Endothelial Adhesion Molecule Expression and Its Inhibition by Recombinant Bactericidal/Permeability-Increasing Protein Are Influenced by the Capsulation and Lipooligosaccharide Structure of Neisseria meningitidis

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Received 19 May 1999/Returned for modification 1 July 1999/Accepted 12 August 1999

Vascular endothelial injury is responsible for many of the clinical manifestations of severe meningococcal disease. Binding and migration of activated host inflammatory cells is a central process in vascular damage. The expression and function of adhesion molecules regulate interactions between leukocytes and endothelial cells. Little is known about how meningococci directly influence these receptors. In this study we have explored the effect of Neisseria meningitidis on endothelial adhesion molecule expression and found this organism to be a potent inducer of the adhesion molecules CD62E, ICAM-1, and VCAM-1. Exposure of endothelium to a serogroup B strain of Neisseria meningitidis, B1940, and a range of isogenic mutants revealed that lipooligosaccharide (LOS) structure and capsulation influence the expression of adhesion molecules. Following only a brief exposure (15 min) to the bacteria, there were large differences in the capacity of the different mutants to induce vascular cell adhesion molecules, with the unencapsulated and truncated LOS strains being most potent ($P < 0.05$). Furthermore, the pattern of cell adhesion molecule expression was different with purified endotoxin from that with intact bacteria. Meningococci were more potent stimuli of CD62E expression than endotoxin, whereas endotoxin was at least as effective as meningococci in inducing ICAM-1 and VCAM-1. The effect of bactericidal/permeability increasing protein (rBPI21), an antibacterial molecule with antiendotoxin properties, was also dependent on LOS structure. The strains which possessed a truncated or nonsialylated LOS, whether capsulated or not, were more sensitive to the inhibitory effects of rBPI21. These findings could have important implications for the use of antiendotoxin therapy in meningococcal disease.

Infections caused by Neisseria meningitidis remain an important cause of mortality and morbidity worldwide (14). It is the organism responsible for the majority of childhood cases of bacterial meningitis in the United Kingdom, and in patients presenting with severe shock, mortality may be as high as 50%. Although prompt recognition, early treatment and intensive care has reduced this figure in recent years (18), survivors may have extensive tissue injury, sometimes requiring amputation and/or skin grafting.

Capillary leak and intravascular thrombosis are serious consequences of meningococcal sepsis and are indicative of widespread vascular endothelial injury (23). Histological studies of meningococcal disease show that cutaneous lesions contain large numbers of organisms that are associated with the vascular endothelium (11). Recent studies have also shown that meningococci have the capacity to bind endothelial cells in a receptor-ligand-specific fashion (33, 34) and indicate that bacterium-endothelium contact may itself be critical in mediating the vascular injury seen in this disease (29). There is evidence that meningococci, both alone and in the presence of neutrophils, can lead to endothelium damage (20, 32). However, there is still very little information on how meningococci may themselves modulate the influx of neutrophils into inflammatory sites.

Expression of adhesion molecules by the vascular endothelium is a critical step in the inflammatory response. Leukocyte adhesion occurs through a complex and multistep process involving initial tethering and then rolling of leukocytes by low-avidity interactions with mainly the selectin family of cell adhesion molecules (e.g., CD62E/E-selectin). This is followed by firmer adhesion, which is mediated largely by higher-affinity interactions involving the members of the immunoglobulin Ig superfamily (e.g., ICAM-1 and VCAM-1) on endothelial cells (30). After firm adhesion, transendothelial and subendothelial migration may occur, a process also involving leukocyte integrins and complex cross talk among leukocytes, the endothelium, cytokines, and chemokines. The initial inflammatory stimulus to this activation cascade is critical since it determines which leukocytes will participate in the subsequent inflammatory response (4). The pattern of endothelial activation seen in response to the proinflammatory cytokines tumor necrosis factor alpha, interleukin-1, and CD40 (17) and bacterial endotoxin has been described (5, 39). There is very limited information on the response of endothelial adhesion molecules to live meningococci.

