.4 s).

In contrast, studies by Bunning et al. [8, 9] have shown that the intracellular position of *L. monocytogenes* does not significantly increase the heat resistance of the organism. It is apparent, however, from most of the above studies that the heat resistance of this organism is greater than many vegetative microbes and that in sufficient numbers may be able to survive a minimal HTST pasteurization treatment.

In contrast to the above studies done using mainly dairy isolates, and raw or sterile milk as the heating menstruum, there has been little data on the

JEFFREY M. FARBER, A. HUGHES, B. BROWN
Health and Welfare Canada, Health Protection Branch, Tunney's Pasture, Banting Bldg. Microbiology
Ottawa, Ontario KIA OL2, Canada

R. HOLLEY
John Labatt Brewing Company Limited
London, Canada N6A 4M3

heat resistance of *L. monocytogenes* in meat or meat products. The object of the present investigation thus was to study the thermal resistance of *L. monocytogenes* in meat.

Materials and methods

The ten *L. monocytogenes* meat isolates used in this study (seven serotype 1, three serotype 4 strains) were from collections maintained at the Health Protection Branch in Ottawa. Each of the 10 strains was subcultured (30 °C for 24 h) in 5 ml of tryptone soya broth containing 0.6% yeast extract. The broths were centrifuged and the pellets resuspended in 0.01 M phosphate buffered-saline (pH 7). Equal amounts (1 ml) of each culture were then mixed together and added to irradiated sausage meat to give a final concentration of approximately 1.0×10^7 cells/g. Meat was obtained from a meat processor in Toronto. Ground meat consisted of 66% pork and 33% beef. Ground meat plus cure contained in addition to the pork-beef mixture, added salt, white pepper, nitrite, dextrose, lactose, and corn syrup. The meat was blended for 5 min in an Oster blender to obtain a homogeneously inoculated meat mixture, and then packed into three-ply laminate flexible pouches (7.5×11.5 cm) which were vaccum sealed (Swissvac). The pouches were placed into wire racks, and then transferred into a Blue M constant temperature bath. Water bath temperatures were monitored with a Kaye Digistrip 4c monitor controller attached to copper/constantan thermocouples. The latter were calibrated using a platinum resistance temperature detector. In addition to pouches containing only meat, several pouches contained (in addition to meat) a thermocouple placed in the geometric centre of the pouch for monitoring the temperature profile. Temperatures and times examined ranged from 56 °C to 68 °C and to 90 min, respectively.

To enumerate listeriae, the pouches were opened, the contents aseptically transferred to a stomacher bag containing 80 ml of tryptose broth, and bags then stomached for 1 min. Suspensions were surface-plated (0.2 ml) onto tryptose agar plates which were incubated at 25–30 °C for 7 days. Typical *Listeria* colonies (bluish gray, ground glass appearance) were selected for confirmation by performing the hanging drop motility test (22 °C), beta-haemoysis test on trypticase soy agar with 7% horse blood and the catalase test.

Results and discussion

The D values obtained for both the ground meat and ground meat plus cure are shown in Table I. Although there is no comparable data in the literature on D values for *Listeria* in meat products, there are reported D values for

Table I

Thermal destruction of L. monocytogenes in ground meat

Temperature (°C)	Ground Meat*		Ground Meat plus Cure**	
	D value (min)	r^2	D value (min)	r^2
56	13.18	0.97	—	—
58	6.39	0.93	50.0	0.96
60	3.12	0.88	16.7	0.95
62	1.01	0.52	7.06	0.98
64	—	—	1.28	0.87

* Z value = 4.92 °C

** Z value = 3.5 °C; cure includes salt, nitrite, dextrose, lactose, and corn syrup

Listeria suspended or grown in other types of food. Reported D values at 56 °C for two *L. monocytogenes* strains in cabbage juice (pH 4.6 or 5.6) ranged from 2.04 to 6.8 min [10], all well below our $D_{56\ °C}$ value (Table I). Donnelly and Briggs [5], looking at the heat resistance of *L. monocytogenes* in whole milk, obtained $D_{62.7\ °C}$ values ranging from 0.35 to 1.0 min. The latter figure was similar to the one we obtained for ground meat, but 7-fold lower than D values obtained for the ground meat plus cure. In studies done with strain Scott A in fluid milk products, $D_{57.8\ °C}$ values ranging from 3.96 to 6.25 min were obtained [3, 4]. Bunning et al. [8, 9] working with freely suspended cells or *Listeria* residing inside murine (raw-shole bovine milk) or bovine phagocytes (sterile whole milk) obtained $D_{57.8\ °C}$ values ranging from 5.5 to 8.2 min. The $D_{57.8\ °C}$ values obtained in both these latter studies agrees well with our results with ground meat ($D_{58\ °C} = 6.39$ min). The cured meat, besides containing added nitrite, had dextrose, lactose, corn syrup and sodium chloride (around 3% w/v) added. It is fairly well established that organisms are more heat resistant in substrates containing added carbohydrates such as glucose. This could be related to the reduced a_w of the heating menstruum, to the solute, or to both. In addition at least one study has shown that a Gram-positive organism *(Staphylococcus aureus)* can have an increased heat resistance in the presence of sodium chloride [11]. It is evident from the results of this study that rare meat (internal temperature < 60 °C), and possibly some cooked cured meats would probably not receive a sufficient heat treatment to inactivate *Listeria*.

REFERENCES

1. Fleming, D. W., Cochi, S. L., Macdonald, K. L., Brondum, J., Hayes, P. S., Plikaytis, B. D., Holmes, M. B., Audurier, A., Broome, G. V., Reingold, A. L.: N Eng J Med **312,** 404 (1985).
2. Beckers, H. J., Soentoro, P. S. S., Delfgou-van Asch, E. H. M.: Int J Food Microbiol **4,** 249 (1987).
3. Bradshaw, J. G., Peeler, J. T., Corwin, J. J., Hunt, J. M., Tierney, J. T., Larkin, E. P., Twedt, R. M.: J Food Protect **48,** 743 (1985).
4. Bradshaw, J. G., Peeler, J. T., Corwin, J. J., Hunt, J. M., Twedt, R. M.: J Food Protect **50,** 543 (1987).
5. Donnelly, C. W., Briggs, E. H.: J Food Protect **49,** 994 (1986).
6. Donnelly, C. W., Briggs, E. H., Donnelly, L. S.: J Food Protect **50,** 14 (1987).
7. Doyle, M. P., Glass, K. A., Beery, J. T., Garcia, G. A., Pollard, D. J., Schultz, R. D.: Appl Environ Microbiol **53,** 1433 (1987).
8. Bunning, V. K., Crawford, R. G., Bradshaw, J. G., Peeler, J. T., Tierney, J. T., Twedt, R. M.: Appl Environ Microbiol **52,** 1398 (1986).
9. Bunning, V. K., Donnelly, C. W., Peeler, J. T., Briggs, E. H., Bradshaw. J. G., Crawford, R. G., Beliveau, C. M., Tierney, J. T.: Appl Environ Microbiol **54,** 364 (1986).
10. Beuchat, L. R., Brackett, R. E., Hao, D. Y. Y., Conner, D. E.: Can J Microbiol **32,** 791 (1986).
11. Hurst, A., Hughes, A.: J Appl Bacteriol **55,** 81 (1983).

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HEAT RESISTANCE OF *LISTERIA MONOCYTOGENES*

G. SUAREZ FERNANDEZ

Department of Animal Pathology I, Veterinary University, Madrid, Spain

Raw milk was inoculated with *Listeria monocytogenes* and heat at 69 °C to 73 °C. The organism was recovered from 46.6% of the heated samples, but not from samples heated at 73 °C, after variable periods at refrigeration temperature. The results suggest that a low number of listeriae survive thermal treatments, but cold enrichment is necessary to repair the thermally injured cells.

The problem of the heat resistance of *Listeria monocytogenes* and the implications in the milk pasteurization process, still continue to be an interesting and controversial topic, despite many credited opinions that consider the fact that *L. monocytogenes* is not able to survive a correct pasteurization process, e. g. 71.7 °C for 15 s.

The investigations on the thermoresistance of *Listeria* began with the discovery of *L. monocytogenes (Bacterium monocytogenes)* by Murray et al. [1].

Several works on the subject were published through the history of *L. monocytogenes* by Murray et al. [1], Özgen [2], Seeliger and Linzenmeyer [3], Potel [4], Dedie and Schulze [5], Bearns and Girard [6] and Donker--Voet [7]. The used methodology was very heterogeneous, with different heating menstrua, methods of heating and strains and the results were very variable but, in general, pointed out an interesting subject on the thermoresistance of *Listeria*. Nevertheless during the following decades, in the 1960's and 1970's, no significant work in this direction was published until the outbreaks of listeriosis in the maritime provinces of Canada in 1981 [8], Massachussetts, 1983 [9] and California, 1985 [10] with 176 persons affected and 60 deaths in total.

Above all, the work of Fleming et al. [9] considering pasteurized milk as a vehicle of infection in the outbreak of listeriosis in Massachussetts (1983) attracted the attention of many reserchers to check the denounced possibility.

We revised the publications of Bradshaw et al. [11], Bunning et al. [12], Donelly and Briggs [13], Terplan et al. [14], Beckers et al. [15], Bradshaw

G. SUAREZ FERNANDEZ
Dept. Patologia Animal I (Sanidad Animal). Unidad de Microbiologia e Immunologia. Faculta de Veterinaria
Universidad Complutense
28040 Madrid, Spain

et al. [16], Dominguez et al. [17], Donnelly et al. [18], Farber et al. [19] and Fernández Garayzabal et al. [20, 21]. Part of the results is shown in Table I.

Again the techniques used by the different authors offer a wide variation including also the heat resistance of phagocytized *L. monocytogenes* (Table II). Consequently, the reports are very conflicting and the results are quite different but many of them indicated the possibility of the survival of *L. monocytogenes* in certain conditions to the time/temperature values officially accepted in the pasteurization process.

Our work with different methods (glass tubes, capillary tubes, Erlenmeyer flask, stainless steel serpentines) gave also some different results and, consequently, we used a more direct method trying to study the survival of *L. monocytogenes* in raw milk treated in a pilot plant pasteurizer.

Materials and methods

The pasteurizer had the following characteristics: plate heat exchanger unit "APV paraflow" type "junior" with a processing capacity of 100 l/h. The milk was heated by water at 80 °C and cooled with chilled water at 1 °C. The pasteurizer consisted of four stainless steel

Table I

Recent investigations and results on the heat resistance of L. monocytogenes

Author(s)	Temperature (°C)	D-value/time	Heating menstrua	c.f.u./ml	Methods
Beckers et al. [15]	80	1 min: survival	tryptose broth	2.7×10^5 5.3×10^3	open test tubes water bath
Dominguez Rodriguez et al. [17]	62.8	low-temperature holding process: survival	naturally contaminated raw milk		covered glass tubes/water bath
		15 s: survival			capillary tubes sealed with a Bunsen burner
	63	D-values 228 s	sterile commercial milk	10^8	Erlenmeyer flask/ /water bath
	72.5	1.8 s			
	72.5	D-values: S* : 1.9 s S**: 1.38 s	sterile commercial milk	10^7	two stainless steel serpentines (S_1 S_2) water bath
	74	S* : 1.59 s S**: 1.43 s		10^8	
Donnelly et al. [18]	72, 82, 92	30 min: survival	sterile whole milk 11% NFMS	5×10^6	open test tubes/ /water bath

* Serpentine 1: wall width 0.4 mm; internal diameter 1.0 mm
** Serpentine 2: wall width 0.4 mm; internal diameter 0.75 mm

Table II

Investigations on the heat resistance of phagocytized L. monocytogenes

Author(s)	Temperature (°C)	D-value/time	Heating menstrua/phagocytizing cells	Methods
Donker-Voet [7]	59	15 s: survival	naturally contaminated raw milk	plate-pasteurizer
	62.3	15 s: no survival	$1-5\times10^5$ PMNL/ml 10^3-10^5 c.f.u./ml	
Bunning et al. [12]	52.2—68.8	$D_{71.1\,°C}$ value 1.9 s	raw whole milk/murine monocytes	sealed glass tubes/ /water bath
Doyle et al. [22]	71.7—73.9	16.4 s: six of nine trials: survival	raw whole milk from listeria-infected cows	HTST-plate heat exchanger pasteurization unit
	76.4—77.8	15.4 s: no survival	$4.5\times10^5-2.4\times10^6$ PMNL/ml $5.1\times10^5-3.8\times10^7$ c.f.u./ml	

hold tubes (each 1 m long, internal diameter 10 mm) connected in series, with connexions of 40 cm and 85 cm to add to the total length of the holding tube.

The holding time efficiency E=0.8 is recommended by APV for this pasteurizer working in a regime of turbulence according to the determined Reynolds number. In these conditions with E=0.8 a minimum holding time of 15.76 s is obtained with an average of holding time of 19.7 s.

Results and discussion

In this work raw milk was inoculated with different initial concentrations of *L. monocytogenes* and heated at temperatures ranging from 69 °C to 73 °C. Listeriae were not isolated from any of the milk samples immediately after thermal treatment. They were isolated from 46.6% of the heated samples (none from samples heated at 73 °C) after variable periods at refrigeration temperature. The results suggest that a low number of listeriae survive some thermal treatments but a cold enrichment is necessary to repair the thermal injured cells and detect these organisms in milk. We must emphasize the importance of the isolation technique in the recovery of listeriae from pasteurized milk samples.

Finally, we must conclude that a normal or correct pasteurization process is usually sufficient to destroy *Listeria* if the number is not over 10^8 per ml. This high quantity is almost impossible to reach in raw milk. Only when feeding animals with contaminated silage or in the case of mastitis produced by *Listeria*, which is not very frequent, can a risk exist.

In this case the relation time/temperature of 71.7 °C for 15 s is not sufficient to guarantee a correct pasteurization.

We consider the techniques used for the recovery, resuscitation, recuperation, revitalization, reparation, etc., of *Listeria* shocked by heat as a very important point.

This point is most important not only from a scientific point of view but also in practice, because *Listeria* can grow after repairing the thermal damage at refrigeration temperatures (4 °C) which does not occur with *Salmonella*, for instance. This property emphasizes, still more, the real interest of the subject of heat resistance of *L. monocytogenes*.

REFERENCES

1. Murray, E. G. D., Webb, R. A., Swann, M. B. R.: J Pathol **29**, 407 (1926).
2. Özgen, H.: Thesis. Giessen 1961.
3. Seeliger, H. P. R. Linzenmeyer, G.: Zschr Hyg **136**, 335 (1953).
4. Potel, J.: Wissenschaftliche Zeitschrift der Martin Luther Universität Halle-Wittenberg. Jahrgang III. Mathematisch-naturwissenschaftliche Reihe. Heft 2. 341 (1953/54).
5. Dedie, K., Schulze, D.: Berlin München Tieräztl Wschr **70**, 231 (1957).
6. Bearns, R. E., Girard, K. F.: Can J Microbiol **4**, 55 (1985).
7. Donker-Voet, J.: In Gray, M. L. (ed.): Proceeding of the Second Symposium on Listeriosis. Montana State College, Bozeman 1962. pp. 133–139.
8. Schlech, W. F., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, A. E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nichols, E. S., Broome, C. W.: N Engl J Med **308**, 203 (1983).
9. Fleming, D. W., Cochi, S. L., MacDonald, K. L., Brondum, J., Hayes, P. S., Plikaytis, B. D., Holmes, M. B., Audurier, A., Broome, C. V., Reingold, A. L.: N Engl J Med **312**, 404 (1985).
10. Communicable Diseases Report 85/26. Public Health Laboratory Service. Atlanta, USA. 1985.
11. Bradshaw, J. G., Peeler, J. T., Corwin, J. J., Hunt, J. M., Tierney, J. T., Larkin, E. P., Twedt, R. M.: J Food Protect **48**, 743 (1985).
12. Bunning, V. K., Crawford, R. G., Bradshaw, J. G., Peeler, J. T., Tierney, J. T., Twedt, R. M.: Appl Environ Microbiol **52**, 1398 (1986).
13. Donnelly, C. W., Briggs, E. H.: J Food Protect **49**, 994 (1986).
14. Terplan, G., Schoen, R., Springmeyer, W., Degle, I., Backer, H.: Arch Lebensmittelhyg **37**, 129 (1986).
15. Beckers, H. J., Soentoro, P. S. S., Denfgou-Van Asch, E. H. M.: Intern J Food Microbiol **4**, 249 (1987).
16. Bradshaw, J. G., Peeler, J. T., Corwin, J. J., Hunt, J. M., Twedt, R. M.: J Food Protect **50**, 543 (1987).
17. Dominguez Rodriguez, L., Fernandez Garayzabal, J. F., Rodriguez Ferri, E., Vazquez Boland, J. A., Gomez-Lucia, E., Suarez Fernandez, G.: Supplement to IDF Circular 87/3 (Microbiological week). 1987.
18. Donnelly, C. W., Briggs, E. H., Donnelly, L. S.: J Food Protect **50**, 14 (1987).
19. Farber, J. M., Sanders, G. W., Emmons, D. B., McKellar, R. C.: 74th Annual Meeting of IAMFES. J Food Protect **50**, 441 (1987).
20. Fernandez Garayzabal, J. F., Dominguez Rodriguez, L., Vazquez Boland, J. A., Blanco Cancelo, J. L., Suarez Fernandez, G.: Can J Microbiol **32**, 149 (1986).
21. Fernandez Garayzabal, J. F., Dominguez Rodriguez, L., Vazquez Boland, J. A., Rodriguez Ferri, E. F., Blanco Cancelo, J. L., Suarez Fernandez, G.: J Appl Bacteriol **63**, 533 (1987).
22. Doyle, M. P., Glass, K. A., Beery, J. T., Garcia, G. A., Pollard, D. J., Schultz, R. D.: Appl Environ Microbiol **53**, 1433 (1987).

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HEAT RESISTANCE OF *LISTERIA MONOCYTOGENES*

VÉRONIQUE LEMAIRE, O. CERF and A. AUDURIER

Microbiology Laboratory, Faculty of Medicine, Tours, and INRA Food Hygiene Laboratory, Massy, France

Two methods were used to study the thermal resistance of *Listeria monocytogenes*. The $D_{60\ °C}$ values ranged from 1.3 to 6.5 s in a open vessel. $D_{72\ °C}$ varied from 0.06 to 1.5 s and z from 3.1 to 6.5 K (38 strains) in capillary tubes. Therefore a conventional pasteurization process of 15 s at 72 °C could assure 10 to 250 decimal reductions, depending on the strain. Six days enrichment at 4 °C in milk prior to incubation at 37 °C did not show any influence on measured heat resistance (4 strains). A significant difference of thermal resistance among strains according to their serotype was noted: strains belonging to serogroup 1 were more heat resistant than those belonging to serogroup 4.

Bacteriological investigations of dairy products have demonstrated the presence of *Listeria monocytogenes* in some samples. This observation raises the question of the origin of these *Listeria:* whether they were present in raw milk and were not destroyed by pasteurization, or if they arose through contamination during processing or packaging, after heat treatment. Results obtained previously were contradictory and it was considered necessary to undertake a study to determine the heat resistance characteristics of a large number of strains.

Materials and methods

The thermal resistance of 38 strains were determined. These strains, of serotype 1/2 and 4, were isolated from dairy products, environmental samples or clinical specimens. They were selected after phage-typing the results of which suggested that they were probably unrelated strains. Two methods of testing were used in order to study a wide temperature range.

The first method was conducted in a double-walled flask. The broth in the inner vessel was heated to the required temperature by circulating water in the outer part. The temperature of the broth was controlled by a thermocouple and made homogeneous by using a magnetic stirrer. At the time t = 0, the bacterial culture was added to the broth. Samples were taken and quickly cooled at intervals determined by the temperature, from 55 to 64 °C. They were then cultured on Tryptose agar and bacterial counts performed after 48 h at 37 °C. The counts of

VÉRONIQUE LEMAIRE
Diagnostics Pasteur
BP 16, Route de Cassel, 59114 Steenvoorde, France

O. CERF
INRA, Laboratoire de Génie de l'Hygiène et des Procédés Alimentaires
91300 Massy, France

ANDRÉ AUDURIER
Laboratoire de Microbiologie, Faculté de Médecine
2 bis Boulevard Tonnelle, 37032 Tours, France

surviving bacteria gave survival curves which allowed the determination of thermal reduction times (D) given by the inverse of the slope. D is the time necessary to reduce the surviving population by a factor of 10. Plotting D versus temperature for each strain gave the z value as the inverse of the slope. Both D and z values are necessary to determine the thermal resistance of a strain. It is possible to extrapolate the D values to 72 °C by considering the curve as a straight line up to 72 °C.

The second method permitted the study of higher temperatures up to 72 °C. Bacterial cultures in litmus milk were sealed in 25 μl capillary tubes. Nine tubes were dipped in a water-bath for each studied temperature. They were removed and quickly cooled at intervals determined by the temperature. They were then left for 48 h at 37 °C before being read: the presence of living listeriae was revealed by the decoloration of the medium. Signs + or — according to the result of the culture were plotted a graph of $\log_{10}$ (time) versus temperature. Two parallel lines were then drawn, one above which there were only — signs, and the other under which there were only + signs. A third parallel line drawn between these lines permitted determination of the z value as the inverse of the slope. The D values could be determined at each temperature by an equation based on the heating-time and the initial inoculum.

Results and discussion

The $D_{60\,°C}$ values obtained by the first method ranged from 1.3 to 6.5 min. The median value was 2.3 min. The z values ranged from 4.5 to 5.8 K (Table I). The $D_{72\,°C}$ values obtained by the second method ranged from 0.02 to 1.54 s. The median value was 0.80 s for the strains of serogroup 1 and 0.08 s for serogroup 4. The general median was 0.10 s. The z values ranged from 3.1 to 6.5 K. (Tables II and III, Fig. 1).

Table I

z and $D_{60\,°C}$ values obtained by the flask method

Strain origin	z (Kelvin)	$D_{60\,°C}$ (min)
Serogroup 1		
Cheese	5.3	3.2
Cheese	4.8	2.2
Environment	5.1	1.3
Environment	5.2	3.8
Meat	5.6	6.5
Pathological	5.8	2.3
Serogroup 4		
Cheese	5.2	2.9
Cheese	4.6	2.0
Cheese	4.7	2.1
Cheese	5.3	3.8
Pathological	4.5	2.7
Scott	4.6	1.6
Unknown	4.5	1.5
Median $D_{60\,°C}$ value, 2.3 min		

Table II

z and $D_{72\,°C}$ values obtained by the capillary tube method

Strain origin	z (Kelvin)	$D_{72\,°C}$ (s)
Serogroup 1		
Cheese	5.1	0.90
Cheese	4.8	0.12
Cheese	6.5	1.41
Cheese	3.4	0.06
Cheese	3.5	0.07
Cheese	6.0	1.08
Cheese	5.6	0.99
Cheese	5.3	0.92
Cheese	4.1	0.17
Cheese	5.3	0.86
Cheese	3.4	0.07
Raw milk	3.4	0.06
Raw milk	4.2	0.10
Raw milk	3.7	0.05
Environment	6.0	1.09
Meat	5.5	1.54
Pathological	6.4	1.41
Unknown	6.3	0.74
Median $D_{72\,°C}$ value, 0.80 s		

The first method did not permit studies at high temperatures because the sampling time was too long with regard to the D values at temperatures higher than 64 °C. It was possible to extrapolate up to 72 °C but the values obtained were then approximate and needed confirmation.

The second method, an end-point technique could be used to reach high temperatures because the survival curves obtained by the first method were straight lines. The culture in capillary tubes which were not opened after heat

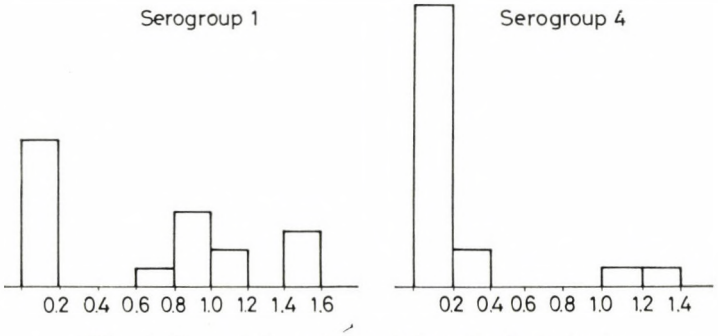

Fig. 1. $D_{72\,°C}$ values obtained from both methods

Table III

z and $D_{72\,°C}$ values obtained by the capillary tube method

Strain origin	z (Kelvin)	$D_{72\,°C}$ (s)
Serogroup 4		
Cheese	4.8	0.02
Cheese	3.9	0.10
Cheese	5.7	0.10
Cheese	4.1	0.32
Cheese	4.8	0.24
Cheese	3.4	0.02
Cheese	3.7	0.07
Cheese	3.7	0.08
Cheese	3.8	0.10
Raw milk	3.1	0.02
Raw milk	3.8	0.07
Dairy product	4.5	1.35
Dairy product	3.4	0.06
Dairy product	3.8	0.08
Environment	4.8	0.19
Pathological	3.8	0.09
Scott	3.1	0.02
Vaccine	4.3	0.13
Unknown	3.6	0.07
Median $D_{72\,°C}$ value, 0.08 s		

treatment avoided the risk of contamination. The required temperature could be reached quickly in the capillary tubes and thus reproduced the heating conditions of an industrial heat exchanger. The medium used was milk. Thus the second method was quite similar to normal pasteurization.

Our results are different from those obtained previously for the Scott strain. This strain was one of the most sensitive in our study. We left four strains at 4 °C after heat processing during six days and we could not detect any difference in the D and z values. This finding disagrees with previous results. The difference of thermal resistance of the serogroup 1 and 4 strains is important as most of the strains isolated from dairy products are of serogroup 1.

Treatment at 72 °C for 15 s resulted in over 10 decimal reductions of 37 of the 38 strains studied. A recent study of the thermal resistance of *Listeria* within leukocytes did not show increased D values. Pasteurization can be consodered an efficient means of eliminating all *Listeria* present in raw milk and does not represent a great risk in the dairy products processing.

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6. Taxonomy and Diagnostic Methods

SPECIES OF THE GENUS *LISTERIA*

JOCELYNE ROCOURT

Unit of Bacterial Ecology, Institute Pasteur, Paris, France

The main purpose of taxonomy is to distinguish one organism from another, providing identification criteria based on a combination of data obtained by various methods. The species, the basic unit of classification, is a group of closely related organisms sharing a given set of properties. As a science, bacterial taxonomy is evolving according to the introduction of new methodologies. With the developments of numerical taxonomy, chemotaxonomy, DNA/DNA- and DNA/RNA hybridizations, and more recently those of ribosomal RNA sequencing, a better understanding of the phylogenetic arrangement of the bacterial world is emerging. A more precise definition of a bacterial species was recently proposed by the "Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics" [1]: "The phylogenetic definition of species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less delta *Tm*. Both values must be considered. Phenotypic characteristics should agree with this definition. Nomenclature should reflect genomic relationships to the greatest extent possible." The recognition of DNA relatedness data as a prerequisite for the description of a new species allows for the first time an accurate delimination of the bacterial species.

Listeria monocytogenes and genomically related species

DNA/DNA hybridization experiments performed by Stuart and Welshimer [2] in 1973 already suggested that the species *L. monocytogenes* was genomically heterogeneous. More recently, DNA homology studies undertaken with 66 strains of *L. monocytogenes* as defined in the 8th edition of the Bergey's Manual of Determinative Bacteriology revealed that this species covered in fact five groups of strains, with DNA homology values below 58% between them. Within each genomic group, DNA reassociation percentages were com-

JOCELYNE ROCOURT
Institut Pasteur, Unité d'Ecologie Bactérienne
25 rue du Dr. Roux, 75724 Paris Cedex 15, France

prised between 63/70 and 100%, with delta *Tm* values below 6 °C, thus indicating that these genomic groups represent distinct bacterial species [3]. These five genomic groups correspond to *L. monocytogenes* (type species), *L. ivanovii* [4], *L. innocua* [5], *L. welshimeri* and *L. seeligeri* [6].

The phenetic realtionships between these species as described in a recent numerical taxonomic study remain still unclear [7]. A few markers are suitable to identify these species: CAMP-test with *Staphylococcus aureus* (or spontaneous haemolysis on sheep or horse blood agar) and with *Rhodococcus equi*, and acid production from D-xylose, L-rhamnose, and alpha-methyl-D-mannoside [8]. Chemotaxonomic properties underline the close genomic relatedness between these species: fatty acids composition (when determined), structure of lipotechoic acids, as well as whole-cell proteins electrophoregrams are similar [7, 9, 10]; different teichoic acids have been described, whose occurrence seems to be more correlated to the serovar than to the species [9]. To date, isoenzymes patterns are the most appropriate chemotaxonomic marker to clearly distinguish these five species [11].

Listeria grayi and Listeria murrayi

On the basis of 70% DNA homology found between *L. grayi* and *L. murrayi* (respectively described in 1966 and 1973 [12, 13]), Stuart and Welshimer [14] proposed to consider them as a single species with two biovars (phenotypically, *L. grayi* differs from *L. murrayi* only by its inability to reduce nitrates). This proposal has never been officially validated.

L. grayi and *L. murrayi* mainly diverge from *L. monocytogenes* and genomically related species by some phenotypic characteristics: nitrate reduction, acid production from mannitol, lack of phosphamidase and acid phosphatase, inability to grow on trypaflavine medium. These two species share the same antigenic structure which distinguishes them from the other *Listeria*. Chemotaxonomic studies pointed out slight differences in the G+C DNA content, nature of the substitutiton of lipoteichoic acids, and electrophoregrams of whole-cell proteins between *L. grayi* and *L. murrayi* on the one hand and the remaining *Listeria* species on the other [2, 10, 14, 15]. Numerical taxonomic results showed *L. grayi* and *L. murrayi* to form a cluster distinct from those grouping the other *Listeria* strains at similarity values from 81 to 87% [14, 16–18], thus leading to the conclusion that these two species belong to this genus. On the basis of low DNA homology percentages found between these two species and the other *Listeria* strains tested, Stuart and Welshimer [14] proposed in 1974 to allocate them to a new genus "*Murraya*", but this proposal remained unvalidated. The possibility to revive the genus "*Murraya*" required a more precise evaluation of the genomic distance between these two groups of species. The 16S rRNA partial sequence of *L. murrayi* was therefore determined

and compared to that of *L. monocytogenes*. The high similarity coefficient found between these two microorganisms (SAB value = 0.72), joint to the lack of important phenetic differences between them as mentioned above, provided no support for the exclusion of these species from the genus *Listeria*. Consequently, *L. murrayi* and *L. grayi* should be presently considered members of the genus *Listeria* [19].

Jonesia (Listeria) denitrificans

L. denitrificans is a species constituted by a single strain which was described in 1948 [20]. Numerous studies pointed out important discordances between this species and the other members of the genus *Listeria*, indicating that this species was misclassified: numerical phenetic studies showed low similarity coefficients; the G+C DNA content of *L. denitrificans* is significantly higher than those of *Listeria;* cell-wall components, menaquinone, fatty acids and polar lipids composition as well as proteins electrophoregrams, are distinct [2, 7, 15, 16, 21, 22]. Low DNA homology values formerly found between *L. monocytogenes* and *L. denitrificans* [2] were further investigated using the 16S rRNA cataloguing approach. While *L. monocytogenes* belong to the *Bacillus –Clostridium* subdivision of Gram-positive bacteria, results unambiguously allocated *L. denitrificans* to one of the several subbranches of the coryneform bacteria-actinomycetes subdivision, defined by *Actinomyces*, *Arthrobacter*, *Micrococcus*, *Cellulomonas*, *Stomatococcus*, *Promicromonospora* and allied taxa. The low SAB-values found between *L. denitrificans* and these genera, associated to important differences in chemotaxonomic properties, infered that this species should be excluded from the genus *Listeria* and transferred to a new genus, named *Jonesia*, as *Jonesia denitrificans* [23].

It may be concluded that the genomic dissection of the genus *Listeria*, as evidenced by DNA hybridization and 16S rRNA cataloguing results, — allowed to exclude *L. denitrificans* from this genus, and to transfer it to a new genus of the coryneform bacteria-actinomycetes subdivision, as *J. denitrificans;* — demonstrated that the genus *Listeria* comprises two distinct lines of descent: one contains *L. monocytogenes* and genomically related species, the other is composed of *L. grayi* and *L. murrayi*.

REFERENCES

1. Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., Trüper, H. G.: Int J System Bacteriol **37,** 463 (1987).
2. Stuart, S. E., Welshimer, H. J.: Int J System Bacteriol **23,** 8 (1973).
3. Rocourt, J., Grimont, F., Grimont, P. A. D., Seeliger, H. P. R.: Curr Microbiol **7,** 383 (1982).
4. Seeliger, H. P. R., Rocourt, J., Schrettenbrunner, A., Grimont, P. A. D., Jones, D.: Int J System Bacteriol **34,** 336 (1984).

5. Seeliger, H. P. R.: Zentralbl Bakteriol (A) **249,** 487 (1981).
6. Rocourt, J., Grimont, P. A. D.: Int J System Bacteriol **23,** 866 (1983).
7. Feresu, S. B., Jones, D.: J Gen Microbiol **134,** 1165 (1988).
8. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134,** 65 (1983).
9. Fiedler, J., Seger, J., Schrettenbrunner, A., Seeliger, H. P. R.: System Appl Microbiol **5,** 360 (1984).
10. Lamont, R. J., Petrie, D. T., Melvin, W. T., Postlewaite, R.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. (eds): Listeriose, Listeria, Listeriosis Proc 9th International Symposium on the Problems of Listeriosis. 1985–1986. Université de Nantes, Nantes 1986.
11. Kressebuch, H., Aeschbacher, M., Bille, J., Bannerman, E., Rocourt, J., Pifaretti, J. C.: Annual Meeting of the American Society for Microbiology, Miami, USA, 1988. Abstract No. D-65.
12. Larsen, H. E., Seeliger, H. P. R.: Proc. Third International Symposium on Listeriosis. Bilthoven, The Netherlands, 1966.
13. Welshimer, H. J., Meredith, A. L.: Int J. System Bacteriol **21,** 3 (1971).
14. Stuart, S. E., Welshimer, H. J.: Int J System Bacteriol **24,** 177 (1974).
15. Ruhland, G., Fiedler, F.: System Appl Microbiol **9,** 40 (1987).
16. Jones, D.: J Gen Microbiol **87,** 52 (1975).
17. Stuart, M. R., Pease, P. E.: J Gen Microbiol **73,** 551 (1972).
18. Wilkinson, B. J., Jones, D.: J Gen Microbiol **98,** 399 (1977).
19. Rocourt, J., Wehmeyer, U., Cossart, P., Stackebrandt, E.: Int J System Bacteriol **37,** 298 (1987).
20. Sohier, R., Benazet, F., Piéchaud, M.: Ann Microbiol (Paris) A **74,** 54 (1948).
21. Collins, M. D., Feresu S.,, Jones, D.: FEMS Microbiol Lett **18,** 131 (1983).
22. Fiedler, F., Seger, J.: System Appl Microbiol **4,** 444 (1983).
23. Rocourt, J., Wehmeyer, U., Stackebrandt, E.: Int J System Bacteriol **37,** 266 (1987).

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DIFFERENTATION OF *LISTERIA* AND *STREPTOCOCCUS* STRAINS

ÁGNES HERENDI and B. RALOVICH

National Institute of Hygiene, Budapest, and Institute of Public Health and Epidemiology, University Medical School, Pécs, Hungary

Colonial morphology of non-beta haemolytic *Listeria* strains is frequently similar to that of non-haemolytic streptococci. Biochemical characteristics, motility, haemolysis on ox blood agar, growth on Clauberg, selective streptococcus (Si) and Mitis-Salivarius agar medium, CAMP test, serological behaviour of 16 *Listeria* strains were studied and the results were compared with the properties of *Streptococcus* strains. Microscopic morphology, motility and catalase activity are useful for distinguishing these strains. To avoid a false diagnosis, latex-agglutination should be supplemented with the above tests.

Differentiation among *Listeria* and *Streptococcus* species sometimes requires more attention as haemolytic and non-haemolytic *Listeria* strains further haemolytic and non-haemolytic *Streptococcus* strains may show similar colonial morphology on different media. Similarities of biological and biochemical properties as well as antigenic cross-reactivity can lead to misidentification and on the basis of that to inappropriate therapy. Therefore, it has been decided to study what kind of characters can be used for the identification of these strains on the simpliest way.

Materials and methods

Biochemical properties set out in Table II, and haemolysis on blood agar containing ox or sheep red blood cells, growth on Clauberg-, Selective Streptococcus (Si)- and Mitis-Salivarius agar media, CAMP-test, latex agglutination (Wellcome), Lancefield precipitation, motility and virulence of 16 *Listeria* strains were studied and the results were compared with those of 182 *Streptococcus bovis* and 137 *Streptococcus faecium* strains.

Listeria strains examined are listed in Table I. *S. bovis* and *S. faecium* strains were isolated from clinical samples in the National Institute of Hygiene.

Standard methods were used to determine cultural, biological and biochemical characters of the strains [1–4]. Serological properties of *Listeria* and *Streptococcus* strains were studied

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

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

with Streptex (Wellcome), Lancefield precipitation using our own and Sevac (Prague, Czechoslovakia) [5] grouping sera and our own *Listeria* immunosera.

Morphology of bacteria was examined with electron microscope (Type JEM 100 C) on copper grid with carbon membrane after negative stain with 2% phosphotungstic acid.

Results and discussion

Colonial morphology of non-haemolytic or haemolytic *Listeria* strains is sometimes similar to that of non-haemolytic or haemolytic *Streptococcus* strains not only no blood agar but on Mitis-Salivarius agar, Selective Streptococcus agar and Clauberg medium, too. Mitis-Salivarius agar inhibited the growth of *Listeria ivanovii* type strain (non-motile).

Beta-haemolytic property on sheep or horse blood agar is a very important marker of virulence of *Listeria* strains. Ox blood should not be used as a substitute to sheep or horse red blood cells, because 3 out of 5 *Listeria* strains showed beta-haemolysis and the other two caused intermediate — between alfa and beta — haemolysis on ox blood agar. These 5 strains showed no haemolysis or weak alfa-haemolysis on sheep blood agar. Virulence of these 5 strains was checked in eyes of guinea pigs and in mice and the results were in accordance with the type of haemolysis observed on blood agar containing sheep red cells.

Table I

List of 16 Listeria strains tested

Designation	Species	Serovar	Remarks	Abbreviation
4067	*L. seeligeri*	?	(No. 5031)*	s. ?
4068	*Listeria sp*	1830	(No. 5180)*	sp. 1830
4069	*L. monocytogenes*	1/2b	(No. 5467)*	m. 1/2b
4070	*L. seeligeri*	1/2b	(No. 5475)*	s. 1/2b
4071	*L. seeligeri*	1/2b	(No. 5559)*	s. 1/2b
4072	*L. seeligeri*	1/2b	(No. 5575)*	s. 1/2b
4073	*L. seeligeri*	6b	(No. 5606)*	s. 6b
4074	*L. seeligeri*	6b	(No. 5626)*	s. 6b
4075	*L. seeligeri*	1/2b—1830	(No. 5628)*	s. 1/2—1830
4076	*Listeria sp.*	1/2b	(No. 5797)*	sp. 1/2b
4535	*L. ivanovii*	5	(Own isolate)	iv. 5
No. 10	*L. innocua*	4ab	(Own isolate)	in. 4ab
1830	*L. welshimeri*	1830	(Own isolate)	w. 1830
G44	*L. murrayi*		(G44 type strain)	mu. G44
C644	*L. innocua*	6a	(NCTC 10889)	in. 6a
Lm5	*L. ivanovii*	5	(type strain non-motile)	iv. 5nm

Note. Strains marked with asteriks were sent by Professor A. L. COURTIEU's (Nantes, France) collection. They were identified as *L. seeligeri* strains. The type strains originated from Professor H. P. R. SEELIGER (Würzburg, FRG)

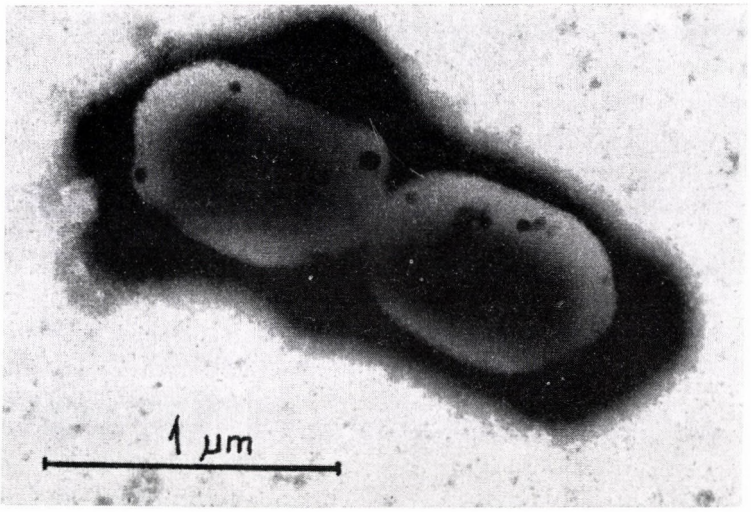

Fig. 1. Electron microscopic picture of S. *pyogenes* (group A, No. 80153) with elongated coccobacillus-like form

Listeria and *Streptococcus* cells are Gram-positive. The former have rod-shape with rounded ends or coccoid forms and can occur singly or in short chains. The latters are usually spherical or elongated in shape and arranged in chains or pairs. Some species have short rod form, others appear to be highly pleomorphic. Therefore, only on the basis of Gram stain sometimes it is difficult to give a correct opinion. In case of our strains we had no diagnostic problems. Electron microscopic picture of these germs rarely causes difficulty. Figure 1 shows a pair of *Streptococcus* having elongated coccobacillus-like shape.

Catalase activity is an important feature for the diagnosis but it is necessary to mention that there are data about streptococci showing catalase or pseudo-catalase activity and also about catalase-negative non-haemolytic *Listeria* strains. Among our 16 *Listeria* strains 1 proved to be catalase-negative.

Motility is a character which cannot be used alone for the diagnosis because *Streptococcus* strains belonging to group D may also have flagella and one non-motile *L. ivanovii* strain has been recognized, too. Examples can be seen in Figs 2 and 3.

Missing arginine hydrolysis is characteristic for *Listeria* strains because positive reaction among them have not been observed yet. However, it is necessary to keep in mind that some of *Streptococcus* species cannot hydrolyse arginine, too. Such results can be found in Table II.

As to acid production from carbohydrates *Listeria* spp. almost never produce acid from D-arabinose and positive reaction indicates a *Streptococcus*. In the case of mannitol *Listeria grayi* and *Listeria murrayi* strains give positive

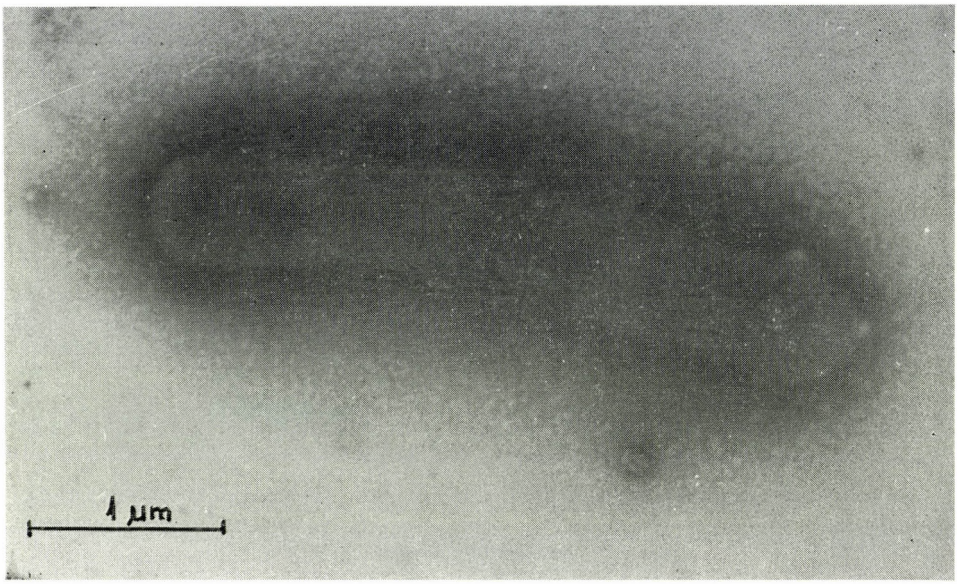

Fig. 2. Electronmicrograph of non-motile *L. ivanovii* strain

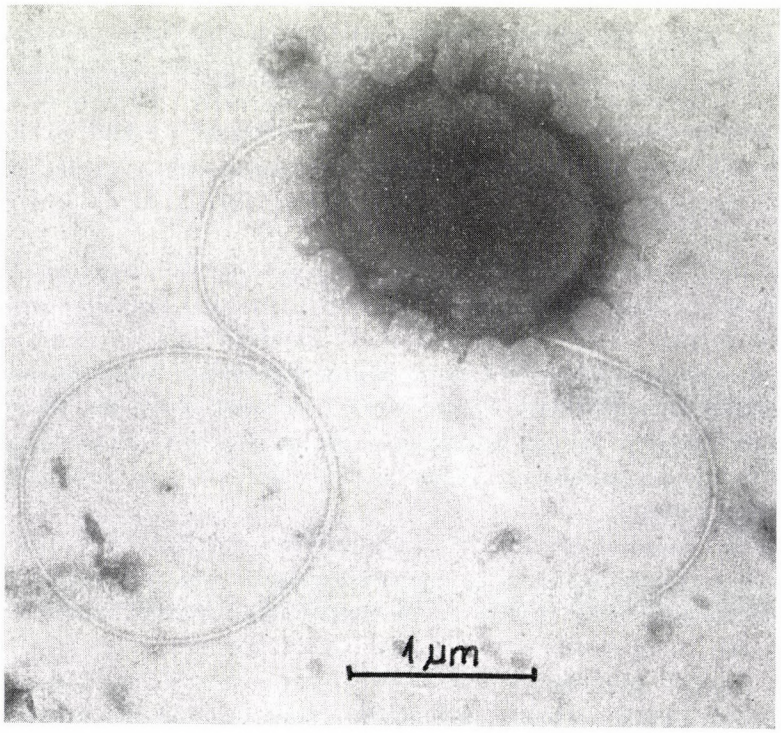

Fig. 3. Electron microscopic picture of motile *S. faecium* (group D, No. 128)

Table II

Biological and biochemical properties of Listeria and Streptococcus strains

Properties	Strains		
Number of	*Listeria* spp 16	*S. bovis* 182	*S. faecium* 137
Gram stain	+	+	+
β-haemolysis*	7	0	0
α-haemolysis*	9	182	50
Catalase activity	15	0	0
Motility	15	0	35
Hydrolysis of			
arginine	0	0	137
esculin	16	182	137
Acid production from			
D-arabinose	0	70	137
glycerol	13	53	0
i-inositol	0	0	0
lactose	9	182	137
maltose	16	182	137
mannitol	1	0	137
raffinose	0	53	0
D-salicin	16	182	137
D-sorbitol	0	36	0
sucrose	5	182	137
D-trehalose	16	182	137
D-xylose**	6	.	.
L-rhamnose**	4	.	.

* Haemolysis was tested on blood agar containing ox red blood cells
** These media were supplemented with rabbit serum
. not tested

results as well as some *Streptococcus* species. The other *Listeria* species are mannitol negative.

All strains were examined with Streptex test. The results are shown in Table III. There were 6 of the 16 *Listeria* strains giving cross-reaction with one or more Streptex reagents. As to the appearance of the positive reactions no rule has been observed yet. All *S. bovis* and *S. faecium* strains agglutinated typically only in Streptex D reagents.

Lancefield extractions of the 16 *Listeria* strains were tested with streptococcus grouping sera A–V and vice versa (Tables IV, V). The number of cross-reactions was less. There was no precipitation with the group B serum in contrast to the latex agglutination. Our own and the commercial sera gave the same results. There was no relationship between virulence or beta-haemolytic ability and capability causing cross-precipitation of *Listeria* strains.

Table III

Latex agglutination of the 16 Listeria strains

Streptex	Abbreviation and designation of strains															
	s. 6b 4073	w. 1830 1830	m. 1/2b 4069	s. 1/2b 4070	s. 1/2b 4071	mu. G44	s. 6b 4074	in. 6a C644	in. 4ab No. 10	iv. 5 4535	iv. 5nm Lm 5	s. ? 4067	sp. 1830 4068	s. 1/2— 1830 4075	sp. 1/2b 4076	s. 1/2b 4072
A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B	—	+	—	+	—	+	—	—	—	—	—	—	—	—	—	—
C	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—
D	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F	—	+	—	—	—	+	—	—	—	—	—	—	—	—	—	—
G	—	+	+	—	—	+	—	—	—	—	—	—	—	—	—	+

Table IV

Precipitation of Listeria Lancefield extracts with Streptococcus grouping sera

Sera for *Streptococcus* serogroups	Abbreviation and designation of strains															
	s. 4073	w. 1830 1830	s. 1/2b 4069	m. 1/2b 4070	s. 1/2b 4071	mu. G44	s. 6b 4074	in. 6a C644	in. 4ab No. 10	iv. 5 4535	iv. 5 nm Lm5	s. ? 4067	sp. 1830 4068	s. 1/2— 1830 4075	sp. 1/2b 4076	s. 1/2b 4072
A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
D	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F	—	+	—	—	—	+	—	—	—	—	—	—	—	—	—	—
G	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—	+
H—V	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Virulence	—	—	+	—	—	—	—	—	—	+	±	—	—	—	—	—
Beta-haemolysis	—	—	+	—	—	—	—	—	—	+	+	—	—	—	—	—

Table V

Precipitation of Streptococcus Lancefield extracts with Listeria sera

Anti *Listeria* immunosera	Extracts of reference *Streptococcus* strains					
	A	B	C	D	F	G
1/2	—	+	—	—	—	++++
4ab	—	—	—	—	—	—
5	—	—	—	—	—	—
6	—	—	—	—	—	—
1830	—	—	—	—	—	—

Lancefield extracts of reference *Streptococcus* strains of group A, B, C, D, F and G were tested with *Listeria* type sera 1/2, 4ab, 5, 6 and 1830. Group G antigen gave a typical, group B antigen a weak and slow precipitation with serum 1/2. None of the antigen extracts reacted with sera 4ab, 5, 6 and 1830. These results seem to be in accordance with those of the latex agglutination and Lancefield precipitation.

The fact of antigenic relationship among *Listeria* and *Streptococcus* strains was first published by Seeliger [6]. Hopfer et al. [7] wrote that 6 out of their 17 *Listeria* strains reacted with Streptex G reagent. They did not mention the serovar of their strains. It is interesting on the basis of our results that *Listeria* antigens 4, 5 and 6 did not with *Streptococcus* reagents or grouping sera. It has not been known yet what is the real importance of these observations. The number of *Listeria* strains tested is too small for a final conclusion.

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REFERENCES

1. Holt, J. G.: In Sneath, P. H. A., Mair, N. S., Sharpe, M. E. (eds): Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore—London—Los Angeles—Sydney 1986.
2. Lányi, B. (ed.): Public Health and Clinical Bacteriology. Standard Methods (Hung.) National Institute of Hygiene, Budapest, 1980.
3. Lennette, E. H., Balows, A., Hausler, W. J., Shadomy, H. J. (eds): Manual of Clinical Microbiology. American Society for Microbiology, Washington D.C. 1985.
4. Ralovich, B.: Listeriosis Research; Present Situation and Perspective. Akadémiai Kiadó, Budapest 1984.
5. Rotta, J., Facklam, R. R.: Manual of Microbiological Diagnostic Methods for Streptococcal Infections and their Sequeale. WHO(BAC) 80. 1.
6. Seeliger, H. P. R.: Zentralbl Hyg (A) **141,** 15 (1955).
7. Hopfer, R. L., Pinzon, R., Wenglar, M., Rolston, K. V. I.: J Clin Microbiol **22,** 677 (1985).

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DIRECT ISOLATION (WITHOUT ENRICHMENT) OF *LISTERIA MONOCYTOGENES* IN MILK

L. Dominguez, V. Briones, M. Blanco, J. F. Fernandez-Garayzabal, J. L. Blanco, J. A. Vazquez and G. Suarez

Department of Animal Pathology, Microbiology and Immunity, Veterinary Faculty, Madrid, Spain

In the present work we have developed a direct method (without enrichment) for the isolation of listeriae in milk, using the combinated action of SDS (sodium dodecyl sulphate), centrifugation and selective media. It is possible to identify even 0.5 listeriae/ml amongst 7.2×10^7 c.f.u. contaminants/ml.

Listeria monocytogenes is a widespread pathogen of concern to public health and food industry. This microorganism has been involved in recent food-associated outbreaks [1, 2] and has been also isolated from raw milk [3, 4] and soft cheeses [2], which shows that a wide variety of foods for human consumption may serve as vehicle of transmission of listeriosis. This fact and its wide distribution in the environment has increased the interest for its detection on foods.

The recovery of *L. monocytogenes* is difficult from samples where other contaminating bacteria are present, as the number of listeriae is usually low and they are overgrown by other bacteria. For this reason, the attempts to isolate them by direct plating are in most cases fruitless. Numerous methods have been described to detect *L. monocytogenes* from environmental and biological specimens and foods [5]. Microbiological methods are generally used, but they require a tedious and lenghty enrichment procedure to isolate listeriae. More rapid procedures for the isolation of *L. monocytogenes* have been developed [6, 7] but these techniques are yet seldom used for routine analysis.

The present work shows a rapid method for the isolation of *L. monocytogenes* from food by direct plating without previous enrichment. This technique is rapid and cheap, and it can be used as a routine method in food industries.

Materials and methods

Organism. The strain used in this work was *L. monocytogenes* serovar 1/2a NCTC 7973. Two flasks containing 1 litre of sterilized milk were inoculated with two distinct amounts of *Listeria* grown on a yeast-glucose-lemco-agar medium to obtain two pure cultures, one with

Lucas Dominguez Rodriguez, V. Briones, M. Blanco, J. F. Fernandez-Garayzabal, J. L. Blanco, J. A. Vazquez, G. Suarez
Microbiologia-Patol. Animal I-Fac. Veterinaria-Univ. Complutense de Madrid
28004 Madrid, Spain

a high and another with a low counts of *Listeria*. These flasks were kept at 4 °C during the course of the experiments (one year). When the stationary phase was reached, the growth was transferred to a new flask, so that listeriae in milk were always in the exponential phase.

Media. All counts were done on the three best media in our hands [8], in order to check the inhibiting action of each medium on the natural milk microflora, allowing at the same time the growth of *Listeria* and the easy identification of their colonies by macroscopic observation. Media used were a yeast-glucose-lemco-agar non selective medium [9] and two *Listeria* selective media: LSAM [10] and LPM [11].

Experimental designe. Three types of milk were examined, following the same scheme.

First of all, we used sterilized milk to see the amount of *Listeria* we could recover at the end of the process when they were in pure culture. In previous experiments we had evaluated the sensitivity of listeriae to the action of SDS (sodium dodecyl sulphate), observing that the listeria-concentration was almost the same in the counts done before and after submitting samples to the SDS action for 0.5 to 1 h at a final concentration of 0.75%.

The remaining experiments were carried out with two kinds of raw milk: one of a high sanitary value from a controlled farm and with a natural contaminating flora of about 3.1×10^5 c.f.u./ml and a second one from the mixing tank of a dairy plant with a count of about 7.2×10^7 c.f.u./ml.

Raw milk was introduced in two sterile bottles with sodium azide at a final concentration of 0.05% to inhibit multiplication of the microflora during transport and handing to warrant the same concentration of bacteria. This sodium azide concentration does not affect listeria-viability but only multiplication capability.

Raw milk was distributed in flasks (300 ml each). They were then inoculated with the *Listeria* stock inoculum decribed previously with a known listeria-concentration. In this way, we obtained the same volume of raw milk with the same microflora charge but with different (and known) listeria-concentration (from 0.5 to 106 c.f.u./ml).

The same treatment was then applied to all the flasks: after inoculating, we made a first count to know the amount of listeriae we could detect by direct plating. Nine ml of an SDS sterile solution (25%) was then added to each flask to obtain a final concentration of 0.75%. In this way, we partially eliminated the microflora without affecting listeriae; this was verified by a second plating.

In the next step we centrifuged 200 ml from each flask at 12 000 rpm for 40 min at 4 °C obtaining three fractions: sediment, supernatant and cream. We observed that bacteria were found at the sediment and cream and therefore the supernatant was discarded. Cream and sediment were then resuspended in 200 ml of a 1% saline solution and a third plating was done. This washing was necessary to eliminate most of SDS that inhibits the growth of bacteria from cream and sediment which were then re-centrifuged in the same way as before. After this centrifugation, the supernatant was discarded and cream and sediment were resuspended in 20 ml of a 1% saline solution. From this final suspension the fourth and final plating was done.

Results and discussion

The results shown in the present work suggest that this technique is a fast and efficient method to detect *Listeria* in raw milk.

By direct plating in the selective media used without enrichment the detection limit of *Listeria* ranged between 1.2×10^2 listeriae/ml of milk heavily contaminated by natural microflora (7.2×10^7 c.f.u./ml) and 2.5 listeriae/ml in milk with a low microbial density (3.1×10^5 c.f.u./ml). However, with the technique we describe it is possible to detect even 0.5 listeriae/ml in samples with a high bacterial charge (7.2×10^7 c.f.u./ml).

First of all, the addition of sodium azide at a concentration of 0.05% as preservative can be very useful for the routine isolation of *Listeria* as it inhibits multiplication but not viability of microorganisms, keeping constant the bacterial population.

