

Course of Infection and Development of Immunity in Experimental Infection of Mice with *Listeria* Serotypes†

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NMRI mice were experimentally infected with *Listeria monocytogenes* serotypes 1/2b, 3a, 4b, and 4d and *Listeria innocua* serotype 6b by different means. The course of infection was monitored, using bacteriological and histological methods. The following typical features of experimental infection with the various *L. monocytogenes* and *L. innocua* serotypes were observed. (i) On the basis of the mean lethal dose, *L. monocytogenes* 4b, 4d, and 1/2b proved to be mouse pathogenic, although to different degrees. *L. monocytogenes* 3a and *L. innocua* can be regarded as nonpathogenic for NMRI mice. The virulence of *L. monocytogenes* serotype 4d was increased 1,000-fold after adaptation to mice. (ii) Primary infection with any serotype of *L. monocytogenes* or *L. innocua* resulted in protection against a lethal challenge with the most virulent serotype, 4b. This protective immunity could be transferred by spleen cells. Compared with the duration of immunity achieved by infection with *L. monocytogenes* serotype 4b, the protection induced by infection with *L. innocua* was short lived and dose dependent. The data obtained also suggest that immunity after experimental infection with any serotype of *L. monocytogenes* or *L. innocua* is produced only when the animal host is filled with bacteria. (iii) The distribution of the germs in the internal organs of the mouse shortly after infection was dependent on the route of infection rather than on the serotype used. (iv) The main difference among the *Listeria* serotypes tested was their ability to multiply within the host and to induce a granulomatous inflammation. The results indicate that mouse pathogenicity and virulence of *Listeria* spp. cannot be defined only by the capacity of the bacteria to infect or kill conventional mice. Such a definition should include an analysis of the immune system of the host, a kinetic study of experimental infection, and a histomorphological evaluation of the lesions induced.

Infections of humans and animals with bacteria of the genus *Listeria* are common throughout the world. Human infection, resulting in severe and sometimes fatal disease, is predominantly caused by *Listeria monocytogenes* serotypes 1/2b and 4b (17). Other serotypes of this species have been described, including serotype 6, now called *Listeria innocua* (18). The experimental infection of mice with *L. monocytogenes* serotype 4b is almost routine in several laboratories, since these bacteria provide an excellent model for studying the mechanism of cell-mediated immunity to infection. Resistance to *L. monocytogenes* is thought to be mediated by macrophages that are activated by specifically sensitized T-lymphocytes (2, 10, 13, 23). The only successful experimental infection with *L. innocua* has been achieved by Patocka et al. (14) with

an intracerebral infection of suckling mice. Since no detailed information about the cellular kinetics and the histological changes during infection with *L. innocua* is available, it seemed reasonable to perform the following experiments. A model for infection with different *Listeria* serotypes including information about the response of the host to the different bacterial challenges would, furthermore, provide information about how to define the pathogenicity and immunogenic capacity of the various serotypes of the species.

MATERIALS AND METHODS

Bacteria. All strains of the various *Listeria* serotypes were kindly provided by H. P. R. Seeliger (Institute for Hygiene and Microbiology, Würzburg). The following serotypes of *L. monocytogenes* and *L. innocua* were used. *L. monocytogenes*: serotype 1/2b, SLCC 2755; serotype 3a, SLCC 2373 = NCTC 5105 = ATCC 19113; serotype 4b, two strains originally isolated from human clinical material (cerebrospinal fluid

† This paper is dedicated to Oswin Günther, Paul-Ehrlich-Institut, Frankfurt (Main), on the occasion of his 75th birthday.

and amniotic fluid), typed by H. P. R. Seeliger; serotype 4d, SLCC 2377 = ATCC 19117. *L. innocua*: serotype 6b, formerly *L. monocytogenes* serotype 4g, SLCC 3423.

The bacteria were grown on blood agar base, unless otherwise indicated, and were stored as a frozen stock culture. Suspensions of bacteria for experimental infections were made from a single colony which was incubated at 37°C for 18 h in tryptose broth. Dilutions, when necessary, were prepared in sterile 0.9% phosphate-buffered saline, pH 7.2.