We have previously shown that encapsulation and lipooligosaccharide (LOS) structure influence the host inflammatory response to N. meningitidis (20). In this study, we have used isogenic mutants of N. meningitidis B1940 to investigate the relationship between bacterial structure and the expression of...
adhesion molecules on cultured human endothelial cells. We have also explored the effect of human recombinant bacterial/ permeability increasing protein (rBPI21) (2) to modulate en- dothelial activation by these organisms.

MATERIALS AND METHODS

Bacterial strains. The parent organism, N. meningitidis B1940, and three isogenic mutants derived from it have been described previously (7). The capsule deficient (siaD) mutant of B1940 was constructed by using an insertional inactivation of the polysialyltransferase gene. Inactivation of the gatE gene by replacement of the cpsD region with a chloramphenicol resistance marker pro- duces a capsulated mutant that expresses a truncated LOS that cannot be sialy- lated. In the cps mutant, the whole cps gene complex is missing, and so it has both defective capsule expression and a truncated LOS. A fourth mutant, the lst mutant of B1940, has a deleted N-2,3-sialytransferase gene and cannot sialylate terminal lacto-N-neotetraose of its LOS (36). The parent bacterium, B1940, and its derived mutants express pilus and both Opa and Opc as indicated by electron microscopy and immunoreactivity with specific monoclonal antibodies (20).

Materials. rBPI21, a recombinant, modified amino-terminal fragment of bacterial/permeability increasing factor, was a kind gift from XOMA (US) LLC, Berkeley, Calif. Escherichia coli lipopolysaccharide (LPS) (protein content <1%), serotype O111:B4, was purchased from Sigma, Poole, United Kingdom. LOS from N. meningitidis serogroup B (strain 44/76) was prepared as previously described (1). Briefly, LPS was extracted by hot aqueous phenol extraction, ultracentrifugation, gel filtration, and cold-ethanol precipitation (10). The final product contained <0.3% protein and was without detectable nucleic acids.

Bacterial culture. All the above strains were grown on gonococcal agar (Difco) supplemented with Vitek (Oxoid) and cultured in 6% CO2 in air at 35°C. In all experiments, the bacteria used were subcultured at least once and were used after 18 h. Suspensions of bacteria were prepared in RPMI 1640 medium with no phenol red (Gibco, Paisley, United Kingdom), and their optical density was measured at 540 nm. Bacterial viability counts were measured by a modification of Miles and Misra technique (24). In some experiments, nonviable bacteria were used. These were killed either with 0.5% paraformaldehyde or by heating at 56°C for 30 min.

Endothelial-cell culture. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel) and subcultured as described previously with some modifications (21). Cells in primary culture were grown in MCDB 131 medium (Gibco) supplemented with 2 mM L-glutamine (Gibco) in 25-cm2 tissue culture flasks (Becton Dickinson, Oxford, United Kingdom). The cells were then passaged into 24-well plates, previously treated with endothelial attachment factor, by using trypsin-EDTA (Sigma). The cells were grown to confluence and then washed thoroughly, 24 to 48 h prior to experiments, in antibiotic-free RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin, streptomycin, and 20% heat-inactivated fetal calf serum (Gibco) (25) in tissue culture flasks (Becton Dickinson, Oxford, United Kingdom). The cells were then transfected into 24-well plates, previously treated with endothelial attachment factor, by using trypsin-EDTA (Sigma). The cells were grown to confluence and then washed thoroughly, 24 to 48 h prior to experiments, in antibiotic-free RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin, streptomycin, and 20% heat-inactivated fetal calf serum (Gibco).

