The Capsule Supports Survival but Not Traversal of *Escherichia coli* K1 across the Blood-Brain Barrier

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The vast majority of cases of gram-negative meningitis in neonates are caused by K1-encapsulated *Escherichia coli*. The role of the K1 capsule in the pathogenesis of *E. coli* meningitis was examined with an in vivo model of experimental hematogenous *E. coli* K1 meningitis and an in vitro model of the blood-brain barrier. Bacteremia was induced in neonatal rats with the *E. coli* K1 strain C5 (O18:K1) or its K1− derivative, C5ME. Subsequently, blood and cerebrospinal fluid (CSF) were obtained for culture. Viable bacteria were recovered from the CSF of animals infected with *E. coli* K1 strains only; none of the animals infected with K1− strains had positive CSF cultures. However, despite the fact that their cultures were sterile, the presence of O18 *E. coli* was demonstrated immunochemistry in the brains of animals infected with K1− strains and was seen by staining of CSF samples. In vitro, brain microvascular endothelial cells (BMEC) were incubated with K1+ and K1− *E. coli* strains. The recovery of viable intracellular organisms of the K1+ strain was significantly higher than that for the K1− strain (P = 0.0005). The recovery of viable intracellular K1− *E. coli* bacteria was increased by cycloheximide treatment of BMEC (P = 0.0059) but was not affected by nitric oxide synthase inhibitors or oxygen radical scavengers. We conclude that the K1 capsule is not necessary for the invasion of bacteria into brain endothelial cells but is responsible for helping to maintain bacterial viability during invasion of the blood-brain barrier.

Meningitis remains a potentially devastating disease. In the neonatal period *Escherichia coli* is the most common gram-negative pathogen responsible for meningitis (9, 31). It is associated with a mortality rate as high as 40%, and more than half of the survivors have neurologic sequelae (9, 31). The poor outcome statistics, despite medical advances, including bactericidal antibiotics and improved intensive-care unit care, point to our incomplete knowledge of the pathogenesis and pathophysiology of neonatal *E. coli* meningitis. It is well documented that the majority of cases of neonatal *E. coli* meningitis are caused by K1-encapsulated bacteria (26). The reasons for this association are myriad and may include the neonatal immune system’s incomplete ability to localize and fight infection and the propensity of certain strains of *E. coli* to invade the central nervous system. In addition, studies of infants with meningitis and animal models of meningitis have shown that a high level of brain endothelial cell infection is required for the development of *E. coli* meningitis (7, 15). Previous investigations have determined that the K1 capsule contributes to this high level of bacteria by virtue of its serum resistance and antiapoptotic properties (15).

In an effort to better understand how systemically circulating *E. coli* bacteria cross the blood-brain barrier, we have used an *in vivo* model of neonatal rat meningitis (13, 15). This model shares several characteristics with human neonatal meningitis, most notably hematogenous infection of the meninges. In addition we have used an *in vitro* model of the blood-brain barrier, with bovine brain microvascular endothelial cell (BMEC) monolayers (11, 25, 29), to examine the process of invasion by K1+ and K1− *E. coli*. While the use of two different species of brain tissues may be questioned, in detailed experiments performed in our laboratory, the interactions between *E. coli* and brain endothelial cells were found to be similar, regardless of the cell’s species of derivation (25, 29). Given that bovine brain cells are more readily available, we opted to use these cells for our *in vitro* experiments. The present research uses these experimental models to examine, in part, the process by which bacteria gain access to the central nervous system and remain viable. We hypothesize that the K1 capsule is not necessary for the invasion of brain endothelial cells. It is, however, an important virulence factor, protecting *E. coli* from host defenses, and thus the bacterium is able to cross the blood-brain barrier alive, ultimately leading to meningitis.

**MATERIALS AND METHODS**

**Bacterial strains.** The clinical isolate of K1-encapsulated *E. coli*, strain C5 (O18:K1), and its unencapsulated mutant CSME have been characterized previously (15). Briefly, strain C5 was isolated from the cerebrospinal fluid (CSF) of a newborn infant with *E. coli* meningitis. Strain CSME was obtained by selection for resistance to the K1-specific bacteriophages. Strain CSME was examined for the loss of capsule production by the antisera agglutination technique, testing for agglutination with an anti-K1 monoclonal antibody as well as by sensitivity to K1-specific bacteriophages, as described previously (15). Extensive investigations have been undertaken to examine known virulence factors in the K1 mutant in order to ensure that these phenotypic characteristics remained intact. There were no phenotypic alterations in virulence factors such as outer membrane protein, S fimbriae, O18 lipopolysaccharide (LPS), and the invasion protein Ibe10 (11, 15, 24). The parent K1+ strain and the K1 mutant strain possess identical genotypes when they were examined by multilocus enzyme electrophoresis (15).

