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NOTES

Haemophilus influenzae Outer Membrane Vesicle-Induced Blood-Brain Barrier Permeability during Experimental Meningitis

BRIAN WISPELWEY,[1] ERIC J. HANSEN,[2] AND W. MICHAEL SCHELD[1,3]*

Departments of Internal Medicine[1] and Neurosurgery,[3] University of Virginia School of Medicine, Charlottesville, Virginia 22908, and Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235[2]

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Haemophilus influenzae **type b (Hib) lipopolysaccharide (LPS) may be present in the cerebrospinal fluid largely as part of outer membrane vesicles (OMV), which could possibly alter its activity. Similar to inoculation of purified Hib LPS, intracisternal inoculation of Hib OMV into adult rats resulted in dose- and time-dependent increases in blood-brain barrier permeability. Polymyxin B, but not an oligosaccharide-specific monoclonal antibody, significantly inhibited the activity of Hib OMV. No change in blood-brain barrier permeability occurred in leukopenic rats inoculated with Hib OMV. Hib OMV was as active as purified Hib LPS on a weight basis and therefore appears to be a relevant vehicle for the delivery of LPS during meningitis.**

The factors, either host or bacterial, responsible for significant and often irreversible brain injury during meningitis continue to be elucidated. *Haemophilus influenzae* type b (Hib) remains the most common bacterial cause of meningitis (15). Previous work from this laboratory has characterized the role of purified Hib lipopolysaccharide (LPS) (20) in the alteration of blood-brain barrier permeability (BBBP) and induction of cerebrospinal fluid (CSF) pleocytosis. Intracisternal inoculation of Hib LPS into adult rats resulted in both dose- and time-dependent alterations in BBBP and CSF inflammation. These effects were significantly attenuated by preincubation of the Hib LPS with either polymixin B (PMB) or neutrophil acyloxyacyl hydrolase but unaffected by LPS preincubation with either of two oligosaccharide-specific monoclonal antibodies prior to inoculation, supporting a dominant role for the lipid A moiety in the biologic activity of LPS. Additionally, intracisternal inoculation of LPS had no effect on BBBP in leukopenic animals, suggesting a crucial role for the host leukocyte in LPS-mediated cerebrovascular injury.

However, it must be emphasized that essentially nothing is known about the physical state of Hib LPS in the CSF during the course of natural infections. Hib gains access to the CSF when Hib cells invade this compartment from the bloodstream. Once present in the CSF, LPS can be released from dying Hib cells as well as from growing cells, in the latter case in the form of blebs, or outer membrane vesicles (OMV) (2). The LPS in these various types of OMV is then free to interact with CSF and host cells. Hib LPS in the form of OMV may therefore be a biologically more relevant form of LPS and could possibly alter the previously characterized activity of LPS. Recently, intracisternal inoculation of Hib OMV into rabbits was observed to be a potent inducer of CSF inflammation (G. A. Syrogiannopoulos, G. H. McCracken, Jr., and E. J. Hansen, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 612, 1987); however, no evaluation of blood-brain barrier integrity was performed. The role of the host leukocyte in the induction of vascular injury during meningitis has been actively debated (10, 17, 20; E. Tuomanen and S. D. Wright, 28th ICAAC, abstr. no. 877, 1988) and therefore raises significant questions concerning the importance of CSF pleocytosis following OMV challenge without a concomitant evaluation of a physiologic variable such as brain edema or BBBP.

The purpose of the current study was to evaluate and compare the effect of intracisternal inoculation of isolated Hib OMV and purified LPS on BBBP in an adult rat model.

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The Hib LPS and OMV were isolated from Hib DL42, a clinical isolate from an invasive infection. The LPS was purified by the hot-phenol water method of Westphal and Jann (19) as modified by Johnson and Perry (9). This purified LPS was resolved by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis. Silver staining (18) revealed a single LPS band migrating at approximately the same position as the LPS of an Rb LPS mutant of *Salmonella typhimurium*. Coomassie blue staining showed no detectable protein in the LPS preparation. Western blots (immunoblots) showed LPS antigenic group 2 as defined by a set of two monoclonal antibodies to the oligosaccharide region previously described by Gulig et al. (5). Group 2 represented the most common LPS type (62%) present in a group of 126 Hib strains isolated from patients with invasive infections (5). Twenty nanograms was calculated to be equivalent to the amount of LPS present in 2×10^6 whole organisms (6).