Incubation of N. meningitidis with HUVEC. Initial experiments were conducted with either heat-inactivated or 0.5% paraformaldehyde-fixed bacteria. In subsequent experiments, HUVEC were stimulated with endotoxin or live meningo- cocci. However, when HUVEC were incubated for 5 h and used for staining, the whole LOS and the capsulated (unencapsulated, truncated LOS, nonsialylated) organisms was always higher than that induced by the parent but not usually to the levels observed with the siaD and cps mutants (Fig. 1). Interestingly, in separate experiments comparing the parent and the cpsD and lst (capsulated, nonsialylated LOS) mutants, the level of cell adhesion molecules seen with the lst organisms was between those seen with the parent and the cpsD mutant. Similar results were obtained when live meningococci were replaced with bacteria that were either heat inactivated or fixed in 0.5% paraformaldehyde (result not shown).

Once the patterns of adhesion molecule expression under these conditions had been established, the influence of bacte- rial concentration was investigated. Figure 2 shows the effect of bacterial concentration on CD62E expression. The pattern of adhesion molecule expression as shown in Fig. 1 was preserved at all the concentrations tested. There was a threshold bacte- rial concentration at which no adhesion molecule expression was observed. This differed between the parent and siaD mut- tant by at least 1 log unit in bacterial concentration (Fig. 2). Similar results were seen with ICAM-1 and VCAM-1 (results not shown).

E. coli LPS and meningococcal LOS cause a different pattern of adhesion molecule expression to N. meningitidis B1940 and isogenic mutants. When HUVEC were incubated with purified E. coli LPS or meningococcal LOS, the profiles of CD62E, ICAM-1, and VCAM-1 expression observed by flow cytomtery were similar (Fig. 3). In comparison, N. meningitidis induced greater expression of CD62E than did purified endo- toxin from either bacterial species, even when very high endo- toxin doses (100 ng/ml) were used. Endotoxin from either source was at least as effective at up-regulating ICAM-1 or VCAM-1 as were the bacteria (Fig. 3).

Results. As shown in Fig. 1 demonstrated that a short exposure of the HUVEC to unencapsulated siaD and cps meningococcal mutants induced higher levels of CD62E expression than did continuous exposure to purified endotoxin, whereas endotoxin induced higher levels of ICAM-1 and VCAM-1 expression. To investigate this further, HUVEC were incubated with either E. coli LPS or the N. meningitidis B1940 parent or siaD mutant for increasing lengths of time, ranging from 1 min to 3 h, before being washed thoroughly to remove free LPS and/or nonadherent organisms. Cultures were incubated for a further 5 h,
after which time adhesion molecule expression was measured by flow cytometry. There was minimal induction of CD62E expression on HUVEC exposed to LPS for less than 15 min (Fig. 4A). For exposures of 15 min or more, CD62E expression could be detected. Similar results were obtained with ICAM-1 and VCAM-1 (data not shown). Increasing the duration of HUVEC exposure to LPS also increased the level of adhesion molecule expression, so that maximal expression was seen at 5 h. When the kinetics of adhesion molecule expression were examined by using the parent organism, the profiles were similar to that seen with LPS (Fig. 4B). The pattern of expression was very different when the experiments were performed with the siaD mutant. CD62E expression was detected after only a 5-min exposure (Fig. 4B), reached a peak at 60 min, and declined progressively by 5 h. Similar differences were observed with ICAM-1 and VCAM-1 (results not shown).