As it is readily seen, the use of SDS as indicated, is a good method for enhancing the detection of listeriae in media heavily contaminated by other microorganisms such as raw milk. In previous experiments we have seen that the effect of SDS on listeriae at the concentration and time stated is irrelevant (data not shown); in these experiments we observed that SDS is less inhibitory to listeriae than to the other contaminants. Thus after adding SDS to raw milk, the natural microflora concentration corresponded to 7.5% of the initial number, while the number of listeriae was 70% of the initial amount.

The combined effect of SDS and centrifugation on the concentration of listeriae and natural microflora of the different fractions and types of milk are shown in Tables I and II. After centrifugation, listeriae concentrated mainly in the cream and in the sediment.

When the cream and the sediment are resuspended and combined, practically the total of the initial listeria—concentration was recovered. In this way listeriae had been concentrated nine to ten times, as the final volume is around 10% of the original. This is the reason for the great sensitivity of the technique, along with the effect of SDS on the natural microflora of milk allowing a better identification of the colonies of *Listeria* as it eliminates most of the competitive microflora.

The importance of the second centrifugation has to be stressed: the first centrifugation does not completely eliminate the SDS and the number of

Table I

Effect of SDS on listeria in sterilized milk. Percentage of the initial bacterial population recovered in the different fractions after centrifugation

	Cream	Sediment	Supernatant	Cream + sediment
With SDS	326	2782	0.4	4025
Without SDS	526	8345	2.4	12200

SDS = sodium dodecyl sulphate

Table II

Effect of SDS on Listeria and raw milk natural microflora. Percentage of the initial bacterial population recovered in the different fractions after centrifugation

	Cream		Sediment		Supernatant		Cream + sediment	
	Microflora	*Listeria*	Microflora	*Listeria*	Microflora	*Listeria*	Microflora	*Listeria*
With SDS	11.4	1300	1.85	386	0.08	1.7	107.1	11700
Without SDS	257.1	2000	25	380	1.04	8.4	1028	12720

Microflora = natural microflora of raw milk; SDS = sodium dodecyl sulphate

Table III

Efficacy of different selective media on the recovery of Listeria in relation to the natural milk microflora at the different stages of the experiment

Listeria/ml	Media	Raw milk 7.2×10^7 c.f.u./ml			Raw milk 3.1×10^6 c.f.u./ml		
		without SDS	with SDS	centri-fugated	without SDS	with SDS	centri-fugated
	NSM	+	+	+			
1.5×10^4	LSAM	++	++	++		NT	
	LPM	c	+++	+++			
	NSM	−	−	−			
1.4×10^3	LSAM	+	+	+		NT	
	LPM	+	+	++			
	NSM	−	−	−	+	+	++
1.8×10^2	LSAM	+	−	+	+	+	++
	LPM	+	+	+	++	++	++
	NSM	−	c	−	+	+	+
1.8×10^1	LSAM	−	−	+	+	−	−
	LPM	−	+	+	++	++	++
	NSM	−	−	−	+	+	+
1.8×10^0	LSAM	−	−	+	−	−	−
	LPM	−	−	+	−	−	+
	NSM	−	−	−	−	−	+
0.5×10^0	LSAM	−	−	−	−	+	−
	LPM	−	−	+	−	−	++

NSM = non-selective medium; LSAM = *Lis eria* selective agar medium; LPM = LiCl-phenylethanol moxalactam; c = contaminated; NT = not tested; + 0—10% of total colonies were *Listeria;* ++ 10—50% of total colonies were *Listeria;* +++ > 50% of total colonies were *Listeria;* — none of the colonies were *Listeria;* SDS = sodium dodecyl sulphate

colonies observed in the media decrease, specially when plated directly without diluting the sample. Frequently we have seen the paradoxical effect that there are more colonies when the sample is diluted 1 : 10 than in the undiluted one. This problem is greatly solved with the second centrifugation.

The joint action of SDS, centrifugation and selective media may be seen in Table III. It can be noticed that before the addition of SDS listeriae may be isolated by direct plating in the non-selective medium only when the difference between the concentrations of listeriae and competitive microflora is less than 10^3. However, in LSAM and LPM listeriae are isolated even when the difference is 10^4 to 10^5 c.f.u./ml.

After the addition of SDS natural microflora decreased ten times of the original amount when plated on the non-selective medium and LSAM, and almost one hundred times on LPM, while the listeria-concentration is scarcely

affected. This means than in LPM the colonies of *Listeria* are more easily seen and identified even when the difference with natural microflora is 10^6 c.f.u./ml. The difference has to be less than 10^4 when plating on the non-selective medium, and less than 10^5 on LSAM, but their identification is easier than before the addition of SDS.

Finally, after centrifugation it is possible to identify even 0.5 listeria/ml amongst 7.2×10^7 c.f.u. contaminants/ml on LPM; in LSAM the difference has be less than 10^7 and in the non-selective medium of 10^6 to detect listeriae. In addition, colonies of *Listeria* were more easily identified on LPM than on the other two media, due to the lower growth of competitive microflora.

REFERENCES

1. Fleming, D. W., Cochi, S. L., MacDonald, K. L., Brondum, J., Hayes, P. S., Plikaytis, B. D., Holmes, M. B., Audurier, A., Broomer, C. V., Reingold, A. L.: N Engl J Med **312,** 404 (1985).
2. James, S. M., Fannin, S. L., Agree, B. A., Hall, B., Parker, E., Vogt, J., Run, G., Williams, J., Lieb, L., Salminen, C., Pendergast, P., Werter, S. B., Chin, J.: Morbidity and Mortality W Rep **34,** 357 (1985).
3. Fernandez, J. F., Dominguez, L., Vazquez, J. A., Rodriguez, E. F., Briones, V., Blanco, J. L., Suarez, G.: J Appl Bacteriol **63,** 533 (1987).
4. Lovett, J., Francis, D. W., Hunt, J. M., Crawford, R. G.: Dairy Food Sanit **5,** 399 (1985).
5. Dominguez, L., Suarez, G., Fernandez, J. F.: 8th ISOPOL, Madrid 1981.
6. Donnelly, C. W., Baigent, G. J.: Appl Environ Microbiol **52,** 689 (1986).
7. Farber, J. M., Peirs, J. I.: J Food Protect **50,** 479 (1987).
8. Dominguez, L., Fernandez, J. F., Briones, V., Blanco, J. L., Suarez, G.: J Dairy Res **55,** (1988).
9. Naylor, J., Sharpe, M. E.: J Dairy Res **25,** 92 (1958).
10. Dominguez, L., Suarez, G., Fernandez, J. F., Rodriguez, E. F.: Appl Environ Microbiol **47,** 1188 (1984).
11. Lee, W. H., Maclain, D.: Appl Environ Microbiol **52,** 1215 (1986).

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THE USE OF MONOCLONAL ANTIBODIES TO DEMONSTRATE *LISTERIA MONOCYTOGENES* IN POST MORTEM TISSUE USING A DIRECT IMMUNOFLUORESCENCE TECHNIQUE, AND TO DETECT A SOLUBLE ANTIGEN IN CSF SUPERNATANTS USING AN ELISA

J. McLAUCHLIN, D. SAMUEL and A. G. TAYLOR

Division of Microbiological Reagents and Quality Control, Central Public Health Laboratory, Colindale, London, England

A direct immunofluorescence technique was developed using FITC conjugates of anti-listeria antibodies (CL2 and CL17). Using this technique *Listeria monocytogenes* (whole cells) were observed in post mortem tissue from 3 patients where listeriosis had been proven by the isolation of the bacterium, and also from a patient where listeriosis was suspected. Using one of these monoclonal antibodies (CL2) an indirect antigen capture ELISA was developed for the detection of soluble antigen. Soluble antigen was detected in cerebrospinal fluid (CSF) from 27% of patients infected with *L. monocytogenes* serogroup 4 and 2% of patients where listeriosis was suspected. The test was 100% specific in that antigen was not detected in 270 other CSF samples tested. Antigen was only detected in CSF samples taken within 7 days after onset of illness.

Listeriosis is a disease with high mortality rates which may show a rapid onset of symptoms [1]. Early diagnosis with appropriate treatment, however, may modify the poor prognosis of this disease [2–5], thus there is a need for rapid methods of laboratory diagnosis. In this report we investigate the presence of *L. monocytogenes* (whole bacteria) in post mortem tissue, and of a *Listeria* serogroup 4 specific soluble antigen in cerebrospinal fluid (CSF) using anti-*Listeria* monoclonal antibodies.

Materials and methods

Direct immunofluorescent test. The specificities of the monoclonal antibodies (designated CL2 and CL17) have been discussed by McLauchlin et al., this symposium. Direct FITC (fluorescein isothiocyanate) conjugates were prepared as described previously [6].

Formalin fixed post mortem tissue was obtained from 4 patients, which were; *Patient 1*, a 12-week-old aborted foetus where *L. monocytogenes* serovar 1/2b was isolated from maternal blood cultures, amniotic fluid, chorioallantoic membrane, and from the thoracic wall of the foetus [7]. *Patient 2*, a 60-year-old with prosthetic heart valves; *L. monocytogenes* serovar 4b was isolated from blood cultures and from heart valve tissue. *Patient 3*, a previosuly healthy 85-year-old with encephalitis and pneumonia; *L. monocytogenes* serovar 1/2a was cultured from the brain-stem and meningeal swabs. *Patient 4*, a diagnosis of herpes encephalitis made in a

JAMES McLAUCHLIN, D. SAMUEL, A. G. TAYLOR
Central Public Health Laboratory, Division of Microbiological Reagents and Quality Control
Colindale Avenue, NW9 5HT London, England

previously healthy 50-year-old who died. Since the brain was fixed at autopsy, culture was not attempted, however, numerous Gram-positive coccobacilli were seen in the mid-brain.

Impression smears of post mortem tissue from the above 4 patients were prepared on microscope slides which were air dried, and fixed in acetone for 10 min. FITC conjugates of CL2 and CL17 were added to the slides at their optimal dilutions, incubated for 30 min at 37 °C, washed in phosphate buffered saline (PBS, Dulbecco A) for 10 min, and examined by fluorescence microscopy.

ELISA test. A total of 399 CSF samples collected from 393 patients were tested, and Table I shows a brief clinical description of these patients. A soluble antigen was prepared from a wild type *L. monocytogenes* serovar 4b strain using a modification of the Fuller method [8]. Eight-well flat bottom ELISA strips were treated for 72 h at 4 °C with a dilution of whole ascitic fluid of CL2 in PBS plus sodium azide (0.08% w/v). The fluid from each well was aspirated, and 200 µl of blocking buffer (1% w/v milk powder, containing 0.08% w/v sodium azide in PBS) was added to each well for 3 h at room temperature. The fluid from each well was aspirated, 200 µl of blocking buffer added to each well, the strips were sealed with adhesive tape and stored for up to 4 weeks at 4 °C.

An anti-FITC monoclonal antibody was prepared as described previously [6], and conjugated with horseradish peroxidase using the method of Wilson and Nakane [9].

When required for use, the strips were washed 6 times (200 µl per well) with wash buffer (0.1% v/v Tween 20 in PBS), and to appropriate wells 100 µl of CSF diluted 1 : 1 (v/v) in buffer 1 (1% w/v milk powder plus 1% v/v Tween 20 in PBS) was added. The strips were then incubated for 3 h at 37 °C, and washed 6 times with wash buffer. CL2/FITC conjugate was added (100 µl) in buffer 2 (buffer 1 plus 5% w/v bovine serum albumin), and reincubated for 1 h at 37 °C. The strips were then washed 6 times as before, anti-FITC/HRP conjugate added in buffer 1, and reincubated for 1 h at 37 °C. Wells were then washed as before, 100 µl of substrate (tetramethylbenzidine) added using the method of Bos et al. [10], and optical density values (OD 450 nm) determined. All CSF samples were tested in duplicate, and results are expressed as a ratio of the mean OD as compared with that obtained for a blank (substrate only) well.

Results and discussion

Reactivity of the monoclonal antibodies in direct immunofluorescence test with post mortem specimens is shown in Table II and Fig. 1. Bacteria were observed in material from patients 1, 2 and 3 which had reacted with the monoclonal antibody of the specificity corresponding to the serogroup of *L. monocytogenes* isolated from each of the patients. Bacilli reacting with CL2 (presumably *L. monocytogenes* serogroup 4) were seen in the material from patient 4.

Soluble antigen was detected using ELISA in the formamide extracted antigen, which was used as a positive control in each assay run. The presence of antigen was taken as corresponding to wells having OD values of > 3 times that obtained with the blank. Antigen was detected in samples taken from patients with *L. monocytogenes* serogroup 4 infections (29%, 18 out of 62 patients), and from patients where listeriosis was suspected (3%, 2 out 60 patients). In the 2 cases where listeriosis was suspected and antigen was detected, a diagnosis of aseptic meningitis and central nervous system "abnormalities" had been made. Antigen was not detected in any of the other 330 CSF samples tested.

The timing when the CSF sample was taken in relation to onset of illness was known in 46 samples from 42 adults and juveniles infected with *L. mo-*

Table I

ELISA results on 399 cerebrospinal fluid samples from 393 patients for the detection of a Listeria specific antigen

No. of patients	No. of CSF specimens	Category of patient	No. of specimens where antigen was		Proportion of patients where antigen was detected
			not detected*	detected+	
15	15	*L. monocytogenes* serogroup 1/2 infections	15	0	0.00
		L. monocytogenes serogroup 4 infections:			
7	7	neonates	6	1	0.14
56	60	adults and juveniles	41	19	0.32
145	145	infections due to other agents§	145	0	0.00
60	62	listeriosis suspected	60	2	0.03
110	110	infection not suspected	110	0	0.00

* Samples wich gave OD 450 nm readings of $\leq$ 3 times the test blank

+ Samples which gave OD 450 nm readings of $>$ 3 times the test blank

§ These comprised infections in: 69 patients with *Neisseria meningitidis*, 41 with *Streptococcus pneumoniae*, 32 with *Haemophilus influenzae*, 3 with *Mycobacterium tuberculosis*

The test blank was obtained by using wells containing substrate and H_2SO_4 only.

Antigen was captured from CSF samples using wells coated with the anti-*Listeria* monoclonal antibody CL2. Bound antigen was detected using a FITC-CL2 conjugate, followed by a peroxidase labelled anti-FITC monoclonal antibody

Table II

Results of direct immunofluorescence tests using FITC conjugates of anti-Listeria monoclonal antibodies tested against post mortem tissue

Tissue sampled	No. of fluorescing bacilli	
	CL17 FITC (anti serogroup 1/2)	CL2 FITC (anti serogroup 4)
Patient 1		
Placenta	++	−
Foetus (abdominal cavity)	++	−
Patient 2		
Aortic valve	−	++
Mitral valve	−	++
Patient 3		
Temporal lobe (surface)	−	−
Brain stem/cerebellum (surface)	+*	−
Brain stem/cerebellum (necrotic area)	++	−
Cortex (surface)	−	−
Patient 4		
Brain stem (necrotic area)	−	++

* Scanty numbers of bacilli only seen

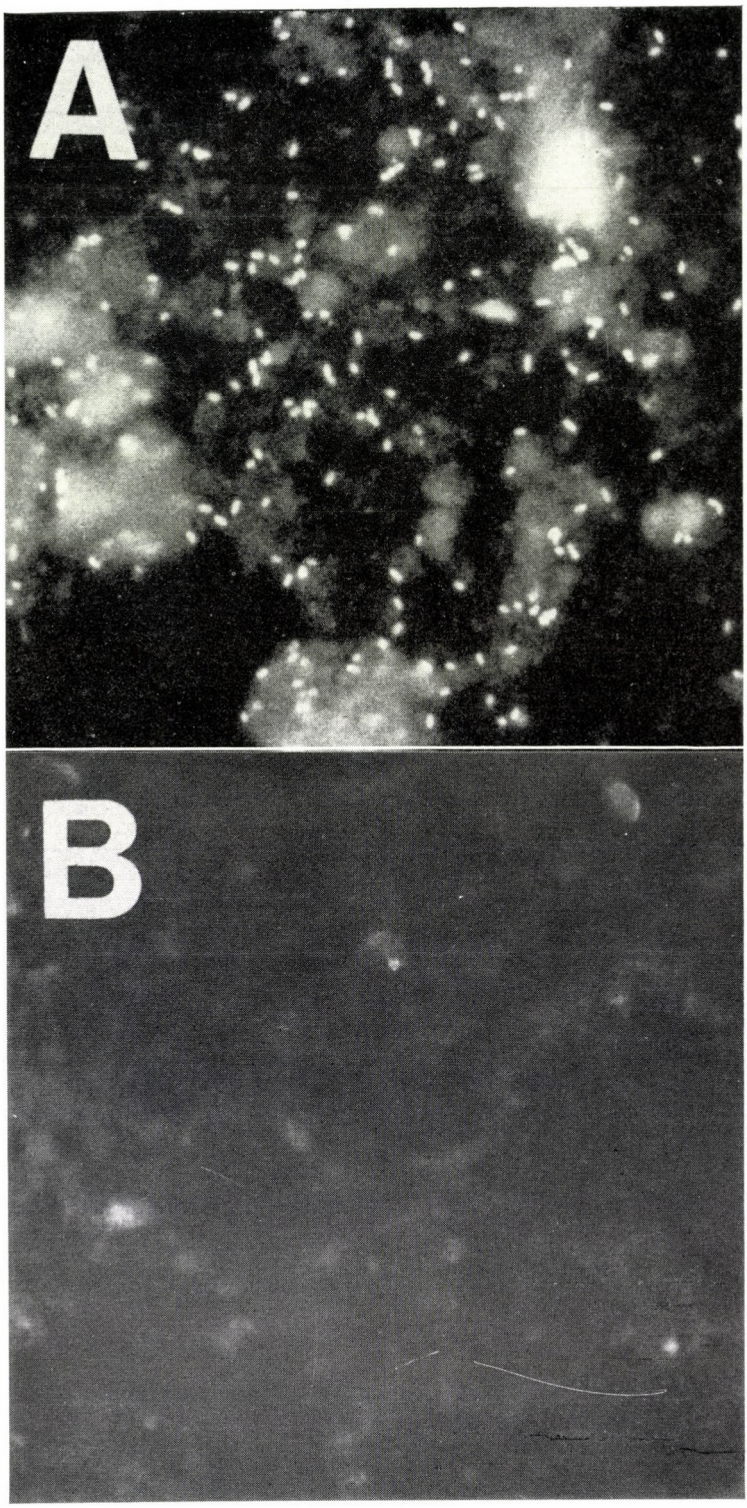
A
B

Table III

Detection of a Listeria specific antigen in CSF samples by ELISA in relation to the timing of the specimen and onset of illness

	Days after onset of illness CSF specimen was collected		
	0–3	4–7	>7
	No. of patients		
Antigen detected*	13	2	0
Antigen not detected+	12	11	8
Proportion of samples where antigen was detected	0.52	0.15	0.00

* OD 450 nm > 3 times test blank
\+ OD 450 nm < 3 times test blank

nocytogenes serogroup 4, and the proportion of these in which antigen was detected is shown in Table III. Antigen was detected most often within 3 days of onset, and not in any of the samples taken later than 7 days after onset. In 2 patients where sequential samples were obtained, antigen was detected in both taken within one day after onset, and not in specimens taken on the 3rd and 5th day after onset of illness.

The frequency with which each serogroup of *L. monocytogenes* causes infection in humans in Britain has been previously reported [11]. Using this information together with the results in Table II, it can be estimated that, when present in clinical specimens, strains of *L. monocytogenes* in over 95% of all patients with listeriosis will be detected using these antibodies. This assumes that the specificities of these antibodies are similar with respect to bacteria grown in vitro and in vivo, and the results of this small series suggests this to be so. It has been reported that *L. monocytogenes* may be difficult to cultivate from body fluids [1], and even if grown may be misidentified and dismissed as a contaminant [12]. Problems in culturing bacteria after patients have started receiving antimicrobial chemotherapy are also well recognized. In addition, it has been advocated that the examination of tissue by conventional techniques may aid diagnosis [3]. The reagents described here may be further helpful in the direct demonstration of *L. monocytogenes* in clinical specimens.

An accurate and rapid diagnosis of bacterial meningitis is highly advantageous in patient management since this may allow early implementation of appropriate chemotherapy. Various immunological techniques have been

Fig. 1. Immunofluorescent photomicrographs of impression smear from patient 1 (the abdominal cavity) stained with FITC labelled anti-*Listeria* monoclonal antibodies. A = stained with CL17 (anti-*L. monocytogenes* serogroup 1 antibody); B = stained with CL2 (anti-*L. monocytogenes* serogroup 4 antibody)

used to detect antigens in samples from patients infected by other agents [13]. There has been one previous report [14] of the detection of listerial antigen in CSF taken from a single patient during infection.

The ELISA described here detected a *L. monocytogenes* serogroup 4 specific antigen in the CSF. Antigen was detected in CSF samples taken from 30% of all patients with culture proven serogroup 4 infections, and also in 2 specimens where listeriosis was suspected. The assay appears to be 100% specific in that antigen was not detected in any of the other CSFs tested. However, the sensitivity of this test was low, since antigen was not detected in CSFs from 70% of patients with culture proven serogroup 4 infections (Table I). A possible reason for this may be that the antigen in sometimes present at concentrations which are below the detection limit of this test. However, the observation that antigen was detected in approximately half the CSF samples collected within 2 days of onset of infection and in none of the samples collected after 7 days (Table III), suggests that antigen concentration rapidly decreases with time after onset of illness. This is also supported by the results obtained with specimens from 2 cases where sequential CSF samples were obtained. In addition, the dates of onset of infection used here may be rather subjective, and represent the date of onset of acute symptoms, admission to hospital or the date when the first specimens were taken. Thus, the levels of sensitivity obtained here may be an underestimate of the potential sensitivity of this assay.

We demonstrate here that serogroup specific antigens are produced by *L. monocytogenes* in vivo both on the bacterial cell surface, and (for serogroup 4 strains) in a soluble form in CSF. The relatively simple tests described here may help in the laboratory diagnosis of human listeriosis.

REFERENCES

1. Gray, M. L., Killinger, A. H.: Bacteriol Rev **30,** 309 (1966).
2. Fleming, D. W., Cochi, S. L., MacDonald, K. L. et al.: N Engl J Med **312,** 404 (1985).
3. Relier, J. P.: J Antimicrob Chemother **5** (Suppl A), 51 (1979).
4. Robertson, M. H., Mussalli, N. G., Aizad, T. A., Okaro, J. M. Banwell, G. S.: Arch Dis Child **54,** 549 (1979).
5. Schlech, W. F., Lavigne, P. M., Bortolussi, R. A. et al.: N Engl J Med **308,** 203 (1983).
6. Samuel, D., Patt, R. J., Abuknesha, R. A.: J Immunol Methods **107,** 217 (1988).
7. Pezeshkian, R., Fernando, N., Carne, C. A., Simanowitz, M. D.: Br J Obstet Gynaecol **91,** 85 (1984).
8. Fuller, A. T.,: Br J Exp Pathol **19,** 130 (1938).
9. Wilson, M. B., Nakane, P. K.: In Knapp, W., Holubar, K., Wick, G. (eds): Immunofluorescence and Related Staining Techniques. Elsevier, Holland 1978. p. 215.
10. Bos, E. S., van der Doelen, A. A., van Rooy, N., Schuurs, A. H. W. M.: J Immunoassay **2,** 187 (1981).
11. McLauchlin, J.: J Appl Bacteriol **63,** 1 (1987).
12. Dee, R. R., Lorber, B.: Rev Infect Dis **8,** 968, (1986).
13. Wilson, C. B., Smith, A. L.: In Remington, J. S., Swartz, M. N. (eds): Current Topics in Infectious Diseases. McGraw-Hill, New York 1986. p. 134.
14. Morel, A., Rigal, P., Barege, H., Lemeland, J. F., Boiron, H.: Nouv Pr Med **7,** 2568 (1978).

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COMPARISON OF IMMUNOFLUORESCENT ANTIBODY TEST AND ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES TO *LISTERIA MONOCYTOGENES*

E. G. Russell

Department of Microbiology, Monash Medical Centre, Clayton, Victoria, Australia

This laboratory has used an immunofluorescent antibody test (IFAT) for the detection of IgG and IgM antibodies to *Listeria monocytogenes*. This test is performed using whole cells of serotypes 1/2a, 1/2b, and 4b as antigen. Sera are pre-absorbed to remove cross-reacting antibodies. This test can diagnose listeriosis by demonstration of seroconversion in appropriately spaced acute and convalescent sera. The disadvantages of this test are the necessity for pre-absorption, the use of three different test antigens and the need for subjective interpretation. An enzyme-linked immunosorbent assay (ELISA) using sonicated listeria cells as the antigen in a microtitre plate system can theoretically overcome these shortcomings. An ELISA for anti-listerial IgG and IgM was compared to the IFAT using sera from bacteriologically confirmed cases as positive controls and sera submitted for routine rubella serology as negative controls. The ELISA method as tested appeared to be less specific than the IFAT. As the serological diagnosis of listeriosis is complicated by the failure of the main antibody response to switch from IgM to IgG after infection, asimilar ELISA system was used to detect anti-listerial IgA antibody. This assay detected IgA in patients with proven listeriosis but not in the control patients. It appears to be sensitive, specific and easy to perform.

The immune response to infection with *Listeria* includes both humoral and cellular components, cross-reacts with other Gram-positive bacteria and remains substantially as IgM after infection [1]. These obstacles have hindered the development of a definitive serological test. The standard test for several years in this laboratory has been the immunofluorescent antibody test (IFAT). Because enzyme-linked immunoassay (ELISA) tests have advantages in that they are usually sensitive and easy to perform, the usefulness of ELISA tests was evaluated for the detection of anti-listerial IgG, IgM and IgA.

Materials and methods

Patient samples. Sera from 24 cases of bacteriologically proven listerial infections were available to test by IFAT and ELISA. Paired sera were available in three cases. Controls were randomly selected from sera sent for routine rubella serology. Additional sera submitted for investigational serology were also tested for anti-listerial antibodies.

IFAT. Listeria monocytogenes strains 1/2a, 1/2b and 4b, obtained from H. P. R. Seeliger were grown overnight in Oxoid Tryptic Soya Broth, washed three times and resuspended in phosphate-buffered saline (PBS) (FA-Buffer, Difco). Slides were prepared of single serotypes or a mixture of all three. The cell density was adjusted to give an even bacterial layer and 50 μl

Edward G. Russell
Department of Microbiology, Monash Medical Centre
246 Clayton Road, Clayton, Victoria, Australia 3168

drops were spread on each well of 8-well Flow Multitest slides. Test sera were diluted 1 : 20 in PBS and absorbed with a slurry of *Staphylococcus aureus* (Oxford strain) and *Streptococcus faecalis* (ATCC 19433) to remove cross-reacting antibodies. After centrifuging, 30 μl drops of appropriate serum dilutions in PBS were added to test wells and slides were incubated in a moist chamber for 15 min at 35 °C. After washing three times in PBS, 30 μl of a working dilution (usually 1 : 400) of fluorescein-conjugated rabbit anti-human IgG or IgM (Silenus Laboratories, Melbourne, Australia), was added to each well. The slides were incubated a further 15-min, washed, and observed with a Leitz Dialux 20 EB fluorescence microscope fitted with a HBO-50 W mercury incident light source and I2 filter system. The end-point was recorded as the highest dilution at which moderately bright apple-green fluorescence occurred.

ELISA. The three strains of *Listeria* were grown as described, suspended in 0.1 M sodium carbonate buffer (pH 9.5) and sonicated three times for a total of 60 s. Equal quantities of the soluble fractions were combined. Flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 50 μl per well of this combined antigen. The wells were washed three times with PBS containing 0.1% Tween 20 (PBS-Tween), and 50 μl of the appropriate serum dilution in ELISA buffer (0.1 M Tris hydrochloride (pH 8.0), 0.5 N NaCl, 2×10^{-3}; M EDTA, 0.05% Tween 20, 5×10^{-5} M thiomersal, 0.2% bovine serum albumin) was added to duplicate wells. Incubation was for 60 min at room temperature. Duplicates of a pool of the three strongest reacting sera were assayed in each run as a positive control. After the wells were washed three times in PBS-Tween, 50 μl of Silenus affinity-purified goat anti-human IgG, IgM, or IgA conjugated to horseradish peroxidase, was added to each well and incubated for 60 min at room temperature. The appropriate dilution of the conjugate was determined by checkerboard titration agains the antigen. Generally, the conjugate was diluted 1 : 1000 for use. After the wells were washed three times in PBS-Tween, 100 μl of 5-aminohydroxybenzoic acid purified by the method of Ellens and Gielkens [2] with hydrogen peroxide at a final concentration of 6×10^{-3}%, was added to each well. After 60 min, optical density at 492 nm was measured with a Flow Uniskan spectrophotometer.

Results

There was no significant difference in IFAT titre whether or not the test antigen was the infecting serotype. To compare ELISA results the positive control was arbitrarily assigned a value of 100 ELISA Unit (100 U) and the

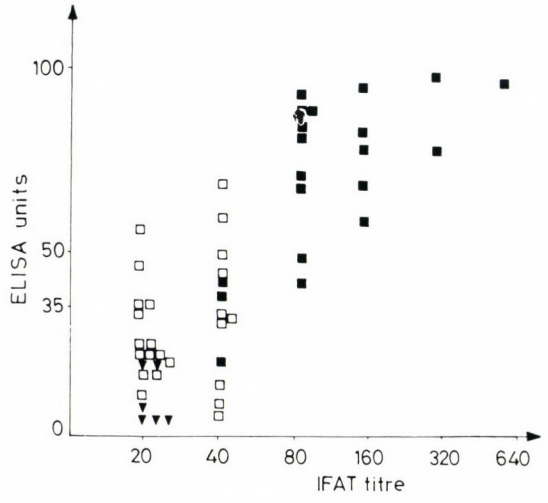

Fig. 1. Comparison of antilisterial IgG levels measured by IFAT and ELISA in 18 adults (■) and 6 neonates (▼) with bacteriological evidence of listeriosis and in 24 adult controls (□)

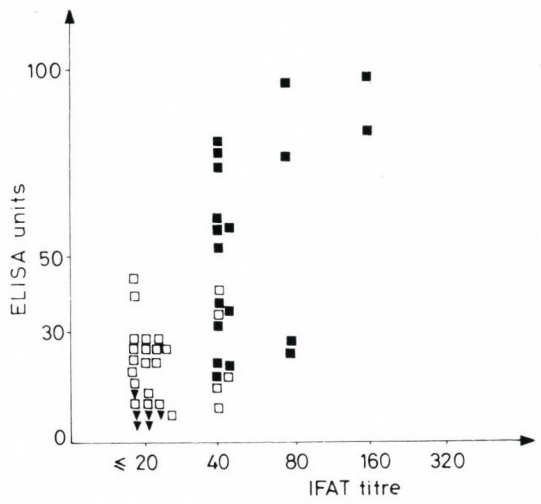

Fig. 2. Comparison of antilisterial IgM levels measured by IFAT and ELISA in 18 adults (■) and 6 neonates (▼) with bacteriological evidence of listeriosis and in 24 adult control (□)

cut-off which would include the most number of positives and exclude the most negatives was established at 35 U for IgG and 30 U for IgM assays. The detection of IgG and IgM antibodies by IFAT and ELISA was compared in 18 adult and 6 neonatal bacteriologically proven cases of listeriosis, and in 24 random controls (Figs 1 and 2). IFAT-IgG titres of >40 were present in 15/18 adult cases but not in controls. Corresponding ELISA values were >35 U in 17/18 cases but 6/24 controls were above this value. IFAT-IgM titres were ≥ 40 in all 18 adult cases and 5/24 controls. ELISA-IgM titres were >30 U in 13/18 adult cases, but also in 4/24 controls. All neonatal sera were negative in all IFAT and ELISA tests. There was a significant rise in both IFAT and ELISA titres when values for the convalescent sera were compared to acute sera in all three adult cases where paired sera were available (Table I).

Table I

Comparison of IgG and IgM levels measured by IFAT (titres) and ELISA (ELISA units) in three pairs of acute and convalescent sera from patients with bacteriological evidence of listeriosis

Patient	Acute/convalescent serum		
	A	B	C
IFAT–IgG	≤20/80	≤20/160	≤20/80
ELISA–IgG	<5/71	<5/94	16/90
IFAT–IgM	≤20/80	40/80	≤20/40
ELISA–IgM	15/92	<5/69	<5/24

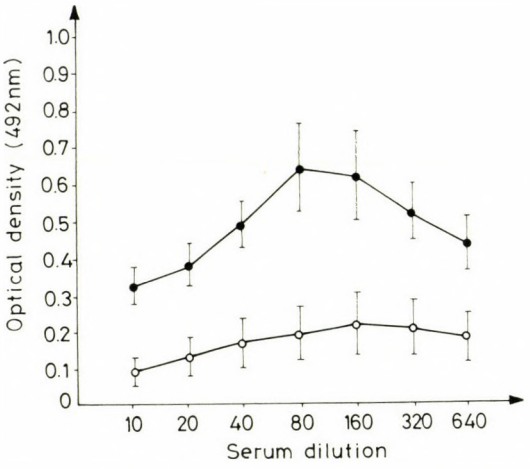

Fig. 3. Titration of antilisterial IgA in two pools of serum with (●) and without (O) bacteriological evidence of listeriosis

Serial dilutions of sera from cases and from controls were tested for specific IgA antibody (Fig. 3). Examination of these dose-response curves indicated that a single dilution at 1 : 100 would most effectively separate case and control sera. The positive control was assigned a value of 100 U and the

Table II

Results of ELISA IgA tests on 118 serum specimens from adults

	Positive (>12 U)	Negative (≤12 U)
Bacterial evidence of listeriosis	34	0
No bacterial evidence of listeriosis	2	82

cut-off made at 12 U, using the same reasoning as before. A total of 118 sera were tested for specific IgA antibody at 1 : 100 dilution (Table II). The control patient sera which were positive for antilisterial IgA comprised 1/36 submitted for listeria investigation and 1/48 for rubella serology. The first patient was a sheep farmer who had been treated with many antibiotics for liver abscesses. Drainage subsequent to therapy was sterile and extensive serology failed to reveal evidence of any other pathogen. The serum also showed high titres of antilisterial IgG and IgM when tested by IFAT and ELISA. The second was an antenatal patient who later suffered spontaneous abortion. Insufficient serum was available for further tests and no further investigations were carried out.

Discussion

Most serological tests for the diagnosis of listeriosis have problems with cross—reactivity and with the overlap of response between normal and infected subjects [3]. The IFAT has comparable sensitivity and specificity to previously described microagglutination [4, 5] and complement fixation [3] tests. The ELISA IgG and IgM test were similar in character to those previously described [1, 6] in that they were very sensitive but lacked predictive power. They were not as effective as the IFAT in distinguishing known infected cases from controls. This may have been due to the greater sensitivity of these tests in detecting small amounts of antibody or because they may detect cross-reacting antibodies. These tests may benefit from pretreatment of sera similar to that used in the IFAT. Neither test detected antibodies in neonates, as has been reported elsewhere [7].

The place of IgA antibodies in the diagnosis of infectious diseases is under investigation. It has been proposed that it correlates particularly well with active infection [8]. In the assay described here, a positive IgA correlated well with bacteriological evidence of listeria infection. Of the two positive sera from the non-proven group, it appears very likely that the first had listeriosis and it is a possibility in the second. Although this study has only looked at small numbers, it appears that this assay may provide a useful method for diagnosing listeriosis.

REFERENCES

1. Hudak, A. P., Lee, S. H., Issekutz, A. C., Bortolussi, R.: Clin Invest Med **7,** 349 (1984).
2. Ellens, D. J., Gielkens, A. L. J.: J Immunol Methods **37,** 325 (1980).
3. Issekutz, T. B., Lee, S. H., Bortolussi, R.: Manual of Clinical Microbiology. American Society for Microbiology, Washington 1986. p. 402.
4. Bodnár, L., Pap, G., Kemenes, F.: Acta Microbiol Acad Sci Hung **19,** 389 (1972).
5. Larsen, S. A., Jones, W. L.: Appl Microbiol **24,** 101 (1972).
6. Meyer, S., Brunner, H.: Zentralbl Bakteriol (A) **248,** 469 (1981).
7. Issekutz, T. B., Evans, J., Bortolussi, R.: Clin Invest Med **7,** 281 (1984).
8. Sarov, I., Insler, V., Sarov, B., Cevenini, R., Rumpianesi, F., Donati, M., Kleinman, D., Piura, B., Lieberman, J., Kimmel, N., Friedman, M., La Placa, M.: New Horizons in Microbiology. Elsevier, Basel 1984. p. 157.

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SEROLOGICAL DIAGNOSIS OF LISTERIOSIS IN MAN, SHEEP AND RABBIT BY IMMUNOPEROXIDASE TECHNIQUE

V. Briones, L. Dominguez, M. Domingo, A. Marco, J. A. Ramos, J. A. Garcia and G. Suarez

Department of Microbiology, Pathology and Immunity, Veterinary Faculty, Madrid, and Department of Anatomy, Pathology and Histology, Veterinary Faculty, Barcelona, Spain

Due to the already demonstrated specificity and usefulness of *Listeria* intracellular antigen in paraffin-embedded tissues for the direct diagnosis, we have used an indirect immunoperoxidase technique for the serological diagnosis of listeriosis in man, sheep and rabbit. This technique has been compared with other immunoperoxidase techniques (PAP) using the same antigen, and with other serlogical techniques — ELISA and microplate agglutination test — using two kinds of *Listeria* antigens, live formalinized and heat at 100 °C for one hour whole cells.

The serological diagnosis of listeriosis is currently done by the microplate agglutination test using a heated and trypsinized antigen or by the indirect immunofluorescence technique using lyophilized antigens. These methods have a very low fidelity because of the high titres found on healthy individuals. Contact with apathogenic listeria-related bacteria might be the cause of this phenomenon, but, in fact, these tests do not discriminate healthy individuals from carriers nor even from bacteriologically diagnosed patients.

The already demonstrated usefulness of formalin-fixed paraffin-embedded tissues as source of antigen in many immunocytological diagnosis procedures [1–7] and the reliability of immunoperoxidase techniques [8–10] have induced us to the application of both to the serological diagnosis of listeriosis. Furthermore, we have developed an in vivo formalinized whole cells antigen for its use in the ELISA technique.

Materials and methods

Antigens. 1. *Intracellular antigen.* Swiss mice of about 20 g weight were inoculated with *Listeria monocytogenes* of serovars 1, 3, 4, 6 and 7, and *Listeria ivanovii* serovar 5. Inoculations were done by the intraperitoneal way with 0.5 ml of a 10×6 c.f.u./ml suspension. After sacrifice

Dieste Victor Briones, L. Dominguez, J. A. Garcia, G. Suarez
Microbiologia Patologia Immunologia Animal I.
Fac. Veterinaria, Univ. Complutense de Madrid
28040 Madrid, Spain

M. Domingo, A. Marco, J. A. Ramos
Dpto Anatomia Patologica e Histologia,
Fac. Veterinaria UAB, 08193 Barcelona, Spain

(48 h post-infection) the liver was removed and adequately formalin-fixed/paraffin-embedded. Histological cuts of about 3 μm thickness were prepared and used as intracellular antigen.

2. *In vivo formalinized whole cells.* Listeriae were grown in a shaken brain heart infusion (HBI) broth for 24 h. A double-salted buffered formalin was added to the broth (1 : 1) and after 3 h it was centrifuged and washed in phosphate buffer solution twice to obtain the cells. They were then resuspended in PBS with a 0.5% of phenol as a preservative agent and kept in refrigeration.

Antisera. Sera from microbiologically (man, sheep) and histopathologically (sheep) diagnosed cases were used as primary antibody. Sera from not diagnosed individuals but positive with the microagglutination and/or IIF tests and negative control sera were also tested. Each rabbit antiserum was obtained against a serovar of *L. monocytogenes* (serovars 1/2a, 3a, 4a, 4b, 4c, 4d and 7) and *L. ivanovii* [5] by hyperimmunization of New Zealand rabbits with live bacteria. Each serum was tested to determine the optimal working dilution. Antisera 3a, 4b and 7 were found to be optimal at 1 : 200; anti 4a, 4c and 5 at 1 : 500; anti 4d at 1 : 1000 and anti 1/2a at 1 : 2000 dilutions. These sera reached an homologous titre in the MA of about 1 : 5120 (data not shown). The control rabbit was not inoculated.

Bacteria. The strains utilized belong to the SLCC and all them were serotyped and kindly by Dr. H. P. R. Seeliger.

Indirect immunoperoxidase. The technique was developed as follows [8–10]: Deparaffinized and rehydrated as usual. Inhibition of endogenous peroxidase was made using a freshly prepared solution of hydrogen peroxide 33% v/v (500 μl) in methanol (15 ml). Normal swine serum (NSS), 10 min. Rinsed twice with Tris buffer solution (TBS). Primary antibody diluted in TBS with a 20% of NSS, 30 min. Rinsed twice with TBS. Horseradish peroxidase conjugated secondary antibody (antispecies) diluted in TBS/20% NSS, 30 min. Washed in three changes of TBS, 5 min each. Developed with DAB in a imidazole-HCl solution prepared freshly as follows (2 min): imidazole, 1.5 g; distilled water, 336 ml; HCl 1 N 11.25 ml; hydrogen peroxide, 33%

Table I

Results of the IIP test with serum dilutions 1 : 50

Ovine sera	Antigens							
	1/2a	3a	4b	4b	4c	4d	5	7
s1	—	—	—	—	—	—	—	—
s2	—	—	—	—	—	—	—	—
s3	—	—	—	—	—	—	—	—
s4	—	—	—	—	—	—	—	—
s5	—	—	—	—	—	—	—	—
s6	—	—	—	—	—	—	—	—
s7	—	—	—	—	—	—	—	—
s8	—	—	—	—	—	—	—	—
s9	—	++	+++	++	—	—	—	—
s10	—	—	++	—	—	—	—	—
s11	—	+	++	++	—	—	—	—
s12	—	—	++	—	—	—	—	—
s13	—	—	++	++	—	—	—	—
s14	—	—	++	+	—	—	—	—
s15	—	—	+++	++	—	—	—	—
s16	—	—	+++	+	—	—	—	—
s17	—	—	++	+	+	+	—	—
s18	—	—	++	+	+	+	—	—
s19	—	—	++	+	+	+	—	—
s20	—	—	++	+	+	+	—	—

v/v 84 μl. Washed in tap water for 10 min. Counterstaining with methylene blue or haematoxylin. Dehydrate and mount. Developed as described, the technique will yield the results shown in Figs 1 and 2.

ELISA technique. Microtiter flat plates were gelantinized to promote the antigen adherence. The previously obtained in vivo formalized antigen, diluted 1 : 2 in TBS, was added to the wells and the microplate incubated stirring at 37 °C for 3 h. Bovine serum albumin (BSA) diluted 1 : 100 in TBS was added to the wells and left in stirring overnight. Inhibition of endogenous peroxidase was carried out as before described. Washed twice in TBS with a 0.1% of Tween 20. Primary antibody diluted 1 : 500 in TBS with a 0.2% of BSA, 15 min. Washed twice with TBS/Tween. Horseradish peroxidase conjugated secondary antibody (antispecies) in the same way than first. Washed with TBS/Tween three times. Developing solution: citrate buffer, 50 ml; orto-phenylene-diamine, 40 μg; hydrogen peroxide, 33% v/v 50 μl. Readings must be done after no more than 2 min. Sulphuric acid may be used to stop the reaction.

Microplate agglutination was carried out as usual [11] but using the in vivo formalized antigen diluted 1 : 10 in PBS with a 0.5% of safranin O.

Results

Results of the IIP test are shown in Tables I, II and III. Sheep sera 1–8 were taken from healthy animals and showed very low titres (less than 1 : 80) with MA test. Sera 9–12, also obtained from healthy animals exhibited MA titres at least 1 : 640. Sera 13–20 were received from two ovine listeriosis outbreaks at Vizcaya and Barcelona (Spain). Human sera 1 and 2 belonged to microbiologically diagnosed human patients who developed clinical signs. Sera 3–6 were obtained from immunodepressed patients who aquired the infection subsequently. Sera 7 and 8 belonged to apparently healthy people with a positive titre in the IIF test of at least 1 : 1200. Sera 9–11 were from healthy people with a positive result in the IIF test at 1 : 600 titre. Finally, 12 and

Table II

Results of the IIP test with serum dilutions 1 : 50

Human sera	Antigens							
	1/2a	3a	4a	4b	4c	4d	5	7
h1	++	+	+++	−	−	−	−	−
h2	++	+	+++	−	+	−	−	−
h3	+	+	+++	−	−	−	−	−
h4	−	−	++	−	−	−	−	−
h5	++	−	+++	−	−	−	−	−
h6	−	−	−	−	−	−	−	−
h7	−	−	+++	−	−	−	−	−
h8	−	−	++	−	−	−	−	−
h9	−	−	−	−	−	−	−	−
h10	−	−	−	−	−	−	−	−
h11	−	−	−	−	−	−	−	−
h12	−	−	−	−	−	−	−	−
h13	−	−	−	−	−	−	−	−

Table III

Results of the IIP test. Sera from exeprimentally inoculated rabbits

Sera*	Antigens							
	1/2a	3a	4a	4b	4c	4d	5	7
r1 (anti 1/2a)	+++	++	+	−	−	−	−	−
r2 (anti 3a)	++	+++	++	−	++	−	−	+
r3 (anti 4a)	−	+	+++	++	+++	−	−	−
r4 (anti 4b)	−	+	++	+++	++	+++	++	+
r5 (anti 4c)		++	++	+	+++	−	−	−
r6 (anti 4d)	−	−	+	++	−	+++	−	−
r7 (anti 5)	−	+	++	++	+++	++	++	−
r8 (anti 7)	−	++	++	++	+++	−	−	+++
r9 (control)	−	−	−	−	−	−	−	−

* Dilution 1 : 200 to 1 : 2000

13 belonged to healthy people who gave a negative result in the IIF and MA tests.

Results with rabbit sera are described in "Materials and methods".

The ELISA technique yielded no representative results; in fact, this technique is not yet well standardized so as to discriminate between sera from healthy and ill individuals, accordingly, the data are not shown. In the same way, the microplate agglutination test was done with the field sera but no relevant differences between really positive and other sera were recorded.

Discussion

The results of the IIP reveal the possibilities of this technique in the serological diagnosis of listeriosis. It is based upon the evidence of those listeria antigens which are expressed when the bacteria are in intracellular localization in the hepatocytes surrounding the necrotic foci. They can be protein (flagellar?) antigens more specific of genus than thermoresistant (somatic) antigens. There was no strict relation between IIP reaction in field sera and the serovar isolated. The high reactivity of the 4a antigen is an impressive fact to consider. All the really positive sera reacted with it. Some of the sera from healthy individuals also reacted with other techniques but only those with a very high titre (at least 1 : 1200 in IIF and 1 : 1280 in MA). When these titres were less than 1 : 1200 and 1 : 640, respectively, no other reactions occurred.

Sera from immunodepressed individuals (transplanted, lymphoma, Hodgkin) also reacted with this technique, showing its high sensitivity.

In respect to the ELISA and MA carried out with the in vivo formalinized antigen, they yielded no significant differences between really positive sera

(sera from microbiologically diagnosed individuals) and other sera, showing the importance of the intracellular localization of antigens to discriminate sera from those individuals which have had a real contact with pathogenic listeriae from others.

Based on these facts we consider a positive reaction in the IIP using our antigens as an indicator of an intensive contact with pathogenic listeriae, regardless whether or not clinical signs developed. At present we cannot differentiate the serovar responsible for an infection.

REFERENCES

1. Domingo, M., Ramos, J. A., Dominguez, L., Ferrer, L., Marco, A.: J Vet Med **33,** 537 (1986).
2. McRill, C. M., Kramer, T. T., Griffith, R. W.: J Clin Microbiol **20,** 281 (1984).
3. Nguyen, H. T., Buergelt, C. D.: Am J Vet Res **44,** 2173 (1983).
4. Parsons, K. R., Wilson, A. M., Hall, G. A., Bridger, J. C., Chanter, N., Reynolds, D. J.: J Clin Pathol **37,** 645 (1984).
5. Ramos, J. A., Domingo, M., Dominguez, L., Ferrer, L., Marco, A.: Avian Pathol **17,** 227 (1988).
6. Rogers, D. G.: J Histochem Cytochem **33,** 762 (1985).
7. Turfrey, B. A.: Br Vet J **141,** 195 (1985).
8. Heyderman, E.: J Clin Pathol **32,** 971 (1979).
9. Sternberger, L. A.: Immunochemistry. 2nd ed. Wiley, New York 1979.
10. Taylor, C. R.: Arch Pathol Lab Med **102,** 113 (1978).

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PHOSPHOLIPASE C IN *LISTERIA*

EVA MENČÍKOVÁ

Institute of Medical Microbiology and Immunology, Charles University School of Medicine, Prague, Czechoslovakia

The cooperative and antagonistic effect of extracellular bacterial proteins of *Streptococcus agalactiae*, *Rhodococcus equi*, *Corynebacterium ovis*, and *Corynebacterium haemolyticum* with proteins of listerial strains of various sources, species and serovars of the membrane of sheep erythrocytes was investigated. *Listeria monocytogenes* and *Listeria ivanovii* produce, beside listeriolysin, further proteins. Their specific effect on the sheep erythrocyte membrane becomes apparent after the appearance of substances produced by the organisms under study. Ten strains of *L. ivanovii* produced phospholipase C responsible for the zone of incomplete haemolysis. It was inhibited by the non-haemolytic sphingomyelinase D of *C. ovis* and *C. haemolyticum*. Chromatographic analysis revealed that phospholipase C splits sphingomyelin in the membrane of sheep erythrocytes. The inhibition of double haemolysis of *L. ivanovii* by sphyngomyelinase D of *C. ovis* on agar plates with washed (!) sheep erythrocytes can be utilized as a specific and rapid identification test of *L. ivanovii*.

Production of haemolysin in *Listeria* is correlated with their virulence. Therefore, in identification and evaluation of their possible pathogenic effect on the host, properties of strains cultivated usually on sheep blood agar are taken into consideration.

On agar with washed sheep erythrocytes cultures of *Listeria monocytogenes* serovar 1/2 and serovar 4a (strain 17/57) are surrounded by a zone of complete haemolysis the extent of which varies. In addition, a second zone of incomplete haemolysis can be observed in *L. ivanovii*. Beta-haemolysis is induced by so-called alpha-listeriolysin which has long been studied [1] and subsequently purified and analyzed [2–13]. Alpha-listeriolysin has been identified as a classical oxygen-sensitive toxin, related with streptolysin O and exhibiting corresponding biological properties. The existence of beta-listeriolysin or listeriolysins has been assumed [10] primarily in human pathogenic strains with a positive CAMP phenomenon and their possible significance for pathogenicity of *Listeria* in man has been suggested [5, 10]. Production of an extracellular phospholipase C (E.C.3.1.4.3.) hydrolyzing lecithine was proved by Leighton et al. [11] in culture filtrate of *L. monocytogenes* Boldy grown anaerobically.

EVA MENČÍKOVÁ
Institute of Medical Microbiology, and Immunology, Charles University School of Medicine
Studničkova 7, 128 00 Prague 2, Czechoslovakia

Specific interactions of bacterial proteins with lipidic components of erythrocyte membranes may lead to their incomplete lysis or the lytic effect may not occur et all (e.g. the sphingomyelinase D of *Corynebacterium haemolyticum* and *Corynebacterium ovis* is non-haemolytic) or their effect is manifested only after interaction with products of other bacteria (e.g. classical CAMP test in *Streptococcus agalactiae* or potentiation of *Listeria* haemolysis by cholesterol oxidase of *Rhodococcus equi*).

Materials and methods

We studied the effect of extracellular products of *S. agalactiae* (CAMP factor), *R. equi* (cholesterol oxidase) and sphingomyelinase D of *C. ovis* and *C. haemolyticum* on the haemolytic activity of various species of *Listeria* [12]. The collection (Table I) included a total of 85 strains

Table I

Modification of haemolytic activity of Listeria by extracellular proteins of different bacterial species (nutrient agar with sheep erythrocytes)

Listeria species	Number of strains	*S. agalactiae*	*R. equi*	*C. ovis* *C. haemo lyticum*
L. innocua				
L. seeligeri	13	—	—	—
L. murrayi				
			−49	−17
L. monocytogenes	62	—	+17	+45
L. ivanovii	10	+	+	*INH*

— negative
\+ potentiation
INH inhibition

of which 13 were non-haemolytic *(Listeria innocua, Listeria seeligeri* and *Listeria murrayi)*, 62 were *L. monocytogenes* serovars 1/2, 4a and 4b, and 10 were *L. ivanovii*. The tests were performed on 2% nutrient agar (TS agar bio-Mérieux) with 2% sheep erythrocytes washed with phosphate physiological saline (sphingomyelinase D is inhibited by the serum). The cultures were incubated 18 h at 37 °C.

Results and discussion

In non-haemolytic strains isolated from carriers or from outer environment, the areas of combined effects of products of the strains tested remained unchanged. Out of *L. monocytogenes* strains isolated from patients and animals, haemolysis was increased in 13 strains by *R. equi* independently of the serovar, and in 45 strains by *C. ovis* or *C. haemolyticum*, also independently of serovar. Different results were obtained in *L. ivanovii*. The collection includes the

prototype strain Ivanov 6613, other strains were isolated by Dr. Elischerová from commercial meat, rectal swabs of patients and animals, one strain was isolated from human placenta after delivery of a dead fetus. In all strains of *L. ivanovii* the incomplete haemolysis was significantly potentiated by CAMP

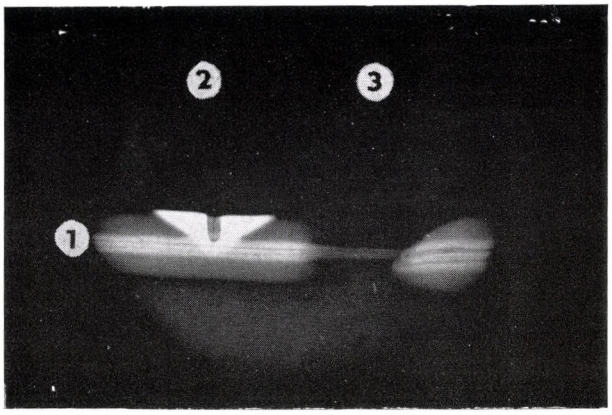

Fig. 1. Cooperative haemolytic effect of phospholipase C of *L. ivanovii* and *S. agalactiae* and its inhibition by sphingomyelinase D *(C. ovis)*. Nutrient agar with 2% washed sheep erythrocytes, incubation at 37 °C for 18 h. 1 = *L. ivanovii*, strain K 54; 2 = *S. agalactiae;* 3 = *C. ovis*

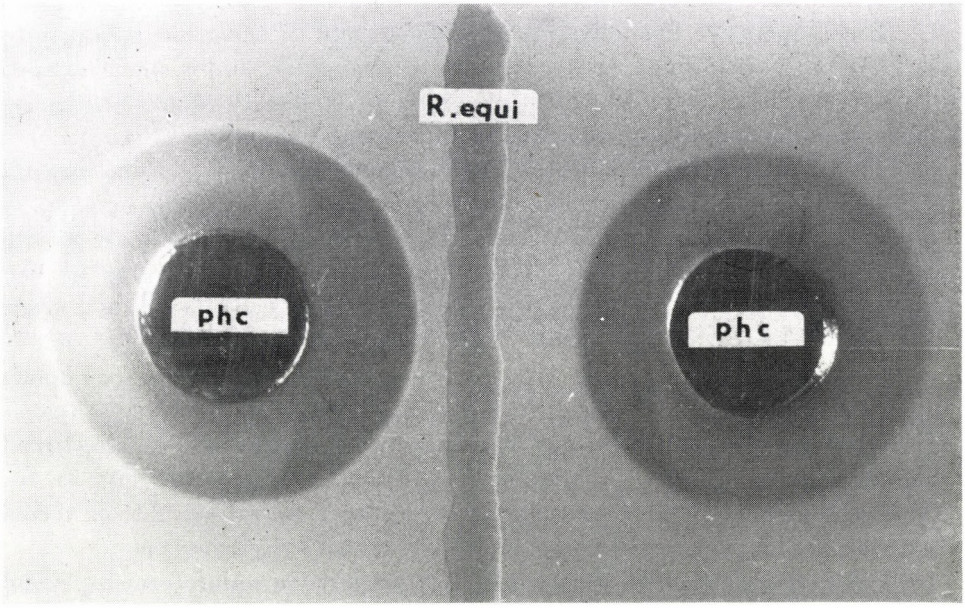

Fig. 2. Cooperative haemolysis of crude phospholipase C *(L. ivanovii)* and extracellular proteins from *R. equi*. Nutrient agar with 2% washed sheep erythrocytes. phc = phospholipase C (*L. ivanovii* strain K 54) purified from broth culture by ammonium sulphate

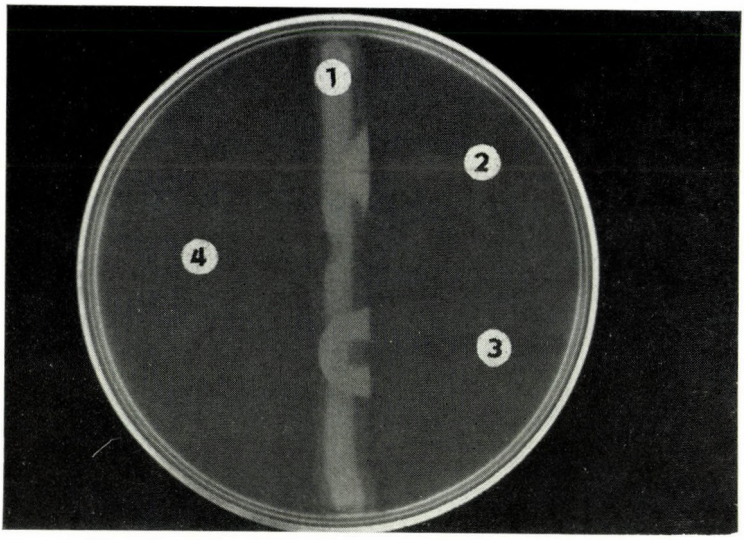

Fig. 3. Demonstration of phospholipase C of *L. ivanovii* by means of cooperative and antagonistic haemolysis with *R. equi*, *S. agalactiae* and *C. ovis*. Nutirent agar with 2% washed shee erythrocytes; incubation at 37 °C for 18 h. 1 = *L. ivanovii*, strain K 11; 2 = *S. agalactiae*; 3 = *R. equi*; 4 = *C. ovis*

factor and cholesterol oxidase of *R. equi* and was inhibited by sphingomyelinase D.