**Animal model for *E. coli* bacteremia and meningitis.** *E. coli* bacteremia and meningitis, defined as a positive CSF culture, were induced in 5-day-old rats by a method described previously (13, 15). Briefly, outbred, specific-pathogen-free, pregnant Sprague-Dawley rats with timed conception were purchased from Charles River Breeding Laboratories (Wilmington, Mass.); the rats delivered in our vivarium 5 to 7 days after arrival. Each adult rat and her pups (average litter size, 10; range, 8 to 16) were housed in an opaque solid-polypropylene cage under a Small Animal Isolator (model 1984; Forma Scientific, Inc., Marietta, Ohio).

At the age of 5 days, all members of each litter were randomly divided into two...
groups to receive E. coli C5 (O18:K1; wild type) or CSME (K1 mutant) subcutaneously. Pilot experiments were performed with each bacterial strain to determine the inoculum size that would induce a level of bacteremia ($10^5$ to $10^8$ CFU/ml of blood) found to be necessary for hematogenous bacteria to enter the central nervous system (15). Inoculum sizes were 1 x $10^3$ to 2.5 x $10^4$ CFU for C5 and 5.6 x $10^3$ to 1.4 x $10^4$ CFU for CSME. Eighteen hours after bacterial inoculation, blood and CSF (approximately 15- to 20-μl) specimens were obtained as described previously (13, 15). A 10-μl portion of each CSF specimen was used for quantitative cultures, and the remaining CSF (5 to 10 μl) from each animal was pooled (total volume, approximately 30 to 50 μl) for the detection of bacteria as described below. Immediately after blood and CSF specimens were obtained, the brains of selected animals were removed for examination of the presence of bacteria by immunocytochemistry (see below). Because the animals soon die after they reach the high degree of bacteremia necessary for the development of meningitis, this model does not permit us to investigate the effect of the duration of high-degree bacteremia on the development of meningitis.

Detection of bacteria in CSF. Two methods were used to demonstrate morphologically the presence of bacteria in CSF. First, selected CSF specimens were pooled and concentrated by cyto spun centrifugation, stained with acridine orange, and examined under a fluorescence microscope (Olympus BH2, equipped with a wide-band BP-Y 95 filter set). Second, single-CSF specimens were pooled and centrifuged, and sediments were fixed with 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 1 h. The sediments were rinsed twice, for 5 min each time, with 0.1 M PBS, and then 1% gelatin (J.T. Parker Chemical Co., Philiburg, N.J.) was added to the vial to form a pellet. The pellet was fixed with 1% OsO4 in 0.1 M PBS for 1 h, dehydrated in graded alcohols, and embedded in Epon 812. One-micron-thick sections were cut and stained with 1% Azur II, 1% methylene blue, and 0.5% basic fuchsin for light microscopy examination.

Immunocytochemical detection of bacteria in brains. Brains from infected animals were embedded in OCT compound (Tissue Tek; Sakura Finetek) and cut by using a B1-H1 cryostat. Sections were fixed in acetone, preincubated with 1% acetic acid to block endogenous alkaline phosphatase activity, and then blocked with 5% heat-inactivated serum to avoid nonspecific binding of immunoglobulin (Ig) to neuronal tissues. The sections were then incubated with the primary antibody to O18 LPS (murine IgG monoclonal anti-O18 antibody) (14), followed by incubation with the secondary antibody (biotinylated sheep anti-mouse IgG). The sections were further incubated with alkaline phosphatase-conjugated streptavidin. Visualization of the antigen-antibody complex (red color) was done with the Alkaline Phosphatase Substrate Kit 1 (Vector Laboratories), and sections were counterstained with hematoxylin. Controls used for tissue specimens as well as for antibodies included uninfected brains and omission of the primary and/or secondary antibody.

