

## Phosphatidylcholine-Specific Phospholipase C from *Listeria monocytogenes* Is an Important Virulence Factor in Murine Cerebral Listeriosis

DIRK SCHLÜTER,<sup>1\*</sup> EUGEN DOMANN,<sup>2</sup> CHRISTINE BUCK,<sup>3</sup> TORSTEN HAIN,<sup>2</sup> HERBERT HOF,<sup>1</sup> TRINAD CHAKRABORTY,<sup>2</sup> AND MARTINA DECKERT-SCHLÜTER<sup>3</sup>

*Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum Mannheim, Universität Heidelberg, Mannheim,<sup>1</sup> Institut für Medizinische Mikrobiologie, Justus-Liebig-Universität Gießen, Gießen,<sup>2</sup> and Institut für Neuropathologie, Universitätskliniken Bonn, Bonn,<sup>3</sup> Germany*

Received 8 July 1998/Returned for modification 18 August 1998/Accepted 25 September 1998

Meningoencephalitis is a serious and often fatal complication of *Listeria monocytogenes* infection. The aim of the present study was to analyze the role of internalin A (InlA) and B, which are involved in the invasion of *L. monocytogenes* into cultivated host tissue cells, and that of phosphatidylcholine-specific phospholipase C (PlcB), which mainly promotes the direct cell-to-cell spread of *L. monocytogenes*, in murine cerebral listeriosis by use of an InlA/B ( $\Delta$ inlAB2)- and a PlcB ( $\Delta$ plcB2)-deficient isogenic deletion mutant strain and the wild-type (WT) *L. monocytogenes* EGD. *Listeria* strains were directly applied to the brain, a technique which has been employed previously to study the pathogenesis of cerebral listeriosis (D. Schlüter, S. B. Oprisiu, S. Chahoud, D. Weiner, O. D. Wiestler, H. Hof, and M. Deckert-Schlüter, Eur. J. Immunol. 25:2384–2391, 1995). We demonstrated that PlcB, but not InlA or InlB, is an important virulence factor in cerebral listeriosis. Nonimmunized mice infected intracerebrally with the  $\Delta$ plcB2 strain survived significantly longer and had a reduced intracerebral bacterial load compared to mice infected with the  $\Delta$ inlAB2 strain or WT bacteria. In addition, immunization with the WT prior to intracerebral infection significantly increased the survival rate of mice challenged intracerebrally with the  $\Delta$ plcB2 strain compared to that of mice infected with the WT or  $\Delta$ inlAB2 strain. Histopathology revealed that the major difference between the various experimental groups was a significantly delayed intracerebral spread of the  $\Delta$ plcB2 mutant strain, indicating that cell-to-cell spread is an important pathogenic feature of cerebral listeriosis. Interestingly, irrespective of the *Listeria* mutant used, the apoptosis of hippocampal and cerebellar neurons and an internal hydrocephalus developed in surviving mice, indicating that these complications are not dependent on the virulence factors InlA/B and PlcB. In conclusion, this study points to PlcB as a virulence factor important for the intracerebral pathogenesis of murine *L. monocytogenes* meningoencephalitis.

*Listeria monocytogenes* is a gram-positive ubiquitous living bacterium that can be isolated from soil, decaying plants, and food and is able to infect a wide variety of organisms, including humans and rodents. *L. monocytogenes* is the causative agent of human listeriosis, which is normally acquired by the consumption of contaminated food, particularly unpasteurized dairy products (11). Persons at risk include the elderly, the immunocompromised, and pregnant women. An important pathogenic feature of this facultative intracellular pathogen is its capacity to infect a broad range of host cells, and in vitro studies have revealed that *L. monocytogenes* induces its own uptake into nonprofessional phagocytic cells, including enterocytes, fibroblasts, dendritic cells, hepatocytes, and endothelial cells (reviewed in references 4 and 7). After internalization, *L. monocytogenes* escapes from the phagosome into the cytosol of infected cells, recruits actin filaments, and is capable of actin-based motility and cell-to-cell spread.

Recently, a chromosomal genetic locus, the internalin operon, has been identified, whose gene products are involved in entry into various cell types (6, 13, 27). The gene products of

this operon, InlA and InlB (InlA/B), are cell wall-associated proteins of 88 and 65 kDa, respectively (27). InlA associates with the bacterial cell wall by a conventional cell wall anchoring motif (25), while the cell wall association of InlB requires a larger segment, comprising the last 232 amino acid residues of the C terminus (3). A striking structural feature of both proteins is the presence of consecutive leucine-rich repeats, present 15 times in InlA and 7 times in InlB (5, 27). Homology searches have revealed that InlA and InlB are members of a superfamily of leucine-rich repeat-containing proteins found only in pathogenic bacteria (24, 29, 39). It has been shown previously that InlA and InlB are differentially required for entry into various tissue and culture cell lines (reviewed in references 4 and 7). InlA mediates the uptake into the epithelium-like cell line Caco-2 via interaction with host cell E-cadherin (30), whereas the eukaryotic receptor for the bacterial ligand InlB is still unknown. It has been shown that pathogenic *Listeria* also harbors an internalin-like protein gene (*irpA*), encoding a secreted protein of 30 kDa (5, 10). Recently, four more internalin-like proteins have been found in *L. monocytogenes*; however, the role of these proteins in infection is at present unclear (8).

The capacity of *L. monocytogenes* to spread efficiently from cell to cell appears to be dependent on the product of the *plcB* gene, a secreted phospholipase with broad-substrate specificity, particularly for phosphatidylcholine. Thus, the highly purified 29-kDa mature form of this enzyme (termed PlcB or

\* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum Mannheim, Universität Heidelberg, Theodor-Kutzer-Ufer 1-3, D-68167 Mannheim, Germany. Phone: (49) 621/383-2036. Fax: (49) 621/383-3816. E-mail: dirk.schluter@imh.ma.uni-heidelberg.de.

PC-PLC) cleaves a variety of phospholipids, including sphingomyelin, in detergent micelles, liposomes, and biological membranes (15, 16, 31). The *plcB* gene is part of the lecithinase operon, consisting of the metalloprotease *mpl*, actin recruiting factor *actA*, and *plcB* (48), and mutations of the *plcB* gene have resulted in a partial defect in cell-to-cell spread and reduced virulence (35, 41, 42). Transmission electron microscopic studies revealed that *plcB*-deficient mutant strains accumulate in double-membrane vesicles in newly infected cells which form as a consequence of cell-to-cell spread (48). Thus, infection of host cells by *L. monocytogenes* can occur by two distinct mechanisms: direct bacterial invasion of the cells in an internalin-mediated fashion and cell-to-cell spread (9). The intracellular lifestyle along with direct cell-to-cell spread is responsible for T cell-mediated immunity to cure the infection, whereas antibodies, albeit induced, are not important for elimination of the bacteria (20, 28).

Whereas most experimental studies with animals have focused on the role of bacterial virulence factors in systemic listeriosis, which involves predominantly the liver and the spleen, the pathogenesis of cerebral listeriosis, a common and life-threatening complication of human listeriosis, has remained largely unexplored (43). Previous studies in an established murine model of cerebral listeriosis (12, 26) have revealed that in the brain most of the bacteria reside intracellularly in plexus epithelial cells, ependymal cells, and neurons as well as macrophages (37). The intracellular location of *L. monocytogenes* indicates that bacterial virulence factors promoting its entry into host cells or facilitating its spread from cell to cell may significantly contribute to the pathogenesis and severity of cerebral listeriosis. The aim of this study was to examine the roles of InlA and InlB, necessary for invasion, and that of PlcB, necessary for the efficient spread of *Listeria* cells, in experimental murine cerebral listeriosis. In the present study we have used *Listeria* mutants lacking InlA and InlB ( $\Delta$ inlAB2) or PlcB ( $\Delta$ plcB2). Our data clearly demonstrate that PlcB, but not InlA/B, is an important virulence factor contributing to murine cerebral listeriosis.

