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Research Article

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Characterization of Antigens from Nontypable *Haemophilus influenzae* Recognized by Human Bactericidal Antibodies

Role of *Haemophilus* Outer Membrane Proteins

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Abstract

Major outer membrane antigens, proteins, and lipopolysaccharides (LPSs), from nontypable *Haemophilus influenzae* were characterized and examined as targets for complement-dependent human bactericidal antibodies. Outer membranes from two nontypable *H. influenzae* isolates that caused otitis media and pneumonia (middle ear and transtracheal aspirates) were prepared by shearing organisms in EDTA. These membranes were compared with membranes prepared independently by spheroplasting and lysozyme treatment of whole cells and found to have: similar sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of the proteins; identical densities (ρ = 1.22 g/cm^3); and minimal *d*-lactose dehydrogenase activity indicating purity from cytoplasmic membranes. Outer membranes were solubilized in an LPS-disaggregating buffer and proteins were separated from LPS by molecular sieve chromatography. The SDS-PAGE patterns of outer membrane proteins (OMPs) from the two strains differed in the major band although other prominent bands appeared similar in molecular weight. LPS prepared by hot phenol water extraction of each of the strains contained 45% (pneumonia isolate) and 60% (otitis isolate) lipid (wt/wt), 49% and 50% carbohydrate (wt/wt), respectively, and <1%, 3-deoxy-*manno* octulosonic acid.

Immunoglobulin M (IgM) purified from normal human serum (NHS) plus complement was bactericidal for both strains. Purified immunoglobulin G (IgG) from NHS killed the middle ear isolate and immune convalescent IgM from the serum of the patient with pneumonia killed his isolate. NHS or convalescent serum were absorbed with OMPs and LPS (0.6–110 μg) from each of the strains and immune specific inhibition of bactericidal antibody activity by each antigen was determined. OMPs from the pulmonary isolate inhibited bactericidal antibody activity directed against the isolate in both NHS (1.5 μg of antigen) and immune serum (0.75 μg of antigen). OMPs (60 μg) from the ear isolate also inhibited bactericidal activity in the respective immune serum. LPSs exhibited minimal inhibition (>110 μg). Three human sera (two normal, one immune) were selectively depleted of 80% of antibody activity against OMPs (measured by enzyme-linked immunosorbent assay) by affinity chromatography using OMPs from the pulmonary isolate coupled to a solid phase. These OMP antibody-depleted sera also showed an 88% reduction of bactericidal activity against this strain. Immunopurified antibody against OMPs eluted from the solid phase was bactericidal.

Introduction

Unencapsulated or nontypable (NT)[1] *Haemophilus influenzae* are isolated frequently from middle ear effusions in acute and recurrent otitis media in children (1) and from lower respiratory tract secretions of adults with chronic bronchitis (2, 3). NT *H. influenzae* is recognized as a pathogen that causes middle ear infection in children and sinusitis in children and adults (4, 5), and increasingly it is being reported as an important cause of pneumonia (6), particularly in the elderly (7, 8) and the hospitalized patient (9). Transmission of NT *H. influenzae* from parturient women to their offspring with consequent bacteremia in the newborn has also been recognized, and indeed bacteremia may also occur in the mother (10, 11). NT *H. influenzae* not only occasionally colonizes the pregnant woman at term, but it may also be recovered from fallopian tubes or peritoneal exudate cultures in a few women with pelvic inflammatory disease (12). Although little is known currently about the unique properties of unencapsulated *H. influenzae* that might enable it to produce these specific types of infections, its antigenicity may prove important in defining its interaction with the human host in a manner similar to that described for type b *H. influenzae* (13–15) and other bacteria (16–19).

The outer membranes of Gram-negative bacteria are immunologically important structures because of their accessibility to host defense mechanisms. As in many Gram-negative bacteria, the major antigenic constituents of the outer membranes of *H. influenzae* are comprised of proteins (OMPs) (15) and lipopolysaccharides (LPSs) (20). Differences in the antigenic make-up of *H. influenzae* have been demonstrated by serologic methods, using antibodies directed against surface antigens including purified LPSs (14, 20) and antigens enriched in protein (21). The identification of size differences in OMPs analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel

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1. *Abbreviations used in this paper:* C', complement; CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; *d*-LDH, *d*-lactate dehydrogenase; KDO, 3-deoxy-*manno* octulosonic acid; LPS, lipopolysaccharide; NaD, sodium deoxycholate; NT, nontypable; OMP, outer membrane protein; PAGE, polyacrylamide gel electrophoresis.

electrophoresis (PAGE) also suggests that OMPs of different *H. influenzae* may contain unique antigenic determinants (15, 22–26).