In vitro invasion assays. An in vitro model of the blood-brain barrier was developed with bovine BMECs. These cells were isolated and cultured as described previously (29) and were used in invasion experiments. Ten million bacteria were added to the vial to form a pellet. The pellet was fixed with 1% OsO4 in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 1 h. The sediments were rinsed twice, for 5 min each time, with 0.1 M PBS, and then 1% gelatin (J.T. Parker Chemical Co., Philiburg, N.J.) was added to the vial to form a pellet. The pellet was fixed with 1% OsO4 in 0.1 M PBS for 1 h, dehydrated in graded alcohols, and embedded in Epon 812. One-micron-thick sections were cut and stained with 1% Azur II, 1% methylene blue, and 0.5% basic fuchsin for light microscopy examination.

Effects of eukaryotic inhibitors on E. coli invasion. Cycloheximide (20 μg/ml), N-nitro-l-arginine (NNLA) (1 mM), N-nitro-l-arginine (NNLA) (1 mM), superoxide dismutase (SOD) (100 μg/ml), or catalase (5,000 U/ml) (all from Sigma) was added to the wells in 400 μl of experimental medium 1 h before the bacteria. None of these inhibitors affected the viability or morphology of the BMEC monolayers. Bacteria were then added in 100-μl volumes to the wells, thereby decreasing the concentrations of inhibitors by 20%. Following this, the procedure was the same as that for the invasion assay described above. Experiments with cycloheximide were run in triplicate of 3, 6, or 9 and repeated five times, while those done with other inhibitors were performed in replicates of 3 or 6 and repeated three times.

RESULTS

Demonstration of live bacteria in the CSF from the animal model. To examine whether the entry of E. coli K1 into the central nervous system requires the capsule, E. coli bacteremia and meningitis were induced in 5-day-old rats with a K1-encapsulated and a K1- E. coli strain. The isolation of E. coli from CSF was, as expected, observed in animals infected with the K1+ strain, who developed a high degree of bacteremia (e.g., $>10^5$ CFU/ml of blood). Overall, 14 of the 24 (58%) animals who were infected with strain C5 and had levels of bacteremia greater than $10^5$ CFU/ml of blood were found to have positive CSF cultures. In contrast, none of the 20 animals infected with the K1+ strain (CSME) were found to have positive CSF cultures, despite the fact that all animals developed similarly high levels of bacteremia. However, we were not able to exclude the possibility that these CSF specimens might contain viable bacterial counts below the lower limit of detection, e.g., $<10$ CFU/total CSF, assuming that the total CSF of a 5-day-old rat is approximately 50 to 100 μl.

Detection of bacteria in CSF. Due to the observation that animals infected with K1+ bacteria did not develop meningitis, despite reaching high levels of bacteremia, we sought to determine if the K1 capsule was necessary for blood-brain barrier traversal or if it might function to facilitate bacterial survival instead. If the latter hypothesis is true, one would expect to find evidence of nonviable K1 E. coli in the CSF or brains of animals infected with this strain. The presence of bacteria was demonstrated in the CSF of these animals by two methods as described above. Figure 1 demonstrates the presence of bacteria in the CSF of animals infected with K1+ E. coli whose CSF cultures were sterile. Similarly, methylene blue staining of semithin sections of pelleted and fixed sterile CSF specimens from animals infected with CSME (K1-) revealed the presence of bacteria with a bacillus morphology consistent with that of E. coli in acridine orange-stained cytospin specimens from animals infected with K1+ E. coli whose CSF cultures were sterile. The demonstration of live bacteria in the CSF of C5ME (K1-) revealed that sterile CSF cultures may not represent failure of the organism to invade the central nervous system. Instead, these organisms may be unable to survive the invasion process.

Immunocytochemical detection of bacteria in the brain. Corroborating evidence that K1+ organisms could be found in the brains of animals infected with the K1 mutant, despite their having sterile CSF cultures, comes from immunocytochemical studies of brain sections from K1+-infected animals. The presence of O18 E. coli was demonstrated in the brain sections of two animals infected with E. coli K1 and two animals infected with its K1- derivative. Figure 2 shows representative brain cortex slices from three different experimental conditions: infection with E. coli K1 C5 (panel A), omission of primary antibody (panel B), and infection with CSME (K1+) (panel C). The brain of an animal with a positive CSF culture infected with strain C5 revealed red precipitates, indicating E. coli stained by an O18 LPS antibody in the brain cortex (Fig. 2A).
Controls (uninfected brains or brain sections for which the primary anti-O18 antibody was omitted) did not show any red precipitates (Fig. 2B), supporting the specificity of O18 E. coli interaction with the anti-O18 antibody. Similarly, E. coli bacteria, stained by O18 LPS antibody, were demonstrated in the brain of an animal infected with strain C5ME (O18 K12) despite the fact that its CSF cultures were sterile (Fig. 2C). This correlates with our in vivo finding that viable bacteria were recovered only from CSF specimens of animals infected with E. coli K1.

