Roles of *Listeria monocytogenes* Virulence Factors in Survival: Virulence Factors Distinct from Listeriolysin Are Needed for the Organism To Survive an Early Neutrophil-Mediated Host Defense Mechanism

J. WAYNE CONLAN* AND ROBERT J. NORTH

Trudeau Institute, Inc., P.O. Box 59, Saranac Lake, New York 12983

Received 9 October 1991/Accepted 11 December 1991

Avirulent mutant strains of *Listeria monocytogenes* which fail to produce phosphatidylinositol-specific phospholipase C, or which produce reduced amounts of hemolytic listeriolysin O, are incapable of causing progressive infection in normal mice. However, both strains can grow progressively in mice that have been rendered incapable of focusing neutrophils at sites of infection as a result of being treated with monoclonal antibody 5C6, specific for the type 3 complement receptor of myelomonocytic cells. In 5C6-treated mice, phospholipase C-negative and listeriolysin-defective mutant strains of *L. monocytogenes*, like the wild-type strain, give rise in the liver to large numbers of discrete foci of infected hepatocytes that retain their morphological integrity during the first 24 h, despite their large bacterial burden. In normal mice, in contrast, sites of infection in the liver are indicated by discrete foci accumulations of neutrophils that occupy the space originally occupied by infected hepatocytes. It is apparent that in normal mice neutrophils function to lyse infected hepatocytes and thereby to release *L. monocytogenes* for ingestion and killing by neutrophils themselves and by macrophages. However, whereas a proportion of wild-type organisms survive this early mechanism of defense to give rise to progressive infection, the phospholipase C-negative organisms are totally eliminated. On the basis of these and other results, it is suggested that virulence factors other than listeriolysin are needed by *L. monocytogenes* to counteract the early neutrophil-mediated mechanism of defense. Listeriolysin, itself, is an intrinsic virulence factor that allows *L. monocytogenes* to survive and multiply in a proportion of the fixed phagocytes of the liver (permissive phagocytes) and which enables the organism to go on to infect and replicate in adjacent hepatocytes.

It was found that a mutant strain of *L. monocytogenes* incapable of producing any listeriolysin was incapable of establishing progressive infection, even in 5C6-treated mice.

Acquired resistance to infection with the facultative intracellular bacterium *Listeria monocytogenes* is considered a convincing model of cellular immunity which illustrates the importance of specifically sensitized T cells in activating the microbicidal mechanisms of macrophages that must ultimately ingest and destroy the organism (9). Although once thought of exclusively as an intramacrophage parasite (8), it is now known that *L. monocytogenes* can parasitize a variety of nonphagocytic mammalian cells in vitro (4, 5, 12) and in vivo (13, 14, 17). Moreover, *L. monocytogenes* can directly infect neighboring cells within a tissue at sites of plasma membrane contact (13, 14) and in this way avoid exposing itself to host cellular defenses.

In experimentally infected mice, over 90% of an intravenous inoculum of *L. monocytogenes* is removed from the circulation within 15 min by Kupffer cells which line the liver sinusoids (8, 11), with most of the phagocyted bacteria being destroyed during the first 6 h. Bacteria which survive this killing mechanism of Kupffer cells give rise to discrete foci of infection in the liver. Because *L. monocytogenes* grows in the liver and spleen progressively during the first 24 h in foci of infection that are heavily populated by neutrophils (8, 10), it has been generally assumed that these host cells play little, if any, role in anti-*L. monocytogenes* defense. However, it was recently demonstrated (2) that if neutrophils are prevented from accumulating at infectious foci in the liver by treating mice with a monoclonal antibody (MAb) specific for the type 3 complement receptor of myelomonocytic cells (16), *L. monocytogenes* grows 50- to 100-fold more in this organ during the first 24 h. It was revealed by the same study that increased *Listeria* growth was due to the failure of neutrophils to make contact with and lyse infected hepatocytes and to thereby cause the release of the organism into the extracellular environment for phagocytosis and killing by neutrophils themselves and by macrophages. This defense mechanism of neutrophils is obviously not absolute, because a proportion of small, as well as large, inocula survive to give rise to established infection. Therefore, in considering the virulence of *L. monocytogenes*, it is necessary to take into account those properties of the organism that enable it to avoid being completely destroyed by Kupffer cells in the first place, and by neutrophils in the second.