#### MATERIALS AND METHODS

**Bacterial strains and media.** The weakly hemolytic *L. monocytogenes* EGD serotype 1/2a was originally obtained from G. B. Mackness and served as the parental strain for construction of the  $\Delta$ plcB2 (18) and  $\Delta$ inlAB2 (32) mutant strains. *Listeria* strains were grown in brain heart infusion broth (Difco, Freiburg, Germany) at 37°C, with vigorous shaking.

**Mice.** Female C57BL/6 mice (6 to 8 weeks old) were obtained from Harlan-Winkelmann (Borchen, Germany). Animals were kept under special pathogen-free conditions before infection with *L. monocytogenes*.

**Experimental procedure.** Mice were infected intracerebrally (i.c.) with  $4 \times 10^2$  organisms of *L. monocytogenes* EGD (wild type [WT]),  $\Delta$ inlAB2 (InlA/B deficient), and  $\Delta$ plcB2 (lecithinase deficient) strains. In addition, experimental groups were actively immunized by intraperitoneal injection of  $5 \times 10^3$  *L. monocytogenes* EGD cells 14 days prior to i.c. infection. The survival rates of the mice in each experimental group were monitored.

The i.c. bacterial load was determined at days 1, 3, and 5 postinfection (p.i.) by plating 10-fold serial dilutions of brain tissue on tryptose agar (Difco). CFU were counted after incubation at 37°C for 24 h. In addition, spleens and livers from five immunized mice were used to assess the bacterial load 14 days after intraperitoneal immunization prior to i.c. challenge infection. After incubation of spleen and liver tissue for 24 h, no CFU could be recovered from these animals.

For histology, mice were sacrificed by CO<sub>2</sub> asphyxiation and immediately thereafter perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) at days 1, 3, 5, and 10 p.i. Brains were dissected and incubated in 4% paraformaldehyde in phosphate-buffered saline at 4°C for 24 h and subsequently embedded in paraffin.

**Histology.** For histology, sections (4  $\mu$ m each) were cut from paraffin-embedded brain tissue. Specimens were stained with hemalum and eosin, cresyl violet, and Giemsa solution (Merck, Darmstadt, Germany). In addition, *L. monocytogenes* was demonstrated immunohistochemically by incubating deparaffinized sections with a polyclonal rabbit anti-*L. monocytogenes* antiserum (Difco) followed by peroxidase-labelled goat anti-rabbit immunoglobulin G F(ab')<sub>2</sub> fragments (Jackson-Dianova, Hamburg, Germany). Peroxidase reaction products

were visualized by 3,3'-diaminobenzidine tetrahydrochloride (Sigma, Deisenhofen, Germany), and H<sub>2</sub>O<sub>2</sub> was used as the cosubstrate.

Histopathological alterations were evaluated in specimens stained with hemalum and eosin, cresyl violet, and Giemsa solution as well as in *L. monocytogenes* immunohistochemically stained sections obtained from various regions of the brain, including the basal ganglia, the hippocampus, the hypothalamus, the cerebellum, the brain stem, the ventricular system at the levels of the lateral, third, and fourth ventricles, the subarchnoid space at the level of the forebrain, the cerebellar cisterns, and the brain stem. To compare the intensity of cerebral listeriosis between the various groups, a semiquantitative grading system was introduced as follows: no pathological alterations, −; single bacteria and small inflammatory infiltrates, +; clusters of bacteria and large infiltrates with no tissue destruction, ++; and huge amounts of bacteria and large numbers of infiltrates with parenchymal destruction, ++++. For the in situ detection of DNA fragmentation, the TUNEL method (14) was employed with paraffin sections. The In Situ Cell Death Detection kit (Boehringer, Mannheim, Germany) was used according to the manufacturer's instructions. Negative control experiments were carried out without terminal transferase in the TUNEL reaction mixture.

**Statistics.** The Wilcoxon test was used to compare statistical differences between the survival times of the various experimental groups as well as differences in the i.c. bacterial load at various time points after infection. *P* values of <0.05 were considered significant. Each experiment was performed at least twice.

#### RESULTS

**Survival rates.** Following i.c. infection with the *L. monocytogenes* WT and  $\Delta$ inlAB2 mutant strains, all nonimmunized mice succumbed to the disease by day 5 p.i. (Fig. 1). In addition, although mice infected with the *L. monocytogenes*  $\Delta$ plcB2 mutant strain inevitably showed a fatal outcome caused by central nervous system (CNS) listeriosis (Fig. 1), death was significantly delayed in these animals (*P* < 0.05 for  $\Delta$ plcB2 versus WT and  $\Delta$ inlAB2).

Mice actively immunized with WT *L. monocytogenes* were significantly protected from i.c. infection with the  $\Delta$ plcB2 strain compared to nonimmunized animals (*P* < 0.05 [Fig. 1]). In contrast, immunization did not significantly protect mice i.c. infected with either WT or  $\Delta$ inlAB2, and mortality rates did not differ significantly from those of nonimmunized mice infected with WT and the  $\Delta$ inlAB2 mutant strain. Thus, survival rates of these mice were significantly reduced compared with those of immunized mice infected with  $\Delta$ plcB2 (Fig. 1 [*P* < 0.05]). Notably, after day 6 p.i., no more animals succumbed to the disease, irrespective of experimental group.

**Kinetics of bacterial load.** Determination of the i.c. bacterial load at days 1, 3, and 5 p.i. revealed that nonimmunized and immunized mice infected with WT and  $\Delta$ inlAB2 had comparable amounts of bacteria in their brains. In both the nonimmunized and immunized mice in these groups, the i.c. bacterial load increased from day 1 to day 3 p.i. Interestingly, immunized WT- and  $\Delta$ inlAB2-infected mice reduced the i.c. bacterial load only moderately from days 3 to 5 p.i. (Fig. 2).

A comparison of WT- and  $\Delta$ inlAB2-infected mice with  $\Delta$ plcB2-infected animals revealed that the bacterial load in the brains of  $\Delta$ plcB2-infected mice was significantly reduced (Fig. 2). This observation was made for both immunized and nonimmunized groups throughout the course of disease. Although the number of bacteria increased in the brains of  $\Delta$ plcB2-infected nonimmunized mice from days 1 to 3 p.i., the bacterial load was significantly lower compared to those in nonimmunized WT- and  $\Delta$ inlAB2-infected mice (*P* < 0.05 for  $\Delta$ plcB2 versus WT and  $\Delta$ inlAB2). In addition, immunized  $\Delta$ plcB2-infected mice had a significantly reduced i.c. bacterial load at days 1, 3, and 5 p.i. compared to immunized WT- and  $\Delta$ inlAB2-infected mice (*P* < 0.05 for  $\Delta$ plcB2 versus WT and  $\Delta$ inlAB2). Interestingly, analysis of nonimmunized and immunized  $\Delta$ plcB2-infected mice revealed that immunization, which significantly protected these mice from the fatal course of disease, did not result in a significant reduction of the i.c. bacterial load before day 5 p.i.