OMV were extracted from Hib DL42 by the lithium chloride-based method of McDade and Johnston (12) as modified by Gulig et al. (4). Differential centrifugation methods were used to separate the vesicles from whole cells and other debris (4). The LPS content of these Hib OMV was 1 μg/3 μg of protein, as determined by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis of sodium dodecyl sulfate-solubilized vesicles, followed by silver staining (18) and densitometry with a Bio-Rad model 620 computer-

* Corresponding author.

TABLE 1. CSF WBC and BBBP at various times following OMV inoculation (20 ng)

h	Mean ± SE			
	CSF WBC/mm^3		BBBP (%)[a]	
	OMV	Control	OMV	Control
2	0 ± 0 (n = 2)	0 (n = 3)	0.37 ± 0.05	0.08 ± 0.04
4	17,411 ± 3277[b] (n = 9)	0 (n = 9)	5.22 ± 0.90[b,c]	0.37 ± 0.05
6	47,128 ± 3252[b] (n = 7)	0 (n = 3)	3.80 ± 0.50[b]	0.35 ± 0.02
8	51,180 ± 10,014[b] (n = 5)	0 (n = 5)	2.04 ± 0.69[b]	0.35 ± 0.06

[a] Calculated as (counts per minute in CSF/counts per minute in blood) × 100.
[b] $P < 0.05$ compared with control.
[c] $P < 0.05$ compared with results at 2 and 8 h.

ized video densitometer (Bio-Rad Laboratories, Richmond, Calif.). Determination of the LPS content of the vesicles was based on quantitative comparison with purified Hib DL42 and LPS resolved in the same polyacrylamide gradient gel.

The DL42 LPS-specific murine monoclonal antibody 4C4 has been characterized as being directed against a cell surface exposed LPS antigenic determinant on the oligosaccharide portion of the LPS (5). Approximately 5 μg of 4C4 was incubated with OMV containing 20 ng of LPS in 0.1% low-pyrogen bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) for 60 min at 37°C before inoculation. One microgram of antibody is calculated to bind at least 20 ng of LPS, assuming the molecular mass of LPS to be ≈4,000 kilodaltons and that of the immunoglobulin G antibody to be 150,000 kilodaltons (8). Polymixin B (2 μg; Pfizer Inc., New York, N.Y.) was incubated with OMV containing 20 ng of LPS for 15 min at 37°C before inoculation. Pyrogen-free saline (Lyphomed Inc., Rosemont, Ill.) was used in all control inoculations.

Leukopenia was induced by intraperitoneal injection of 100 mg of cyclophosphamide (Adria Laboratories, Columbus, Ohio) per kg of body weight into rats. Leukopenia, defined as a peripheral leukocyte count (WBC) of <1,000/mm^3, occurred reproducibly 4 days after cyclophosphamide treatment (nadir).

The adult-rat model of meningitis was used as previously described (14). Normal or leukopenic (100- to 125-g) Wistar rats (Hilltop Farms, Scottsdale, Pa.) were anesthetized intramuscularly with 100 mg of ketamine (Parke, Davis and Co., Detroit, Mich.) and 7 mg of xylazine (Miles Laboratories, Swanee, Kans.) per kg. Cyclophosphamide-treated rats had an evaluation of peripheral WBC with a hemacytometer at the start of the experiment to ensure leukopenia (WBC, <1,000/mm^3). The rats were then inoculated intracisternally with a 25-gauge butterfly needle fitted into an micromanipulator. After removal of 50 to 75 μl of CSF, 50 μl of a given inoculum (described above) was injected into the cisterna magna. Only inoculations without visible blood contaminations were evaluated. The rats were then allowed to progress for various times (as outlined below). At a given evaluation time, the rat was again anesthetized and the CSF was sampled by the above-described method. Micropipette (25 μl; Dade Scientific Corp., Miami, Fla.) samples were collected for evaluation of CSF WBC and BBBP (see below).