The cooperative haemolysis of *L. ivanovii* and *S. agalactiae* similarly to the CAMP test with *Staphylococcus aureus*, is triangular, and is shovel-shaped with *R. equi*. The antagonistic effect of sphingomyelinase D induces an inverse CAMP phenomenon only in the zone of incomplete haemolysis (Fig. 1).

The results obtained indicate that *L. ivanovii* produces phospholipase C. In order to demonstrate the enzyme supernatants of stationary cultures of *L. ivanovii* strains incubated in BHI for 48 h at 37 °C were precipitated with ammonium sulphate, dialyzed against distilled water (18 h at 4 °C) and dialysates were used in tests on agar with washed sheep erythrocytes as a crude toxin. After incubation the purified preparation induced a zone of double haemolysis at the periphery of wells to which it was added. The incomplete haemolysis was inhibited in the diffusion area of sphingomyelinase D of *C. ovis* NCTC 4655 purified by Dr. Souček of this Institute. It cleared a sickle-shaped area after treatment with proteins of *R. equi* and *S. agalactiae* (Fig. 2). An analogous purification of *L. monocytogenes* serovar 4a (strain 22/72) tested simultaneously induced only beta-haemolysis of sheep erythrocytes.

Using the method of Nelson [13] we prepared a chloroform-methanol extract of lipids from sheep erythrocytes and, after treatment with phospholipase of *L. ivanovii*, analyzed it by thin-layer chromatography on silicagel impregnated paper Whatman No. 3 [14] developed in a solvent system diisobu-

tylketone-acetic acid—water (40 : 30 : 7) and detected by Rhodamine B. As compared with control (lipids incubated with heat-inactivated purified preparation), native phospholipase degrades sphingomyelin in the extracted lipids whose spot almost disappeared on the chromatogram.

It can thus be conluded that synthesis of phospholipase C is a characteristic marker of *L. ivanovii* (Fig. 3). Demonstration of the enzyme in freshly isolated strains by means of the test of cooperative or antagonistic haemolysis with *R. equi*, *S. agalactiae* and *C. ovis* can be used as a specific and fast identification test for *L. ivanovii*. In experiments with animals (rabbit, mouse), a direct effect of *Listeria* phospholipase (lethal, dermonecrotic, capillary-toxic) could not be demonstrated.

REFERENCES

1. Fraser, G.: J Pathol Bacteriol **85,** 349 (1964).
2. Geoffroy, Ch., Gaillard, J. L., Alouf, J. E., Berche, P.: Infect Immun **55,** 1641 (1987).
3. Girard, K. F., Sbarra, A. J., Bardawil, W. A.: J Bacteriol **85,** 349 (1963).
4. Jenkins, E. M., Watson, B. B.: Infect Immun **3,** 589 (1971).
5. Khan, M. A., Seaman, A., Woodbine, M.: Zentralbl Bakteriol (A) **225,** 66 (1973).
6. Njoku-Obi, A. N., Jenkins, E. M., Njoku-Obi, J. C., Adams, J., Covington, V.: J Bacteriol **86,** 1 (1963).
7. Parisius, J., Bhakdi, S., Roth, M., Tranum-Jensen, J., Goebel, W., Seeliger, H. P. R.: Infect Immun **51,** 314 (1986).
8. Siddique, I. H., Liu, I. F., Chung, R. A.: Am J Vet Res **35,** 289 (1974).
9. Skalka, B., Smola, J., Elischerová, K.: J Clin Microbiol **15,** 503 (1982).
10. Watson, B. B., Lavizzo, J. C.: Infect Immun **7,** 753 (1973).
11. Leighton, I., Threfall, D. R., Oakley, C. J.: In Woodbine, M. (ed.): Problems of Listeriosis. Leicester University Press, Leicester 1975. p. 239.
12. Menčíková, E.: Folia Microbiol **32,** 514 (1987).
13. Nelson, G. J.: Biochim Biophys Acta **144,** 221 (1967).
14. Rouser, G., Siakotos, A. N., Fleisher, S.: Lipids **1,** 85 (1966).

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7. Vaccination and immunity

A TEN YEAR'S EXPERIENCE WITH INACTIVATED VACCINE AGAINST LISTERIOSIS OF SHEEP

GY. SZEMERÉDI and M. PADÁNYI

Central Veterinary Institute, Budapest, and Phylaxia Veterinary Biologicals Co., Budapest, Hungary

Efficacy of preventive use of an inactivated vaccine was controlled through several years in 24 farms on 32 124 vaccinated and 35 880 unvaccinated (control) sheep. Death due to listeriosis was 273 (0.85%) in the vaccinated and 462 (1.29%) in the non-vaccinated groups. Vaccination in the acute phase of the disease was performed in 5 flocks, on 10 303 animals, leaving 9 781 as unvaccinated controls. In most flocks, deaths due to listeriosis ceased or became minimized 7–10 days post-vaccination. In total 59 (0.57%) of the vaccinated and 251 (2.56%) of the non-vaccinated animals died as a result of listeriosis.

The failure of therapeutic attempts against listeriosis of sheep has prompted researchers to develop specific control procedures.

Certain researchers recommend live vaccines made either from avirulent or from moderately virulent strains. Olson et al. [1] and Ivanov [2] conducted successful experiments with attenuated listeriae. Kotylev et al. [3] and Idrisov and Sedov [4] reported extensive studies on the immunogenicity of an attenuated strain designated AUF from the Soviet Union. Mayer and Steng [5] found an avirulent strain suitable for vaccine production and obtained good results.

Dijk et al. [6] studied the immunogenicity of an inactivated vaccine and established that with effective adjuvants a vaccine of good antigenicity could be produced. Polish researchers [7, 8] achieved good results with autovaccines. Vockel [8] reported that autovaccines had been used in Poland since 1975 and their use significantly reduced losses by listeriosis. Kempski [7] confirmed these observations, adding that the disease cannot be eradicated with formol vaccines; however, losses can be reduced substantially; the inactivated vaccine elaborated by Graham and Lewis in 1961 was similarly effective.

In experiments conducted in 1962, we achieved good results with a formol vaccine adsorbed to aluminium hydroxide gel [9]. Experience gained during the past 10 years has encouraged us to report these results.

GYULA SZEMERÉDI
Central Veterinary Institute
Tábornok u. 2, H-1149 Budapest, Hungary

MÁRIUS PADÁNYI
Phylaxia Veterinary Biologicals Co.
Szállás u. 5–7, H-1107 Budapest, Hungary

The direction of research in Hungary has been determined by the unequivocal statement made by the veterinary authorities according to which no new live bacterial vaccines shall be put into circulation.

Vaccine production comprised the following studies. (i) Selection of strains with good immunogenic potential. (ii) Study of the inactivating effect of various concentrations of different preservatives on *Listeria* cultures, with special regard to mild inactivation and to immunogenicity of the vaccine. (iii) Determination of optimum vaccine density. (iv) Selection of expedient vaccine adjuvating methods.

Materials and methods

Organisms. From strongly haemolytic strains of higher virulence vaccine of better immunogenicity can be produced. Four *Listeria monocytogenes* strains (three of serovar 1/2 and one of serovar 4) were used for vaccine production.

Inactivating effect of various concentrations of different preservatives. It was surprising that *Listeria* cultures proved to be highly resistant. Phenol, beta-propiolactone, and ethylene imide did not kill these bacteria even in high concentrations. Merthiolate in a concentration of 1 : 10 000 and 0.2% formalin inactivated the cultures in 72 h. Merthiolate-inactivated cultures proved to possess better antigenic properties.

Vaccines were of optimum density if they were prepared from cultures containing 3×10^9 bacteria per ml. A good adjuvating effect was achieved if 1 ml of the vaccine contained 0.007 to 0.008 g $Al(OH)_3$. The vaccine was prepared and used in the practice on the basis of the above experience.

Vaccination was carried out primarily in farms where listeriosis occurred every year and gave rise to substantial losses. A precondition of the vaccination experiments was to use unvaccinated control animals in the same number as vaccinated sheep. This is difficult to carry out under practical conditions. Therefore, vaccination of a whole flock was also accepted if sheep kept under the same managemental, feeding and hygienic conditions were used as controls.

The dose of the vaccine was 5 ml. To prolong immunity, another vaccination is recommended 3 to 6 weeks after the first one. Lambs younger than 6 weeks shall not be vaccinated.

Results

The experiment comprised a total of 47 farms: 38 agricultural co-operatives, 8 state farms and a sheep enterprise. A total of 63 639 sheep (55 290 mature animals, 6128 growing sheep and 2221 lambs) were vaccinated twice. 57 878 sheep (49 241 mature animals, 5994 growing sheep and 2643 lambs) were kept under the same managemental, feeding and hygienic conditions and served as unvaccinated controls.

In 24 farms preventive vaccination was used: here listeriosis appeared only later, thus the experiment could be evaluated in the vaccinated and control group. On these farms 32 124 sheep were vaccinated and 35 880 served as unvaccinated control. In the vaccinated group 273 (0.85%), whereas in the control group 462 sheep (1.29%) died of listeriosis (Table I). Statistical evaluation of the data indicated that this difference was highly significant ($P<0.001$).

Table I

Vaccination of sheep with an inactivated vaccine against listeriosis

	Number of sheep dying of listeriosis	Percentage
I. Number of sheep vaccinated twice with preventive purposes		
32 124	273	0.85
Number of control animals		
35 880	462	1.29
II. Number of sheep vaccinated twice against listeriosis (evaluated from day 8 as of the first vaccination)		
10 303	39	0.37
Number of control sheep		
10 711	321	2.996

In 18 farms the test vaccinations could not be evaluated since neither cases of listeriosis nor deaths caused by this disease occurred among the vaccinated and control animals.

Although our vaccine was recommended for preventive vaccination only, in 5 farms 10 303 sheep suffering from acute listeriosis were vaccinated. The most spectacular results were achieved precisely in these farms: while prevaccination losses amounted to 3%, after the first vaccination losses decreased to 0.36% and 2 weeks later deaths ceased to occur.

In the majority of cases vaccination reactions were observed neither after the first nor after the second vaccination. On certain farms some of the vaccinated sheep showed a transient elevation of body temperature and listlessness lasting a few days. The vaccine did not have any tissue-irritative effect, no abscess formation occurred, only the subcutaneous node characteristic of the adsorbing organ was observed.

Discussion

In our opinion the test vaccination have reached their goal: on large numbers of animals, under large-scale conditions it has been proved that the test vaccine effectively reduces the losses caused by listeriosis.

Active immunization is recommended in sheep flocks where listeriosis occurs from year to year and causes substantial losses. In such farms preventive vaccination is recommended: where possible, the first vaccination shall be completed by the end of November at the latest, since at that time the sheep are not yet kept in closed barns. Experience and practical observations made

by veterinarians suggest that at that time the sheep posses a firm resistance which is a precondition of satisfactory immunity. The second vaccination sohuld be carried out about 2 to 3 weeks before term. By this method passive immunity can be conferred on the highly susceptible lambs via the colostrum.

Vaccination is recommended not only for preventive purposes but also to control outbreak of listeriosis. Experience shows that 7 to 10 days after the first vaccination a substantial decrease occurs in the losses, and losses are practically eliminated within 2 weeks, especially after the second vaccination.

For mature sheep the vaccine dose is 5 ml subcutaneously, as far as possible in the cervical region. Lambs younger than 2 months fail to develop immunity, presumably because of their young age. Therefore, active immunization of this age group cannot be recommended.

Vaccinations shall be carried out every year as specified in the recommendations.

Field experience has convinced us that suitable control measures make vaccinations more effective (e.g. if sheep are kept under closed conditons for the shortest possible time). However, daily exercise of the animals can only be omitted if it is made impossible by unfavourable weather conditions. The sheep can be kept in good body condition by offering them feeds rich in vitamins and supplemented with selenium salts. Good-quality silage can be fed without any risk. Should disease cases associated with silage feeding occur, it is advisable to interrupt silage feeding for 2 to 3 weeks. Subsequently, however, small quantities of silage can be given again. As far as possible, forage and silage shall be placed on a feeding rack rather than directly on the ground or litter, to prevent contamination with earth and faeces which might contain listeriae. In the spring, sheep shall be turned out to pasture as early as possible.

In our days artificial synchronizasion of lambing is a distinct possibility. Attempts shall be made to time lambing so as to avoid the most hazardous periods.

REFERENCES

1. Olson, J. C., Cook, R. H., Bagdonas, V.: Am J Vet Res **45,** 306 (1961).
2. Ivanov, I. V.: VIIIth International Symposium on the Problems of Listeriosis. Madrid 1981.
3. Kotylev, O. A., Uchenye zapiski kazanskovo veterinarnogo instituta 126 (1976).
4. Idrisov, G. Z., Sedov, N. K.: Veterinariya (Moscow) **2,** 37 (1975).
5. Mayer, H., Steng, G.: Prakt Tierärzt **56,** 149 (1975).
6. Dijk, H. van., Nature (London) **286,** 713, (1980).
7. Kempski, W.: Życie Wet (Warsaw) **56,** 141 (1981).
8. Vockel, L.: Życie Wet (Warsaw) **52,** 294 (1977).
9. Szemerédi, Gy., Nagy, G.: Magyar Állatorvosok Lapja; Phylaxia special issue. 1962. p. 43.

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EXPERIMENTAL LISTERIOSIS IN IMMUNIZED SHEEP

EVA MENČÍKOVÁ, J. ŠNIRC, D. GUBRAN, J. SMOLA and M. MÁRA

Institute of Medical Microbiology and Immunology, Charles University School of Medicine, Prague Bioveta, Nitra, and School of Veterinary Medicine, Brno, Czechoslovakia

Sheep immunized with live or inactivated vaccine were challenged with a virulent strain of *Listeria monocytogenes*. Clinical manifestations, penetration of listeriae into the mucous membranes and persistence in organs were noted. Bacteriaemia caused by intravenous injection of high doses of virulent strain disappears in 3 days. Readily after inoculation, listeriae penetrate into the gastrointestinal tract where they persist for 8 days. Listeriae appear from the 3rd day in the conjunctiva and nasal mucous membranes with clinical symptoms of conjunctivitis and rhinitis whence they are eliminated in 8 to 14 days. The brain, liver and spleen of animals that died in 5 to 7 days were loaded with listeriae. Organs from immune animals sarificed 14 days after challenge were negative by cultivation even when listeriomas were in the liver and spleen. Rapid penetration and persistence of listeriae are an expression of their increased affinity to epithelia of systems which are not only the portal of their entry but even the site of their secondary propagation and thus a source of infection.

In industrial meat production, large-scale sheep breeds may be potential sources of an increased occurrence of infective diseases. In Czechoslovakia, sheep mortality caused by listeria-infections has an increasing tendency. An inactivated vaccine in a lipoid adjuvant (paraffine oil with Arlacel and Tween 80) "Listakol" was developed for immunization of sheep in large-scale breeds. Its efficiency was compared with that of a live vaccine prepared from *Listeria innocua* ("Welshimer") administered with the identical adjuvant in divided doses.

Materials and methods

Groups of 3-month-old lambs from healthy breeds, in which listeriosis did not occur according to veterinary examination, were used for testing both vaccines. After immunization with 2 doses of a given vaccine administered at 14 days intervals intramuscularly (Listakol) or subcutaneously (Welshimer) the lambs (both experimental and control) were infected with 10^9–10^{10} c.f.u. of the virulent *Listeria monocytogenes* RNV-1 (serovar 1/2a) isolated from the brain of a lamb with listeric encephalitis. The infective dose was applied to the jugular vein, 14 days after the recall vaccine dose.

EVA MENČÍKOVÁ, M. MÁRA
Institute of Medical Microbiology and Immunology, Charles University School of Medicine
Studničkova 7, Prague 2, Czechoslovakia

J. ŠNIRC, D. GUBRAN
Bioveta
Nitra, Czechoslovakia

J. SMOLA
School of Veterinary Medicine,
Brno, Czechoslovakia

Temperature, clinical state (conjunctivitis, nasal discharge, respiratory disorders, ataxia, etc.) were followed in individual animals. At suitable intervals blood was collected for cultivation (0, 1, 2, 3, 5 days) and smears were taken from the conjunctive sac, nose and rectum. Surviving sheep were killed 14 days after the infection and their brains, livers and spleens were examined by cultivation. The Czechoslovak standard method was used for haemocultivation. Swabs with mucous membrane samples were first cultivated in V1 broth (Michalany) at 4 °C and then examined as described by Fenlon [1, 2] using sheep blood agar and selective media. Growth of *L. innocua* Welshimer was inhibited in the selective medium.

Results

Bacteraemia induced by the intravenous administration of 2×10^{10} of *L. monocytogenes* RNV-1 lasted for 2 days in control animals, and, on the third day, listeriae were eliminated from the circulation. Until 8 h after infection listeriae entered the gastrointestinal tract, where they persisted for up to 8 days. In tunica conjunctiva the bacteria were demonstrated beginning with the 2nd day and up to 6th day after the infection, even after disappearance of clinical symptoms. Cultivation findings of smears from nasal mucous membrane correlated with the maximum positivity (100%) after 4 days. The presence of

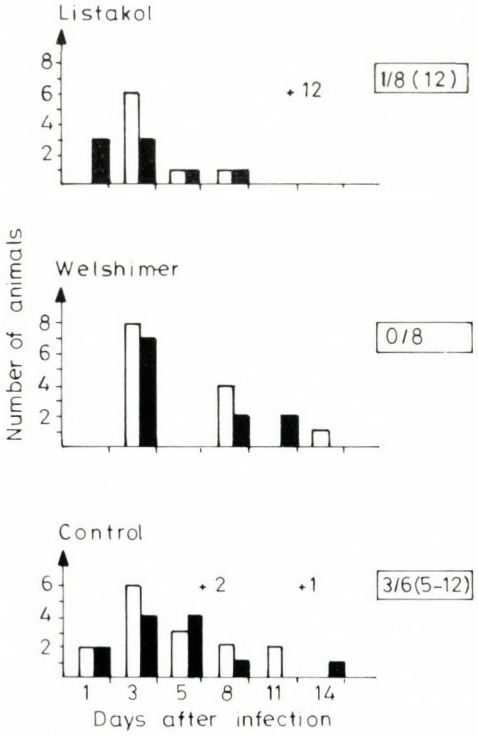

Fig. 1. Listeriae on mucous membranes of lambs immunized with inactivated (Listakol) or live vaccine (Welshimer) challenged intravenously with 10^9 c.f.u. *L. monocytogenes* RNV 1. + no animals died; framed figures, dead animals/total (days); solid columns, rectum; open columns, nose

listeriae on this type of mucous membrane is prolonged (8 days, rarely 14 days) and continuous.

Neither of the vaccines induced a sufficient immunity against the lethal effect of the high infective dose administered. Listeriae were eliminated from the blood two days after the infection only in lambs that had died after longer time intervals or in animals that had survived for 14 days. The invasion and persistance of listeriae in target mucous membranes were also not influenced by the immunization. The onset of clinical symptoms in animals that subsequently died had been fast (beginning with the 2nd day). The disease proceeded as sepsis, the mucous membranes studied were significantly affected.

Organs of animals that had died up to 7 days were flooded with listeriae. Mean values reached 1.3×10^8 in the brain, 1.9×10^7 in the spleen and 2.8×10^5 in the liver (c.f.u. per 1 mg of homogenized tissue). The organs of immunized animals killed at the end of the experiment were negative according to cultivation tests, even in the case of a rare histological finding of listerioms in the spleen and liver.

Figure 1 shows results of an analogous experiment in which the sheep were infected with 10^9 c.f.u. of *L. monocytogenes* RNV-1 corresponding to LD_{50} for lamb. In both groups of immunized animals, all blood cultures taken 1–5 days after the infection were negative, mortality decreased considerably and the infection was of a latent type. In 3 of 6 control animals a secondary transient bacteraemia associated with clinical manifestations and subsequent death within 5–12 days could be detected two days after the infection. Colonization of mucous membranes by the virulent strain was not substantially influenced even in this experiment.

Discussion

The role of alimentary tract as an entrance gate in natural listeriosis has been demonstrated and verified esperimentally [3–7]. Colonization of this tract can be favourably influenced by the indigenous bacterial flora [6]. On the basis of the study of affinity of listeriae to cells of human enterocyto like cell line CaCo-2 that are permissive for haemolytic listeriae [7] suggested that enterocytes are not only a portal of entry of virulent strains but also sites of their primary multiplication. Conjunctiva, nasal mucous membrane and respiratory tract are less frequently referred to as the portal of entry of natural or experimental infections. The present observation of the rapid invasion by the virulent strain and its persistence on mucous membranes as a result of induced bacteraemia can be considered as a further demonstration of affinity of listeriae to epithelia of these systems that can be a portal of entry and also the site of secondary mulitplication and thus a source of the infection.

REFERENCES

1. Fenlon, D. R.: J Applied Bacteriol **59,** 537 (1985).
2. Smola, J., Šnirc, J., Gubran, D.: Folia Microbiol **32,** 518 (1987).
3. Gray, M. L., Killinger, A. H.: Bact Rev **30,** 309 (1966).
4. Miller, J. K., Jeans Burns: Appl Microbiol **19,** 772 (1970).
5. Rácz, P., Tenner, K., Mérő, E.: Lab Invest **26,** 694 (1972).
6. Zachar, Z., Sawage, D. C.: Infect Immunol **23,** 168 (1979).
7. Gaillard, J. L., Berche, P., Mounier, J., Richard, S., Sansonnetti, P.: Infect Immunol **55,** 2822 (1987).

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GENETIC REGULATION OF MACROPHAGE PRODUCTION IN RESPONSE TO SURFACE COMPONENTS OF *LISTERIA MONOCYTOGENES*

SARA B. GALSWORTHY, S. TING, D. FEWSTER and A. PAVAN

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

Resistance to murine listeriosis requires humoral factors which alter production or delivery of monocytes to infective foci. Production of such factors is under genetic control and may be modulated by bacterial components. Two components from *Listeria monocytogenes*, monocytosis producing activity (MPA) and immunosuppressive activity (ISA) were used to study monocytopoiesis in mice with known abnormalities in microphage-related functions. *Listeria*-sensitive A/J mice fail to produce or respond to MPA or an endogenous mediator of monocytosis (EF). These studies provided evidence that a second humoral factor, decreases monocytopoiesis. Sera from A/J mice are more active in this respect and MPA treatment increases the amount of inhibitory factor in A/J mice. A polyclonal B cell activator, ISA, which induces suppressor splenic macrophages, suppresses the anti-SRBC response in resistant B10. A mice but not in A/J mice. ISA induced no change in prostaglandin E2 production by spleen cells of either strain. One interpretation of these findings is that A/J mice after stimulation by ISA or MPA, produce a substance which inhibits development of mononuclear phagocytes.

During listeriosis macrophages act as a site for bacterial replication, protect the host, and suppress humoral and cell-mediated immunity [1–3].

Two separable components from *Listeria* affect production and function of macrophages. A monocytosis-producing agent (MPA) induces a transient endogenous factor (EF) which increases proliferation of monocyte precursors in the bone marrow [4]. MPA-treated mice exhibit enhanced phagocytic activity but no immunosuppression. A polyclonal B cell activator suppresses the immune response in vivo [5] by inducing splenic macrophages (Otokunefor and Galsworthy, submitted).

The heterogeneity of macrophages has been reviewed [6]. We do not know whether the production of distinct subclasses of macrophages is regulated by common endogenous mediators. We approached this question by measuting the effect of MPA and ISA on macrophage function of inbred strains of mice.

Materials and methods

Bacterial extracts. MPA and ISA were prepared form *Listeria monocytogenes* serotype 1/2a as described [7].

Mice. Mice of the B10. A/SgSn and A/J were obtained from Jackson laboratoires, Bar Harbor, Maine, and used at 8 to 12 weeks of age.

SARA B. GALSWORTHY, S. TING, D. FEWSTER, A. PAVAN
Departmen of Microbiology and Immunology, University of Western Ontario
London, Ontario, Canada N6A 5C1

Table I

Effect of mixing fractions from MPA treated mice c.f.u.-m in bone marrow of B10.A mice

Substance injected	Addition to serum	c.f.u. m/10^4 Bone Marrow cells 12 h after injection; n = 12
B10.A-MPA S12	B10.A-PBS S12	
0.1 ml	nil	17.63±1.85
0.05 ml	0.05 ml	24.80±3.41
0.01 ml	0.09 ml	26.63±4.63
0.005 ml	0.095 ml	15.88±3.56
	0.10 ml	9.8±2.71
	A/J-PBS S12	
0.05 ml	0.05 ml	10.10±3.0
0.01 ml	0.09 ml	10.62±2.98
0.005 ml	0.095 ml	12.50±2.98
nil	0.10 ml	2.62±1.19
	A/J-MPA S12	
0.05 ml	0.05 ml	5.12±1.72
0.01 ml	0.09 ml	4.5±1.9
0.005 ml	0.095 ml	2.9±1.6
nil	0.10 ml	2.9±1.1

Preparation of serum samples. Mice were injected intraperitoneally with 0.5 mg MPA or phosphate-buffered saline (PBS). At 12 h after the injection mice were anaesthetized with ether and blood was obtained by cardiac puncture [4]. Serum from MPA treated mice was designated B10. A MPA S12 or A/J-MPA-S-12 and serum from PBS-injected mice B.10.A PBS S12 or A/J-PBS-S-12.

Measurement of c.f.u. Mice were injected intraperitoneally with 0.5 mg MPA in PBS or PBS, or intravenously with 0.1 ml serum. Bone marrow cells were obtained 24 h after injection of MPA or 12 h after injection of serum and cultured for 7 days in growth medium as described [8]. At the end of the incubation period the cultures were rinsed, and stained by Giemsa's method and the number of macrophage colonies counted as described [8].

Measurement of immune reponse. The in vivo immune response to SRBC was assessed by measuring the number of IgM plaque-forming cells (p.f.c.) in spleens 4 days after immunization using the Cunningham slide modification [9] of the Jerne plaque assay technique [10].

Measurement of prostaglandin synthesis in splenocyte cultures. Splenocytes (3×10^6 cells/ml) were cultured at 37 °C as described [5] with the following modification. Standard culture medium was supplemented with FCS which was treated with activated charcoal to remove endogenous prostaglandins, centrifuged for 30 min to remove the charcoal, and sterilized by filtration. Quadruplicate cultures were used for each experimental point. At the appropriate time, incubation mixtures were centrifuged at 1700 *g* for 10 min and the amount of PGE-2 in supernatant was determined by radioimmunoassay [11].

Results

Results are shown in Table I and Figs 1 and 2. We tested for the presence of an inhibitor in sera of A/J mice. The results, in Table I, suggest that an inhibitory factor is present in even B10.A-MPA-S12 since dilution in normal se-

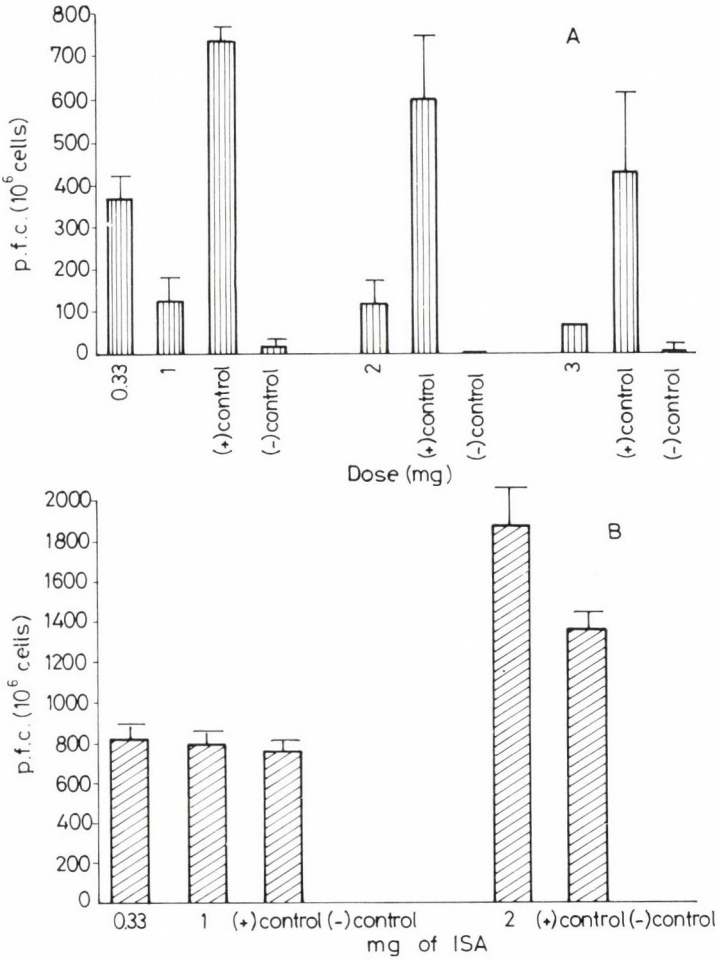

Fig. 1. Effect of ISA on the anti-SRBC response. Mice (4 per group) were injected intraperitoneally with the indicated dose of ISA and immunized with 2×10^8 SRBC 24 h later. The number of SRBC plaque forming cells was determined 96 h after immunization. Panel A: B10. A mice; B: A/J mice

rum enhances activity. Sera from untreated A/J mice decreased the activity of B10.A-MPA-S-12 and also suppresses the baseline response of B10.A mice. Sera from MPA-treated A/J mice caused an even greater inhibiton. Treatment of mice with ISA 24 h prior to immunization suppressed immune responses to SRBC in B10. A but not in A/J mice. Treatment of B10.A or A/J mice with suppressive doses of ISA did not alter the subsequent pattern of PGE-2 production by splenocytes. Indomethacin failed to alter suppression (or lack of it) in either strain (results not shown). These results are consistent with the notion that suppression is not mediated by PGE-2.

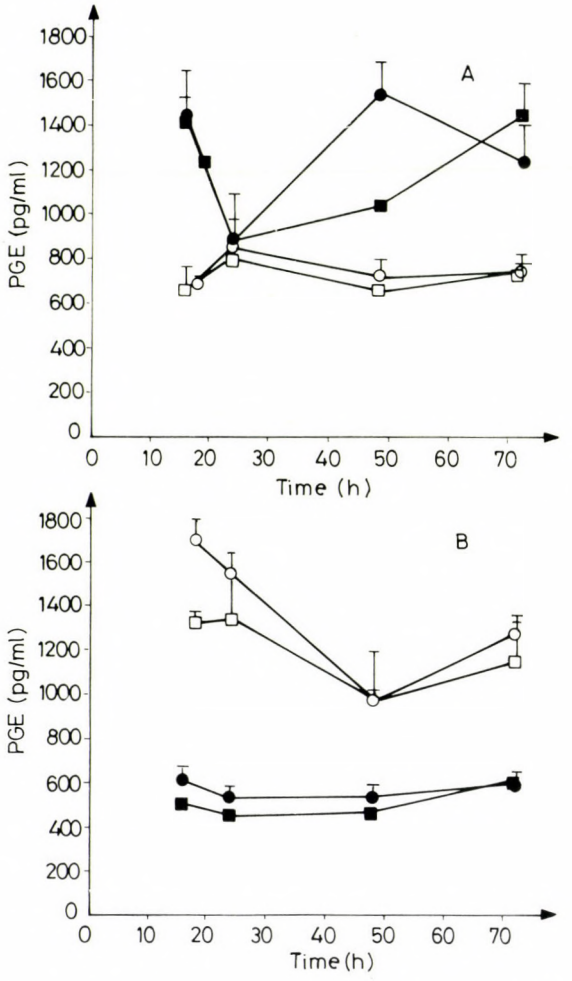

Fig. 2. Prostaglandin production in splenocyte cultures. Mice were injected with ISA (1 mg intraperitoneally) or PBS. Twenty-four hours after splenocytes were placed in culture medium. Supernatant fluids were obtained from cultures at the times indicated, pooled and PGE-2 levels measured by radioimmunoassay. Values represent the mean for 4 cultures ± standard deviation. O— —O ISA-treated mice, stripped FCS; △— —△ PBS-treated mice, stripped FCS; ●— —● ISA-treated mice, normal FCS; ▲— —▲ PBS-treated mice, normal FCS. Panel A: A/J mice; B: B10. A mice

Discussion

MPA causes increased production of macrophages which do not suppress the immune response. We have shown elsewhere that MPA acts by stimulating production of an endogenous factor, EF, which in turn enhances replication of macrophage precursors in the bone marrow [4, Fewster and Galsworthy,

submitted]. We have also shown that A/J mice are defective in their ability to produce or respond to EF. The present results suggest that A/J mice possess a humoral factor which abrogates the activity of EF and that injection of MPA increases the production of the inhibitory factor.

ISA induces suppressor macrophages in the spleen. ISA suppressed the -SRBC response in B10.A but not in A/J mice. In fact, the response was enhanced under these circumstances, possibly due to the B cell mitogenic effect of this preparation. Others have attributed to prostaglandins a role in the macrophage-mediated suppression induced by immune complexes [12] or by infection with *Listeria* [13]. We were unable to demonstrate any suppressor factor in supernatants of spleen cell cultures from ISA-treated mice (Otokunefor, unpublished). We found that levels of PGE-2 produced by spleen cells from B10.A and A/J mice were comparable and were not altered by pretreatment of mice with high doses of ISA. Pretreatment of mice with indomethacin did not alter the anti-SRBC response in either strain or the effect of ISA. We conclude that suppression is not mediated by PGE-2.

These results suggest that failure of A/J mice to develop suppressor macrophages after ISA treatment and their failure to enhance monocytopoiesis after MPA treatment may be due to a common inhibitory factor.

REFERENCES

1. Kongshavn, P. A. L., Ho, A., Sebaldt, R. J.: Immunology **28,** 284 (1977).
2. North, R. A.: J Exp Med **138,** 342 (1973).
3. Takeya, K., Shimotori, S., Taniguchi, T., Nomoto, K.: J Gen Microbiol **100,** 373 (1977).
4. Shum, D. T., Galsworthy, S. B.: Immunology **46,** 343 (1982).
5. Otokunefor, T. V., Galsworthy, S. B.: Can J Microbiol **28,** 1373 (1982).
6. Unanue, E. R., Rosenthal, A. S.: In Unanue, E. R. (ed.): Macrophage Regulation of Immunity. 1980. pp. 73–86.
7. Galsworthy, S. B.: J Clin Invest Med **7,** 223 (1984).
8. Stewart, C. C., Yen, S., Senior, R. M.: Manual of Macrophage Methodology: Collection, Characterization, and Function. Marcel Dekker, New York 1980. p. 290.
9. Cunningham, A. J., Szenberg, Z.: Immunology **14,** 599 (1968).
10. Jerne, N. K., Nordin, A. A.: Science **140,** 405 (1963).
11. Evans, C. A., Kennedy, T. G., Patrick, J. E., Challis, J. R. G.: Endocrinology **109,** 1533 (1981).
12. Virgin, H. W., IV, Wittenberg, G. F., Bancroft, G. J., Unanue, E. R.: Infect Immun **50,** 343 (1985).
13. Petit, J-C., Richard, C., Burghoffer, B., Daguel, C-L.: Infect Immun **49,** 383 (1985).

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CELLULAR IMMUNE RESPONSE TO *LISTERIA* IN GENETICALLY RESISTANT AND SUSCEPTIBLE MOUSE STRAINS

WIESLAWA RUDNICKA, H. DŁUGOŃSKA, M. CHMIELA, B. PAZIAK-DOMAŃSKA and A. WIEWIÓRA

Department of Immunology, University of Łódź, Łódź, Poland

Relatively *Listeria*-resistant C57Bl/6 mice and more susceptible to the infection DBA/2 mice were immunized with *Listeria*-antigen (LA). Immunized DBA/2 mice developed weaker delayed hypersensitivity to LA and still eliminated listeriae less effectively than identically immunized C57Bl/6 mice. An accessory function of LA-pulsed macrophages of normal C57Bl/6 mice was only slightly enhanced as compared with LA-pulsed macrophages of DBA/2 mice. It is suggested that some suppressor lymphocytes, IgM^+ and/or FcR^+, could be responsible for the enhanced susceptibility of DBA/2 mice to listeriosis.

Greater anti-listerial resistance of C57Bl/6 mice as compared with DBA/2 mice is associated with an increased accumulation of inflammatory macrophages and neutrophils in their peritonea and an increased capacity of their neutrophils to restrict listeriae growth [1]. On the other hand innately resistant C57Bl/6 mice have in their spleens a higher number of B cells binding *Listeria* antigen (LA) than more susceptible DBA/2 mice [2]. It has been suggested that some antigen specific mechanisms can be also involved in an enhanced resistance of C57Bl/6 mice to listeriosis. The present work was designated to compare the effect of immunization with LA on the expression of anti-listerial resistance of C57Bl/6 and DBA/2 mice and the development of delayed hypersensitivity to LA. An accessory function of the macrophages of immunized mice and the proliferation of their spleen cells in the presence of LA was also evaluated.

Materials and methods

Strains and antigens. An avirulent Welshimer's strain of *Listeria ioncua* and a virulent strain of *Listeria monocytogenes* were used. The bacteria were grown overnight at 37 °C in trypticase soy broth supplemented with vitamin B. A soluble fraction of Welshimer's strain was obtained by the method of Woan et al. [3].

Mice, immunization and protection. Inbred C57Bl/6 and DBA/2 male mice 2–4 months old were used. The average lethal dose of virulent *L. monocytogenes* was 10^7 organism for normal C57Bl/6 mice and 10^5 for DBA/2 mice. LA (50 μg emulsified with incomplete Freund's adjuvant) was subcutaneously injected into the forefeet and both sides. Six days later normal and immunized mice were intraperitoneally infected with virulent *L. monocytogenes*. The numbers of live bacteria in the spleens were estimated [1].

WIESLAWA RUDNICKA, H. DŁUGOŃSKA, M. CHMIELA, B. PAZIAK-DOMAŃSKA, A. WIEWIÓRA
Department of Immunology, University of Łódź
Banacha 12/16, 90-237 Łódź, Poland

Delayed hypersensitivity. Delayed reaction was elicited on the 6th day of immunization by injecting 50 μg of LA in 0.02 ml of saline into a hind footpad. The footpad thickness was measured with dial gauge calipers before and after (24 h, 48 h) injection.

Spleen cells proliferation. Spleen cells were harvested from the mice of the 6th day of immunization. They were cultured in Medium 199 containing 0.2–100 μg of LA/ml and supplemented with 8% fetal calf serum, 2% mouse serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 20 mM HEPES, 100 U penicillin, 100 μg streptomycin per ml. The cells (10^6/well) were cultured in 96-well microtiter plates (Falcon) for 3 or 4 days with the addition of 0.5 μCi of ^{3}H-thymidine on the 2nd or 3th day. The incorporation of ^{3}H-thymidine was determined by using a scintillation counter. Spleen cells were fractionated in the following way. Incubation of the cells in Petri dishes (Greiner) for 1.5 h at 37 °C, yielded plastic non-adherent cells. A part of such cells was additionally purified over a nylon wool column according to the method of Julius et al. [4]. Another part of plastic non-adherent cells was incubated for 2 h at 37 °C in Petri dishes (Greiner) coated with rabbit IgG antibodies, anti-mouse IgM (100 μg/ml, 24 h at 4 °C) — anti mouse IgM coated plastic non-adherent cells.

Accessory function of macrophages. Peritoneal adherent macrophages (1×10^4) from the mice being 5 days previously intraperitoneally injected with 10 μg of Con. A were dispensed in flat-bottomed 96-well microtiter plates (Falcon), pretreated with LA (100 μg/ml) for 1.5 h and then washed prior to addition of T cells. Lymph node cells obtained from the mice 6 days after immunization, were depleted in plastic adherent cells and then purified over a nylon wool column — T immune cells. 1×10^6 of T cells were added to each well containing LA-pulsed macrophages. The cells were cultured for 3 days, with the addition of 0.5 μCi of ^{3}H-thymidine on the 2nd day.

Results

Six days immunization with 50 μg of LA with incomplete Freund's adjuvant enhanced the elimination of *L. monocytogenes* from the spleen of both listeria-resistant C57Bl/6 and listeria-susceptible DBA/2 mice (Table I). However, immunized C57Bl/6 mice eliminated *L. monocytogenes* more effectively than immunized DBA/2 mice.

Immunized DBA/2 mice developed weaker delayed reaction than identically immunized C57Bl/6 mice (Table II). The accessory function of the macrophages of immunized DBA/2 mice was also slightly decreased as compared with the macrophages of C57Bl/6 mice (Table III).

There was not any difference in the proliferation of the spleen cells from immunized C57Bl/6 and DBA/2 mice if they were cultured during 3 days in

Table I

The effect of immunization on the elimination of L. monocytogenes from the spleens

Mice	Priming	Challenge	Number of bacteria $\times10^3$ spleen	
			48 h	96 h
C57Bl/6	none	*Listeria*	51.0±16.0	48.9±11.9
C57Bl/6	LA	virulent (10^5)	5.9±2.6	2.3±0.3
DBA/2	none	*Listeria*	50.1±15.9	399.7±250.6
DBA/2	LA	virulent (10^4)	9.0±0.6	130.5±81.2

Mice were immunized with 50 μg of LA with incomplete Freund's adjuvant; 6 days later they were i.p. infected with *L. monocytogenes*

Table II

Delayed hypersensitivity to LA

Mice	Footpad thickness (mm)	
	24 h	48 h
C57B1/6	1.01±0.22	0.52±0.28
DBA/2	0.64±0.12	0.24±0.08

Mice were immunized with 50 μg of LA with imcomplete Freund's adjuvant; 6 days later they were challenged with 50 μg of LA

Table III

Accessory function of the macrophages

Cells	cpm×10²/culture	
	C57B1/6	DBA/2
LA-pulsed macrophages and T immune lymphocytes	184.3±25.4	116,6±10.5
Non-pulsed macrophages and T immune lymphocytes	18.8±1.8	14.4±4.6
Non-pulsed macrophages and T immune lymphocytes and LA	328.8±22.9	241.8±10.9

Peritoneal macrophages (1×10^4) from normal mice were pulsed with LA (100 μg/ml) and the cultures with lymph node T cells (1×10^6) from the mice immunized with LA 6 days previously

the presence of LA. The response was dose dependent and the optimal concentration of the antigen was between 20–100 μg/ml (Fig. 1). However, in 4 days cultures the cells of C57B1/6 mice still responded intensively to such doses of

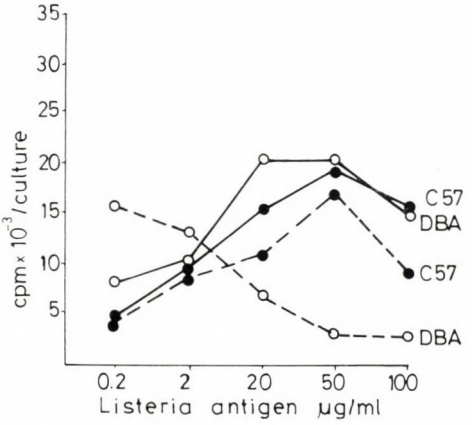

Fig. 1. Proliferation of immune spleen cells in the presence of LA. Spleen cells from LA immunized mice were cultured for 3 (———) or 4 (— — —) days in the medium containing LA

Table IV

Proliferation of immune spleen cells of DBA/2 mice in 4 days cultures

Cells	cpm × 10^2/culture	
	LA	non LA
Unfractionated	7.4±0.6	18.3±1.0
Plastic non-adherent	22.4±1.8	11.4±6.9
Anti-mouse IgM coated plastic non-adherent	74.9±9.6	37.6±11.5
Nylon wool non-adherent	163.9±20.9	4.8±1.9

The spleen cells were obtained from DBA/2 mice 6 days after immunization with 10^3 *L. inocua cells*. They were cultured for 4 days in the medium containing 20 μg of LA/ml

LA while the proliferation of the cells of DBA/2 mice was inhibited. The inhibition of the proliferation of the spleen cells of immunized DBA/2 mice was probably caused by the nylon wool adherent cells because removing of such cells restored the ability of DBA/2 spleen cells to respond well to 20 μg of LA/ml (Table IV). The reaction of DBA/2 spleen cells to this dose of LA was also increased after removing the cells adhesive to the plastic coated with rabbit IgG antibodies anti mouse IgM.

Discussion

The results showed that innate difference in anti-listerial resistance between C57B1/6 and DBA/2 mice was maintained after immunizing them with soluble LA. Also the delayed hypersensitivity to LA, considered as indispensable for the appearence of acquired anti-listerial resistance [5], was weaker in DBA/2 mice. It can be explained by a tendency of DBA/2 mice to switch on suppressor mechanisms, specially in the presence of higher doses of LA. The suppressor cells were among the nylon wool adherent lymphocytes, IgM^+ and/or FcR^+. An increase in the number of FcR^+ cells in the tissues of *Listeria* injected susceptible to listeriosis Balb/c mice was demonstrated [6].

REFERENCES

1. Długońska, H., Chmiela, M., Kierońska, D., Rózalska, B., Rudnicka, W.: Arch Immunol Ther Exp **36,** 33 (1987).
2. Rudnicka, W., Chmiela, M., Długońska, H.: Immunology Letters **11,** 57 (1985).
3. Woan, M. C., Forsum, U. K., McGregor, D. D.: Infect Immun **29,** 1102 (1980).
4. Julius, M. H., Simpson, E., Herzenberg, L. A.: Eur J Immunol **3,** 645 (1973).
5. Kaufmann, S. H. E., Simon, M. M., Hahn, H.: J Exp Med **150,** 1033 (1979).
6. Gościcka, T., Walencka, M.: Arch Immunol Ther Exp **34,** 489 (1986).

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THE IMMUNOLOGICAL PROPERTIES OF LISTERIAL COMPLEX EI

M. MÁRA, K. NOUZA, C. JOHN, J. PRŮCHOVÁ, J. OČENÁŠKOVÁ, H. ŠESTÁKOVÁ, J. HŘIBALOVÁ and E. MENČÍKOVÁ

Institute of Medical Microbiology and Immunology, Charles University School of Medicine, ÚEM ČSAV, Prague, SÚKL, Prague, and IHE, Prague, Czechoslovakia

Factor Ei, besides exerting toxic reactions, is amphipathic, antigenic, chemotaxinogenic, causes blastic transformation, adjuvant effect, hypersensitivity (MIF, skin test), activates the RES (splenomegaly), increases the macrophage production, prevents listerial infection in mice, mycobacterial infection in guinea pigs and enhances the effect of BCG experimental tuberculosis and neoplasia in mice (Sa 180).

Studying the pathogenicity of *Listeria monocytogenes* we used the modification of Ribi's method for the preparation of crude endotoxin [1]. The same preparation elaborated by Srivastava and Siddique [2] was used one year later. The preparation made by the ether-water method and finished by precipitation of the active agent by adjustment of pH to 3.6 and redissolution of the precipitate in phosphate buffer pH 7.2 was designated factor Ei. The advantage of this factor was that it was free of viable cells, relatively well soluble in water solvents and due to its complex nature it comprised all biological qualities characteristic of *Listeria* which we describe in this communication. A disadvantage of this factor was that it was not stable after lyophilization or longer storage in the frozen state. The complex is made up of proteins, lipids and polysaccharides [3].

Factor Ei causes erythema and oedema in the skin of rabbits, is pyrogenic [4], highly active (10^{-9} g) in coagulation of haemolymph of *Limulus polyphenus*, [5], it is not lethal, but kills mice with sublethal doses of actinomycine D [1], causes a local Shwartzman reaction but only after intravenous induction with LPS [4], enhances the infection of *L. monocytogenes* virulent strains in mice [4], causes monocytosis, leucocytosis and lymphocytosis in mice after intraperitoneal infection [6], is cytopathogenic to human embryonal fibroblasts [7], after intravenous administration causes hyperaemia, haemorrhagia

M. MÁRA, C. JOHN, J. OČENÁŠKOVÁ, H. ŠESTÁKOVÁ, E. MENČÍKOVÁ
Institute of Medical Microbiology and Immunology, Charles University School of Medicine
Studničkova 7, Prague 2, Czechoslovakia

K. NOUZA
ÚEM ČSAV, Prague, Czechoslovakia

J. PRUCHOVÁ
SÚKL, Prague, Czechoslovakia

J. HRIBALOVÁ
IHE, Prague, Czechoslovakia

and death of chick embryos [7], encephalitis in neonatal mice, potentiation of erythrogenic toxin in rabbits and damages erythrocytes.

Factor Ei is antigenic and precipitates with antibacterial sera and anti Ei sera. It is amphipatic [1], it has an adjuvant capacity with sheep red blood cells (SRBC), is polyclonally active using SRBC, FITC, TMP [8], causes delayed hypersensitivity detected by skin test in rabbits and guinea pigs and by MIF in mice, rabbits and guinea pigs [9].

Materials and methods

Factor Ei was prepared from *L. monocytogenes* by ether–water extraction and redissolution of the precipitate at pH 3.6 from the aqueous phase.

Results and discussion

Here we wish demonstrate some important and newly recognized properties of this complex. First of all we observed that the administration of factor Ei three to one day before infection causes prevention of lethal effect of virulent strain of *L. monocytogenes* in mice. The simultaneous or postinfection application is without effect (Table I) [4].

We determined the capability of the factor to inhibit the production of cytochrome P-450 in the liver microsomal fraction in mice. This reaction evoked within a few hours with a maximum after 24 h was determined by the method of Omura and Sato [10]. The sensitivity of the reaction is smaller in comparison with classical endotoxin. For the reaction 10^{-3} g of factor Ei is necessary (endotoxin is active up to 10^{-7} g). The factor activates the monocyte-macrophage system in mice determined by splenomegaly after intraperitoneal administration of 0.5 mg of the complex. Hepatomegaly was not observed (Fig. 1).

Factor Ei causes spontaneous and chemotaxigenic activity of polymorphonuclear leucocytes in comparison with *Escherichia coli* endotoxin. The

Table I

Prevention of L. monocytogenes infection by factor Ei

Days of administrarion of Ei	No. of mice	No. of deaths
3	5	1
2	5	0
1	5	1
0	5	5
Infection only	5	4

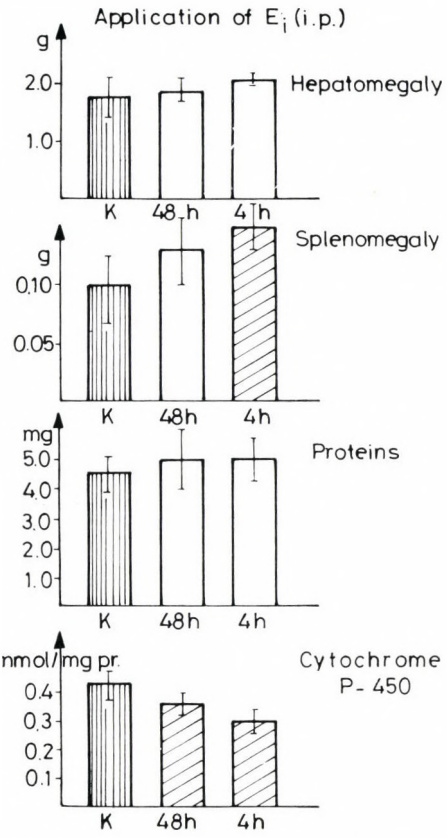

Fig. 1. Activation of RES and inhibition of cytochrome P-450 in mouse liver. Application of Ei factor intraperitoneally

concentration of endotoxin and listerial factor Ei was 240 μg/ml and the concentration of polymorphonuclear leucocytes was 2×10^7/ml (Fig. 2) [11].

Factor Ei causes the mitogenicity of human peripheral blood cells (Fig. 3).

Factor Ei increases prolipherative response of guinea pig thymocytes with phytohaemagglutinin (PHA) or concavaline A (Con A). Comitogenic effect is qualitatively and quantitatively comparable with the effect of MDP (Fig. 4).

The simultaneous administration of the factor Ei with ethanol extracted lipids of BCG or BCG vaccine enhances significantly their immunogenicity determined by spleen weight and Feldman index calculated according to the number of the tuberculous changes in some organs [12, 13]. This model was elaborated on guinea pigs and represents the immunogenic form of experimental tuberculosis which is used for determining the efficacy of BCG vaccines [14].

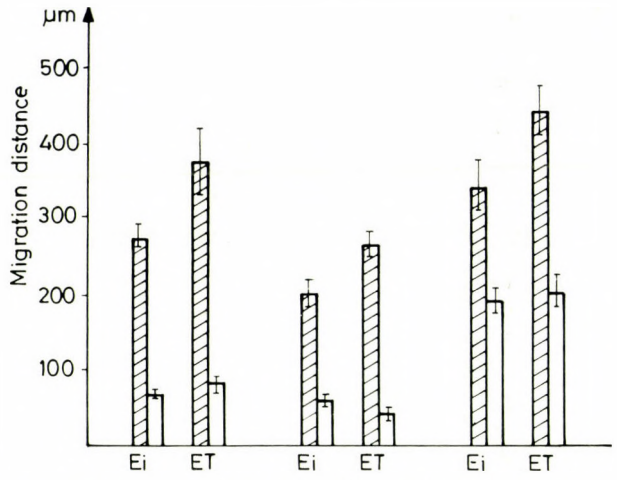

Fig. 2. Chemotaxigenic effect of LPS *E. coli* (ET) and listerial factor Ei. Shaded columns, endotoxin or factor Ei; open columns, control

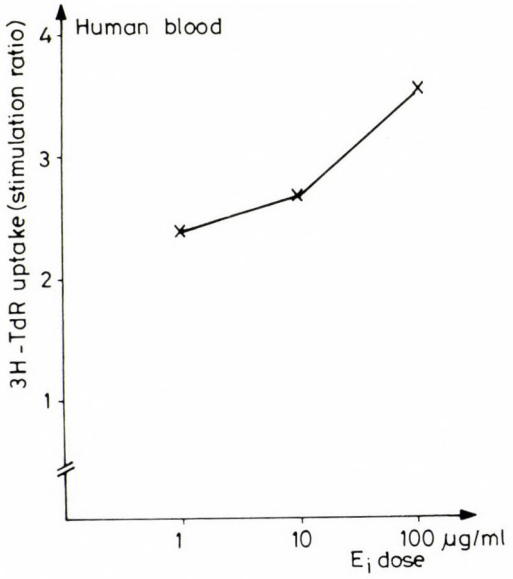

Fig. 3. Blast transformation of human blood lymphocyte

In the same manner factor Ei potenciates the stimulating effect of BCG to protect mice to transplantation of sarcoma 180 (Fig. 5). The factor administered one day after transplantation increased the survival time of mice. The combination of Ei with BCG vaccine is preferable too (Fig. 6) [15].