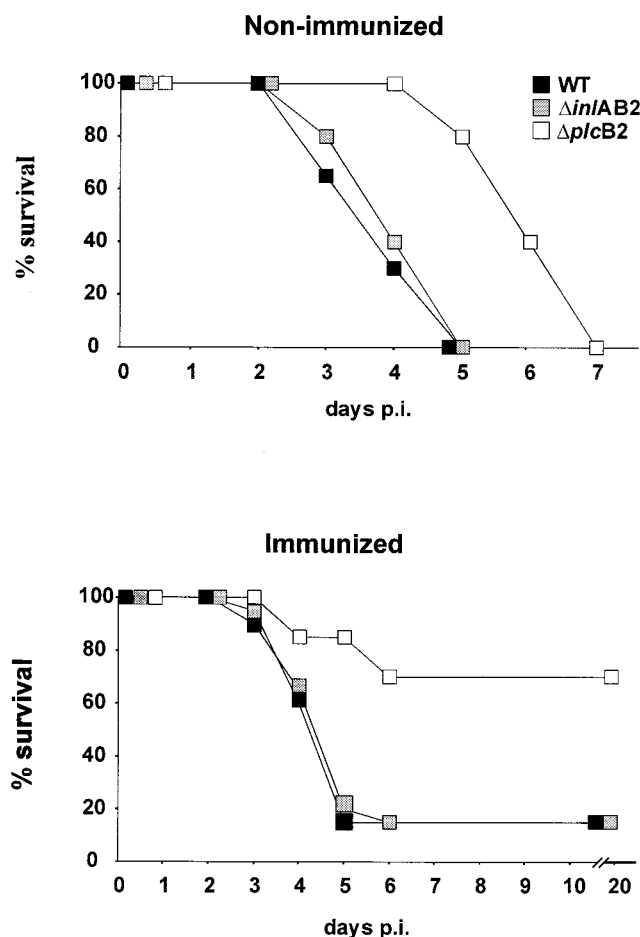


FIG. 1. Survival rates of nonimmunized (upper panel) and immunized (lower panel) mice infected i.c. with *L. monocytogenes* WT,  $\Delta inlAB2$ , and  $\Delta plcB2$ . Non-immunized mice infected i.c. with  $\Delta plcB2$  survived significantly longer than mice infected with WT or  $\Delta inlAB2$  ( $P < 0.05$ ). Immunized mice infected i.c. with  $\Delta plcB2$  had a significantly increased survival rate compared to that of mice infected with WT or  $\Delta inlAB2$  *L. monocytogenes* ( $P < 0.05$ ). Data represent survival rates of 10 mice per experimental group. The results of one of two experiments which gave comparable results are shown.

**Histopathology.** After i.c. application of *L. monocytogenes* WT,  $\Delta inlAB2$ , and  $\Delta plcB2$  strains, all mice developed cerebral listeriosis. Whereas the same neuroanatomical structures were affected in all groups, there were major differences in the kinetics and severity of inflammation (Table 1).

In general, following the i.c. application of *L. monocytogenes* onto the rostral basal ganglia, nonimmunized WT- and  $\Delta inlAB2$ -infected mice developed similar pathologies. At day 1 p.i., bacteria and inflammatory cells were present in the ventricular system associated with the choroid plexus and the ependyma. The  $\Delta plcB2$  mutant strain was observed in slightly reduced numbers in the same structures. However, there was no evidence of an invasion of the ependyma by the  $\Delta plcB2$  strain at this stage of the disease.

At day 3 p.i., cerebral listeriosis had significantly progressed in all experimental groups. *L. monocytogenes* WT and  $\Delta inlAB2$  strains had caused severe meningitis and ventriculitis. The lateral, third, and fourth ventricles were completely obstructed by large numbers of polymorphonuclear leukocytes and macrophages (Fig. 3a and c). Interestingly, numerous elongated, rod-shaped WT bacteria and  $\Delta inlAB2$  mutants adhered to plexus epithelial cells and formed large clusters (Fig. 3b and d). They

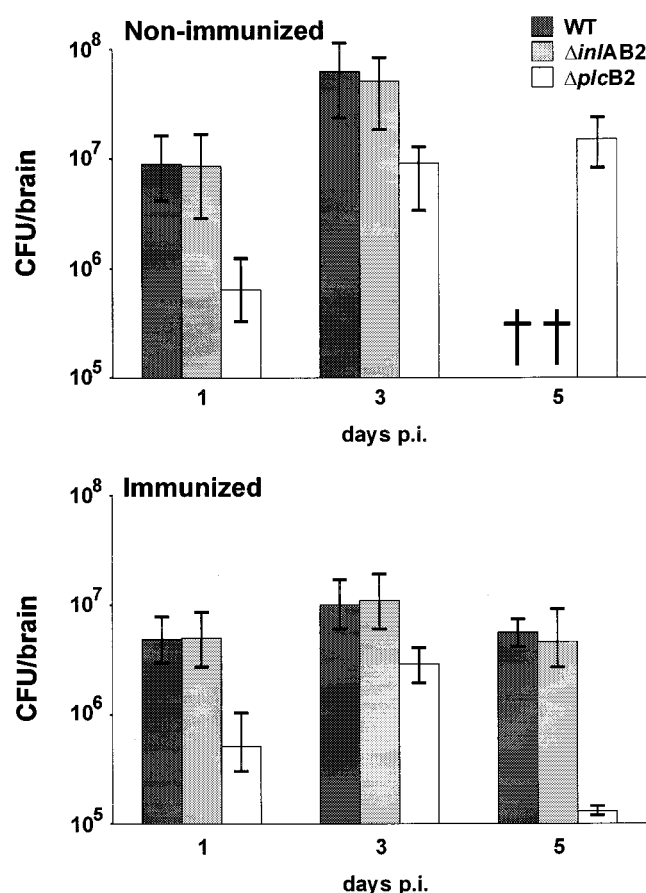


FIG. 2. Parasitic load of nonimmunized (upper panel) and immunized (lower panel) mice i.c. infected with *L. monocytogenes* WT,  $\Delta inlAB2$ , and  $\Delta plcB2$ . At each time point after infection, the i.c. bacterial load of mice infected with  $\Delta plcB2$  was significantly lower than that of mice infected with WT or  $\Delta inlAB2$  ( $P < 0.05$ ). †, mice were already deceased. Five mice per group were analyzed, and the median  $\pm$  the highest and lowest values of each group are shown.

had destroyed the choroid plexus and the ependyma (Fig. 3a to d), allowing the bacteria access to the periventricular tissue, where inflammatory infiltrates were associated with the bacteria and small foci of necrosis (Fig. 3a and c). Invasion of WT and the  $\Delta inlAB2$  strain was most prominent in the brain stem, where bacteria were also detected in the cytoplasm of neurons. These *Listeria*-infected neurons were surrounded by neutrophils and macrophages (Fig. 3c).

In contrast to WT and  $\Delta inlAB2$ , the  $\Delta plcB2$  mutant strain caused a course of disease that was significantly less severe (Fig. 3e). The number of  $\Delta plcB2$  bacteria was much lower than those of WT and  $\Delta inlAB2$ , and consequently there were fewer inflammatory infiltrates. Interestingly,  $\Delta plcB2$  bacteria did not take an elongated shape or form large clusters in the ventricular system but were isolated or present in small groups (Fig. 3f). The choroid plexus was much less severely destroyed, and the intact ependymal layer precluded significant bacterial invasion of the adjacent brain tissue (Fig. 3e). Up to day 5 p.i., when mice infected with WT and  $\Delta inlAB2$  had already succumbed to necrotizing brain stem encephalitis, the disease was still progressing in animals infected with the  $\Delta plcB2$  strain. At this stage of infection, histopathological alterations of  $\Delta plcB2$ -infected mice were comparable to those in mice infected with WT and  $\Delta inlAB2$  bacteria at day 3 p.i.

