Antibodies Present in Normal Human Serum Inhibit Invasion of Human Brain Microvascular Endothelial Cells by *Listeria monocytogenes*

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*Listeria monocytogenes* causes meningitis and encephalitis in humans and crosses the blood-brain barrier by yet unknown mechanisms. The interaction of the bacteria with different types of endothelial cells was recently analyzed, and it was shown that invasion into, but not adhesion to, human brain microvascular endothelial cells (HBMEC) depends on the product of the *inlB* gene, the surface molecule InlB, which is a member of the internalin multigene family. In the present study we analyzed the role of the medium composition in the interaction of *L. monocytogenes* with HBMEC, and we show that invasion of HBMEC is strongly inhibited in the presence of adult human serum. The strong inhibitory activity, which is not present in fetal calf serum, does not inhibit uptake by macrophage-like J774 cells but does also inhibit invasion of Caco-2 epithelial cells. The inhibitory component of human serum was identified as being associated with *L. monocytogenes*-specific antibodies present in the human serum. Human newborn serum (cord serum) shows only a weak inhibitory activity on the invasion of HBMEC by *L. monocytogenes*.

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*Listeria monocytogenes*, a gram-positive, facultatively intracellular bacterium, is known to cause meningitis, encephalitis, and brain abscesses, mainly in immunocompromised individuals (21). Central nervous system (CNS) penetration by *L. monocytogenes* suggests that invasion of brain microvascular endothelial cells may be an important way of crossing the blood-brain barrier. During the last couple of years, several groups have reported on the capacity of *L. monocytogenes* to invade different types of human endothelial cells. However, the absolute values of invasion, as well as the dependency of invasion on the *inlB* gene product, differed markedly among the studies (5, 11, 12, 17, 22). It has previously been shown that invasion of, but not adhesion to, human brain microvascular endothelial cells (HBMEC) by *L. monocytogenes* is strictly dependent on the presence of the product of the *inlB* gene (2, 10, 11). InlB is a 630-amino-acid protein of the internalin family of leucine-rich repeat proteins which is found at the cell surface (17). *L. monocytogenes* causes meningitis and encephalitis in humans and crosses the blood-brain barrier by yet unknown mechanisms. The interaction of the bacteria with different types of endothelial cells was recently analyzed, and it was shown that invasion into, but not adhesion to, human brain microvascular endothelial cells (HBMEC) depends on the product of the *inlB* gene, the surface molecule InlB, which is a member of the internalin multigene family. In the present study we analyzed the role of the medium composition in the interaction of *L. monocytogenes* with HBMEC, and we show that invasion of HBMEC is strongly inhibited in the presence of adult human serum. The strong inhibitory activity, which is not present in fetal calf serum, does not inhibit uptake by macrophage-like J774 cells but does also inhibit invasion of Caco-2 epithelial cells. The inhibitory component of human serum was identified as being associated with *L. monocytogenes*-specific antibodies present in the human serum. Human newborn serum (cord serum) shows only a weak inhibitory activity on the invasion of HBMEC by *L. monocytogenes*.

**MATERIALS AND METHODS**

Cell culture and infection. Culture of HBMEC, Caco-2 epithelial cells, and J774 macrophages and their infection with *L. monocytogenes* have been described in detail recently (2, 11). *L. monocytogenes* strain EGD was cultured aerobically in brain heart infusion (BHI) broth (Difco) at 37°C until it reached the mid-log phase of growth. After the bacteria were washed twice with phosphate-buffered saline (PBS), they were stored in aliquots in PBS with 20% (vol/vol) glycerol at −80°C until they were used for the infection experiments. HBMEC were isolated from a brain biopsy specimen of an adult female with epilepsy and were cultured by methods described previously (19). HBMEC were subsequently immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages (20). HBMEC were cultured in gelatin-coated flasks without the addition of antibiotics in complete HBMEC medium (RPMI 1640 medium [Gibco] supplemented with FCS [10%] [Gibco or Sigma], NuSerum IV [10%] [Becton Dickinson, Bedford, Mass.], nonessential amino acids [1%] and vitamins [1%], heparin [5 U/ml], sodium pyruvate [1 mM], l-glutamine [2 mM], and endothelial cell growth supplement [30 μg/ml] [all from Sigma]) and were incubated at 37°C under a humid atmosphere of 5% CO₂. Caco-2 epithelial cells and J774 macrophages were cultured in RPMI 1640 medium supplemented with FCS (10%) according to standard procedures (2).