Using a special model of multiple immunoassay (MIA), factor Ei was tested for delayed hypersensitivity (DTH), the amount of IgG and IgM anti-

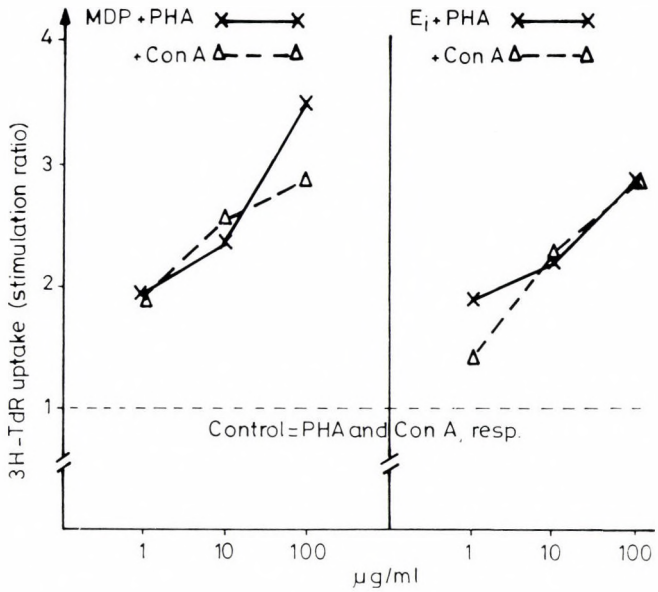

Fig. 4. Comitogenic effect of guinea pig thymocyte

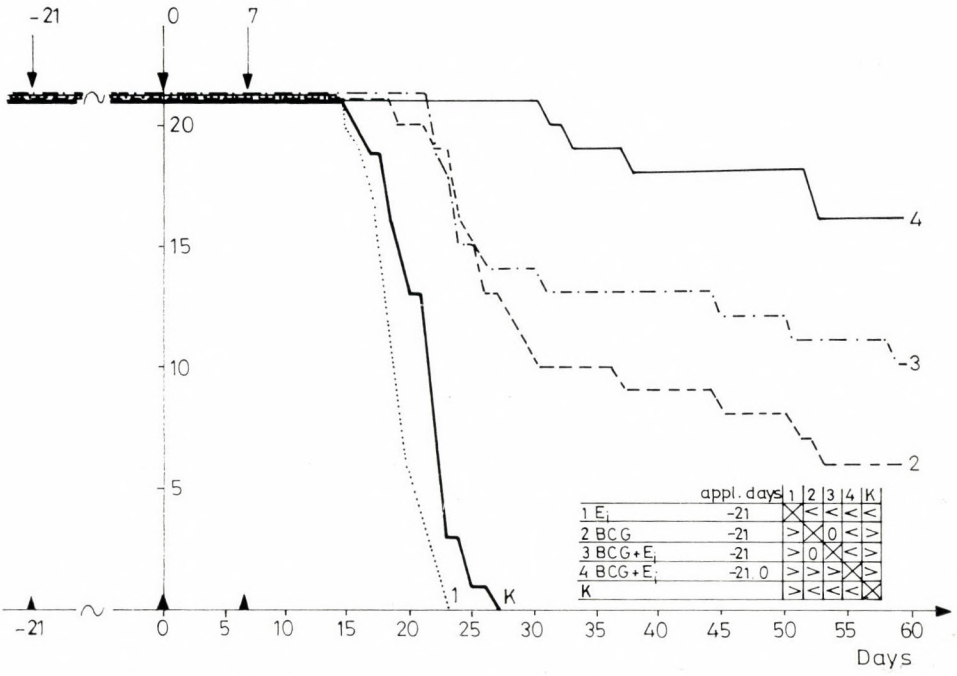

Fig. 5. Immunostimulation of mice against sarcoma 180

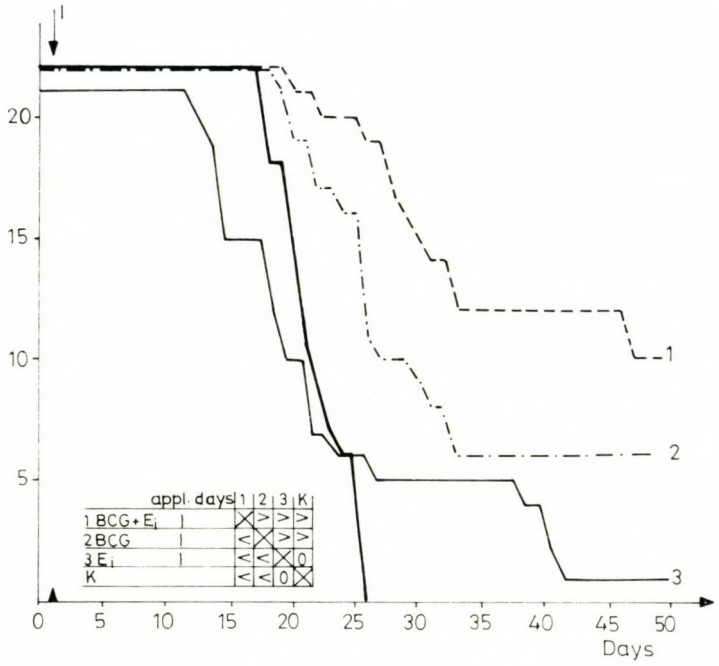

Fig 6. Immunotherapy of mice against sarcoma 180

bodies against SRBC, phagocytosis, blast transformations with PHA, Con A and LPS, and increase of weight of thymus, spleen and lymph nodes [16].

Factor Ei evokes detectable hypersensitivity after 8 days. Increases the amount of anti-SRBC antibodies especially after repeated (4×) application. It enhances phagocytosis significantly and causes spleno- and thymomegaly. After immunosuppression caused by antithymus monoclonal antibodies, cyclophosphamide (CY), dexamethazone (DX) or irradiation factor Ei restores the destroyed immunity especially in the field of DTH, antibody production, phagocytosis and splenomegaly. The negative effect on blast transformation is reversed in the case of restoration after artificial immunosuppression. The effect of chemical immunosuppressives is more easily restored by the factor Ei in the case of transformation and increase of the weight of organs and in the case of phagooytosis. The restoration of immunity altered by monoclonal antibodies or irradiation, using factor Ei only was observed in DTH, antibody production and phagocytosis.

REFERENCES

1. Patočka, F., Mára, M.: J Hyg Epidemiol Microbiol Immunol **17,** 457 (1973).
2. Mára, M., Patočka, F., Julák, J., Potužníková, B.: J Hyg Epidemiol Microbiol Immunol **18,** 29 (1974).

3. Mára, M., Julák, J., Patočka, F.: J Hyg Epidemiol Microbiol Immunol **18,** 359 (1974).
4. Patočka, F., Mára, M., Potužníková, B., Jirásek, A.: J Hyg Epidemiol Microbiol Immunol **18,** 365 (1974).
5. Průchová, J., Mára, M., Meisel-Mikolajczyk, F.: Čs Epidemiol **28,** 130 (1979).
6. Mára, M., Menčíková, E., Julák, J.: Čs Epidemiol **23,** 257 (1974).
7. Mára, M., Menčíková, E., Julák, J., Meisel-Mikolajczyk, F., Průchová, J., Hofman, J., Kotelko, K.: Abstracts on International Symposium "Biological Active Products of Microorganisms in Pathogenesis of Human Diseases". Prague-Liblice 1978. p. 44.
8. Hofman, J., Pospíšil, M., Mára, M., Hříbalová, V.: Folia Microbiol **30,** 231 (1985).
9. John, J., Sprátková, I., Morávková, M., Patočka, F., Mára, M.: J Hyg Epidemiol Microbiol Immunol **18,** 369 (1974).
10. Omura, T., Sato, R.: J Biol Chem **239,** 2370 (1964).
11. John, C., Šestáková, H., Mára, M.: Folia Microbiol **30,** 224 (1985).
12. Mára, M., Průchová, J., John, C., Šmerák, P., Šir, Z.: Folia Microbiol **26,** 52 (1981).
13. Mára, M., Průchová, J., Šmerák, P., John, C., Miková, Z., Nedvědová, V., Šír, Z.: International Symposium on BCG Vaccines and Tuberculins, Budapest, Hungary, 1983. Develop Biol Standard 38. S. Karger, Basel 1986. p. 379.
14. Průchová, J., Hejdová, E.: Symposium Ser Immunbiol Standards **17,** 251 (1971).
15. Mára, M., Nedvědová, V., Miková, Z., Hynčica, V.: Neoplasma **26,** 703 (1979).
16. Nouza, K. et al. 1987 In press.

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Round Table Discussions

INTRODUCTION

METHODS OF ISOLATION, CHARACTERIZATION AND CONTROL OF *LISTERIA*

During the Tenth International Symposium on Listeriosis in Pécs, Hungary (August 22–26, 1988), two discussion sessions were held on methods of isolation, characterization and control of *Listeria* (August, 21 and 25, 1988). The intent of the round tables was to arrive at a consensus of the status of knowledge in these fields. During the sessions 45 presentations were given by leading scientists representing 10 countries. They were asked to present the latest information available on the detection, enumeration, identification and control of *Listeria monocytogenes*. Submitted abstracts of the papers of invited speakers will be published here, together with the various elements of analytical methodology for *L. monocytogenes*. Full texts of these subjects have been published in International Journal of Food Microbiology.

In a plenar session the conclusions were discussed in detail by the Scientific Committee of the Symposium.

ABSTRACTS

THE USDA FOOD SAFETY AND INSPECTION SERVICE METHOD FOR THE RECOVERY OF *LISTERIA MONOCYTOGENES*

W. H. Lee

USDA, FSIS, Beltsville, Maryland, USA

The USDA, FSIS method was developed originally for recovery of low numbers of naturally occurring *Listeria monocytogenes* from raw meat, and it performs well even when large numbers of microbial competitors are present. Other workers have extended its use to other types of food and to environmental sampling in food plant quality control programs. Use of a two-stage enrichment procedure, which was 42% better than a single stage, highly selective

LPM agar, and thin-layer horse blood agar plates for the detection of surface--streaked beta-haemolytic colonies are the important steps of this method. *L. monocytogenes* can be presumptively identified in 3–4 days. We have detected it in 39/86 raw meat samples. For regulatory purposes, our Agency tests cooked meat for the presence of *L. monocytogenes* at point of production by culturing a 10 ml portion (one gram) of a 1 : 10 dilution of blended sample. The method has been shown to be effective. Other laboratories, as well as my own, routinely use direct plating of decimal dilutions onto LPM agar to detect high levels ($>$ 100) of *L. monocytogenes*. Most of the plates will be blank, indicating absence of high numbers, but rarely a nearly pure lawn of *Listeria* colonies can be seen (with magnification) in 24 h and counted in 48 h. However, LPM is a very selective medium, and inhibits injured cells. The latter require resuscitation in non-selective media.

SELECTIVE AND DIAGNOSTIC ENUMERATION OF LOW NUMBERS OF *LISTERIA* SPP. AT 30 °C

P. VAN NETTEN, I. PERALES and D. A. A. MOSSEL

Governmental Food Inspection Service, 's-Hertogenbosch, The Netherlands, Health Service Direction of Vizcaya, Bilbao, Spain, and Department of Science of Food of Animal Origin, Faculty of Veterinary Medicine, The University of Utrecht, The Nethdrlands

A selective-diagnostic *Listeria* enrichment broth named L-ALCEFTY has been elaborated. The medium allows reliable enrichment at 30 °C. It is based on sugar-free peptone yeast extract broth with 2.5% egg yolk emulsion. The diagnostic traits of this medium were attained by incorporation of aesculin plus ferreous salt and mannitol plus phenol red. Selective agents were 0.005% ceftazidime, 0.001% acriflavine and 1% lithium chloride. L-ALCEFTY resulted to (i) a productivity ratio; and (ii) growth rate which was of the same order as attained in Colombia broth. The growth of virtually all other bacteria of common occurrence in fresh foods was inhibited.

SELECTIVE AND DIAGNOSTIC ENUMERATION OF *LISTERIA* SPP. FROM HEAVILY CONTAMINATED FOODS

P. VAN NETTEN, A. VAN DE MOOSDIJK, I. PERALES and D. A. A. MOSSEL

Governmental Food Inspection Service, 's-Hertogenbosch, The Netherlands; Health Service Direction of Vizcaya, Bilbao, Spain, and Department of Science of Food of Animal Origin, Faculty of Veterinary Medicine, The University of Utrecht, The Netherlands

A new medium called ALPAMY agar was elaborated for the isolation of *Listeria* spp. and their enumeration. ALPAMY is based on Colombia agar with 2.5%egg yolk emulsion, made selective by the addition of 0.001% acriflavine, 1.5% lithium chloride and 0.25% 2-phenyl ethanol and given diagnostic traits by the incorporation of 0.05% aesculin plus 0.05% ferrous salt and 1% mannitol plus 0.008% phenol red. ALPAMY medium recovered *Listeria monocytogenes* and some strains of *Listeria seeligeri* quantitatively, whereas suppressing virtually all other bacteria of common occurrence in fresh foods. *Listeria* colonies were approximately 2 mm, dark-grey with a black centre on a cherry red background (aesculin-positive, mannitol-negative).

A REVIEW OF CURRENT METHODS USED IN THE UNITED STATES FOR ISOLATING *LISTERIA* FROM FOOD

D. J. PUSCH

Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota, USA

The two methods most commonly used in the United States for detecting *Listeria* in food have been developed by the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA), the two federal agencies which regulate the nation's food supply. These methods were developed to shorten the time of recovery over the traditional cold enrichment technique. Although the two procedures use different media, both make use of selective enrichments, streaking on selective media, purifying on a non-selective medium and final identification using physical, biochemical and serological tests. Recently two rapid methods (results within three days) have become commercially available. One employs a nucleic acid hybridization technique which uses a *Listeria*-specific probe labelled with a radioisotope. The other

procedure utilizes monoclonal antibodies and an enzyme-linked immunosorbent assay (ELISA). Both of these rapid tests are said to be specific for all species and serotypes of *Listeria*. Therefore, any positive results must be confirmed using conventional procedures to determine whether the results were due to a pathogenic or non-pathogenic *Listeria* species or a false positive.

METHODS TO CULTIVATE AND ISOLATE *LISTERIA* STRAINS

B. Ralovich

Institute of Public Health and Epidemiology, University Medical School, Pécs, Hungary

Cultivation and isolation of listeriae from contaminated materials — faeces, foods, feeds, environmental samples, pathological materials — are not simple tasks. The procedure needs special efforts to promote growth of *Listeria* strains and to inhibit that of the concomitant bacteria. For this purpose the following techniques and substances were proposed: cold enrichment, oblique light technique, tellurite, thioglicollate, guanofuracin, NaCl, furacin, thiocyanate, polymyxin B, thallous acetate, acridine dyes, nalidixic acid, cyclohemixide, dichromate, chromic acid, thionine, Tween 80, azide, chloramphenicol, glycine, lithium chloride, viomycin, gallocyanine, pyronine, phenyl ethanol, glycine anhydride, moxalactam, oxolinic acid, colistin, ferric ammonium citrate, indigocarmine, tartrazine, propolis and ceftazidime. There is no official method to control foods for listeriae. Usefulness of Levinthal broth, Holman medium, TNSA plate McBride agar, RAPAMY medium, Rodriguez's procedure, UVM enrichment broths and LPM agar will be discussed.

SEROLOGICAL ANALYSIS OF THE GENUS *LISTERIA* (ITS VALUE AND LIMITATION)

H. P. R. Seeliger and B. Langer

Institute of Hygiene, Medical Faculty, University of Würzburg, FRG

A valid scheme for antigenic analysis of *Listeria monocytogenes* has been elaborated by Paterson and was later extended by Seeliger and Donker-Voet. It is, however, not possible to propose a serological differentiaton between pathogenic *L. monocytogenes* and non-pathogenic *Listeria seeligeri*, as they are indistinguishable and both are beta-haemolytic. The H-antigens of *L. mono-*

cytogenes and the more closely related species offer a high degree of generic specificity. The usefulness of antigenic analysis of listeriae lies in its value to confirm suspected listeriae and to help in their classification on the species level. Although antigenic identity would be a good indicator for possible epidemiological links — at the best —, serological analysis of isolates may help to rule out supposed chains of infections instead of providing evidence for direct connections. The breakdown of apparently homogeneous serovars has been effected by recognition of numerous lysovars (phage types) composing each serovar of *L. monocytogenes*.

PHAGE-TYPING OF *LISTERIA* STRAINS

S. Ortel

Institute of Medical Microbiology and Epidemiology, Reference Laboratory for Listeriosis, Martin Luther University, Halle/S., GDR

The systematic search for *Listeria* phages (since 1977) and the Multi Centre Study (1982) gave the basis for typing *Listeria* strains. The results in the laboratories have shown that the number of typeable strains is different and fluctuates between 25–54% (serovar 1/2a) and 66–89% (serovar 4b) and often depends on geographical territories. *Listeria* strains from sheep and cattle were typeable (75% in 1/2a- and 85% in 4b-serovars) usually more frequently than strains from human beings. The experiences with phage-typing have as yet shown that this method is valuable in epidemiological work. But phage-typing is not always successful because the rate of non-typeable strains is sometimes too high so that epidemiological conclusions cannot be done. Therefore, it seems necessary to isolate new and better phages to complete the set and exclude phages which give only few reactions. Further problems in phage typing are the alteration of phage patterns, the decision whether strains or phagovars are identical, similar or different and if there are phage patterns which are specific for a definite illness.

LISTERIOCINS (MONOCINS)

S. Ortel

Institute of Medical Microbiology and Epidemiology, Reference Laboratory for Listeriosis, Martin Luther University, Halle/S., GDR

Listeriocins or monocins are substances which were isolated from *Listeria* and have an antibiotic effect against *Listeria monocytogenes*. Monocins were mostly isolated from non-lysogenic strains after UV or mitomycin induction.

Our results have shown that from altogether 162 strains of *L. monocytogenes* 123 (76%) were monocinogenic, serovar 4b produced in 94% and serovar 1/2a in 67% monocins. Serovar 5 is a good indicator strain for monocins isolated from 1/2a and 4b serovars. Our monocins were classified as A, B, C, D, E, F concerning their action on different indicator strains. The titres of monocins must be high to build up a typing scheme for *Listeria*.

ANALYSIS OF *LISTERIA MONOCYTOGENES* BY MULTILOCUS ENZYME ELECTROPHORESIS AND APPLICATION OF THE METHOD TO EPIDEMIOLOGIC INVESTIGATIONS

W. F. Bibb, B. Schwartz, B. Gellin, P. Plikaytis, and R. Weaver

Centers for Disease Control, Atlanta, Georgia, USA

We examined 310 strains of *Listeria monocytogenes* by multilocus enzyme electrophoresis. Fifty-six electrophoretic types (ETs) of the organism were defined. Ten ETs were defined among serovar 4b strains, 11 ETs among serovar 1/2b strains, and 30 ETs among serovar 1/2a strains. Strains of serovars 1/2c., 3a, 3b, and a non-typeable strain were distributed among the remaining five ETs. Mean genetic diversity of the species was 0.41. Principal coordinates analysis revealed a sharp division among ETs which divided the species into two major clusters. Ninety-seven per cent of all ETs representing serovar 1/2a strains (29 of 30) were in one cluster while all serovar 4b, 1/2b, and 3b ETs were in the second cluster. Except for two ETs which contained strains from both serovar 1/2b and 3b, no ET contained strains from more than one serovar. The method has proved useful in facilitating the analysis of epidemiologic data. In three separate epidemiologic investigations electrophoretic typing confirmed the probability of a common source outbreak, while in a fourth investigation a common source as a cause of an outbreak was effectively ruled out. It was also useful in documenting potential links between contaminated food items and patients who consumed those products.

IDENTIFICATION AND ENUMERATION OF VIRULENT *LISTERIA* STRAINS

A. DATTA and B. WENTZ

Division of Microbiology, CFSAN, Food and Drug Administration, Washington DC, USA

Recent outbreaks and the continuous increase in cases of listeriosis underscore the need for rapid, sensitive and reliable techniques to detect *Listeria*. Of the species of *Listeria*, only *Listeria monocytogenes* has been found to be associated with human and animal infections. One factor which definitely contributes to it's pathogenicity is the presence of haemolysins, though *Listeria ivanovii* and *Listeria seeligeri* also elaborate haemolysins. Based upon cloned haemolysin genes, we have developed DNA probes specifically for detecting *L. monocytogenes*. The technique combines growth of bacterial colonies on selective agar plates and DNA hybridization of these colonies on a solid matrix. This technique permits identification and enumeration and the entire procedure can be completed in 3–4 days. Our method was found to be suitable to identify and enumerate this organism in various foods, the main vehicle of human infection. Advantages and disadvantages of this technique will be discussed and compared with the other existing techniques.

A NEW NUCLEIC ACID PROBE ASSAY FOR *LISTERIA* IN FOODS AND FOOD PROCESSING ENVIRONMENTS

J. KLINGER, D. HALBERT, A. JOHNSON, M. KIMBALL, W. KING, D. LANE and J. LAWRIE

GENE-TRAK Systems, Framingham, Massachusetts, USA

Outbreaks of foodborne human listeriosis and discovery of listeria-contaminated food products in North America and Europe have increased the need for rapid and sensitive screening assays for the presence of *Listeria*. We have developed a hybridization assay which utilizes highly specific DNA oligomer probes to detect unique sequences of 16S ribosomal RNA present in all *Listeria* spp. The initial version of the assay included ^{32}P-labelled probes in a 4 ha filter hybridization format following approximately 48 h of cultural enrichment. Extensive comparisons have been made of standard procedures (e. g., FDA method) and the GENE-TRAK hybridization assay with environmental samples and uninoculated and inoculated dairy products. In the vast majority of cases the hybridization assay had a lower false negative rate than

the reference method, with an unconfirmed positive rate of approximately 3%. Results were equivalent to those of the USDA procedure when used to test meats or seafoods. Initial result susing a new non-isotopic (colorimetric) hybridization assay indicate the potential for even greater sensitivity.

DIAGNOSTIC IMPORTANCE OF NUCLEIC ACID PROBES AND MONOCLONAL ANTIBODIES FOR *LISTERIA*

B. Swaminathan

Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia, USA

There is an urgent need for rapid *Listeria* detection methods which can give reliable results in 24 to 48 h without extensive culture and subculture. Based on experience with o spectrum of other pathogenic microorganisms, ranging from viruses to parasites, nucleic acid probe based hybridization assays and monoclonal antibody based immunoassays appear to offer the most promise. Currently available assays need further refinement and validation before they become more widely accepted.

PROBLEMS OF DETERMINATION OF VIRULENCE OF *LISTERIA* STRAINS

B. Ralovich

Institute of Public Health and Epidemiology, University Medical School, Pécs, Hungary

Before measuring the virulence of *Listeria* strains, it is necessary to determine the term *Listeria* virulence. Listeria-infection can be characterized by the adhesion, induction of endo- or phagocytosis, multiplication in cells and other effects of the organism in the host. For characterization of virulent *Listeria* strains it is obligatory to know properties of these germs and the differences existing among virulent, avirulent or non-virulent listeriae. In case of *Listeria* strains the following markers of virulence can be mentioned: beta-type haemolysis on blood agar, keratoconjunctivitis-causing capacity in rabbit and guinea pig, ability to kill mice or chicken embryos for the virulence and missing of monocytosis producing activity, negative VP and MR reactions, presence of antigen noted 1830 for avirulence or non-virulence.

POSSIBILITIES OF DIFFERENTIATION OF LISTERIAL HAEMOLYSINS BY SYNERGISTIC HAEMOLYTIC REACTIONS (CAMP-REACTIONS)

J. Smola

University of Veterinary Science, Brno, Czechoslovakia

From the published information it is evident that haemolytic active substances from different strains of *Listeria* should not be necessarily identical. Different modifications of CAMP reactions employing prepurified bacterial exosubstances or productive strains were carried out on the blood agar prepared from Columbia agar (Oxoid) with the addition of 5 v/v% washed ovine erythrocytes. With the exception of *Listeria innocua* and *Listeria welshimeri* (all CAMP reactions negative), the results show different reactions of all the tested strains (species) of *Listeria*. We would like to emphasize that characteristically *Listeria monocytogenes* (sensu stricto) gives a positive CAMP reaction with erythrocytes affected by beta-toxin, equi-factor and haemolysin of *L. ivanovii*. While strains of *L. ivanovii* reacted characteristically in the zone of equi-factor and strains of *L. seeligeri* only in the zone of beta-toxin. The described differences show that the active haemolytic substances of *Listeria* species have different characters. These differences can be used in practice for presumptive identification of isolated *Listeria* strains before biochemical examination and test on mice. We considered, that not haemolysis, but haemolysin is the marker of pathogenicity.

INVESTIGATIONS ON THE PATHOGENICITY OF *LISTERIA* SPP. BY EXPERIMENTAL INFECTION OF THE CHICK EMBRYO

G. Terplan and S. Steinmeyer

Chair of Hygiene and Technology of Milk, University of Munich, Munich, FRG

The infection of chick embryo is considered to be a possibility to replace the mouse pathogenicity test. The chorioallantois membrane of 10 days old chicken embryos (5 per strain) was inoculated with 22 reference strains, 86 strains isolated from cheese and 31 strains from meat and meat products of different *Listeria* spp. All embryos infected with the pathogenic species *(Listeria monocytogenes, Listeria ivanovii)* died within 72 h, whereas embryos infected with the non-pathogenic species *Listeria innocua, Listeria seeligeri*, and *L.*

welshimeri survived. The results indicate that the infection of the chorioallantois membrane of chick embryos seems to be a suitable method for determining the pathogenicity of *Listeria* spp.

IMPEDIMETRIC DETERMINATION OF ACTIVITY OF DISINFECTANTS AND DETERGENTS ON *LISTERIA:* A PRELIMINARY STUDY

J. L. CORDIER, T. PUTALLAZ and L. J. COX

Nestec S. A., Quality Assurance Department, Vevey, Switzerland

The efficiency of several disinfectants or detergents against 3 strains of *Listeria monocytogenes*, 1 strain of *Listeria innocua*, and 2 strains of *Streptococcus* D was tested in water as well as in the presence of milk, whey and salt by an impedimetric method using a Bactometer M120. Certain synergistic effects between active agents and matrix could be observed. Differences in sensitivities were noticed amongst the tested strains. Products containing iodine, peroxide or quaternary ammonium as active agents were shown to be efficient even at relatively low concentrations.

CONCLUSIONS

1. *Essentials of analytical methodology*

1. *Sampling*

   — Homogenization and randomization
   — Size of sample
   — Size of aliquot to be subjected to examination

2. *Resuscitation*

   — Essential role
   — Choice of medium
   — LMR vs SMR
   — Extrinsic conditions and duration
   — Sequences

3. *Enrichment*

   — Choice of medium
   — Proportion sample: medium
   — Selection of culture conditions

4. *Isolation*

— Choice of medium; cave: differential traits !
— Mode of inoculation
— Choice of culture conditions

5. *Biochemical identification*

— Choice of criteria
— Interpretation of gallery results

6. *Serological examination*

— Procurement of sera
— Techniques
— Interpretation of results
— Exchange of data with other Institutes

7. *Phage typing*

— Procurement of phages
— Techniques
— Interpretation of readings
— Exchange of data

8. *Techniques based on gene probes*

— Selection of gene probes; cave: colour label preferable !
— Selection of a procedure allowing non-geneticists to use the technique and obtain unequivocal results

9. *Virulence markers*

— Reach consensus of validity of markers
— Choice of procedures
— Interpretation of collective results 1–9

10. *Other procedures or criteria suggested by RTD-members:*

use of IF techniques, isoenzymes, phospholipases, flagellins, listeriocins, etc. etc.

11. *Attempts to elaborate an internationally agreed set of procedures*

— Participants: IDF, IUMS, WHO Reference Centres, ILSI, others
— Baseline
— Operating procedure
— Other facets
— Constitution of a Working Party

2. *Sampling for L. monocytogenes*

The objective of sampling for *Listeria monocytogenes* in foods is to protect the consumer. At present it is difficult to make a rational or final decision on the sample size necessary to adequately protect public health. Data required for such a decision include (i) the human infective dose for healthy consumers; (ii) the "normal" intake of *L. monocytogenes* from raw foods as salads and the like.

Sampling of foods at the point of production for low numbers of L. monocytogenes by enrichment procedures. At present for regulatory purpose, the FDA samples 25 g of food, whereas the USDA/FSIS blends 25 g of food but takes a 10 ml portion, or 1 g sample to determine whether the food samples are positive or negative. Sampling at the 1 g level allows the FSIS to identify problem plants and take corrective actions. Both FDA and USDA enrichment procedures are not quantitative and take several days to perform.

"Rapid" sampling of foods containing large numbers, i.e. over 100/g of L. monocytogenes by direct planting. The organism grows in foods at refrigeration temperatures of 5 °C and a few foods may contain 10^{2-6} listeriae/g. These foods can be considered more of a human health risk than those with low numbers i. e. below 1/g. It is very easy to detect the high number of *Listeria* by direct plating of decimal dilutions of food on LiCl–Phenylethanol–Moxalactam (LPM) agar, and by the "swab and streak" direct plating method which is suitable for extensive screening of large numbers of retail food samples.

3. *Resuscitation*

L. monocytogenes incurs sublethal injury upon exposure to heating, freezing, or low pH. This results in inability of cells to form colonies on selective media that would otherwise support growth. Moreover, such sublethal injuries may interfere with the functioning of diagnostic systems. Since sublethally injured cells maintain their pathogenic properties, resuscitation is required to include these in colony counts or P–A tests. When this is ignored, underestimation of the efficacy of procedures for processing food for safety will result.

Resuscitation conditions for injured listeriae are yet to be developed. This should be done relying on assessment of so-called destruction-repair-curves. Although it would be unwise to predict any results at this stage, the limited amount of data available indicates that it is well-nigh impossible to elaborate selective differential media that allow sublethally injured cells to repair lesions adequately.

4. *Culturing*

Methods primarily used for enrichment of *L. monocytogenes* are the UVM and EB broths. Differential media have been developed more recently whose merits and shortcomings are yet to be evaluated, preferably by referee procedures.

Several isolation media are presently being used to isolate *L. monocytogenes* and several more have been suggested for use. However, no one medium can be expected to function well for use with all foods in all situations. Such a medium should be highly selective against contaminants but retain maximum recovery of listeriae regardless of the food being analyzed. An indicator system would always be helpful in facilitating recognition of *L. monocytogenes* colonies.

Loopfuls from appropriate selective enrichment broths are always streaked onto suitable isolation media including Modified McBride agar, Acriflavine–Nalidixic Acid–Serum agar, LPM.

After incubation at 37 °C for 48 h colonies typical of *L. monocytogenes* will be picked off and their identity confirmed by further examination; see below.

5. *Biochemical identification*

The genus *Listeria* presently consists of seven species on the basis of DNA relatedness. These include *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi* and *L. murrayi*. Morphological characteristics and biochemical tests used for genus and species identification are described.

Species identification is based on haemolysis, determined either on blood agar plates or by the CAMP-test with *Staphylococcus aureus* or with *Rhodococcus equi;* acid production from D-xylose, L-rhamnose, alpha-methyl-D-mannoside and mannitol; and nitrate reduction. Further investigations are required to improve species identification, especially the detection of haemolysis.

6. *Serotyping*

A distinction should be made between serogroup and serovar determination. Serogroups of listeriae 1/2 and 4 are determined using unabsorbed or roughly absorbed sera, which are commercially available. The reference method for serovar determination 1/2 a, b, c, 3a, b, c, 4a, b, ab, c, d, e, 5, 6a, b is based on the detection of a combination of antigenic factors using absorbed sera. Methods for the preparation of antigen and antisera, agglutination methods and a list of reference strains have been published.

7. *Phage typing*

A working group on *L. monocytogenes* phage typing was constituted by the ICSB Subcommittee on Taxonomy of *Listeria*, *Erysipelothrix*, and *Brochothrix* during ISOPOL 8 held in Madrid, Spain in 1981. Standardization of the methods used and selection of the phages were studied during a multicenter study.

Phage typing is presently done in 3 laboratories: A. Audurier, Tours, France; S. Ortel, Halle, GDR; and J. Rocourt, Paris, France. A fourth laboratory will be in Washington DC, USA.

The members of the working group will discuss on phages delivery, comparison of results on a given set of strains, interpretation of results and nomenclature of phage types.

For a more reliable association of *L. monocytogenes* strains isolated from patients with those isolated from foods involved in outbreaks, results of serovar and lysovar determinations should be linked to those obtained by methods based on for molecular markers which will be discussed in the sections which follow.

8. *Gene probe based techniques*

Gene probe hybridization assay may well provide the most specific means of identification and enumeration of *Listeria*, provided conditions and reagents are standardized. These assays can be used to obtain genus designation, species identification and/or to verify the presence of virulence-related genes, e. g. haemolysin genes.

Techniques have recently been increasingly simplified and with the availability of non-isotopic, colorimetric detection systems, wide-spread use of this technique is anticipated.

New hybridization assays should always be extensively compared with several "gold standard" conventional methods to verify efficacy.

9. *The use of restriction fragment length polymorphisms (RFLPs) for typing L. monocytogenes*

The basis of the typing method is the detection of inter-strain RFLPs within the species *L. monocytogenes* using a cloned DNA probe. DNA from strains of interest is cut with a restriction endonuclease. The fragments generated are separated according to size, blotted onto membrane filters and probed. The probes (2 sequences of approximately 10 kb each) were selected from a lambda-phage DNA library on the basis of their ability to hybridize with

polymorphic restriction fragments of all *L. monocytogenes* strains and to generate reproducible patterns of bands. The typing method takes at least 2 days to complete from harvesting cells to development of the blot and furthermore considerable manipulation is required.

Preliminary studies suggest that the reproducibility of the method is excellent.

10. *Immunoassay techniques*

Recent experience with various bacterial pathogens indicates that monoclonal antibody-based immunoassays are specific, sensitive, as well as easy to perform and thus are invaluable in the rapid detection of such pathogens. Monoclonal antibodies have been prepared against antigens of *L. monocytogenes* and *L. innocua*. All monoclonal antibodies prepared thus far react with *L. monocytogenes* and at least one other *Listeria* species.

Monoclonal antibodies specific for *L. monocytogenes* are not yet available. Attempts to prepare such an antibody by immunization with inactivated whole cells or cell extracts have not been successful. A concerted effort needs to be made to identify antigenic components unique to *L. monocytogenes*. Specific extracellular proteins of *L. monocytogenes*, e. g. haemolysin, are good candidates for the generation of species-specific monoclonal antibodies.

A monoclonal antibody-based sandwich immunoassay is commercially available for the detection of *Listeria* spp. in food products.

11. *Isoenzyme analysis*

Application of multilocus enzyme electrophoresis to the species *L. monocytogenes* has resulted in the establishment of a scheme for subtyping the organism. It further provides a framework for the study of the population genetics of the organism. Fifty-six electrophoretic types (ETs) were defined. With two exceptions no ET contained strains from more than one serogroup. The strong association of ET to serogroup is further illustrated by the deep separation of those ETs representing strains with a H-antigen of a and those ETs representing strains with a H-antigen of b.

This method has been successfully applied in studies on the epidemiology of listeriosis.

12. *Virulence markers*

Several virulence markers for *Listeria* strains have been proposed. Presence or absence of antigenic markers associated with virulence factors has

not been proved yet. Differences in cell-wall structures may be responsible for differences between virulent, weakly virulent and avirulent strains. Phage-typing, while a useful epidemiologic marker, appears to be unrelated to virulence. Biochemical reactions also do not appear to reflect virulence, with the possible exception of VP and MR reactions.

Beta-haemolytic property of *L. monocytogenes* and *L. ivanovii* is the only proven virulence factor. The molecular background of listeriolysins is under examination. Anton's test, mouse test, chick-embryo test and cell cultures can be used to detect virulence of *Listeria* strains.

Conclusions of the Panel Meetings held at the Tenth International Symposium on Listeriosis

1

Studies are being carried out to develop and improve media for the detection of *L. monocytogenes* and other *Listeria* species. However, on elaborating new or modifying existing methods the literature on available methods should be comprehensively reviewed.

2

Quality control of all liquid and solid detection and identification media is essential for proper assessment of contaminaton of food by *L. monocytogenes* and other *Listeria* species.

3

Repairing procedure may be necessary to recover injured *L. monocytogenes* cells from foods in all instances.

4

The wide-spread use of gene probes to identify, enumerate and type *L. monocytogenes* can be expected in the near future. Other promising techniques include immunofluorescence methods, isoenzyme analysis, ELISA procedures, latex agglutination tests and the restriction fragment length polymorphism (RFLP) approach.

5

Results of serovar and lysovar determinations as well as those from the methods mentioned under 4 above, should be used in epidemiological investi-

gations. It should thus be possible to determine with greater certainty relationships between strains of *L. monocytogenes* isolated from patients and suspect foods. If a direct relationship between patient and food in this sense cannot be confirmed than there is no epidemiological evidence for foodborne listeriosis.

6

Virulence factors of *L. monocytogenes* are not fully understood. Therefore elucidation of these factors through continued studies of in vivo or in vitro models of infection is important in establishing how strains of *L. monocytogenes* cause human listeriosis. Despite the availability of abundant data on the usefulness of beta-haemolytic properties of *L. monocytogenes* as virulence markers, further research into the molecular basis of haemolytic phenomena is required.

7

Correct pasteurization of milk sensu IDF will "eliminate" *L. monocytogenes* from milk at the level that is likely to be present in raw milk obtained by similar sound practices. Hence there should be no *L. monocytogenes* in pasteurized milk and ice cream.

8

Listeriae often occur on raw meats and poultry. So far no case of human listeriosis associated with the ingestion of raw meats has been reported. However, this provides no reassurance in view of (i) the well known underreporting of food transmitted infectious diseases in general; (ii) the lack of awareness in many areas of the role of food in the transmission of listeriosis; (iii) inadequate bacteriological and epidemiological methods. (See Note.)

9

The numbers of *L. monocytogenes* on raw meat and poultry can and should be markedly reduced by following optimal husbandry and slaughtering practices. Similar problems in seafoods may be solved by the application of appropriate measures during production and preparation.

10

Although it is impossible to avoid occasional low level occurrences of *L. monocytogenes* in raw foods, meticulous attention to hygiene measures in

animal production, slaughter, food processing, catering and home preparation must be maintained. However, the presence of *L. monocytogenes* in products processed for microbiological safety is unacceptable.

11

In the development of food products intended to be stored for long periods at refrigeration temperature a thorough risk analysis followed by design of GMP's is essential to prevent food-borne listeriosis.

Note. Since the Panel Meetings there cases of human listeriosis conveyed by meat products have been reported.

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HAEMOLYSIN PRODUCING CAPACITY AND MOUSE-PATHOGENECITY OF *LISTERIA MONOCYTOGENES*

S. Bernáth and Maria Pitron

Veterinary and Food Control Service,
Control Institute for Veterinary Biologicals
and Pharmaceuticals, Budapest

(Received February 23, 1988)

For studying the relationship between their haemolysin producing capacity and pathogenicity on mice, 12 *Listeria* strains were investigated, and 9 of them were reinvestigated one year later. Haemolysin production was measured on ^{51}Cr-labelled sheep erythrocytes and the 50% haemolysis-inducing dilution of the bacterial suspensions was calculated. Pathogenicity of the strains was estimated by intraperitoneal injection of mice. In both experimental series the correlation coefficients were calculated ($r = 0.408$, $t = 1.41$ and $r = 0.163$, $t = 0.43$). Using this experimental system, no correlation existed between the haemoysin producing capacity and mouse-pathogenicity of these strains.

Haemolysin production is registered as an important indicator for the virulence of *Listeria monocytogenes* strains. However, literary data are contradictory where the real role of haemolysin in the pathogenesis of listeriosis is concerned.

Hunter et al. [1] have assumed that a correlation exists between haemolysin production and virulence. Later Girard et al. [2] found that purified and concentrated haemolysin is relatively harmless for rabbits, mice and guinea-pigs. On the basis of their experiments they have concluded that no haemolysin-virulence correlation can be set up for *Listeria* strains. Njoku-Obi and Jenkins [3] and Lemeland et al. [4] came to the same conclusion. Other authors [5] injected a partly purified haemolysin to rabbits intravenously and observing a febrile temperature and cytotoxic effect, they assumed that this factor may have some role in the pathogenicity. According to Ralovich [6] one of the most important — however not absolutely determinant — virulence markers of *L. monocytogenes* is the beta-haemolysing capability of the strains that correlates well with the provocation of kerato-conjunctivitis.

On the basis of the above-mentioned results we have tried to set up numeric relation between the haemolysin producing capacity and mouse-virulence of different listerial strains.

Sándor Bernáth, Maria Pitron

Veterinary and Food Control Service, Control Institute for Veterinary Biologicals and Pharmaceuticals
H-1581 Budapest, P.O.B. 2, Hungary

Materials and methods

Bacterial strains. Twelve *L. monocytogenes* strains were investigated in the first experiment. One of them, used in vaccine production, had a decreased virulence, the others were previously or newly isolated from man, from the faeces and vagina of healthy sheep, and from sheep died of listeriosis (Table I). One year later we repeated the experiment with 9 of the strains (Nos 1, 2, 3, 6, 7, 8, 9, 11 and 12). Control of the haemolysing capability of the strains was performed on blood agar plates following one passage in mice and then culturing the strains on Tryptic Soy Agar (TSA) in Kolle bottles. After culturing for 24 h, the cultures were washed off with 5 ml physiological saline per bottle (basal suspension). The colony-counting, the haemolysis and mouse virulence tests were performed for all suspensions in one series of experiment. Colony counting was based on the determination of colony forming units (c.f.u.) on TSA agar.

Virulence determinations. Serial 10-fold dilutions of the individual strains were prepared in horse meat broth. Each of five mice, each weighing 18–20 g, were inoculated with 0.5 ml bacterial suspension intraperitoneally. The observation period lasted for one weak. The infective strain was reisolated from the mice perished. The LD_{50} values of the strains were calculated by the Reed-Muench method.

Radioisotope labelling of sheep erythrocytes. Sheep erythrocytes were washed three times with physiological saline. To the centrifuged sediment 100 ml 2% erythrocyte suspension containing 12 MBq ^{51}Cr was added (Na_2CrO_4, Isotope Institute, Hungarian Academy of Sciences, Budapest; specific activity 1046 GBq/g). The erythrocyte suspension was incubated for one hour at 37 °C with temporary stirrings (52% of the radioactivity was bound to the erythrocytes). After washing three times, the erythrocyte suspension was diluted up to 2% with saline (98.9% of the radioactivity was bound to the erythrocytes). The radioactivity of the samples was measured with a NZ 310 type Autogamma spectrometer (Gamma Works, Budapest).

Determination of the haemolysis. To 500 μl portions of serial 3-fold dilutions of the bacterial suspensions were incubated with the same quantity of labeled erythrocyte suspensions for one hour at 37 °C. After centrifugation the radioactivity of the supernates were measured. The maximum radioactivity released after total haemolysis was determined by using erythrocyte samples lysed with freezing-thawing three times followed by a one-hour incubation in distilled water. Spontaneous isotope-release was measured in the supernate of an equal mixture of labelled sheep erythrocytes and saline incubated at 37 °C as well. Three parallel investigations were made on each sample.

The degree of haemolysis was calculated according to Polos and Gallaher [7]:

$$\text{Percentage of radioactivity released due to haemolysis} = \frac{\text{radioactivity of the sample supernate (cpm)} - \text{spontaneous release of radioactivity (cpm)}}{\text{maximum released radioactivity (cpm)} - \text{spontaneous release of radioactivity (cpm)}} \times 100$$

Correlation coefficient was calculated on the basis of the dilutions of listerial strains provoking 50% haemolysis and the LD_{50} values for mice.

Results

Figure 1 shows the experimental results on the haemolysin production of the 12 strains investigated under the same conditions in the first experiment. The percentage of haemolysis was expressed for the undiluted bacterial suspension as well as for its 3-fold serial dilutions.

It is obvious from Fig. 1 that two of the strains produced little, nine of them medium and one of them great amounts of haemolysin. On the basis of the degree of haemolysis we have calculated the very dilution of the bacterial

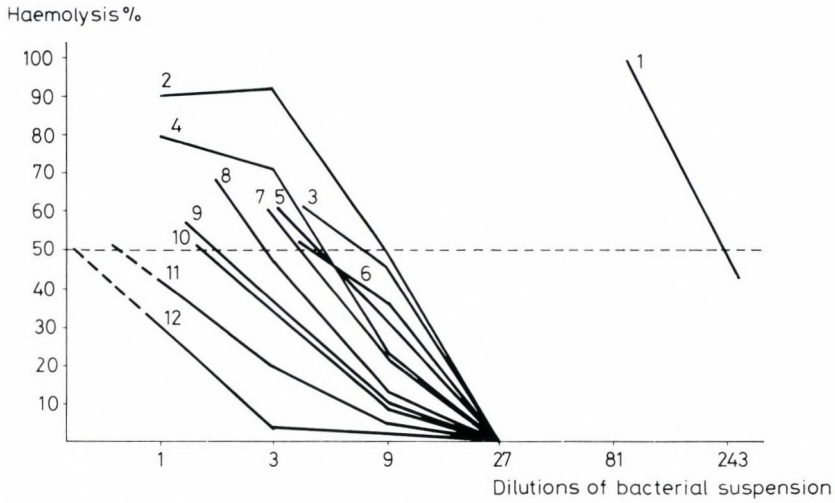

Fig. 1. Haemolysin-producing capacity of 12 *Listeria monocytogenes* strains. The dotted line represent the 50% haemolysin-producing capacity of the individual strains

suspension that might have resulted in 50% haemolysis in the given system. The results of the calculations together with the colony countings of the undiluted bacteria suspensions and the mouse LD_{50} values are represented in Table I. The strains are ranged according to their haemolysin production. The

Table I

Haemolysing activity and mouse LD_{50} values of L. monocytogenes strains

No.	Strain: Designation	Strain: Sero-type	c. i. u. in 0.5 ml baëal bacterial suspension	50% haemolysis*	Monse LD_{50}/0.5 ml**
1	Sheep lymph node	1/2	3.8×10^9	237.6	10^4
2	R-18	1/2	3.6×10^9	8.8	$10^{1.7}$
3	Bp-20[1]	4	3.6×10^9	8.0	$10^{3.5}$
4	Karancs[1]	1/2	4.3×10^9	5.4	$10^{2.84}$
5	Sur*	1/2	7.0×10^9	5.2	$10^{2.85}$
6	Sárszentágota[1]	1/2	2.0×10^{10}	4.8	$10^{2.5}$
7	22 Oe sheep faeces	1/2	2.4×10^9	3.8	10^4
8	AU	1/2	3.3×10^9	2.93	$10^{1.1}$
9	Pásztó[1]	1/2	2.7×10^9	1.79	$10^{2.5}$
10	Bodajk[1]	1/2	2.9×10^9	1.73	$10^{3.17}$
11	22 Ou sheep vagina	1/2	3.8×10^9	-1.73[2]	$10^{3.37}$
12	OU sheep lymph node	1/2	2.2×10^9	-2.47[2]	10^1

* Reciprocal dilution of the basal bacterial suspension

** Dilution of the basal bacterial suspension that killed 50% of the inoculated mice. Coefficient: r = 0.41, t = 1.42

[1] Isolated from sheep brain

[2] Calculated value

differences in the colony counts of the undiluted suspensions of the individual strains are relatively small.

The correlation coefficients for the haemolysis and mouse-pathogenicity of the 12 strains were $r = 0.408$ and $t = 1.41$, respectively. If only those 9 of the 12 strains were considered which were used in the second experiment one year later, the values were $r = 0.448$ and $t = 1.34$, respectively. One year later when these 9 strains were repeatedly investigated, these coefficients were $r = 0.163$ and $t = 0.43$.

Discussion

For ensuring similar conditions, all strains were investigated in the same experimental series. The differences in the colony counts of the undiluted bacterial suspensions had no adverse effect on the study of correlation between haemolysis and mouse-pathogenicity, because both values were determined by investigating the same bacterial suspensions.

From data in the Table it is obvious that in some cases high haemolysis values are connected with low LD_{50} values. This fact and the low correlation data calculated ($r = 0.448$ and $r = 0.163$, respectively) point out that in the given system there is no numeric relationship between the haemolysin producing capacity and mouse-pathogenicity of the individual listerial strains. Accordingly, not only the extent of haemolysis but other virulence markers (Rocourt and Berche [8]) have to be taken into consideration when testing the virulence of listeriae.

REFERENCES

1. Hunter, M. C., Stahly, G. L., Myers, W. M. G.: Ohio J Sci **50,** 253 (1959).
2. Girard, K. F., Sbarra, A. J., Bardawill, W. A.: J Bacteriol **85,** 349 (1963).
3. Njoku-Obi, A. N., Jenkins, E. M.: Bacteriol Proc **77,** M57 (1962).
4. Lemeland, J. F., Allaine, R., Boiron, H.: Pathologie-Biologie **22,** 763 (1974).
5. Siddique, I. H., Ying, L. C., Robinson, B. B.: Can J Comp Med **33,** 292 (1969).
6. Ralovich, B.: Listeriosis Research. Present Situation and Perspective. Akadémiai Kiadó, Budapest 1984.
7. Polos, P. G., Gallaher, W. R.: J Gen Virol **52,** 259 (1981).
8. Rocourt, J., Berche, P.: Ann Microbiol (Paris) A **138,** 241 (1987).

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NEW DEVELOPMENTS ON THE GENERATION OF MUTATIONS IN *ESCHERICHIA COLI* LYSOGENS

M. LIEBER

Department of Microbiology,
Stritch School of Medicine, Loyola University
Maywood, Illinois, USA

(Received February 2, 1988)

Through the lytic process, Pl is a bacteriophage or virion capable at very low frequency of carrying out generalized transduction between strains of *Escherichia coli.* When P1 is not involved in lytic functions, it exists as a prophage in the form of circular DNA molecule, persisting in an extra-chromosomal or plasmid state, and not integrating into the host chromosome. *E. coli* carrying such plasmids have been referred to as lysogens. Earlier research dealth with P1 plasmids carrying drug-resistant factors. Up to the present study, P1 plasmids of any type were not known to have any mutagenic effect on the *E. coli* chromosome (genome), nor was it known that generalized transduction can be associated with mutagenicity. From P1 plasmids carrying a chloramphenicol resistance-factor, mutant plasmids of a particular type were isolated in the present study. P1 plasmids of this type carried a mutant factor which greatly impaired the capacity of phage derived from such plasmids, upon the completion of the lytic cycle, to lysogenize *recA E. coli* through phage promoted recombination. The plasmid of this mutant type is referred to as P1CMrec, and lysogens carrying such are referred to as P1CMrec lysogens. This paper describes the history of these P1CMrec lysogens and the genetic mutability within the *E. coli* chromosome of such P1CMrec lysogens, and the relationship of such genetic mutability (instability) to the incorporation of virion-DNA, following the absorption of P1 phage by these lysogens. As illustrated in the paper, a mutagenic effect was generated within the *E. coli* chromosome of P1CMrec lysogens by means of the P1CMrec plasmid. Furthermore, this mutagenic effect was found to be greatly, non-locally, and uniformly enhanced as a consequence of P1 virion incorporation by, or likely generalized transduction of, such lysogens. More specifically, the plasmid of this mutant type (P1CMrec) is responsible for the creation of a wide range of genetic mutabilities (instabilities) of differing degree within the *E. coli* genome (not carrying *recA*), some mutabilities being very high upon extended incubation. The P1CMrec plasmid was also involved in the creation of new mutant genes within the *E. coli* chromosome, some of which manifested high mutability. Evidence indicated that parts (or apart) of P1CMrec were transposed to the *E. coli* chromosome and that such transposed element(s) were responsible for the organized creation of new mutant genes and mutabilities throughout the *E. coli* genome. Connected to this, it was found that virion-DNA incorporation by (or very likely generalized transduction via P1 of) such lysogenic bacteria carrying P1CMrec greatly enhanced the various mutabilities mediated by P1CMrec, as well as making the P1CMrec-generated mutant regions (many of which were originally stable, or slightly mutable) unstable. In fact, all mutabilities of various regions and of various degrees were significantly enhanced to the same degree, and in much shorter time, as opposed to what occurs without such virion-DNA incorporation, even though some regions were widely separated in the genome. It was thus clear that virion-DNA incorporation, or very likely generalized transduction, not only enhanced P1CMrec-mediated mutabilities in a shorter period, but uniformized them as well. Processes involving the creation of non-uniform force configurations within the *E. coli* genome are postulated as underlying such phenomena.

MICHAEL LIEBER
1619 Hopkins St., # 101, Berkeley, CA 94707, USA

Through the lytic process, P1 is a bacteriophage or virion capable at very low frequency of carrying out generalized transduction between different strains of *Escherichia coli* [1, 2]. In generalized transduction a rare DNA segment of the bacterial chromosome, equivalent in length to about 2 minutes of the bacterial chromosome (Fig. 1, [3]), is incorporated into a protein capsule (capsid) which goes to make up a P1 virion. (A virion can either be just a phage-protein capsul containing bacterial DNA or such a capsul containing phage

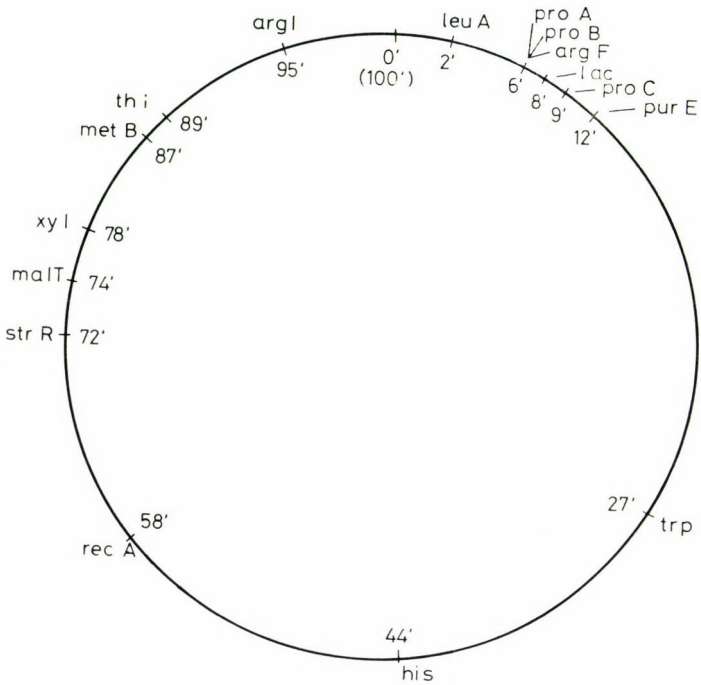

Fig. 1. The *E. coli* K12 chromosomal map [3, 69] with genetic factors referred to and used in this study

DNA.) Upon lysis and adsorption, the bacterial DNA of such is injected into a recipient bacterium where the segment is incorporated through recombination into a homologous region of the recipient's chromosome [2, 4–10]. When P1 is not involved in lytic functions, it exists as a prophage in the form of a circular DNA molecule, persisting in an extrachromosomal or plasmid state [11], and not integrating into the host chromosome. Up to the present study, P1 was not known to have any mutagenic effect on the host genome. Though such a plasmid is a self-replicating entity, only one P1 plasmid can exist per bacterial cell [11].

This plasmid can be transferred from one strain to another also by way of the lytic process. Upon infection, after the release of mature phage (virions) following lysis, the linear, terminally-redundant phage DNA of a given virion is injected into a bacterium and is converted into a circular form by means of recombination between redundant ends [11]. *E. coli* carrying such plasmids are referred to as lysogens. One group of lysogens that has been studied contains the plasmid P1CM [12, 13]. This plasmid carries a genetic region *(CM)* conferring resistance to the antibiotic chloramphenicol, and lysogens carrying P1CM can grow very well on media having various concentrations of chloramphenicol. P1CM also carries the genetic factor, c1, which allows the initiation or induction of the lytic process upon the elevation of temperature from 32 °C to 42 °C, whereupon the release of phage and subsequent infection of chloramphenicol-sensitive (cm) strains results in the transduction of a very high frequency of bacteria for chloramphenicol-resistance *(CM)*. The high frequency transduction (transmission) of a genetic marker from one bacterial strain to another is referred to as specialized transduction and indicates that the marker in question resides on the plasmid rather than on the chromosome of the *E. coli*, which is the donor strain. P1CMc1 virions can carry out generalized and specialized transduction upon thermo-induction. Generalized transduction of markers deleted in recipients does occur, but at a relatively very low frequency [9].

In a study by Rosner [13], lysogens were found in about 30% of *rec*$^+$ (capable of recombination) cells and 3% of *recA* (recombination-deficient) cells infected with P1CMc1, irrespective of the multiplicity of infection. Therefore, according to Rosner, the recombination function of *E. coli* is not essential for, but does facilitate P1CM lysogen formation. An implication of this, as pointed out by Rosner, is that P1CM itself carries a factor for phage-promoted recombination which enables it to circularize as a process of lysogenization. In the present study, such a predicted factor (in mutant form) was detected.

The present study grew out of the isolation of a particular mutant class of P1CMc1 plasmid in connection with other research objectives. Members of this class of plasmids, referred to as P1CMrec, (this class of plasmid is still thermo-inducible but the c1 factor will not always be indicated), introduced as infective phage into *E. coli recA* (recombination-deficient, UV-sensitive), were unable to form *CM* lysogens. That is, they were not able to transduce *recA* bacteria for *CM*, as opposed to normal P1CM. In contrast, they could transduce *recA*$^+$ bacteria for *CM*, since they could form P1CMrec lysogens of *recA*$^+$ bacteria. This strongly indicated, in view of Rosner's work, that members of this mutant-plasmid class contained an impairment in recombination ability, so that they were unable to circularize in most or all cases upon infection (or incorporation) in *recA* bacteria, thereby precluding the formation of plasmids in such bacteria. The term "*rec*" refers to or is a symbol of that

impairement. This property of impaired lysogenization of P1CMrec of *recA* bacteria was inheritable. Moreover, this deficiency carried by the various lysogenic strains was not functionally negated through complementation between such strains, suggesting it was within the same locus in all such strains. All studied lysogens carrying P1CMrec were found to have unforseen and unusual genetic mutabilities within the *E. coli* chromosomes which could be further modified in a genetically unstable manner upon the incorporation of virion DNA, and thus upon the likely generalized transduction of these P1CMrec lysogens. This was exemplified in the following way. By means of the P1CMrec plasmid, a mutagenic effect was generated within the *E. coli* chromosome of P1CMrec lysogens. Furthermore, this mutagenic effect was found to be greatly, non-locally, and uniformly enhanced as a consequence of P1 virion incorporation by, or very likely generalized transduction of, such lysogens. In this paper, these phenomena are described, detailed, and developed in an historical manner. In so doing, the reader will better understand and appreciate the unfolding of the total thinking required to give a complete picture of these phenomena.

Materials and methods

Media. The various types of media used, including media designed to select for various classes of prototrophs and media designed to detect various carbohydrate-mutants (such as strains unable to utilize lactose) were prepared according to methods described in Luria et al. [8] and Stodolsky et al. [9]. Minimal media contained thiamine, complete (Luria) media designed to select for chloramphenicol-resistant (*CM*) colonies or transductants contained 60 μg/ml chloramphenicol. Complete media used for the detection of kanamycin-resistant (*KN*) transductants contained 50 μg/ml kanamycin and complete media used for the detection of streptomycin-resistant (str^R) strains contained 50 μg/ml streptomycin.

Strains of E. coli K12: see Table I. Additional strains, which are mutants, will be described in the course of this paper. Unless otherwise indicated, incubation was at 31 °C.

Lysates. Lysates (P1-virions) were prepared from thermoinducible lysogens according to procedures described by Rosner [13]. Phages were titred by mixing appropriate lysate-dilutions and 1×10^8 exponential growing bacteria (strain 1800) in 3 ml of soft-nutrient-agar, containing 0.005 M Ca^{++}, and embedding (pouring) such agar on complete-media plates, whereupon such plates were incubated at 42 °C overnight, after which plaques were scored.

