INFECTION AND IMMUNITY, May 1993, p. 2245–2248
0019-9567/93/052245-04$02.00/0

Vol. 61, No. 5

# Characterization of an Aromatic Amino Acid-Dependent *Listeria monocytogenes* Mutant: Attenuation, Persistence, and Ability To Induce Protective Immunity in Mice

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Received 6 November 1992/Accepted 8 February 1993

**A transposon insertion mutant of *Listeria monocytogenes* was shown to be deficient in prephenate dehydratase, an enzyme acting late in the pathway for biosynthesis of phenylalanine. This mutant had reduced virulence in mice. The mutant and parent strains persisted to the same extent in the tissues of infected mice and elicited similar degrees of splenomegaly. Mice vaccinated with the mutant were protected significantly from subsequent challenge with virulent *L. monocytogenes*.**

In farm animals, especially sheep, losses due to deaths from listeriosis can be considerable (21). To protect against infection, killed and live attenuated strains have been used as vaccines in animals, but with little or no success (1, 3, 8, 13). The failure of previous live attenuated strains as vaccines is probably due to the method of attenuation used (8, 13).

An alternative to the procedures previously used for making attenuated strains is the generation of strains with a defined mechanism of attenuation. This approach is the basis of the suggestion that hemolysin-deficient strains of *Listeria monocytogenes* could be suitable as attenuated strains for vaccines (15). However, use of these strains would exclude protection from the effects of hemolysin, an important virulence factor (18). Therefore, we decided to assess the potential of aromatic amino acid-dependent, hemolytic strains of *L. monocytogenes* for use as live vaccines. Aromatic amino acid-dependent (*aro*) strains of *Salmonella typhimurium* and *Salmonella typhi* are very successful examples of the use of defined mutants as vaccines (12, 17, 19) against salmonellosis. We assessed an aromatic amino acid-dependent transposon insertion mutant of *L. monocytogenes* as a candidate vaccine in a mouse model of listeriosis.

*L. monocytogenes* Lm.918.6 is an aromatic amino acid-dependent mutant of the wild-type strain Lm.1070138 (2). Both strains were obtained from D. Portnoy, University of Pennsylvania, and maintained as described before (20). When necessary, minimal medium (6), supplemented with the appropriate amino acid at 20 μg/ml, was used. Cells for inoculation into mice were grown at 30°C to late log phase in tryptose soya broth (Difco), supplemented when appropriate with erythromycin (25 μg/ml). The cells were then resuspended in tryptose soya broth containing 10% (vol/vol) glycerol and stored at −20°C for several months without significant loss of viability. When required, the bacteria were thawed rapidly, harvested by centrifugation, and resuspended in sterile distilled water. The specific activity of prephenate dehydratase was assayed by the method of Nester and Jensen (16). Female MF1 outbred mice, ca. 30 g in weight (Harlan Olac Ltd., Shaw's farm, Bicester, United Kingdom), were used throughout. Virulence was estimated by determining the 50% effective dose ($ED_{50}$). Doses of bacteria between $10^3$ and $10^8$ CFU in 100 μl of distilled water were administered intravenously to groups of five mice. The number of mice per group that reached the end point of the assay (i.e., became moribund) between days 0 and 6 was recorded and used to calculate the $ED_{50}$ by the log-probit method (the percentage of mice surviving to end point versus the log of the CFU inoculated) (5).

In experiments to ascertain the numbers of bacteria in the livers and spleens, infected mice were killed by cervical dislocation at the desired times after infection. The spleens and livers were removed, weighed, and homogenized separately in 10 ml of sterile distilled water in a Stomacher-Lab blender (Seward Medical), and viable counts on tryptose agar were obtained (20). Results were expressed as mean counts of viable listeriae per gram of tissue. To determine whether vaccination with Lm.918.6 conferred resistance to subsequent infection, mice were first vaccinated (intravenously) with $10^4$ CFU of Lm.918.6 in water. The booster vaccination was an identical dose of Lm.918.6 given 14 days after the initial vaccination. At 28 days after the initial vaccination with Lm.918.6, the $ED_{50}$ of Lm.1070138 for the mice was determined as described above. Data were analyzed by the Mann-Whitney U test (10) or the Kolmogorov-Smirnov test (10).