According to the studies of others (1, 3, 6), the virulence of *L. monocytogenes* depends on its ability to synthesize at least two proteins: a hemolytic listeriolysin and a phosphatidylinositol-specific phospholipase C. It seems certain, moreover, that listeriolysin is an intrinsic virulence factor that enables *L. monocytogenes* to escape from the phagocytic vacuole of host cells and consequently to grow intracytoplasmically (4, 19) and that this is an absolute requirement for the organism to cause infection. Listeriolysin also seems to be needed by *L. monocytogenes* to infect neighboring host cells at points of plasma membrane contact. The functions of phospholipase C and other virulence factors, however, are less certain.

* Corresponding author.
To date, the functions of *Listeria* virulence factors have only been demonstrated in vitro. The purpose of the present study was to examine how virulence factors function in vivo to allow *L. monocytogenes* to establish infection, in the face of efficient early host defenses. This study showed that whereas listeriolysin enables *L. monocytogenes* to avoid destruction by Kupffer cells and consequently to establish infection in adjacent hepatocytes, other virulence factors, including phospholipase C, are needed by the organism to avoid complete destruction by an early neutrophil-mediated mechanism of defense.

**MATERIALS AND METHODS**

**Mice.** B6D2F1 (C57BL/6 × DBA/2) adult female mice (11 to 13 weeks old) were obtained from the Trudeau Institute Animal Breeding Facility (Saranac Lake, N.Y.). Mice were reared under barrier-free conditions and were free of common viral pathogens according to tests performed by the diagnostic testing services of Charles River Professional Services, Wilmington, Mass.

**Bacteria.** The streptomycin-resistant strain 10403S of *L. monocytogenes* (serotype 1) was the wild-type organism from which the transposon mutants were derived. Wild-type and mutant strains of *L. monocytogenes* were generously provided by D. A. Portnoy, Department of Microbiology, University of Pennsylvania. The listeriolysin-negative strain (DPL-1044) and the listeriolysin-defective strain (DPL-1049), capable of producing 25% of the amount of listeriolysin as the wild-type organism, and their relevant characteristics have been previously described by others (18). The mutant *Listeria* strain DPL-1054 has a mutation in a gene encoding a phosphatidilinositol-specific phospholipase C enzyme, the activity of which is completely abolished in this strain (1, 18). Additionally, it is now known (1a) that this mutation exerts a polar effect on the expression of a downstream gene called prfA which is a positive regulator of transcription that controls the expression of listeriolysin (7) and other gene products (3a). Hence, mutant strain DPL-1054 may be defective in its expression of factors other than phospholipase C, although it produces the same amount of listeriolysin as the wild-type strain (18). For convenience, and in keeping with previously published work, strain DPL-1054 is designated as a phospholipase C-negative mutant in the present study. All bacterial strains were grown to the mid-log phase in static cultures of Trypticase-soy broth (BBL Microbiology Systems, Cockeysville, Md.), dispensed into 1-ml volumes (approximately 5 × 10^8 CFU/ml), and frozen at −70°C until required. For experimental use, these stock cultures were thawed, washed once in saline, and diluted appropriately in the same solution for intravenous i.v. inoculation in a volume of 0.2 ml. Bacteria were enumerated in the liver by plating 10-fold serial dilutions of organ homogenates on Trypticase-soy agar. Colonies were counted after incubation for 24 h at 37°C. For 50% lethal dose (LD$_{50}$) determinations, groups of five mice were inoculated with various dilutions of bacteria in the range of 10^2 to 10^8 CFU, and deaths were recorded daily. The LD$_{50}$ of each *Listeria* strain was calculated by the method of Reed and Muench (15).