One hour before each evaluation time, each rat was given ≈10 μCi of ^{125}I-labeled bovine serum albumin (ICN Pharmaceuticals, Inc., Irvine, Calif.) via intracardiac injection. At a given evaluation time, 25 μl each of both CSF and blood was obtained and placed in individual 1-dram vials, and samples were counted in a gamma 300 counter (Beckman Instruments, Inc., Irvine, Calif.) for 10 min. The percent permeability of the blood-brain barrier was then calculated as follows: (counts per minute in CSF/counts per minute in blood) × 100. This method has previously been shown to correlate positively with the degree of morphologic change in the blood-brain barrier during experimental meningitis (14).

Statistical comparisons between groups were done by Student's t test, and $P \leq 0.05$ were considered significant.

Evaluations were done at several intervals to assess the kinetics of onset and reversal in BBBP following intracisternal inoculation of OMV containing 20 ng of LPS (Table 1). Two hours after inoculation, neither CSF WBC nor BBBP was different from those of saline-challenged controls. The maximal increase in BBBP was observed at 4 h, with evidence of partial reversal at 6 h and a significant decrease at 8 h ($P < 0.04$) from the observed 4-h peak value. The number of CSF WBC progressively increased from 4 to 8 h following inoculation.

Inoculation of multiple doses of OMV was evaluated at 4 h (the point of maximal BBBP with OMV containing 20 ng of LPS (Table 2). Inoculation of OMV containing 200 pg of LPS resulted in a modest but significant increase in both CSF WBC and BBBP. A peak and statistically equivalent increase in BBBP was observed following inoculation of OMV containing 20 ng, 400 ng, or 1 μg of LPS. A significant attenuation in this peak response was observed following inoculation with OMV containing 4 μg of LPS ($P < 0.05$).

Rats were inoculated intracisternally with OMV containing 20 ng of LPS 4 days following cyclophosphamide treatment (mean peripheral WBC, <300/mm^3) and then evaluated 4 h later for assessment of CSF WBC and BBBP. No CSF pleocytosis was detected, and BBBP was no different in the leukopenic animals ($P = 0.75$) and the saline-challenged controls, as previously observed with purified LPS. In addition, BBBP following inoculation with OMV containing

TABLE 2. *H. influenzae* OMV dose response after 4 h of incubation

Dose	Mean ± SE	
	CSF WBC/mm^3	BBBP (%)[a]
200 pg (n = 5)	2,220 ± 728[b]	1.46 ± 0.21[b]
20 ng (n = 9)	17,411 ± 3,277[b]	5.22 ± 0.9[b]
400 ng (n = 4)	22,900 ± 4,876[b]	5.44 ± 0.9[b]
1 μg (n = 4)	18,025 ± 7,670[b]	7.37 ± 3.2[b]
4 μg (n = 5)	2,850 ± 703[b]	1.14 ± 0.12[b]
Saline (n = 9)	0.0 ± 0.0	0.37 ± 0.05

[a] Calculated as (counts per minute in CSF/counts per minute in blood) × 100.
[b] $P < 0.05$ compared with saline.

TABLE 3. CSF WBC and BBBP with purified LPS and OMV in normal and leukopenic rats

Inoculum	Mean ± SE			
	CSF WBC/mm³		BBBP (%)[a]	
	Normal	Leukopenic	Normal	Leukopenic
Hib LPS (20 ng)	24,294 ± 3,252 (n = 17)	0 (n = 8)	7.97 ± 0.99[b] (n = 17)	0.43 ± 0.08[c] (n = 8)
Hib OMV (20 ng)	17,411 ± 3,277 (n = 9)	0 (n = 4)	5.22 ± 0.90 (n = 9)	0.34 ± 0.07[c] (n = 4)
Saline control	0.0 ± 0.0 (n = 9)	0 (n = 6)	0.37 ± 0.05[c] (n = 9)	0.39 ± 0.08[c] (n = 6)

[a] Calculated as counts per minute in CSF/counts per minute in blood) × 100.
[b] P = 0.09 compared with OMV.
[c] $P < 0.05$ compared with normal LPS and OMV.

20 ng of LPS was statistically equivalent to that previously observed following inoculation with 20 ng of purified LPS (P = 0.09) (Table 3).

Intracisternal inoculation of OMV containing 20 ng of LPS preincubated with 2 μg of PMB resulted in a significant attenuation ($P < 0.01$) in the observed OMV effect on BBBP at 4 h. Preincubation of OMV containing 20 ng of LPS with the 4C4 monoclonal antibody before inoculation did not result in attenuation in the OMV effect on BBBP at 4 h (P = 0.8) (Table 4).