To evaluate the role of surface antigens of NT *H. influenzae,* we used an immunologic assay that detects functioning antibodies that might contribute a host-protective effect against the organism, complement-mediated bactericidal antibody activity (27). Although these antibodies are prevalent in adult human sera (28), children with otitis media caused by NT *H. influenzae* lack these antibodies against NT *H. influenzae* more often than children with non-*Haemophilus* otitis media. However, most infected children develop bactericidal antibodies in response to the infection (27). Although in humans the bactericidal antibody activity against encapsulated *H. influenzae* (e.g., *H. influenzae* type b) often is specific for the capsule, other noncapsular antigens located in the outer membrane are also targetted for attack by bactericidal antibodies (13, 14); in unencapsulated strains, noncapsular antigens may be the major targets for bactericidal antibody. Here we report methods for the separation of NT *H. influenzae* outer membranes which can then be fractionated into an array of proteins, separate from LPS. These outer membrane proteins isolated from two respiratory isolates of NT *H. influenzae* were shown to contain major antigenic targets for human immune and natural antibody killing.

Methods

Sources of antibodies

Normal human sera were obtained from 10 adult volunteers; immune sera were obtained from a child with otitis media and an adult patient with lobar pneumonia 2 and 3 wk after the onset of their acute illnesses, respectively. Each were infected with NT *H. influenzae.* These sera and their derivatives were used as sources of antibodies.

Strains

We employed two strains: one was a β-lactamase producing strain isolated in pure culture from the transtracheal aspirate of the adult with pneumonia (BCH-1). This patient showed neither a clinical response nor clearing of the organisms when he was treated initially with ampicillin, but he rapidly improved when treated with chloramphenicol. The second isolate (BCH-2) was the only organism isolated upon tympanocentesis of the young child with acute otitis media. This strain was referred to as strain 37567 in a previous publication (27).

NT *H. influenzae* were identified according to standard methods (29); the organisms failed either to show positive quellung or agglutination reactions when tested with a panel of antisera a–f (typing sera obtained from the Centers for Disease Control). In addition, saline suspensions of each of the two strains failed to show lines of precipitation in counterimmunoelectrophoresis (30) compared to similar suspensions of known serotypes. A latex agglutination assay (31) for capsular antigen of *H. influenzae* type b was also negative when suspensions of organisms were compared with similar suspensions of *H. influenzae* type b. Both strains were biotype III (29). Organisms were stored frozen at −70°C in brain heart infusion broth supplemented with horse blood lysate and NAD (13) after two subpassages from the clinical isolate.

Media and growth conditions

For mass culture of organisms, one loopful of thawed organisms was transferred to chocolate agar plates and grown for 16 h at 37°C. The plates were swabbed with a moistened cotton tipped swab and inoculated into 40 ml of brain heart infusion broth, supplemented as indicated above (13). The 40-ml aliquots were grown (37°C) in 2-liter Erlenmeyer flasks and were stirred with a magnetic stirrer to produce aeration.

Total volumes (10^8 colony-forming units [CFU]/ml) were harvested by centrifugation at 12,000 *g* (4°C) and washed three times with 0.15 M NaCl. Contamination was checked at the end of each growth cycle. For experiments that required the production of radiolabeled antigen, intrinsic labeling of the organisms was accomplished by growing the bacteria in the presence of 5 mCi of [^{3}H]sodium acetate (sp act, 75–150 mCi/mmol, New England Nuclear, Boston, MA) per liter of broth. We also used solid media (chocolate agar [1%]) to grow organisms for antigen production; these were grown for 16 h at 37°C, harvested by scraping organisms off the plates with rubber spatulas, and subsequently prepared identically to broth grown organisms.

Isolation and purification of cell wall antigens

Preparation of outer membranes by EDTA incubation and shearing of whole organisms. Pelleted organisms were suspended at room temperature in a buffer containing 0.05 M Na_2PO_4, 0.15 M NaCl, and 0.01 M EDTA, adjusted to pH 7.4 (EDTA buffer). The suspension of organisms was incubated at 60°C for 45 min, subjected to mild shearing by passage through a 25-gauge hypodermic needle attached to a 50-ml syringe (manual pressure), and mixed for 10 s in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, CT). In experiments designed to test the purity of outer membranes, which included *d*-lactate dehydrogenase (*d*-LDH) assays performed on membrane preparations, the suspensions were not heated prior to shearing. Whole organisms were separated from the suspension by centrifugation at 12,000 *g* (4°C) for 20 min. Ultracentrifugation of the supernatant at 80,000 *g* for 2.5 h (4°C) was performed to pellet the outer membranes (EDTA preparations).

Assessment of purity of outer membranes prepared by EDTA incubation and shearing of whole organisms with cell wall fractions prepared by spheroplasting and lysozyme treatment of whole organisms. The purity of outer membranes prepared by EDTA incubation and shearing of whole organisms (EDTA preparations, above) was compared with fractionated cell wall membranes prepared by spheroplasting and lysozyme treatment of organisms (spheroplast/lysozyme preparations) followed by density gradient centrifugation of recovered cell walls to separate outer and cytoplasmic membranes. Measurements performed to assess purity of EDTA preparations included: (*a*) the determination of their density and comparison with fractions obtained from the spheroplasting experiments; (*b*) the comparison of *d*-LDH activity, a marker for cytoplasmic membranes, in all the preparations; and (*c*) comparisons of protein patterns and presence or absence of lipopolysaccharides by SDS-PAGE.