Lysogen formation through P1-virion infection. P1CMcl and P1KNcl lysogens were prepared according to methods described in Rosner [13].

Generalized transduction through P1-virion infection (or incorporations). Respective classes of recipients for generalized transduction were grown in respective tubes containing 3 ml Luria Broth, with aeration, to the same concentration in each experiment as determined by the use of a klettmeter. Cultures were then placed at room temperature (21 °C). Ca^{++} was added (to enhance phage adsorption) to a final concentration of 0.005 M, and any given lysate was added with the same multiplicity of infection (m.o.i.) as any other in a given experiment, as indicated in the appropriate experimental table. After a given time of adsorption, as indicated in appropriate tables, concentrations of bacteria were again measured to confirm equal concentrations and equal samples from the tubes were plated on appropriately supplemental media. Sterility controls were done in parallel.

The production of mutant plasmids using the mutagen nitrosoguanidine (NSG). The procedure suggested by Stodolsky was used. Rather than treating a lysate with NSG, 1802-CM lysogens were exposed to NSG. Ten ml of tris-malenate buffer, pH 6, containing 100 μg/ml NSG and 2×10^8/ml 1802-lysogens were prepared (in a tube) and left at 21 °C for 20 min. The solution was then centrifuged a 8000 rpm for 5 min, supernatant containing mutagen was removed, inside of tube was rinsed with buffer, being careful not to disturb bacterial plug. Upon rinsing, 20 ml of Luria Broth (LB) were added, mixed with bacteria, and the entire con-

tents was placed in an Erlenmeyer flask, and incubated at 32 °C in a shaker-waterbath. During incubation, Klett readings were taken. A 101 m sample of bacterial solution was removed from the flask into a test tube just about the time bacteria began to show increased turbidity (which indicated bacterial coats were normal, not clear, and hence bacteria were healthy and on the verge of multiplying, P1 plasmid dividing in synchrony with bacterial division [11] and thermally-induced at 42 °C in a waterbath-shaker, adding 0.01 M Mg^{++} to the sample bacterial solution in the test tube to stabilize phage lysates. The lysate of such treated lysogens was isolated according to methods previously referred to. Such a lysate containing likely mutated phage (and hence future mutant plasmids) was added to strain 1800 in order to produce *CM* lysogens of 1800 carrying such likely mutated P1CMcl plasmids. Such *CM* lysogens (in colonial form), and which were also *CM* transductants, were detected at very high frequency, using complete media + chloramphenicol as the selective media. (Strain 1800 controls did not grow on such media.) A large sample of such colonies, each being well spaced from any other colony, was isolated and purified for further characterization, that is, to look for P1CM plasmids having particular mutant properties.

Lysates (which were shown respectively to give rise to continuous plaques on a lawn of 1800 at 42 °C) were prepared from a number of such P1CM lysogens contained in that sample. In this case, lysates were prepared (induced to form) at 42 °C in small wells containing growing cultures of the lysogens in LB + Mg^{++}. Upon 2 h incubation, a small drop of chlorofrom (to kill any remaining lysogens) was added to each well, and drops of lysates therefrom (as well as 1802-lysate) were replicated (using pin-heads on a wood block) onto 2.5 ml top, soft-nutrient agar containing respectively C600 and 1800 overlaid on a complete media + chloramphenicol, and incubated overnight at 32 °C. Plates containing overlaid 1800 were also incubated at 42 °C to test for (confirm) plaque formation. Upon incubation, CM lysogens of 1800 (at 32 °C) were evident in the form of many large colonies (many confluent) growing where the drops of lysates had been placed. On the C600 plates some of the corresponding drops (but not that from 1802) had not given rise to any areas of growth (transductants). (This procedure, using larger drops, was repeated twice with the same results.) On the strain 1800 plates at 42 °C, there were clear regions (plaque confluence) in the bacterial lawn in all places where lysate drops were placed. Sterility controls were done in parallel. Control plates containg C600 and 1800 overlaid (in soft agar) on chloramphenicol media did not grow in repeated tests. This procedure was suggested by M. Stodolsky.

The lysates were added to the *recA* strain (C600) and to 1800 *(recA+)* in order to determine whether one could isolate P1CM lysogens of the *recA* strain. It was found that lysates (virions) from particular 1800-P1CMcl lysogens, as opposed to others, could not repeatedly transduce C600 for *CM* (i.e., produce C600-P1CMcl lysogens) whereas they could always transduce 1800 for *CM*. Such results, especially in light of Rosner's findings [13] indicated that there is a particular sub-class of 1800-P1CMcl lysogens which carry a mutated plasmid of a particular type, a type whose phage-offspring are unable to transduce for CM (lysogenize) a bacterial strain deficient in recombination capacity. Additional information pertaining to this sub-class of mutant-lysogens will be presented in the Results section.

Determination of nutritional requirements of specific strains. In order to determine whether certain strains only required certain amino acids, bacteria from such strains were put in test tubes containing liquid minimal media and in test tubes containing liquid minimal media plus all amino acids. The amino acids were L-amino acids and each was at a concentration of 20 μg/ml. (Such supplemental media is referred to as AM.) Test tubes were then left in a 31 °C roller-drum incubator for about 7 h, upon which times tubes were observed for growing cultures. Specific amino-acid requirements were then determined by adding strains to test tubes of AM respectively minus a given amino acid and to an AM control. The tubes were then incubated in the 31 °C roller-drum for about 7 h, observed for growing cultures.

Location of nutritional markers through conjugal crosses. General procedures for the location of markers are described in Stodolsky et al. [9]. In this study, HfrH-P1KNcl (3000/P1KNcl) was used as the male donor and it was grown at 34 °C. This strain was used as a donor to preclude the P1-restriction system in the recipient [14]. (Stodolsky, personal communication). The F-, *CM* recipient was grown at 32 °C. Conjugation was carried out at 34 °C. The *E. coli* map is based upon conjugation at 37 °C. However, in this study, control experiments at 34 °C, using markers of known location, indicated there was no significant difference in time of entry of markers at this lower temperature. Selection plates containing chloramphenicol were incubated at 31 °C for 30–34 h before scoring.

Viability of strains. By low density platings onto complete medium from a given concentration, the respective viabilities of different strains were determined. Respective strains were found to have high, comparable viabilities. Also, on complete medium plus chloramphenicol, the various *CM* strains, including 1802, had comparable viabilities.

Results

I. *Enhanced production of prototrophs confined to P1CMrec lysogens*

Observations focused on M35, one of twenty P1CMrec-mutant lysogens originally isolated from the large group of presumptive mutant transductants (i. e., transductants with presumptive-mutant plasmids). The bacteria so transduced were of bacterial strain 1800; this strain has the same genotype as lysogen-strain 1802. In a study designed to determine the effect of the *rec* factor of P1CM on the generation of various types of transductants, M35 and 1802 were infected with P1 phage (virions) from the prototrophic lysogen 7103 in two sequential experiments. Following the incorporation of virion DNA from 7103 (lysate) by M35 and 7103 lysate by 1802 which in two separate aliquots, carries a non-mutant P1CM, there were far greater frequencies of *Pro*$^+$ and *arg*$^+$ prototrophs in each experiment amongst M35 lysogens than amongst the same number of 1802 lysogens (see Tables IIa, IIb). Among the *pro*$^+$ prototrophs alone, the difference between the frequencies of M35 *pro*$^+$ colonies and 1802 *pro*$^+$ colonies was at least 160-fold in each experiment. The difference between the frequencies of M35 *arg*$^+$ colonies and 1802 *arg*$^+$ colonies was not as great. With regard to the frequencies of M35 prototrophs, it is suggested that the brief transient induction (which, however, is not enough to induce lysis [10]), at high temperature had a depressive effect. Testing 200 of the *pro*$^+$ M35 prototrophs for arginine requirement, all were *arg*$^+$. Testing 200 of the original *arg*$^+$ M35 prototrophs, all were *pro*$^+$. Also, all M35 prototroph were *CM*, indicating the continued presence of P1CMrer. (A velvet-enclothed, circular replicator was used is such a test.) Eighty per cent of 1802 *arg*$^+$ prototrophs were *pro*.

The preceding findings, subsequent to an incorporation of virion DNA, or subsequent to a likely generalized transduction, were clearly unexpected and unprecedented. The thereby warranted further investigation. M35 and 1802 were again infected with phage from 7103. (Phage produced by the lysogen 7103 is referred to as 7103 φ.) At the same time, seven other P1CMrec lysogen-strains were also subjected to infection by 7103 φ. This time one also looked for *his*$^+$ prototrophs. The results are in Table III. The frequencies of *pro*$^+$, *arg*$^+$, and *his*$^+$ prototrophs amongst M35 lysogens were clearly far greater than the frequencies of such found among the same number of 1802 lysogens. Of *his*$^+$ colonies tested, all were *pro*$^+$ *arg*$^+$; of *pro*$^+$ and *arg*$^+$ colonies tested, all were *his*$^+$. These colonies were *CM*. Furthermore, there were exceedingly far greater frequencies of *pro*$^+$, *arg*$^+$, and *his*$^+$ prototrophs amongst the lysogens of each of the seven other P1CMrec lysogen-strains than amongst the same number of 1802-lysogens. In fact, for each of the three different proto-

Table I

Strain	Sex	Releveant characteristics and nomenclature[a]	Source
1800	F—	[*argI*][c] [*proA ProB argF lac*][b] *his metB malT xyl* str^R	Stodolsky
1802[d]		Same genotype as 1800 + P1CMcl	Rosner Stodolsky
8800/PlKNcl		Same genotype as 1800 + P1KNcl	From 1800 Stodolsky
8330	F—	*leuA proC purE trp* str^R P1 (defective)	Markowitz
7103[e]	F—	Prototroph-Plcl	Stodolsky Rosner
3000/PlKNcl	HfrH	Prototroph-P1KNcl	From 3000 (Luria)
C600	F—	*LeuA lac recA su* str^R	Rosner

[a] All strains require thiamine, symbolized by *thi; argI* and *argF*, each determining requirement for arginine (though different loci, each is responsible for a deficiency of the same enzyme); *proA*, *proB*, and *proC* genetic regions or loci, each determining a requirement for proline; *leuA*, *trp*, *purE*, *metB*, and *his*, genetic regions or loci determining requrements for respectively leucine, tryptophane, purine (adenine), methionine, and histidine. *Thr*, *phe*, *try*, and *ilv*, mutant regions or loci determining requirements respectively for threonine, phenylalanine, tyrosine, and isoleucine-valine; *lac*, *malT*, and *xyl*, loci determining an inability to respectively utilize lactose, maltose, and xylose as carbon sources. str^R, locus determining resistance to streptomycin *(*str^S, allele determining streptomycin-sensitivity); *recA*, inability to eventuate recombination, UV sensitive; *su*, unable to surpress amber mutations in Pl. P1 is a temperate bacterio-phage existing as a plasmid in *E. coli* K12. This plasmid can be induced to produce mature, infective phage in a lysate. Plcl is a plasmid which is thermo-inducible at 42 °C to generate infective phage and lysis. P1CM1 is a P1 plasmid carrying a chloramphenicol resistance-factor *(CM)* and which is thermoinducible. P1KNcl is a plasmid carrying a kanamycin resistance-factor *(KN)* and which is thermoinducible. *E. coli* strains carrying such Pl plasmids are referred to as lysogens. Only one plasmid can exist or be maintained per bacterium.
Additional nomenclature: mutability of *arg* means that mutability of non-deleted genetic region(s) resulting in the negation of arginine-requirement, and mutability of *pro* means that mutability of non-deleted genetic region(s) resulting in the negation of proline-requirement. Furthermore, mutability of respectively *his*, *met*, *leu*, *trp*, and *ilv* will be taken to mean mutability of genetic region(s) involved in allowing respective requirements for histidine, methionine, leucine, tryptophane, and isoleucine-valine

[b] Genes within brackets refer to a deletion containing such genetic regions. The deletion is about 1′ in map-length [11]. It has been referred to as X111. 1800 is also auxotrophic for those deleted markers

[c] *argI* is deleted. 1800 is also auxotrophic for this deleted marker

[d] Transduction of deleted markers occurs, but comparable strains without the deletions are transduced at a much greater frequency for the same markers [9]

[e] This lysogen produces wild-type Pl carrying the thermo-inducible gene

trophic classes (also including those in M35), the differences in frequencies, compared to 1802, were at least 100-fold in the majority of cases.

In other experiments, M35(P1CMrec) and 1802(P1CM) were again infected with 7103 φ. In Tables IV, V and VII, we again see the phenomenon of very

Table IIa

Results of an infection of specific strains with P1 virions in order to bring about generalized transduction for prototrophic markers on all-x media[a]

Strains infected and virion class used[c]	Frequency of prototrophs (No. of prototrophic colonies per plate and per bacteria per plate)[b]		
	Selection for:	*pro*+	*arg*+ :Prototrophic classes
1802 + P1 — 7103[d]		10	87
M35 + P1 — 7103		1600	1580
1802 + P1 — 7103 (1802 transiently induced at 42 °C for 5′ before adsorption)		4	8
M35 + P1 — 7103 (M35 transiently induced at 42 °C for 5′ before adsorption)		850	900
Strains not infected			
M35		0	0
1802		0	0

[a] Minimal media plus histidine, proline, arginine, methionine minus a given amino-acid
[b] 2.5×10^7 bacteria plated per plate
[c] M.o.i. = 0.9. Adsorption was for 40′ at 21 °C. period of incubation: 2 days
[d] Phage of a lysate derived from the prototrophic lysogen, 7103

Table IIb

Strains infected and virion class used[b]	Frequency of prototrophs (No. of prototrophic colonies per plate and per bacteria per plate)[a]		
	Selection for:	*pro*+	*arg*+ :Protrophic classes
1802 + P1 — 7103		0	85
M35 + P1 — 7103		2200	1800
1802 + P1 — 7103 (1802 transiently induced at 42 °C for 5′ before adsorption)		1	17
M35 + P1 — 7103 (M35 transiently induced at 42 °C for 5′ before adsorption)		710	650
Strains not infected			
M35		0	0
1802		0	0

[a] 2.5×10^7 bacteria plated per plate
[b] M.o.i. = 0.9. Adsorption was for 40′ at 21 °C. Period of incubation: 2 days

Table III

Results of an infection of specific strains with Pl virions in order to bring about generalized transduction for prototrophic markers on all-x media

Strains infected and virion class used[a]	Frequency of prototrophs (No. of prototrophic colonies per plate — 3.4×10^7 bacteria per plate)[b]			
Selection for:	*his*$^+$	*pro*$^+$	*arg*$^+$	:Prototroph classes
1802 + Pl — 7103 (A)	0	0	0	
1802 + Pl — 7103 (B)	0	0	0	
M35 + Pl — 7103	110	67	70	
M781 + Pl — 7103	82	87	72	
M534 + Pl — 7103	65	82	56	
M542 + Pl — 7103	70	74	68	
M364 + Pl — 7103	114	40	75	
M294 + Pl — 7103	120	110	100	
M797 + Pl — 7103	112	124	95	
M536 + Pl — 7103	54	70	104	
Strains not infected				
1802	0	0	0	
M35	0	0	0	
M781	0	0	0	
M534	0	0	0	
M542	0	0	0	
M364	0	0	0	
M294	0	0	0	
M797	0	0	0	
M536	0	0	0	

[a] M.o.i. = 0.05. Adsorption was for 40′ at 21 °C
[b] Period of incubation was 2 days

enhanced prototrophic-production in M35 relative to the degree of such in 1802, subsequent to virion infection. All experiments, thus far, have shown this phenomenon to be reproducible.

The degree of enhanced prototroph-production is dependent upon the m.o.i. Nevertheless, the frequencies of the different M35 prototrophic classes in any experiment are comparable, indicating a uniformity in the generation of different prototrophies. Through extrapolation, M35 prototrophs are very probably all *arg*$^+$ *pro*$^+$ *his*$^+$. This further indicates an uniformity in the generation of different prototrophies.

It is clear from all preceding results that the PlCMrec-plasmid is in some way responsible for this very enhanced production of prototrophs. If some form of plasmid-enhanced generalized transduction was involved in the production of those very high frequencies, it is clear that *lac*$^+$ was not being transduced or was not implicated in the enhanced changes (data not tabulated).

Possibly, some form of enhanced transformation involving 7103-donor DNA was implicated. To rule out the possibility that 7103 bacterial-transforming DNA was also contained in the 7103 φ lysate, the 7103 lysate was treated with DNase before the lysate was added to M35 and 1802. A control in which 7103 lysate was not treated with DNase was also performed. It is clear from Table V that the DNase treatment had no effect on the very enhanced production of prototorophs. Clearly, the phenomenon of enhanced-prototroph production does not involve a bacterial-DNA transforming-vector.

II. *Increased prototrophy related to chromosomal changes induced by mutant plasmid*

Though the P1CMrec-plasmid is implicated in the enhancement of prototroph frequency, it was still not clear in what manner it was responsible. Nor was it clear whether by transferring P1CMrec by way of transduction of 1800 (lysogenization of 1800), one could derive a new set of M35 lysogens, which would in turn give rise to a very high frequency of M35 prototrophs

Table IV

Results of an infection of specific strains with P1 virions in order to bring about generalized transduction for prototrophic markers on all-x media

Strains infected and virion class used[a]	Frequency of prototrophs (No. of prototrophic colonies per plate — 3.4×10^7 bacteria per plate)[b]			
Selection for:	*his*$^+$	*pro*$^+$	*arg*$^+$	:Prototroph-classes
1802 + P1 — 7103	16	0	0	
	14	0	0	
M35 + P1 — 7103	50	78	63	
	51	62	64	
Strains not infected				
M35	0	0	0	
	0	0	0	
1802	0	0	0	
	0	0	0	

[a] M.o.i. = 0.05. Adsorption was for 45' at 21 °C
[b] Period of incubation was 2 days

Table V

Results of an infection of specific strains with Pl virions using all-x media

Strains infected and virion class used[a]	Selection for:	Frequency of prototrophs (No. of prototrophic colonies per plate — 3.4×10^7 bacteria per plate)[b]			:Prototrophic class
		his$^+$	*pro*$^+$	*arg*$^+$	
1802 + P1 — 7103		48[f]	0	3	
		43[f]	0	2	
1802 + P1 — 7103 (DNase treated)[d]		50[e,f]	0	4	
		46[f]	0	3	
M35 + P1 — 7103 (DNase treated)[d]		154[e]	172[c]	153[c]	
		136[e]	153	147	
M35 + P1 — 7103		148[e]	127	159	
		144[c]	150[c]	152[c]	
Strains not infected					
1802		0	0	0	
		0	0	0	
M35		0	0	0	
		0	0	0	

[a] M.o.i. = 0.1. Adsorption was for 45' at 21 °C
[b] Period of incubation was 2 days
[c] These colonies were tested for *CM;* all were *CM*
[d] 0.1 mg DNase per ml of lysate. Treatment was for 1 h before lysate was added to bacteria
[e] All *his*$^+$ colonies were found to be *arg*$^+$
[f] All 1802-*his*$^+$ colonies were *arg*

relative to 1802 upon infection with 7103 φ. Namely, is the factor (or factors) promoting enhanced prototroph production inherited exclusively by way of P1CMrec? To test this idea a phage-lysate was prepared from M35; 1800 was infected with such, using given media to isolate *CM* transductants, that is, 1800-P1CMrec lysogens which received P1CMrec from M35. From five such CM lysogens, designated respectively as 35t-1, 35t-2, 35t-3, 35t-4, and 35t-5, cultures were prepared, and 7103 φ were added for infection. An 1802 control was prepared and infected. Results are in Table VI. It is clear that the promoting-property of enhanced prototroph production cannot be transferred by way of the plasmid *alone* from one group of bacteria (I) to another (II). The keyword is "alone", for it is clear that P1CMrec is somehow implicated in enhanced prototroph production in group I; its mode of action may be contingent or dependent on genomic changes it induced within the genome of group I bacteria, changes which for some reason it could not induce in group II bacteria. It is possible that the putative changes initially created by P1CMrec in the chromosomes of group I bacteria, M35(P1CMrec), would be sufficient by

themselves, and hence without the plasmid, to be responsible for the induction of increased prototrophy. This position can be tested by replacing the PlCMrec plasmid with another plasmid, P1KNcl, known not to be responsible for any unusual properties. As only one Pl plasmid can be maintained in any *E. coli* bacterium, one can infect M35 with virions, prepared from lysogens containing P1KNcl, a plasmid carrying a kanamycin-resistance factor, and select for kanamycin-resistant (KN) transductants. A P1KNcl plasmid present in 1800 was introduced into M35, replacing PlCMrec. *KN* colonies (*KN* transductants of M35) were observed on kanamycin plus complete media. One of these transductants, 35KN-a, was further purified, producing a group of 35 KN-a colonies. An isolate was obtained and a culture of 35KN-a was infected with 7103 φ. Control cultures of M35, 1802, and P1KNcl/1800 were also infected with 7103 φ, with the objective of seeing the effects of virion DNA incorporation. Results are in Table VII.

The frequencies of 35Kn-a prototrophs were extremely high, comparable to those of M35 prototrophs, while 1802 and P1KN/1800 lysogens had no

Table VI

Results of an infection of specific strains with P1 virions using all-x media

Strains infected and virion class used[a]	Frequency of prototrophs (No. of prototrophic colonies per plate — 3.1×10^7 bacteria per plate)[b]		
	Selection for	*pro*+	*arg*+ :Prototrophic class
1802 + P1 — 7103		0	12
		0	10
35t-1 + P1 — 7103		0	6
		0	8
35T-2 + P1 — 7103		0	7
		0	9
35t-3 + P1 — 7103		0	5
		0	8
35t-4 + P1 — 7103		0	8
		0	10
35t-5 + P1 — 7103		0	8
		0	11
Strains not infected			
1802		0	0
		0	0
M35		0	0
		0	0

[a] M.o.i. = 0.1. Adsorption was for 45′ at 21 °C
[b] Period of incubation was 2 days

Table VII

Results of an infection of specific strains with P1 virions using all-x media

Strains infected and virion class used[a]	Frequency of prototrophs (No. of prototrophic colonies per plate — 4×10^7 bacteria per plate)[b]			
Selection for:	*pro*+	*arg*+	*his*+	:Prototrophic class
1802 + P1 — 7103	0	1	30[e]	
	0	2	35[e]	
P1KN/1800 + P1 — 7103	0	4	39[e]	
	0	3	45[c]	
35KN-a + P1 — 7103	128[c]	130[c]	144[d]	
	124	133	139[d]	
M35 + P1 — 7103	126	101	110[d]	
	129[c]	119[e]	120[d]	
Strains not infected				
1802	0	0	0	
	0	0	0	
P1KN/1800	0	0	0	
	0	0	0	
35KN-a	0	0	0	
	0	0	0	
M35	0	0	0	
	0	0	0	

[a] M.o.i. = 0.1. Adsorption was 45′ at 21 °C
[b] Period of incubation was 3 days
[c] All were *his*+
[d] All were *pro*+ *arg*+
[e] All were *pro arg*

significant frequencies of prototrophs amongst themselves. It is thus quite evident, in view of the results, that some types of change related to P1CMrec were initiated in the M35 chromosome and that such changes can allow or induce a major enhancement in prototroph production, even without the presence of the plasmid initially responsible for those changes. What is the nature of those changes? A significant clue is offered by a phenotypic characteristic of 35KN-a.

Inocula from the twenty-five 35KN-a colonies and from the 35KN-a isolate-culture grew on chloramphenicol plus complete media, though clearly not as well as either M35 or 1802. Inocula from P1KN/1800 and P1KN/1802 (a lysogen originally carrying P1CM, but which was replaced by P1KN) did not grow on such media. This indicates that 35KN-a carries a factor (or factors) giving 35KN-a a significant degree of resistance of chloramphenicol, and that this factor (or factors) was derived or originated from the P1CMrec plasmid.

That this factor of factors must be within the 35KN-a chromosome follows from the fact that upon induction and lysis, 35KN-a does not yield a lysate bringing about high-frequency-transduction (HFT) for CM; it does yield a HFT for KN, however. It would if the chloramphenicol resistant factor (or factors) was integrated into the P1KN plasmid.

It is indicated in view of what has been found that the chloramphenicol resistant factor (or factors), derived from the original P1CMrec plasmid, and integrated into the 35KN-a bacterial chromosome, is a manifestation or cause of those changes in the M35 (or 35KN-a) chromosome responsible for the enhanced production of prototrophs. More will be said about this *CM* factor (or factors) in the Discussion. Shortly, the phrase "P1CMrec mediated (or generated) mutability" will be used. This will mean mutability which is generated by means of genetic segments having their origin in the PlCMrec plasmid.

II. *Other genetic changes induced by PlCMrec within the bacterial chromosome*

Acquisition of the chloramphenicol resistant factor(s) by the M35 chromosome is only one of the changes that occurred within the M35 chromosome. It was also found that M35 had aquired additional auxotrophies, as inferred from the fact M35 did not grow on medium supplemented with all nutrients required by 1800, from which M35 was derived. Also, M35 was found to be no longer str^R. Moreover, the original chromosome of M35 (or 1800) carried *mal* and *xyl*. Now, upon incubation, most colonies of M35 were mal^+, while the remaining colonies were *mal*. Also, all resulting colonies of M35 were now found to be xyl^+. Another P1CMrec lysogen, M294, referred to earlier, was also found to have acquired additional auxotrophies, as well as being str^S. Additionally, all M294 colonies were now xyl^+, while many colonies were *mal*, the remaining being mal^+. In testing 6 other P1CMrec lysogens (most of those referred to in Table III), 4 cultures each differed regionally for utilization capacities of maltose and xylose respectively, as indicated by stained regions within a given culture; and 2 cultures were both *mal xyl* (1802 was still *mal xyl*). Furthermore, these 6 lysogens, M294, and 5 other PlCMrec lysogens (included in this 12 are all strains referred to in Table III) were each lac as 1800, the strain into which PlCMrec was introduced (1802 is still *lac*). However, these same 6 strains (and 6 other tested P1CMrec lysogens) are now str^S, unlike 1800 and 1802 (PlCM) which are still str^R. Moreover, out of these tested 12 P1CMrec lysogens, including M35 and M294, all were found to have acquired additional requirements. Whereas, out of a tested sample of 21 other types of mutant-lysogens (these to be the subject of other papers), obtained from the very same population of transductants which included the original P1CMrec group, only 5 were found to have additional requirements. Subsequently, one of those 5

lysogens, M446, was also found to carry *rec* on its CM plasmid. Because of the nature of the other 4 mutant-lysogens, it is possible those carried an undetectable *rec* factor on their plasmids. Moreover, out of a large sample of the mutant-lysogens which are not P1CMrec, all were still str^R, as was 1802. Such overall results clearly indicate that the creation of additional requirements and the negation of str^R is linked with the P1CMrec plasmid. It also follows from the data that the induction of a stable xyl^+ phenotype and the variation in maltose and xylose utilization (i.e., the instability in such utilization-capacity) was determined by the P1CMrec plasmid. It is also to be noted that all members of a sample of the mutant-lysogens not P1CMrec, and not having additional requirements, did not respectively produce a high frequency of prototrophs upon virion-DNA incorporation. The respective frequencies were comparable to those of the frequency of prototrophs amongst 1802 subsequent to virion-DNA incorporation.

It was found that M35 had only acquired additional amino-acid requirements: *trp*, *try*, *phe*, *leu*, *ilv*, and *thr*. Most important, M35 still had its original auxotrophies (also, still *lac*) namely, *his*, *pro*, *arg*, and *met*. Furthermore, M294 and ten other P1CMrec lysogens were each found to have the *same* additional amino-acid requirements as M35. M294 was found to still be *pro arg his met*. Testing a sample of the other P1CMrec lysogens for the afore-mentioned auxotrophies, all members were still *pro arg his met*. Mutant-changes in M35 and M294 responsible for the additional auxotrophies mapped approximately 0′–5′ from the origin. Genes for such auxotrophies are known to exist within such a map region [3].

IV. *P1CMrec generated mutagenesis leading to prototrophy; the enhancement of such mutagenesis by generalized transduction; the connection of enhancement to growth-period*

Because from the earliest experiments M35 was found to have the additional auxotrophies based upon the genetic changes within the M35 chromosome, the high-frequency and uniform generation of prototrophy to his^+, pro^+, and arg^+ would also be (in all experiments) the high-frequency negation of the additional auxotrophies, as well. This generation and negation would point to far-reaching genetic changes, which could only have their basis in some type of generally enhanced mutability, a mutability somehow related to the P1CMrec plasmid and to virion-DNA incorporation, or generalized transduction. (Mutability resulting in arginine and proline prototrophy would have to occur in the non-deleted genetic regions.) To investigate this question further, additional experiments were carried out, in which, among other things, the

spontaneous mutability of non-infected M35 was further studied. Since all requirements of M35 were now known, it was now possible to observe the reversion frequency for particular genetic regions, or phenotypic characteristics, on appropriately supplemented media (referred to as 13 all-x media in addition to other nutrients used previously, this media contained phenylalanine, tyrosine, tryptophane, leucine, isoleucine, valine, threonine, and adenine, minus any given amino-acid(s). In non-infected strains, the mutability of two of the mutant-regions, *leu* and *trp*, responsible for two of the additional requirements, was studied as well as the mutability of *his*, *pro*, (mutability resulting in the negation of proline requirement, see Materials and methods), and *arg* (mutability resulting in the negation of arginine requirement). Also, the degree of prototrophy to *leu*$^+$ and *trp*$^+$ upon virion-DNA incorporation by M35 was studied, transducing as well a known *leuA trp* strain, 8330, as an indicator of the highest level of *trp*$^+$ *leu*$^+$ prototrophic-production basically due to transduction. Though *trp* in 8330 is located in a different chromosomal region with respect to the mutant *trp* region in M35, non-closely linked genes or genetic regions have almost the same probability of being transduced by way of Pl, that is, they are transduced with about equal frequency [1, 15, 16], thus using 8330 is a valid control or reference. In fact, control data in all tables of this paper collectively indicate this. The locus of the *leuA* gene of 8330 is close to the newly generated *leu* region of M35, both being near the origin. Overall results of the latest study are in Table VIII.

It is clear that upon 2–3 days incubation *leu* in M35 is significantly more mutable than *leu* in 8330. No significant difference exists between the degrees of *trp* mutability in both strains. The degree of *his*, *pro*, and *arg* mutability was far greater in M35 than in 1802. It is strongly indicated that parts of P1CMrec have greatly increased the mutability to *his*$^+$, *pro*$^+$, and *arg*$^+$. When one compares this data with data in Tables IIa, IIb, V and VII, where the frequencies of *pro*$^+$, *arg*$^+$, and *his*$^+$ prototrophs upon virion-DNA incorporation are presented, it is seen that such incorporation must have further enhanced *pro*, *arg*, and *his* mutability. The presence of P1CMrec has not only created a mutant *leu* region, but it has made such a region more mutable to prototrophy than *leu* in another strain. A response by the parts of the P1CMrec plasmid to virion-DNA incorporation is to make *leu* in M35 even more mutable to prototrophy. The greatest enhancement of such mutability occurs within 0–2 days. The presence of PlCMrec's parts (or their induced changes) in conjunction with virion-DNA incorporation is responsible for a major enhancement in the frequency of *trp*$^+$ prototrophs. This major enhancement occurs in 0–2 days. In view of this and subsequent data, the process of virion-DNA incorporation somehow allows P1CMrec's chromosomally-incorporated parts (or induced changes) to greatly enhance mutability to *leu*$^+$, *trp*$^+$, *pro*$^+$, *arg*$^+$, *his*$^+$, and *met*$^+$. Most *leu*$^+$, *trp*$^+$, *his*$^+$, *pro*$^+$ and *arg*$^+$ colonies observed upon virion-DNA

Table VIII

Frequencies of prototrophs on 13 all-x media obtained with and without P1 virion infection of specific strains

Strains not infected[b]	Frequency of prototrophs (No. of prototrophic colonies per plate — 4×10^7 bacteria per plate)					
	Selection for:			:Prototrophic class		
	pro$^+$	*arg*$^+$	*pro*$^+$ *arg*$^+$	*leu*$^+$	*trp*$^+$	*his*$^+$
8330	—	—	—	9	15	—
				10	12	
1802	0	0	0			0
	0	0	0	—	—	0
M35	29	27	30	33	8	24
	31	32	28	31	9	22
Strains infected and virion class used[a,b]						
8330 + P1 — 7103	—	—	—	18	10	
				16	12	
M35 + P1 — 7103	—	—	—	50	48	—
				54	50	
Strains not infected[c]						
8330	—	—	--	9	15	
				10	12	
1802	0	0	0	—	—	0
	0	0	0			0
M35	30	28	32	33	8	25
	32	33	30	31	9	24
Strains infected and virion class used[c]						
8330 + P1 — 7103	—	—	—	18	10	—
				16	12	
M35 + P1 — 7103	—	—	—	62	59	—
				67	63	

[a] M.o.i. = 0.1. Adsorption for 45′ at 21 °C
[b] Two days incubation
[c] Three days incubation

incorporation were much larger for the same incubation-period than the prototrophs of lysogens not involved in virion-DNA incorporation. This indicates that virion-DNA incorporation not only enhances P1CMrec mediated (determined) mutability, but somehow allows it to occur earlier within a given period.

V. *Enhancement of mutability mediated by PlCMrec, its dependency on time and infection: further examples*

In order to determine other ways in which PlCMrec-mediated mutability is related to growth-period, other experiments were performed, in which the behaviour of other genetic regions was also studied. Results are in Tables IX, X and XI. It is again seen that spontaneous mutability to *pro*$^+$ and *arg*$^+$ is

Table IX

Frequencies of prototrophs on 13 all-x media obtained with and without P1 virion infection of specific strains

Strains not infected[b]	Frequency of prototrophs (No. of prototrophic colonies per plate — 3.6×10^7 bacteria per plate)				
	Selection for:				:Prototrophic class
	arg$^+$	*pro*$^+$	*pro*$^+$ *arg*$^+$	*met*$^+$	*met*$^+$ *ilv*$^+$ *leu*$^+$ *trp*$^+$
1802	0	0	0	0	—
	0	0	0	0	
M35	30	36	35	10	0
	28	31	32	8	0
Strains infected and virion class used[a,b]					
1802 + P1 — 7103	0	1	0	29	—
	0	0	0	25	
M35 + P1 — 7103	71	51	65	60	59
	73	53	68	57	61
Strains not infected[c]					
1802	0	0	0	0	—
	0	0	0	0	
M35	87	62	74	15	0
	92	69	81	18	0
Strains infected and virion class used[c]					
1802 + P1 — 7103	0	1	0	36	—
	0	0	0	39	
M35 + P1 — 7103	161	145	163[e]	77	76
	175	157	171[d]	82	83

[a] M.o.i. = 0.1. Adsorption was for 50′ at 21 °C
[b] Two days incubation
[c] Three days incubation
[d] Found all to be *his*$^+$ when tested for histidine requirement, only
[e] Found all to be *his*$^+$ *ilv*$^+$ *met*$^+$ *trp*$^+$

Table X

Frequencies of prototrophs on 13 all-x media obtained with and without P1 virion infection of specific strains

Strains not infected[b]	Frequency of prototrophs (No. of prototrophic colonies per plate — 4×10^7 bacteria per plate)				
	Selection for:				:Prototrophic class
	leu$^+$	*trp*$^+$	*ilv*$^+$	*met*$^+$	*met*$^+$ *ilv*$^+$ *leu*$^+$ *trp*$^+$
8330	0	7	—	—	—
	0	5			
1802	—	—	—	0	—
				0	
M35	45	4	6	9	0
	42	4	7	8	0
Strains infected and virion class used[a,b]					
8330 + P1 — 7103	26	19	—	—	—
	23	17			
1802 + P1 — 7103	—	—	—	12	—
				15	
M35 + P1 — 7103	44	92	47	78	72
	48	85	51	73	76
Strains not infected[c]					
8330	0	7	—	—	—
	0	5			
1802	—	—	—	3	—
				1	
M35	61	5	16	17	0
	58	4	14	15	0
Strains infected[c]					
8330 + P1 — 7103	26	19	—	—	—
	23	17			
1802 + P1 — 7103	—	—	—	25	—
				14	
M35 + P1 — 7103	61	100	55	88	85
	55	96	60	84	87
Strains not infected[d]					
8330	0	7	—	—	—
	0	5			
1802	—	—	—	3	—
				1	
M35	78	8	29	30	3
	83	9	26	27	0
Strains infected and virion class used[d]					
8330 + Pl — 7103	26	19	—	—	—
	23	17			
1802 + Pl — 7103	—	—	—	25	—
				16	
M35 + Pl — 7103	74	105	94	126	107
	79	98	97	115	100

[a] M.o.i. = 0.1. Adsorption was for 45′ at 21 °C
[b] Two days incubation
[c] Three days incubation
[d] Seven days incubation

Table XI

Multabilities of PlCMrec lysogens on 13 all-x media upon an extended period of incubation

Strains[a] not infected	Frequency of prototrophs (No. of prototrophic colonies per plate — 4×10^7 bacteria per plate)						
	Selection for:					:Prototrophic class	
	pro+ *arg*+	*his*+	*met*+	*thr*+	*trp*+	*phe*+	*tyr*+
M35	129	54	31	1	8	0	0
	138	60	28	0	7	0	0
M294	70	89	35	0	6	1	0
	74	83	30	1	8	2	1
M364	33	97	41	3	9	0	1
	30	95	39	1	9	0	1
1802	0	0	0	—	—	—	—

[a] Six days incubation

markedly enhanced by the parts of PlCMrec or their effected genomic changes. Moreover, the degree of such mutability is a function of growth-period, a major increase in *pro arg* mutability occurring within a 2–3 day period. Furthermore, it is seen that virion-DNA incorporation enables in some manner P1CMrec's parts or their effected changes, to further enhance such mutability (i.e., that of *arg*, and *pro*). The degree of such further enhancement is also contingent upon growth-period, a very large increase in *pro arg* mutability occurring within a specific period, i.e., 2–3 days. Also, judging from the comparative sizes of prototrophs, of different classes, noted between 0–2 days, it appears that within that period, the effect of virion-DNA incorporation is to allow the parts of P1CMrec to evoke mutability at a time earlier than one in which mutability would occur without such an effect.

Including data from Table XI, where M35 was grown for an extended period (6 days), one sees that upon such an extended period, the overall degree of mutability of *pro arg* evoked by the parts of P1CMrec, without the influence of virion-DNA incorporation, has become equivalent to the degree of such mutability occurring within a much earlier period, when virion-DNA incorporation enhances the mutagenic action of P1CMrec or its induced-changes. Virion-DNA incorporation has the effect of inducing the parts of P1CMrec to eventuate far more *pro arg* mutability at an earlier period in general growth than they otherwise would do without the influence of such virion incorporation. Thus, taking all data into account, the influence of virion incorporation, or likely generalized transduction, on P1CMrec-mediated *pro arg* mutability is not only to enhance it, but to enhance it within a much shorter period of general growth.

As with *pro arg* mutability in M35, extended growth is associated with a marked increase in *his* mutability in M35 (Tables VIII and XI). This, in

connection with other data related to the effect of virion incorporation on P1CMrec-mediated *his* mutability, would suggest that virion incorporation allows major increases in such *his* mutability to occur much earlier in growth. Upon increased incubation, the degree of *his* mutability in M35, though quite high, is seen without infection, to be less than one-half that of *pro arg* mutability (Table XI).

Met mutability to prototrophy appears greater in M35 than in 1802, indicating the mutagenic effect of P1CMrec's sections. It is also clear that virion-DNA incorporation as a process markedly enhances the degree of *met* mutability that can be evoked by the parts of P1CMrec (Tables IX, X). In other words, virion-DNA incorporation, very probably in the form of generalized transduction, triggers the chromosomally-incorporated parts of the plasmid to evoke far greater mutability of *met*. This enhancement of *met* mutability is greatest during the 0–2 day period of three days growth (Tables IX and X). This enhanced *met* mutability increases as in non-linear function of growth-period. During seven days incubation approximately 60% of such enhanced mutability occurs during the 0–2 day period. Thus, not only does virion-DNA incorporation enhance P1CMrec-mediated *met* mutability, but allows or stimulates it to occur primarily during early growth. Even without infection, extended incubation is associated with an increase in *met* mutability, judging from the increase in the frequency of prototrophs over time (Tables IX, X and XI). This information, in comparison with other data related to the effect of virion-DNA incorporation on P1CMrec-mediated *met* mutability during a given growth period, would suggest that virion-DNA incorporation allows major increases in such *met* mutability to occur much earlier in general growth. In general, enhanced mutability by way of virion incorporation would appear to occur earlier than non-enhanced P1CMrec-mediated mutability within a given period.

Upon increased incubation, and without infection, *met* mutability (in. M35) appears less than *his* mutability, which is certainly less than *pro arg* mutability in M35 (Table XI). *Thr* is the least mutable of all, appearing quite stable (Table XI). *Trp*, *phe*, and *tyr* have very low mutabilities upon extended incubation (6 days), comparable to that of *thr*. When incubation was for 8 days, the same relative difference between P1CMrec-mediated mutabilities were noted, without infection (data not tabulated). Namely, *pro arg* mutability was the highest, the degree of *his* and *leu* mutability being about 1/2 that of *pro arg* mutability, and *met* and *ilv* mutability being about 1/2 that of *his* mutability, with *thr*, *phe*, *tyr* and *trp* displaying very little (if any) mutability.

In another experiment (data not tabulated), M35 was grown for 7 days. No infection was involved. Arg^+ pro^+ and his^+ prototrophs were noted. The frequency of arg^+ pro^+ prototrophs was very high and the frequency of his^+ prototrophs was about 50% of the former. Upon replication, it was found that

55% of the *arg*$^+$ *pro*$^+$ prototrophs were *his*$^+$, 42% were *leu*$^+$, 31% were *ilv*$^+$, and 10% were *trp*$^+$. P1CMrec-mediated *his* and *leu* mutability is thus again shown to be about 1/2 that of *arg pro*, *ilv* mutability being about 1/3 that of *arg pro*, and *trp* being the least mutable of all. In a subsequent test, it was found that a small percentage of the *arg*$^+$ *pro*$^+$ prototrophs was *met*$^+$. Data in Table IX also indicate that *arg* and *pro* in M35 are far more mutable than *met* in M35. Data in Table IX further indicate that the respective, P1CMrec-mediated mutabilities to *arg*$^+$ and *pro*$^+$, where virion incorporation is not involved, are of equal degrees. This is also the case where P1CMrec-mediated mutability to *pro*$^+$ and *arg*$^+$ is enhanced through virion-DNA incorporation.

It is again seen that *trp* mutability, though initially low, is greatly enhanced through a process dependent upon virion-DNA incorporation. It can be seen that the degree of greatest enhancement occurs during 0–2 days. Moreover, the degree of enhanced mutability of *trp* is comparable to that of *met*. In fact, following virion-DNA incorporation by M35, the frequency of *ilv*$^+$ *trp*$^+$ *met*$^+$ *leu*$^+$ prototrophs is comparable to the respective frequencies of *ilv*$^+$, *met*$^+$, and *trp*$^+$ prototrophs amongst M35. The frequencies of these respective prototrophic classes are very high (Tables IX and X). The major enhancement of P1CMrec-mediated mutability by virion-DNA incorporation has resulted in the same degree of prototrophic change for various genetic regions, the mutations very probably occurring near the same time.

At this stage of the work (Table X), P1CMrec-mediated *leu* mutability is now very high without the influence of virion-DNA incorporation. This suggests further enhancement of *leu* mutability does not always require the influence of virion-DNA incorporation. Though from the earliest results, virion incorporation appears to modify *leu* mutability in such a way where it is synchronous with the enhanced mutability of other genetic regions. Certainly, (Tables IX and X) the influence of virion-DNA incorporation is to greatly increase the level of mutability of various genetic regions, such as *met* and *ilv*, to a point where it is comparable to that of *leu* mutability. It is appreciated in the case of *ilv*, no *ilv* strain other than M35 was transduced (or infected) as a reference. However, taking into account what has been said regarding the equal probability of transduction of a given chromosomal marker and the control data in this paper pertaining to such, it can be concluded that *ilv* mutability is also enhanced by virion-DNA incorporation or likely generalized transduction.

Conclusions

The general enhancing effect of virion-DNA incorporation, or very likely generalized transduction, upon the P1CMrec-mediated mutability of many different genetic regions, in which, some (if not more) are widely separated in

the chromosome (genome) of M35, is very evident. This is so, when in view of recent findings, one looks at the data from the earliest experiments (Tables IIa, IIb, III, IV, V and VII), in which the very high frequencies of *pro*$^+$, *arg*$^+$, and *his*$^+$ prototrophs were also in effect and respectively very high frequencies of prototrophs of the *trp*$^+$ *phe*$^+$ *thr*$^+$ *leu*$^+$ *tyr*$^+$ *ilv*$^+$ class, on media which was not 13 all-x. Thus, the major enhancement of *pro*, *arg*, and *his* mutability occurred along with the major enhancement of respectively *trp*, *tyr*, *phe*, *thr*, *leu*, and *ilv* mutabilities. The degree of the latter mutabilities was apparently comparable to that of the enhanced *pro arg his* mutability, and hence comparable to one another. Furthermore, with the influence of virion-DNA incorporation, it was found that all 163 *pro*$^+$ *arg*$^+$ prototrophs referred to in Table IX were also *ilv*$^+$ *his*$^+$ *met*$^+$ *trp*$^+$ prototrophs, again suggesting, in view of earlier data, all differing mutabilities were enhanced to a comparable level upon virion incorporation. In this connection, we have seen that the differences between the degrees of *met* and *trp* mutability, between *leu* and *trp* mutability, and between *leu* and *met* mutability also cease to exist upon virion-DNA incorporation. In the earliest experiments, we have seen that, upon virion-DNA incorporation, all *arg*$^+$ and *pro*$^+$ prototrophs amongst M35 were also *his*$^+$, and conversely, all *his*$^+$ prototrophs on media lacking histidine were *arg*$^+$ *pro*$^+$. This further indicates that different P1CMrec-mediated mutabilities were enhanced to a comparable level upon virion-DNA incorporation. Thus, in enhancing or stimulating P1CMrec-mediated mutability, virion-DNA incorporation negates the major differences between the different gene mutabilities, differences which were previously shown to exist without the influence of virion-DNA incorporation. This also means, in view of all data, that virion-DNA incorporation not only greatly increases P1CMrec-mediated (or controlled) mutabilities (in M35) and promotes them to occur earlier in time, but greatly makes uniform such mutabilities for various genetic regions, irrespective of what previous differences existed in their mutabilities, and irrespective of the location of such regions. Furthermore, considering the very low mutabilities of *trp*, *phe*, *tyr*, and *thr* in M35 (Table XI), past data pertaining to the influence of virion-DNA incorporation upon the relative stability of these genetic regions in M35 would indicate that such virion incorporation evokes the chromosomally incorporated parts of the P1CMrec plasmid to uniformly initiate mutabilities of such regions to the same very high level.

In M294 and M364, the presence of P1CMrec also leads to the generation of high mutabilities to *pro*$^+$ *arg*$^+$ and *his*$^+$, some increased mutability to *met*$^+$, with no effect on the relative stabilities of *trp*, *phe*, *tyr*, and *thr* (Table XI). In contrast to M35, the engendered mutability in M294 to *his*$^+$ is comparable to that mutability to *pro*$^+$ *arg*$^+$, while in contrast, *his* mutability in M364 is far greater than *pro arg* mutability. In repeating this experiment, comparable results pertaining to different strain-mutabilities were obtained, it also being

found again that in M294 and M364, as in M35, *phe*, *tyr*, *thr*, and *trp* displayed very little, if any, mutability.

This data again show that the chromosomally incorporated parts of the P1CMrec plasmid, or the induced changes due to such incorporation, generate high mutability in particular genetic regions of strains whenever such a plasmid has maintained itself in such strains. In view of early data (Tables III and VII), it is thus clear that virion-DNA incorporation, operating through P1CMrec's parts, greatly enhances or evokes mutabilities in other strains. Views as to the means by which such a plasmid can generate various mutabilities and why such P1CMrec-mediated or controlled mutability is greatly influenced by virion-DNA incorporation, or very likely generalized transduction, will be presented in the Discussion.

Discussion

When the very high frequency of prototrophs among M35 lysogens, compared to that of 1802 prototrophs, was first observed following virion-DNA incorporation or generalized transduction from a prototrophic strain, it was originally thought that the particular plasmid carried by M35, P1CMrec, greatly enhanced in some manner the actual or normal frequency of generalized transduction, that is, enhanced the frequency in which donor genetic material was incorporated into the recipient's genome. However, when one considers collectively all the data presented in this paper, effectively pooling the data, such an inclusive consideration makes it quite clear this was not the case. There was definitely an enhancement in the degree and scope of genomic change in lysogens carrying the particular plasmid, P1CMrec, upon virion-DNA incorporation, but this had its basis in two connected processes not involving the enhancement of transduction. First, P1CMrec indirectly generated or mediated major increases in the mutabilities of various genetic regions throughout the *E. coli* genome, some regions being widely separated from one another, and second, those mutabilities were in turn further enhanced in some manner through the process or influence of virion-DNA incorporation or likely generalized transduction. As earlier experiments pointed out, these broadly occurring mutabilities were generated by or resulted from genomic changes within the *E. coli* chromosome induced by P1CMrec, and there was strong indication that those changes involved incorporation of a part or parts of the P1CMrec plasmid into the bacterial genome.

The chloramphenicol region of P1CMrec was involved in the incorporation, but even so, when lysates of (from) M35 are prepared, such lysates give rise to a high frequency of CM transductants, indicating that P1CMrec still retains a factor conferring resistance to chloramphenicol. This shows that *CM* has a multilocus basis and that parts of this multilocus can be transposed. In *Pro-*

teus mirabilis, there is a plasmid which contains multiple, tandem copies of the gene conferring resistance to chloramphenicol [17]. Part of this region can remove and re-insert itself. This is a type of transposition.

The *CM* multilocus in P1CMrec is additive in its effects. This is suggested by the fact that 35KN-a, the strain not carrying P1CMrec, but resistant to chloramphenicol, displayed a degree of resistance to chloramphenicol which was clearly less than that displayed by either M35 or 1802. It is quite probable that transposition elements, transposons, from the *CM* multilocus and from other P1CMrec regions were released as a group by means of intra-molecular recombinations between repetitive ends, possibly through the agency of the *rec* factor, and in turn incorporated through recombinations into various regions of the *E. coli* chromosome, leading to the creation of the mutable regions determining the original auxotrophies and to the generation of the new mutant and mutable regions. Alternatively, though not mutually exclusive, such transposons could have been incorporated as a group into various regions of the *E. coli* chromosomes by means of recombinations between plasmid and chromosome, leading to the mutator effects.

It is known that plasmids such as P1 and Gram-negative bacteria such as *E. coli* have very small genetic segments capable of transposition within a given genome or from one genome to another, and these segments have been referred to as IS insertion sequences [18]. It is also known [18] that R factors have small translocatable drug-resistance elements (larger than IS sequences) capable to transpositions to other plasmids and to the chromosomes of Gram-negative bacteria, where in the latter, they are capable of inducing mutation. In Pl, IS sequences (ISl) have in fact been found located at or near the site of the *CM* marker [19], this site being referred to as *cat-gene 2* [20].

In bacteria, a transposon, Tn9, exists which carries a *cat* locus [21]. And evidence indicates some Tn elements (at least) in bacteria are transposed by means of particular types of recombination between genomic entities [22].

Transposable genetic elements with mutator effect have also been found in other organisms, such as protozoa [23, 24], insects [25–28], nematodes [29], maize [30–32], and yeast [33–37]. Transposon-like elements also exist in humans, monkeys, and prosimians, but so far, no mutator effect has been noted in vivo [38, 39].

A specific example of transposon phenomena in insects occurs in *Drosophila mauritiana*. In this organism, a mutation resulting from the insertion of a transposon exhibits genetic instability. This instability is greatly enhanced in the presence of a trans-acting autosomal factor [26] and has a maternal effect when transmitted to another *Drosophila* species. If this autosomal factor gives rise to a transposon, this situation would show a clear parallel to the *E. coli*-P1CMrec system, as would the situation in *Drosophila simulans* wherein a transposon introduced into the genome via transfection induced a mutation

effect of another transposon [28]. An analogous situation also prevails in *Drosophila melanogaster* [27]. In this connection, transposable elements from the T1 plasmid residing in higher plant cells (after infection by agrobacteria) become inserted into the plant chromosome, generating mutations leading to tumours [40]. In animal and human cell lines, transposons, or transposon-like elements, have been implicated in neoplasia through the process of insertional mutagenesis [41]. And in human lymphoblastoid cell lines, the human transposable element, THE-1, is present [39]. In general, the various transposons found in different organisms can induce various genetic changes through their insertion in and removal from chromosomes, and a number can do so in a regulatory manner. Many of these genetic changes can affect or regulate gene expression; and thus in a related mode, transposons can behave as factors in genetic regulation [24, 32].

Transposons from various organisms are similar to one another in molecular/genetic structure [32]. For example, the Tn3 transposon in bacteria and the Ac controlling element in maize have similar genetic/molecular organization [32]. These transposons and others produce an enzyme, referred to as transposase, apparently, a type of recombinase involved in transposition [22, 24, 32]. It and related enzymes appear to be involved in various types of recombination-events necessary for transposition [22, 24, 36, 42]. A number of these events would be related to the type of recombination-events involved in the release and incorporation of a prophage such as lambda [43], though some transposon-systems utilize another type of recombination known as gene conversion [24]. Various transposons may in fact be related to or derived from viruses, inasmuch as the structure of retroviral DNA resembles the structure of transposable elements present in bacteria, yeast, and insects [42]. Proviruses (the integrated retroviral DNA) exhibit insertional mutagenesis and excision by recombination between terminally repeated sequences, as "expected of other transposable elements" [42]. In maize, a certain mutator activity was found to be caused by the transposition of various bacteriophage elements, known as Mu [44–47]. Small segments of Mu (mini-Mu) also transpose in bacteria [47], and Mu elements are known to have a mutator effect in *E. coli* [47]. In this connection, an invertible region of Mu, possibly a mini-Mu, was found to be identical in DNA sequence to that of an invertible region found in P1 [48]. Perhaps such a mini-Mu is also implicated in the P1CMrec-*E. coli* mutator system.

With regard to the various transposon-systems, the picture is still left incomplete, especially in the following, important respect. How are the behaviour and molecular organization of respective transposons subsumed under the general and local regulatory constraints guiding and structuring transposon phenomena? Such regulatory constraints are quite relevant to the *E. coli*-P1CMrec system, and the subsequent discussion will address this matter.

In this regard, it is theorized [49] that various transposons from P1CMrec integrated into various, widely spread regions of the *E. coli* chromosome, causing mechanical distortions, configurational changes in the genome, leading to dynamical stresses or non-uniform pressures within regions through the *E. coli* genome resulting in non-conformity in replication (or misreplication) and misrepair, with the consequent generation of high mutability and new mutant genes, some of which being also mutable. The fact that the same mutant changes (to *leu*, *ilv*, *thr*, *phe*, *trp*, *tyr*, and to str^S) occurred in 12 independently arising transductants, each arising from the separate infection and lysogenization by a different P1CMrec phage, points to a directed or programmed generation of new mutations by PlCMrec. (Being in the same amino-acid family, *phe*, *trp* and *tyr* requirements might be due to one pleiotropic mutation.) In eight such strains, the generation of similar mutant changes involved in the utilization of particular carbohydrates also supports the conclusion of directed mutation by PlCMrec. Inasmuch as these new mutant genes, with the exception of str^S, mal^+, and xyl^+, the latter two being highly mutable, mapped within the same relatively small section in two tested strains, it is quite possible that this directed mutation in the P1CMrec lysogens can be explained (in part) by given transposons from P1CMrec plasmids always being dynamically drawn to and integrated, by means of recombination, within the same *E. coli* chromosomal regions of given configurations, with the consequent generation of the same fields of non-uniform force configurations within the genome, leading to the same genomic changes. Also, particular recombinations between plasmid and chromosome at specific configurational regions could account for such integrations, leading to directed mutator effects. The generation of respectively high mutabilities of *his* and *pro arg* in respectively M35, M294, and M364 would also suggest that in these cases the generation of mutability was directed, though it is appreciated that the degree of such varies with the strain, suggesting that the integration of the transposons was not in exactly the same places in the different strains.

As one will recall, the genetic section including *proA*, *proB*, and *argF* is deleted in the P1CMrec lysogens, as is a region including the *argI* locus. In this connection, one may ask how there could be high-frequency reversion to arg^+ and pro^+ prototrophy if the genes for *proA*, *proB*, *argF*, and *argI* are deleted. Clearly, as indicated earlier, the reversion to pro^+ and arg^+ involves another genetic region (or regions), possibly one(s) between the ones that have been deleted, and that a transposon, by integrating into that region(s), which would not be far from either the *argI* deletion or the deletion including *proA proB argF*, has made it unstable, with the consequent suppression of *pro arg* auxotrophy. The location of this presumed, unstable, suppressor-like region (or regions) could very well be near the section where the other, newly created mutant genes map (this would be close to both [*argI*] and to [*proA proB*

arg F], and the directed creation of such mutant genes could have occurred along with the directed creation of the presumed unstable *pro arg* suppressor. (The site of *mutT* mutator gene [3, 50, 51] also maps close to the region of the presumed unstable suppressor and it is possible that a newly created *mutT* mutator gene in PlCMrec lysogens due to an insertion of a transposon is also implicated in *pro arg* mutability). In this connection, it is possible that some of the other, induced mutabilities, such as that of *his*, may also involve regulatory or suppressor-type regions. As noted earlier, transposons in other organisms do in fact implicate genetic regulatory regions, or function as extensions of genetic regulation. And enhanced mutagenesis invoked in the *E. coli*-P1Mrec system generally occurred within a specific period, suggesting a regulation.