During actual infection, it is unlikely that LPS is present in a purified state. Gram-negative bacteria (including Hib) have been shown to release LPS as part of OMV during specialized growth conditions (1–3, 7, 11). One report (16) described the presence in the CSF of both cell-associated and free-floating outer membrane blebs during meningococcal meningitis.

It has been noted by others that the physical state of LPS might alter its biologic behavior. Munford et al. (13) compared the biologic activity, high-density lipoprotein binding, and fate following intravenous injection of phenol-extracted *Salmonella typhimurium* LPS, outer membrane fragments, intact outer membranes, or whole bacteria. The biologic activity was only slightly greater with the phenol-extracted than with the outer membrane-fragment LPS as measured by *Limulus* lysate assay and rabbit pyrogen test. However, both the phenol-extracted and outer-membrane fragment LPS were equivalent with regard to in vitro and in vivo high-density-lipoprotein binding, with both showing significantly greater high-density-lipoprotein binding and tissue uptake than either intact outer membranes or whole organisms. Therefore, the only native LPS which resembled extracted LPS in activity was that present as outer membrane fragments. This observation correlated with decreased amounts of protein and phospholipid in these fragments compared with that in intact outer membranes. We previously have shown (20) that intracisternal inoculation of purified Hib LPS into rats resulted in significantly greater increases in CSF WBC and BBBP than inoculation with the live Hib, despite the fact that the amount of LPS (20 ng) in each challenge was calculated to be equivalent. This observation may be understandable in light of the study by Munford et al. (13) and prior observations that only 18% of the total LPS of log-phase cultures of *Neisseria meningitidis* was present in the form of blebs from the cell walls (3). These data cannot be directly applied to infection with Hib; however, if only purified LPS or LPS in the form of OMV is biologically active, then it is clear that on a weight basis only a small percentage of the LPS is present in a biologically active form during actual infection.

TABLE 4. Effect of PMB and LPS-specific monoclonal antibodies on Hib OMV-induced CSF WBC and BBBP

Inoculum	Mean ± SE	
	CSF WBC/mm³	BBBP (%)[a]
Saline (n = 9)	0[b]	0.37 ± 0.05[b]
OMV (n = 9)	17,411 ± 3277	5.22 ± 0.90
PMB (2 μg) (n = 4)	25 ± 14[b]	0.97 ± 0.25[b]
PMB (2 μg) + OMV (n = 13)	5776 ± 2022[b]	2.13 ± 0.65[b]
4C4 (n = 4)	0 ± 0[b]	0.35 ± 0.14[b]
4C4 + OMV (n = 5)	14,322 ± 4000[c]	5.60 ± 1.02[c]

[a] Calculated as (counts per minute in CSF/counts per minute in blood) × 100.
[b] $P < 0.05$ compared with OMV.
[c] $P > 0.1$ compared with OMV.

Our study confirms previous observations (13; Syrogiannopoulos et al., 27th ICAAC) that LPS in the form of OMV is similar in activity to purified LPS. Hib OMV inoculation resulted in dose-dependent increases in BBBP and CSF pleocytosis; however, the peak activity was sustained over a broader range of doses than observed following purified LPS inoculation (20). Because of this sustained peak, the attenuation in activity previously noted with higher doses of purified LPS (500 ng and 1 μg) was not observed until doses of >1 μg were inoculated. The explanation for this difference is not readily apparent. However, the amount of LPS in any OMV preparation that is available to interact with the host may vary, and subtle or slight differences in activity between OMV and purified LPS might actually exist despite the fact that such differences did not attain statistical significance at the 20-ng-dose level. As noted above, the biologic activities of extracted *S. typhimurium* LPS and outer membrane fragment LPS were slightly different (13), but no statistical evaluation of this difference was described. As noted in our previous study, the attenuation in biologic activity at higher doses of LPS and now OMV is not without precedent (20), but further study is necessary to explain the mechanism.

In summary, Hib OMV inoculation into rats resulted in both dose- and time-dependent increases in CSF WBC and BBBP. These effects were inhibited by PMB, which specifically interferes with lipid A and depends on an intact neutrophil response, as previously observed with purified Hib LPS (20). Hib OMV appears to be as active on a weight basis as purified Hib LPS and therefore is a relevant vehicle for the delivery of LPS in vivo during meningitis.