Bacterial concentration, LOS structure, and capsulation influence the inhibitory effect of rBPI21 on the induction of HUVEC adhesion molecule expression by meningococci. The effects of the endotoxin antagonist rBPI21 on the induction of HUVEC adhesion molecule expression were investigated. When 10 μg of rBPI per ml was added prior to or at the same time as 100 ng of E. coli LPS per ml, adhesion molecule expression was completely inhibited (Fig. 5). However, when rBPI21 was added to meningococci, the pattern of cell adhesion molecule expression was more complicated. First, the bacterial concentration was found to be important. When 10 μg of rBPI21 per ml was added to either the parent B1940 or siaD mutant at 10^4 organisms/ml, there was a marked reduction in adhesion molecule expression. However, when the bacterium was present at 10^6 organisms/ml, inhibition was only between 20 and 50%. Little effect was observed at 10^8 organisms/ml (Fig. 6). Second, LOS structure and encapsulation also influenced the efficacy of rBPI21. When 10^8 organisms of N. meningitidis B1940 or the isogenic mutants per ml were incubated with rBPI21, the levels of CD62E expression were more efficiently inhibited for the cps and cpsD mutants than for either the parent or siaD mutant (Fig. 7A). In separate experiments, the level of inhibition seen with the lst mutant was similar to that observed with the cps and cpsD mutants (Fig. 7B), indicating that LOS sialylation may be the major determinant of this effect. rBPI21-mediated inhibition of either ICAM-1 or VCAM-1 up-regulation by meningococci was similar to that seen with CD62E expression (results not shown).

The influence of rBPI21 on induction of HUVEC adhesion molecule expression after exposure to N. meningitidis or endotoxin. The capacity of rBPI21 to influence adhesion molecule expression after bacterial exposure was investigated. HUVEC were exposed to either the parent (nonadherent) or the siaD mutant (adherent) for 15 min before washing. rBPI21 was then added at 15, 60, and 120 min after bacterial exposure. A reduction of between 40 and 60% in the expression of CD62E was observed when rBPI21 was added after a 15-min exposure to the bacteria (Fig. 8). This level of inhibition was similar even if rBPI21 was added at 60 or 120 min. While the siaD mutant induced higher levels of CD62E than the parent organism, the relative effect of rBPI21 was similar for both organisms.
DISCUSSION

The nature and degree of the host inflammatory response to *N. meningitidis* may determine the fate of an infected individual. While host factors such as cytokine gene polymorphisms are likely to be determinants of this response, there is increasing recognition that bacterial properties are also important. This study provides evidence that bacterial composition can influence host endothelial cell responses. In view of the importance of vascular injury in this condition, these findings may be pertinent to understanding the pathophysiology of meningococcal disease.

In this study, we have used HUVEC to investigate the expression of adhesion molecules in response to *N. meningitidis*. We found this organism to be a potent inducer of the major vascular endothelial cell adhesion molecules, CD62E, ICAM-1, and VCAM-1, even after only limited periods of exposure. Our previous studies have shown that meningococci also markedly enhance the expression of the β2-integrin CD11b/CD18 and diminish the expression of the selectin CD62L on neutrophils (20). Since these counterreceptors on both cell types are responsible for neutrophil adhesion and transmigration through vascular endothelia, these findings provide a mechanism to account for the presence of neutrophils within meningococcal lesions (29).

Capsulation and LOS structure have been shown to be determinants of meningococcal survival in human serum, in whole blood, and in the infant-rat model of meningococcal disease (16, 36–38). We have been able to show that these bacterial properties can also influence the degree and pattern of endothelial adhesion molecule expression. Interestingly, the organisms that induced the highest levels of adhesion molecules in this study were also those that we have previously shown to cause the largest reduction in neutrophil CD62L expression (20). These experiments were performed with whole blood and appeared to be related to the degree of bacterial killing. In the present study and in a previous study of meningococcal induction of endothelial cell tissue factor (10), bacterial killing was not the explanation for the differing effects of LOS structure and capsulation on endothelial cell activation. The reasons for the influence of these bacterial structures on endothelial cell activation are complex.