In *Salmonella*, there is a generally non-revertible strain incapable of leucine biosynthesis due to the deletion of a gene involved in the leucine-biosynthesis pathway. However, certain point mutations were found to restore the ability of this strain to synthesize leucine [52]. Thus, in *Salmonella*, there exists an avenue, most likely regulatory, which can permit or allow the generation of a degree of *leu* mutability to prototrophy even though a gene involved in leucine biosynthesis is deleted. It is quite possible that such *leu* mutability could be enhanced. In fact, there exists in *Salmonella* a plasmid, different from P1, which can generate high mutability within a specific locus, and when introduced into *E. coli*, this same plasmid can also generate high mutability within a specific locus [53]. In *E. coli*, this plasmid, pKM101 enhances the mutagenic effect of a chemical mutagen and UV on this particular locus, and it was postulated that this plasmid acts mutagenically by increasing the process of misrepair.

Where this other plasmid in *E. coli* enhances the mutagenic effects of known mutagens or mutagenic processes, we have seen that the P1CMrec mutagenic effect is itself greatly enhanced by another process, and this one not even mutagenic itself. It is not certain how this process of P1-virion-incorporation does this. First, is it enough for virion-DNA from the donor bacteria to merely enter and reside in the recipient's cytoplasm for the enhanced mutability to be eventuated? Unlikely, as such exposed linear DNA would be digested by DNase. Or, second, must DNA fragments of donor bacteria (included among the virion-DNA) integrate into the recipient chromosome in order for the enhanced P1CMrec-mediated mutability to take place? In view of the postulated and quite possible dynamical basis for P1CMrec-generated mutability, it would seem that the integration of additional (bacterial) genetic material into a chromosome, already made dynamically distorted or stressed (dynamically non-uniform) by previous integration of transposons into critical regions, would have the chromosome incur further distortions (or configurational changes) and hence additional internal, dynamical stresses, making it even more dynamically non-uniform in various regions, with the consequent generation of even greater, though uniform, mutability. It is not clear why this additional integra-

tion would make uniform the increased mutabilities, unless the degree of increased internal, dynamical stress was made uniform for all the genetic regions in question.

A significant implication of the foregoing is that insertion of genetic elements can have a global and co-ordinate, mutagenic effect on a chromosome as a whole. This is especially so as some of the sites of induced, co-ordinated mutagenesis were widely separate from one another. A further implication of this and the data is that the insertion of a given transposon or genetic element can have a mutagenic/regulatory effect far beyond its site of insertion. That is, it can eventuate a controlled mutagenesis at a distance through which, among other genetic effects, gene expression could become active or inactive. This would suggest more of a physical process being involved than a chemical one, though the latter is seen as a significant complement, or marker, nevertheless.

Such insertions of genetic elements into new genetic regions are a type of genetic-re-arrangement, itself a genetic change. In this situation, these are genetic re-arrangements which have regulatory/mutagenic effects on the chromosome beyond the genetic loci wherein they are inserted.

This phenomenon is analogous, if not related, to position-effect variegation (P-EV), a phenomenon known for many years [54]. When a genetic rearrangement involving heterochromatin — an intensely coiled section of the chromosome — takes place, active genes, many units distal to the site of heterochromatin breakage, become repressed in sequence, and thus in a polarized fashion, as the regions they are in become progressively heterochromatic. There is a polarized spreading of the heterochromatic state from the site of heterochromatin breakage, i.e., the site of heterochromatin re-arrangement, to the euchromatic region of the chromosome. And phenotypically, this is expressed as variegation. Many cases of genetic instability are derived from or implicate chromosomal re-arrangements involving heterochromatin (for a discussion of this, see 55), including the controlling elements in maize [30]. In some organisms, heterochromatin, itself, is subject to preferential loss at particular periods and in specific regions as an aspect of development [56].

In the Ac-Ds system in maize, a type of P-EV can be generated in turn. For example, the Ds element, once inserted in a given position, brings about an unstable expression or mutation to a series of genes adjacent to the insertion site [57], this effect thus spreading from the position of the Ds element.

In yeast, a type of P-EV is also related to its transposon system involving mating type, i.e., the MAT locus [33, 35, 37]. Repressed or silent copies of mating type genes exist at two other genetic localities (HML and HMR) on the same yeast chromosome. Copies of these silent genes can frequently be inserted into the MAT locus, with their resultant expression. Though not expressed, the HMR and HML genes have completely functional genetic in-

formation, including a promoter. Both of these repressed genes are kept silent by two DNA sequences that are many hundreds of base pairs away from the genes in question. Comparisons of the chromatin structure at the MAT locus, where genetic expression occurs, with that of the two repressed genes, show significant differences in chromatin structure. Moreover, in yeast, the insertion of the Ty transposition element hundreds of base pairs from a gene can cause the gene to be expressed [34]. Alternatively, such an insertion can turn off a gene's expression.

Many transposable elements in bacteria exert a strong polar effect on the expression of genes located in the same operon distal to the insertion [58]. In chickens, lymphomas can be generated through the activation of a cellular gene, *c-myc*. This activation is due to the insertion of retroviral DNA, a transposon-like element, at a distance from the gene in question [41]. In *Drosophila*, there are separable control elements present 1.1 to 1.9 kilo bases from the white gene, and their presence is required for the expression of this gene [59]. They are thus *cis*-acting at a distance.

Also relevant is that supercoiling of DNA has been shown to affect transposon-mediated recombination [36]. And it is proposed [36] that suppressor-mutators (Spms) in yeast can affect the superhelicity of DNA in and around a Ty element. These Spms, themselves, could very well be transposition elements. In this connection, a mutation in bacteria decreases DNA supercoiling and shows a concomitant decrease in the transposition frequency of Tn5 and Tn3 [22]. As Borst and Greaves state [24], "chromatin structure is .... an important element in the decision about which gene will be rearranged (transposed) and it may co-determine the direction of gene transposition".

Clearly, these P-EV type, mutagenic/regulatory effects of transposons, and the unifying control of such genetic elements, involved alterations in the configurational structure of the chromosome, the *E. coli*-P1CMrec system being the prime example of such control of gene expression/mutagenicity at a distance encompassing the genome as a whole. It would appear this unifying control operates through different levels of genomic organization. (At a given level of genomic organization controlled-gene instability and regulated-gene expression would become identical.)

By so involving these different levels, changing configurations within or of a chromosome could provide the physical, dynamical connections or the unifying, dynamical nexus through which transposition-mutator phenomena are generated, co-ordinated, and controlled. The enzymes involved in transposition and consequent phenomena would be subsumed under this dynamical nexus. For example, such enzymes could be dynamically co-ordinated/synchronized by means of given chromatin configurations, making the enzymes dynamically accessible in various ways to the chromatin regions in question. If made accessible in a particular manner, mutagenicity at the DNA level

might ensue. As it pertains to transposition elements and their co-ordination or synchrony, there is in fact evidence for a relationship between such enzyme accessibility and chromatin configuration [24]. The importance of such co-ordination or synchrony of transposition phenomena, and its implications, are seen as follows.

Though the influence of P1-virion-incorporation or generalized transduction was to make uniform the degree of P1CMrec-mediated mutability, data in Table IX suggested an apparent incongruity pertaining to such uniformity. Though all tested (163) *pro*$^+$ *arg*$^+$ colonies (in Table IX) were also *trp*$^+$ *ilv*$^+$ *his*$^+$ *met*$^+$ upon virion-DNA incorporation, and though, as noted earlier, the frequencies of *met*$^+$, *trp*$^+$, *leu*$^+$, *ilv*$^+$, and *trp*$^+$ *met*$^+$ *ilv*$^+$ *leu*$^+$ prototrophic classes are comparable to one another upon virion-incorporation (generalized transduction), it is nevertheless seen in Table IX that the respective frequencies of *met*$^+$ and *trp*$^+$ *ilv*$^+$ *leu*$^+$ *met*$^+$ prototrophs (prototrophic classes) are one-half that of the frequency of *pro*$^+$ *arg*$^+$ *his*$^+$ *ilv*$^+$ *trp*$^+$ *met*$^+$ prototrophs upon virion-incorporation. Perhaps the former two classes are just at a selective disadvantage compared to the latter. However, in view of the fact that prototrophs of different phenotypic classes resulting from virion-incorporation are generally recoverable at comparable frequencies, even when one is selecting for prototrophs of more than one amino-acid, this explanation is not likely.

Considering another explanation, mutability to *pro*$^+$ *arg*$^+$ upon virion-incorporation on supplemental media (13 all) lacking proline and/or arginine may be greater due to a special interaction between a uniquely mutable genome and a particular micro-environment; and since, as we have seen, the increased mutability within one region is associated with comparable increases in other regions upon virion incorporation, increased reversion to *pro*$^+$ and *arg*$^+$ may have stimulated, through changes in chromosomal configuration, increased, and hence comparable, reversion to *met*$^+$, *ilv*$^+$, *leu*$^+$, and *trp*$^+$ on that particular media lacking proline and/or arginine. The special interaction referred to, and the synchrony of mutagenesis involved, suggest an environmentally dependent, yet inner-directed, adaptive-mutagenesis, the adaptiveness contingent upon the state of co-ordinately enhanced mutabilities involving most, if not all, of the bacterial genome. Such synchrony would have been mediated through changes in the dynamical configuration of the chromosome. Yet, clearly, such changes, implicating the insertion of genetic elements, were linked to the nutritional stress of the growth environment. Data present in other tables are certainly also in accord with an insertion-element-controlled mutagenesis adaptively responsive to the nutritional stress of the growth environment.

In various organisms, including maize, transposable elements are activated when the genome (or organism) is stressed [32], a dynamical situation,

and they can serve an important role in restructuring the genome under conditions of environmental stress [60, 61]. Recent research with *E. coli* by Cairns et al. [62] has shown that bacterial cells can mutate adaptively in response to a nutritional stress. In given strains, transposition elements were not detected in this process. However, in a strain containing a bacteriophage Mu element, located near the genetic region under study, the Mu element was clearly implicated in the adaptive mutagenesis. When another Mu element was also present elsewhere on the chromosome, the adaptive mutagenesis did not occur, clearly indicating an action-at-distance effect on adaptive mutagenesis. Other, significant parallels were also found between the genetic situation described by Cairns et al. and the one reported in this paper.

Transposon behaviour involving regulatory regions would appear to be an adaptive response to stress, and such an adaptive response would also appear to operate through the dynamical avenues of chromosomal configurations. In eukaryotes, the dynamical configurations of cellular membranes would also be involved, these providing more effective links between chromosome and environmental stress.

With regard to environmental stress, a high frequency of controlled, genetic re-arrangements involving recombinase activity is at the basis of immunological diversity [24]. It is such which enables the immune system to be adaptively responsive to environmental stress, i.e., infection. Such a system of controlled, genetic re-arrangements might have evolved from a system of transposons. In fact, the generation of immunological diversity in chicken B cells and antigen diversity in some protozoa and eubacteria involve the alteration of a gene in an expression site through a type of transposition utilizing gene conversion [24], a form of recombination. The protozoa in question, *Trypanosoma*, and the eubacteria in question, *Borrelia*, can respectively evade the host immune system, itself an environmental stress for those organisms, by repeatedly changing, through genetic transposition, the antigens making up their surface coats [24].

In this regard, the effects of environmental stress on the *E. coli*-P1CMrec system should be investigated more comprehensively and in more detail. The effects of environmentally stressful factors such as the lack of specific nutrients, high temperatures, UV, and chemical mutagens, would prove important to know. (Temperature does have an influence on this system, as we have seen, and Tn3 transposition in bacteria is temperature-sensitive [58].) The effects of genetic stress within this system, such as the inclusion of repair deficient markers, e.g., *recA*, and the incorporation of other types of viruses, such as retroviruses, would be of significant interest.

Some of the phenomena associated with this mutability system have significant medical relevance. For example, the phenomenon of the directed negation by way of a particular plasmid of antibiotic resistance in bacteria has

important medical implications, as this could be developed into a controlled means of eliminating drug-resistant bacteria.

Transfection, by way of a vector, of transposable elements from a P1CMrec-type plasmid into the chromosome(s) of neoplastic cells (at least in vitro) may generate transposon-based, genomic changes, eventually leading to the reversal of the neoplastic state. Transfection has successfully been used to genetically transform mammalian cells in vitro (e.g., 63. 64). It has the clear potential of generating transposon-based genomic instability in such cells.

Another point should be made with regard to application. Other workers have shown that the P1 plasmid system can incorporate bacterial genes [10, 20, 65]. Being so, this system can be regarded as a naturally occurring recombinant-DNA system. It is known that all bacterial genes carried by respective P1 plasmids, including the genetic region for chloramphenicol-resistance, (the *CM* region being acquired from a bacterial genome by way of an R factor), exist within the same particular region in the P1 plasmid [20]. This region is referred to as *cat-gene* 2. However, it was not known by what means such bacterial DNA was incorporated into the *cat-gene* 2 region of the P1 plasmid. It was thought that such incorporation is related to concatemer formation during the phage-producing cycle of P1, though there was no evidence of this.

Certain results of this current study strongly indicate what is involved in such incorporation. One will recall the strong genetic evidence that transposition or insertion elements compose, or are included in, the chloramphenicol-resistance region of P1CMrec, meaning that the *cat-gene* 2 region in P1CMrec is composed of, or includes, transposition elements. Hence, in this connection, it is very likely that incorporation of bacterial genes into the P1 plasmid occurs by means of transposition elements. Genes from bacteria are likely transposed into the *cat-gene* 2 region of the P1 plasmid by such elements. Subsequent research has shown this to be in fact the case [19]. And, as we have seen, such elements can return to the bacterial genome, transposing genetic material that had been residing in the plasmid.

In this and other studies, there was no evidence that the existence of bacterial genes (DNA) in the P1 plasmid made the plasmid itself mutagenetically unstable, whereas the incorporation of transposition elements from P1CMrec into key regions of the bacterial genome made the latter unstable. The apparent genetic stability of the P1 plasmid system — in spite of it containing integrated bacterial DNA — may be tied to the fact that such DNA always resides in a particular genetic region of the P1 plasmid. Incorporation of foreign or bacterial DNA into this key region would allow the plasmid to maintain its dynamical integrity, and hence allow it to remain genetically stable. However, it is quite possible that were bacterial genes incorporated into other regions of the plasmid, dynamical instability (or dynamical non-uniformity) of the entire plasmid would be generated, leading to genetic instability and consequent

or associated mutagenesis. Moreover, this dynamical instability could, as a facet of such, lead to the activation of transposition phenomena previously dormant, bringing about transpositions of genetic material between widely separate regions of the Pl genome, leading to mutagenesis in Pl, and transpositions between the plasmid and the bacterial genome, leading to genetic instabilities in the latter.

All of this has significant implications for recombinant-DNA technology, which is based upon artificially introducing foreign DNA into plasmids and into eukaryotic chromosomes through transfection. The introduction of such foreign DNA into key or critical regions of a plasmid or chromosome could eventually generate wide-spread and unwanted mutagenesis within the plasmid or chromosome; and in the case of the plasmid, this could lead to the induction of mutagenesis within the genomes of the bacteria which carry such plasmids.

As pointed out by R. W. Old and S. B. Primrose [66], plasmid stability has been a factor to consider in recombinant-DNA research. They refer to the structural instability of some plasmids that have been used, pointing out that structural instability may be mediated by transposons resident in the host chromosome or in the plasmid. Also, transposons used for the purpose of transfecting the eukaryotic genome could generate unwanted mutations or genetic instability. In this regard, germ line abnormalities occurred in *Drosophila simulans* when it was transfected with a transposable element [28], and this element appeared to induce, over the generations, a mutational activity of another transposon already present in the genome. An increase in the number of other transposons in the genome was also noted subsequent to transfection. Furthermore, a Tn5-transposon induced mutant of *Rhizobium* resulted in unexpected genetic changes leading to pathogenic-like responses in a legume containing the bacteria [67]. As a possibility, one should consider that the Tn5 element unexpectedly transposed from the bacteria into the legume genome where it generated the unexpected and unwanted mutations. This is especially relevant in view of the use of transposable elements in the genetic engineering of plants [68], though it is encouraging that such use, involving the maize transposable element Mul, can result in situations where there is an absence of mutator effects [68].

Perhaps, based on these successes, such technology has generally assumed that incorporation of foreign DNA can have no effects upon the genetic integrity of the plasmid and chromosome. Such technology has generally been fortunate, so far, in that incorporation of foreign DNA has been in those regions of the plasmid or chromosome which allowed such to maintain its dynamical, and hence, genetical integrity.

Finally, the existence of the type of mutability system as described in this study, especially its ability to confer almost immediate adaptiveness to the organism carrying it, has important implications for the evolution of the

prokaryotes. Moreover, one must not rule out the significant role that such an adaptive-conferring system could play in the evolution, e.g., punctuated evolution, of eukaryotes in general, and in higher organisms in particular.

Addendum

This paper is based on research completed in July 1977. Because this work anticipated subsequent developments in molecular biology, it was considered incomprehensible at that time and its publication was consequently delayed. In this regard as in others, there is a similarity between the work of B. McClintock and the present work. This concerns their content, their mode of scientific investigation which ascribes primacy to experimentally established phenomenological facts relative to any particular mode or convention used for their interprepations, as well as their respective histories leading to their eventual reconciliation with significant findings in Molecular Biology which they anticipated phenomenologically.

The primacy of an experimentally established phenomenological fact relative to any particular mode, convention or theoretical model used for its comprehension and interprepation is manifested throughout the development of Science. It is significantly exemplified by the constancy and thus primacy of the phenomenological thermodynamical theories of Carnot and Clausius relative to the model dependent and thus limited theories of Statistical Thermodynamics which changed in order to accommodate new phenomenological facts that led to Quantum Mechanics and Relativity Theory. The primacy of phenomenology relative to a chemical description of nature is further exemplified by Catalysis which as a most fundamental aspect of chemical phenomena remains inexplicable in chemical terms. These observations do not detract from the power and significance of the chemical mode of description of nature and of biological phenomena in particular. They are presented here to help put matters in proper perspective.

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REFERENCES

1. Lennox, E. S.: Virology **1,** 190 (1955).
2. Ozeki, H., Ikeda, H.: Ann Rev Genetics **2,** 245 (1968).
3. Bachmann, B. J., Low, K. B., Taylor, A. L.: Bacteriol Reviews **40,** 116 (1976).
4. Arber, W.: Virology **11,** 273 (1960).
5. Franklin, N. C., Luria, S. E.: Virology **15,** 209 (1961).
6. Ikeda, H., Tomizawa, J.: J Mol Biol **14,** 85 (1965).
7. Ikeda, H., Tomizawa, J.: J Mol Biol **14,** 120 (1965).
8. Luria, S. E., Adams, J. N., Ting, R. C.: Virology **12,** 348 (1960).

9. Stodolsky, M., Rae, E. M., Mullenbach, E.: Genetics **70,** 495 (1972).
10. Stodolsky, M.: Virology **53,** 471 (1973).
11. Ikeda, H., Tomizawa, J.: Cold Spring Harbor Symp Quant Biol **33,** 791 (1968).
12. Kondo, E., Mitsuhashi, S.: J Bacteriol **88,** 1266 (1964).
13. Rosner, J. L.: Virology **49,** 679 (1972).
14. Wu, T. T.: J Bacteriol **98,** 314 (1969).
15. Wu, T. T.: Genetics **54,** 405 (1966).
16. Goodenough, U., Levine, R. P.: Genetics. Holt, Rinehart, and Winston, Inc., New York 1973.
17. Perlman, D., Stickgold, R.: Proc Natl Acad Sci USA **74,** 2518 (1977).
18. Kleckner, N.: Cell **11,** (1977).
19. York, M. K., Stodolsky, M.: Mol Gen Genet **181,** 230 (1981).
20. Schultz, G., Stodolsky, M.: Virology **73,** 299 (1976).
21. Possot, O., Appl Environ Microbiol **54,** 734 (1988).
22. Herfron, F.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 223–260.
23. Borst, P.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 621–659.
24. Borst, P.: Science **235,** 658 (1987).
25. Rubin, G. M.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 329–361.
26. Bryan, G. J., Hartl, D.: Science **240,** 215 (1988).
27. Cooley, L., Science **239,** 1121 (1988).
28. Scavarola, N. J., Hartl, D. L.: J Genet **66,** 1 (1987).
29. Link, C., Proc Natl Acad Sci USA **84,** 5340 (1987).
30. McClintock, B.: Cold Spring Harbor Symp Quant Biol **16,** 13 (1951).
31. McClintock, B.: Brookhaven Symp Biol **18,** 162 (1965).
32. Federoff, N. V.: Scientific American **250,** 84 (1984).
33. Nasmyth, K. A., Nature **239,** 255 (1981).
34. Beier, D. R., Young, E. T.: Nature **300,** 724 (1982).
35. Nasmyth, K. A.: Cell **30,** 567 (1982).
36. Roeder, G. S., Fink, G. R.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 299–328.
37. Abraham, J. J., Cold Spring Harbor Symp Quant Biol **47,** 989 (1983).
38. Lloyd, J., Mol Biol Evol **4,** 85 (1987).
39. Misru, R., J Mol Biol **196,** 233 (1987).
40. Zambryski, P., In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 505–533.
41. Bishop, J. M.: Science **235,** 305 (1987).
42. Varmus, H. E.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 411–503.
43. Campbell, A.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 65–103.
44. Walbot, V.: Genetics **114,** 1293 (1986).
45. Alleman, M., Freeling, M.: Genetics **112,** 107 (1986).
46. Robertson, D. S.: Mol Gen Genet **200,** 9 (1985).
47. Toussaint, A., Resibois, A.: In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 105–158.
48. Chow, L. T., Bukhari, A.: Virology **74,** 242 (1976).
49. Lieber, P., Lieber, M. M.: In press.
50. Cox, E. C., Yanofsky, C.: J. Bacteriol **100,** 390 (1969).
51. Siegal, E. C., Bryson, V.: J Bacteriol **94,** 38 (1967).
52. Kemper, J.: Bacteriol **120,** 1176 (1974).
53. Walker, G. C.: Molec Gen Genet **152,** 93 (1977).
54. Lewis, E. B.: Advances in Genetics **3,** 73 (1950).
55. Lieber, M. M.: Mutation Research **37,** 33 (1976).
56. Beerman, S.: Genetics **54,** 567 (1966).
57. McClintock, B.: Proc Natl Acad Sci USA **36,** 344 (1950). (Reprinted in Peters, J. A. (ed.): Classical Papers in Genetics, Prentice-Hall, 1959).
58. Iida, S., In Shapiro, J. A. (ed.): Mobile Genetic Elements. Academic Press, New York 1983. pp. 159–221.
59. Levis, R., Eur Mol Biol Organ J **4,** (13A) 3489 (1985).
60. McClintock, M.: Science **226,** 792 (1984).

61. Courage, K., Cold Spring Harbor Symp Quant Biol **49,** 329 (1984).
62. Cairns, J., Nature **335,** 142 (1988).
63. Mulligan, R., Berg., P.: Proc Natl Acad Sci USA **78,** 2072 (1981).
64. Southern, P. J. Berg, P.: J Molec Applied Genet **1,** 327 (1982).
65. Rae, M. E., Stodolsky, M.: Virology **58,** 32 (1974).
66. Old, R. W., Primrose, S. B.: Principles of Gene Manipulation: An Introduction to Genetic Engineering. Third Edition. Published as Studies in Microbiology. Volume 2. Blackwell Scientific Publications, Oxford 1985.
67. Djordjevic, S. P., J Bacteriol **170,** 1848 (1988).
68. Zhang, H., Somerville, C.: Plant Science **48,** 165 (1987).
69. Taylor, A. L., Trotter, C. D.: Bacteriol Rev **36,** 504 (1972).

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CLINICAL OCCURRENCE AND VIRULENCE TESTING OF COAGULASE-NEGATIVE STAPHYLOCOCCI*

I. Barcs, T. Vályi-Nagy and J. Pánovics

László Central Hospital for Infectious Diseases, National Institute for Dermatology and Venereology, and Department of Urology, Semmelweis University Medical School, Budapest

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A total of 650 coagulase-negative staphylococcal strains of miscellaneous clinical origin were isolated and identified during a 7-year-period. In all kinds of samples *Staphylococcus epidermidis* was the most frequently found species. In wounds the incidence of haemolytic species *(S. cohnii, S. epidermidis, S. haemolyticus, S. simulans, S. warneri)* was 79.3%. Among strains derived from male genitals (urethra, semen, etc.) *S. epidermidis* and *S. simulans* predominated. Only 8.2% of the strains were found not to harbour resistance determinants, while 63% proved to be multiply resistant. Only the incidence of resistance to oxacillin and first and second generation cephalosporins was low (12.8, 14.9 and 4.5%, respectively). Vancomycin was the only drug effective against all strains. Extracellular slime was produced by 42.2% of the strains. The frequency of slime-positivity among isolates causing clinical infection was twice as high as among contaminations. Slime production seems to be a stable characteristic of the isolates. The comparison of two methods used for detection of slime factor showed that incubation in test tube in Tryptone Soya Broth, or its application to microtiter plates evaluated by photometry are of equal diagnostic value.

Coagulase-negative members of the genus *Staphylococcus* are by now widely acknowledged to have the potential to cause human disease under certain conditions. In general, actual state of host resistance and virulence of the infectious agent are the factors determining the outcome of an infectious process. Staphylococci lacking the enzyme coagulase may still produce certain virulence-factors enabling them to invade human tissues [1, 2].

Coagulase-negative staphylococci (CNS) are responsible for a significant portion of infections seen in immunocompromised individuals, but they can

István Barcs

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

Tibor Vályi-Nagy

National Institute for Dermatology and Venereology, Mária u. 41, H-1085 Budapest, Hungary

József Pánovics

Department of Urology, Semmelweis University Medical School, Mária u. 39, H-1085 Budapest, Hungary

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cause disease in the normal host as well [2–5]. These organisms may play an important role in infections following surgery or associated with implanted prosthetic devices [1, 2, 6–9]. It is extremely difficult to determine the clinical significance of the recovery of CNS from clinical specimens as they are inhabitants of the normal skin and mucous membranes. The question as to whether the isolated strain is a contaminant or plays a causative role in the given disease process, can only be answered after thorough analysis of all clinical and laboratory data available [3, 10]. An important piece of information for this analysing process is provided by the determination of the given strain's slime producing ability.

Materials and methods

Bacterial strains. Between 1982 and 1986, 609 CNS strains were isolated in the Department of Microbiology of the László Central Hospital for Infectious Diseases, Budapest, from miscellaneous clinical samples sent by the hospital's wards and from heart valve specimens sent by the National Institute of Cardiology, Budapest. In 1987, 41 CNS strains were isolated from male urethra, semen, prostatic fluid or urine of outpatients in the Department of Bacteriology, National Institute for Dermatology and Venereology Budapest. Culturing and identification was performed by biochemical tests and novobiocin sensitivity [11].

Antibiotic sensitivity tests. In agar diffusion tests [12] the following set of Resistest (Human, Budapest–Gödöllő) disks was used on Mueller-Hinton agar: penicillin, oxacillin, cephaloridine, cefamandole, gentamicin, tobramycin, chloramphenicol, tetracycline, erythromycin, lincomycin, co-trimoxazole, vancomycin, novobiocin. Macrolide-lincosamide cross-resistance of the strains was tested by the method of Weisblum and Demohn [13].

Clinical significance. Isolation in pure culture with significant counts was the precondition for judging a given strain of urogenital origin to represent a probable infection. [6]. Clinical significance was attributed to those blood culture isolates that (i) were recovered repeatedly, (ii) were recovered parallel from other sites (cerebrospinal fluid, heart valves, venous catheters, sternal punctates), or (iii) sourced from patients with a clinical picture suggesting CNS-infection [10, 14, 15].

Slime test. Detection of slime production was performed by two methods simultaneously. The standard tube method (ST) was carried out in 5 ml Tryptone Soya Broth (TSB, Oxoid, London) at 35 °C for 48 h. The cultures were removed, the tubes washed twice with distilled water and stained with 0.25% safranin T (Reanal, Budapest). A continuous film on the surface of the glass tube was considered positive [15, 16]. The microtitre plate method (SM) was performed by the modified procedure of Christensen et al. [17]: overnight broth cultures of the strains were diluted hundredfold with fresh TSB and 200 μl portions were placed into holes of microtitre plates (Non-coated pre-incubation strips, Organon Teknika, Boxtel) and incubated at 37 °C for 18 h. As negative control, sterile TSB was used. After washing with phosphate buffered saline for three times the plates were dried, fixed in methanol, stained with 2% crystal violet and detected with Organon Teknika Stripreader 330 type photometer at 492 nm. A three-grade scale was used to evaluate the slime producing ability of strains: SM− = negative, $OD_{492} < 0.060$; SMw = weakly positive, OD_{492} 0.060−0.200; SM + = strongly positive, $OD_{492} > 0.200$.

Results

Clinical occurrence. A total of 650 CNS strains were isolated and identified in our laboratories from different clinical sources during a 7-year-period. In all kinds of samples *S. epidermidis* occurred the most frequently as shown

in Table I. Of all isolates, 76.4% belonged to haemolytic species *(S. cohnii, S. epidermidis, S. haemolyticus, S. simulans, S. warneri)* while among strains recovered from wounds and other purulent laesions their ratio was 79.3%. Novobiocin resistant species *(S. cohnii, S. saprophyticus, S. sciuri, S. xylosus)* occurred only in 8.3%. In male genitals *S. epidermidis* and *S. simulans* predominated (30.6% each).

Antibiotic susceptibility. The results of antibiotic sensitivity tests are demonstrated in Table II. Only 8.2% of the isolates were sensitive to all tested antimicrobials, and 63% of them proved to be resistant to three or more drugs.

Slime production. Altogether 166 randomly chosen CNS strains were tested for slime production with the parallel use of the two methods. Of these isolates 66 were derived from blood cultures, 3 each from venous cannulas and mitral or aortic valves, 5 from wound exudates, 34 from male urethras, and 55 from miscellaneous other sites, mainly from the throat and respiratory tract of patients.

OD values obtained by the SM method were compared to ST test results. Negative control OD values were found to show a variation between 0.039 and 0.042, while the detected highest OD value was 0.950. Examination of strains negative with the ST method yielded OD values under 0.060 in the SM test. Higher than 0.060 OD values were detected in all cases when ST method-positive strains were tested. Extinction was found to be over 0.200 with strains strongly positive in the ST test. In summary, the two methods seem to be of equal value. First and repeated experiments yielded identical results, thus slime production proved to be a stable characteristic of the strains.

Table III summarizes our results concerning CNS strains. Of the tested isolates 42.2% were found to produce slime material. The ratio of slime factor producing strains among blood isolates of clinical significance was found twice as high as among contaminants (Table IV). Because of the small number of studied samples, Table IV does not show the recovery frequency of slime producing strains from some sites. These were according to site of recovery as follows: (i) valve vegetation: 3 strains (1 SM+, 1 SMw, 1 SM—); (ii) wound exudate: 2 strains with clinical significance (1 SM+, 1 SMw) and (iii) from catheter tips 2 strains, SM+ both. Out of the remaining strains in this group, only three were attributed pathogenic significance based on pertinent clinical information. These were isolated from cerebrospinal fluid of patients with brain abscess or generalized sepsis. Two of them were found to be SM+, one SMw. In the group of urogenital isolates half of the strains produced slime factor.

Although the number of examined strains was not big enough for exact statistical evaluation, we mention that the occurrence rate of slime positivity seems to be the highest in the species *S. epidermidis* and *S. simulans.*

Table I

Frequency and distribution of coagulase-negative staphylococcal

Species	No. (%) of isolates				
	Total	Wound*	Urine	Blood	I.v. catheter
S. capitis	42 (6.5)	12 (13.1)	1 (1.0)	10 (4.5)	1 (3.3)
S. cohnii	17 (2.6)	4 (4.3)	3 (3.0)	4 (1.8)	—
S. epidermidis	273 (42.0)	27 (29.3)	32 (32.7)	127 (57.0)	13 (43.4)
S. haemolyticus	76 (11.7)	23 (25.0)	13 (13.3)	16 (7.2)	2 (6.7)
S. hominis	67 (10.3)	6 (6.5)	16 (16.3)	13 (5.8)	6 (20.0)
S. hyicus	3 (0.5)	1 (1.1)	—	—	—
S. saprophyticus	34 (5.2)	—	9 (9.2)	23 (10.3)	1 (3.3)
S. sciuri	1 (0.2)	—	—	—	—
S. simulans	77 (11.8)	9 (9.8)	13 (13.3)	20 (9.0)	3 (10.0)
S. warneri	54 (8.3)	10 (10.9)	11 (11.2)	6 (2.7)	4 (13.3)
S. xylosus	2 (0.3)	—	—	1 (0.4)	—
Uuidentified	4 (0.6)	—	—	3 (1.3)	—
Summarized	650 (100)	92 (100)	98 (100)	223 (100)	30 (100)

* Pus, abscesses, fistula, papula, pyoderma, etc.
** Cerebrospinal fluid, lochia, sinus punctates
*** Prostatic fluid, semen, urethra
+ Bone marrow, sternal punctate, ear, nasopharynx

Discussion

The different *Staphylococcus* species are the indigenous members of the resident flora of the skin and mucous membranes. All species are considered to be potentially pathogenic microorganisms, i.e. under certain conditions may cause human disease. They are responsible for 5–25% of all urinary tract infections [1, 2, 6, 9, 18], the most frequently occurring species being *S. saprophyticus* and *S. epidermidis*. As the László Hospital has no Urological care unit, it is understandable that the detected range of recovered species differs somewhat from corresponding data of the literature [19–26].

Following *Chlamydia trachomatis* and the *Mycoplasma/Ureaplasma* group, CNS (first of all *S. epidermidis*) are accounted for chronic prostatitis [27]. In present study *S. simulans* was detected almost as frequently as *S. epidermidis*. Also our preliminary data suggest an important role of CNS in the aetiology of urogenital infections. CNS were detected in 16% of specimens obtained from men with chronic prostatitis, while mycoplasmas were isolated in 21%, *Streptococcus faecalis* in 18%, beta-haemolytic streptococci in 6%, Gram-negative bacilli in 19%, and *C. trachomatis* only in 5% [28].

species isolated from clinical specimens

Species	No. (%) of isolates				
	Heart valve	Sterile fluids**	Eye	Male genitals***	Other+
S. capitis	4 (13.8)	2 (8.7)	2 (5.6)	1 (2.8)	9 (10.8)
S. cohnii	—	1 (4.3)	1 (2.7)	—	4 (4.8)
S. epidermidis	14 (48.3)	6 (26.1)	20 (55.6)	11 (30.6)	23 (27.7)
S. haemolyticus	1 (3.4)	3 (13.1)	3 (8.3)	5 (13.8)	10 (12.1)
S. hominis	6 (20.7)	2 (8.7)	4 (11.1)	3 (8.3)	11 (13.3)
S. hyicus	—	—	—	—	2 (2.4)
S. saprophyticus	—	—	—	1 (2.8)	—
S. sciuri	—	—	—	—	1 (1.2)
S. simulans	2 (6.9)	5 (21.7)	2 (5.6)	11 (30.6)	12 (14.5)
S. warneri	2 (6.9)	4 (17.4)	4 (11.1)	4 (11.1)	9 (10.8)
S. xylosus	—	—	—	—	1 (1.2)
Unidentified	—	—	—	—	1 (1.2)
Summarized	29 (100)	23 (100)	36 (100)	36 (100)	83 (100)

CNS cause 2–4% of wound infections and the rate of their incidence is increasing [1, 2, 9, 18]. Recovery rate of the different species in our study was congruent with other authors' data [19–26].

CNS play often a role in nosocomial infections, in particular in those related to foreign bodies. They are responsible for the majority of infected cerebrospinal shunts and intravenous catheters [2, 3, 6, 7, 18, 29] and are also the most frequent causes of prosthetic valve endocarditis [18, 29].

The most serious CNS infection is septicaemia. The aetiological evaluation of blood cultures is extremely difficult: 80–90% of the isolates are in no relation to the disease process as their presence is due to transient bacteraemia or contamination [10, 14, 29–32]. Also in our preliminary study the majority of strains from blood cultures was assumed to be contaminants, they having been isolated also from other samples (sternal punctate, intravenous catheter tip, heart valve) or from subsequent blood cultures only in 8.8% [30, 31].

Detection of extracellular slime material provides important information on the isolates and helps the clinical interpretation of results. Slime producing ability is an important factor of virulence: it makes possible adherence to hydrophobic surfaces, thus the colonization of prosthetic devices [7, 8, 15]. Furthermore slime protects bacteria against antibacterial agents and against both specific and aspecific factors of defence of the host [8, 15, 33–35].

Detection of the slime factor is simple in a liquid medium based on casein hydrolyzate that contains the necessary precursors of slime material in high

Table II

Susceptibility of coagulase-negative staphylococcal isolates to 13 antibacteria agents

Species	No.	Per cent of resistant strains to												
		P	OX	CD	MA	GM	TM	C	TE	E	LM-c	LM-i	SXT	VA
S. capitis	42	67.5	7.5	5.2	2.6	2.5	2.8	20.0	55.0	33.3	14.2	12.5	26.3	—
S. cohnii	13	76.9	7.6	7.6	—	15.3	15.3	46.1	61.5	53.8	30.7	—	—	—
S. epidermidis	224	92.8	8.4	12.3	2.8	24.1	27.0	35.9	80.3	50.8	20.5	25.7	46.7	—
S. haemolyticus	64	82.8	15.6	16.3	10.3	18.7	20.7	26.9	59.3	33.3	21.8	4.6	23.4	—
S. hominis	52	90.3	13.4	9.6	6.3	21.1	24.0	32.6	76.9	46.1	21.1	17.3	56.7	—
S. hyicus	3	3/3*	1/3	1/3	—	0/3	0/2	1/3	1/3	0/3	0/3	—	0/3	0/3
S. saprophyticus	29	93.1	6.8	3.8	3.5	20.6	28.5	34.4	93.1	31.0	20.6	6.8	39.2	—
S. sciuri	1	1/1	0/1	0/1	—	0/1	—	0/1	1/1	1/1	1/1	1/1	0/1	0/1
S. simulans	71	98.5	28.1	35.0	8.5	40.0	34.6	58.3	86.4	72.8	41.4	27.1	40.0	—
S. warneri	42	65.0	15.0	20.5	5.0	32.5	28.0	47.5	70.0	38.0	23.8	10.5	28.9	—
S. xylosus	2	2/2	0/2	0/2	0/2	1/2	1/1	0/2	2/2	0/2	0/2	—	1/2	0/2
Unidentified	4	2/4	1/4	1/4	1/4	0/4	0/4	2/4	1/4	2/4	1/4	—	1/4	0/4
Summarized	547	87.6	12.8	14.9	4.5	23.3	24.3	36.8	75.1	47.5	23.4	17.7	39.5	—

Abbreviation: P = penicillin G; OX = oxacillin; CD = cephaloridine; MA = cefamandole; GM = gentamicin; TM = tobramycin; C = chloramphenicol; TE = tetracycline; E = erythromycin; LM-c = lincomycin (constitutive); LM-i = lincomycin (inducible); SXT = co-trimoxazole; VA = vancomycin

* No. of resistant strains/all tested strains

Table III

Slime production of clinical isolates of coagulase negative staphylococcal strains

Species	No. (%) of isolates				
	No.	Clinical significance	Slime positive		
			ST+	SM+	SMw
S. capitis	4	1	1	—	—
S. epidermidis	63	39	36	15	21
S. haemolyticus	26	11	6	2	4
S. hominis	27	2	7	2	5
S. saprophyticus	3	1	1	—	1
S. simulans	29	18	15	4	11
S. warneri	14	8	4	3	1
Summarized	166 (100)	80 (48.2)	70 (42.2)	26 (15.7)	44 (26.5)

Abbreviations: ST+ slime producer by tube test; SM+ strong slime positive; SMw weak slime positive by microtiter plate method

Table IV

Occurrence of slime producer strains in clinical samples

Source	No. (%) of isolates				
	Total	Possible clinical significance		Slime positive	
		+	−		
Blood	66			31	(46.9)
		39		23	(58.9)
			27	8	(29.6)
Male genitals	34			16	(47.1)
		30		14	(46.6)
			4	2	(50.0)
Other	66			23	(34.8)
		11		10	(90.9)
			55	13	(23.6)
Summarized	166	80	86	70	(42.2)

concentrations [8, 16, 32]. Application of photometry makes the test quantitative. Adapting the method of Christensen et al. [17] we found the two tests to be of equal value. Neither methods gave false positive or negative results in our hands. As the more labour- and energy-consuming quantitative test takes the same 48 h to carry out, we recommend the tube test for routine use.

Slime factor positivity was found to be two times more frequent among blood-derived CNS strains with clinical significance than among contaminants.

Christensen et al. [14, 16] found 63% slime positivity among strains derived from patients with symptomatic infection, while 35% of blood culture contaminants and 38% of skin isolates proved to be slime producer. More recent results of the same team show that among skin isolates and contaminants the ratio of slime negativity is 50% (weak and strong positivity is approximately 25% each). The strains isolated from catheter-dependent patients or from infected cerebrospinal shunts show strong slime positivity around 60%, while in bacteraemia weakly positive strains predominated [17]. Davenport et al. [15] found 70–80% slime factor positivity among strains of clinical significance while, depending on the kind of samples, among contaminants 15–60% positivity was detected. The most explicit difference was found with strains in connection with infected foreign bodies. Slime positivity was four times more frequent among strains with attributed clinical significance than among contaminants. Inaccurate judgement of certain strains' clinical importance seems to be the most likely explanation of our somewhat differing findings.

Slime production may also play a role in the development of infectious endocarditis [36]. Among the three CNS strains we isolated from heart valves one *S. epidermidis* was slime negative, one *S. hominis* weakly and another strongly positive.

The occurrence of slime positivity may also vary in the different species. According to Christensen et al. [14, 17] this marker is most frequently present among strains of *S. capitis*, *S. epidermidis*, *S. hominis*, *S. saprophyticus*. On the other hand 93% of the 95 *S. aureus* isolates they tested was slime negative. Our results are in good congruency with their data [37].

In comparison with relevant data in the literature, CNS strains isolated in our laboratories proved to be above the average resistance to antimicrobial agents [20–23, 25, 26, 38]. Inducible resistance to lincosamides, resistance to oxacillin and second generation of cephalosporins (cefamandole) were the only markers found somewhat less frequently than excepted. Relative to our previous studies [30, 31], isolates resistant to first generation cephalosporins (cephaloridine), aminoglycosides and co-trimoxazole were found with an increased frequency. In our study cefamandole and vancomycin proved to be the most effective among the tested agents.

Only 8.2% of the studied strains were found to be susceptible to all antibiotics tested. The found frequency is considerably lower than the 14% rate shown by Marples and Richardson [22] and the 33% reported by Brun et al. [21]. On the other hand the ratio of multiple resistant strains in our study (63%) was similar to the corresponding findings of Akatov et al. [20] and Marples and Richardson [22].

We could not detect any correlation between slime positivity and carriage of resistance determinants. This is in partial contradiction to the results of Christensen et al. [14] who detected gentamicin resistance in 70% of the

slime positive and in 30% of the slime negative isolates. We found the occurrence rate of gentamicin resistance in the two groups only insignificantly different.

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REFERENCES

1. Gemmel, C. G., Thelestam, T. M., Wadström, T.: In Jeljaszewicz, J. (ed.): Staphylococci and Staphylococcal Diseases. Gustav Fischer, Stuttgart–New York 1976. p. 133.
2. Parker, M. T.: In Macdonald, A., Smith, G. (eds): The Staphylococci. Aberdeen University Press, Aberdeen 1981. p. 156.
3. Marples, R. R.: J Hosp Infect **5,** (Suppl A) 51 (1984).
4. Fehér, L., Barcs, I.: Orv Hetil **126,** 1599 (1985).
5. Feigin, R. D., Shearer, W. T.: J Pediat **87,** 852 (1975).
6. Editorial: Lancet **1,** 139 (1981).
7. Price, E. H.: J Hosp Infect **5,** 7 (1984).
8. Peters, G.: In Jeljaszewicz, J. (ed.): The Staphylococci. Zentralbl Bakteriol Suppl 14. Gustav Fischer, Stuttgart–New York 1985. p. 515.
9. Pulverer, G., Peters, G., Schumacher-Perdreau, F.: Zentralbl Bakteriol (A) **264,** 1 (1987).
10. Weinstein, M. P., Reller, L. B., Murphy, J. R., Lichtenstein, K. A.: Rev Infect Dis **5,** 35 (1983).
11. Barcs, I.: Orvosképzés **64,** 265 (1989).
12. Matsen, J. M.: In Sonnenwirth, A. C., Jarett, L. (eds): Grandwohl's Clinical Laboratory Methods and Diagnosis. 8th ed. C. V. Mosby, St. Louis 1980. p. 1937.
13. Weisblum, B., Demohn, V.: J Bacteriol **98,** 447 (1969).
14. Christensen, G. D., Parisi, J. T., Bisno, A. L., Simpson, W. A., Beachey, E. H.: J Clin Microbiol **18,** 258 (1983).
15. Davenport, D. S., Massanari, R. M., Pfaller, M. A., Bale, M. J., Streed, S. A., Hierholzer, W. J. Jr.: J Infect Dis **153,** 332 (1986).
16. Christensen, G. D., Simpson, W. A., Bisno, A. L., Beachey, E. H.: Infect Immun **37,** 318 (1982).
17. Christensen, G. D., Simpson, W. A., Beachey, E. H.: In Jeljaszewicz, J. (ed.): The Staphylococci. Zentralbl Bakteriol Suppl 14, Gustav Fischer, Stuttgart–New York 1985. p. 471.
18. Pulverer, G.: In Jeljaszewicz, J. (ed.): The Staphylococci. Zentralbl Bakteriol Suppl 14. Gustav Fischer, Stuttgart–New York 1985. p. 1.
19. Nord, C-E., Holta-Öie, S., Ljungh, Å., Wadström, T.: In Jeljaszewicz, J. (ed.): Staphylococci and Staphylococcal Diseases. Gustav Fischer, Stuttgart–New York 1976. p. 105.
20. Akatov, A. K., Khatenever, M. L., Devriese, L. A.: In Jeljaszewicz, J. (ed.): Staphylococci and Staphylococcal Infections. Zentralbl Bakteriol Suppl 10, Gustav Fischer, Stuttgart–New York 1981. p. 153.
21. Brun, Y., Freurette, J., Forey, F., Gonthier, B.: In Jeljaszewicz, J. (ed.): Staphylococci and Staphylococcal Infections. Zentralbl Bakteriol Suppl 10. Gustav Fischer, Stuttgart–New York 1981. p. 137.
22. Marples, R. R., Richardson, J. F.: In Jeljaszewicz, J. (ed.): Staphylococci and Staphylococcal Infections. Zentralbl Bakteriol Suppl 10. Gustav Fischer, Stuttgart–New York 1981. p. 175.
23. Marsik, F. J., Brake, S.: J Clin Microbiol **15,** 640 (1982).
24. Marrie, T. J., Kwan, C.: Antimicrob Agents Chemother **22,** 395 (1982).
25. Gill, V. J., Selepak, S. T., Williams, E. C.: J Clin Microbiol **18,** 1314 (1983).
26. Iwantscheff, A., Kühnen, E., Brandis, H.: Zentralbl Bakteriol (A) **260,** 41 (1985).
27. Weinberger, M., Cytron, S., Samra, Z., Servadio, C., Pitlik, S.: Third European Congress of Clinical Microbiology. Book of Abstracts. The Hague 1987. p. 44.
28. Pánovics, J., Vajda, Z., Molnár, Gy., Barcs, I.: Seventh Congress of the Hungarian Society of Urology. Book of Abstracts, Szeged 1988.
29. Smith, I. M.: In Jeljaszewicz, J. (ed.): Staphylococci and Staphylococcal Infections. Zentralbl Bakteriol Suppl 10. Gustav Fischer, Stuttgart–New York 1981. p. 765.
30. Barcs, I.: Egészségtudomány **27,** 366 (1983).

31. Barcs, I.: Antibiotika Monitor **2,** 6 (1986).
32. Hansen, B. G.: Acta Path Microbiol Immunol Scand Sect B **93,** 1 (1985).
33. Ludwicka, A., Uhlenbruck, G., Peters, G., Seng, P. N., Gray, E. D., Jeljaszewicz, J., Pulverer, G.: Zentralbl Bakteriol (A) **258,** 256 (1984).
34. Solymossy, M., Mandl, J., Nagy, Zs., Antoni, F.: Acta Microbiol Acad Sci Hung **29,** 277 (1982).
35. Solymossy, M., Nagy, Zs., Antoni, F.: Acta Microbiol Acad Sci Hung **29,** 27 (1982).
36. Baddour, L. M., Christensen, G. D., Bisno, A. L., Simpson, W. A., Hester, M. G., Beachey, E. H.: Program and Abstracts of the 24th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D. C. 1984. p. 328.
37. Barcs, I.: Egészségtudomány **32,** 209 (1988).
38. Hamilton-Miller, J. M. T., Iliffe, A.: J Med Microbiol **19,** 217 (1985).

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ANTIMICROBIAL AND IMMUNOMODULATING EFFECTS OF SOME PHENOLIC GLYCOSIDES

J. Molnár, Gyöngyi Gunics, Ilona Mucsi, M. Koltai, Ildikó Petri, Yukihiro Shoyama, Masami Matsumoto and Itsuo Nishioka

Institute of Microbiology, University Medical School, Institute of Pharmacology, University Medical School, Blood Transfusion Centre, University Medical School, Szeged, Hungary, and Institute of Pharmacognosy, Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka 812, Japan

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Several phenolic glycosides, i.e. acteoside, desrhamnosyl acteoside, and purpureaside A, B and C, exerted weak antibacterial effects on *Escherichia coli*. Acteoside had antiplasmid effects, including F'lac plasmid elimination, and inhibited kanamycin resistance transfer in *E. coli*. Acteoside, desrhamnosyl acteoside and purpureaside A displayed antiviral effect on Aujeszky virus. All of the phenolic glycosides decreased some human leucocyte functions, including rosette formation, mitogen-induced blast transformation and phagocytic activity in vitro. The purpureaside C had significant proinflammatory action, however, other phenolic glycosides showed neither proinflammatory nor antiinflammatory effect on carrageenin-induced inflammation in vivo.

Various derivatives of caffeic acid and structurally related phenolic glycosides occur widely in plants [1]. Some of these compounds are part of the natural defence mechanisms of plants against infectious diseases [2, 3] and these compounds inhibit the multiplication of plant and human pathogenic bacteria [4], and viruses in vitro [5–8]. Phenolic glycosides as stress compounds have important role in the natural host defence mechanism of plants. Therefore it was worth-while to study their antimicrobial effect and immune modulating effects in vitro. The effect of newly isolated phenolic glycosides was tested on several infectious agents like bacteria, viruses, plasmids and on several leucocyte functions.

Joseph Molnár, Gyöngyi Gunics, Ilona Mucsi

Institute of Microbiology, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

Mathias Koltai

Institute of Pharmacology, University Medical School
Dóm tér 12, H-6720 Szeged, Hungary

Idikó Petri

Blood Transfusion Centre, University Medical School
Dóm tér 10, H-6720 Szeged, Hungary

Yukihiro Shoyama, Masami Matsumoto, Itsuo Hishioka

Institute of Pharmacognosy, Faculty of Pharmaceutical Sciences. Kyushu University
Maidashi, Higashi-ku, Fukuoka 812, Japan

For this purpose we have already set up the preparation of phenolic glycosides using a cell culture [9] besides the clonal micropropagation system of various medicinal plants [10–14]. In this paper, we report on the antimicrobial (antibacterial, antiplasmid and antiviral) and leucocyte function modulating (rosette formation, blast transformation, ADCC and phagocytosis) effects of some phenolic glycosides, i.e. acteoside, desrhamnosyl acteoside, and purpureaside A, B and C.

Materials and methods

Compounds. Acteoside, desrhamnosyl acteoside, purpureaside A, purpureaside B and purpureaside C produced by cell cultures of *Rehmannia glutinosa* and *Digitalis purpurea* cells were isolated by Shoyama et al. [4, 9]. The chemical structures of the compounds are shown in Fig. 1.

Bacterial strains. Escherichia coli K12 LE 140 F'lac and *E. coli* K12/R144 drd-3 were kindly provided by I. B. Holland (Department of Genetics, University of Leicester). *E. coli* K12, (lac^-, F^-) sodium azide resistant, were applied.

The antibacterial effect was examined in tube dilution test as previously described [10].

Elimination of the F'lac plasmid was carried out as described earlier [15].

Inhibition of R-plasmid transfer. E. coli K12 R 144 kanamycin resistant strain was used as donor, and *E. coli* K12 W1 sodium azide resistant strain was used as recipient [16].

Erythrocyte rosette formation assay. The number of rosette-forming T-cells was determined by the erythrocyte rosette formation assay [17] and the number of Fc-bearing lymphocytes was estimated in the EA-rosette test [18].

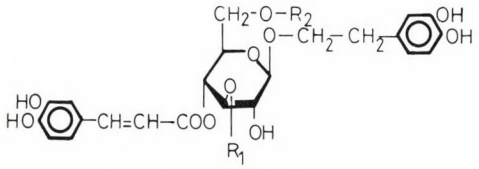
Blast transformation of T-lymphocytes in relation to the capacity of cellular immune response. Mononuclear cells were separated on Ficoll-Uromiro gradient, and 2×10^5 cells were treated with the acteoside and purpureaside derivatives at 1–5 μg/ml and cocultured in the presence of 10 μg/ml PHA and 5 μg/ml Con A and PWM for 72 h in RPMI medium. Cells were cultivated in Greiner microtitre plates. The transformation rate was estimated after the addition of 0.5 μCi ^{3}H-thymidine to the samples, which were incubated for 5 h. Results were expressed in cpm as the radioactivity incorporated by the mitogen stimulated cells minus the radioactivity of the control cells (without mitogens).

ADCC (antibody-dependent cytotoxicity) assay. O Rh-positive human red blood cells were used as target, and a monocyte-free mononuclear cell suspension was treated with phenolic glycosides as effector in 1 : 10 ratio. The reaction was mediated by red blood cell specific anti-D antibody in Greiner microtitre plates. The results were expressed in terms of the amount of ^{51}Cr released during the lysis of the target [19].

Assay for phagocytotic activity of neutrophilic granulocytes. The granulocytes obtained were activated with ^{125}I-labelled zymosan [20] in the presence of the tested compounds in concentrations of 0.5 to 5.0 μg/ml. The phagocytosis index was calculated as the quotient of zymosan-activated sample cpm and basic activity cpm [21]. All immunological assays were performed in three independent experiments with cells from three different healthy persons.

Assay of antiviral activity. A pseudorabies virus (Aujeszky) was propagated in primary chick embryofibroblast (CEF) cells. The infectivity of the virus was estimated by the plaque method on CEF [22]. The virus titre was expressed as log 10 p.f.u. (0.1) ml. The *virostatic effect* of the compounds on the multiplication of pseudorabies virus was investigated in secondary CEF cells by the yield reduction test in Eagle's basal medium [23]. For the *virucidal effects* of compounds equal volumes of drug solutions and the virus suspension having a titre of $10^{5.94}$ p.f.u./0.1 ml were mixed. The mixture was incubated at room temperature for 24 h and after that the infectivity of the mixtures was measured by the plaque method. For *cytotoxicity* of compounds, the secondary CEF cells were cultured in the presence of the compounds at different concentrations at 37 °C for 24 h. The cells were examined under light microscope and compared with the untreated cells.

Assay for antiinflammatory effect. Eight-week-old randomly-bred female CFLP mice weighing 30.6 + 1.46 g, fed commercial food pellets and tap water ad libitum, were used to detect the effects of the compounds on the acute inflammatory response induced in the left footpad by 300 μg carrageenin [24] (Viscarin 402, Marine Colloids, Inc.) dissolved in 0.03 ml isotonic saline.



1. R_1=H, R_2=H: desrhamnosyl acteoside
2. R_1=rha, R_2=H: acteoside
3. R_1=glc, R_2=H: purpureaside A
4. R_1=glc, R_2=rha: purpureaside B
5. R_1=rha, R_2=gal: purpureaside C

(rha=rhamnosyl, glc=glucosyl, gal=galactosyl)

Fig. 1. Chemical structures of phenolic glycosides

Results and discussion

Several phenolic glycosides, as stress components, may prevent the infections of various plants, due to their antibacterial effects [5, 25]. The antibacterial effects of acteoside, desrhamnosyl acteoside and purpureaside A, B and C were studied when a few thousand test bacteria were cultivated in the presence of the compounds at various concentrations, the *E. coli* K12 LE 140 laboratory strain was shown to be sensitive to the growth inhibitory effect of the compound (Table I). Phenolic glycosides exerted the same antibacterial effects despite they have different sugar moieties on the middle ring of the tricyclic skeleton.