Lm.918.6 had been described previously as a Tn*917* insertion mutant that required aromatic amino acids for growth (2). To ensure that Lm.918.6 contained a single Tn*917* insertion, a Southern blot was performed to *Eco*RI-digested chromosomal DNA with a radiolabelled Tn*917*-specific probe as described in reference 2. Uncut plasmid pTV32 was included as a positive control, as it carries a single copy of Tn*917* (22). The probe hybridized to a single *Eco*RI fragment (Fig. 1). Since there are no recognition sites for *Eco*RI within Tn*917* (2), Lm.918.6 appears to contain a single Tn*917* insertion. To characterize more precisely the biochemical defect, aromatic amino acids and intermediates in the aromatic amino acid pathway were assayed for their ability to support the growth of Lm.918.6 in minimal medium. While phenylalanine and its immediate precursor, phenylpyruvic acid, supported growth, neither tyrosine nor prephenate (a precursor of both tyrosine and phenylpyruvic acid) did. To explain this, the activity of prephenate dehydratase, the enzyme that catalyzes the conversion of pre-

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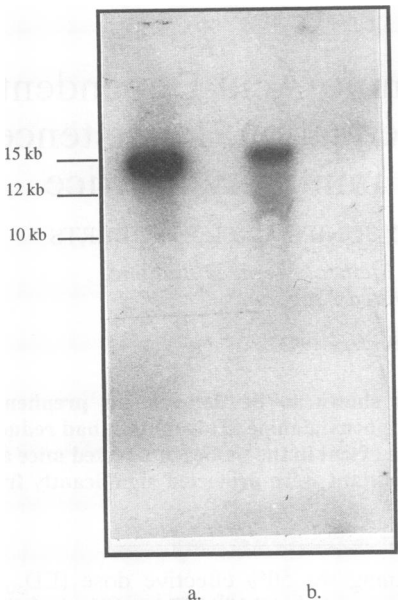


FIG. 1. Southern blot of *Eco*RI-digested Lm.918.6 chromosomal DNA (a) and uncut plasmid pTV32 DNA (b), probed with a 1.5-kb transposon Tn*917*-specific DNA probe (2). Lambda DNA digested with either *Xho*I or *Hin*dIII was used as molecular weight markers.

phenic acid to phenylpyruvic acid, in both Lm.1070138 and Lm.918.6 was determined. In Lm.918.6, the activity was 0.02 U/mg of protein, whereas the wild-type level was 7.00 U/mg of protein. This suggested that the single transposon insertion in Lm.918.6 was affecting expression of the *pheA* gene, which encodes prephenate dehydratase. As measured by $ED_{50}$, strain Lm.918.6 was significantly ($P < 0.05$) attenuated compared with the wild-type strain, Lm.1070138, in the mouse model of listeriosis (Table 1). Lm.918.6 multiplied to significantly ($P < 0.05$) lower numbers in both the liver and the spleen than Lm.1070138 (Fig. 2A and B). After a dose of $10^4$ viable cells of Lm.1070138, peak counts of $6 \times 10^9$ CFU/g of spleen tissue and $7 \times 10^7$ CFU/g of liver tissue were obtained after 3 days. At the same dose, Lm.918.6 grew more slowly, reaching peak counts of $2 \times 10^6$ and $9 \times 10^5$ CFU/g of spleen and liver tissue, respectively, at day 5 (Fig. 2A and B). No significant difference ($P > 0.05$) in the clearance of the two strains between days 1 and 9 was noted, and by day 11, both strains had been cleared (Fig. 2A and B). Maximum splenomegaly, as measured by the mean spleen weight of the five animals at each time point, occurred 4 days after maximum numbers of listeriae were detected, i.e., on day 7 postinfection with the wild-type strain Lm.1070138 and on day 9 postinfection with Lm.918.6 (Fig. 2C). The extent of splenomegaly induced by Lm.918.6 was not significantly

TABLE 1. Comparison of $ED_{50}$s of Lm.1070138 and Lm.918.6

| Strain | $ED_{50}$ (CFU) | | |
|---|---|---|---|
| | Mean[a] | SEM | 95% CL[b] |
| Lm.1070138 | $5.51 \times 10^4$ | $4.97 \times 10^2$ | $1.28 \times 10^4$–$2.37 \times 10^5$ |
| Lm.918.6 | $2.61 \times 10^{6*}$ | $3.67 \times 10^3$ | $7.00 \times 10^5$–$9.71 \times 10^6$ |

[a] Mean dose required to render 50% of the mice moribund. *, significantly different ($P < 0.05$) from value for Lm.1070138.
[b] CL, confidence limits.