Forty-eight hours prior to infection, cells were split and seeded into normal
PBS, and 1010 bacteria were incubated with 0.5 ml of the immunoglobulins (5 
strain- or species-specific antibodies from the immunoglobulin preparation. Bac-
H9004 were incubated overnight with either 
land) (Sandoglobin; complement free) or from Sigma Chemicals. Human new-
primary antisera in Western blots with the separated listerial proteins as targets.

tions were then used either as supplements in cellular infection assays or as

membranes, and detection with horseradish peroxidase-conjugated secondary anti-
were precipitated by addition of 10% (vol/vol) trichloroacetic acid, harvested by
a lysate of total listerial proteins. The proteins present in the culture supernatant
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bacteria per cell.

to invade the cells. One milliliter of RPMI medium containing 100 μg of gen-
tamicin (Sigma)/ml was then added to the washed monolayers to kill extracellular
, and the plates were further incubated for 1 h at 37°C. After the cells were
were washed twice with PBS, they were lysed and plated on BHI agar. All cellular
association and invasion assays were performed in triplicate and repeated at least
three times. The absolute numbers of intracellular bacteria were always around 10
bacteria per well, which means that about 5% of the bacteria added to the
cells were taken up by the HBMEC.

For statistical analysis, the two-tailed, unpaired Student t test was applied, and
P values of ≤0.01 were considered statistically significant. Invasion and early
association efficiencies were always compared to those for the untreated control,
which was set to 100% invasion or association. Values for invasion upon addition
of serum or immunoglobulins are presented relative to the control.

Preparation of bacterial proteins and Western blot analysis. One milliliter of
L. monocytogenes grown overnight in BHI broth (Difco) was centrifuged, and the
pellet was resuspended in sodium dodecyl sulfate-polyacrylamide gel electro-
phoresis (SDS-PAGE) buffer and heated to 100°C for 10 min in order to prepare a lysate of total listerial proteins. The proteins present in the culture supernatant
were precipitated by addition of 10% (vol/vol) trichloroacetic acid, harvested by
centrifugation, and also resuspended in SDS-PAGE buffer and heated as de-
described above. SDS-PAGE, transfer of the proteins onto nitrocellulose mem-
branes, and detection with horseradish peroxidase-conjugated secondary anti-
bodies were carried out by standard procedures (18).

HS and human immunoglobulins. Commercially available pooled HS prepa-
ations (Sigma Chemicals, St. Louis, Mo.), heat inactivated for 30 min at 50°C,
were used in all experiments. HS was fractionated by centrifugation for 30 min
through Centricon filters which retain proteins larger than 100 kDa. Both frac-
tions were then used either as supplements in cellular infection assays or as
primary antisera in Western blots with the separated listerial proteins as targets.
Human immunoglobulins (5 mg/ml) were either from Sandoz (Basel, Switzer-
land) (Sandoglobin; complement free) or from Sigma Chemicals. Human new-
born serum (cord serum) was obtained as described previously (14).

Preadsorption of immunoglobulins. Immunoglobulins (Sigma Chemicals)
were incubated overnight with either L. monocytogenes EGD, L. monocytogenes
ΔnAB (12), Bacillus subtilis 166, or Escherichia coli K-12 in order to remove strain-
or species-specific antibodies from the immunoglobulin preparation. Bac-
teria were grown to mid-log phase as described above and washed twice with
PBS, and 1010 bacteria were incubated with 0.5 ml of the immunoglobulins (5
mg/ml) overnight at 4°C with shaking. The bacteria were then removed by two
rounds of centrifugation, and the supernatant (preadsorbed immunoglobulins)
was tested for inhibitory activity in the invasion assay.