**MAb.** The 5C6 MAb (16) was given i.v. in a dose of 0.5 mg 1 h prior to infection. Antibody was obtained from the 5C6 hybridoma (a gift from H. Rosen and S. Gordon, University of Oxford, Oxford, United Kingdom) growing as ascites in CD2F1 mice. It was purified by a one-step chromatographic procedure by using an Avidal affinity column (Bio-

---

**TABLE 1.** LD$_{50}$ of *Listeria* strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Listeriolysin activity$^a$</th>
<th>Phospholipase C activity$^b$</th>
<th>LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10403S</td>
<td>Wild type</td>
<td>100</td>
<td>+</td>
<td>10^4.5</td>
</tr>
<tr>
<td>DPL-1044</td>
<td>Listeriolysin negative</td>
<td>0</td>
<td>+</td>
<td>&gt;10^6</td>
</tr>
<tr>
<td>DPL-1049</td>
<td>Listeriolysin defective</td>
<td>20</td>
<td>+</td>
<td>&gt;10^9</td>
</tr>
<tr>
<td>DPL-1054</td>
<td>Phospholipase C</td>
<td>100</td>
<td>-</td>
<td>10^7.8</td>
</tr>
</tbody>
</table>

$^a$ Expressed as percent wild-type activity.

$^b$ Based on data from Sun et al. (18).

$^c$ This study. B6D2F1 mice were inoculated i.v.

---

**RESULTS**

LD$_{50}$ of wild-type and mutant strains of *L. monocytogenes*. The *Listeria* strains used in this study and their relevant characteristics have been described in Materials and Methods and elsewhere (18) and are summarized in Table 1. The wild-type strain (10403S) had an LD$_{50}$ of 10^4.5 CFU for B6D2F1 mice. The mutant listeriolysin-negative strain (DPL-1044) and the listeriolysin-defective strain (DPL-1049) were avirulent for control mice at a dose of 10^6 CFU. The phospholipase-negative strain (DPL-1054), which expresses normal levels of listeriolysin, was slightly more virulent than the other two strains, causing some mice to die at an inoculating dose of 10^4 CFU. The LD$_{50}$ for this strain was 10^7.8 CFU.

**Inhibition of early host defenses permits the growth of the phospholipase C-negative and listeriolysin-defective strains but not the listeriolysin-negative strain of *L. monocytogenes in the livers of mice.** Figure 1 shows that all three mutant strains of *L. monocytogenes* failed to grow in the livers of control mice after i.v. inoculation of approximately 10^7 CFU. By contrast, the wild-type strain showed growth kinetics which were typical of virulent *L. monocytogenes*, in that it grew progressively after the first 6 h of infection. The uptake of all *Listeria* strains by the livers of control mice, however, was similar. The mutant strains were also similar in terms of the rates at which they were initially inactivated in the livers of normal mice.

In the livers of mice that were treated with MAb 5C6 to inhibit the focusing of neutrophils at sites of infection, the situation was quite different. From Fig. 1, it can be seen that treatment with this MAb allowed the listeriolysin-defective and the phospholipase-negative strains to grow logarithmically in the liver over a 48-h period. Additionally, treatment with 5C6 promoted the growth of the wild-type organism such that there were 500-fold more listeriae in the livers of the treated mice than in the livers of control mice by 24 h.
However, in 5C6-treated mice, the wild-type organism grew more quickly than the phospholipase C-negative organism, which in turn grew faster than the listeriolysin-defective strain. Note that, because most mice treated with 5C6 and infected with wild-type *L. monocytogenes* were dead by 48 h, the growth curves for this strain only extend to 24 h. Treatment of mice with 5C6 failed to permit infection with the listeriolysin-negative strain.

**Wild-type and phospholipase C-negative strains, but not listeriolysin-negative or listeriolysin-defective strains, kill mice treated with 5C6.** Pilot experiments (unpublished data) showed that the LD₅₀ of wild-type *L. monocytogenes* was approximately 100-fold higher for control mice than for 5C6-treated mice. It was decided to determine, therefore, whether the same treatment with 5C6 would enable the mutant strains of *L. monocytogenes* to grow progressively to kill mice. Figure 2 shows that at an inoculating dose of 10⁶ CFU, the phospholipase C-negative strain was completely avirulent for control mice but caused rapid death of 5C6-treated mice. The failure of this strain to grow in the livers of control mice (Fig. 1) and the speed at which it killed 5C6-treated mice indicate that exacerbation of infection in the latter mice was not due to the reversion to the wild-type phenotype.