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LITERATURE CITED

1. **Bishop, D. G., and E. Work.** 1965. An extracellular glycolipid produced by Escherichia coli grown under lysine limiting conditions. Biochem. J. **96**:567–575.
2. **Deich, R. A., and L. C. Hoyer.** 1982. Generation and release of DNA-binding vesicles by *Haemophilus influenzae* during induction and loss of competence. J. Bacteriol. **152**:855–864.
3. **Devoe, I. W., and J. E. Gilchrist.** 1973. Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J. Exp. Med. **130**:1156–1167.
4. **Gulig, P. A., G. H. McCracken, Jr., C. F. Frisch, K. H. Johnston, and E. J. Hansen.** 1982. Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic *Haemophilus* disease. Infect. Immun. **37**:82–88.
5. **Gulig, P. A., C. C. Patrick, L. Hermanstorfer, G. H. McCracken, Jr., and E. J. Hansen.** 1987. Conservation of epitopes in the oligosaccharide portion of the lipooligosaccharide of *Haemophilus influenzae* type b. Infect. Immun. **55**:513–520.
6. **Ingraham, J. L., O. Maatoe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinaur Associates, Inc., Sunderland, Mass.
7. **Ingram, J. M., K. J. Cheng, and J. W. Costerton.** 1973. Alkaline phosphatase of Pseudomonas aeruginosa: the mechanism of secretion and release of enzyme from whole cells. Can. J. Microbiol. **19**:1407–1415.
8. **Inzana, T. J., W. E. Seifert, Jr., and R. P. Williams.** 1985. Composition and antigenic activity of the oligosaccharide moiety of *Haemophilus influenzae* type b lipooligosaccharide. Infect. Immun. **48**:324–330.
9. **Johnson, K. G., and M. B. Perry.** 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. **22**:29–34.
10. **Lesse, A. J., E. R. Moxon, A. Zwahlen, and W. M. Scheld.** 1988. Role of cerebrospinal fluid pleocytosis and Haemophilus influenzae type b capsule on blood-brain barrier permeability during experimental meningitis in the rat. J. Clin. Invest. **82**:102–109.
11. **Lindsay, S. S., B. Wheeler, K. E. Sanderson, and J. W. Costerton.** 1973. The release of alkaline phosphatase and of lipopolysaccharide during growth of rough and smooth strains of Salmonella typhimurium. Can. J. Microbiol. **19**:335–343.
12. **McDade, R. L., Jr., and K. H. Johnson.** 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. **141**:1183–1191.
13. **Munford, R. S., C. L. Hall, J. M. Lipton, and J. M. Dietchy.** 1982. Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of Salmonella typhimurium lipopolysaccharides. J. Clin. Invest. **70**:877–888.
14. **Quagliarello, V. J., W. J. Long, and W. M. Scheld.** 1986. Morphologic alteration of the blood-brain barrier with experimental meningitis in the rat. Temporal sequence and role of encapsulation. J. Clin. Invest. **77**:1084–1095.
15. **Schlech, W. F., J. I. Ward, J. D. Band, A. Hightower, D. W. Fraser, and C. V. Broome.** 1985. Bacterial meningitis in the United States, 1978 through 1981. The national bacterial meningitis surveillance study. J. Am. Med. Assoc. **253**:1749–1754.
16. **Stephens, D. S., K. M. Edwards, F. Morris, and Z. A. McGee.** 1982. Pili and outer membrane appendages on Neisseria meningitidis in the cerebrospinal fluid of an infant. J. Infect. Dis. **146**:568–574.
17. **Tauber, M. G., U. Borschberg, and M. A. Sande.** 1988. Influence of granulocytes on brain edema, intracranial pressure, and cerebrospinal fluid concentrations of lactate and protein in experimental meningitis. J. Infect. Dis. **157**:456–464.
18. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
19. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohyd. Chem. **5**:83–91.
20. **Wispelwey, B., A. J. Lesse, E. J. Hansen, and W. M. Scheld.** 1988. Haemophilus influenzae lipopolysaccharide-induced blood-brain barrier permeability during experimental meningitis in the rat. J. Clin. Invest. **82**:1339–1346.