To maintain the virulence of the various serotypes, passages through mice were performed at intervals of 10 to 14 days.

The experiments described below were performed with *L. monocytogenes* serotype 4b, passages number 94 to 135, *L. monocytogenes* serotypes 1/2b, 3a, and 4d, passages number 0 to 16, and *L. innocua*, passages number 0 to 16.

Mice. Throughout the study, we used female NMRI/Han mice weighing 22 to 28 g and 8 to 16 weeks old. All animals were purchased from the Central Institute for Laboratory Animals (Hannover, Federal Republic of Germany), where they were raised under specific pathogen-free conditions. Upon arrival in our laboratory, the mice were infected immediately and were kept under conventional conditions.

In addition, for some experiments, we used newborn NMRI mice which were bred in our own laboratory.

The animals were fed with ready-made pellets (Ssniff, Soest, Federal Republic of Germany) and drank water ad libitum.

Bacterial count in organs. Since the spleen and, to a lesser extent, the liver are optimally suited for following the course of experimental listeriosis, both organs were removed under aseptic conditions at certain intervals after infection with a sublethal dose of listeriae. The organs were homogenized in tryptose broth (Difco Laboratories, Detroit, Mich.) with an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.). The homogenate was plated in 10-fold dilutions on tryptose agar (Difco), and the CFU were counted after incubation for 20 h at 37°C.

Mean lethal dose. The mean lethal dose (LD₅₀) for all *Listeria* serotypes used was determined after intraperitoneal (i.p.) or intravenous (i.v.) infection according to the method of Reed and Muench (15). Groups of 10 mice were injected with 0.5 log-decreasing doses of viable bacteria. The experiments were repeated four times.

Histological evaluation. Mice were infected with either 3.5×10^3 *L. monocytogenes* serotype 4b or with 6.8×10^{11} *L. innocua* serotype 6b. Noninfected NMRI mice served as controls. On days 0, 1, 2, 3, and 9 after infection, autopsy was performed, and the livers and spleens were removed. The organs were fixed in 2.5% buffered Formalin. Sections of both organs were embedded in paraffin and stained with standard dyes (hematoxylin-eosin) (16). A semiquantitative evaluation of the histological lesions in the spleen was performed as follows: (+), single, poorly defined, inflammatory focus; +, up to 10; ++, 10 to 20; +++, 20 to 50; +++++, more than 50 distinct pyogenic or granulomatous lesions per histological section (5).

Radiolabeling of bacteria. *L. monocytogenes* sero-

TABLE 1. LD₅₀ of different serotypes of *L. monocytogenes* and *L. innocua* for NMRI/Han mice after i.p. infection

Serotype	LD ₅₀ (CFU)
1/2b	10 ⁷
3a	10 ¹²
4b	10 ⁴
4d	10 ¹¹
6b (<i>L. innocua</i>)	10 ¹²

types 1/2b, 3a, 4b, and 4d and *L. innocua* serotype 6b were incubated during the late phase of growth with 5.0 µCi of ¹²⁵I-iododeoxyuridine (New England Nuclear Corp., Dreieich, Federal Republic of Germany) for 2 h at 37°C. After incubation, the bacteria were pelleted, washed three times in phosphate-buffered saline containing 25 mmol of deoxyuridine (Sigma Chemical Co., Munich, Federal Republic of Germany), and diluted in phosphate-buffered saline plus deoxyuridine.

Autopsy was performed 2 h after infection of mice. Peritoneal exudate cells were harvested after peritoneal lavage with 1.0 ml of RPMI 1640 medium (Mediaparm, Munich, Federal Republic of Germany) containing 2.0 USP-E of heparin per ml. The liver, spleen, kidneys, pancreas, and lungs were also recovered, and the gamma radiation of the organs was determined. The results were expressed as the percentage of radioactivity of the injected dose.