Unlike the phospholipase C-negative strain, the listeriolysin-defective strain did not kill 5C6-treated mice at an inoculating dose of 10⁷ CFU despite the fact that it grew progressively in the livers of these mice for 48 h as shown in Fig. 1. However, its growth rate was slower than that of the phospholipase-negative strain after the first 24 h of infection. Perhaps this strain did not multiply to lethal numbers before the in vivo action of MAb 5C6 decayed.

**Progressive growth of the phospholipase C-negative mutant in 5C6-treated mice results from failure of the host to mount a neutrophil-mediated mechanism of defense.** It was shown in an earlier publication (2) that increased growth of wild-type *L. monocytogenes* in the livers of mice treated with MAb 5C6 results from a failure of neutrophils and other host cells to accumulate at infectious foci and to lyse infected hepatocytes. Consequently, *L. monocytogenes* is not liberated from permissive host cells for ingestion by neutrophils. This means that the organism is left free to multiply intracellularly and to directly infect neighboring hepatocytes. It was predicted, therefore, that failure of neutrophils to lyse infected hepatocytes and to ingest *L. monocytogenes* would explain the ability of the phospholipase-negative and the listeriolysin-negative strains to grow progressively in the livers of 5C6-treated mice.

**FIG. 1.** Evidence that mice treated with MAb 5C6 permit progressive growth in their livers of avirulent phospholipase C-negative (PLC⁻) and listeriolysin-defective (LLY⁻def) but not listeriolysin-negative (LLY⁻) *L. monocytogenes*. Treatment with 5C6 also increased the rate of growth of the wild-type organism. Mice were injected i.v. with 10⁵ CFU of one or another strain of *L. monocytogenes*, and growth of bacteria in the liver was monitored over 48 h. Means of five mice per group are shown. Standard error of the mean was <0.25 log₁₀. CONT, control.

**FIG. 2.** Groups of mice were injected i.v. with the numbers of bacteria indicated, and deaths were recorded daily. Infection with phospholipase C-negative *L. monocytogenes* (PLC⁻) was lethal for mice treated with 5C6 but not for control mice. In contrast, listeriolysin-defective *L. monocytogenes* (LLY⁻def), even at the high inoculum given, failed to kill 5C6-treated mice within the 10-day period of observation. The wild-type organism was lethal for control as well as for 5C6-treated mice. Five mice were used per group.
ylosin-defective mutants to grow progressively in the livers of 5C6-treated mice. To investigate this possibility, the livers of control and 5C6-treated mice infected with the wild type or one or other of the mutant strains of *L. monocytogenes* were examined histologically. As shown by Fig. 3, within 10 min of i.v. inoculation into control and 5C6-treated mice, all *Listeria* strains were found to be exclusively associated with Kupffer cells. This was expected because 5C6 has been shown (17) not to interfere with the clearance of *L. monocytogenes* from the blood. This was the case with small inocula, as well as with inocula as large as 5 × 10⁸ CFU. It seems fairly certain, therefore, that other cells of the liver do not remove *L. monocytogenes* from the circulation. By 24 h postinoculation in 5C6-treated mice (Fig. 4), the wild-type organism and the phospholipase C-negative and the listeriolysin-defective mutants grew to large numbers in foci of infected hepatocytes. It can be seen, moreover, that the infected hepatocytes retained their morphological integrity. This was expected because of the failure of neutrophils to accumulate at foci of infected hepatocytes. However, whereas wild-type and phospholipase-negative *L. monocytogenes* showed obvious spread from foci of heavily infected hepatocytes to adjacent hepatocytes, cell-to-cell transmission of listeriolysin-defective *L. monocytogenes* was less pronounced. In the livers of control mice, in contrast, all three strains of *L. monocytogenes* caused lesions at 24 h which were densely populated by neutrophils that occupied space originally occupied by infected hepatocytes. Therefore, with all three *Listeria* strains neutrophil accumulation at infectious foci resulted in dissolution of infected hepatocytes and the liberation of *L. monocytogenes* into the extracellular environment. In mice infected with the wild-type organism, however, liver lesions contained large numbers of bacteria, whereas in liver lesions of mice infected with the phospholipase C-negative or listeriolysin-defective mutant, intact organisms were difficult to find, particularly for the latter strain. It was obvious from the histology that the phospholipase C-negative mutant had caused extensive infection of hepatocytes before neutrophils became involved, in that infectious foci were large and the accumulations of neutrophils at these foci were extensive. This aspect of infection with the phospholipase C-negative and wild-type organisms is shown in more detail in Fig. 5.