With either bacterial strain, the anatomic structures that



TABLE 1. Semiquantitative histopathological grading of cerebral listeriosis in mice infected with *L. monocytogenes* WT,  $\Delta$ *inlAB2*, or  $\Delta$ *plcB2*

Day p.i.	<i>Listeria</i> strain	Results for indicated group of mice <sup>a</sup>					
		Nonimmunized			Immunized		
		Meningitis	Encephalitis	Ventriculitis	Meningitis	Encephalitis	Ventriculitis
1	WT	+	+	++	+	—	++
	$\Delta$ <i>inlAB2</i>	+	+	++	++	+	++
	$\Delta$ <i>plcB2</i>	+	+	+	+	+	+
3	WT	+++	++	+++	+++	+++	+++
	$\Delta$ <i>inlAB2</i>	+++	++	+++	++	++	+++
	$\Delta$ <i>plcB2</i>	+	+	++	+	+	+
5	WT	DEC	DEC	DEC	++	++	++
	$\Delta$ <i>inlAB2</i>	DEC	DEC	DEC	+	++	+++
	$\Delta$ <i>plcB2</i>	+	++	++	+	+	+

<sup>a</sup> Three mice per group were analyzed, and results from one of two experiments with similar results are shown. —, no pathological alterations; +, presence of single bacteria and small inflammatory infiltrates; ++, clusters of bacteria and large infiltrates in the absence of tissue destruction; +++, large numbers of bacteria and infiltrates with tissue destruction; DEC, deceased.

were affected in actively immunized mice were the same as those affected in nonimmunized mice. However, the severity of cerebral listeriosis, particularly in mice infected with mutant strain  $\Delta$ *plcB2*, was reduced (Table 1 and Fig. 4). The involvement of the periventricular basal ganglia, the fourth ventricle, and the adjacent brain stem tissue, especially, was less severe (Fig. 4a to d). Nevertheless, most of the immunized mice infected with the WT and  $\Delta$ *inlAB2* strains still developed progressive encephalitis, although it was somewhat delayed, and subsequently the vast majority of these animals succumbed to the disease.

Immunized  $\Delta$ *plcB2*-infected mice also developed meningitis and ventriculitis; however, these diseases were much less severe than in nonimmunized  $\Delta$ *plcB2*-infected or immunized WT- or  $\Delta$ *inlAB2*-infected animals (Fig. 4e and Table 1). The most-striking finding was the lack of disease progression from days 3 to 5 p.i. in most animals (Fig. 4f and Table 1). In addition, only a small number of immunized  $\Delta$ *plcB2*-infected mice developed brain stem encephalitis, and inflammation was restricted mostly to the periventricular brain tissue. In particular, necrosis did not occur in the brains of these animals. Interestingly, the  $\Delta$ *plcB2* mutant strain was observed less often in neurons than were bacteria from the other experimental groups.

At day 10 p.i., surviving mice had developed postinflammatory internal hydrocephalus of the lateral, third, and fourth ventricles (Fig. 5a) due to partial destruction of the ependymal lining and formation of delicate sheets of glial-ependymal tissue in the fourth ventricle, irrespective of the injected *Listeria* mutant (Fig. 5c).

**Apoptosis.** To determine whether apoptosis of CNS cells contributes to the pathogenesis of cerebral listeriosis and whether apoptosis is influenced by virulence factors of *L. monocytogenes*, brain sections from the various experimental groups were analyzed by the TUNEL technique.

At day 1 p.i., no apoptotic cells were detectable in the brains of any of the *Listeria*-infected mice. Starting at day 3 p.i., apoptosis of hippocampal neurons was observed in all experimental groups. The vast majority of neurons of the CA1 segment of the hippocampus exhibited small, pyknotic nuclei and an eosinophilic cytoplasm, and these neurons were strongly labelled in the TUNEL reaction. Apoptotic neurons were confined to the CA1 segment and were sharply demarcated from the adjacent intact hippocampus (Fig. 5d). In addition, surviving mice exhibited a small but significant number of apoptotic

Purkinje cells in the cerebellum at day 10 p.i. (Fig. 5c). In contrast to hippocampal and cerebellar neurons, other structures of the CNS exhibited only a marginal number of apoptotic cells. Thus, the destruction of the plexus epithelium, the ependymal layer as well as parts of the brain parenchyma, was due to *L. monocytogenes*-induced necrosis rather than to apoptosis. Furthermore, at days 3 and 5 p.i., some apoptotic inflammatory cells in the ventricular lumen were detected by TUNEL labelling.

## DISCUSSION

In this study we have assessed the properties contributing to the virulence of the *inlA*, *inlB*, and *plcB* genes of *L. monocytogenes* in the CNS following the crossing of the blood-brain barrier (BBB). We employed *L. monocytogenes* isogenic deletion mutant strains lacking the main virulence factors necessary for invasion and cell-to-cell spread, *inlA/B* and *plcB*, respectively.

We demonstrated that PlcB, but not InlA or InlB, is an important determinant of the virulence of *L. monocytogenes* in cerebral listeriosis. The delayed intracerebral spread and reduced multiplication of the *L. monocytogenes*  $\Delta$ *plcB2* mutant strain indicated that cell-to-cell spread, which is dependent mainly on lecithinase activity (48), is important for the rapid dissemination of the bacterium in the brain. Consequently, nonimmunized mice infected i.c. with  $\Delta$ *plcB2* survived significantly longer than mice infected with the WT and  $\Delta$ *inlAB2* strains. However, it should be stressed that although its spread was delayed,  $\Delta$ *plcB2* finally gained access to the same anatomic structures of the CNS, i.e. the plexus epithelium, the ependyma, the leptomeninges, and the brain parenchyma, as the WT and  $\Delta$ *inlAB2* strains did. Thus, PlcB seems to be required for efficacious and fulminant infection in cerebral listeriosis.

In immunized mice the delayed spread of  $\Delta$ *plcB2* enabled the immune system to prevent a significant spread of the bacterium to the brain stem, resulting in an outcome that was significantly more favorable, with an increased survival rate, compared to that of mice infected with WT and  $\Delta$ *inlAB2*. The improved prognosis of immunized *L. monocytogenes*  $\Delta$ *plcB2*-infected mice can be explained by two complementary factors. For infection of host cells to be ongoing, the  $\Delta$ *plcB2* mutant strain must continuously leave the host cells and enter the extracellular space. This enables macrophages, stimulated by