Cell transfer. Cell transfer was performed with purified spleen cells. After aseptic removal of the spleen, it was homogenized (glass homogenizer) in 2.0 ml of RPMI 1640 medium containing 1.0 USP-E of heparin per ml. The cells were filtered through gauze and purified over a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). The recovery was 85 to 90% viable cells. Viability was assured by dye exclusion tests. The cells were transferred in a suspension of 1.5×10^7 to 2.0×10^7 cells per mouse in RPMI 1640 medium.

Statistical evaluation. Differences between mean values were tested for significance with Student's *t* test after analysis of variance. Differences at the $2P < 0.05$ level were regarded as significant.

RESULTS

LD₅₀. The resistance to infection with *L. monocytogenes* is thought to be mediated by a single gene, *Lr* (19), not linked to the major histocompatibility complex. With respect to this genetic determinant, mouse strains can be divided into susceptible and resistant strains. The NMRI mice used throughout this study belong to the susceptible variants. Table 1 displays the numbers of CFU for the different serotypes of *L. monocytogenes* and *L. innocua* necessary to kill 50% of mice after i.p. injection (LD₅₀). It is obvious that only *L. monocytogenes* serotype 4b can be considered as a mouse pathogen. Serotype 1/2b is not so pathogenic, and all other serotypes can be considered to be nonpathogenic. Infective doses of 10¹² living bacteria normally only occur in experimental infection and not

TABLE 2. Distribution of different serotypes of *L. monocytogenes* and *L. innocua* after i.p. and i.v. infection^a

Serotype	(% Activity)	
	Liver	Peritoneal cells
i.p. infection		
1/2b	10	74
3a	10	81
4b	20	72
4d	17	80
6b (<i>L. innocua</i>)	18	70
i.v. infection		
1/2b	68	4
3a	84	4
4b	83	3
4d	80	4
6b (<i>L. innocua</i>)	76	4

^a Autopsy was performed 2 h after infection. Results are expressed as the percentage of recovered radioactivity of ¹²⁵Iododeoxyuridine-labeled bacteria.

during naturally acquired disease. The second experiment was performed with the same suspensions of bacteria given i.v. into the tail vein. No significant differences in respect to LD₅₀ could be observed between the i.p. and the i.v. route of infection for serotypes 1/2b, 3a, and 4d and for *L. innocua* serotype 6b. Serotype 4b, however, displayed a significantly lower (2.5 × 10³ bacteria) LD₅₀ upon i.v. infection.

Since Patocka et al. (14) used corticosteroid-treated suckling mice to achieve an intracerebral infection with *L. innocua*, the LD₅₀ for 7-day-old mice injected i.p. was determined. Previous studies had shown that the LD₅₀ of *L. monocytogenes* serotype 4b for NMRI mice aged 1 to 10 days is less than 10² viable bacteria (22). However, we found the LD₅₀ of *L. innocua* for mice of the same age to be >10⁹ viable bacteria.

The third route of infection was performed via an intragastric tube. Neither the pathogenic serotype 4b of *L. monocytogenes* nor any other serotype was able to initiate a reproducible lethal infection via the oral route. Even a dose of 10⁹ living *L. monocytogenes* serotype 4b was unable to kill the mice.

Distribution of *Listeria* sp. in internal organs.

The distribution of radiolabeled bacteria in spleen, kidneys, lungs, or pancreas showed that none of the organs incorporated more than 5% of the total activity. Significantly higher values could be recovered from the liver and from peritoneal exudate cells. Table 2 gives the percentage of radioactivity recovered from both materials after i.p. or i.v. infection. As can be seen, the liver is the main clearing organ after i.v. infection, whereas peritoneal exudate cells

serve as the initial barrier after i.p. infection. No significant differences could be observed with respect to the initial clearing of bacteria after infection with any of the serotypes of *L. monocytogenes* and *L. innocua*.