Last, an extensive search for signs of infection caused by the listeriolysin-negative mutant in the livers of control and 5C6-treated mice was unsuccessful. It was apparent that this organism failed to infect hepatocytes and, hence, failed to stimulate neutrophil accumulation.

**DISCUSSION**

The purpose of this study was to show how virulence factors function in vivo to enable *L. monocytogenes* to evade early host defense mechanisms and consequently to establish progressive infection in the liver. This study shows that whereas listeriolysin allows *L. monocytogenes* to spread from the Kupffer cells that removed it from blood to liver hepatocytes, other virulence factors are needed for *L. monocytogenes* to survive subsequent neutrophil-mediated defenses. The results make it clear that an understanding of bacterial virulence requires a detailed knowledge of the early host defenses that a bacterium needs to overcome to establish infection.

It is known (8, 11) that after i.v. inoculation, 95% of *L. monocytogenes* is rapidly cleared from the blood, mainly by perivascular granulocytes of the liver. A large proportion of the bacterial load in liver macrophages is destroyed over the following 6 h, with surviving organisms giving rise to foci of infected hepatocytes (2). It is apparent, therefore, that a proportion of liver Kupffer cells are permissive for *L. monocytogenes* and that the organism can go on from these cells to infect neighboring hepatocytes. Indeed, *L. monocytogenes* is well equipped for intracellular parasitism. It has the capacity to escape from the phagocytic vacuole of permissive macrophages and to multiply freely in their cytoplasm (12, 19). Moreover, it is capable of entering and multiplying in a variety of nonphagocytic mammalian cells in vitro (4, 5, 12) and to spread directly from cell to cell at points of plasma membrane contact (19). This ability to spread directly from cell to cell within a tissue in vivo was convincingly demonstrated for intestinal epithelium and corneal epithelium by Racz et al. (13, 14) using electron microscopy. More recently, it was demonstrated that the target cell for *L. monocytogenes* in the liver of mice is the hepatocyte (17).

According to studies of others (4, 18, 19), the ability of *L. monocytogenes* to infect mammalian cells and to spread from cell to cell in culture depends on its ability to synthesize the virulence factor, listeriolysin O. This protein is also needed by *L. monocytogenes* to establish infection in vivo, because *Listeria* strains that are unable to make it fail to multiply in mice (1, 3, 6). It has been shown, moreover, that listeriolysin O is needed by *L. monocytogenes* to escape the phagocytic vacuole of its host cell and to multiply intracellularly (4, 12). However, other virulence factors are undoubtedly needed by *L. monocytogenes* to avoid being destroyed by host defenses. One such defense mechanism...
that needs to be avoided is mediated predominantly by neutrophils during the first 24 h of infection. In this regard, it is shown in a recent publication (2), and by the histological results presented here, that in mice inoculated with wild-type \textit{L. monocytogenes}, neutrophils accumulate in large numbers at sites of infection in the liver during the first 24 h of infection and that the arrival of these cells is followed by the lysis of infected hepatocytes. If neutrophils are prevented from accumulating during this period of infection, hepatocytes do not undergo lysis and \textit{L. monocytogenes} grows unrestrictedly within them and spreads to neighboring cells to cause extensive foci of infection. Under these conditions, \textit{L. monocytogenes} grows an extra 100-fold in the liver in less than 24 h. It was concluded, therefore, that neutrophils play an important early antimicrobial role by lysing infected hepatocytes and, by doing so, break the chain of cell-to-cell transmission of infection within the liver parenchyma. At the same time, \textit{L. monocytogenes} is released into the extracellular environment where it can be ingested and inactivated by neutrophils themselves, and later by macrophages. Although neutrophils constitute the overwhelming population of recruited host cells at sites of infection in the liver during the first 24 h, it is possible that other CR3-positive cells, such as monocytes, contribute to anti-\textit{L. monocytogenes} defenses during this time. However, the possibility that other mobile host cells such as T cells and natural killer cells were involved is made very unlikely given the results of recent in vivo depletion studies (unpublished data) which show that removal of these cells does not exacerbate infection in the liver during the first 24 h.