RESULTS

HS inhibits invasion of HBMEC and Caco-2 cells by L. monocytogenes but not uptake by J774 macrophages. Measuring early association of L. monocytogenes EGD with HBMEC by live cell counts, we found that the presence of 10% heat-
inactivated HS during the infection period reduced the associ-
ation of the bacteria with HBMEC close to 100-fold. In contrast, addition of FCS reduced the association of L. mono-
cytogenes with HBMEC only about fivefold (Fig. 1). The effect of HS on invasion was even more dramatic than the effect on adhesion: increasing concentrations of HS resulted in a de-
crease in L. monocytogenes invasion of HBMEC. The presence of 10% HS reduced L. monocytogenes invasion of HBMEC
more than 200-fold (Fig. 2), but even the presence of HS concentrations as low as 1% in the infection medium resulted in significant reductions in invasion. FCS only weakly (about fivefold) inhibited L. monocytogenes invasion of HBMEC un-
der the same experimental conditions (presence of 10% FCS) (Fig. 2), and lower concentrations of FCS had no effect in contrast to HS. As shown in Fig. 3, HS also strongly inhibited the invasion of Caco-2 epithelial cells by L. monocytogenes in a concentration-dependent manner, showing that the inhibitory ef-
effect of HS on invasion is not observed only in endothelial cells. In contrast, the uptake of L. monocytogenes by profes-
sional phagocytic cells such as J774 macrophages was not in-
hibited by the presence of 10% HS (Fig. 3). To test whether the inhibitory component of the HS was acting on the endothelial cells or on the bacteria, we incubated either L. monocytogenes or the endothelial cells for 1 h with 10% HS prior to infection and found that only the preincubation of the bacteria with HS resulted in a significant inhibition of invasion (Fig. 4), indicat-
ing that the effective component of the serum interacts with L. monocytogenes and not with the target cells.

The inhibitory effect is due to anti-Listeria antibodies present in HS. In order to identify the inhibitory component of the HS, we first used purified human serum albumin free of immunoglobulins (Sigma) at concentrations resembling those present in serum (approximately 20 mg/ml) and found no inhibitory effect on L. monocytogenes invasion of HBMEC (data not shown), ruling out the possibility that this main protein fraction of HS is inhibitory. In a second step, we separated the serum into a fraction containing only high-molecular-weight proteins larger than 100 kDa, which should harbor mainly the immunoglobulins, and a fraction containing all components with molecular sizes below 100 kDa. Both fractions were recon-
stituted with PBS to their original volumes to avoid dilution effects and tested either for inhibition of HBMEC invasion or for recognition of listerial proteins in Western blots. As shown in Fig. 5, only the high-molecular-weight fraction contains ant-
ibodies recognizing listerial proteins. Furthermore, this high-
molecular-weight fraction is also able to inhibit L. monocytogenes invasion of HBMEC (Fig. 5) to the same extent as the whole HS prior to fractionation.

FIG. 1. Early association of L. monocytogenes EGD with HBMEC in the presence of HS and FCS. Cells were infected with L. monocytogenes, and associated bacteria were counted at 60 min postinfection. Association after infection in RPMI medium was compared with association after infection in the presence of 10% HS or 10% FCS. Associated bacteria were enumerated, and the numbers of bacteria recovered (expressed as percentages of the number recovered for the untreated control, taken as 100%) are shown. Values are means and
standard deviations (error bars) of the results of a representative experiment. In all figures, open bars indicate that differences from the control are statistically significant; shaded bars represent experimental results that are not statistically significantly different from those for the control.
Similarly, the immunoglobulin fractions of normal HS from different sources are able to significantly inhibit the invasion of HBMEC by *L. monocytogenes* in a concentration-dependent manner, as demonstrated in Fig. 6. It has previously been shown that this immunoglobulin fraction contains antibodies specific for several listerial antigens, since it recognizes listerial proteins derived from whole-cell lysates as well as proteins from listerial supernatants (15). To further demonstrate that the inhibiting activity is due to *Listeria*-specific antibodies present in the immunoglobulin fraction, we preadsorbed the immunoglobulins by overnight incubation with either *L. monocytogenes* EGD, *B. subtilis*, *E. coli*, or *L. monocytogenes* ΔinLAB. As expected, preincubation with *L. monocytogenes* EGD or *L. monocytogenes* ΔinLAB resulted in a significant reduction in the inhibitory activity of the immunoglobulins (Fig. 7) in the invasion assay. Preattachment of the immunoglobulins with either *E. coli* or *B. subtilis* resulted in no reduction in the inhibitory activity (Fig. 7). These results clearly show that the removal of the *Listeria*-specific antibodies alone leads to a loss of inhibitory activity.

**Human newborn serum only slightly influences *L. monocytogenes* invasion of HBMEC.** Since newborns are especially susceptible to *L. monocytogenes* infections, we also tested human serum derived from newborns (cord serum) for its ability to inhibit *L. monocytogenes* infection of HBMEC. Human cord serum (14) was heat inactivated as described above, and its activity was tested in an invasion assay at concentrations of 0.1 and 1%. In contrast to adult HS, which at similar concentrations resulted in dramatic inhibition of *L. monocytogenes* uptake by HBMEC (Fig. 2), the uptake of *L. monocytogenes* was minimally influenced by the presence of the cord serum (Fig. 8). At a concentration of 1% cord serum, the invasion efficiency was still around 55% of that for the untreated control, compared to less than 1% with the same concentration of adult HS (Fig. 2), clearly showing that human newborn serum is largely devoid of the inhibiting activity found in adult serum preparations.