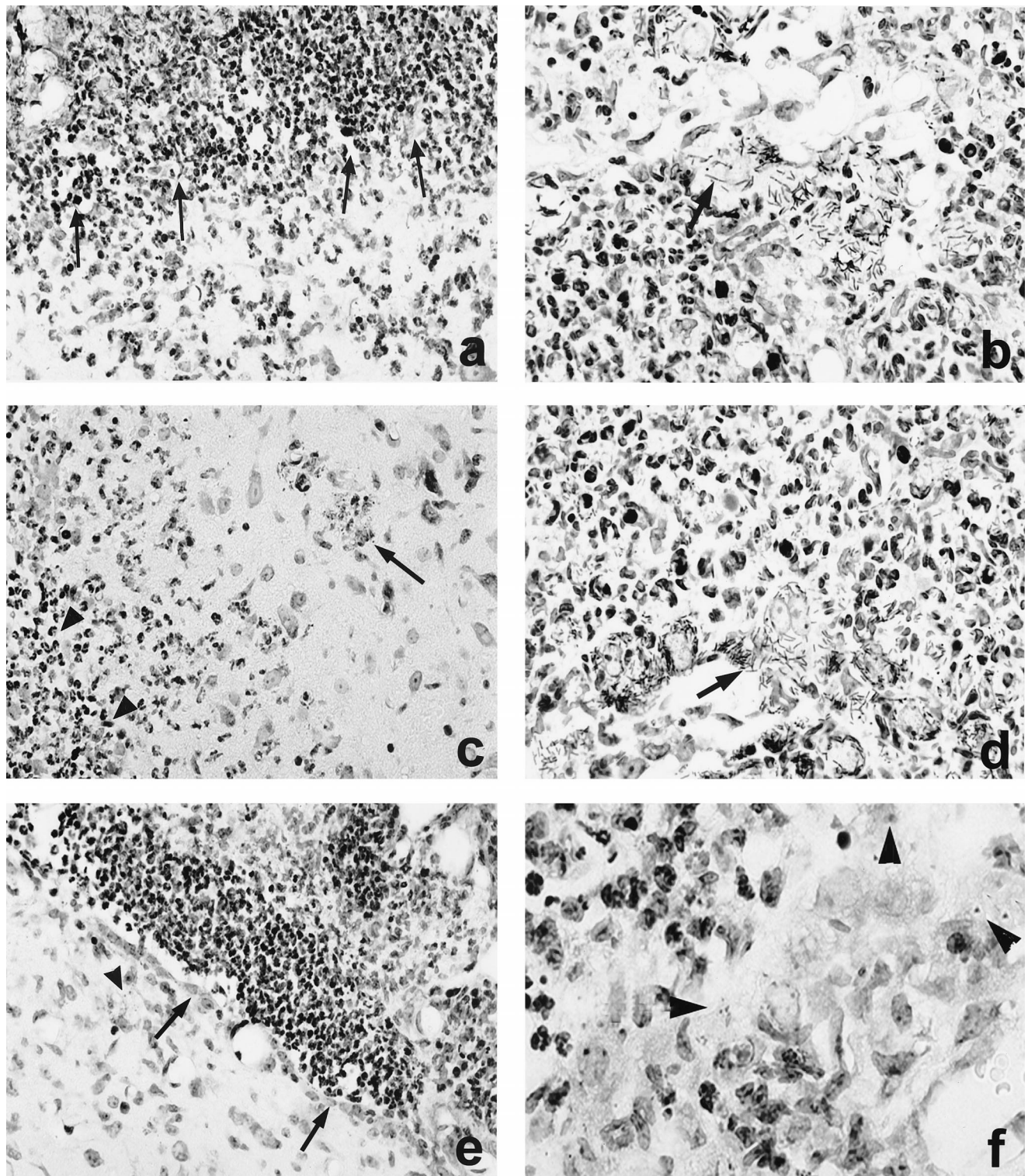


FIG. 3. CNS pathology of nonimmunized mice i.c. infected with *L. monocytogenes* WT (a and b),  $\Delta$ *inlAB2* (c and d), and  $\Delta$ *plcB2* (e and f) at day 3 p.i. (a) Severe ependyma of the ventricle (area above the arrows). The ependymal wall is largely destroyed and barely discernible, and the periventricular brain stem is infiltrated by numerous neutrophils and macrophages. (b) *L. monocytogenes* WT cluster in the largely destroyed plexus. The arrow points to a group of remarkably elongated WT bacteria. (c)  $\Delta$ *inlAB2* has also largely destroyed the ependyma of the ventricular wall (area left of the arrowheads) and has invaded the adjacent brain parenchyma. Brain stem neurons are surrounded by inflammatory leukocytes (arrow). (d)  $\Delta$ *inlAB2* cluster in the partially necrotic choroid plexus. Note that the elongated shape of the  $\Delta$ *inlAB2* strain (arrow) is identical to that of the WT in panel b. (e)  $\Delta$ *plcB2* has also infected the fourth ventricle, and the bacteria are accompanied by intraventricular neutrophils and macrophages. In contrast to WT (a) and  $\Delta$ *inlAB2* (c), the ependymal lining is largely intact (arrows). Very few bacteria are detectable in the periventricular tissue (arrowhead). (f) Some  $\Delta$ *plcB2* are detectable either as single or small groups of coccoid bacteria (arrowheads) in the choroid plexus. The choroid plexus is infiltrated by neutrophils and macrophages, but in contrast to the images shown in panels b and d, its structure is largely preserved. Specimens in panels a to f were stained with cresyl violet. Magnification is as follows: for panels a and e,  $\times 260$ ; b and d,  $\times 1,250$ ; c,  $\times 520$ ; f,  $\times 1,470$ .



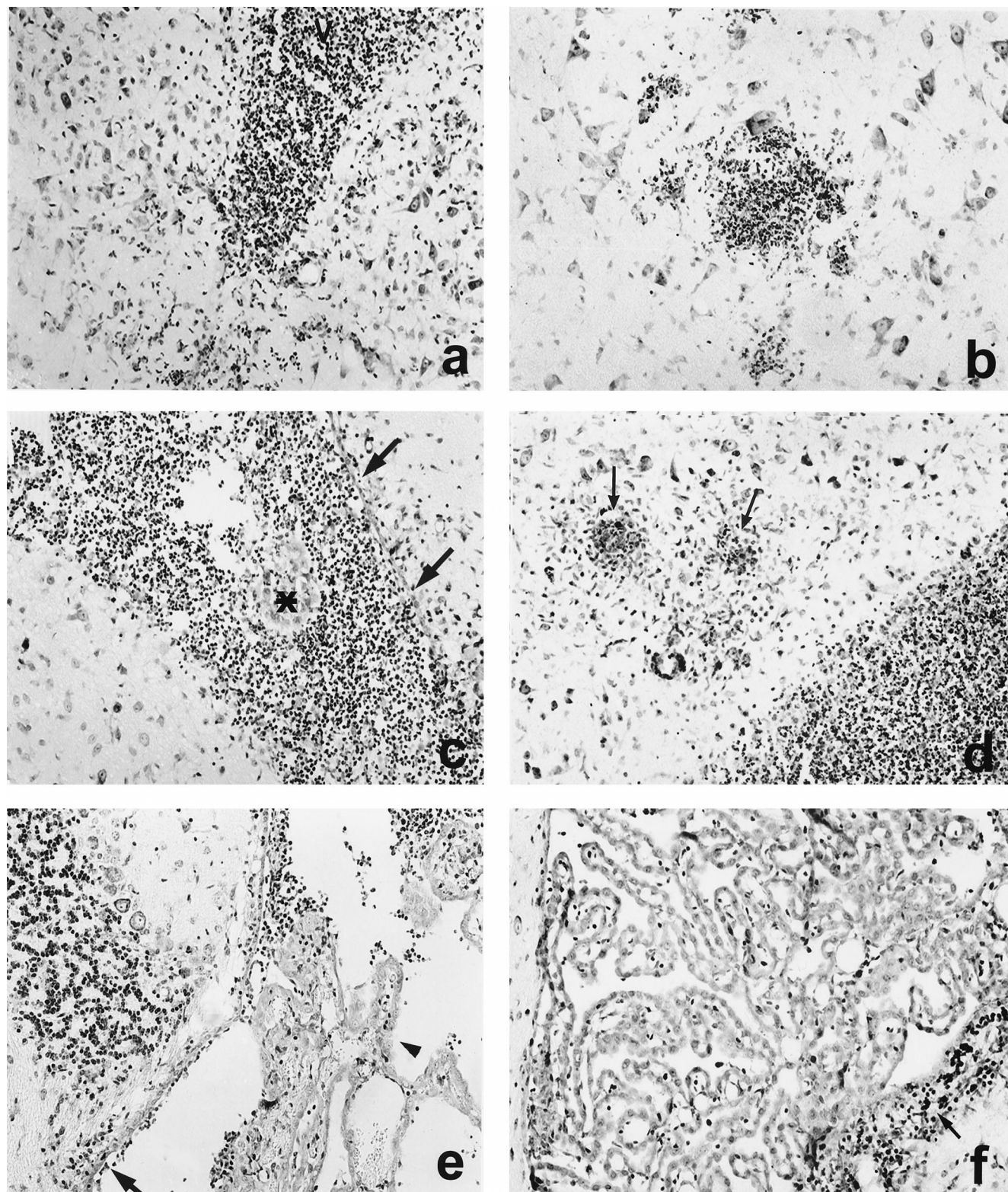


FIG. 4. CNS pathology of immunized mice i.c. infected with *L. monocytogenes* WT (a and b),  $\Delta inlAB2$  (c and d), and  $\Delta plcB2$  (e and f) at days 3 (a, c, and e) and 5 (b, d, and f) p.i. (a) Inflammation of the lateral ventricle (V) in a mouse infected with *L. monocytogenes* WT. Additional infiltrates are present in the adjacent brain parenchyma. (b) Small, circumscribed infiltrates are present in the brain stem in the vicinity of neurons. (c) In  $\Delta inlAB2$ -infected mice, inflammation is also largely confined to the lateral ventricle, and small parts of the choroid plexus are preserved (asterisk). A significant part of the ependyma is still intact (arrows). (d) From days 3 to 5 p.i., disease progressed in  $\Delta inlAB2$ -infected mice. Purulent ventriculitis and focal brain stem encephalitis (arrows) developed. (e) Only discrete infiltrates were detectable in the largely normal fourth ventricle in a  $\Delta plcB2$ -infected mouse at day 3 p.i. The choroid plexus was largely preserved (arrowhead), and ventricular empyema was absent. The ependyma was only focally destroyed, and small, single infiltrates were present in the periventricular tissue (arrow). (f) At day 5 p.i., the inflammation was largely resolved, and only small residual infiltrates were present (arrow) in the wall of the fourth ventricle. Specimens in panels a to f were stained with cresyl violet. Magnification,  $\times 260$ .



