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Virulence of different strains of *Listeria monocytogenes* serovar 1/2a*

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Abstract. Different strains of *Listeria monocytogenes* serovar 1/2a were used to infect mice. A smooth, haemolytic strain multiplied in the spleen of normal adult mice and induced a long-lasting immunity to subsequent challenge infection. When the animals were treated with dextran sulphate (DS) *Listeriae* multiplied more rapidly and death followed within a few days. When normal baby mice were infected with this organism, fatal infection occurred. In nude mice a chronic infection developed.

Secondly, a rough, haemolytic strain was used to infect mice. In normal adult mice no multiplication of this strain was observed. This holds true also for DS-treated animals. In nude mice the bacteria were eliminated slowly. Normal baby mice could only be killed if the infective dose was increased. This strain was considered to be intermediate in virulence. The infection stimulated a considerable immune response in mice, although to a lesser degree than the smooth, haemolytic *Listeria* strain.

Thirdly, a smooth, non-haemolytic strain of *L. monocytogenes* serovar 1/2a was used. The bacteria were rapidly eliminated in normal, in DS-treated and in nude animals. Normal baby mice did not develop fatal disease, so it was considered that this strain of *L. monocytogenes* serovar 1/2a was avirulent. This variant was found to be non-immunogenic. A boosting of immunity of animals by this avirulent *Listeria* strain was, however, found to be possible.

A reasonable explanation for the rapid elimination of avirulent *L. monocytogenes* serovar 1/2 a from mice cannot be presented at this time. Whereas it is known that the macrophage system and the T-lymphocytes play an essential role in the resistance to virulent *Listeriae,* there is no increased susceptibility of the avirulent bacteria to these defence mechanisms. Other bacterial properties, such as serum sensitivity and lysozyme susceptibility, are likewise considered to be unimportant.

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Introduction

Since the early studies of Seeliger (1961) it has been known that certain strains of *Listeria monocytogenes* cause disease in animal and man. Serovars 1/2a and 4b are most frequently encountered (Seeliger and Finger 1979). It has also been recognized since 1963 (Kautter et al. 1963) and confirmed by several authors (Audurier et al. 1981; Rocourt et al. 1983; Wirsing von König et al. 1983) that there is a marked difference in virulence between strains. The virulent variants, when injected into mice, multiply rapidly in the reticuloendothelial system and eventually lead to death of the animals. Once the animals have overcome the primary infection, they will be highly protected against a challenge dose.

Mackaness (1962, 1969) has clearly shown that cellular rather than humoral mechanisms are responsible for this type of protective immunity. This model of murine listeriosis has hence been used by several authors to explore the principles of cell-mediated immunity (North 1973; Zinkernagel 1974; Hahn and Kaufmann 1981). In these studies most often one single strain of *L. monocytogenes,* that is, the EGD strain, belonging to serovar 1/2, was tested.

The aim of this study was, first, to investigate other strains belonging to the same serovar and to detect possible differences in their virulence, and secondly, to study their respective immunogenic capacities.

Materials and methods

Bacteria. Strains of *L. monocytogenes* (Table 1) were obtained from the Special Listeria Culture Collection (SLCC) (H.P.R. Seeliger, Institute of Hygiene and Microbiology, University of Würzburg, FRG). The EGD strain was kindly supplied by S. Kaufmann (Max Planck Institute, Freiburg, FRG). Strains SLCC 5779, a rough variant, was obtained from J. Potel (Institute for Medical Microbiology, Medical Academy, Hannover, FRG), who derived this variant from a smooth, haemolytic strain of serovar 1/2a (Potel, 1963). Strain SLCC 4013 serovar 4b was used for challenge infections. Serotyping was performed according to the method of Seeliger and Höhne (1979). The rough strain, however, was not typable by this method. Bacteria were grown for 18 h at 37°C in tryptose bouillon. An appropriate suspension was then prepared in phosphate-buffered saline (PBS), pH 7.2.