**DISCUSSION**

For many years, *L. monocytogenes* has been known as a pathogen that is able to infect and to grow in a number of mammalian cell types including macrophages, epithelial cells, hepatocytes, dendritic cells, and fibroblasts (16, 21). In recent years it was also shown that *L. monocytogenes* efficiently infects human endothelial cells of different origins, including HUVEC.
shown to be necessary for efficient invasion (2, 10, 11). The InlB protein was also derived from the inlB protein inlB, since deletion of the inlB gene in L. monocytogenes for 60 min as described in Materials and Methods. The importance of InlB for HBMEC invasion, InlB was found to be totally ineffective in mediating the early association of L. monocytogenes with HBMEC (2, 10). Surprisingly, despite the role of InlB in the invasion of microvascular endothelial cells in vivo is not known. However, the ability to invade and grow in endothelial cells is believed to be an important feature that is necessary for crossing the blood-brain barrier or the placental barrier (21). Knowledge about factors modulating this InlB-dependent endothelial cell invasion in vitro and in vivo would therefore be of great importance for understanding the mechanisms used by L. monocytogenes to interact with endothelial cells in order to cross the blood-brain barrier.

The data presented here clearly demonstrate that HS significantly impairs L. monocytogenes invasion of HBMEC. The finding that the presence of 1% HS reduces the invasive capacity of L. monocytogenes for HBMEC more than 200-fold now challenges the in vivo relevance of the in vitro finding on endothelial cell invasion by L. monocytogenes. As it is known from animal studies that septicemia and hence the presence of bacteria in the bloodstream is believed to be an important feature that is necessary for crossing the blood-brain barrier or the placental barrier (21), the data presented here question whether free L. monocytogenes in the bloodstream might be able to directly infect microvascular endothelial cells in vivo.

FIG. 4. Invasion of HBMEC by L. monocytogenes EGD after pre-incubation of either the bacteria or the HBMEC with HS. Prior to infection, either the bacteria (left) or the HBMEC (right) were incubated in RPMI medium containing 10% HS for 1 h at 37°C. Preincubated HBMEC were washed once and infected with untreated L. monocytogenes for 60 min as described in Materials and Methods. Preincubated listeriae were collected by centrifugation, resuspended in RPMI medium, and used for infection of untreated HBMEC for 60 min as described in Materials and Methods. Intracellular bacteria were enumerated after an additional 60 min of incubation in the presence of gentamicin. The numbers of bacteria recovered (expressed as percentages of the number recovered for the untreated control, taken as 100%) are shown. Values are means and standard deviations (error bars) of the results of a representative experiment. Open versus shaded bars are explained in the legend to Fig. 1.

and HBMEC (5, 11, 12, 17, 22). Invasion of HBMEC by L. monocytogenes is strictly dependent on the listerial surface protein InlB, since deletion of the inlB gene causes a >200-fold reduction in invasion (2, 10, 11). The InlB protein was also shown to be necessary for efficient invasion of HUVEC (17), but several other studies on endothelial cell invasion by L. monocytogenes presented conflicting data on the role of the proteins internalin A and InlB (5, 12, 22). Surprisingly, despite the importance of InlB for HBMEC invasion, InlB was found to be totally ineffective in mediating the early association of L. monocytogenes with HBMEC (2, 10). Adhesion is obviously mediated by L. monocytogenes structures other than the internalins, since even the nonpathogenic species Listeria innocua binds efficiently to HBMEC (10).

The role of InlB in the invasion of microvascular endothelial cells in vivo might be able to directly infect microvascular endothelial cells in vivo in the presence of septicemia. Possibly, other mechanisms such as cell-to-cell spread from infected monocytes or leukocytes into endothelial cells, as already described in reports of in vitro (5, 11) and in vivo (4) studies, might be more important for the crossing of the blood-brain barrier by L. monocytogenes than direct infection of microvascular endothelial cells by bacteria that are free in the bloodstream.