Course of infection in spleen. Figure 1 shows the course of infection with the different *Listeria* serotypes in the spleen, expressed as numbers of CFU recovered from the spleen. It can be seen that the injection of 3.5 × 10³ *L. monocytogenes* serotype 4b cells resulted in multiplication of the bacteria in the host, reaching peak values at day 3 after infection. Injection of 1.8 × 10⁹ *L. innocua* cells, however, resulted in recovery of only 3.0 × 10⁴ live bacteria 1 day after the infection, with a subsequent rapid decline. At day 6 after infection, no viable bacteria could be detected. The other serotypes displayed a course of infection in between the two possibilities mentioned above. Serotype 1/2b gave only poor evidence for multiplication in the host, since the numbers of CFU increased only between days 3 and 6 after infection. Serotype 3a showed a recovery of 3.0 × 10⁶ viable bacteria 1 day after i.p. injection of 2.4 × 10⁹ organisms. Numbers of CFU increased marginally on day 2 and then rapidly decreased until day 6. Serotype 4d, however, was able to multiply in mice. The numbers of CFU increased from 4.0 × 10⁵ on day 1 to 1.1 × 10⁸ on day 3 after infection.

Consequently, two basically different courses of experimental infection could be observed. Since *L. monocytogenes* serotype 4b and *L. innocua* can serve as prototypes for either course, only these two serotypes were used for some of the following experiments.

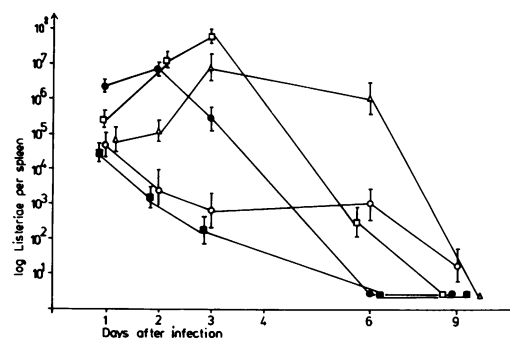


FIG. 1. Course of infection with different serotypes of *L. monocytogenes* and *L. innocua* as measured by the number of CFU in the spleens of NMRI mice after i.p. infection. Five to six mice were used per point. Results are expressed as log CFU per spleen ± standard error of the mean. Symbols: ○, *L. monocytogenes* serotype 1/2b, 6.0 × 10⁶ bacteria; ●, *L. monocytogenes* serotype 3a, 2.4 × 10⁹ bacteria; △, *L. monocytogenes* serotype 4b, 3.5 × 10³ bacteria; □, *L. monocytogenes* serotype 4d, 2.5 × 10⁸ bacteria; ■, *L. innocua* serotype 6b, 1.8 × 10⁹ bacteria.

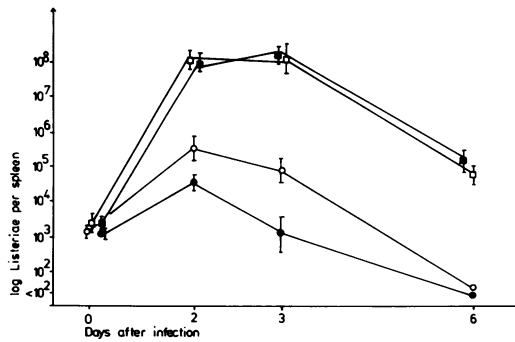


FIG. 2. Adaption of *L. monocytogenes* serotype 4d to mice during serial infections. Five to six mice were used per point. Results are expressed as log CFU per spleen \pm standard error of the mean. Infectious dose of *L. monocytogenes* serotype 4d given i.p.: passage 0, 1.0×10^9 bacteria (●); passage 1, 1.0×10^9 bacteria (○); passage 2, 0.8×10^9 bacteria (■); passage 5, 0.9×10^9 bacteria (□).

Adaption of *Listeria* spp. to mice. As none of the various listeriae was originally isolated from mice, the alteration of the LD₅₀ during the adaption of the bacteria to mice was monitored for all strains. Although *L. monocytogenes* serotypes 1/2b, 3a, and 4b and *L. innocua* showed no difference in LD₅₀ for NMRI mice between passages number 0 and 10, *L. monocytogenes* serotype 4d was able to adapt to mice. This was evident by a sharp decrease in the LD₅₀ from 1.0×10^{11} CFU at passage 0 to 1.0×10^8 CFU at passage 2. After this initial 1,000-fold increase of

virulence, no significant differences could be observed among the following passages number 2 through 16. Figure 2 demonstrates the course of infection with *L. monocytogenes* serotype 4d, using bacteria of different passages. Since infection was performed with a constant dose of 0.8×10^9 to 1.0×10^9 bacteria, the numbers of CFU in the spleens could be compared. Peak values reached 4.5×10^4 CFU in animals infected with bacteria of passage 0, but climbed to 2.0×10^8 CFU in animals infected with bacteria of passage 2 or 5. Although none of the animals infected with bacteria of passage 0 succumbed to the infection, 80% of the animals infected with bacteria of passage 2 or 5 died within the observation period.

