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Effect of *Haemophilus influenzae* Type b Lipopolysaccharide on Complement Activation and Polymorphonuclear Leukocyte Function

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ABSTRACT. Purified lipopolysaccharide (LPS) from *Haemophilus influenzae* type b (Hib) was examined for its capacity to interact with human hemolytic complement, generate conversion products of C3, C4, and factor B, stimulate C5a activity, and affect human neutrophil chemiluminescence and phagocytosis. *Salmonella typhimurium* LPS and *Salmonella minnesota* Rb LPS (R345 mutant) were examined for comparison. Incubation of Hib LPS with human serum deficient in γ-globulin or with normal human serum containing 10 mM EGTA and 7 mM $MgCl_2$ resulted in some depletion of hemolytic complement and conversion of C3 to degradation products (determined by inhibition of passive hemolysis and electrophoresis/immunofixation, respectively), indicating that complement activation occurred by the alternative pathway. Complement activation by Hib LPS and *S. minnesota* Rb LPS was similar, but significantly less effective than by *S. typhimurium* LPS ($p < 0.01$). Solubilized Hib lipid A, but not LPS, induced conversion products of C4 in hypogammaglobulinemic serum, indicating activation of the classical pathway. Similar levels of C5a activity were generated by incubation of Hib LPS and *S. tryphimurium* LPS in hypogammaglobulinemic serum, as determined by neutrophil shape change and neutrophil aggregation. Hib LPS directly stimulated neutrophil chemiluminescence, whereas *S. typhimurium* LPS had little effect. Phagocytosis of radiolabeled, opsonized Hib by neutrophils was diminished by *S. minnesota* Rb LPS, Hib LPS, or solubilized Hib lipid A ($p < 0.001$), but was slightly increased by *S. typhimurium* LPS. Neither the oligosaccharide of Hib LPS or Hib capsular polysaccharide was capable of interacting with complement or altering neutrophil chemiluminescence or phagocytosis. These results indicate that in comparison to *S. typhimurium* LPS, Hib LPS was less effective at activating complement, but more effective at impairing polymorphonuclear leukocyte function (*Pediatr Res* 22: 659–666, 1987)

Abbreviations

LPS, lipopolysaccharide
Hib, *Haemophilus influenzae* type b
PMN, polymorphonuclear leukocyte
Lipid A-BSA, the lipid moiety of Hib LPS complexed to bovine serum albumin
HγS, hypogammaglobulinemic serum
VBS, veronal buffered saline
NHS, normal human serum
NHS-EM, normal human serum containing 10 mM EGTA and 7 mM $MgCl_2$
PBS, Dulbecco's phosphate-buffered saline containing 0.2% D-glucose
HBSS-gel, Hanks' balanced salt solution containing 0.1% gelatin.
CPM, counts per minute

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Hib is the etiologic agent of a variety of systemic diseases of children, including meningitis, epiglottitis, pneumonia, and septicemia (1). As for other gram-negative bacteria, Hib has an outer membrane containing an endotoxic LPS. The lipid A component is similar in composition and biologic activity to the lipid A of Enterobacteriaceae (2), but the carbohydrate moiety is an oligosaccharide containing less 3-deoxy-D-*manno*-2-octulosonic acid than enteric LPS and no O side chains (3) (although Hib endotoxin is actually a lipooligosaccharide, the conventional nomenclature LPS will be used in this report) (4).

The repeating O side chains of enteric LPS are capable of activating the alternative complement pathway (5). However, the structure of the carbohydrate, more so than the size, is critical for efficient activation of complement (6–8). In the absence of specific antibody, activation of complement by the alternative pathway can result in bacterial killing (C5b-9), opsonization (C3b), and chemotaxis and activation of PMNs (C5a) (9). However, the O side chains also promote bacterial resistance to the lethal action of normal serum (10, 11), to bactericidal proteins of PMNs (12), and inhibits opsonization and phagocytosis of the bacteria by PMNs (13, 14).

Hib LPS lacks O side chains, however, the polyribosylribitol phosphate capsule protects bacteria from the cidal action of normal serum and antisomatic antibodies and complement (15–18). Depletion of complement in infant rats by cobra venom factor enhances bacteremia and meningitis by Hib (19), indicating the importance of complement in host resistance to Hib infection. Intact Hib is capable of activating the alternative complement pathway (20) and generating C5a activity in normal serum (21). The component of Hib responsible for complement activation has not been identified, but does not appear to be the capsule (20). In addition, serum opsonization and phagocytosis of Hib by PMNs, rather than serum bactericidal activity, appears

to be the most important complement-mediated factor in host defense against systemic infection (22, 23). The effect of Hib components on PMN function, however, has not been examined.

Herein we describe the capability of purified Hib LPS, *Salmonella typhimurium* LPS, and *Salmonella minnesota* Rb LPS to interact with complement, generate conversion product of C3, C4, and factor B, induce C5a activity in the absence of antibody, and to alter PMN chemiluminescence and phagocytosis. Our results indicate that in contrast to *S. typhimurium* LPS, Hib LPS activates complement relatively poorly, and significantly reduces the capability of PMNs to phagocytize opsonized Hib.

MATERIALS AND METHODS

Bacteria and culture conditions. Hib strain Eag was kindly provided by Dr. Porter Anderson, University of Rochester Medical Center, Rochester, NY, and stored at −70° C in sterile skim milk (15). A small aliquot was thawed and streaked on brain heart infusion agar supplemented with 2 mg/liter factor V (nicotinamide adenine dinucleotide) and 5 mg/liter factor X (hemin) (24). Several colonies were picked and grown in brain heart infusion broth supplemented with factors X and V at 37° C with shaking to mid log phase (10^9 colony forming units/ml), determined spectrophotometrically.