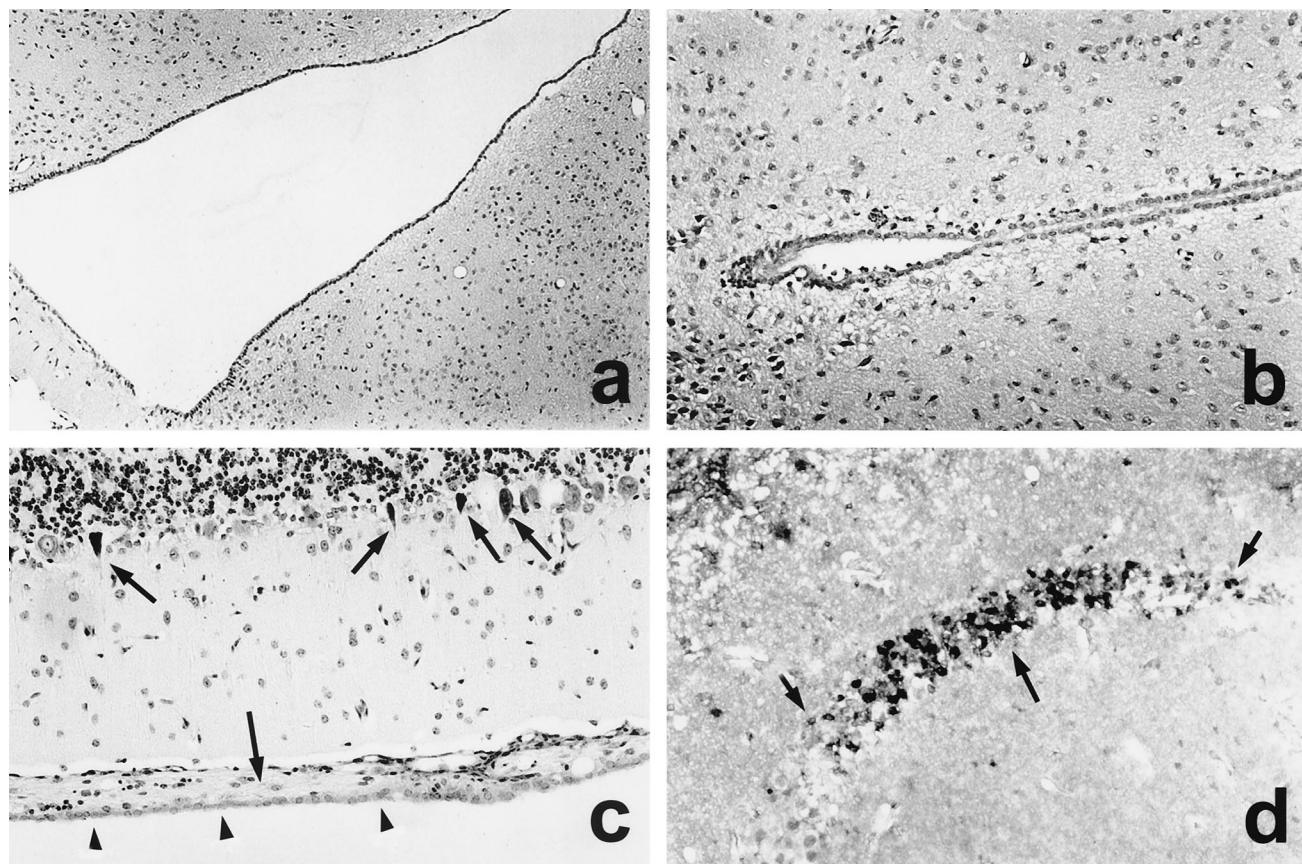


FIG. 5. CNS complications in *L. monocytogenes* meningoencephalitis at day 10 p.i. (a and b) A severe obstructive hydrocephalus developed at day 10 p.i. Note the massive enlargement of the third ventricle (a) compared to the normal size of the third ventricle of an immunized mouse i.c. infected with  $\Delta plcB2$  at day 1 p.i. (b). (c) Postinflammatory scarring of the ventricular wall of the fourth ventricle. Subependymal formation of a membranous gliotic tissue (large arrow). In this area, the ependyma is largely intact (arrowheads). In addition, some Purkinje cells in the adjacent cerebellum have small, pyknotic nuclei with condensed chromatin (small arrows), which is characteristic of apoptotic cells. (d) Apoptotic, TUNEL-positive neurons with small, pyknotic nuclei in the CA1 segment of the hippocampus (large arrow). Note the relatively sharp demarcation from the adjacent hippocampal segments (small arrows). Specimens in panels a to c were stained with cresyl violet, and panel d shows TUNEL staining. Magnification for panels a to d,  $\times 260$ . Images shown in panels a, c, and d are from immunized mice i.c. infected with  $\Delta plcB2$  at day 10 p.i. Similar observations were made about the brains of surviving mice infected with *L. monocytogenes* WT and  $\Delta inlAB2$  at day 10 p.i.

immunization-induced *L. monocytogenes*-specific T cells, to phagocytize and degrade extracellular  $\Delta plcB2$  mutant listeriae. In fact, previous studies have demonstrated that systemic immunization results in the increased activation of i.c. macrophages in cerebral listeriosis (37). In addition, the lack of cell-to-cell spread results in the reduced growth of *L. monocytogenes*  $\Delta plcB2$  in the brain, independent of a *Listeria*-specific T-cell response (Fig. 2, nonimmunized mice). Therefore, immunization-induced *Listeria*-specific T cells have to combat a significantly reduced number of i.c. bacteria in mice infected with the  $\Delta plcB2$  strain compared to the number in mice infected with the WT or the  $\Delta inlAB2$  strain, significantly facilitating control and elimination of the pathogen. These findings extend those of previous in vitro studies, which have indicated that the lecithinase expressed by mature PlcB is essential for cell-to-cell spread in various tissue culture cell lines (35, 42, 48).

In contrast, the cell wall-associated virulence factors InlA/B were unimportant for the efficient spread and multiplication of *L. monocytogenes* in the brain. Thus, while InlA is necessary in mediating the invasion of intestinal Caco-2 cells, and InlB is required for the invasion of hepatocytes and human umbilical cord endothelial cells (6, 13, 30, 32), these virulence factors are dispensable for the efficient spread and multiplication of

*L. monocytogenes* in the brain. In addition, the experiments of Gregory et al. (17) have revealed that InlA and InlB are not required for the entry of *L. monocytogenes* into hepatic cells in vivo.