Histological evaluation. Mice infected with *L. monocytogenes* serotype 4b developed an intense inflammation corresponding to that previously described by others (9, 11, 13) and by our group (4, 6). Three days after infection, numerous pyogenic foci with or without necrosis were present in the livers of experimental animals (Fig. 3A). Nine days after infection, lesions frequently showed a distinct granulomatous character (Fig. 4A). In contrast, mice infected with *L. innocua* serotype 6b showed a weak inflammatory response. Three days after infection, single pyogenic foci were detectable in the liver (Fig. 3B), whereas after nine days, no lesions at all could be found in this organ (Fig. 4B). The poor reaction induced by *L. innocua* did not result in a granulomatous type of inflammation at any time during observation. The

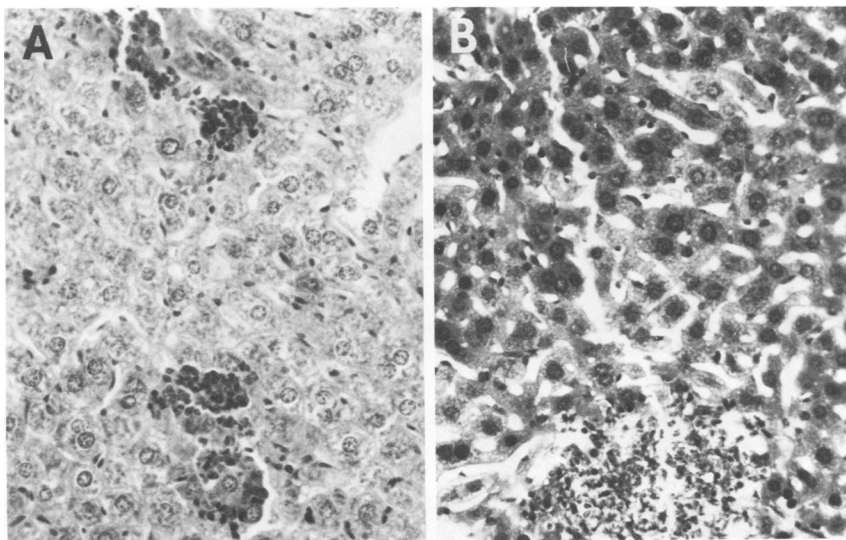


FIG. 3. Histological changes in livers of mice 3 days after infection with either *L. monocytogenes* serotype 4b (A) or *L. innocua* serotype 6b (B). Numerous pyogenic foci can be seen in (A); only single lesions can be seen in (B). (Hematoxylin-eosin stain; $\times 220$)