LPS and capsular polysaccharide. LPS from Hib strain Eag was isolated by enzyme digestion and phenol/water extraction (3). The chemical and physical properties of Hib Eag LPS have been previously described (3). Lipid-free oligosaccharide and lipid A was obtained by hydrolysis of a 1% solution of LPS in 1% acetic acid for 3 h at 100° C. The carbohydrate moiety was purified by gel filtration on Sephadex G-50 (3) and the lipid was washed in distilled water, dried, and stored at −70° C. The lipid was solubilized with triethylamine and complexed to bovine serum albumin (lipid A-BSA) as described by Galanos *et al.* (25). LPS from *S. typhimurium* and from *S. minnesota* R345 (Rb LPS) was obtained from Sigma Chemical Co., St. Louis, MO, and List Biological Laboratories, Campbell, CA, respectively. Capsular polysaccharide from Hib was obtained from Lilly Research Laboratories, Indianapolis, IN.

Inhibition of hemolytic complement. Interaction of LPS with human complement was measured by inhibition of complement-mediated lysis of opsonized sheep red blood cells as described (26), except that human HγS was used in place of guinea pig serum. The HγS was obtained from a patient with common variable immune deficiency: γ-globulin levels were 125 mg/dl for IgG, 12 mg/dl for IgM, and 7 mg/dl for IgA. The 50% hemolytic complement activity of this serum was previously determined (27) and was normal. Various concentrations of LPS suspended in 20 μl of distilled water were incubated in 100 μl of HγS for 30 min at 37° C. The samples were diluted to 300 μl, and 100 μl were added to 1.5 ml of 1.5% opsonized sheep red blood cells in VBS (M.A. Bioproducts, Walkersville, MD). The mixtures were incubated for 1 h at 37° C and centrifuged at 800 × *g* for 5 min. The absorbance at 546 nm (A_{546}) was determined with a Gilford Rapid Sampler (Gilford, Oberlin, OH). The 100% lysis control included distilled water in place of buffer and the 0% lysis control contained heat inactivated HγS (56° C for 30 min). The anticomplementary activity of various concentrations of LPS was expressed as the percent inhibition of hemolysis. Control samples containing no LPS caused 70% lysis of the erythrocytes. Inhibition of complement mediated lysis by a direct effect of LPS on the erythrocytes was examined by addition of LPS to red blood cell samples for 30 min at 37° C prior to addition of fresh complement. The A_{546} for all samples preincubated with LPS was within 10% of the serum control without LPS. Alternatively, activation of complement by the alternative pathway was measured using pooled NHS chelated with NHS-EM and unopsonized rabbit erythrocytes as described by Riches and Stanworth (28).

Cleavage of complement proteins. Generation of conversion of products of C3, C4, and factor B was determined by electrophoresis of activated serum, followed by immunofixation of complement components. A modification of the method described by Johnson (29) was used. One percent agarose (FMC Corporation, Marine Colloids Division, Rockland, ME) gels (0.1-cm thickness) in barbital buffer (pH 8.6 and ionic strength 0.075) containing 0.058% calcium lactate was solidified on 8 × 9 cm "Gel Bond" mylar sheets (FMC Corporation, Rockland, ME). Concentrations ranging from 0 to 100 μg of LPS in VBS were incubated in 1 ml of NHS, NHS-EM, or HγS for 1 h at 37° C. Activated serum was applied to a well in the sample mask on the paper-blotted cathode side of the gel: for C3 and C4, 1 μl of serum was applied to a 7 × 0.5 mm well, and for factor B, 3 μl was applied to a 10 × 1 mm well. Serum was electrophoresed at 40 mA constant current for 1 h. A 1:1 mixture of anti-C3 serum in saline, neat anti-C4 serum (Atlantic Antibodies, Scarborough, ME) or neat anti-factor B serum (donated by Dr. Gregory Buffone, Department of Clinical Chemistry, Texas Children's Hospital, Houston, TX) was then spread on the gel and covered with parafilm. The gel was incubated in a humid chamber for 1 h, pressed with a sheet of no. 40 Whatman filter paper and several sheets of no. 1 Whatman filter paper, and covered with a weighted glass plate for 10 min. The gel was incubated in saline overnight with shaking, pressed with filter paper as described, thoroughly dried onto the mylar with a hair dryer, and stained with Coomassie Brilliant Blue R-250. The relative densities of the stained complement component bands were determined by densitometric scanning using a Quick-Scan Jr. thin-layer chromatographic densitometer and Quick-Quant III Computer set to the relative percent mode (Helena Laboratories, Beaumont, TX).

Generation of C5a activity in HγS by LPS diluted in VBS was measured by PMN shape change (21, 30) and by PMN aggregation (31, 32). These assays were used because they provide direct measurement of C5a activity (30–32) and because a functional analysis was desired concerning the interaction of Hib LPS with PMNs. A dose response curve plotting percent change in bipolar morphology of PMNs against the concentration of Hib LPS in NHS indicated that the percent of PMNs that underwent shape change increased in a linear fashion above background levels at LPS concentrations of 1 μg/0.2 ml NHS (49 ± 2%) through 6 μg/0.2 ml NHS (80 ± 3%). Concentrations of more than 6 μg/0.2 ml did not increase the percent of PMNs that underwent shape change. The same concentrations of *S. typhimurium* LPS gave results similar to those of Hib LPS. Therefore, additional experiments were performed with 5 μg of LPS/0.2 ml of serum in order to remain below the plateau of the dose response curve.