We then endeavored to define the mechanism of E. coli killing by a putative substance produced de novo by BMECs. To inhibit eukaryotic protein synthesis, BMECs were preincubated with cycloheximide (20 \( \mu \)g/ml) for 1 h prior to the addition of the bacteria. When BMEC monolayers were preincubated with cycloheximide prior to the invasion experiment, there was a statistically significant 2.5-fold increase (\( P = 0.0059 \)) in the intracellular recovery of K12 E. coli (C5ME) organisms but not in that of K11 E. coli (C5) organisms (Fig. 3). These findings suggest that a newly synthesized endothelial-cell product might be responsible for the decreased recovery of the K12 strain. Nitric oxide (NO) and oxygen intermediates have been shown to have antimicrobial properties (2, 8, 20). NO and/or nitric oxide synthase (NOS) has been isolated from a variety of different types of endothelial cells (12, 23), including cerebrovascular endothelium (5, 21). Superoxide and hydrogen peroxide have also been detected in cultured endothelial cells (4, 27). To see if NO and/or oxygen radicals produced by BMECs might be involved in the killing of K1- organisms, one of the NOS inhibitors NNLA (1 mM), NMLA (1 mM), SOD (100 \( \mu \)g/ml), and catalase (5,000 U/ml) (all from Sigma) was added to wells 1 h before the bacteria. None of these inhibitors affected the viability or morphology of the BMEC monolayers. Bacteria were then added in 100-\( \mu \)l volumes to the wells. As shown in Fig. 4, the addition of either of two NOS inhibitors (NNLA and NMLA), SOD–catalase, or a combination of SOD–catalase with NNLA or NMLA did not affect the intracellular recovery of K1- E. coli; the level of bacterial killing was similar to that in the standard invasion assay. There-
fore, these agents could not duplicate the survival benefit seen with cycloheximide.

**DISCUSSION**

In the study of the pathogenesis of *E. coli* meningitis, we employed both in vitro and in vivo models of the blood-brain barrier, using BMEC monolayers and experimental hematogenous meningitis in neonatal rats. Our present findings revealed that both K1+ and K1− *E. coli* bacteria can invade the central nervous system and BMECs; however, significantly fewer K1+ bacteria remain alive after invasion. The CSF clinical isolate of wild-type O18:K1+ *E. coli*, strain CS, and its K1− derivative, CSME, have been shown to be identical in terms of several phenotypic properties examined (e.g., outer membrane protein, O18 LPS, S fimbriae, and invasion proteins) except for the presence of the capsule (11, 15, 24). Therefore, the differences between the recovery of live K1+ and K1− *E. coli* bacteria can likely be ascribed to the capsule. Because the genetic basis of the loss of the K1 capsule for the K1 phage-derived CSME, undetermined, work to construct a genetically defined K1 mutant strain is in progress. This strain will contribute to further understanding of the role of the K1 capsule in central nervous system invasion by *E. coli* K1. Despite this limitation, we believe that the present study supports our hypothesis that the capsule is a crucial component of the bacterium’s armamentarium, allowing it to cross the blood-brain barrier and remain viable.