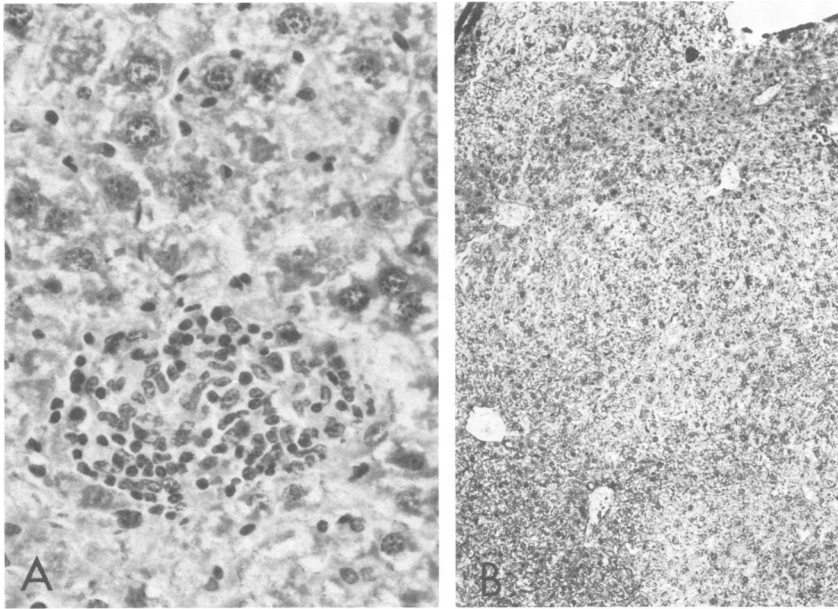


FIG. 4. Histological changes in livers of mice 9 days after infection with either *L. monocytogenes* serotype 4b (A) or *L. innocua* serotype 6b (B). Granuloma can be seen in (A); no signs of inflammatory reaction can be seen in (B). (Hematoxylin-eosin stain; (A) $\times 250$; (B) $\times 85$)

histomorphological findings in the spleen corresponded largely to those described for the liver.

The observation of a profound difference in the tissue changes induced by *L. monocytogenes* serotype 4b and *L. innocua* serotype 6b

could be further substantiated by semiquantitative histological evaluation of the lesions. Results of these studies for the spleen are depicted in Fig. 5. As is evident from the data presented, the time course as well as the extent of the

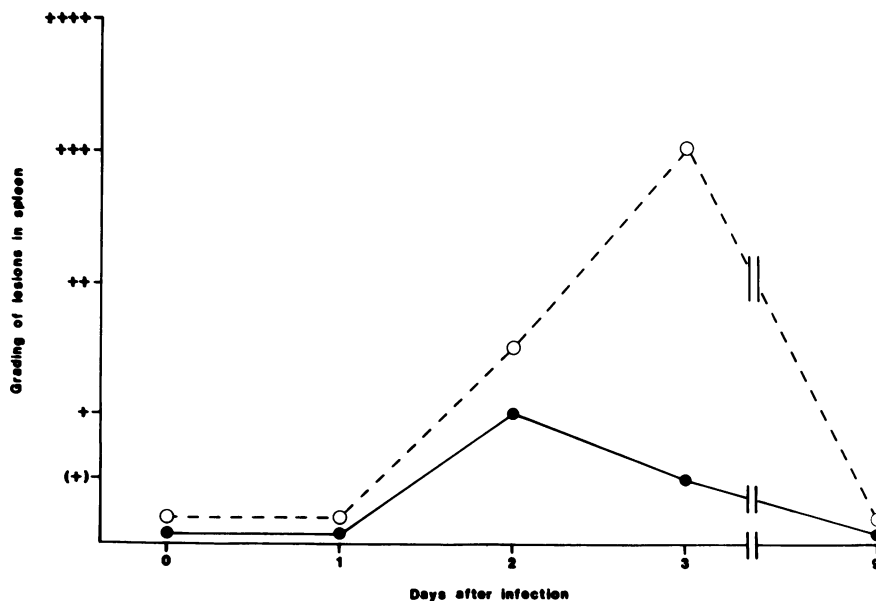


FIG. 5. Time course and extent of inflammatory lesions in spleens of NMRI mice infected with either *L. monocytogenes* serotype 4b (O) or *L. innocua* serotype 6b (●). Each point represents the mean of results from five mice. (+), single, poorly defined, inflammatory focus; +, up to 10 foci; ++, 10 to 20 foci; +++, 20 to 50 foci; +++++, more than 50 distinct foci.