Mice. Female outbred NMRI mice weighing 20–25 g were obtained from the Central Institute for Laboratory Animals, Hannover, FRG, in a specified pathogen-free (SPF) state. After infection they were kept in conventional conditions of controlled temperature, moisture and light intensity. They were fed with pellets and water ad libitum.

Athymic, nude mice of the same mouse strain were purchased from the same breeder. Baby mice were infected at the age of 5 days. Macrophage-depleted animals were obtained by injecting intraperitoneally (IP) into adult animals 1 mg of dextran sulphate 500 (DS) (Serva Feinbiochemica, Heidelberg, FRG) 18 h before infection.

Infection. Adult mice were infected intravenously (IV) with an appropriate suspension of bacteria in 0.2 ml PBS. At different intervals 4–6 mice of each group were killed.

Table 1. Virulence of different strains of *Listeria monocytogenes* serovar 1/2a

Bacterial strain	Phenotype	Infective dose	Bacterial counts ($\times 10^3$) per spleen 2 days after iv infection (means and mean errors); 4 mice per group
SLCC 2371 = NCTC 7973	haemolytic, smooth	180,000	3,500 ± 1,300
SLCC 5764 = EGD strain	haemolytic, smooth	170,000	3,100 ± 1,800
SLCC 2715	haemolytic, smooth	160,000	2,900 ± 1,300
SLCC 2713	haemolytic, smooth	180,000	13,700 ± 5,700
SLCC 5025	haemolytic, smooth	75,000	15,300 ± 4,200
SLCC 5581	haemolytic, smooth	130,000	14,900 ± 2,000
SLCC 5598	haemolytic, smooth	85,000	13,000 ± 3,800
SLCC 5779	haemolytic, rough	670,000	58 ± 16
SLCC 53 = NCTC 10357	non-haemolytic, smooth	190,000	0.5 ± 0.2

Spleen and occasionally liver were removed aseptically and homogenized. Serial dilutions in PBS were prepared and poured with tryptose agar in Petri dishes. After 24 h incubation at 37°C, a colony count was carried out. The means and mean errors of the bacterial counts per spleen and liver were calculated. The survival rates of baby mice which had been infected IP were recorded daily for 21 days.

Serum sensitivity test. Fresh mouse serum was obtained from non-infected NMRI mice. Rat serum from Sprague Dawley rats was also used. Fresh human serum (group O Rh^-) was obtained from a healthy donor. One part of serum was mixed with one part of an appropriate suspension of bacteria in PBS. The mixture was incubated at 37°C, and at different times the bacterial numbers were determined by plating.

Lysozyme susceptibility. Egg white lysozyme (Sigma, Munich, FRG) was dissolved in a buffer (pH 8) containing 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCL. To 9

ml buffer 1 ml of an appropriate bacterial suspension in PBS was added. At different intervals the number of bacteria was determined by plating as above.

Results

Virulence of various strains of L. monocytogenes serovar 1/2 a

Most of the strains tested belonging to this serovar were found to multiply within the murine host. Spleen counts of adult mice showed an increase in numbers of bacteria 2 days after infection. The differences among these strains were found to be small. The rough strain tested was apparently less virulent. The non-haemolytic strain (SLCC 53) was unable to multiply, since only a few bacteria were recovered from the spleen (Table 1).

Infection of baby mice

Animals were infected with the smooth-haemolytic strain SLCC 2371. Only small doses were given, but the mice succumbed to the infection. The rough strain used was only able to kill baby mice when given in much higher doses. The non-haemolytic strain tested was unable to kill baby mice even at large doses (Table 2).

Course of infection in normal adult mice

The smooth-haemolytic strain SLCC 2371 multiplied within the spleen during the first 3 days. After this period the bacterial counts declined. When animals were challenged 2 weeks after the primary infection, they were protected, since only low bacterial counts were found. This state of protection gradually declined with time (Fig. 1).

Secondly, mice were infected with the rough variant. No significant bacterial multiplication could be detected in the spleen. Bacterial counts remained stable during the first 3 days and then declined. Thereafter, mice were considerably protected against challenge infection.