In the present study, *L. monocytogenes* was directly applied to the brain, a technique which is widely used to study the pathogenesis of cerebral listeriosis (12, 26, 36, 37) as well as that of other bacterial (reviewed in reference 46) and viral (26, 45) cerebral infections. This approach guaranteed that i.c. listeriosis started at the same time point in all groups of mice, with exactly the same amounts of the various *Listeria* mutants, a prerequisite for evaluating the roles of the different virulence factors for the i.c. pathogenesis of listeriosis in the CNS. However, it must be remembered that cerebral listeriosis is, in general, a food-borne disease acquired by the consumption of contaminated food. After the intestinal barrier has been passed, bacteremia enables *L. monocytogenes* to cross the BBB and to infect the CNS, generating characteristic cerebral symptoms (1). The specific listerial determinants that are required for the crossing of the intestinal barrier and the infection of the CNS are still unknown. Two general mechanisms for traversing the BBB can be envisaged as follows: first, transcytosis through the endothelial cells lining the barrier, and second, paracellular infection, either directly or via infected phagocytic cells be-

tween vascular endothelial cells. We have recently shown that *L. monocytogenes* can invade endothelial cells directly and that this invasion is mediated by the *inlB* gene product (32). Other studies showed that the interaction of *Neisseria meningitidis* with components of the BBB correlates with the increased expression of PilC (34) and that the outer membrane protein A of *Escherichia coli* contributes to the invasion of brain microvascular endothelial cells (33). Both bacterial proteins may be important factors for the crossing of the BBB, allowing pathogens access to the normally sterile CNS. Another mechanism by which bacteria might gain access to the CNS is the modulation of the BBB permeability during systemic bacterial infections such as those caused by nitric oxide, tumor necrosis factor alpha (21, 23, 38, 40), and bacterial products (44, 47). We speculate that paracellular forms of listerial transfer may also occur via changes in tight junction physiology, probably mediated by the action of listeriolysin.

In the present study, a common feature of cerebral listeriosis was the capacity to induce the apoptosis of neurons in the hippocampus. Interestingly, the apoptosis of hippocampal neurons was confined to the CA1 segment, which is particularly vulnerable to a variety of lesions. Purkinje cells in the cerebellum, which are also highly vulnerable to stress, underwent apoptosis as well. Since the same apoptotic changes were observed in the mice infected with the  $\Delta$ *inlAB2* and  $\Delta$ *plcB2* mutant strains, these virulence factors of *L. monocytogenes* are not critical for the induction of apoptosis in neurons. The fact that the apoptosis of hippocampal neurons was also observed in other experimental models of bacterial meningitis, including pneumococcal and streptococcal meningitis (2, 49), indicates that a common pathogenic mechanism underlies the induction of neuronal apoptosis in bacterium-induced meningoencephalitis. Candidate molecules, which have been shown to induce neuronal apoptosis in experimental group B streptococcal meningitis as well as in neuronal cell cultures stimulated with *Streptococcus pneumoniae* or group B streptococcal cell walls, are tumor necrosis factor alpha and nitric oxide (2, 22), which are also produced in cerebral listeriosis (26, 36). The assumption that immune reactions may be critically involved in the induction of neuronal apoptosis is further evidenced by our observation that apoptotic cells were often observed in the vicinity of inflammatory infiltrates. In addition, it has been demonstrated recently that a main virulence factor from *L. monocytogenes*, listeriolysin, is able to induce the apoptosis of mouse dendritic cells (19). Since the  $\Delta$ *inlAB2* and  $\Delta$ *plcB2* mutant strains we used in this study produce listeriolysin (data not shown), we can assume that this toxin may also contribute to the apoptotic process of neurons in the hippocampus and cerebellum.

Furthermore, a common feature of all surviving mice, irrespective of the type of *L. monocytogenes* that was applied, was the development of a hydrocephalus. This hydrocephalus was caused by the destruction of the ependymal lining and the formation of sheets of glial-ependymal scar tissue in the ventricles, interfering with cerebrospinal fluid outflow. Since the development of a hydrocephalus was independent of the listerial virulence factors InlA/B and PlcB, the infection and subsequent destruction of specific anatomic structures such as the ependyma seems to be the main pathogenic factor. In this respect, murine CNS listeriosis closely resembles human bacterial meningitis, in which postinflammatory hydrocephalus is a common complication.

In conclusion, the present study identifies PlcB as a virulence factor in CNS listeriosis, which is important in determining the course and final outcome of the disease. Since the  $\Delta$ *plcB2* mutant strain was not completely attenuated, additional, as-

yet-undefined virulence factors may play a significant role in the i.c. pathogenesis of this life-threatening disease.

#### ACKNOWLEDGMENTS

The expert technical assistance of Nadja Kaefer and Andrea Rang is gratefully acknowledged.

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft through SFB 535 (TP/A5) to E.D.

#### REFERENCES

- Berche, P. 1995. Bacteremia is required for invasion of the murine central nervous system by *Listeria monocytogenes*. *Microb. Pathog.* **18**:323-336.
- Bogdan, I., S. L. Leib, M. Bergeron, L. Chow, and M. G. Täubner. 1997. Tumor necrosis factor-alpha contributes to apoptosis in hippocampal neurons during experimental group B streptococcal meningitis. *J. Infect. Dis.* **176**:693-697.
- Braun, L., S. Dramsi, P. Dehoux, H. Bierne, G. Lindahl, and P. Cossart. 1997. InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. *Mol. Microbiol.* **25**:285-294.
- Chakraborty, T., and J. Wehland. 1997. The host cell infected with *Listeria monocytogenes*, p. 271-290. In S. H. E. Kaufmann (ed.), *Host response to intracellular pathogens*. R. G. Landes Company, Austin, Tex.
- Domann, E., S. Zechel, A. Lingnau, T. Hain, A. Darji, T. Nichterlein, J. Wehland, and T. Chakraborty. 1997. Identification and characterization of a novel PrfA-regulated gene in *Listeria monocytogenes* whose product, IrpA, is highly homologous to internalin proteins, which contain leucine-rich repeats. *Infect. Immun.* **65**:101-109.
- Dramsi, S., I. Biswas, E. Maguin, L. Braun, P. Mastroeni, and P. Cossart. 1995. Entry of *Listeria monocytogenes* into hepatocytes requires expression of inlB, a surface protein of the internalin multigene family. *Mol. Microbiol.* **16**:251-261.
- Dramsi, S., M. Lebrun, and P. Cossart. 1996. Molecular and genetic determinants interfere in invasion of mammalian cells by *Listeria monocytogenes*. *Curr. Top. Microbiol. Immunol.* **209**:61-77.
- Dramsi, S., P. Dehoux, M. Lebrun, P. L. Goossens, and P. Cossart. 1997. Identification of four new members of the internalin multigene family of *Listeria monocytogenes* EGD. *Infect. Immun.* **65**:1615-1625.
- Drevets, D. A., R. T. Sawyer, T. A. Potter, and P. Campbell. 1995. *Listeria monocytogenes* infects human endothelial cells by two distinct mechanisms. *Infect. Immun.* **63**:4268-4276.
- Engelbrecht, F., S.-K. Chun, C. Ochs, J. Hess, F. Lottspeich, W. Goebel, and Z. Sokolovic. 1996. A new PrfA-regulated gene of *Listeria monocytogenes* encoding a small, secreted protein which belongs to the family of internalins. *Mol. Microbiol.* **21**:823-837.
- Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* **55**:476-511.
- Frei, K., D. Nadal, H. W. Pfister, and A. Fontana. 1993. *Listeria* meningitis: identification of a cerebrospinal fluid inhibitor of macrophage listericidal function as interleukin-10. *J. Exp. Med.* **178**:1255-1261.
- Gaillard, J.-L., P. Berche, C. Frehel, E. Gouin, and P. Cossart. 1991. Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* **65**:1127-1141.
- Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**:493-501.
- Geoffroy, C., J. Raveneau, J.-L. Beretti, A. Lecroisey, J.-A. Vazquez-Boland, J. E. Alouf, and P. Berche. 1991. Purification and characterization of an extracellular 29-kilodalton phospholipase C from *Listeria monocytogenes*. *Infect. Immun.* **59**:2382-2388.
- Goldfine, H., N. C. Johnston, and C. Knob. 1993. Nonspecific phospholipase C of *Listeria monocytogenes* activity on phospholipids in Triton X-100-mixed micelles and in biological membranes. *J. Bacteriol.* **175**:4298-4306.
- Gregory, S. H., A. J. Sagnimeni, and E. J. Wing. 1996. Expression of the *inlAB* operon by *Listeria monocytogenes* is not required for entry into hepatic cells in vivo. *Infect. Immun.* **64**:3983-3986.
- Guzman, C. A., E. Domann, M. Rohde, D. Bruder, A. Darji, S. Weiss, J. Wehland, T. Chakraborty, and K. N. Timmis. 1996. Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of *Listeria monocytogenes*. *Mol. Microbiol.* **20**:119-126.
- Guzman, C. A., M. Rohde, T. Chakraborty, E. Domann, M. Hudel, J. Wehland, and K. Timmis. 1995. Interaction of *Listeria monocytogenes* with mouse dendritic cells. *Infect. Immun.* **63**:3665-3673.
- Kaufmann, S. H. E. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol.* **11**:129-163.
- Kim, K. S., C. A. Wass, and A. S. Cross. 1997. Blood-brain barrier permeability during the development of experimental bacterial meningitis in the rat. *Exp. Neurol.* **145**:253-257.
- Kim, K. S., C. A. Wass, A. S. Cross, and S. M. Opal. 1992. Modulation of blood-brain barrier permeability by tumor necrosis factor and antibody to