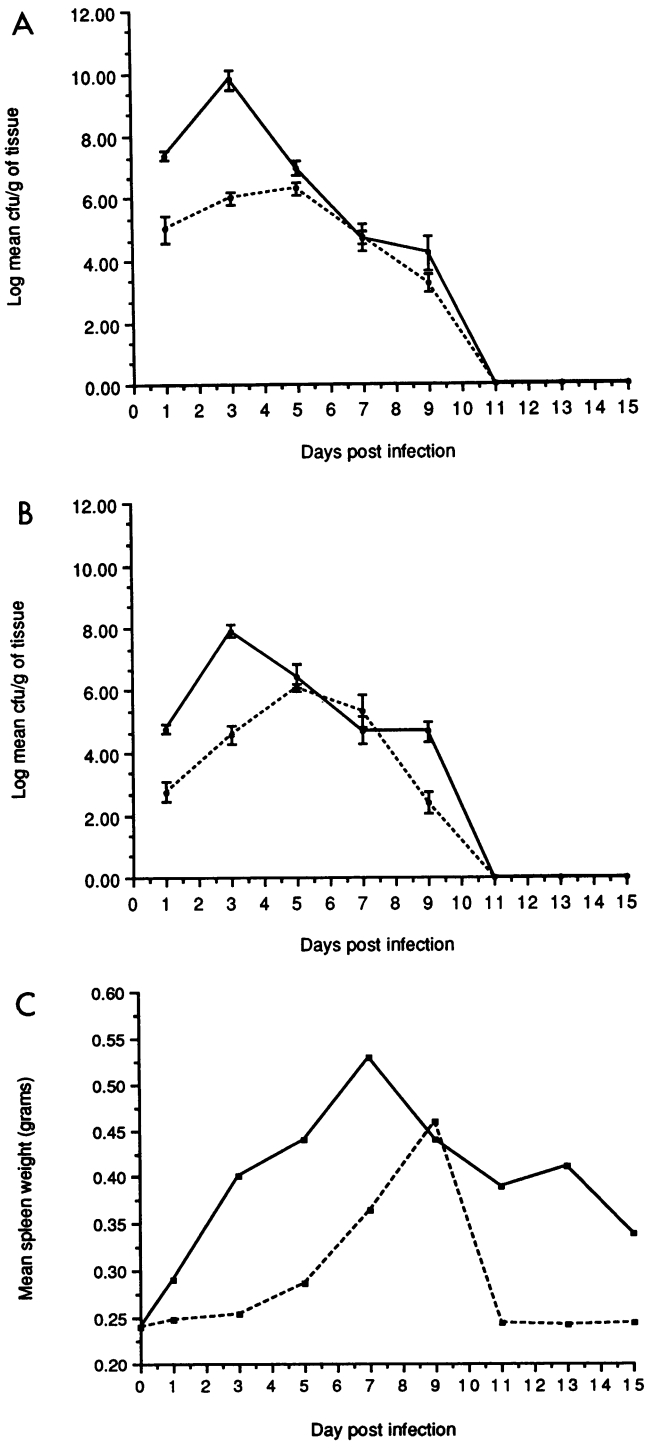


FIG. 2. (A and B) Concentrations of viable Lm.1070138 (——) and Lm.918.6 (– – –) in spleens (A) and livers (B) of MF1 mice over 15 days after intravenous infection with $10^4$ CFU. Each point (A and B) represents the geometric mean for five mice. (C) Splenomegaly (expressed as mean spleen weight, in grams) induced over 15 days by infection with $10^4$ CFU of Lm.1070138 (——) and Lm.918.6 (– – –). Each point represents the arithmetic mean for five mice.