The protective immunity in mice achieved after infection with the rough variant disappeared more rapidly (Fig. 1).

The non-haemolytic strain was not able to multiply in mice. After injection, the bacterial counts steadily decreased, and no immunity could be detected (Fig. 1). Not even a high dose of bacterial cells of this strain could produce an immune response. Even repeated injections of 10^9 viable bacteria for 5 consecutive days did not induce protective immunity (data not shown). This strain was, however, able to trigger an

Table 2. Mortality of baby mice infected with different strains of *L. monocytogenes*

Bacterial strain	Infective dose	Number of surviving animals / Number of infected animals
SLCC 2371	10^3	2/10
SLCC 5779	10^5	9/10
	10^7	5/10
SLCC 53	10^7	10/10

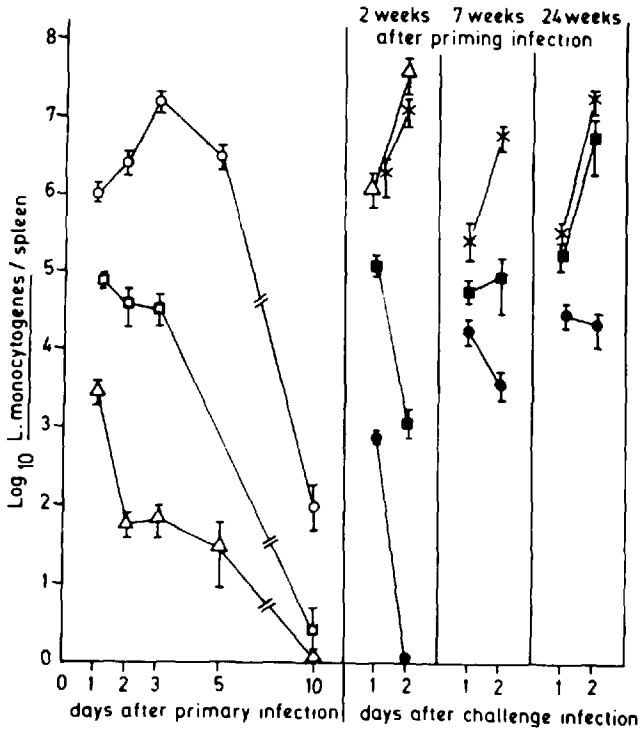

Fig. 1. Course of primary and secondary infection of adult mice with strains of *Listeria monocytogenes* of different virulence. Mice were infected primarily with 10^5 viable bacteria of the haemolytic strain SLCC 2371 (○), or with 5×10^5 viable bacteria of the rough strain SLCC 5779 (□), or with 10^6 viable bacteria of the non-haemolytic strain SLCC 53 (△). Challenge infection with 4×10^4 viable bacteria of the strain SLCC 4013 (serovar 4b) was done either 2, 7 or 24 weeks, respectively, after primary infection. Non-primed mice (X) were used as controls

immune response in those animals which had lost their immune status 24 weeks after priming with the rough strain SLCC 5779 (Table 3).

The role of macrophages

The treatment of mice with DS removed their resistance to infection with the smooth-haemolytic strain SLCC 2371. The organisms multiplied even more rapidly than in the untreated controls. Mice succumbed to infection within 3 days after administration of 7×10^3 living bacterial cells. The rough strain could not even multiply in DS-treated animals (Fig. 2). Non-haemolytic bacteria did not multiply after treatment with DS. These organisms were eliminated, though the process was somewhat slower.