Capsulation is an important determinant of B1940 adhesion to HUVEC. Capsule-deficient mutants are more adherent to endothelial cells than are the capsulated parent strain and the nonsialylated, capsulated organism, B1940 ΔsiaD (20). Possession of a truncated, nonsialylated LOS also influences bacterial adhesion to HUVEC, as indicated by enhanced binding of the B1940 Δcps mutant compared to the B1940 ΔsiaD mutant. In this study we show that adhesion molecule expression is directly correlated with the adherence of these bacteria to endothelial

![Flow cytometric analysis of expression of CD62E, ICAM-1, and VCAM-1 on HUVEC following 5 h of incubation with 100 ng of *E. coli* O111:B4 LPS, 100 ng of meningococcal LOS, and 10⁷ *N. meningitidis* B1940 parent and ΔsiaD mutant organisms per ml. Shaded areas represent CD62E, ICAM-1, and VCAM-1 staining in response to stimuli; the solid line represents cell adhesion molecule staining in unstimulated cells; and the dotted line represents staining with an irrelevant mouse immunoglobulin G1 antibody. Numbers on each plot are median fluorescent intensities and percent positive events for stimulated cells. Data presented here is representative of three experiments yielding similar results.](http://iai.asm.org/)
cells, which indicates that the adhesive capacity of *N. meningitidis* is likely to be a major factor in determining endothelial adhesion molecule expression.

One interpretation of our data is that the more adherent organisms effectively provide a higher dose of endotoxin to the endothelial cells. This appeared to be the explanation for the enhanced tissue factor expression seen in this study. Meningococci, especially the unencapsulated mutants compared to the parent strain (10). However, the dose of endotoxin does not fully account for the differences in adhesion molecule expression seen in response to the meningococcal mutants. This is presumably because, in unencapsulated strains, there may be enhanced interaction between opacity proteins and their ligands. It may also explain the differential patterns of CD62E, ICAM-1, and VCAM-1 seen in response to the bacteria and purified endotoxin. It has been demonstrated recently that invasive strains of *N. meningitidis* cause greater expression of epithelial ICAM-1 than do noninvasive strains. Interestingly, this was not associated with an increase in transcription of ICAM-1 mRNA, indicating that ICAM-1 expression can be modulated by bacteria at the translational or post-translational level (13). We suggest that there are multiple signalling mechanisms that occur when meningococci interact with vascular endothelial cells. Variations in bacterial structure could affect these mechanisms. We are currently undertaking studies to investigate the signal transduction pathways induced in activation of endothelial cells by the meningococcal mutants.

A further explanation for our results comes from the recent discovery of human Toll-like receptors that transduce signals in response to endotoxin in both CD14+ and CD14− cells (43). There are at least five human homologues of the Toll receptor, and two of these, TLR2 and TLR4, transduce endo-

![FIG. 4. Brief exposure of the unencapsulated siaD mutant to HUVEC is a potent inducer of expression of CD62E. (A) N. meningitidis B1940 siaD mutant at 10^6 organisms per ml or 10 ng of *E. coli* LPS was added to HUVEC, which were then washed thoroughly with fresh medium at 5, 15, 60, 120, and 240 min. (B) N. meningitidis B1940 siaD and parent organisms were incubated at 10^6 organisms per ml and washed at 15, 60, 120, and 240 min. CD62E expression was determined by flow cytometry after 5 h of incubation. The results shown here are representative experiments from at least three separate experiments that yielded similar results.](http://iai.asm.org/)

![FIG. 5. Effect of rBPI 21 on *E. coli* O111:B4 LPS-induced HUVEC expression of CD62E, ICAM-1, and VCAM-1. *E. coli* LPS (100 ng/ml) was added to HUVEC preincubated with 10 μg of rBPI21 per ml. Cell adhesion molecule determination was determined by flow cytometry after 5 h of incubation. The results shown are representative of multiple experiments with same level of inhibition.](http://iai.asm.org/)
toxin signals (28). The demonstration of these receptors has highlighted the potential complexity of endotoxin signalling to host cells. Initial findings indicate that different Toll receptors may have different properties and may relate to observed differences in signal transduction (19). It is becoming clear that the location and form of endotoxin are central to how it interacts with host endotoxin recognition molecules (42). This may be critical in how host cells interact with whole bacteria such as *N. meningitidis*. Our results demonstrate that LOS structure is an important factor in determining the pattern of vascular endothelial adhesion molecule expression. The *cpsD* mutant, which possesses a truncated LOS, and the *lst* mutant, which lacks just the terminal sialic acid of the α-oligosaccharide chain, induced higher levels of adhesion molecules than did the parent organism. This indicates that even subtle changes in LOS structure can influence endothelial-cell activation, possibly by influencing the interactions between the bacteria and endothelial-cell endotoxin recognition receptors.