Isolation of PMNs. Human PMNs from normal adults were separated from heparinized venous blood by sedimentation of erythrocytes in 0.6% dextran. The cells from the resulting leukocyte-rich plasma were centrifuged over a Ficoll-Hypaque solution, as previously described (21). The PMNs were incubated with LPS at this time, as described for each assay. After incubation with LPS, the remaining red blood cells were lysed with distilled water and the mixture containing the PMNs (>95 by Wright stain) was isotonically restored with an equal volume of 1.8% NaCl. PMN suspensions were incubated with LPS in the presence of red blood cells to more closely simulate conditions *in vivo*. Control experiments in which the red blood cells were lysed prior to incubation with LPS indicated that red blood cells had no significant effect ($p < 0.01$) on LPS interaction with PMNs. The effect of LPS on PMNs was, however, slightly more pronounced in the absence of red blood cells (data not shown). Red blood cells were removed to avoid interference in the assays (*e.g.* quenching during chemiluminescence). Following removal of red blood cells the PMNs were sedimented at 800 × *g* for 10 min and resuspended in Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY), with 0.2% D-glucose, pH 7.2 (PBS).

Chemiluminescence. Neutrophils (10^6 in 300 μl of PBS) were incubated with PBS alone or with 1, 10, or 100 μg of LPS for 4

h at 37° C. After incubation, the red blood cells were hypotonically lysed, the PMNs restored to isotonic conditions, and 1.5 ml of PBS was added. One-half ml of each suspension was then dispensed in triplicate to vials that contained 0.1 ml of 10^{-3} M luminol (5-amino-2, 3-dihydroxy, 4-phthalizinedione; Sigma Chemical Co., St. Louis, MO). Quantitation of chemiluminescence in these mixtures (final volume, 1.0 ml) was performed in a liquid scintillation counter (model C2425 Tri-Carb; Packard Instrument Company, Downers Grove, IL) in the out of coincidence mode. Values were obtained from the area under the curve in counts per 60 min with 10-min intervals between counts (33). Data were expressed as the area under the curve for PMNs incubated with LPS minus the area under the curve for PMNs incubated with PBS, divided by the area under the curve for PMNs incubated with PBS, times 100.

PMN phagocytosis of opsonized Hib. PMN suspensions were adjusted to 10^7 cells/ml in RPMI-1640 medium (Gibco) and 0.4 ml of cells were incubated with PBS or 40 μg of LPS in PBS for 16 h in closed 50 × 9 mm Petri plates (Falcon, Oxnard, CA) at 37° C in a humidified chamber without shaking, as described by Hendricks *et al.* (34). After incubation with PBS or sample, distilled water was added to PMN suspensions to lyse red blood cells and the PMNs were restored to isotonic condition with an equal volume of 1.8% NaCl. The PMNs were sedimented at 800 × *g* for 5 min and resuspended in the original volume with HBSS-gel (Gibco). Preliminary experiments were performed to assess the effect of incubation of LPS on the viability of PMNs. On three separate occasions PMNs were incubated for 16 h with 0, 10, or 100 μg of LPS exactly as described above, followed by trypan blue exclusion. Viability of control cells and cells incubated with 10 or 100 μg of LPS was 95% ± 4%. Phagocytosis of opsonized Hib by incubated control cells was 10.5 ± 0.7%. Therefore, the capability of incubated PMNs to phagocytize opsonized Hib in our assay was identical to that previously reported for fresh PMNs (27).

H. influenzae type b was grown overnight in BHI-XV containing 10 μCi ^{3}H-thymidine/ml (New England Nuclear, Boston, MA, specific activity 77.2 Ci/mmol) as previously described (27). The bacteria were adjusted to 10^9 colony forming units/ml, washed three times in PBS (10,000 × *g* for 10 min), resuspended in PBS, and opsonized by incubation in pooled, heat-inactivated NHS (1:1, vol/vol) for 45 min at 37° C with shaking. The opsonized bacteria were washed three times in PBS, resuspended in the original volume with HBSS-gel, and held on ice until needed (<1 h). The NHS used for phagocytosis of Hib had an antibody titer of 1:512 to Hib capsule, determined by enzyme immunoassay (35).

Opsonized bacteria (0.1 ml) were added to 0.25 ml of PMNs at a final ratio of 80:1 in 1.5-ml microfuge tubes (Bel Art Products, Pequannock, NJ) and gently tumbled for 30 min at 37° C. The PMNs were washed three times in HBSS-gel at 150 × *g* for 5 min and the inside of the tube was wiped dry following the last wash. Bacterial uptake was differentiated from adherence by incubation of control PMNs with bacteria at 0° C. Incubation of phagocytic cells with Salmonella at 4° C or less has been documented to inhibit phagocytosis (8). Experiments in our laboratory showed that incubation of opsonized Hib with PMNs for 20 min at 37° C resulted in about 11% association of bacteria with PMNs, whereas incubation at 0° C resulted in less than 3% association after washing. The cells were resuspended in 0.35 ml of PBS and added to 10 ml of Scinti Verse II scintillation cocktail (Fisher, Houston, TX) and counted in a liquid scintillation counter. For each series of experiments, the percentage of uptake of ^{3}H-Hib by neutrophils was determined from the ratio of CPM of washed neutrophils exposed to opsonized bacteria to the CPM of the total number of opsonized bacteria exposed to neutrophils × 100. The CPM of PMNs incubated with ^{3}H-Hib at 0° C was subtracted from the CPM of treated and untreated PMNs incubated with ^{3}H-Hib at 37°C.

Statistics. Significance was determined using the paired Student's *t* test on three or more separate experiments performed in triplicate (36).