The present study shows that a phospholipase C-negative \textit{Listeria} mutant that produces normal amounts of listeriolysin in vitro but which is incapable of causing infection in normal mice can grow rapidly and establish infection in the
livers of mice that have been treated with MAb 5C6 to prevent neutrophils from accumulating at foci of infected hepatocytes. Under these conditions, the mutant organism multiplies progressively in hepatocytes and causes extensive lesions. With the doses of *L. monocytogenes* employed in this study, the main difference between the lesions caused by the wild-type organism and the phospholipase C-negative mutant in the livers of normal mice was the almost complete absence of *L. monocytogenes* in lesions caused by the latter organism. This strongly suggests that, on lysing infected hepatocytes and releasing mutant *L. monocytogenes* into the extracellular space, neutrophils can ingest the organism and progressively inactivate it.

Despite the fact that the phospholipase C-negative strain is able to spread from hepatocyte to hepatocyte, its rate of growth in the livers of 5C6-treated mice is less than that of the wild-type organism. This finding is in keeping with results published by others (18) showing that the mutant strain has a reduced ability to spread from cell to cell in monolayers of bone marrow-derived macrophages in vitro. A reduced ability to spread to neighboring hepatocytes in vivo could limit the mutant organism's access to nutrients which, in turn, could limit its rate of multiplication.

In the phospholipase C-negative strain used in the present study, the mutation in the phosphatidylinositol-specific gene also exerts polar effects on the *prfA* gene of *L. monocytogenes*. Because the *prfA* gene is a positive regulator of transcription (7) which controls the expression of several gene products, the altered behavior of this mutant strain cannot be ascribed with certainty solely to its failure to make phosphatidylinositol-specific phospholipase C. Therefore, the precise nature of the virulence factors that enable *L. monocytogenes* to resist total destruction by neutrophils will require the development of more-specific mutations. Nevertheless, it is clear from the present study that the phospholipase C-negative strain is avirulent because of an inability to escape host defense, rather than because of an inability to survive and multiply intracellularly.

In contrast, the listeriolsin-negative mutant failed to give rise to any infection at all, even in mice treated with the 5C6 MAb. Indeed, at 24 h postinoculation in 5C6-treated and control mice, there were neither foci of infected hepatocytes nor accumulations of neutrophils, respectively. This suggests that this mutant did not escape from the Kupffer cells that removed it from the circulation. Furthermore, the finding that this strain is inactivated at a similar rate in the livers of control and 5C6-treated mice indicates that this defense mechanism is CR3 independent. This is not surprising given the reportedly low to undetectable levels of CR3 expressed on Kupffer cells (17). Unlike the listeriolsin-negative strain, the listeriolsin-defective mutant did grow progressively and give rise to foci of infected hepatocytes in the livers of mice treated with 5C6. However, it is apparent from the histological study that this organism had a limited ability to spread from hepatocyte to hepatocyte in the livers of normal and 5C6-treated mice. This is in accordance with an in vitro study (18) which showed that this listeriolsin-defective strain had a severely reduced ability to spread from cell to cell. Because its possession of other virulence factors, including phospholipase C, presumably enabled it to resist total elimination by neutrophils, its reduced ability to cause infection is almost certainly due to its reduced ability to multiply and spread within permissive cells.

**ACKNOWLEDGMENTS**

We thank D. A. Portnoy for a critical reading of this manuscript; Linda Schaefer, Ron LaCourse, Debra Duso, and Lynn Ryan for expert technical assistance; and Mary Durett for typing.

This work was supported by an internal grant from the Trudeau Institute.

**REFERENCES**


SURVIVAL ROLES OF L. MONOCYTOGENES VIRULENCE FACTORS