Table 3. Reinduction of lost immunity by avirulent bacteria

	Infective dose of *L. monocytogenes* serovar 4b SLCC 4013	Bacterial counts ($\times 10^3$) per spleen 2 days after iv infection (means and mean errors); 4 mice per group
Unprimed control animals	5×10^3	596 ± 96
Animals infected with 10^6 *L. monocytogenes* SLCC 5779 24 weeks before	5×10^3	405 ± 136
Animals infected with 10^6 *L. monocytogenes* SLCC 5779 24 weeks before + 10^6 *L. monocytogenes* SLCC 53 3 weeks before	5×10^3	0.2 ± 0.05

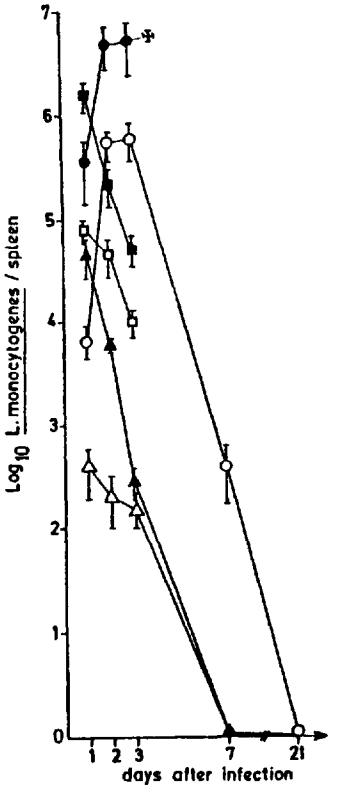

Fig. 2. Effect of dextran sulphate on infection with *L. monocytogenes* of different virulence. Mice were infected with 7×10^3 viable bacteria of the strain haemolytic SLCC 2371 (○;●), or with 10^5 viable bacteria of the rough strain SLCC 5779 (□;■), or with 10^6 viable bacteria of the non-haemolytic strain SLCC 53 (△;▲). One half of each group (*closed symbols*) was pretreated with dextran sulphate

The role of T-lymphocytes

The smooth-haemolytic strain SLCC 2371 induced a chronic infection in nude mice. Bacterial counts were higher in the spleen than in the liver. The non-haemolytic strain from which even higher doses were injected was recoverable in the internal organs shortly after injection, whereby the bacterial counts were higher in the liver than in the spleen. However, the bacteria were totally eliminated at day 11. When the rough strain was used, only a relatively slow elimination of the bacteria took place (Fig. 3).

Serum sensitivity

Incubation of suspensions of *Listeriae* with fresh mouse or human serum did not influence the bacterial counts. There was no difference in serum sensitivity between the haemolytic-smooth, the rough and the non-haemolytic strain. Fresh rat serum, how-

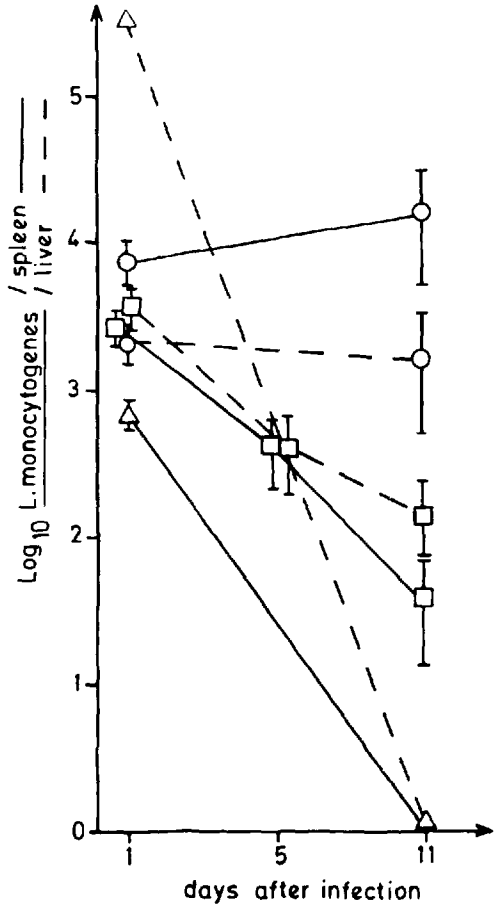

Fig. 3. Course of infection of nude mice with strains of *L. monocytogenes* of different virulence. Mice were infected with either 10^5 viable bacteria of the haemolytic strain SLCC 2371 (○), or with 5×10^5 viable bacteria of the rough strain SLCC 5779 (□), or with 10^7 viable bacteria of the non-haemolytic strain SLCC 53 (△). Bacterial counts of spleen (——) and liver (– – –) were determined

ever, killed *Listeriae*. Both the smooth-haemolytic and the non-haemolytic strain were sensitive (Table 4).