In order to acquire information on the antiplasmid effects of the phenolic glycosides upon plasmid replication, *E. coli* F'lac cultures were grown in the presence of the acteoside and purpureaside derivatives and tested on EMB differential media. The presence of this plasmid was easy to test, because it codes the lactose fermentation ability of chromosomally lactose-negative bacteria. Acteoside had an antiplasmid effect, while its desrhamnosyl derivative and the purpureasides were ineffective (Table I). In addition, acteoside treatment of plasmid-harbouring bacteria leads to some inhibition of conjugational plasmid transfer from the donor to the recipient bacteria. At the same time, the mating pair treatment did not inhibit the plasmid transfer. It is possible that the lipophilic sugar (rhamnosyl) moiety makes the molecule able to bind to bacterial pili and the inhibition of the conjugal transfer of the bacterial plasmid is probably due to the inactivation of the pili (Table II). Purpureaside A, with a glucosyl moiety instead of rhamnosyl, was ineffective, because glucosyl substitution makes the molecule more hydrophobic than in the case of acteoside. Desrhamnosyl acteoside was also ineffective. Purpureaside B and C exerted weak inhibitory effects on the conjugation process (Table II). As a consequence

Table I

Elimination of F'lac plasmid and antibacterial effect of phenolic glycosides on E. coli

Compounds concentration μg/ml	F' lac plasmid elimination %	Antibacterial effect No. of viable cells *E. coli* Le 140×10^8
Desrhamnosylacteoside		2.6
100	0	2.1
200	0.1	2.0
400	0.1	0.6
1000	0	MIC
1800	—	
Purpureaside A		
100	0	1.2
200	0	0.7
400	0	0.5
1000	0.25	0.3
1800	—	MIC
Purpureaside B		
100	0	0.8
200	0	0.62
400	0	0.5
1000	0.3	0.4
1600	—	MIC
Purpureaside C		
100	0.1	1.8
200	0.0	1.5
400	0.0	1.0
1000	0.3	0.2
1500	—	MIC
Acteoside		
100	0	2.0
200	4.2	2.0
400	2.3	1.6
1000	0	0.5
2000	—	MIC
Control	0	3.2

of inactivation of the bacterial pili, the mating pair formation is inhibited, which is essential for bacterial conjugation.

Because of the direct antimicrobial effect of compounds it was worthwhile to study how the phenolic glycosides can modify the immune response of leucocytes, which represents the first line of defence mechanism of the infected host. The effects of these compounds were tested on the cell surface properties E and EA rosette formation of leucocytes, the response capacities of leucocytes in blast transformation, the ADCC activity of the mononuclear cells and the phagocytic activity of human granulocytes in the presence of the compounds in vitro.

Table II

Effects of some phenolic glycoside compounds on R144 plasmid transfer by pretreatment of the donor strain prior to mating and by treatment of preformed mating pairs

Compounds	Treatment	No. of transconjugants $\times 10^4$	No. of donors $\times 10^6$	No. of recipients $\times 10^6$
Desrhamnosyl-acteoside	donor-pretreatment	0.48	100	90
	pair treatment	0.67	120	98
Purpureaside A	donor-pretreatment	0.41	110	80
	pair treatment	0.70	113	102
Purpureaside B	donor-pretreatment	0.39	160	105
	pair treatment	0.65	138	102
Purpureaside C	donor-pretreatment	0.37	130	90
	pair treatment	0.65	138	95
Acteoside	donor-pretreatment	0.26	110	106
	pair treatment	0.73	117	95
Control		0.78	140	103

Donor *E. coli* R144 and the recipient *E. coli* W1 azir were grown in MTY broth for 8 h then cultures were diluted 1 : 10 in MTY broth. The diluted donor culture was distributed into 0.5 ml aliquots then tested compounds were given to donor cells or in some experiments 0.5 ml of recipient cell culture was added to the donor cells to allow mating pair formation at 37° C for 5 minutes then the compounds were given. Each of phenolic glycosides was added to the samples at 200 μg/ml final concentration. The samples were incubated at 37 °C for 120 min with the compounds then dilutions were plated into selective media. The plates were incubated at 37 °C and the number of colony formers was counted after 48 h.

In the in vitro immunological tests, only acteoside and purpureaside C affected the T-cell membrane structure, purpureaside C inhibited the E- and acteoside inhibited the EA-rosette formation. Acteoside, desrhamnosyl acteoside, and purpureaside A and B lowered the phagocytic activity of human neutrophilic granulocytes, while none of the compounds affected the ADCC (antibody-dependent cytotoxicity) of lymphocytes. All of the phenolic glycosides tested decreased the mitogen induced response capacity of T and B-lymphocytes in blast transformation experiments (Table III). Our experiments demonstrated that phenolic glycosides tested decreased the activities of human leucocytes in vitro and in addition all of the compounds had a weak antiinflammatory effect in vivo. It is not easy to differentiate the chemical structure-dependent effects of the compounds on the various immunofunctions of the leucocytes, since the receptors, if any, and biochemical processes differ greatly as concerns the type of cellular responses measured under special conditions.

Table III

The capacity of cellular immune response of human leucocytes in the presence of phenolic glycosides

Compounds	Rosette formation, E (T cells)	EA (FC bearing)	Blast transformation of lymphocytes cpm/10^5 cells			ADCC antibody dependent cytotoxicity	Phagocytic index J^{125} labelled zymosan phagocytosis by neutrophylic granulocytes
	%	%	PHA	Con A	PWM	%	
Desrhamnosyl-acteoside	64	44	10483	11302	24165	64	4.13
Purpureaside A	70	44	12143	18600	26410	63	4.37
Purpureaside B	70	48	17146	20130	26160	64	4.35
Purpureaside C	26	46	12180	26130	27159	65	8.54
Acetoside	53	27	15250	17435	24842	73	6.74
Control	70	46	17253	19822	32159	62	8.59

Each test was made with 3 different cell lines where the compounds were applied in 5 μg/ml final concentration
PHA — acts on T- and B-lymphocytes
con A — acts on T lymphocytes
PWM — acts mainly on B lymphocytes

Before the antiviral study, the cytotoxicity of the phenolic glucosides was examined on CEF cells by light microscope. No visible morphological difference was observed between treated and untreated cells. The minimal toxic dose of CEF cells for acteoside, desrhamnosyl acteoside, and purpureaside A and B was found to be 500 μg/ml, but purpureaside C was not toxic at this concentration. At first the multiplication of virus on tissue culture was determined in the presence of compounds then the direct virucidal effect was tested.

The virostatic effects of the compounds are presented in Table IV. It can be seen that acteoside, desrhamnosyl acteoside and purpureaside A have only a slight inhibitory effect on the multiplication of pseudorabies virus. These compounds at a concentration of 100 μg/ml reduced the virus yield by 0.4–0.5 log.

Leaf phenols with similar chemical structures have been shown to have moderate antiviral effects on potato virus X [6] and *Herpesvirus hominis* [8]. In the case of purpureaside B and C, the two sugar moieties modify the conformation of the molecules and abolish the antiviral effect on pseudorabies virus.

The inactivating effects on the infectivity of pseudorabies virus can be observed at the high concentrations of desrhamnosyl acteoside, and purpureaside A while the purpureaside C had no effect. The effective antibacterial and antiviral structures apparently differ.

Some experiments were performed on the inflammatory response in vivo in the presence of these compounds, because this type of defence mechanism plays an important role during infection. The results obtained with the car-

Table IV

Effect of compounds on the multiplication of pseudorabies virus

Compounds	Concentration (μg/ml)	Virus titre (log p.f.u./0.1 ml) p. f. u.	Inhibition[1] (log)	Inhibition[2] (log)
Acteoside	100	5.3	0.4	0.2
	50	5.4	0.3	0.2
	10	5.7	0.0	0.2
	1	5.7	0.0	0.0
Desrhamnosyl-acteoside	100	5.2	0.5	0.3
	50	5.2	0.2	0.3
	10	5.7	0.0	0.3
	1	5.5	0.2	0.0
Purpureaside-A	100	5.2	0.5	0.4
	50	5.6	0.1	0.4
	10	5.6	0.1	0.3
	1	5.6	0.1	0.0
Purpureaside-B	100	5.5	0.2	0.4
	50	5.7	0.0	0.3
	10	5.7	0.0	0.2
	1	5.7	0.0	0.0
Purpureaside-C	100	5.7	0.0	0.0
	50	5.7	0.0	0.0
	10	5.7	0.0	0.0
	1	5.7	0.0	0.0
Virus control	—	5.7	—	0

[1] Assay for virostatic effect
[2] Assay for virucidal effect

Table V

Effect of the compounds on carrageenin-induced acute inflammatory response in CFLP mice

Compounds tested (n)	Percentage increase in paw weights as compared to controls means $\pm$ S.E.M.	Percentage difference as compared to controls	P
Control (13)	24.4$\pm$1.67	—	—
Desrhamnosyl-acteoside (10)	24.9$\pm$2.76	+2.3	n.s.
Purpureaside			
A (10)	21.5$\pm$2.45	−11.6	n.s.
B (10)	19.7$\pm$2.98	−19.1	n.s.
C (10)	32.9$\pm$4.90	+35.3	0.05
Acteoside (10)	21.2$\pm$3.28	−12.6	n.s.

n = number of CFLP mice;
n.s. = not significant;
The mice were treated in their left hind paws with 300 μg carrageenin plus 30 μg compound dissolved in 0.03 ml isotonic saline; the inflammatory response was evaluated 3 h after the intrapedal injections

rageenin paw oedema test in CFLP mice showed that none of the compounds tested exerted a significant effect on the acute inflammatory process except purpureaside C, which increased the paw swelling by 35.3% as compared with the control value (Table V).

Despite of the in vitro observed weak antibacterial, antiviral and antiplasmid effects of these phenolic glycosides, the proinflammatory effect of purpureaside C may have theoretical interest.

REFERENCES

1. Ellis, B. E.: Phytochemistry **22,** 1941 (1983).
2. Hayashi, T., Matsui, C.: Phytopathology **55,** 387 (1965).
3. Legrand, M., Fritig, B., Hirth, L.: Phytochemistry **15,** 1353 (1976).
4. Shoyama, Y., Matsumoto, M., Nishioka, I.: Phytochemistry **26,** 983 (1987).
5. Nishibe, S., Okabe, K., Tsukamoto, H., Sakushima, A., Hisada, S., Baba, H., Akisada, T.: Chem Pharm Bull **30,** 4548 (1982).
6. Pierpoint, W. S., Ireland, R. J., Carpenter, J. M.: Phytochemistry **16,** 29 (1977).
7. Shoyama, Y., Matsumoto, M., Nishioka, I.: Phytochemistry **25,** 1633 (1986).
8. Thiel, K.D., Helbig, B., Klöcking, R., Wutzler, P., Sprössig, M., Schweizer, H.: Pharmazie **36,** 50 (1981).
9. Matsumoto, M., Koga, S., Shoyama, Y., Nishioka, I.: Phytochemistry **26,** 3225 (1987).
10. Shoyama, Y., Nagano, M., Nishioka, I.: Planta Medica **48,** 124 (1983).
11. Matsumoto, M., Nagano, M., Shoyama, Y., Nishioka, I.: Shoyakugaku Zasshi **40,** 193 (1986).
12. Hatano, K., Kamura, K., Shoyama, Y., Nishioka, I.: Planta Medica (1987).
13. Hatano, K., Shoyama, Y., Nishioka, I.: Plant Cell Report **6,** 446 (1987).
14. Shoyama, Y., Tareno, R., Nishioka, I.: Shoyakugaku Zasshi **45,** 313 (1987).
15. Mándi, Y., Molnár J., Holland, I. B., Béládi, I.: Genet Res Camb **26,** 109 (1976).
16. Molnár, J., Schneider, B., Mándi, Y., Farkas, S., Holland, I. B.: Acta Microbiol Acad Sci Hung **27,** 315 (1980).
17. Aiuti, F., Lacava, V., Garofalo, J. A.: Clin Exp Immunol **15,** 43 (1973).
18. Macleod, A. M., Mason, R. J., Stewart, Kn. N.: Transplant Proc **15,** 1019 (1983).
19. Urbanaik, S. J.: Br J Haematol **33,** 409 (1976).
20. Klebanoff, S. J., Clark, R. A.: J Lab Clin Med **89,** 675 (1977).
21. Molnár, J., Petri, I., Berek, I., Shoyama, Y., Nishioka, I.: Acta Microbiol Hung **34,** 306 (1987).
22. Béládi, I.: Acta Vet Acad Sci Hung **12,** 417 (1962).
23. Macpherson, I., Stoker, M.: Virology **16,** 147 (1962).
24. Levy, L.: Life Sci **8,** 601 (1969).
25. Kitagawa, S., Hisada, S., Nishibe, S.: Phytochemistry **23,** 1635 (1984).

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INTERFERON-INDUCIBILITY IS DIMINISHED IN TUMOUR-BEARING MICE

PHAM LE HUNG, IVANA STŐGER, ERIKA KARCZAG, J. MINÁROVICS and I. FÖLDES

Microbiological Research Group of the National Institute of Hygiene, Budapest

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Inducibility of interferons was studied in tumour-bearing mice. Using five different transplantable tumour-models, diminishing of interferon-inducibility was revealed during tumour progression, irrespectively of the tumour-model used. Diminishing of interferon-inducibility runs parallel with development of the tumours.

A considerable body of experimental and clinical data referring to the anti-tumour effects of interferons (IFNs) have been accumulated in the last years [1–15]. One can hardly find, however, data, whether inducibility of IFNs undergoes changes in tumour-bearing individuals [16–18]. It seemed to be important, therefore, to analyze inducibility of IFNs during carcinogenesis, as putative changes in this respect could contribute to understand the pathogenesis of tumour development. By the use of 5 different transplantable tumour-models we have analyzed IFN-inducibility in mice.

Materials and methods

Experimental animals. Seven to 8 weeks old Balb/c, C57B1/6 and DBA/2 mice (LATI, Gödöllő, Hungary) of both sexes were used.

Tumours. (1) Lewis lung carcinoma (LL) was maintained by serial in vivo passages inoculating 10^5 LL cells intramuscularly into C57B1/6 females.

(2) BaF1 tumour, a benzpyrene induced transplantable fibrosarcoma established in a Balb/c mouse was kindly provided by J. Fachet (Institute of Pathophysiology, University Medical School, Debrecen, Hungary). It is maintained by in vivo transplantations since years in our laboratory.

(3) Sp4, a transplantable spontaneous adenocarcinoma originated from a Balb/c female mouse in our laboratory. It is maintained in syngeneic female mice by injecting 2×10^5 cells intramuscularly.

(4) P815, a methylcholantrene induced mastocytoma was maintained in ascites form by weekly passages of 5×10^5 tumour cells into the peritoneal cavity of DBA/2 females. Intramuscular injection of 5×10^5 P815 cells resulted in solid tumours with a 100% take.

(5) P388 leukemia was also maintained in DBA/2 mice inoculating 5×10^5 cells intraperitoneally. This tumour also grows in ascitic form.

Chemical. Poly I : C (Calbiochem, sodium salt A grade Lot 702045) dissolved in sterile saline (100 μg/mouse) was used intraperitoneally to induce IFN.

PHAM LE HUNG, IVANA STŐGER, ERIKA KARCZAG, JÁNOS MINÁROVITS, ISTVÁN FÖLDES

Microbiological Research Group of the National Institute of Hygiene
Pihenő út 1, H-1529 Budapest, Hungary

Interferon titration. Pooled sera from 4–4 control and poly I : C induced mice were kept at −20 °C until titration. Titration was performed by measuring the anticytopathic effect in L929 fibroblast cell cultures using encephalo-myocarditis virus (EMC) as challenge. Maintaining and culturing L929 cells and details of the assay are described in [19]. The titres were compared to WHO International Reference Preparation, Mouse IFN, 12 000 IU/ml, prepared and distributed by NIAID-NIH, Bethesda, USA. Interferon titres were expressed in international units (IU). EMC used was originally isolated from mouse-brain suspension and adapted to L929 cells.

Evaluation of the experiments. All kinds of experiments were performed at least three times. We would like to emphasize that IFN-inducibility in tumour-bearing mice showed the same tendency in every experiment as compared to that of control mice. In "Results" we show, therefore, "representative" experiments; according to our opinion no statistical analysis was needed in the experimental conditions given.

Results

Effect of poly I : C on the serum IFN levels of different inbred mouse strains. As the 5 different tumour models use 3 different kinds of mouse strains, we had to compare, first of all, the IFN titres of these strains after induction with poly I : C. Table I shows that (i) there is no detectable IFN in sera of control (non-induced) mice; (ii) two hours after induction a maximal IFN-level can be detected; (iii) the maximal IFN-level in DBA/2 mice is lower than that of C57B1/6 and Balb/c mice; (iv) there is no difference in IFN-levels of male and female mice within the same strain.

Effect of poly I : C on the serum IFN levels of mice transplanted with different kinds of tumours. Tables II, III, IV, V and VI show the poly I : C induced IFN titres of mice at different intervals after tumour-transplantation. IFN-titres proved to be lower in tumour-bearing mice as compared to the control (tumour-free) ones, irrespectively of the tumour-model used. It is also obvious that diminishing of IFN titres runs parallel with tumour-progression; the larger tumour were detected, i.e. the more time elapsed since transplanting the tumours, the lower IFN-titres could be measured.

In the P815 and P388 tumour-models tumour cells were transplanted intraperitoneally, thus tumour cells growing in the peritoneal cavity might interfere with the effects of poly I : C given also intraperitoneally. In these tumour-models, therefore, the experiments were also performed after transplanting the tumour cells intramuscularly. The same diminishing of IFN-titres was observed as after intraperitoneal transplantation of the tumour cells (data not shown).

Having observed lower poly I : C induced IFN-titres in tumour-bearing than in the control mice, the question arose whether this phenomenon reflects (i) really lower IFN quantities produced in the tumour-bearing mice or (ii) some materials produced in the tumourous hosts interfere with IFNs induced, thus inhibiting their detectability. To solve this problem the following experiments were performed.

Table I

Serum IFN titres of male and female Balb/c, C57Bl/6 and DBA/2 mice 0, 2, 6 and 24 h after induction with 100 μg/mouse poly I : C

Strain	Sex	Time after induction (h)	IFN titres (IU)
Balb/c	female	0	0
		2	960
		6	960
		24	60
C57Black/6	female	0	0
		2	960
		6	960
		24	30
DBA/2	female	0	0
		2	480
		6	480
		24	30
Balb/c	male	0	0
		2	960
		6	960
		24	60
C57Black/6	male	0	0
		2	960
		6	960
		24	30
DBA/2	male	0	0
		2	480
		6	480
		24	30

Table II

Serum IFN titres of C57Bl/6 mice transplanted with Lewis lung carcinoma 2 h after induction with 100 μg/mouse poly I : C*

Time after transplantation (days)	Mean tumour diameter (mm)	IFN titres (IU)
5	Ø	960
11	14	480
18	17	240
31	21	120
Control non transplanted) mice	0	960
Control (transplanted, but not treated with poly I : C) mice	17	Ø

* 10^5 tumour cells were given to each mouse intramuscularly

Table III

Serum IFN titres of Balb/c female mice transplanted with Sp4 adenocarcinoma 2 h after induction with 100 μg/mouse poly I : C*

Time after transplantation (days)	Mean tumour diameter (mm)	IFN titres (IU)
2	Ø	960
7	Ø	480
14	4.5	480
21	10.5	240
28	14.5	60
28		
Transplanted mice with no tumor-take	Ø	960
Control mice (non-transplated)	Ø	960

* 2×10^5 tumour cells were given to each mouse intramuscularly

Table IV

Serum IFN titres of Balb/c male mice transplanted with BaF1 fibrosarcoma 2 h after induction with 100 μg/mouse poly I : C*

Time after transplantation (days)	Mean tumour diameter (mm)	IFN titres (IU)
7	Ø	480
13	7.5	240
21	16.5	160
Control (non transplanted) mice	Ø	960
Control (transplanted, but non-induced) mice	7.5	Ø

* 10^5 tumour cells were given to each mouse intramuscularly

Table V

Serum IFN titres of DBA/2 female mice transplanted with P815 mastocytoma 2 h after induction with 100 μg/mouse poly I : C*

Time after transplantation (days)	IFN titres (IU)
3	120
7	Ø
Control (non transplanted) mice	480

* 5×10^5 cells were given to each mouse intraperitoneally

Table VI

Serum IFN titres of DBA/2 female mice transplanted with P388 leukemia 2 h after induction with 100 μg/mouse poly I : C*

Time after transplantation (days)	IFN titres (IU)
3	240
7	30
Control non transplanted) mice	480

* 5×10^5 cells were given to each mouse intraperitoneally

(1) We added sera from progressor BaFl-, Sp4- and LL tumour-bearing mice to sera obtained from poly I : C induced normal mice with known IFN-titres (1 : 1 ratio). In these mixed sera similar IFN-titres could be measured as in the control sera (calculated to similar serum quantities; data not shown).

(2) We incubated sera with known IFN-titres in the presence of LL cells under appropriate conditions for 2 and 18 h. After incubation, IFN-titres of cell-free supernatants were measured and compared to control sera (i.e. incubated similarly in the absence of LL cells). No differences were found, thus effects of putative proteolytic enzymes released from the tumour cells could be excluded. In a series of experiments sera from tumour-bearing mice, or cell free ascitic fluid from mice bearing P815 or P388 tumours in ascitic form were injected to healthy mice and after 24 h IFN was induced with poly I : C. Two hours after induction similar IFN-titres were measured as in control mice not treated with sera or ascitic fluid (data not shown). Thus, a direct effect of materials liberated from the tumour cells or induced by them on IFN-producer cells can be excluded.

Discussion

The experiments performed by the use of five different transplantable tumour-models unanimously show that inducibility of IFNs markedly decreases in tumour-bearing mice. This decrease runs parallel with progression of the tumours. Within this tendency dynamics of the observed phenomenon differs in the tumour-models investigated. By the use of special experiments we could state that the lower IFN-titres measured in tumour-bearing mice reflect a real decrease of IFN production, as we have found no biologically active substances in sera of tumour-bearing mice, or in cell-free supernatants of tumour cells interfering with IFN-determination.

It is a question of utmost importance, whether the phenomenon observed, i.e. a diminished inducibility of IFNs does have some role in tumour development, or it is only a finding having no real meaning in tumourigenesis. No final answer can be given to this question. Taken into consideration, however, that disorders of gene regulation have a central role in cancerogenesis and IFNs are among the most important regulatory proteins, underestimating of the phenomenon observed would be a mistake. Further experiments are necessary to study interrelationships between tumour progression and IFN-inducibility and to analyze the IFN system in individuals with tumours of different histologic types.

REFERENCES

1. Gresser, I.: In Sikora, K. (ed.): Interferon and cancer. Plenum Press, New York and London 1983. pp. 65–76.
2. Borden, E. C.: In Gresser, I. (ed.): Interferon 5. Academic Press, London 1983. pp. 43–83.
3. Oldham, R. K.: In Gresser, I. (ed.): Interferon 6. Academic Press, London 1985. pp. 127–143.
4. Hofmann, V., Fehr, J., Sauter, C., Ottino, J.: Cancer Treatment Reviews **12,** (Suppl. B) 33 (1985).
5. Creagen, E. E., Ahmann, D. L., Frytak, S., Long, H. J., Chang, M. N., Itri, L. M.: Cancer **59,** 638 (1987).
6. Cooper, M. R., Welander, C. E.: Cancer **59,** 594 (1987).
7. Foon, K. A., Roth, M. S., Bunn, P. A.: Cancer **59,** 601 (1987).
8. Glaspy, J. A., Jacobs, A. D., Golde, D. W.: Cancer **59,** 652 (1987).
9. Groopman, J. E.: Cancer **59,** 633 (1987).
10. Krown, S. E.: Cancer **59,** 647 (1987).
11. Sarna, G., Figlin, R., de Kernion, J.: Cancer **59,** 610 (1987).
12. Steis, R. G., Foon, K. A., Longo, D. L.: Cancer **59,** 658 (1987).
13. Talpaz, M., Kantarjian, H., McCredie, K., Trujillo, J., Keating, M., Gutterman, J. U.: Cancer **59,** 664 (1987).
14. Thompson, J. A., Fefer, A.: Cancer **59,** 605 (1987).
15. Volberding, P. A., Mitsuyasu, R. T., Golando, P. J., Spiegel R. J.: Cancer **59,** 620 (1987).
16. Wheelock, E. F.: Proc Natl Acad Sci USA **55,** 774 (1966).
17. Vandeputte, M., De Lafontayne, J., Billiau, A., De Somer, P.: Gesamte Virusforsch **20,** 235 (1967).
18. De Mayer-Guignard, J.: Science **177,** 797 (1972).
19. Finter, N. B.: In Interferons and Interferon Inducers. North-Holland Publishing Co. Amsterdam–London–New York 1973. pp. 135–169.

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EFFECT OF DETOXIFICATION PROCESSES ON THE INTERFERON-INDUCING ACTIVITY OF BACTERIAL ENDOTOXINS

J. Šourek, J. Jandejsek and J. Levin

Institute of Hygiene and Epidemiology, Prague, Czechoslovakia, and Veterans Administration, Medical Center and University of California, School of Medicine San Francisco, CA, USA

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The interferon (IFN)-inducing activity of detoxified lipopolysaccharide (LPS) was tested in rabbits treated with LPS preparation derived from *Escherichia coli, Salmonella typhi, Salmonella enteritidis* and *Shigella dysenteriae* serovar 1. Of the detoxification procedures used, alkaline hydrolysis, hydroxylaminolysis, formolization, treatment with sodium deoxycholate and the radiodetoxification (fast or slow) methods had no appreciable effects on the IFN-inducing potential of LPS. In contrast, acetylation or prolonged alkaline hydrolysis of LPS resulted in up to a 9-fold reduction of IFN-induction capacity and effects of Cu^{++} or Fe^{++} cations bound to LPS were clearly inhibitory (Fe more than Cu).

Endotoxins-lipopolysaccharides (LPSs) of Gram-negative bacteria of the family *Enterobacteriaceae* belong to cell products that are potent inducers of interferon (IFN) production [1, 2]. LPS carries various structural regions of diverse biological function. The principal endotoxic component of this complex is lipid A, the centre of all important biological activities of LPS. Bacterial LPS are generally accepted as effective immunomodulators of non-specific resistance mechanisms, triggering in the macroorganism a variety of favourable immunologic responses by enhancing macrophage stimulation, induction of IFN, anti-tumour activity, etc. [3–5]. However, therapeutic applications of LPS are discouraged by their adverse effects such as toxicity or lethality. To overcome this, many approaches have been chosen in an attempt to remove undesirable toxic effects of LPS while preserving their biological activities favourable for the macroorganism [6]. The methodology used ranges from employment of various chemical detoxification techniques such as acetylation, alkaline hydrolysis or hydroxylaminolysis [7] to attempts at synthesizing the lipid A component of a suitable immuno-modulation characteristic [8–10].

One of the favourable activities of LPS is their capacity to induce IFN. There is relatively little knowledge on the effect of detoxification procedures

J. Šourek, J. Jandejsek
Institute of Hygiene and Epidemiology, Šrobarova 48, 100 42 Prague 10, Czechoslovakia

J. Levin
Veterans Administration, Medical Center and University of California, School of Medicine San Francisco, California, USA

on the IFN-inducing activity of LPS. In the present study we compared the interferongenicity of intact and detoxified LPS preparations derived from four enterobacterial species belonging to the genera *Escherichia*, *Salmonella* and *Shigella*. The degree of LPS endotoxicity was measured in the *Limulus* amebocyte lysate test which is about 100 times as sensitive as the rabbit pyrogen test [11].

Materials and methods

LPS preparations and their subunits. The preparations used were obtained by phenol-water extraction according to Westphal (W) or by trichloroacetic acid extraction according to Boivin (B): LPS (W) from *Salmonella typhi* No. 3124–25, LPS (W) from *S. enteritidis* No. 3126–25, LPS (B) from *E. coli* O128 : B12 No. 3924–25, all provided by Difco, Detroit, MI, USA [12]; laboratory-prepared LPS (W) from *Shigella dysenteriae* serovar 1, R-growth form (strain-o, 58) and its lipid A [13].

Detoxification and other procedures. S. typhi LPS was detoxified by acetylation [14], alkaline hydrolysis [15], alkaline hydroxylaminolysis, hydroxylaminolysis in absence of NaOH [15] or by formolization (final formaldehyde concentration, 0.5%; 1 or 7 days at 37 °C); LPS from *S. typhi* and *S. enteritidis* was altered by binding Fe^{++} or Cu^{++} in absence of EDTA to LPS [16]; LPS from *S. dysenteriae* 1 was treated with sodium deoxycholate according to Ribi [17] and used undialyzed or after 24-hour dialysis at 4 °C.

LPS preparations from *E. coli* and *S. enteritidis* and the lipid A of *S. dysenteriae* 1 LPS were also subjected to radiodetoxification procedures. The source of ionizing radiation was a linear accelerator (4 MeV, Tesla, Prague) or a ^{60}Co source (^{60}Co Rosa, USSR); the samples suspended in saline (concentration 1 mg/ml) were irradiated with a dose of 50 kGy.

Induction of interferon. The IFN-inducing activity of LPS was tested in chinchilla rabbits (weight 2.5 kg) intravenously injected with 100 μg/ml, i.e. 40 μg/kg, of LPS dissolved in pyrogen-free saline (pH 7.2); lipid A was solubilized by the addition of triethylamine (final concentration 0.1%). Production of IFN was measured in the serum of blood obtained by cardiac puncture 2 h after injecting the IFN inducer [18].

Interferon assay. IFN was assayed by means of plaque-reducing method [19] on rabbit embryo fibroblast monolayers in 60 mm plastic Petri dishes using vesicular stomatitis virus (strain Indiana, prepared on L 929 cells, titre 2×10^8 p.f.u./ml) as the challenge virus. The IFN titres established according to the method of Langford et al. [20] were expressed as units/ml. One IFN unit in this test was equal to 2 units of the IFN reference rabbit standard (NIH Reference Reagent, Catalogue No. GO19–902–528). The titres in the Tables are means measurements in three animals.

Limulus test. All specimens were tested for endotoxicity using *Limulus* amebocyte lysate assay [21] and the *E. coli* B, O55 : B5 LPS (DIFCO) as a standard. The LPS detectability limit was as low as 0.000001 — 0.00000001 μg/ml.

Results

Effect of chemical detoxification. As it is evident from Table I data, interferongenicity of LPS remained unaffected by alkaline hydrolysis, hydroxylaminolysis and formolization. In contrast, acetylation reduced the activity of *S. typhi* LPS 5 times and prolonged alkaline hydrolysis 9 times. The inhibitory effects of Cu and Fe cations bound to *S. typhi* and *S. enteritidis* LPS preparations were even more pronounced: the Cu-treated LPS produced 10 times less IFN than untreated preparations following the administration of Fe-treated LPS only trace amounts of IFN could be detected in the recipient rabbits.

Table I

Effect of chemical detoxification procedures on IFN-inducing activity of LPS S. typhi, S. enteritidis, and S. dysenteriae 1

Group	LPS (40 μg/kg weight)	Chemical treatment	LT (μg LPS per ml)	IFN (titre)
A	*S. typhi*		10^{-8}	1033
		acetylation	10^{-6}	193
		alkaline hydroxylaminolysis	10^{-7}	1206
		hydroxylaminolysis	10^{-7}	1600
		alkaline hydrolysis (60 min)	10^{-6}	820
		alkaline hydrolysis (6 days)	NT	144
		formolization (24 h)	10^{-6}	1024
		formolization (7 days)	10^{-3}	1370
		binding Cu^{2+} (1)	10^{-4}	105
		binding Fe^{2+} (1)	10^{-2}	16
B	*S. enteritidis*		10^{-6}	2536
		binding Cu^{2+} (2)	5×10^{-2}	215
		binding Fe^{2+} (2)	10^{-2}	8
C	*S. dysenteriae 1*		10^{-6}	250
		sodium deoxycholate (undialyzed)	NT	380
		sodium deoxycholate (dialyzed)	10^{-5}	260
		alkaline hydrolysis (6 days)	NT	64

Content of cations following binding: (1) 37.4 μg Cu/mg LPS, 58.9 μg Fe/mg LPS; (2 32.4 μg Cu/mg LPS, 65.5 μg Fe/mg LPS
NT = non tested; LT = *Limulus* test (lowest concentration of LPS that produced a positive test, using *E. coli* B (Difco) as the standard). High concentrations indicate low biological activity, as measured in the LT

Table II

IFN induction by LPS or lipid A (LA) preparations radiodetoxified by ionizing radiation

Substance 40 μg/kg weight		Titre IFN (U/ml)		
		linear accelerator	^{60}Co	Control (unirradiated)
E. coli	LPS	314	236	430
S. enteriditis	LPS	4096	1296	2800
S. dysenteriae 1	LA	387	280	193

Effect of ionizing radiation. The data presented in Table II provide evidence which shows that fast and slow radiodetoxification procedures had relatively little effect on the IFN-inducing activity of LPS and lipid A. The preparations irradiated with ^{60}Co gamma rays were somewhat less active than those exposed to high-energy radiation from a linear accelerator, but the difference did not reach the level of statistical significance.

Discussion

The detoxification procedures used tended to vary in their effect on the IFN-inducing activity of LPS, showing no linear correlation between the efficacy of LPS detoxification and the capacity of LPS to induce the production of IFN. It became clearly evident that the IFN-inducing potential of LPS was primarily reduced by the detoxification processes that involved pyrophosphate groups and lipid A fatty acid chains (e.g. alkaline hydrolysis, acetylation, Cu^{++} or Fe^{++} binding). This is fully consistent with the previous observations which showed that inactivations of primary toxic structure of lipid A, represented e.g. by glycosidic-bound phosphate or certain fatty acids [22], resulted in a complete loss of LPS potential to induce IFN. This implies the existence of a close association between the endotoxic and IFN-inducing potentials of LPS. Under these circumstances it is highly unlikely to expect that a completely detoxified LPS might retain its full capacity for inducing IFN [9]. It seems likely that the amount of IFN produced depends rather on the intrinsic structure of the inducer and alterations of molecular symmetry [23] that its toxicity [7]. Nevertheless, these and further relations among toxicity, chemical structure and immunomodulating property of LPS will become of interest to the specialists concerned and, undoubtedly, these relations will also be studied on the molecular basis.

Ionizing radiation has a detoxifying effect on LPS [24–26], suppresses its pyrogenicity and reactivity in the *Limulus* test [27], but has no appreciable effect on its IFN-inducing activity. The available evidence suggests that the induction of IFN is primarily affected by the lipid A constituent of the LPS structure [18]. The synthesized lipid A analogues prepared to have the identical chemical structure as the natural lipid A isolated e.g. from LPS of *E. coli Salmonella typhi-murium* or *Proteus mirabilis* strains have been reported to display the identical spectrum of activities such as IFN induction, tumour regression, mitogenicity, adjuvanticity and pyrogenicity, but these activities were never as strong as activities of natural lipid A [9, 19].

LPSs of Gram-negative bacteria act as immunomodulators or macrophage-derived stimulators. In this context it will no doubt be of interest to compare the regulatory interactions of interleukins, tumour necrosis factor and IFN and to define the extent to which their induction might be influenced by detoxification. These problems will be object of further studies.

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REFERENCES

1. Stewart, W. E. (ed.): The Interferon System. Springer-Verlag, Wien 1981. p. 1.
2. Ho, M.: In Billiau, (ed.): Interferon 1. General and Applied Aspects. Elsevier, Amsterdam 1984. p. 79.
3. Bertók, L.: In Nowotny, A. (ed.): Beneficial Effects of Endotoxins. Plenum Press, New York and London p. 213.
4. Galanos, C., Lüderitz, O.: In Rietschel, E. T. (ed.): Chemistry of Endotoxins. Vol. 1. Elsevier, Amsterdam 1984. p. 46.
5. Homma, J. Y., Kanegasaki, S., Lüderitz, O., Shiba, T., Westphal, O. (eds): Bacterial Endotoxin. Chemical, Biological and Clinical Aspects. Verlag Chemie Weinheim, Deerfield Beach, Basel 1984. p. 205.
6. Rietschel, E. T., Brade, H.: Infection **15,** 3 (1987).
7. Galabov, A. S., Galabov, S. M.: Acta Virol **17,** 493 (1973).
8. Galanos, C., Lüderitz, O., Rietschel, E. T., Westphal, O., Brade, H., Brade, L., Freundenberg, M., Schade, U., Imoto, M., Yoshimura, H., Kusumoto, S., Shiba, T.: Eur J Biochem **148,** 1 (1985).
9. Kanegasaki, S., Kojima, Y., Matsuura, M., Homma, J. Y., Yamamoto, A., Kumazawa, Y., Tanamoto, K., Yasuda, T., Tsumita, T., Imoto, M., Yoshimura, H., Yamamoto, M., Shimamoto, T., Kusumoto, S., Shiba, T.: Eur J Biochem **143,** 237 (1984).
10. Shiba, T., Kusumoto, S.: In Rietschel, E. T. (ed.): Chemistry of Endotoxin. Vol. 1. Elsevier, Amsterdam 1984. p. 234.
11. Levin, J.: In Watson, S. W., Levin, J., Novitsky, T. J. (eds): Endotoxins and their Detection with the Limulus Amebocyte Lysate Test. Alan R. Liss, Inc., New York 1982. p. 12.
12. Westphal, O., Lüderitz, O., Staub, A. M., Nikaido, H.: In Weinbaum, G., Kadis, S., Ajl, S. J. (eds): Microbial Toxins. Vol. IV. Academic Press, New York 1971. p. 145.
13. Šourek, J., Trnka, T., Digeon, M., Raynaud, M.: J Hyg (Prague) **19,** 356 (1975).
14. Martin, W. I., Marcus, S.: J Bacteriol **91,** 1453 (1966).
15. Niwa, M., Milner, K. C., Ribi, E., Rudbach, J. A.: J Bacteriol **97,** 1069 (1969).
16. Šourek, J., Tichý, M., Levin, J.: Infect Immun **21,** 648 (1978).
17. Ribi, E., Anacker, R. L., Brown, R., Haskins, W. T., Malgren, B., Milner, K. C., Rudbach, J. A.: J Bacteriol **92,** 1493 (1966).
18. Jandejsek, J., Šourek, J.: Zentralbl Bakteriol (A) **259,** 390 (1985).
19. Wagner, R. R.: Virology **13,** 323 (1961).
20. Langdorf, M. P., Weigent, D. A., Stanton, G. J., Baron, S.: In Colowick, S. P., Kaplan, N. O. (eds): Methods in Enzymology. Vol. 78. Academic Press, New York 1981. p. 339.
21. Levin, J., Bang, F. B.: Thromb Diath Haemorrh **19,** 186 (1968).
22. Previte, J. J., Chang, Y., El-Bisi, H. M.: J Bacteriol **93,** 1607 (1967).
23. Nowotny, A.: In Rietschel, E. T. (ed.): Chemistry of Endotoxin. Vol. 1. Elsevier, Amsterdam 1984. p. 308.
24. Füst, G., Bertók, L., Juhász-Nagy, S.: Infect Immun **16,** 26 (1977).
25. Bertók, L.: Persp Biol Med **24,** 61 (1980).
26. El Sabbagh, M., Galanos, C., Bertók, L., Füst, G., Lüderitz, O.: Acta Microbiol Acad Sci Hung **29,** 255 (1982).
27. Imoto, M., Yoshimura, H., Kusumoto, S., Shiba, T.: Proc Japan Acad **60**B, 285 (1984).

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RELATIONSHIP OF E1 AND E3 REGIONS OF HUMAN ADENOVIRUS 35 TO THOSE OF HUMAN ADENOVIRUS SUBGROUPS A, C AND D

W. Gy. Kang, Gy. Berencsi, A. Bánrévi, Z. Ascher, G. Fejér, Mária Takács, A. Kiss and I. Nász

Virology Branch, Joint Research Organization of the Hungarian Academy of Sciences at the Semmelweis University Medical School, Budapest

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Cloned *Pst*I fragments of human adenovirus 35 (AV35) genome were compared with the DNA of representatives of human adenovirus subgroups *A* (type 12), *B* (type 7), *C* (types 1, and 5), *D* (type 8), and *E* (type 4), using blot hybridization techniques. The *E1b* region of AV35 was found to be more distantly related to those of other subgroups than *E1a* regions sequences and examined by others. DNA hybridization was observed only between *E1b* of AV35 and the DNA of AV4, thus the recombinant constructed might be applied as B-subgroup-specific diagnostic probe. Common nucleotide sequences were detected within the *E3* regions of serotypes 1, 4, 5, 7, 8, and 35. On the basis of inter-subgroup homology, and *Pst*I-fragments it may be concluded, that the structure of *E3* sequences of AV7 and AV35 DNA are closely related to those of AV3 DNA sequenced by Signäs et al. [18]. *E4* regions were compared only of serotypes representing subgroups *B*, *C*, and *D*. These sequences were subgroup specific, similarly to *E1b* regions.

The homology of DNA has been examined using different members of subgroups of human adenoviruses repeatedly by heteroduplex mapping of the DNA [1], by liquid hybridization procedures [2, 3] and by DNA sequencing [4, 5]. Serotypes above type 31 had not been included into these studies. Developments during the last decade emphasized the biological importance of AV type 35 which was isolated in 1977 [6] and found to affect immunocompromized population more frequently [7, 8] leading to fatal consequences [7–10]. Therefore the DNA, and several genes of AV35 have been studied extensively in recent years [11–14].

There are two gene clusters among early regions of the adenovirus genome, which possess outstanding role in the modulation of host cell properties being important from the immunological point of view: E1 and E3 [15–21]. Interactions with the expression or processing of MHC class I glycoproteins,

W. Gy. Kang

Institute of Experimental Biology, Academy of Sciences of the Democratic People's Republic Korea
Pyogyang, DPRK

György Berencsi, Andrea Bánrévi, Zoltán Ascher, György Fejér, Mária Takács, Alexi Kiss, István Nász

Institute of Microbiology,
Semmelweis University Medical School
H-1445 Budapest, P.O.B. 370, Hungary

and tumour necrosis factor were shown as the main molecular biological mechanisms of these biological events [15–17, 21, 22].

In order to explain the prevalence of AV35 in patients suffering from immunosuppression sequences unique for this adenovirus serotype, it has to be identified, cloned and sequenced. Elb region of the DNA was found to be only distantly related to that of other subgroups. An other region of E3 was found to be partially conserved in all subgroups tested, and very similar to other members of subgroup B (AV7) using blot hybridization for DNA mapping.

Materials and methods

Viruses and tissue cultures. The prototype strain of AV35 was obtained from M. N. Oxman (San Diego, California, USA). The prototype strain of AV7 (Gomen) was obtained from G. Wadell (Ůmeå, Sweden). The Hungarian subtype of AV8 was used as the representative of subgroup D [23, 24]. The prototype strain of AV1 (AD71) has been characterized previously from different respects [25, 26]. The DNA yield from the prototype strain of AV4 was very low in our hands, under the conditions of suspension culture preparation from the "Cincinnati line" of HEp2 cells [14]. Therefore, a field strain was selected from an outbreak of keratoconjunctivitis, which produced the highest yield of unit length DNA using the TritonX-100/sodium chloride extraction procedure described by us [14, 26]. The isolates of type 4 had been obtained from E. Szöllősy (Szeged, Hungary), and were typed serologically and using restriction endonucleases [10, 27]. El region of AV12 and AV5 (subgroup A and C of human adenoviruses) was examined using the cloned EcoRI-C fragment (recombinants pAd12RIC and pAd5XhoI-C from ref. [28]). Details of tissue culture techniques, and preparation of virus stocks were described in detail in the preceding paper [14].

Purification of viral DNA, digestion with restriction endonucleases, and separation of the fragments were also described in the preceding paper [14]. The clones and restriction endonuclease maps were reported and discussed, too [11, 13, 14].

Restriction endonucleases *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I and *Sal*I were purchased from Reanal (Budapest) and the *Hind*III endonuclease used regularly for the preparation of M_r markers from AV type 1 DNA [25] was of the same origin.

Techniques used during fragmentation, separation and detection of DNA were taken from standard protocols [29].].

Blot hybridization technique. Nitrocellulose filters were purchased either from Sartorius GmbH (Göttingen, FRG) or Synpor (Prague, Czechoslovakia). Modified Southern protocols were followed [29] except in cases when duplicate blots had to be obtained from the same gel [30].

The nick-translation kit of Bethesda Research Laboratories (Gaithersburg, Maryland, USA) was regularly used with adenosine-5'-alpha-(^{32}P)-triphosphate of 110 TBq/m M specific radioactivity, but accidentally 13 TBq/mM preparations were also incorporated according to the protocols given by the manufacturers (Izinta, Budapest).

The hybridization was performed at 37 °C either in 0.5 or in 0.55 parts of formamide completed with non-fat dry milk [31], 20 mg/ml sodium laurylsulphate (SDS) and 7×SSC [29]. Other ingredients usually added to the mixtures were ommitted. The filters were washed at 60 °C initial temperature of the 1×SSC solution prepared in 1 mg/ml SDS. At least two subsequent washes were performed before the cleaning of filters with 1×SSC + 1 mg/ml SDS at room temperature.

Autoradiography was performed at room temperature for several days if required with duplicate films of HS11 (Orwo, Wolfen, GDR) or Medifort (Forte, Vác, Hungary). Occasionally the films were traced using a Kipp-Zonen (Delft, Holland) microdensitometer.

Source of sequence data and restriction endonuclease maps. The nucleotide sequence of AV2 DNA was obtained from R. Roberts (Cold Spring Harbor Laboratories, NY, USA). The sequences were analysed using a personal computer (Commodore 64) with programme packages prepared by one of us (M. T.). Physical maps of AV1 and 2 were experimentally compared earlier [25]. Restriction endonuclease maps of DNA cluster I and II of subgroup B adenoviruses were taken from many different sources [10, 11, 14] summarized in our preceding publication

[14]. The data of experimental comparison of AV7 and 35, and the physical maps of AV8 DNA with patterns obtained with other restriction endonucleases were also published earlier [14, 24, 27]. Short regions of sequences were examined without computer processing [4, 5, 18, 32, 33], since those of E1 and E3 of subgroup B members were not available on magnetic discs.

Results

Search for unique regions of AV35 DNA. The direct comparison of electrophoretic patterns of AV1, and AV2 DNA cut with *Pst*I restriction endonuclease allowed the determination of the physical map with this enzyme using the computer derived sites of AV2 DNA. The *Pst*I-fragments of AV1 DNA were blotted (Fig. 1) and the blot was hybridized with labelled AV5 probe ($^{32}P_5$ in Fig. 1) and with AV35 probe ($^{32}P_{35}$ in Fig. 1). The conditions of hybridization were corresponding to intermediate stringency (0.5 formamide) in order to detect only very different regions of the genome. The results are summarized in the lower part of Fig. 1. The *Pst*I map of AV1 DNA is shown in the middle part of the figure. Dark, and open boxes indicate the presence of sequence homology detectable under intermediate stringency of hybridization with (Ad 35) and (Ad 5) probes labelled accordingly in Fig. 1. The numbers indicate the regions (kb), of AV1 DNA, which possess no detectable homology to AV35 DNA. The right terminal fragment carrying E4 sequences showed no hybridization.

Large asterisks indicate fragments of similar size giving positive autoradiographic signals, thus verifying the absence of homology in the neighbouring regions. As shown in Fig. 1, five regions were identified in this system, (*Pst*I-fragments: O, I, R, K, J, and G from left to right according to the physical map) which possessed intra-subgenus homology, but intersubgenus homology was absent.

In order to see the locations of similar regions in subgroup B (AV35) and subgroup D (AV8), a similar blot hybridization experiment is presented, too. In Fig. 2 the *Pst*I-fragments of AV35 DNA (lanes *c*) and cloned fragments of AV35 (lanes *a*, *b*, *g*, and *h*) DNA, *Pst*I-cut AV5 DNA and linearized pBR322-Ad8 recombinant DNA are shown. The viral DNA of AV8 was labelled (Ad h 8). The comparison of ethidium bromide fluorescence to the autoradiographic picture indicates that only *Pst*I-fragments A, B, (D-E), F, and (H-I) possess detectable sequence homology. *Pst*I-fragments of AV35 cloned, and recut by *Pst*I verify the homology between fragments B, and F (E3 region) of AV35 (lane *a*), but only *Pst*I-A gave positive signal when the recombinant PstI—A+K+L was blotted (lane *g*). The results obtained with AV35, and AV5 probes were verified in the experiment shown in Fig. 2. The recombinant pAd5-XhoC recut with *Pst*I (duplicate lanes *d* + *e)* gave positive signals only with AV5 sequences at the junction sites of pBR-related sequences, since the probe was viral DNA of AV8. The fragments of *Pst*I-I and O (shown in Fig. 1,

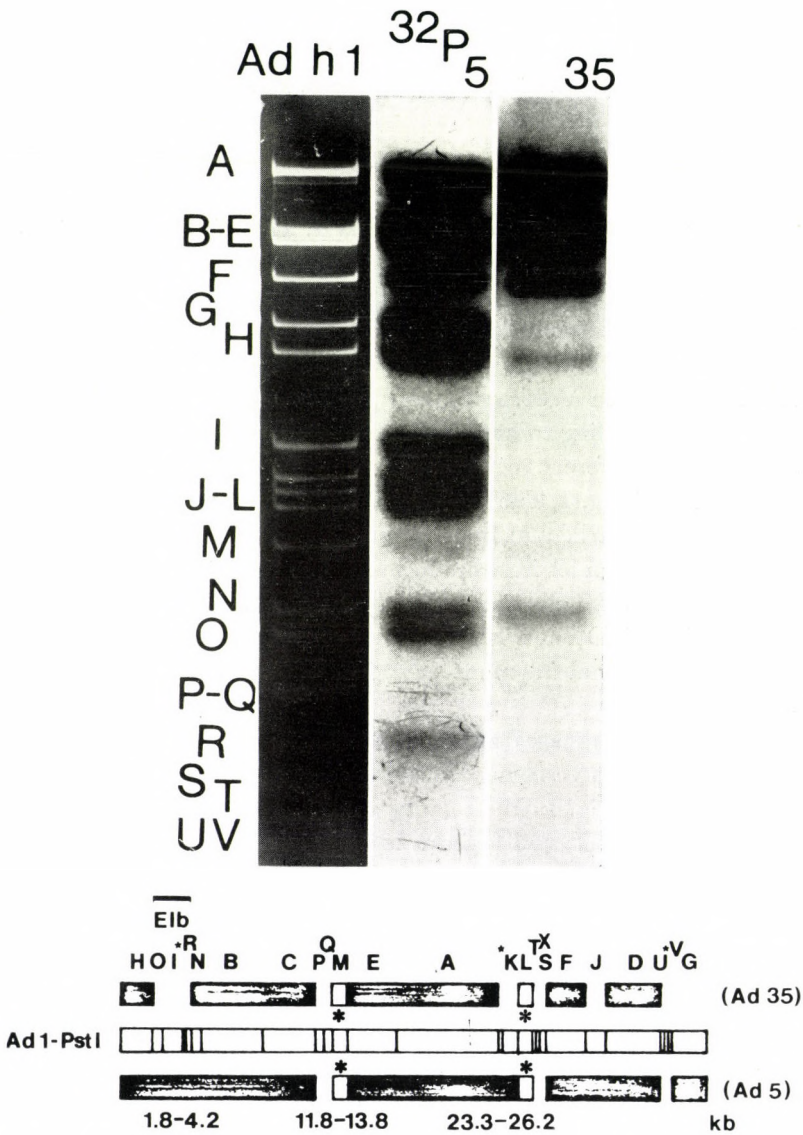


Fig. 1. Intra-subgroup DNA homology of human adenovirus types 1 and 5, and intersubgroup homology of the DNA of types 1 and 35. The *Pst*I fragments of adenovirus type 1 DNA were separated in 20 mg/ml slab gels (Ad h 1). The fragments were blotted [30] using the sandwich technique, and hybridized under less stringent conditions (0.5 parts of formamide at 37 °C) to nick-translated viral DNA of type 5 ($^{32}P_5$), and type 35 ($^{32}P_{35}$). The specific activity of the probes were controlled on the basis of Cerenkov counts [29]. The capital letters show *Pst*I fragments of type 1 DNA belonging to subgroup *C*. Type 5 was also the member of subgroup *C*. Adenovirus type 35 was the representative of subgroup *B* (DNA cluster II, [10]). The autoradiography was performed following differentiation of the blots in 0.1×SSC at 60 °C. The *Pst*I map of type 1 DNA is shown below (Ad-1-*Pst*I). The regions of detectable autoradiographic signals are shown by dark boxes above (Ad35), and below the physical map (Ad5). The fragments carrying Elb sequences are indicated by a horizontal mark. Small asterisks indicate very short *Pst*I fragments of adenovirus type 1. Large asterisks, and open boxes indicate hybridization signals above DNA fragments of identical electrophoretic mobility. Kilobase values (kb) are depicted corresponding to "unique" fragments of type 35 DNA other than E3 and E4

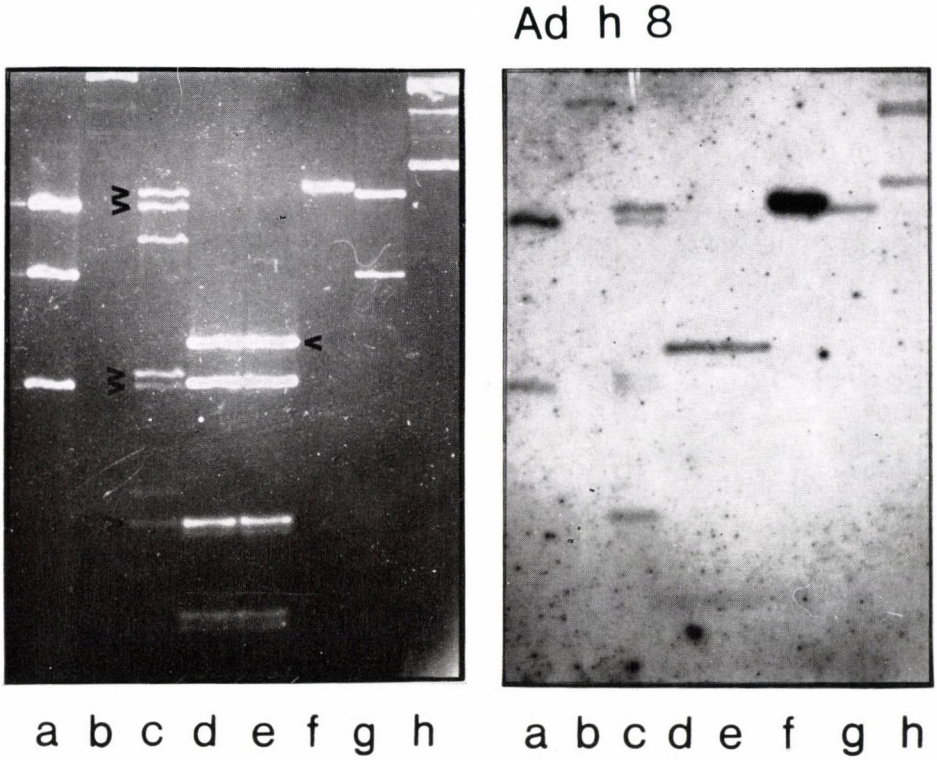


Fig. 2. Blot hybridization of nick-translated adenovirus type 8 (Ad h 8) DNA to cloned DNA fragments of other subgroups. The hybridization was performed in 0.5 parts of formamide using non-fat dry milk [31]. The filter was washed with 1×SSC at 60 ° after hybridization. Arrowheads indicate signals of intersubgroup homology to AV8 (Ad h 8) probe, which is the representative of subgroup D. *Lane a:* cloned *Pst*I—B+F of type 35 DNA; *lane b:* the same uncut recombinant; *lane c:* control viral DNA cut with *Pst*I (type 35); *lanes d* and *e: Pst*I-digested recombinant pAd5XhoI-C [28]; *lane f:* control recombinant of pBR322 carrying 12 kb of the left half of adenovirus 8; *Pst*I-digested recombinant carrying *Pst*I-^{A} of type 35 DNA; *lane h:* undigested recombinant of pAd35PstI-^{A}

which are identical with the AV5 fragments examined) possess no detectable homology to AV8 either. The absence of detectable homology was characteristic at the right end of the genome (E4 region, *Pst*I-C of AV35), and at a few central fragments (*Pst*I-G, K, and L). The E1 region was carried by *Pst*I-(J + + E). The positive signal of *Pst*I-(D + E) cannot be evaluated, because of the identical electrophoretic mobility of the two DNA fragments, therefore cloned *Pst*I-E was used in the following experiments.

Blot hybridization of representatives of other adenovirus subgenera to E1b region of AV35. In order to detect intersubgroup homology of Elb regions the recombinant pAd35*Pst*IE was labelled by nick translation and hybridized under stringent conditions to DNA fragments of AV1, AV2, AV5 (subgroup C), AV7 (Gomen strain, subgroup B), AV35 (homologous DNA) and cloned El region

of AV12 (pAd12EcoRI-C, subgroup A). The results are shown in Fig. 3. The DNA of AV35 cut with *Kpn*I, *Hin*dIII, and *Bam*HI was blotted in lanes *1*, *4*, and *9*, respectively. The DNA of AV7 cut with *Hin*dIII (lane *5)* and *Bam*HI (lane *10)* gave somewhat weaker, but significant signals upon autoradiography with all DNA fragments carrying sequences of E1b.