RESULTS

Inhibition of complement-mediated hemolysis. The capability of Hib LPS, *S. typhimurium* LPS, or *S. minnesota* Rb LPS to interact with complement in the absence of antibody is shown in Figure 1. The concentration of *S. typhimurium* LPS required to inhibit 50% of the hemolytic activity of HγS was 5 μg/0.1 ml. In contrast, neither Hib LPS or *S. minnesota* Rb LPS caused 50% inhibition of hemolysis at any concentration tested. At 45 μg/0.1 ml Hib LPS inhibited less than 45% of the hemolytic activity of HγS. Concentrations of any LPS of more than 45 μg/0.1 ml of serum did not increase inhibition of hemolysis (data not shown). Hib LPS was somewhat more active than Salmonella Rb LPS, but significantly less active than *S. typhimurium* LPS at all concentrations tested ($p < 0.01$). In experiments not shown, similar differences in inhibition of hemolysis were seen when each LPS was incubated with NHS-EM followed by incubation of the serum with rabbit erythrocytes, which are deficient in sialic acid and activate the alternative complement pathway directly (28). Therefore, although *S. typhimurium* LPS (containing 0 side chains) interacted with most of the available hemolytic complement, Hib LPS (lacking 0 side chains) also had some anticomplementary activity, but only at relatively high LPS concentrations. Inhibition of complement-mediated hemolysis by LPS in NHS-EM indicated that complement activation was by the alternative pathway.

Cleavage of complement proteins by LPS. Quantitative measurement of conversion products of C3, C4, and factor B were made by electrophoresis of activated serum, followed by immunofixation and densitometry. The capability of each LPS to generate conversion products of C3 is shown in Figure 2. Densitometric scanning of dried, stained gels provided quantitative measurement of C3 conversion products by various carbohydrates or LPHs in NHS, NHS-EM, or HγS (Table 1). The capsular polysaccharide of Hib and the lipid-free oligosaccharide

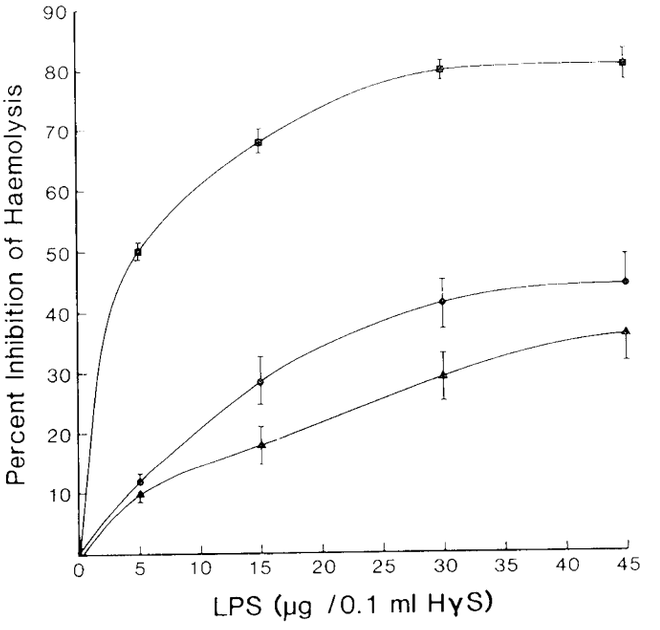

Fig. 1. Inhibition of hemolytic complement activity by LPS. The LPS (5–45 μg in 20 μl of water) was incubated with 0.1 ml HγS for 30 min at 37° C. The serum was diluted to 300 μl with VBS and 100 μl was added to 1.5% sensitized sheep red blood cells in 1.5 ml VBS. *S. typhimurium* LPS, (■– – –■); *S. minnesota* Rb LPS (▲– – –▲); or Hib LPS, (●– – –●). *Vertical bars* represent the SEM of four determinations.

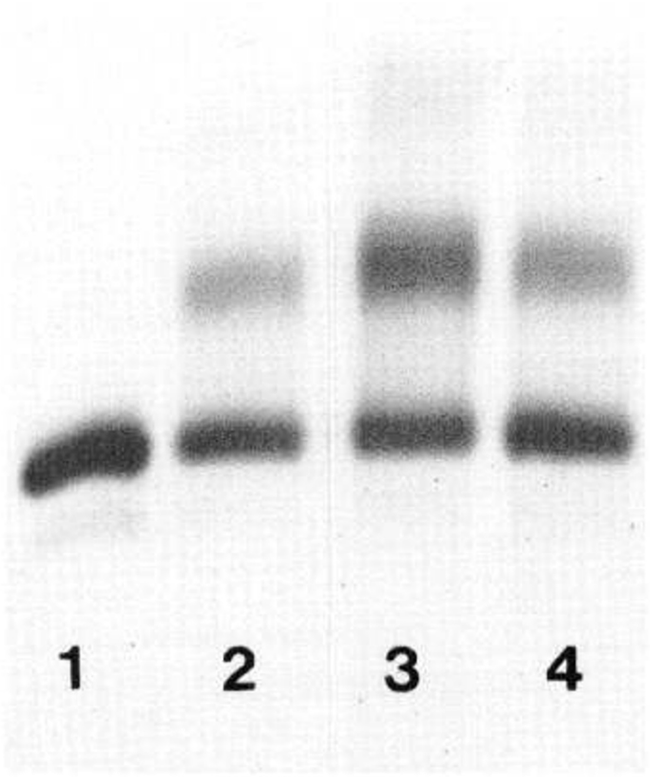

Fig. 2. Immunofixation of C3 conversion products following treatment of HγS serum with LPS. VBS or LPS was incubated in HγS for 1 h at 37° C. The serum was then electrophoresed in 1% agarose and incubated with anti-C3 serum. The gel was washed, dried, and stained with Coomassie Brilliant Blue R250. Lanes and serum treatment: *1*, VBS; *2*, 100 μg/ml Hib LPS; *3*, 100 μg/ml *S. typhimurium* LPS; *4*, 100 μg/ml *Salmonella* Rb LPS.