Prior in vivo studies of *E. coli* K1 meningitis have shown that the K1 capsule is a critical factor in the development of meningitis by virtue of its serum resistance and antiphagocytic properties (15). This is again demonstrated in this study by the differences in inoculum size required for the K1+ and K1− strains to reach high levels of bacteremia in neonatal rats. An inoculum approximately 102–103-fold greater was required for the K1− strain to achieve a high degree of bacteremia (e.g., >105 CFU/ml of blood) compared to the parent K1+ strain. The sterile CSF cultures from animals infected with K1− strains were previously interpreted to mean that the K1 capsule was necessary for the bacterial crossing of the blood-brain barrier (15). Several lines of evidence presented in this paper now suggest that the capsule is not necessary for invading BMECs but is responsible for maintaining the viability of the bacteria inside the BMECs. This is supported by the in vivo observation that while both K1+ and K1− *E. coli* bacteria are found in brains by immunocytochemical assays and in CSF by acridine orange and methylene blue staining, CSF cultures did not reveal any viable K1− *E. coli* organisms. These bacteria were therefore able to enter the central nervous system but were presumably killed in the process. We cannot completely exclude the possibility that sterile CSF cultures from the animals infected with K1− *E. coli* might represent viable counts below the limit of detection, e.g., <102 CFU/ml of CSF. We also recognize that the use of pooled specimens may not be the optimal experimental design; however, the volume of CSF that can be removed from a rat pup is small (15 to 20 μl) and necessitates the pooling of specimens. Therefore, to support these data, several different techniques (e.g., cytopsins and fixed sections of pelleted CSF, and O18 LPS monoclonal antibody staining of brain sections) were used to demonstrate the presence of O18 bacteria in the central nervous systems of these animals, despite their having sterile CSF cultures. The concept that bacteria can be demonstrated in the central nervous systems of K1− *E. coli*-infected animals, without the evidence of viable bacteria, is a novel observation and potentially important for our understanding of the pathogenesis of *E. coli* meningitis.

Corroborating the in vivo finding are our tissue culture invasion data, which show that fourfold-fewer K1+ bacteria can be recovered from BMECs in invasion assays. In addition, our in vitro data showed that by inhibition of BMEC protein synthesis with cycloheximide, the K1− strains were protected from killing. Our interpretation of these data is that brain endothelial cells may produce a substance that is bactericidal to *E. coli* strains without a capsule. The nature of this eukaryotic substance is unknown. Nitric oxide has been found to possess antimicrobial properties (8, 20) and is produced by endothelial cells via NOS (12, 23). NO has been shown, in vitro and in animal models, to be active against a wide variety of pathogens, including, but not limited to, the following organisms: bacteria (*Mycobacterium* spp., *E. coli*, *Salmonella typhimurium* [19, 22, 30]), viruses (herpes simplex virus type 1 and Japanese encephalitis virus [6, 18]), fungi (*Cryptococcus neoformans* [1]), and parasites (*Leishmania major* [17, 28]). We examined the possibility that NO or oxygen intermediates might be the agent of *E. coli* killing in our in vitro BMEC invasion assays. Using two different NOS inhibitors, which are analogues of the NOS substrate l-arginine, at concentrations found to inhibit NO production in endothelial cells (10), we were unable to reproduce the survival advantage to K1− *E. coli* seen with cycloheximide. This suggests that neither NO nor peroxynitrites, which are formed when NO reacts with oxygen radicals (3, 32), are responsible for the bacterial killing in this system. We also examined the possible effects of superoxides and other oxygen radicals, which are known antimicrobial products of professional phagocytes (2) and are produced by endothelial cells (4, 27), in the invasion process of meningitic *E. coli* K1 and its capsule-negative derivative. Experiments using SOD and catalase, which are scavengers of oxygen radicals, were performed. In contrast to the results of experiments with cycloheximide-treated BMECs, there was no increase in levels of viable intracellular K1− *E. coli* bacteria in these experiments. Because SOD and catalase are large proteins, it is possible that they do not freely enter endothelial cells, the putative site of the bacterial killing. There is some evidence that certain cells, such as hepatocytes, do actively take up SOD (16), but this has not been investigated in BMECs. Further experiments are needed to correlate BMEC oxygen radical production with bacterial...
killing and its inhibition with an increase in the survival of unencapsulated bacteria.

In summary, using an in vivo neonatal rat model of hematojenous meningitis and an in vitro model of the blood-brain barrier, we showed that both K1+ and K1− E. coli strains were able to penetrate BMECs and enter the central nervous system. However, only infections caused by K1+ strains resulted in positive CSF cultures in our animal model, and in vitro experiments yielded a significantly higher recovery of viable K1+ intracellular organisms compared to that of K1− strains. This strongly suggests that the K1 capsule has, in addition to its well-recognized serum resistance and antiphagocytic properties, a novel role in the transversal of E. coli K1 across the blood-brain barrier. It serves to protect K1-encapsulated E. coli strains from killing during invasion of the central nervous system, thus helping to explain the predominance of K1-encapsulated bacteria in neonatal E. coli meningitis.

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