TABLE 2. Effect of vaccination with Lm.918.6 on the $ED_{50}$ of wild-type Lm.1070138 in mice

| Lm.918.6 vaccination[a] | Lm.1070138 $ED_{50}$ (CFU) | | |
|---|---|---|---|
| | Mean[b] | SEM | 95% CL[c] |
| None | $5.51 \times 10^4$ | $4.79 \times 10^2$ | $1.28 \times 10^4$–$2.37 \times 10^5$ |
| 1 dose | $1.73 \times 10^{6*}$ | $5.35 \times 10^3$ | $1.73 \times 10^5$–$8.82 \times 10^6$ |
| 2 doses | $6.00 \times 10^{6**}$ | $6.09 \times 10^3$ | $1.37 \times 10^6$–$2.62 \times 10^7$ |

[a] Each vaccinating dose contained $10^4$ CFU of Lm.918.6, given intravenously.

[b] Mean dose required to render 50% of the mice moribund. *, Significantly different ($P < 0.05$) from value for unvaccinated mice; **, significantly different ($P < 0.05$) from value for mice receiving one dose.

[c] CL, confidence limits.

different ($P > 0.05$) from that induced by Lm.1070138 (Fig. 2C), but the duration was significantly reduced, with spleens returning to normal weight 11 days postinfection (Fig. 2C). In contrast, with Lm.1070138, splenomegaly was maintained for at least 15 days postinfection (Fig. 2C). In all these experiments, the phenotype and genotype of Lm.918.6 recovered from the infected animals were confirmed by growth on minimal medium and by Southern blotting with Tn*917*-specific DNA probes. In all cases, Lm.918.6 remained $PheA^-$ with a single transposon insertion (data not shown).

To determine whether vaccination with Lm.918.6 conferred protection against subsequent infection, mice were first vaccinated with Lm.918.6 and then challenged 28 days later with Lm.1070138. The $ED_{50}$ of Lm.1070138 was significantly ($P < 0.05$) greater in mice vaccinated with one dose of Lm.918.6 than in nonvaccinated mice (Table 2). In addition, a significant ($P < 0.05$) booster effect was seen when mice were given a second dose of Lm.918.6 14 days after the initial vaccination (Table 2). Therefore, strain Lm.918.6 with a transposon insertion which affects *pheA* expression is attenuated in the mouse model for listeriosis. In the same system, it confers significant protection against infection by virulent *L. monocytogenes*, with a pronounced booster effect upon second vaccination. This is the first evidence that an auxotrophic mutant of *L. monocytogenes* can be attenuated in a manner similar to that described for *aro* mutants of *S. typhimurium* (17). Why a marked reduction in *pheA* gene expression should result in attenuation is not yet clear. It has been hypothesized that in *aroA* mutants of *S. typhimurium* attenuation may be due to an inability to synthesize *p*-aminobenzoate and dihydroxybenzoate (7). Since Lm.918.6 should be capable of making both *p*-aminobenzoate and dihydroxybenzoate, attenuation in this case may reflect the poor availability of phenylalanine in mammalian cells. In addition, feedback inhibition of earlier aromatic pathway enzymes by the accumulation of prephenate, the substrate of prephenate dehydratase (9), may also be occurring. Reduced virulence of *pheA* mutants of gram-negative or gram-positive bacteria has not, to our knowledge, been reported previously. Studies of *aro* mutants of *Salmonella* spp. (4, 11) suggest that a strain of *L. monocytogenes* with multiple lesions in the pathway of aromatic amino acid synthesis might prove to be more attenuated than Lm.918.6. Future studies will attempt to introduce such additional mutations into Lm.918.6. This should serve to increase the attenuation and also reduce the risk of possible reversions in vivo. It is entirely feasible that such strains could be useful as vaccines in farm animals.

In addition to their potential as vaccine strains, nonreverting aromatic amino acid-dependent mutants of *L. monocytogenes* may be exploited as carriers of cloned gram-positive virulence factors and protective antigens of other pathogens, as has been reported for similar strains of *Salmonella* (4, 14).

We thank D. Portnoy, University of Pennsylvania, for strains Lm.918.6 and Lm.1070138.

One of us, J.E.A., thanks the Medical Research Council of Great Britain for the award of a postgraduate studentship.

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