Table 1. *Conversion of C3 to degradation products in serum by LPS or carbohydrates*

Sample	Percent conversion of C3 to degradation products*		
	NHS	NHS + 10 mM EGTA-Mg^{++}	HγS
Veronal buffered saline	<1	<1	<1
Capsular polysaccharide (100 μg/ml)	<1	<1	<1
Hib (Eag) lipid-free oligosaccharide (100 μg/ml)	<1	<1	<1
Hib (Eag) LPS (100 μg/ml)	41 ± 2.9	36 ± 4.0	38 ± 1.5
S. minnesota Rb LPS (100 μg/ml)	ND†	ND	40 ± 1.9
S. typhimurium LPS (100 μg/ml)	66 ± 1.4	56 ± 1.3	55 ± 1.5

* Percent conversion was determined by densitometry of dried, stained gels following electrophoresis and immunofixation.

† Not determined.

derived from Hib LPS did not cleave C3 in any serum used, whereas each intact LPS cleaved C3 in all sera tested. There was not a significant difference in the quantity of C3 conversion products formed in serum with only alternative pathway activity (NHS-EM) or in serum with classical and alternative pathway activity (NHS). However, significantly greater amounts of C3 conversion products were formed by *S. typhimurium* LPS compared with Hib LPS ($p < 0.01$ in NHS), whereas the amount of C3 conversion products generated by Hib LPS and *S. minnesota* Rb LPS was similar.

Lipid A from Hib Eag solubilized with bovine serum albumin cleaved most of the C4 present in HγS (Table 2), indicating Hib lipid A activated the classical pathway, as does enteric lipid A (5, 25). Each intact LPS failed to generate any detectable level of C4 conversion products, but did generate conversion products of factor B, a component of only the alternative pathway (Table 2). Therefore, the carbohydrate moieties of both rough and smooth LPSs apparently blocked activation of the classical pathway by lipid A.

Generation of C5a activity was assayed by alteration of PMN bipolar morphology or aggregation after incubation of HγS with Hib LPS or *S. typhimurium* LPS. Preliminary experiments demonstrated that when the concentration of Hib LPS was increased from 0.1 to 6 μg/0.2 ml, the percent of PMNs undergoing bipolar shape change increased linearly in a manner similar to that seen with increasing C5a concentration (30). This effect was not due to a direct effect of LPS on the PMNs because the percent change in bipolar morphology of control PMNs incubated with LPS and heat-inactivated serum (56° C for 30 min) remained constant; percent shape change in these controls was similar to control PMNs incubated with fresh serum without LPS. Serum incubated with either Hib LPS or *S. typhimurium* LPS induced a similar increase in the percentage of PMNs that underwent shape change (Fig. 3). Incubation of fresh HγS with anti-C5 serum, after incubation with either *S. typhimurium* LPS or Hib LPS, reduced activity nearly to that of HγS incubated with buffer only, indicating the change in PMN morphology was most likely associated with generation of C5a. In experiments not shown herein, HγS incubated with Hib LPS or *S. typhimurium* LPS could also induce PMN aggregation, another method for determination of C5a activity (31, 32). As for alteration in bipolar morphology, serum incubated with Hib LPS or *S. typhimurium* LPS did not differ in capacity to induce PMN aggregation.

Effect of LPS on PMN chemiluminescence. In preliminary experiments, 10^6 PMNs were incubated with 1, 10, or 100 μg of Hib LPS or *S. typhimurium* LPS for 1, 4, or 16 h, followed by measurement of the chemiluminescence response in the presence of luminol. The optimal incubation time was 4 or 16 h, although a response occurred after at least 1 h; 4 h was used for additional experiments. One or 10 μg of either Hib LPS or *S. typhimurium* LPS had no significant effect on PMN chemiluminescence ($p > 0.05$), whereas 100 μg of Hib LPS—but not *S. typhimurium* LPS—significantly increased chemiluminescence compared with control PMNs incubated with PBS ($p < 0.001$) (Table 3). Therefore, Hib LPS was capable of directly stimulating a PMN metabolic burst, whereas *S. typhimurium* LPS did not.

Effect of LPS on PMN phagocytosis. Neutrophils (10^7/ml) were pretreated with Hib LPS (100 μg/ml) or buffer (RPMI-1640 medium) and incubated with opsonized, ^{3}H-labeled Hib at 37 or 0° C for 5, 10, or 20 min; CPM of PMNs incubated with ^{3}H-Hib at 0° C was subtracted from CPM of treated or untreated PMNs incubated with Hib at 37° C to differentiate adherence from uptake. Phagocytosis of opsonized Hib by PMNs pretreated with buffer was 10–11%, which is identical to the extent of uptake previously reported for this bacterium at this bacterium:PMN ratio (27). There was a significant ($p < 0.001$) decrease in the rate of phagocytosis of opsonized Hib by PMNs pretreated with Hib LPS in comparison to controls incubated with buffer, and the decrease was linear with respect to time of incubation (Fig. 4). Therefore, incubation of human PMNs with Hib LPS was detrimental to PMN phagocytic activity for opsonized Hib.

Comparative analysis of each compound's effect on PMN phagocytosis is shown in Figure 5. In comparison with buffer, Hib LPS significantly decreased PMN phagocytosis of opsonized Hib ($p < 0.001$), whereas *S. typhimurium* LPS moderately increased phagocytosis ($p > 0.05$). *S. minnesota* Rb LPS and the

Table 2. *Cleavage of C4 and factor B by LPS or Hib lipid A in HγS*

Sample	% conversion of C4 to degradation products*	% conversion of factor B to degradation products*
Veronal buffered saline	<1	<1
Hib (Eag) LPS + LPS antibody	27 ± 1.7	ND†
Hib (Eag) Lipid A-BSA‡ (100 μg/ml)	83 ± 1.5	ND
Hip (Eag) LPS (100 μg/ml)	<1	15 ± 1.2
S. minnesota R345 LPS (100 μg/ml)	<1	12 ± 2.1
S. typhimurium LPS (100 μg/ml)	<1	33 ± 1.5

* Percent conversion was determined by densitometry of dried, stained gels following electrophoresis and immunofixation.