Lysozyme susceptibility

The smooth-haemolytic, the rough and the non-haemolytic strains of *L. monocytogenes* all showed resistance against the action of lysozyme for a period of at least 5 h (data not shown).

Table 4. Serum sensitivity of bacteria

		Survival rate of inoculum		
		Mouse serum	Rat serum	Human serum
SLCC 2371				
Smooth-haemolytic	0.5 h	95%	10%	90%
	1 h	95%	5%	90%
	2 h	100%	5%	100%
SLCC 5779				
Rough	0.5 h	70%	–	110%
	1 h	110%	–	100%
	2 h	120%	–	130%
SLCC 53				
Non-haemolytic	0.5 h	100%	10%	90%
	1 h	110%	10%	90%
	2 h	120%	10%	100%

Discussion

The genus *Listeria* (Rocourt et al. 1983) comprises various species which vary considerably in their virulence (Kautter et al. 1963; Weiss and Seeliger 1975; Audurier et al. 1980; Rocourt et al. 1983; Wirsing von König et al. 1983). But even within a species or a single serovar considerable differences in virulence can be observed, as, for example, in the serovar 1/2a of *L. monocytogenes* (Table 1). This indicates that the components of the outer membrane which represent the antigenic structures – presumably the teichoic acids (F. Fiedler, Munich, FRG, personal communication) – are not the only virulence factors, although it has been observed that certain serovars, especially serovars 1/2 and 4b, predominate in clinical isolates (Seeliger and Finger 1979).

On the other hand, changes in the surface composition may principally condition a loss of virulence. A rough variant of *L. monocytogenes* serovar 1/2a was found to be considerably less virulent for mice than the smooth, haemolytic strains (Potel 1963; Table 1). The exact chemical basis for the rough character of the *Listeriae* is, however, not yet known, as it is for *Salmonellae* (Lüderitz et al. 1971). It was found that in normal adult mice no multiplication of this rough strain took place, in contrast to a virulent strain (Fig. 1). Whereas baby mice were found to be extremely susceptible to virulent *Listeriae* (Wirsing von König et al. 1978; Table 2), they could only be killed

with high doses of the rough strain (Table 2). Furthermore, the rough strain was unable to multiply in the DS-treated mouse (Fig. 2). This macrophage-depleted host (Hahn 1974) was highly susceptible to a virulent strain (Fig. 2). Whereas in nude, athymic mice a chronic course of infection with a virulent *Listeria* strain occurred (Emmerling et al. 1975; Fig. 3), the rough variant was slowly eliminated (Fig. 3).

Besides the surface properties of *L. monocytogenes*, there must be other factors determining virulence. Haemolysin has been regarded as an important virulence factor for *Listeriae* (Lemeland et al. 1974; Ralovich et al. 1977; Skalka et al. 1982). Since haemolysin was found to be not only toxic for erythrocytes but also for leukocytes (Njoku-Obi et al. 1963), it could be speculated that the haemolytic property may enable the microorganisms to withstand phagocytosis. Indeed non-haemolytic strains have been found to be avirulent (Audurier et al. 1980; Wirsing von König et al. 1983). On the other hand, it is known that there are few haemolytic *Listeriae* which are completely avirulent (Jenkins et al. 1964. Lemeland et al. 1974; Weiss and Seeliger 1975; Rocourt et al. 1983; Knorz and Hof, unpublished results).