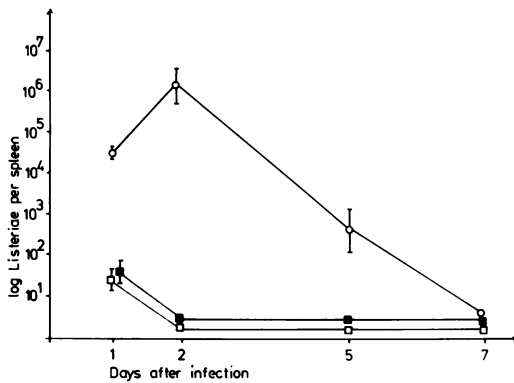


FIG. 6. Course of secondary infection with 2.2×10^4 *L. monocytogenes* serotype 4b cells performed i.p. 10 days after primary infection with either 4.5×10^3 viable *L. monocytogenes* serotype 4b (□) cells or 2.5×10^9 viable *L. innocua* serotype 6b (■) cells. The third group (○) was infected primarily with 2.2×10^4 *L. monocytogenes* serotype 4b cells and served as a control for the course of primary infection. Results are expressed as log CFU per spleen \pm standard error of the mean. Five to six mice were used per point.

inflammatory processes in the two experimental animal groups differed considerably. Whereas serotype 4b organisms caused extensive lesions in the spleen (Fig. 5), serotype 6b organisms induced only a minimal inflammatory reaction which rapidly disappeared (Fig. 5).

Secondary infection. The following experimental schedules were designed to test whether a primary infection with the nonvirulent *L. innocua* serotype 6b could induce protection against a lethal challenge with the virulent *L. monocytogenes* serotype 4b. Ten days after primary infection with 2.5×10^9 *L. innocua* cells, mice were challenged i.p. with twice the LD_{50} of viable *L. monocytogenes* serotype 4b. The course of the secondary infection is depicted in Fig. 6, showing that a primary infection with either *L. monocytogenes* serotype 4b or *L. innocua* induced protection against the lethal challenge. It could also be shown that primary infection with *L. monocytogenes* serotype 1/2b, 3a, or 4d was able to induce a similar form of protection against the lethal challenge. To exclude the possibility that the large numbers of bacteria used for primary infection with *L. innocua* could produce an increase in nonspecific resistance, we immunized mice 10 days before the lethal challenge with 10^{10} killed (70°C, 60 min) *L. monocytogenes* serotype 4b or *L. innocua* serotype 6b cells. As had been previously reported (21), the injection of killed bacteria did not result in a measurable degree of protection against the challenge.

Since the immunity against *L. monocytogenes* serotype 4b can be passively transferred only by

viable cells (10, 12, 13, 23), purified spleen cells were recovered from mice 10 days after infection with *L. monocytogenes* serotype 4b or *L. innocua* serotype 6b. Spleen cells from noninfected donors served as a control. Figure 7 demonstrates that cells from both *L. monocytogenes* serotype 4b- and *L. innocua* serotype 6b-infected animals were able to transfer protective immunity, although to a different degree. As the absolute numbers of transferred cells were almost the same for all three groups of mice (1.5×10^7 to 2.0×10^7), it can be concluded from Fig. 7 that spleen cells from donor animals infected with *L. monocytogenes* serotype 4b confer a higher degree of protection than cells from *L. innocua* serotype 6b-infected mice. Consequently, the next experiments were performed to determine the duration of protective immunity after primary infection with either *L. monocytogenes* serotype 4b or *L. innocua* serotype 6b. Challenge with 20 times the LD_{50} of the virulent strain (4b) was performed after 1, 2, 4, and 6 months. It could be demonstrated that protective immunity lasted for at least 6 months after infection with *L. monocytogenes* serotype 4b, whereas immunity after infection with *L. innocua* serotype 6b had already decreased after 2 months and had completely vanished 6 months after the primary infection. Furthermore, we observed that the protection achieved after infection with *L. innocua* was dose dependent, since the injection of less than 10^8 viable bacteria did not induce any measurable protection against a challenge with *L. monocytogenes* serotype 4b.