† Not determined.

‡ Free lipid A was solubilized with 1 μl of triethylamine/ml of lipid A (2 μg/ml), mixed with 2 μg/ml of BSA, and the lipid A-BSA were complexed by lyophilization.

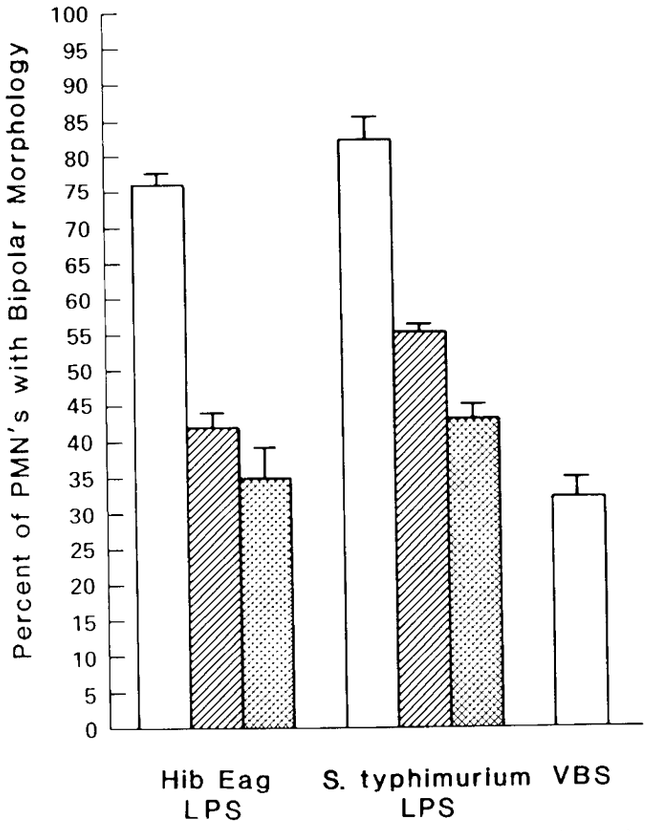

Fig. 3. Change in bipolar morphology of PMNs following incubation of HγS with LPS. Purified LPS from Hib Eag or *S. typhimurium* (5 μg) was incubated with untreated (no lines) or heat-inactivated (*single slashed lines*) HγS for 1 h at 37° C before incubation of serum with PMNs. Anti-C5 serum was incubated with fresh HγS (*cross-hatch lines*), at 37° C for 30 min immediately following incubation with LPS. VBS was used as diluent and to control for nonspecific activity of HγS. *Vertical bars* represent the SEM of three determinations.

solubilized lipid moiety of Hib LPS also decreased phagocytosis to a level similar to that induced by Hib LPS. Preincubation of PMNs with Hib lipid-free oligosaccharide or capsular polysaccharide prior to incubation with opsonized, ^{3}H-Hib, however, did not enhance or diminish PMN phagocytosis (data not shown). Therefore, the lipid moiety of Hib LPS had a detrimental effect on PMN phagocytosis, and its activity was not substantially blocked by the oligosaccharide moiety. Similarly, the oligosaccharide of Salmonella Rb LPS did not influence the effect of lipid A on PMN phagocytosis, whereas the high molecular weight polysaccharide of *S. typhimurium* LPS did appear to exert a moderating effect.

Table 3. *PMN chemiluminescence in response to Hib LPS or S. typhimurium LPS**

LPS	Concentration (μg/10^6 PMNs)	
	10	100
	% increase in chemiluminescence†	
H. influenzae b (Eag)	18 ± 10.4	68 ± 10.6
S. typhimurium	27 ± 10.5	13 ± 11.5

* LPS (10 or 100 μg in PBS) or PBS only was incubated with 10^6 PMNs for 4 h at 37° C. The red blood cells were lysed in distilled water to avoid quenching, and the PMNs isotonically restored and diluted in PBS. Luminol was added and the total counts per 60 min determined. Values are expressed as the percent increase in chemiluminescence by PMNs incubated with LPS relative to PMNs incubated with PBS.

† Values represent the mean of at least three experiments performed in triplicate ± SEM.

DISCUSSION

Functional complement activity is essential for host resistance to Hib infections (19). Hib has been reported capable of activating the alternative complement pathway (20). However, anticapsular antibody is required for bactericidal activity in normal serum (37, 38); antisomatic antibodies to Hib do not activate complement-mediated bactericidal activity by the alternative pathway (37). There is evidence that the most critical complement-mediated event in host resistance to Hib infection is opsonization and phagocytosis by PMNs, rather than bactericidal antibody (22, 23). Therefore, study of the interaction of purified Hib components with complement may aid in understanding the pathogenesis of Hib disease. Hib LPS is a biologically active endotoxin (2), however, the interaction of purified Hib LPS with host defense systems has not previously been reported. We examined the capability of Hib LPS to interact with human PMNs in comparison to *S. typhimurium* LPS and Salmonella Rb LPS. Since the physical and chemical properties of LPS may influence their biologic activity, Hib Eag LPS was used because it has been characterized more extensively than any other Hib LPS (2, 3). Furthermore, Rb LPS from Salmonella R345 was used for comparison because the molecular weight and solubility of the Rb LPS was similar to that of Hib Eag LPS (3) (Inzana T, unpublished data), and because the interaction of rough and smooth Salmonella LPS for complement and PMNs has been characterized (5, 26, 39–41).