Among the several hundreds of strains of *L. monocytogenes* serovar 1/2a of the SLCC, there was only one single non-haemolytic strain. This old laboratory strain SLCC 53 was isolated by E.G.D. Murray in the 1920s (Murray et al. 1929). Barber reported in 1939 that this particular isolate was non-haemolytic and avirulent. Only Ralovich et al. (1977) described this strain as avirulent but haemolytic. Because of its outstanding property this non-haemolytic strain has been replaced as reference strain for *L. monocytogenes* by the strain SLCC 2371 (Jones and Seeliger 1983), which is haemolytic and virulent (Fig. 1). The strain SLCC 53 was unable to multiply in normal, adult mice (Fig. 1). Baby mice could not be killed (Table 2). Furthermore, in DS-treated mice these bacteria were unable to multiply. The elimination rate was, however, somewhat reduced (Fig. 2). And finally, in nude mice this strain was also eliminated (Fig. 3). In summary, one can say that this non-haemolytic strain of *L. monocytogenes* serovar 1/2a is completely avirulent in contrast to the haemolytic strains, which are highly virulent, and the rough strain, which displays intermediate virulence. Whether this loss of virulence of the non-haemolytic strain is solely due to the absence of haemolysin production cannot, however, be stated with absolute certainty. In the course of time, this old laboratory strain may have undergone still more fundamental genotypic alterations, which themselves contribute to the loss of virulence.

Wheras the survival of the virulent *Listeriae* is counteracted by a cooperation between macrophages and T-lymphocytes (Mackaness 1969), the killing of the bacteria with reduced virulence is achieved by other, yet unknown mechanisms. The lack of macrophages in DS-treated mice could reduce the elimination rate, but ultimately both the rough and the avirulent bacteria were killed by other mechanisms (Fig. 2). The T-lymphocytes, too, play no obvious role during healing of infection with the avirulent bacteria. The elimination begins rather early before any immune response could have taken place (Fig. 1). Furthermore, the avirulent bacteria are killed even in nude mice (Fig. 3). While rough *Salmonellae* with chemically defined cell wall deficiencies (Lüderitz et al. 1971) display increased susceptibility to fresh serum, this is not so for rough or avirulent *Listeriae*. These are killed only by fresh rat serum but not by fresh mouse or fresh human serum (Czuprinski and Balish 1981), irrespective of their virulence (Table 4). Equally, another potent non-specific defence mechanism, i.e. lysozyme

(Fleming 1922) is possibly not involved in the defence against *Listeria* infection. Virulent *Listeriae* are held to be resistant to lysozyme (Gosh and Murray 1967). This also holds true for the avirulent bacteria. Consequently, it is at present not clear what prevents the avirulent *Listeriae* multiplying within a host.

Primary infection of mice with a non-lethal dose of a virulent strain of *L. monocytogenes* results in a protective immunity (Mackaness 1969; Fig. 1). Rough strains have been used successfully as a living vaccine for immunization of sheep against listeriosis (Wachendörfer and Zirpel 1976). Mice have also been protected against a challenge infection when primed with the rough strain of *L. monocytogenes* serovar 1/2a (Potel 1963; Fig. 1), but the acquired immunity was less than after infection with the virulent strain and disappeared more rapidly. The avirulent, nonhaemolytic strain SLCC 53 was completely unable to stimulate any protective immunity. Even the injection of high doses of living, avirulent *Listeriae* did not lead to a measurable immune response. This indicates that the quantity of antigen alone is not responsible for the induction of immunity. Also in the *Salmonella* model, only virulent strains which were able to multiply within the host induced strong immunity (Collins 1974). Thus, one can conclude that immunity to *L. monocytogenes* – and probably to other intracellular pathogens as well – can only be achieved by living vaccines having retained a certain degree of virulence which implies a risk of vaccination. Completely avirulent, living bacteria are ineffective for vaccination purposes (Coppel and Youmans 1969; Wirsing von König et al. 1983) as are killed bacteria (Wirsing von König et al. 1982).

The protective immunitiy after a primary infection is limited in time (Emmerling et al. 1979, Fig. 1). But even after the disappearance of protection there remains a residual memory, since avirulent *Listeriae*, which are unable to induce a primary immune response (Fig. 1) were able to restimulate the declined immunity (Table 3). This is in accordance with in vitro findings that even killed *Listeriae* can stimulate primed T-lymphocytes (Farr et al. 1979).

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