- tumor necrosis factor in the rat. Lymphokine Cytokine Res. **11**:293–298.
23. Kim, Y. S., and M. G. Täuber. 1996. Neurotoxicity of glia activated by gram-positive bacterial products depends on nitric oxide production. Infect. Immun. **64**:3148–3153.
  24. Kobe, B., and J. Deisenhofer. 1994. The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. **19**:415–421.
  25. Lebrun, M., J. Mengaud, H. Ohayon, F. Nato, and P. Cossart. 1996. Internalin must be on the bacterial surface to mediate entry of *Listeria monocytogenes* into epithelial cells. Mol. Microbiol. **21**:579–592.
  26. Leist, T. P., K. Frei, S. Kam-Hansen, R. M. Zinkernagel, and A. Fontana. 1988. Tumor necrosis factor  $\alpha$  in cerebrospinal fluid during bacterial, but not viral, meningitis. Evaluation in murine model infections and in patients. J. Exp. Med. **167**:1743–1748.
  27. Lingnau, A., E. Domann, M. Hudel, M. Bock, T. Nichterlein, J. Wehland, and T. Chakraborty. 1995. Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. Infect. Immun. **63**:3896–3903.
  28. Mackaness, G. B. 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–406.
  29. Makhov, A. M., J. H. Hannah, M. J. Brennan, B. L. Trus, E. Kocsis, J. F. Conway, P. T. Wingfield, M. N. Simon, and A. C. Steven. 1994. Filamentous hemagglutinin of *Bordetella pertussis*. A bacterial adhesin formed as a 50-nm monomeric rigid rod based on a 19-residue repeat motif rich in beta strands and turns. J. Mol. Biol. **241**:110–124.
  30. Mengaud, J., H. Ohayon, P. Gounon, R. M. Mege, and P. Cossart. 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *Listeria monocytogenes* into epithelial cells. Cell **84**:923–932.
  31. Niebuhr, K., T. Chakraborty, P. Köllner, and J. Wehland. 1993. Production of monoclonal antibodies to the phosphatidylcholine-specific phospholipase C of *Listeria monocytogenes*, a virulence factor for this pathogen. Med. Microbiol. Lett. **2**:9–16.
  32. Parida, S. K., E. Domann, M. Rohde, S. Müller, A. Darji, T. Hain, J. Wehland, and T. Chakraborty. 1998. Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells. Mol. Microbiol. **28**:81–93.
  33. Prasadarao, N. V., C. A. Wass, J. N. Weiser, M. F. Stins, S. H. Huang, and K. S. Kim. 1996. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect. Immun. **64**:146–153.
  34. Pron, B., M. K. Taha, C. Rambaud, J. C. Fournet, N. Pattey, J. P. Monnet, M. Musilek, J. L. Beretti, and X. Nassif. 1997. Interaction of *Neisseria meningitidis* with the components of the blood-brain barrier correlates with an increased expression of pilC. J. Infect. Dis. **176**:1285–1292.
  35. Raveneau, J., C. Geoffroy, J.-L. Beretti, J.-L. Gaillard, J. E. Alouf, and P. Berche. 1992. Reduced virulence of a *Listeria monocytogenes* phospholipase-deficient mutant obtained by transposon insertion into the zinc metalloprotease gene. Infect. Immun. **60**:916–921.
  36. Schlüter, D., S. Chahoud, H. Lassmann, H. Hof, and M. Deckert-Schlüter. 1996. Intracerebral targets and immunomodulation of murine *Listeria monocytogenes* meningoencephalitis. J. Neuropathol. Exp. Neurol. **55**:14–24.
  37. Schlüter, D., S. B. Oprisiu, S. Chahoud, D. Weiner, O. D. Wiestler, H. Hof, and M. Deckert-Schlüter. 1995. Systemic immunization induces protective CD4+ and CD8+ T cell-mediated immune responses in murine *Listeria monocytogenes* meningoencephalitis. Eur. J. Immunol. **25**:2384–2391.
  38. Sharief, M. K., M. Ciardi, and E. J. Thompson. 1992. Blood-brain barrier damage in patients with bacterial meningitis: association with tumor necrosis factor- $\alpha$  but not interleukin-1  $\beta$ . J. Infect. Dis. **166**:350–358.
  39. Shevchenko, D. V., D. R. Akins, E. Robinson, M. Li, T. G. Popova, D. L. Cox, and J. D. Radolf. 1997. Molecular characterization and cellular localization of TpLRR, a processed leucine-rich repeat protein of *Treponema pallidum*, the syphilis spirochete. J. Bacteriol. **179**:3188–3195.
  40. Shukla, A., M. Dikshit, and R. C. Srimal. 1995. Nitric oxide modulates blood-brain barrier permeability during infections with an inactivated bacterium. Neuroreport **6**:1629–1632.
  41. Smith, G. A., H. Marquis, S. Jones, N. C. Johnston, D. A. Portnoy, and H. Goldfine. 1995. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect. Immun. **63**:4231–4237.
  42. Songer, J. G. 1997. Bacterial phospholipases and their role in virulence. Trends Microbiol. **5**:156–161.
  43. Southwick, F. S., and D. L. Purich. 1996. Intracellular pathogenesis of listeriosis. N. Engl. J. Med. **334**:770–776.
  44. Spellerber, B., S. Prasad, C. Cabellos, M. Burroughs, P. Cahill, and E. Tuomanen. 1995. Penetration of the blood-brain barrier: enhancement of drug delivery and imaging by bacterial glycopeptides. J. Exp. Med. **182**:1037–1043.
  45. Stohman, S. A., C. C. Bergmann, M. T. Lin, D. J. Cua, and D. R. Hinton. 1998. CTL effector function within the central nervous system requires CD4+ T cells. J. Immunol. **160**:2896–2904.
  46. Tunkel, A. R., and W. M. Scheld. 1993. Pathogenesis and pathophysiology of bacterial meningitis. Clin. Microbiol. Rev. **6**:118–136.
  47. Tuomanen, E. 1996. Entry of pathogens into the central nervous system. FEMS Microbiol. Rev. **18**:289–299.
  48. Vazquez-Boland, J.-A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart. 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60**:219–230.
  49. Zysk, G., W. Brück, J. Gerber, Y. Bruck, H. W. Prange, and R. Nau. 1996. Anti-inflammatory treatment influences neuronal apoptotic cell death in the dentate gyrus in experimental pneumococcal meningitis. J. Neuropathol. Exp. Neurol. **55**:722–728.

Editor: E. I. Tuomanen