Activation of the alternative complement pathway has been shown to be a function of the O side chain repeating units of LPS (5). Therefore, as expected, *S. typhimurium* LPS was highly active in interacting with complement. The concentration of *S. typhimurium* LPS required for 50% inhibition of complement-mediated hemolysis in our study was similar to that reported by Galanos and coworkers (26, 39). Salmonella Rb LPS has previ-

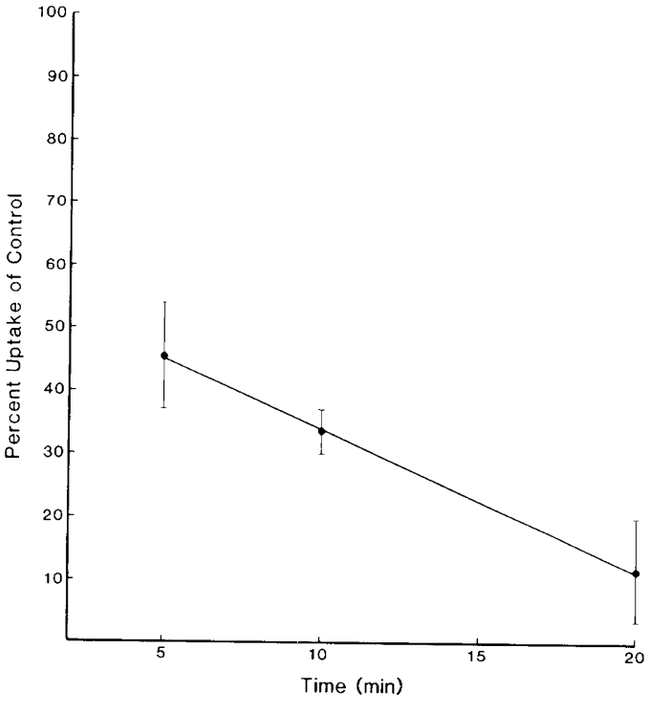

Fig. 4. Effect of Hib LPS on kinetics of PMN phagocytosis of opsonized Hib. LPS (40 μg) or PBS was incubated with 4×10^6 PMNs for 16 h at 37° C in 5% CO_2. The red blood cells were lysed and the washed PMNs were incubated with opsonized, radiolabeled Hib (1:80) at 37 or 0° C for 5, 10, or 20 min. The PMNs were washed three times and the percent uptake of radiolabeled bacteria by PMNs was determined by liquid scintillation counting relative to total bacterial counts. The CPM of PMNs incubated with ^{3}H-Hib at 0° C was subtracted from the CPM of PMNs incubated with ^{3}H-Hib at 37° C, and the percent uptake of Hib was determined. Values are expressed as the percent uptake of ^{3}H-Hib by PMNs treated with LPS relative to control PMNs incubated with PBS. The *vertical bars* represent the SEM of three determinations.

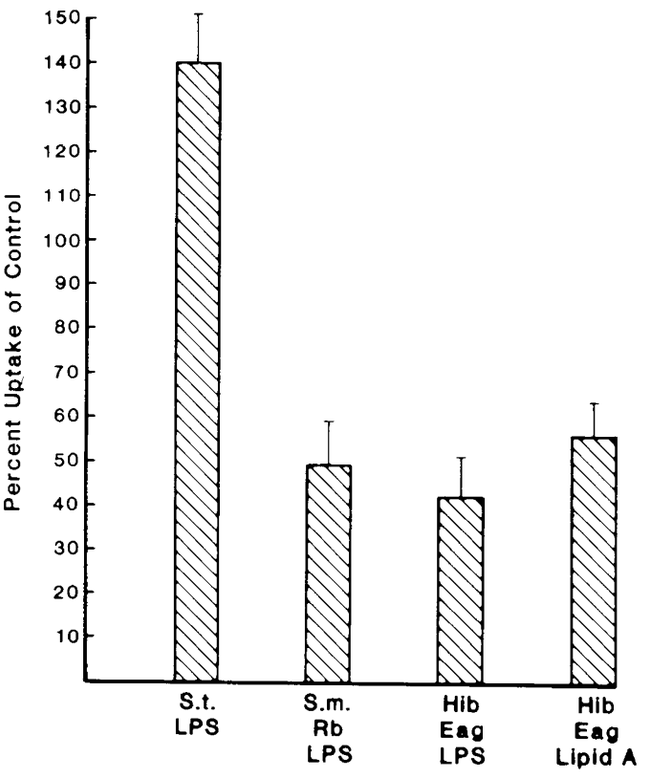

Fig. 5. Effect of LPS or Hib lipid A on PMN phagocytosis. PMNs were pretreated with each LPS or solubilized lipid A (40 μg), or PBS, as described in Figure 4, and incubated with radiolabeled, opsonized Hib for 20 min at 37 or at 0° C. The PMNs were washed and assayed as described in Figure 4. *Vertical bars* represent the SEM of four determinations.

ously been shown to be a poor activator of the complement system (26, 39), which was confirmed by our results. Hib LPS was capable of initiating activation of the alternative complement pathway, but only at relatively high concentrations. In addition to consumption of hemolytic complement, each LPS generated conversion products of C3 and factor B in sera with only alternative complement pathway activity. As for inhibition of complement consumption, however, immunofixation indicated that *S. typhimurium* LPS was significantly more active than Hib LPS or Salmonella Rb LPS. Although immunofixation cannot be used to quantitate generation of C3b, the technique is highly efficient and easily interpreted. When combined with scanning densitometry, immunofixation provides accurate quantitative measurement of complement activation and conversion to degradation products (42). However, C3d and other low molecular weight conversion products may not be detected by the assay. Failure of bacteria to efficiently activate complement results in poor opsonophagocytosis and may thereby enhance virulence (43). In addition, although 0 side chains activate complement they also provide the bacterium protection against the bactericidal activity of serum (10, 11). Hib LPS lacks 0 side chains (3), however, the capsule provides protection to Hib against the bactericidal activity of antisomatic antibodies and complement (16–18). Therefore, the combination of a protective capsule and an LPS that is a poor activator of complement may act together to enhance bacterial resistance to host defense mechanisms.