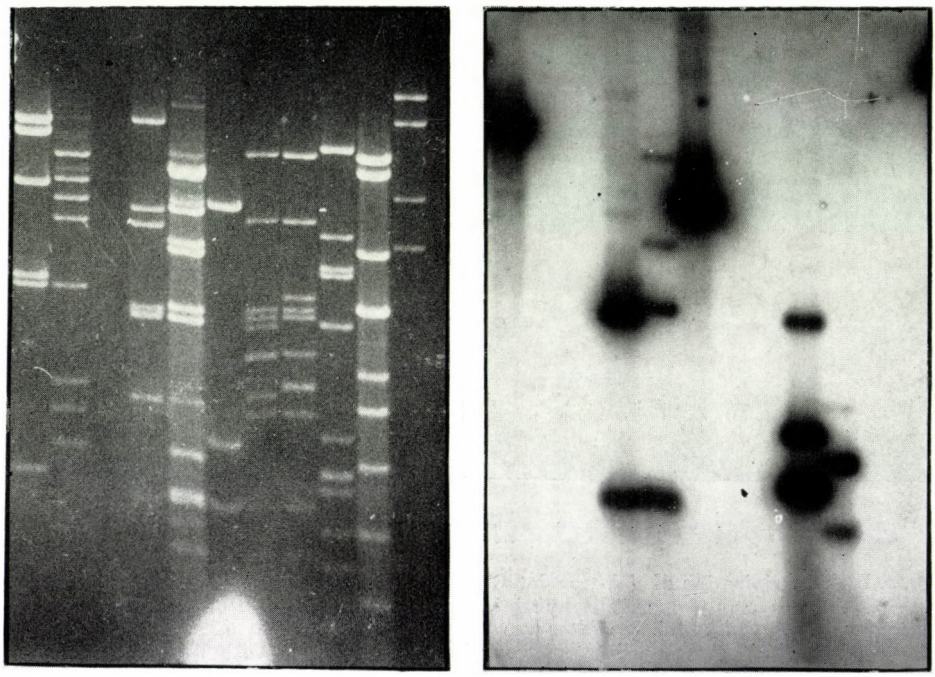


Fig. 3. Blot hybridization of cloned E1B DNA of type 35 to blotted DNA fragments of representatives of other adenovirus subgroups. The hybridization was performed in 0.55 parts of formamide [29]. *Lane 1: Kpn*I-digested DNA of type 35; *lane 29:* DNA of type 2; *lane 3:* no DNA; *lane 4: Hin*dIII-cut DNA of AV35; *lane 5:* AV7 (subgroup B) cut with *Hin*dIII; *lane 69:* pAd12EcoRI-C (28; representative of subgroup A) digested with *Hin*dIII; *lanes 7* and *8: Hin*dIII-cut AV2, and 1; *lanes 9* and *10: Bam*HI-digested DNA of AV 35, and AV7 (Gomen) belonging to subgroup B; *lane 11: Bam*HI-fragmented DNA of type 2 (representing subgroup C). No intersubgroup homology was detected under stringent conditions. PBR322-related sequences gave signals in *lane 6*

Well visible signal was obtained in lane *6*, since pBR322 sequences were separated from AV12 DNA by *Hin*dIII digestion. No detectable homology was obtained to these fragments and to other fragments either, which were carrying E1b sequences of AV1 (lane *8)* or AV2 (lanes *2*, *7*, and *11*). Representative serotypes of subgroup D, and E were also included in the experiment shown in Fig. 4. The same ^{32}phosphate-labelled pAd35*Pst*IE recombinant was used for hybridization under stringent conditions. Homologous DNA frag-

ments of AV35 and AV7 were blotted in lanes *2* and *7*. Only the pBR322 related sequences were hybridizing in the lanes of pAd12EcoRI-C (lane *4)* and pAd5XhoI-C (lane *8*). There was no DNA band visible in lane *8* of the photograph documenting ethidium bromide fluorescence, therefore the lowest weak signal in lane *8* of the autoradiograph was caused by aberrant, deleted or mu-

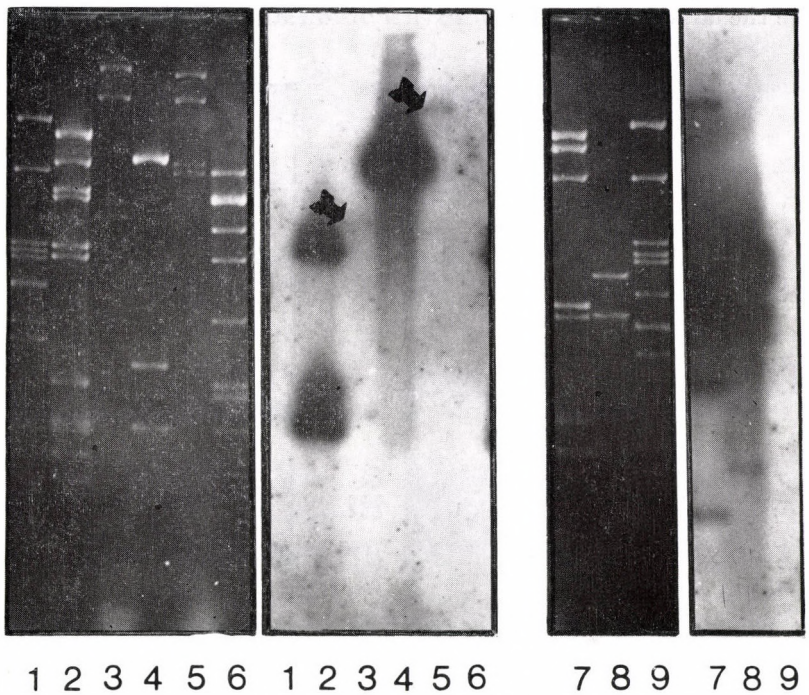


Fig. 4. Hybridization of cloned E1B DNA of type 35 to blotted DNA fragments of representatives of other adenovirus subgroups. The hybridization was performed in 0.55 parts of formamide [29, 31]. *Lane 1: Hin*dIII-digested AV2 DNA; *lane 2: Hin*dIII-cut AV7 (Gomen) DNA; *lane 3:* HindIII fragments of AV4 DNA; *lane 4: Hin*dIII-digested pAd12EcoRI-C; *lane 5: Sal*I-digested AV4 DNA; *lane 6: Hin*dIII-fragments of AV8 DNA; *lane 7: Pst*I-cut AV35 control; *lane 8:* pAdXhoI-C [28] cut with *Pst*I; *lane 9: Hin*dIII fragments of AV1 control. Only pBR322-related sequences gave signals in *lanes 4* and *8*. The black arrows indicate the only inter-subgroup hybridization between AV35 and AV4 in *lanes 3* and *5*

tated population of pBR322-related DNA. The existance of such populations may be seen in Fig. 2 (lanes $d + e$).

No detectable signals were present in lanes *1* (Av2 + *Hin*dIII), *6* (AV8 DNA + *Hin*dIII) and *9* (AV1 + *Hin*dIII). Intersubgroup homology was detected under stringent conditions only to E1b region of AV4, being the single representative of subgroup E. The DNA of AV4 (lane *3*) was digested with *Hin*dIII, and fragment *Hin*dIII-C gave signal with the *Pst*I-E probe of AV35 origin (arrow). The DNA was cut by *Sal*I in lane *5*, and only *Sal*I-B (arrow in lane 5 in Fig. 4) gave specific hybridization signal to AV35 E1b.

Blot hybridization of representative serotypes of adenovirus subgroups with DNA probes carrying E3 sequences. Two recombinants were available for this purpose [14], which carried E3 of AV35 [18]. Since E3 of subgroup B was shown to be 1 kb longer than that of subgroup C, the first experiments were done using *Pst*I-(F + B) recombinant in order to registrate intersubgroup homology. Only the results of AV7, and AV35 comparison are presented (Fig. 5), since the larger probe gave positive hybridization to all virus DNA patterns tested.

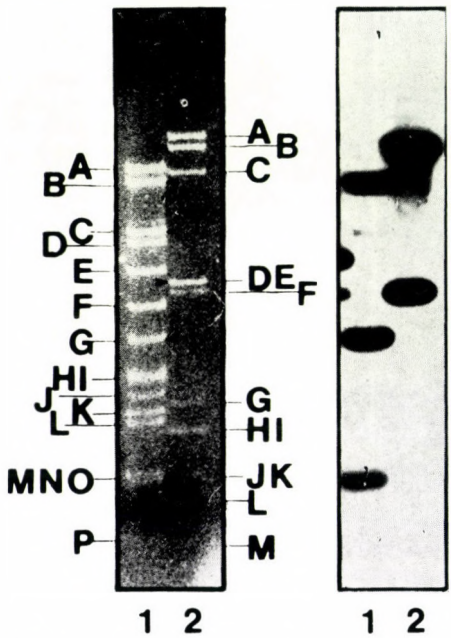


Fig. 5. DNA homology of E3 regions of AV7 and AV35 DNA. The blot hybridization was performed under stringent conditions. *Pst*I-fragments B + F (9.4 kb) was labelled by nick-translation to 10^7 counts μg, and hybridized to *Pst*I-fragments of AV7 DNA *(lane 1)* and AV35 *(lane 2)*. Capital letters indicate *Pst*I-fragments of the correspondingviral DNA

The hybridization pattern shown in Fig. 5 indicates, that *Pst*I-fragments B, C, G, and (M + N + O) of AV7 DNA (lanes *1*, and *1'*) are carrying the corresponding sequences related to *Pst*I-(B + F) of AV35 (lanes *2*, and *2'*).

The intersubgroup homology is demonstrated in Fig. 6. The *Eco*RI-C fragment of AV1 DNA was isolated from an agarose gel, and used as the nick translated probe. This fragment is carrying the majority of E3 region of subgenus C (between 0.76 and 0.84 map units; [25]). This probe gave positive signal only to *Pst*I-B of AV35, (lanes *2*, and *2'*). The *Pst*I-G fragment of AV7 DNA (lanes *3–6*) was the only fragment giving weak signals with AV1 E3. The difference between subgroup B and C is visualized in lanes *7*, and *7'*, which

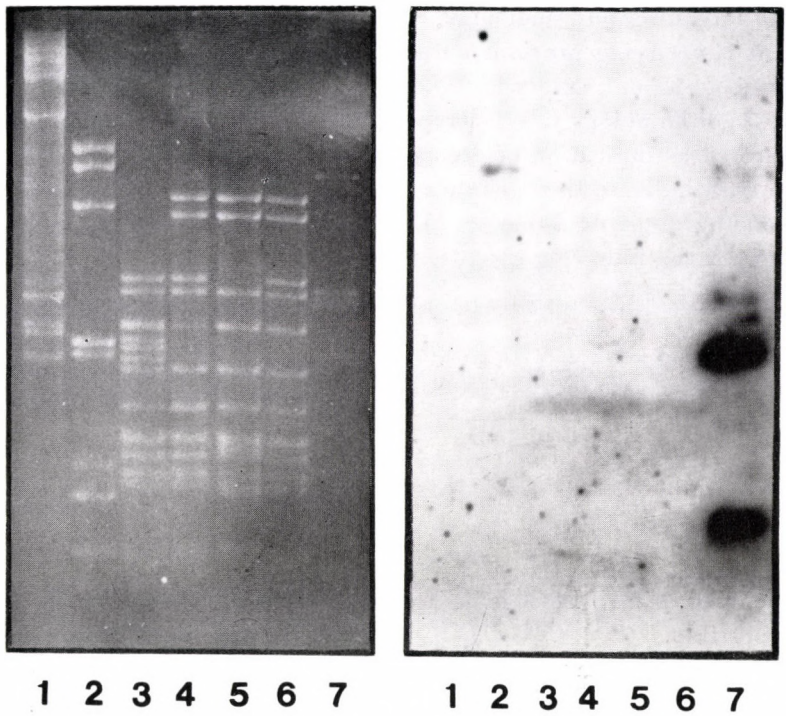


Fig. 6. Intersubgroup homology of E3 regions. Blot-hybridization of *Eco*RI-C fragment of AV1 DNA to representatives of subgroups B and D. *Lane 1: Sal*I-fragments of AV8 DNA; *lane 2: Pst*I-fragments of AV35 DNA; *lanes 3* to *6:* fragments of AV7 DNA cut with *Pst*I [6], *Pst*I + *Sal*I [3], *Pst*I + *Kpn*I, [4], and *Pst*I + *Eco*RI [5]. Subgroup C control: *Pst*I-fragments of AV1 in *lane 7*

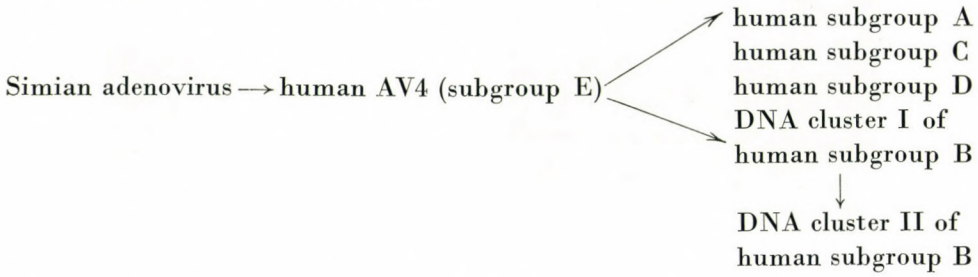
contained the *Pst*I digested AV1 DNA. The *Sal*I-digested AV8 DNA (lanes *1*, and *1')* gave no positive hybridization, but the incomplete digestion pattern makes the evaluation of this result impossible.

Discussion

The blot-hybridization experiments presented were concentrated to E1b, and E3 regions of AV35 DNA. The results revealed that, in contrast to E1a sequences, which had been found to be highly conserved [2, 3, 4, 22, 28, 32, 33] E1b regions seem to be more distantly related to other subgenera.

The E1b recombinant of AV35 made direct comparison of intersubgroup homology possible (Figs 3 and 4). Under stringent conditions no detectable homology was found to representatives of subgroups A, C and D. A high degree of homology was observed between DNA fragments carrying E1b of DNA clusters I and II of subgroup B. The single detectable hybridization signal

indicating intersubgroup homology, was observed in lanes *3*, and *5* of Fig. 4. This result is verifying previous data suggesting human adenovirus type 4 to be the ancient human serotype, descendant of simian adenoviruses [10, 32]. The absence of homology to other representatives of different subgroups might be interpreted as indication of divergent evolution of AV35. Results of DNA sequence data indicate the existance of conserved regions of E1 of previously examined subgroups, A, B (cluster I), and C [4, 5, 28 33]. The results obtained might suggest the following evolution tree:



The sequencing of E1b DNA is before completion in order to make computer analysis possible.

Blot hybridization with nick-translated fragments of AV12 (experiments not shown) supported the above assumptions. No detectable homology was observed to DNA fragments carrying E1b sequences. In contrast to E1b regions, the intersubgroup homology was readily detectable under less stringent conditions of hybridization (Figs 1 and 2).

There was a *Pst*I-fragment (*Pst*I-J in Fig. 1) detected, which seemed to be unique for AV35 within the E3 region. Unfortunately the recombinants available were covering much longer regions of the genom than E3. The DNA homology between AV7 and AV35 (i.e. DNA clusters I, and II) was shown to be at least as high, as in the case of E1b regions (Fig. 5). Since AV7 has not been examined earlier, and *Pst*I cut the DNA of this serotype into several smaller fragments, the intersubgroup homology was examined using samples from both serotypes (Fig. 6). The intersubgroup homology was detected only within the region of 2.0kb (i.e. *Pst*I-G of AV7 DNA).

While this manuscript was in preparation, the nucleotide sequence of the E3 region of AV3 has been published [18], and the major glycoprotein of AV35 coded for by the E3 was shown to be of M_r 27 000 [12]. The nucleotide sequence data provided explanation for the difference of E3 regions in size [4, 12, 18]. About 1 kb was found to be inserted into the E3 region in comparison to subgroup C adenoviruses [18, 20]. The results presented here complete the picture from several respects. The *Sal*I site was shown to be identical in the DNA molecules of AV3, and AV7 [10, 13, 24]. The *Sal*I-site shown in Fig. 7 allow the colinear drawing of E3 sequences of AV3 (nucleotide No. 232 in

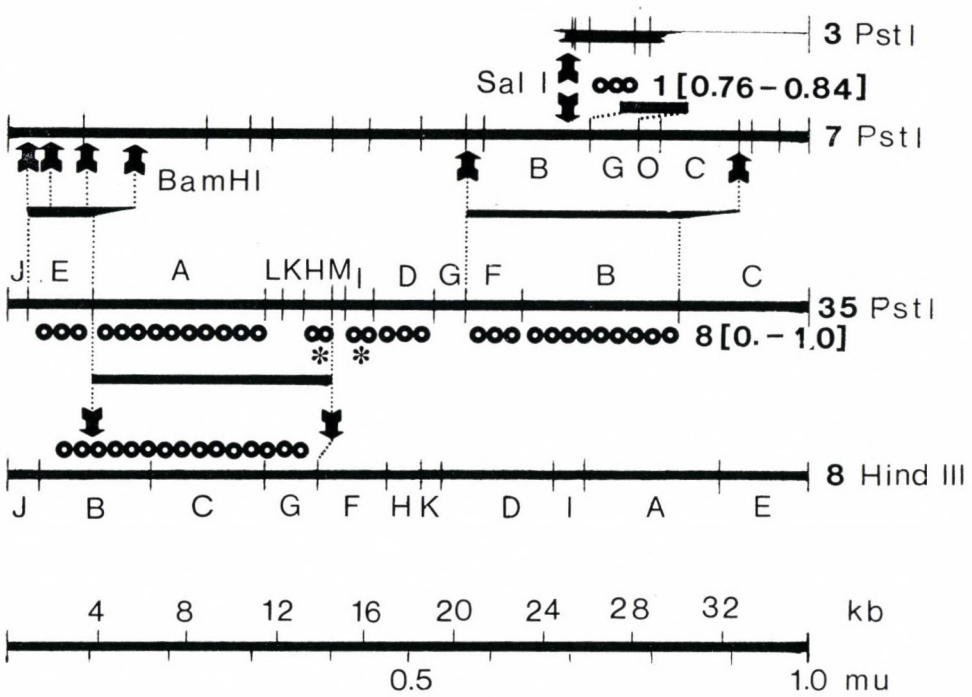


Fig. 7. Colinear comparison of E1b and E3 regions on the basis of DNA-homologies within the subgroup B of adenoviruses, and intersubgroup homologies of subgroups B, C, and D. The horizontal lines represent *Pst*I, adn *Hind*III maps of the DNA of serotypes indicated by arabic numerals. The locations of restriction endonuclease sites are proportional to the marker line below indicating map units (mu) and kilobase values (kb). Intersubgroup homology is indicated by circles. The labeled DNA is indicated by map units in brackets. The sequence of AV3 is localized in the map of AV7 using the *Sal*I site. Cloned fragments of AV35 DNA used as probes are indicated by black horizontal bars, connected with the *Pst*I map by vertical dotted lines. The vertical arrows indicate fragments of othe viral DNA molecules, which were found to be related to AV35 by blot hybridization. Capital letters show restriction endonuclease fragments examined in this study. Other sites were taken from references [14, 18, 24, 36]

reference [18] and the *Pst*I fragments of AV7. The AV3 specific 1 kb insert is exactly in the middle of the *Pst*I fragment corresponding to *Pst*I-G of AV7. The size, and location of *Pst*I fragments of AV3 and AV7 DNA in addition to the blot-hybridization between AV7 and AV35 fragments carrying E3 suggest high degree of homology of AV3, AV7 and AV35 within this region of the genomes. The results presented in Fig. 6 are supporting this assumption in the light of available DNA sequence data [4, 18, 20]. The fragment *Eco*RI-C of AV1 DNA was shown to be very similar to fragment *Eco*RI-D of AV2 of known sequence [1, 4, 25]. The nucleotide sequences of AV2 *Eco*RI-D, and the *Pst*I-G related region of AV3 DNA (nucleotides Nos 1284 and 3389 in reference [18])

possess several identical octa- to dodecanucleotides at both ends. Such sequences may be detected at low stringency of blot hybridization. The results shown in Figs 6 and 7 suggested that the sequence homology between AV1, AV7 and AV35 is probably more than in the case of AV3 and AV2, on the basis of the conditions of blot hybridizations.

Subgroup-specific character of E4 regions is also a new finding of this work. Inter-subgroup homologies are summarized in Figs 1 and 7. Under the conditions of hybridization which could not detect cross-hybridization between E1b regions of different subgenera, no detectable homology was observed between E4 of representatives of subgroups B, C, and D. Unfortunately in the absence of available cloned fragments of AV4 and AV12, this comparative analysis could not be completed.

The results presented show, that *Pst*I-fragments of AV1, and AV35 are short enough to provide subgroup specific results if applied for DNA diagnostics within several regions of the genomes (E1b, E4, and minor central fragments). The elaboration of such DNA probes is continued.

The choice of AV8 as representative of subgroup *D* requires further discussion. On the basis of differences of DNA restriction endonuclease patterns, AV8 was shown to be unique among subgroup *D* adenoviruses [27]. In contrast to the results obtained with *Bam*HI, *Bst*EII and *Hin*dIII, *Sma*I and *Bgl*II did not show these profound differences in the work cited in accordance with our results obtained with *Sal*I [24]. Therefore, type 8 may stay as representative of subgroup *D* as the single type cloned and mapped with restriction endonuclease [24, 27, 34–36].

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REFERENCES

1. Garon, C. F., Berry, K. W., Hierholzer, J. C., Rose, J. A.: Virology **54,** 414 (1973).
2. Mackey, J. K., Wold, W. S. M., Ridgen, P., Green, M.: J Virol **29,** 1056 (1979).
3. Green, M., Mackey, J. K., Wold, W. S. M., Ridgen, P.: Virology **93,** 481 (1979).
4. Van Ormondt, H., Galibert, F.: Curr Topics Microbiol Immunol **110,** 73 (1984).
5. Fujinaga, K., Yoshida, K., Yamashita, T., Shimizu, Y.: Curr Topics Microbiol Immunol **110,** 53 (1984).
6. Stalder, H., Hierholzer, J. C., Oxman, M. N.: J Clin Microbiol **6,** 257 (1977).
7. DeJong, P. J., Valderrama, G., Spigland, I., Horwitz, M. S.: Lancet **1,** 1293 (1983).
8. Horwitz, M. S., Valderrama, G., Korn, R., Spigland, I.: UCLA Symp Molec Cell Biol **16,** 187 (1984).
9. Kim, S. K., Gohd, R. S.: Amer J Dis Child **135,** 473 (1981).
10. Wadell, G.: Curr Topics Microbiol Immunol **110,** 191 (1984).
11. Valderrama-Leon, G., Flomenberg, P., Horwitz, M. S.: J Virol **56,** 647 (1985).

12. Flomenberg, P. R., Chen, M., Horwitz, M. S.: J Virol **61,** 3665 (1987).
13. Kang, W. G.: Ann Immunol Hung **26,** 1003 (1986).
14. Kang, W. G., Berencsi, Gy., Takács, M., Ascher, Z., Fejér, G., Nász, I.: Acta Microbiol Hung **36,** (1989).
15. Lewis, A. M. Jr., Cook, J. L.: Science **227,** 15 (1985).
16. Schrier, P. I., Bernards, R., Vaessen, R. T. M., Houweling, A., Van Der Eb, H. J.: Nature **305,** 771 (1983).
17. Tollefson, A. N., Wold, W. S. M.: J Virol **62,** 33 (1988).
18. Signäs, C., Akusjärvi, G., Pettersson, U.: Gene **50,** 173 (1986).
19. Pääbo, S., Weber, F., Nilsson, T., Schaffner, W., Peterson, P. A.: EMBO J **5,** 1921 (1986).
20. Wold, W. S. M., Cladaras, C., Magie, S. C., Yacoub, N.: J Virol **52,** 307 (1984).
21. Andersson, M., Pääbo, S., Nilsson, T., Peterson, P. A.: Cell **43,** 215 (1985).
22. Byrd, P. J., Grand, R. J. A., Breiding, D., Williams, J. F., Gallimore, P. H.: Virology **163,** 155 (1988).
23. Lengyel, A., Nász, I.: J Virol **6,** 406 (1970).
24. Takács, M., Berencsi, Gy., Lengyel, A., Nász, I.: Acta Virol **27,** 289 (1983).
25. Medveczky, P., Zavizion, B. A., Berencsi, Gy., Chaplygina, N. M., Szomolányi, É., Naroditsky, B. S., Nász, I., Tikhonenko, T. I.: Arch Virol **67,** 85 (1981).
26. Berencsi, Gy., Dyachenko, N. S., Tarassishin, L. A., Vantsak, N. P., Kovalishin, G. G., Kiseleva, E. K., Zhovnovataya, V. Z., Ádám, É., Nász, I.: Acta Microbiol Hung **33,** 233 (1986).
27. Adrian, Th., Wadell, G., Hierholzer, J. C., Wigand, R.: Arch Virol **91,** 277 (1986).
28. Bos, J. R., Polder, L. J., Bernards, R., Schrier, P. I., Van Der Elsen, P. J., Van Der Eb, A. J., Van Ormondt, H.: Cell **27,** 121 (1981).
29. Maniatis, T., Fritsch, E. F., Sambrook, J.: Molecular Cloning — Laboratory Manual. Cold Spring Harbour Press, New York 1982.
30. Smith, G. E., Summers, M. D.: Anal Biochem, **109,** 123 (1980).
31. Johnson, D. A., Gauttsh, J. W., Sportsman, J. R., Elder, J. H.: Gene Anal Techn **1,** 3 (1984).
32. Dekker, B. M. M., Konings, D. A. M., Denisova, T. S., Gibadulin, R. A., Van Ormondt, H. J Gen Virol **65,** 1699 (1984).
33. Kimura, T., Sawada, Y., Shinagawa, M., Shimizu, Y., Shiroki, K., Shimojo, H., Sugisaki, H., Takanami, M., Uemizu, Y., Fujinaga, K.: Nucleic Acid Res **9,** 6571 (1981).
34. Kemp, M. C., Hierholzer, J. C.: J Clin Microbiol **23,** 469 (1986).
35. Nász, I., Lengyel, A., Cserba, I.: Arch Ges Virusforsch **36,** 80 (1972).
36. Takács, M.: Thesis. Budapest 1988.

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THE USE OF MONOCLONAL ANTIBODIES IN THE CHARACTERIZATION AND PURIFICATION OF CELL SURFACE ANTIGENS OF *LISTERIA MONOCYTOGENES* SEROGROUP 4*

J. McLauchlin and A. G. Taylor

Division of Microbiological Reagents and Quality Control, Central Public Health Laboratory, Colindale, London, Britain

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Soluble antigen was prepared from *Listeria monocytogenes* serovar 4b using a formamide extraction method. Antigenic material was detected in this extract by gel diffusion and ELISA using anti-*Listeria* monoclonal antibodies. Using this ELISA, antigen was detected in cerebrospinal fluid (CSF) during infection due to *L. monocytogenes* serovar 4b with wells coated with three of the monoclonal antibodies (CL1, CL2 and CL3). The antigen binding of CL3 was found to be greatly influenced by changes in ionic strength, and these properties were utilized in an affinity purification method. An analysis of the antigens was carried out using crossed immunoelectrophoresis and this indicated that the crude formamide extract contains a number of distinct antigens. A group of three antigens of differing electrophoretic mobilities were demonstrated in the affinity purified material.

We previously reported the production of mouse monoclonal antibodies which recognize epitopes on the surface of *Listeria monocytogenes* serogroup 4 [1]. We report here on an investigation of soluble antigens recognized by these antibodies.

Materials and methods

A panel of 15 mouse hybridoma cell lines secreting antibodies (designated CL1 to CL15) with specificities against *L. monocytogenes* serogroup 4 were described previously [1]. Rabbit polyclonal anti-*Listeria* antisera were produced as described by Seeliger and Hohne [2]. Conjugates of horseradish peroxidase (HRP) to antibodies was carried out using the method of Wilson and Nakane [3].

A soluble antigen was prepared from a strain of *L. monocytogenes* serovar 4b (isolated from the cerebrospinal fluid of a patient with meningitis) using a modification of the Fuller method [4]. This consisted of treating an acetone powder of bacterial cells twice with formamide at 160 °C for 15 min. Cellular debris was removed by centrifugation (10 000 *g* for 20 min). The supernatant was extensively dialysed against phosphate buffered saline (PBS, Dulbecco A) and finally filtered through a 0.22 μm nitrocellulose filter. ELISAs were carried out as described by McLauchlin et al. [5], and double gel diffusion, rocket and crossed immunoelectrophoresis as described by Johnstone and Thorpe [6].

Antibody was bound to CNBr activated Sepharose 4B (Pharmacia) as the manufacturers instructions. All columns were run at a flow rate of 1 ml/min, and regenerated with 20 ml of 4 M $MgCl_2$, 20 ml of 0.1 M Tris/HCl pH 2.0, 20 ml 5 M urea, and finally with 250 ml of 1/10 normal strength PBS.

J. McLauchlin, A. G. Taylor

Division of Microbiological Reagents and Quality Control, Central Public Health Laboratory
Colindale London NW9 5HT, Britain

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

Results

Antigen was detected in the formamide extract by double gel diffusion, and immunoprecipitates were obtained with 7 of the 15 cell lines secreting anti-*Listeria* antibodies (Table I). An ELISA was then developed where microtitre wells were coated with monoclonal anti-*Listeria* antibodies, which were used to capture soluble antigen. The sequential addition of rabbit anti-*Listeria* antisera and goat anti-rabbit-HRP conjugate (Tago) was used to detect captured antigen. Using this ELISA, antigen was detected in the formamide extract by 13 of the 15 anti-*Listeria* antibodies (Table I). Also by this ELISA, antigen was detected in 3 out of 5 CSF samples (collected from 5 patients with *L. monocytogenes* serovar 4b meningitis) with wells coated with 3 of the anti-*Listeria* antibodies (CL1, CL2, and CL3). Using these same CSF samples, antigen was not detected with wells coated with 6 other anti-*Listeria* serogroup 4

Table I

Results of detection of soluble antigen in Fuller (formamide) extract, and in cerebrospinal fluid using anti-Listeria monoclonal antibodies

Monoclonal antibody number*	Antigen detected in formamide extract by		Antigen detected in cerebrospinal fluid samples*** by ELISA**
	Immuno-precipitation	ELISA**	
CL1	−	+	+
CL2	+	+	+
CL3	+	+	+
CL4	+	+	NT
CL5	−	+	−
CL6	−	+	−
CL7	+	+	NT
CL8	−	+	NT
CL9	−	+	NT
CL10	+	+	−
CL11	−	−	NT
CL12	−	+	−
CL13	−	−	NT
CL14	+	+	−
CL15	+	+	−
LPC1	−	−	−

NT = not tested

* The monoclonal antibodies have specificities against: CL1 to CL15 *L. monocytogenes* serogroup 4, LPC1 *Legionella pneumophila*

** ELISA test comprised wells coated with monoclonal antibody, which captures soluble antigen. Captured antigen was detected by the sequential addition of rabbit anti-*Listeria* antisera, followed by horseradish peroxidase conjugated goat anti-rabbit antisera

*** Cerebrospinal fluid samples collected from patients with meningitis due to *L. monocytogenes* serogroup 4b

antibodies, or a monoclonal antibody with a specificity for another genus of bacteria (anti-*Legionella pneumophila* antibody) (Table I).

It was then attempted to produce HRP conjugates with CL1, CL2 and CL3, suitable for use in an ELISA. CL1-HRP conjugate did not retain antigen binding activity. CL2-HRP conjugates precipitated on storage at 4 °C and could not be resolubilized. Successful CL3-HRP conjugates were produced, and an ELISA was developed where wells were coated with the antibody CL2, and captured antigen detected using this conjugate. However, highly inconsistent results were obtained. It was later observed that CL3-HRP conjugate showed great differences in antigen binding with relatively small changes of ionic strength. A 90% loss in signal in the ELISA was obtained when the conjugate was added in 1/10 normal strength PBS compared with 10× normal strength PBS. Similar effects of ionic strength on antigen binding by CL3 were observed in an indirect immunofluorescent antibody test, and in gel diffusion. Thus CL3 was unsuitable for use in this ELISA.

The binding properties of CL3 were, however, utilized in an affinity purification method. The formamide extract was dialyzed against 1/10 strength PBS. To remove non-specifically binding material, the antigen was circulated overnight three times through a CL17 column (an anti-*Listeria* serogroup 1/2 and 3 monoclonal antibody, 18.4 mg of antibody linked to 3 ml of gel). The antitatgen was then circulated overnight in a CL3 column (9.9 mg of antibody linked to 1.3 ml to gel), and treated as outlined in Table II. The fraction eluted at 5× normal strength PBS was dialysed against 1/10 strength PBS, and the process repeated. The fractions were bulked from 6 runs, dialysed against deionised water and freeze dried. Fractions were tested against 6 of the immunoprecipitating antibodies by gel diffusion. The freeze dried material reacted with CL2, CL3 and CL7 but not with CL10, CL14 and CL15 (Table II).

To further assess this purification technique, rocket immunoelectrophoresis was carried out. From the results obtained by the relative heights of the rockets using dilutions of the crude and affinity purified material (Table III, Fig. 1), a purification of at least 20-fold was achieved with respect to antigenic moieties not found in the affinity purified material (i.e. reacting with CL10, CL14 and CL15).

An analysis of this antigenic material was carried out using crossed immunoelectrophoresis. The pattern of immunoprecipitation of the affinity purified material with CL3 is shown in Fig. 2, Gel C. Crossed immunoelectrophoresis with intermediate gels containing antibodies CL2, CL3 and CL7 showed that these three antibodies have different specificities, and recognise at least 3 related antigens with differing electrophoretic mobilities (e.g. Fig. 2 Gel D for CL3/CL2 gel). Intermediate gels also confirmed that antigens recognised by CL10, CL14 and CL15 are different to those recognized by CL3 (results not given).

Table II

Results of optimization of running conditions for affinity chromatography using Sepharose 4B/CL3 antibody column

Running conditions	Results of immunodiffusion with the anti-*Listeria* antibodies					
	CL2	CL3	CL7	CL10	CL14	CL15
5 ml crude formamide extract in 1/10 strength PBS circulated through column overnight	+	+	+	+	+	+
↓→ crude extract	+	+	+	+	+	+
10 ml 1/10 strength PBS	—	+/—	+	—	+/—	+
↓ 10 ml 1/10 strength PBS	—	—	—	—	—	—
↓ 10 ml 1/10 strength PBS	—	—	—	—	—	—
↓ 10 ml of 5×normal strength PBS	+	+	+	—	—	—
↓ 10 ml of 5×normal strength PBS	—	—	—	—	—	—
↓ 10 ml of 5×normal strength PBS	—	—	—	—	—	—
↓ regenerate column						

Table III

Results of rocket immunoelectrophoresis with crude formamide extract and affinity purified material

Antibody in gel	Height of rocket (mm)					
	crude extract				affinity purified*	
	1 : 2	1 : 5	1 : 10	1 : 20	neat	1 : 5
CL2	29	18	9	4	6	3
CL3	41	35	22	13	25	7
CL7	24	18	6	4	11	2
CL10	12	6	3	1	0	0
CL14	11	5	3	1	0	0
CL15	16	9	6	2	0	0

* Affinity purified material = 12.5 mg/ml dry weight of material ie. neat = 120 μg, and 1 : 5 dilution = 24 μg of material.

Discussion

A soluble antigen extract was produced using a Fuller extraction (hot formamide) method. This extract was a good source of antigenic material since of the 15 cell lines producing anti-*Listeria* antibodies, immunoprecipitates were produced with 7, and 13 reacted in the ELISA. Using three of the

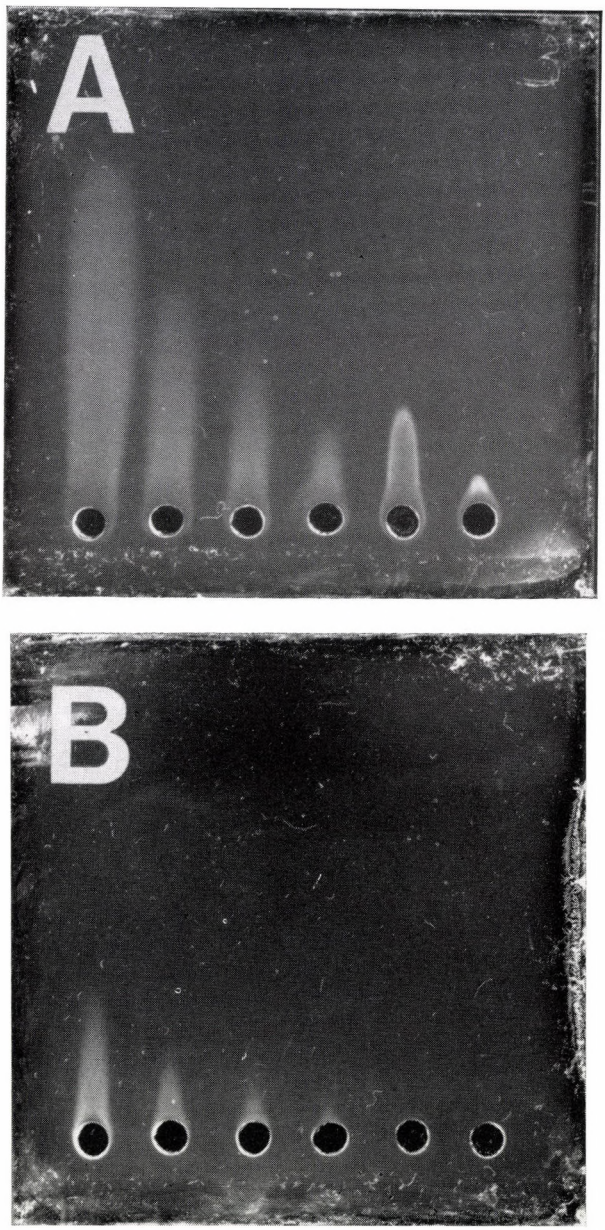

Fig. 1. Assessment of affinity purification of Sepharose 4B/CL3 column fractions by rocket immunoelectrophoresis. A = CL3, B = CL15. Wells contain (left to right): crude formamide extract diluted 1 : 2, 1 : 5, 1 : 10, 1 : 20, and affinity purified material diluted neat and 1 : 5

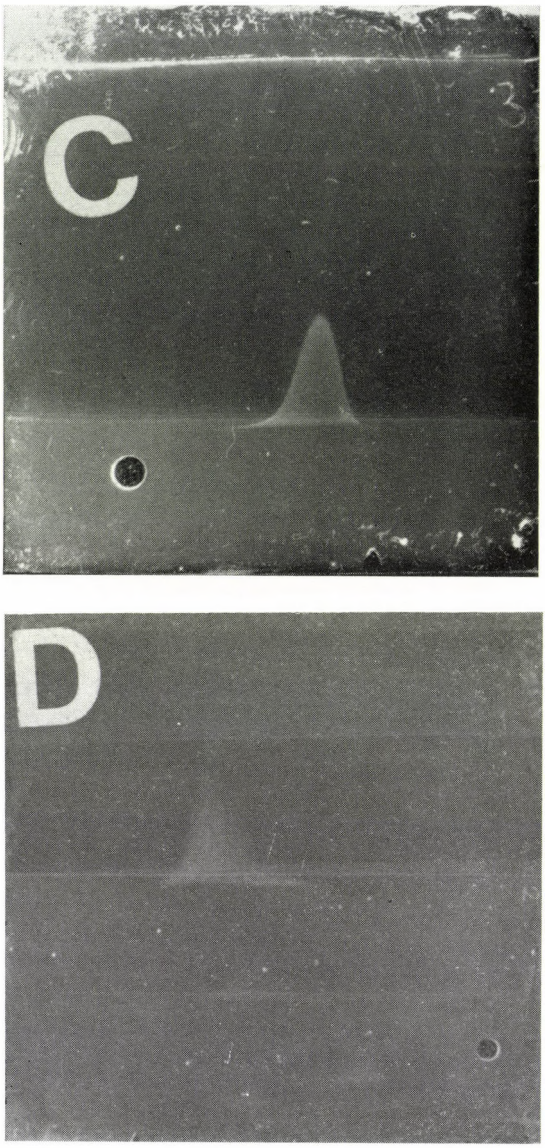

Fig. 2. Patterns of precipitation in crossed immunoelectrophoresis. C = affinity purified material against CL3, D = crude formamide extract tested against CL2 (bottom gel) and CL3 (top gel)

monoclonal antibodies in this ELISA, a *L. monocytogenes* cell surface antigen was identified which occurs in a soluble form in CSF during listeriosis. Thus further attention was focussed on purification and analysis of antigens recognized by these antibodies. A test for this antigen in CSF was further developed [5].

The binding characteristics of the antibody CL3 allowed the affinity purification of a similar antigen to that occurring in CSF from the formamide extract. The chemical nature of these antigens is under investigation. However, preliminary results indicate that these are surface located non-protein and carbohydrate containing antigens, which are of high molecular weight, highly acidic and covalently linked to the cell wall.

REFERENCES

1. McLauchlin, J., Beesley, J. E., Betts, M. P.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): Proceedings of the 9th International Symposium on Problems of Listeriosis. Université de Nantes, Nantes 1986. p. 102.
2. Seeliger, H. P. R., Hohne, K.: In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Academic Press, London 1979. p. 31.
3. Wilson, M. B., Nakane, P. K.: In Knapp, W., Holubar, K., Wick, G. (eds): Immunofluorescence and Related Staining Techniques. Elsevier, Amsterdam 1978. p. 215.
4. Fuller, A. T.: Br J Exp Pathol **19,** 130 (1938).
5. McLauchlin, J.: Acta Microbiol Hung **36,** 303 (1989).
6. Johnstone, A., Thorpe, R.: Immunochemistry in Practice. Blackwell, Oxford 1982.

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THE USE OF MONOCLONAL ANTIBODIES AGAINST *LISTERIA MONOCYTOGENES* IN A DIRECT IMMUNOFLUORESCENCE TECHNIQUE FOR THE RAPID PRESUMPTIVE IDENTIFICATION AND DIRECT DEMONSTRATION OF *LISTERIA* IN FOOD*

J. McLauchlin, A. M. Ridley and A. G. Taylor

Division of Microbiological Reagent and Quality Control, Central Public Health Laboratory, Colindale, London, Britain

(Received September 16, 1988)

Two monoclonal antibodies which recognise serogroup specific cell surface antigens of *Listeria monocytogenes* are described. The antibodies were specific for *Listeria* (i. e. did not react with 87 strains of bacteria from 14 other genera). In an indirect immunoflourescent test the antibodies had similar specificities when tested against strains of *L. monocytogenes* from human infections grown under a number of different growth conditions, and serological reference strains. A direct immunofluorescent antibody test was developed, and similar specificities of the antibodies found with strains of *Listeria* which had been isolated from food and grown under 2 different growth conditions. This technique is also used to directly demonstrate *Listeria* in soft cheese.

The isolation and identification of *Listeria* sp. from environmental specimens is labour intensive and time consuming. Hence there is a need for rapid identification techniques. We reported here the specificities of antibodies from 2 cell lines secreting monoclonal antibodies against *Listeria monocytogenes*, describe their use as an aid to a rapid presumptive identification of cultures growing in vitro, and also their use in directly demonstrating *Listeria* in soft cheese.

Materials and methods

From a panel of seventeen stable mouse hybridomas secreting anti-*Listeria* antibodies which were produced as described previously [1], antibody from 2 cell lines (designated CL2 and CL17) were selected.

A total of 187 strains of *Listeria* (Table I) were grown at 37 °C on either Difco Tryptose Agar, Horse Blood Agar, nutrient broth or brain heart infusion broth. Bacteria from 14 other genera (87 strains) were grown on blood agar as appropriate. The genera of bacteria tested were (numbers of strains tested are shown in parentheses): *Arthrobacter* (1), *Bacillus* (15), *Bifidobacterium* (1), *Brevibacterium* (2), *Brochothrix* (1), *Cellulomonas* (2), *Clostridium* (15), *Corynebacterium* (6), *Erysipelothrix* (7), *Escherichia* (5), *Kurthia* (1), *Lactobacillus* (3), *Staphylococcus* (14), *Streptococcus* Group B (5), *Streptococcus* Group D (9). All strains of bacteria were

J. McLauchlin, A. M. Ridley, A. G. Taylor

Division of Microbiological Reagents and Quality Control,
Central Public Health Laboratory, Colindale,
London NW9 5HT, Britain

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killed in formalin, and tested in an indirect immunofluorescent antibody test as described previously [1]. Fluorescein isothiocyanate (FITC) conjugates were prepared as described previously [2] and a direct immunofluorescent antibody test developed.

Subcultures of *Listeria* which had been isolated from food and submitted to DMRQC were identified and serogrouped as described previously [3]. Strains were cultured overnight at 37 °C on either Modified McBride Agar or Blood Agar. Bacterial growth was emulsified to give a slightly turbid suspension in PBS (Dulbecco A), normal yolk sac (prepared in DMRQC) added (final concentration 0.02%, v/v), and a direct fluorescent antibody test carried out as above.

Soft cheeses were obtained from retail outlets in Britain and were examined and quantitatively enumerated for *L. monocytogenes* by The Food Hygiene Laboratory, Colindale, using conventional techniques [4]. A sample of soft cheese was emulsified into peptone water (1 : 10, w/v), centrifuged at 2000 *g* for 15 min, and the supernatant discarded. A suspension from the top of the deposit was made, and this was added at several dilutions to wells on PTFE coated miscrocope slides. Slides were air dried, fixed by total immersion in acetone for 10 min, antibody-FITC conjugate added to each well, and incubated at 37 °C for 30 min. The slides were finally washed in PBS (10 min), air dried, and examined by fluorescence microscopy. The antibodies were added to wells separately and also as a mixture of the 2 antibodies. All tests were carried out where the results of culture and identification were unknown to the reader of the slides.

Results

The reaction of the antibodies with strains of *Listeria* grown on Difco Tryptose Agar, together with other species of bacteria is shown in Table I. No differences were observed between the reactions of the strains of *Listeria* serogroups 1/2, 3 or 4 when grown on Difco Tryptose, Blood Agar, Nutrient Broth or in Brain Heart Infusion Broth. The monoclonal antibody CL17 reacted with almost all strains of *L. monocytogenes* serogroup 1/2 and serogroup 3, and the type strain of *Listeria seeligeri*. The antibody CL2 reacted with all strains of *L. monocytogenes* serovar 4b, some *L. monocytogenes* serogroup 4, and all *Listeria innocua* serovar 6a. Neither antibody reacted with any of the other species of *Listeria*, nor with the strains from other genera tested. Strains of *Staphylococcus aureus*, however, did react non-specifically in the immunofluorescence test. These reactions were not observed when the *S. aureus* cultures were pre-treated with normal rabbit sera and direct FITC/antibody conjugates used. Since these strains are known to contain Fc receptors on the cell surface, these reactions were probably of a non-immunospecific nature.

The specificities of the monoclonal antibodies against strains of *Listeria* isolated from food and cultivated on Modified McBride's Agar (110 strains) and Blood Agar (192 strains) are shown in Table II, and these were similar to those previously reported for clinical isolates (Table I).

Results of direct examination of 35 soft cheeses is shown in Table III. Similar results were obtained when the antibodies were added to wells together or separately. *Listeria* of the corresponding serogroup to the strains of *L. monocytogenes* isolated using conventional techniques were directly demonstrated using the antibodies in 7 samples of cheese. *Listeria* were not detected using this method in 20 cheese samples where *L. monocytogenes* was present in low numbers ($<10^2$/g), or was not isolated.

Table I

Results of specificity testing of two anti-Listeria monoclonal antibodies against 187 strains of Listeria and 87 strains of bacteria from 14 other genera

Strains of bacteria	No. of strains of bacteria which reacted with the antibody/total number of strains	
	Anti-*Listeria monoclonal* antibody	
	CL2	CL17
L. monocytogenes serogroup 1/2	0/14	50/52
L. monocytogenes serogroup 3	0/12	11/12
L. monocytogenes serovar 4b	77/77	0/12
L. monocytogenes serogroup 4 not 4b	13/24	0/6
L. monocytogenes serovar 7	0/1	0/1
L. innocua serovar 6a	5/5	0/5
L. innocua serovar 6b	0/5	0/5
L. welshimeri	0/1	0/1
L. seeligeri	0/1	1/1
L. ivanovii	0/6	0/6
L. murrayi	0/1	0/1
L. grayi	0/1	0/1
L. denitrificans	0/1	0/1
Other species of bacteria	0/87	0/87

Discussion

When tested against strains of *L. monocytogenes*, the two monoclonal antibodies were broadly serogroup specific, however, neither reacted identically to the factors described in the Donker-Voet/Seeliger antigenic scheme [5]. This suggests that the antigens detected here are related to, but are not necessarily identical with those recognized in this scheme.

Antigenic cross reactions between *L. monocytogenes* and many other species of bacteria have been reported. The monoclonal antibodies described here were shown to be specific for certain serovars of *Listeria*. However, the possibility of cross-reactions with other genera can not be entirely excluded since only a limited range of bacteria were tested here. In addition, these strains were cultured using a single growth medium and it should be borne in mind that the cell surface antigens of bacteria may change with culture conditions [6]. The monoclonal antibodies described here, however, reacted with *L. monocytogenes* when grown under a number of different conditions (including in vivo, [7]) which suggests that the antigens detected are stable characters.

These reagents would give a rapid presumptive identification for over 90% of *L. monocytogenes* isolated from food when growing on Modified McBride

Table II

Reaction of monoclonal antibodies with strains of Listeria isolated from food using a direct immunofluorescent antibody test

No. of strains	Species of *Listeria* (serogroup)	No. of cultures which reacted with the monoclonal antibodies			Proportion reacting with either antibody
			CL17	CL2	
Haemolytic strains cultured on blood agar (n = 102)					
96	*L. monocytogenes*	total	53	40	0.97
54	*L. monocytogenes*	(1/2)	53	0	0.98
1	*L. monocytogenes*	(3)	0	0	0.0
40	*L. monocytogenes*	(4b)	0	40	1.0
1	*L. monocytogenes*	(NT)	0	0	0.0
6	*L. seeligeri*		3	0	0.5
Non-haemolytic strains cultured on blood agar (n = 90)					
84	*L. innocua*		0	6	0.07
6	*L. welshimeri*		0	0	0.0
Strains cultures on Modified McBridge's agar (n = 110)					
56	*L. monocytogenes*	total	28	24	0.93
30	*L. monocytogenes*	(1/2)	28	0	0.93
1	*L. monocytogenes*	(3)	0	0	0.0
25	*L. monocytogenes*	(4)	0	24	0.96
54	other species of *Listeria*		0	2	0.03

NT = non typable

Strains of *Listeria* were incubated overnight at 37 °C either on Horse Blood Agar, or on Modified McBride's Agar

Table III

Results of the direct examination of 35 samples of soft cheeses for the presence of Listeria using a direct immunofluorescent antibody test

No. of cheeses tested	Results of culture (viable count of *L. monocytogenes*/g of cheese)	Results of the IFA test with the antibodies		
		CL17	CL2	Both
2	*L. monocytogenes* 1/2 ($> 10^4$/g)	+	−	+
2	*L. monocytogenes* 1/2 ($< 10^3$/g)	+	−	+
3	*L. monocytogenes* 4b ($> 10^7$/g)	−	+	+
8	*L. monocytogenes* ($> 10^4$/g)	−	−	−
10	*L. monocytogenes* ($< 10^2$/g)	−	−	−
10	Not detected	−	−	−

Agar, while less than 10% of strains of other species of *Listeria* would be misidentified. *L. innocua* and *L. monocytogenes* are the species of *Listeria* most frequently isolated from soft cheese [4]. These two species can be differenciated by demonstration of haemolysis on the blood agar. Combining the results of the tests for haemolysis with those for the immunofluorescence, just over 5% of all strains tested here would be misidentified.

Two monoclonal antibodies conjugated to FITC were successfully used in a direct immunofluorescence test to demonstrate *Listeria* in 7 samples of soft cheese where *L. monocytogenes* had been cultured by conventional techniques. *Listeria* was not detected in 18 cheese samples from which *L. monocytogenes* had been cultivated, or was present in low numbers ($<10^2$). It is of note that the 8 cheese samples containing $>10^4$ *L. monocytogenes*/g and where *Listeria* was not demonstrated using the antibodies, all contained rinds, and the cheeses where *Listeria* was demonstrated were of a homogeneous nature. Work is currently underway to modify the sample preparation to allow the detection of *Listeria* in soft cheeses with rinds, as well as other environmental samples.

Although both these antibodies appeared specific for *Listeria* (Table I), fluorescent bacteria of differing morphology were seen in some samples of cheese with CL17. This fluorescence may have been due to non-specific interactions, or represent true serological reactions which have previously not been detected.

The tests described here for presumptive identification and direct demonstration in soft cheese can both be performed in under 2 h, and we believe that these may be of use in the rapid identification of environmental samples contamined with *L. monocytogenes*.

REFERENCES

1. McLauchlin, J., Beesley, J. E., Betts, M. P.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): Proceedings of the 9th International Symposium on Problems of Listeriosis. Université de Nantes, Nantes 1986. p. 102.
2. Samuel, D., Patt, R. J., Abuknesha, R. A.: J Immunol Methods **107,** 217 (1988).
3. McLauchlin, J.: J Appl Bacteriol **63,** 1 (1987).
4. Pini, P. N., Gilbert, R. J.: Int J Food Microbiol **6,** 317 (1988).
5. Seeliger, H. P. R., Hohne, J. R.: In Bergan, T., Norris, J. R. (eds): Methods in Microbiology. Vol 13. Academic Press, New York 1979. p. 31.
6. Ellwood, D. C., Tempest, D. W.: In Rose, A. H., Tempest, D. W. (eds): Advances in Microbial Physiology. Vol 7. Academic Press, London 1972. p. 83.
7. McLauchlin, J.: Acta Microbiol Hung **36,** 303 (1989).

BOOKS RECEIVED

DECHEMA Biotechnology Conferences Vol. 2 (D. Behrens and P. Krämer, Eds.) Lectures held at A C H E M A '88, June 5/11, 1988 BIOREACTORS, DOWNSTREAM PROCESSING, PROCESS AND REACTOR MODELLING, BIOPROCESSES V C H Verlagsgesellschaft, Weinheim—Basel—Cambridge—New York, December 1988 Paperback, 467 pages; 207 figures and 50 tables — DM 199.00 or US $ 69.00.

The vertical development of Biotechnology has been overviewed at the Conference at Achema (FRG). The majority of the papers has been completed with English, German, and French summaries. Most of the presentations are written in English with two exeptions (these manuscripts use German language instead).

Oxygen uptake rates measured, and the lines of operation were calculated for pilot scale bubble column or airlift bioreactors of 2000 l, and compared with the data of a production scale bioreactor of 32 000 l by BAUER et al. Productivity of *Pseudomonas fluorescens*, and that of *Methylomonas clara* was measured continuously cultivated under increased pressure by LIEFKE and ONKEN.

The BODENSTEIN-NUMBER was calculated for the characterization of growth of *Zymomonas mobilis* in pilot scale horizontal tubular plug flow bioreactors by KÜNG et al. These details of industrial importance are followed by the work of SÁRA et al., who reported on an exciting new development of general biological importance, i.e. the practical use of external, bacterial crystalline protein layers (S-layers) for molecular ultrafiltration. Other interesting alternatives of downstream processing were treated by BOMBERG et al. (cell disintegration), BUCHHOLZ et al. (enzyme immobilization), ENGLAND et al., and MARTINEZ et al. (product purification) followed by the paper of GANETSOS et al. (reactor-separator).

Problems of mixing, on line monitoring, and process control of fermentation processes were modelled by the third group of authors (JURY et al., VALERO et al., and SCHÖNE et al.). Bioprocesses of microbial and mammalian cell cultures are interesting for the virologist reviewer. CZERMAK et al. described membrane reactors; NARODOSLAWSKY et al. two phase system-reactors. N-demethylation of codein and tertiary amines (ENGLAND et al.), conditions of oxidation of aldehydes by xanthine oxidase in reversed micelles (BOMMARIUS et al.), enzymatic formation of oligosaccharides (THIEM et al.), and successful leaching of Fe, Cu, and Zn with mixtures of *Thiobacillus*- and *Leptospirillum*-like bacteria (HELLE et al.) are summarized.

HÄGGSTRÖM and ARTHUN describe aseptic processing conditions for mass production of hybridoma cells applied in the probably largest Swedish plant, which could be run 6 month without contamination; distilled water, filtration, valves, mixing, distribution, pumping and harvesting of mammalian cells is mentioned. According to the conclusion of TOLBERT "A large number of biopharmaceuticals will require mammalian cell culture production, resulting in a dramatic increase in the demand for this capability . . .". Finally HALMANN et al. reports on mesh-aeration of suspension cultures (BHK-21), and GOLDRING et al. on large scale purification of monoclonal antibodies.

The papers on bioprocesses are covering two topics, both of outstanding importance. These problems may determine the future of life on the Globe. (1) The production of fluid fuels, i.e. ethanol from different sources by various procedures including genetic improvement of soja as source of fuel. (2) Environmental biotechnology, i.e. elimination of organic compounds from air, heavy metals and sulphur from waste water by aerobic and anaerobic procedures applying different mixing and areation technologies.

The Reviewer has to refer to the book itself concerning details and authors. The volume is recommended for all who are in contact with biotechnology, industrial microbiology, vaccine production, separation technology and for those who are teaching or interested in problems of the environment.

(Dr. György Berencsi)

Acta Microbiologica Hungarica 36, 1989
Akadémiai Kiadó, Budapest

INSTRUCTIONS TO AUTHORS

Manuscripts are accepted on the basis of scientific significance and suitability for publication on the understanding that they have not been published, submitted or accepted for publication elsewhere. Acceptance depends on the opinion of one or more referees and the decision of the Editorial Board. Papers accepted for publication are subject to editorial revision.

MANUSCRIPT STYLE

Manuscripts must be in English and clearly and concisely written. They should be typed double spaced with wide margins. Two copies of the manuscript should be submitted.

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Title. The title should be a clear and concise statement of the contents in not more than 14 words. A short running title of not more than 40 letters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided.

Introduction. This part should state briefly the nature and purpose of the work and cite recent important work by others.

Materials and methods. Describe microorganisms, methods, apparatus, procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings like "Bacterial strains" or "Culture media".

Results. The experimental data should be presented clearly and concisely. Avoid repeating information presented in tables and figures.

Discussion should be focussed on the interpretation of experimental findings. Do not repeat literary data presented in the Introduction or information given in Results.

Acknowledgement of grants and technical help.

References. Cite only essential references. They should be arranged in numerical sequence as cited in the text, where the numbers appear (in parentheses) with or without the author's name. e.g. Eden and Hansson [1], Frankel [2]. Gelbart et al. [3] or [1, 2]. The reference list at the end of the paper should contain

- for journals: names and initials of all authors, journal title abbreviated according to the style used in Index Medicus, volume number, pagination, year.
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