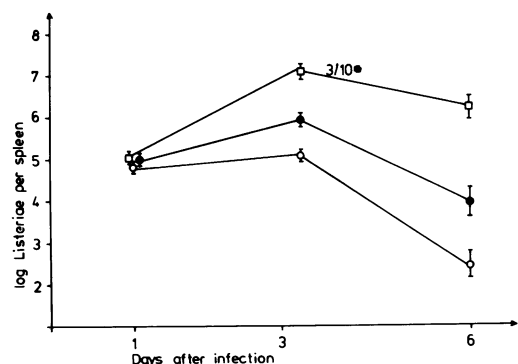


FIG. 7. Course of infection with 4.5×10^3 *L. monocytogenes* serotype 4b cells given i.v. into NMRI mice which had previously received 1.5×10^7 to 2.0×10^7 spleen cells from *L. monocytogenes* serotype 4b-immune (○), *L. innocua* serotype 6b-immune (●), or nonimmune (□) donor animals. Results are expressed as log CFU per spleen \pm standard error of the mean. Four to five mice were used per point. Results of control animals without cell transfer (not shown) were identical to those of the nonimmune group. †, Animals died between days 3 and 6.

DISCUSSION

Our experimental data can be condensed into several features, which differentiate the *L. monocytogenes* and *L. innocua* serotypes.

(i) All strains behaved similarly with respect to their distribution in mice after infection by different routes.

(ii) *L. monocytogenes* serotypes 4b, 4d, and 1/2b may be considered as mouse pathogenic. *L. monocytogenes* serotype 4d was able to increase its virulence significantly during adaptation to mice.

(iii) Primary infection with any of the serotypes of *L. monocytogenes* and *L. innocua* tested resulted in protection against a lethal challenge with the virulent strain. This protection could be transferred by spleen cells. The immunity against a lethal challenge established by *L. innocua*, however, was short lived and dose dependent.

(iv) The distinguishing difference between the strains tested seems to be the capacity to multiply in the host and to induce a chronic granulomatous inflammation as demonstrated for *L. monocytogenes* serotype 4b. Experimental infection with *L. innocua* serotype 6, using the method of intracerebral challenge of corticosteroid-treated suckling mice, has been described previously (14). The results obtained have led to a discussion about how to define *Listeria* pathogenicity and virulence. Weiss and Seeliger (20) as well as Khan et al. (8) define virulence with respect to the ability of the bacteria to kill conventional mice or to be recovered from the organs of symptomless animals after certain intervals postinfection. Patocka et al. (14) stress the importance of the host for the definition of bacterial pathogenicity. They define *L. innocua* as pathogenic (infective) for suckling mice upon intracerebral injection. Audurier et al. (1), also working with *L. innocua*, emphasize the importance of enumeration of bacteria in the spleen and the use of different routes for infection. In accordance with these findings, the results of our experiments suggest that the definition of *Listeria* pathogenicity and virulence cannot be made only by the ability to kill or infect conventional mice. Since resistance and susceptibility of different mouse strains against *Listeria* are controlled by a single gene, *Lr* (19), statements on *Listeria* virulence should be made with regard to the mouse strain being tested. Furthermore, the age of the animal host is important for resistance against infection (3, 22), and this information should also be included. Our experiments indicate that the main feature of a pathogenic *Listeria* sp. is its ability to multiply in the susceptible host. This results in a granulomatous type of inflammation, mainly characterized by an infiltration with monocytes and macrophages

(6, 9, 11, 13). Consequently, the definition of *Listeria* pathogenicity and virulence should include a kinetic study of an experimental infection and a histomorphological evaluation.

The induction of protective immunity to a lethal challenge with *L. monocytogenes* serotype 4b by a primary infection with any serotype of *L. monocytogenes* and *L. innocua* tested suggests a common immunogenic principle for all listeriae. It could be shown by the passive transfer of spleen cells that the mechanism of protective immunity is basically identical to the mode of action first described by Miki and Mackaness (12). Protective immunity, however, is rather short lived after primary infection with *L. innocua*, a finding that has been described by Kearns and Hinrichs (7) for infection with *L. monocytogenes* serotype 1/2b. The dose dependency of protective immunity after primary infection with *L. innocua* furthermore suggests that immunity can be acquired only when the animal host contains great numbers of microorganisms. This is made possible either by the injection of large doses of viable *L. innocua* or by multiplication of initially smaller numbers of *L. monocytogenes* serotype 4b in the host.

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