Hib lipid A was shown to be capable of activating the classical complement pathway, but only after the carbohydrate moiety was removed and the lipid complexed to bovine serum albumin. These results are identical to those reported by Galanos *et al.* (25) for enteric lipid A. Studies have shown that even a relatively small amount of carbohydrate can block the activation of C1 by lipid A (44). Therefore, it would be unlikely that activation of the classical complement pathway by Hib lipid A would occur *in vivo*.

Intact Hib has previously been shown to be capable of generating C5a in serum, but the somatic components responsible were not identified (21). C5a is chemotactic for PMNs and activates them, resulting in aggregation and changes in their bipolar morphology (30–32). These assays were selected because they directly measure the functional activity of C5a, which was more relevant to these studies than immunochemical quantitation. Inhibition of change in bipolar morphology with anti-C5 serum or heated HγS further indicated that changes in bipolar morphology were due to C5a. Endotoxins have been reported to generate C5a activity (41) and therefore it was not unexpected to find that Hib LPS could generate C5a activity. Of interest was that C5a activity in serum incubated with Hib LPS was as great as that of serum incubated with *S. typhimurium* LPS. We found PMN shape change to be a very sensitive assay for C5a activity. Since nanogram quantities of either LPS incubated in serum could induce PMN shape change, only small amounts of C5a may need to be generated to demonstrate activity in this assay.

Generation of C5a by bacterial components would normally operate as a defense mechanism against the bacteria by attracting PMNs to the site of infection. Bacterial components that interfere with clearance or killing of bacteria by PMNs, however, may be important virulence factors. The effect of Enterobacteriaceae

LPS on PMN function has been well studied and recently reviewed (41). We examined the direct effect of Hib LPS on PMN function, as determined by measurement of chemiluminescence and phagocytosis. Hib LPS, but not *S. typhimurium* LPS, could directly stimulate chemiluminescence by human PMNs. In addition, Hib LPS significantly decreased the capability of PMNs to ingest opsonized Hib. Salmonella Rb LPS and Hib lipid A also diminished PMN phagocytosis, whereas the capsular polysaccharide and the lipid-free oligosaccharide of Hib LPS had no effect. Therefore, the lipid A of Hib would appear to be the component responsible for the detrimental effect of LPS on PMN phagocytosis. *S. typhimurium* LPS, in contrast, had a slight stimulatory effect on PMN phagocytosis. These results are consistent with those reported by Hendricks and coworkers (34, 45), who compared th effect of *Escherichia coli* J5 LPS to that of the smooth parent, *E. coli* 0111:B4. *E. coli* J5 LPS, but not 0111:B4 LPS, diminished PMN phagocytosis and chemotaxis, and directly stimulated PMNs in chemiluminescence studies. The lipid A was determined to be the active moiety and the detrimental effect of the LPS could be prevented by scavengers of oxygen radicals. The authors presented evidence that generation of oxygen radicals, through stimulation of the PMNs by lipid A, was toxic for the phagocytic cells and inhibited their antibacterial functions. The large amount of carbohydrate present on *E. coli* 0111:B4 LPS appeared to block the activity of the lipid A on PMNs, but the core oligosaccharide of *E. coli* J5 LPS did not. Enterobacteriaceae lipid A has been shown to bind to receptor sites on PMNs and thereby influence PMN function (40). It is conceivable that a large polysaccharide, but not an oligosaccharide, can block the binding of lipid A to the receptor sites on PMNs. The antigenic (46) and biologic (2) similarity of Hib lipid A to enteric lipid A would suggest the same mechanism of action is responsible for affecting PMN function by Hib lipid A, and that the oligosaccharide does not block this activity.

Hib LPS has been shown not be microheterogenous in respect to molecular weight (47). This variation in molecular weight (and probably structure) may play a partial role in influencing the relative virulence of some Hib strains. Zwahlen *et al.* (48) showed that when a virulent strain of Hib was transformed with a 10 kilobase-pair fragment of Hib DNA, the transformant developed a lower molecular weight LPS, an alteration in one outer membrane protein, and became serum-sensitive *in vitro* and avirulent in rats; there was no change in capsular polysaccharide content. Kimura and Hansen (49) isolated two strains of Hib that failed to react with two monoclonal antibodies reactive with all other strains tested and were less virulent than other Hib strains for infant rats. Isogenic variants were isolated that reacted with the monoclonal antibodies and that were virulent in rats. There was no change in the content of capsular polysaccharide or in the outer membrane protein profile, but the LPS of each variant was of higher molecular weight than the LPS of the parent strains. These results indicated an alteration in the structure or molecular weight of the LPS appeared to play some role in increasing the virulence of Hib. Furthermore, Grossman and Levie (6) and Liang-Takasaki *et al.* (7, 8) have shown that for Salmonella LPS the structure of the 0 side chains is more important than length in regard to complement activation and generation of opsonic C3b. In regard to Hib, different strains with structural differences in their LPS may interact with complement and affect PMN functions differently, which in turn may influence the virulence of individual strains.

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