In the light of these findings, we investigated the capacity of rBPI21, a recombinant form of BPI, a host defense protein with antibacterial and antiendotoxin activities, to modulate the endothelial adhesion molecule response to these different bacterial strains. rBPI21 kills gram-negative bacteria and decreases tumor necrosis factor alpha production induced by gram-negative bacteria or LPS in whole blood (41). rBPI21 also binds to purified endotoxin and abrogates the degree of endothelial-cell activation (2). In our study, we found that 10 μg of rBPI21 per ml could completely abolish the induction of cell adhesion molecules on HUVEC when given prior to or very early following exposure to high doses of purified *E. coli* endotoxin.

**FIG. 6.** Effect of rBPI21 on HUVEC expression of CD62E by various doses of the *N. meningitidis* B1940 *siaD* mutant. rBPI21 (10 μg/ml) or medium was added to HUVEC, and then either 100 ng of *E. coli* O111:B4 LPS or various concentrations of bacteria were added as shown. CD62E expression was determined after 5 h. The data presented here are histograms of fluorescence intensity of phycoerythrin fluorochrome plotted against the number of events. The dotted line represents resting CD62E expression; the shaded area represents CD62E expression with LPS or bacteria; and the continuous black line represents CD62E expression when LPS or bacteria were preincubated with rBPI21.

**FIG. 7.** Effect of rBPI21 on induction of CD62E expression on HUVEC in response to *N. meningitidis* B1940 parent and *cps, siaD*, and *cpsD* mutants (A) and B1940 parent and the *lst* mutant (7B). HUVEC were pre-incubated with 10 μg of rBPI21 per ml, and then 10⁵ organisms/ml were added. CD62E expression was measured after 5 h by flow cytometry. The results shown are the mean median fluorescence intensity (MFI) and standard error of the mean from three separate experiments.
found that rBPI_{121} could influence adhesion molecule expression even when added several hours after the organisms. This was true whether the organism was adherent or nonadherent. These results are consistent with previous studies involving E. coli LPS, which have shown that rBPI_{121} can reverse the LPS-induced expression of CD62E (E-selectin) after several hours of continuous exposure (12).

Taken together, these results indicate that LOS structure and the presence of a capsule can influence the level, kinetics, and profile of endothelial adhesion molecule expression induced by N. meningitidis. The apparent ability of pathogenic Neisseria strains to down-regulate their capsule and desialylate the LOS in natural meningococcal infections would indicate that the endothelial-cell response to invading organisms may be variable even for a single strain (8, 9). It would appear that even limited exposure to some organisms might be able to induce a rapid influx of inflammatory cells. This may be beneficial in killing invading organisms but could influence the degree and extent of endothelial cell injury from activated inflammatory cells (27). Strategies to combat meningococcal disease should take into account the multiple signalling pathways that may be activated during the course of this potentially fatal disease.

ACKNOWLEDGMENTS

This work was supported by grants from Royal College of Physicians and Children Nationwide. Karolina Katowicz and Dominic Jack are funded by The Wellcome Trust.

We thank Mark Peters and the staff of Clinical Microbiology laboratory at Great Ormond Street Hospital. We thank XOMA for providing us with the rBPI_{121} and Russ Dedrick for his valuable comments on the manuscript.

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Editor: E. I. Tuomanen