In contrast to HS, FCS, which is regularly used as an additive in cell culture media, inhibited L. monocytogenes uptake by HBMEC only slightly and at much higher concentrations, demonstrating the specificity of the inhibitory activity of HS. This
activity of HS on invasion was found not to be restricted to HBMEC: invasion of Caco-2 epithelial cells was also inhibited by HS and not by FCS. However, epithelial cells are normally not in contact with serum compounds as are endothelial cells, and hence it is not clear whether serum-dependent inhibition of epithelial cell invasion could play any role during L. monocytogenes infection in vivo. In contrast to uptake by epithelial cells, uptake of L. monocytogenes by macrophages, which is independent of the internalins, is not reduced the presence of HS.

In the search for the inhibitory component of HS, we first tested human albumin fractions, which were, however, inactive. Secondly, we fractionated HS by centrifugation and found that only the high-molecular-weight fraction inhibited L. monocytogenes invasion of HBMEC. This high-molecular-weight fraction also contains high concentrations of anti-Listeria antibodies. To confirm that the antibodies are responsible for the inhibition, we tested two commercial preparations of human immunoglobulins. Both inhibited L. monocytogenes invasion of HBMEC in a dose-dependent manner. In immunoprecipitation experiments with either L. monocytogenes, B. subtilis, or E. coli, we could significantly deplete the inhibitory antibodies in the immunoglobulin fraction only by pretreatment with L. monocytogenes, confirming our assumption that inhibition of L. monocytogenes invasion is not a general effect of any type of antibodies present but that specifically the anti-L. monocytogenes antibodies are inhibitory in the HBMEC invasion assay. Preincubation of the immunoglobulins with the L. monocytogenes ΔinlAB mutant also led to a reduction in the inhibitory activity of the immunoglobulins. Initially we expected that preincubation with an L. monocytogenes strain lacking the InlA and InlB proteins would result in an immunoglobulin fraction still harboring the potentially inhibitory anti-InlB antibodies. However, one must keep in mind that L. monocytogenes harbors 20 genes coding for internalin-like molecules, some of which are highly homologous to the inlB gene (3, 9). Preincubation of the immunoglobulins with a strain expressing these closely related internalins and internalin-like proteins might hence result in the removal of InlB-specific antibodies.

In the legend to Fig. 1. (Top) IgG from Sandoz; (bottom) IgG from Sigma.
antibodies even in the absence of InIB, due to a cross-reactivity of the respective antibodies.

Anti-Listeria antibodies are regularly found in HS from healthy donors (8, 15). The finding that these antibodies drastically inhibit the invasion of HBMEC by L. monocytogenes is very interesting for several reasons. On the one hand, it has previously been shown that anti-Listeria antibodies in HS act as opsonins in the uptake of L. monocytogenes by dendritic cells, thereby increasing invasion of these antigen-presenting cells significantly (15). On the other hand, the humoral immune response to infection with L. monocytogenes was for a long time regarded as not being involved in the clearance of an infection (13). However, it was recently demonstrated that monoclonal anti-listitialysin antibodies can provide resistance in mice to L. monocytogenes infection when administered prior to challenge with virulent listeriae (6). Our data on the inhibitory effect of anti-Listeria antibodies present in HS on the direct infection of HBMEC by L. monocytogenes in vitro suggest that the progression of an infection from septicaemia to meningitis and encephalitis could be strongly impaired by anti-Listeria antibodies present in the blood of most adult humans.

Human newborns or fetuses lack a fully functional immune system. Human newborn serum derived from umbilical cords (cord serum) was hence tested for inhibition of L. monocytogenes invasion of HBMEC and found to be much less active (only 45% inhibition at a concentration of 1% in contrast to more than 99% inhibition with adult serum at the same concentration). This result might help to explain the high susceptibility of newborns to L. monocytogenes infections, since their endothelial cells are obviously much less protected against invasion by L. monocytogenes free in the bloodstream than are the endothelial cells of adults (at least if our in vitro data also hold true for the in vivo situation). This putative increased ability of L. monocytogenes to invade endothelial cells in newborns may contribute directly to the higher susceptibility of newborns, compared to adults, to severe, life-threatening L. monocytogenes infections.

The data presented here could also help to clarify some recent conflicts in determining the role of the internalins InlA and InIB in endothelial cell invasion (5, 11, 12, 17, 22). Some of the conflicting results can probably be explained by the use of different infection media containing either FCS or HS at different concentrations, variables which, as we now know, significantly influence the outcome of standard gentamicin-based infection assays with endothelial cells as targets.

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