In preparing cell wall membranes for fractionation, whole NT *H. influenzae* cells (prepared as described above) were suspended in 0.01 Tris–HCl in 25% (wt/wt) sucrose (pH 7.5) (32) at a concentration of 1 g of cells (wet wt) per 16.7 ml of buffer (33). The suspension was kept chilled (4°C) for 5 min (34), then lysozyme (Sigma Chemical Co., St. Louis, MO) was added to a concentration of 10 μg/ml (35), the suspension was mixed gently with a glass stirring rod for an additional 2 min and EDTA (disodium salt) added to a final concentration of 0.01 M. Spheroplasting was monitored by measuring a decrease in OD 650 nm of the suspension (performed on aliquots diluted 1:25 in water) and by 5 min 90% of the decrease in OD had been achieved (33). DNAase (Sigma Chemical Co.) was added to a concentration of 100 μg/ml (22) and 4 vol of cold deionized and distilled water were added (32). The mixture was stirred with a magnetic stirrer (4°C) for 90 min, then centrifuged at 1,470 *g* for 20 min (4°C) to remove remaining intact cells. The supernatant was then ultracentrifuged at 105,000 *g* (4°C) for 1 h to pellet the crude cell wall membranes (spheroplast/lysozyme preparations).

Cell wall membranes prepared by spheroplasting and lysozyme

treatment of whole cells, and recovered by ultracentrifugation, were resuspended in a solution (diluted 1:4 with cold deionized and distilled H_2O) consisting of 0.01 M Tris–HCl, 0.01 M EDTA (pH 7.5) (Tris/EDTA buffer), and 25% (wt/wt) added sucrose. These membranes were ultracentrifuged at 105,000 *g* (4°C) for 1 h. 85 mg of membrane protein (36) recovered from the pellet was suspended in 4.89 ml of 0.01 M Tris–HCl (pH 7.5) and layered onto a discontinuous gradient ($3\frac{1}{2} \times \frac{9}{16}$-in Ultra-Clear M tubes, No. 344059, Beckman Instruments, Spinco Div., Palo Alto, CA) consisting of a 2.3-ml cushion (bottom) of 70% sucrose (wt/vol) in Tris/EDTA buffer overlaid by 4.32 ml of 15% (wt/vol) sucrose in the same buffer. Outer membranes separately prepared by EDTA incubation and shearing of whole organisms (EDTA preparations, above) were similarly applied to separate tubes containing the same gradient. These gradients were centrifuged in a swinging bucket rotor, SW-41 Ti, (Beckman Instruments, Inc., Fullerton, CA) for $4\frac{1}{2}$ h at 102,000 *g* (4°C). Material that was centrifuged to the interface between the sucrose layers was collected from above, brought up to 3.74 ml with 0.01 M Tris–HCl buffer (pH 7.5) and then transferred to a second discontinuous gradient consisting of a 2.01-ml cushion (bottom) of 70% (wt/vol) sucrose in Tris/EDTA buffer, overlaid by 4.31 ml of 50% sucrose (wt/vol) in the same buffer, which was, in turn, overlaid by 1.44 ml of a third sucrose solution, 15% (wt/vol) in the buffer (37). These gradients were centrifuged for 14 h (4°C) at 102,000 *g*. The 11.5 ml were fractionated into 26 0.44-ml aliquots and protein concentration was determined for each fraction (36).

Protein containing fractions from the gradients of the spheroplast/lysozyme preparations were located at each of the two interfaces and from the EDTA preparation at the lower interface only. Spheroplast/lysozyme fractions, located at each of the interfaces, and EDTA fractions located at the lower interface, were separately pooled to create three new preparations. Each of these was combined with similar fractions containing the same starting material run in other tubes, and dialyzed against 0.01 M Tris–HCl (pH 7.5) to remove the sucrose; combined fractions were each concentrated to 1.6 mg/ml of protein by pressure filtration (Amicon Corp., Lexington, MA) to be applied to a third gradient. This gradient was composed of seven 1.3-ml sucrose fractions ranging in concentrations from 30 to 60% (wt/vol), advanced in increments, in Tris/EDTA buffer. 1-ml fractions from each of the three newly created preparations were layered onto separate gradients and centrifuged at 102,000 *g* (4°C) for 10 h to isopycnic conditions. The tubes were fractioned into 26 0.43-ml aliquots starting at the top and protein and *d*-LDH concentrations determined for the fractions. Bouyant densities of protein fractions were determined by measuring the index of refraction of sucrose solutions in corresponding fractions (refractometer, Bausch & Lomb Inc., Rochester, NY) and the densities were calculated from standard tables.

d-LDH assays were performed on protein containing fractions by using reaction mixtures to which increasing concentrations of membrane fractions were added to a final volume of 1 ml. First, 30 μg of 2,5-diphenyl-3-4,5 dimethyl-2-thiazolyl monotetrazolium bromide (thiazolyl blue), 10 μg of phenazine methosulfate, and 200 μg of *d*-lactate (Sigma Chemical Co.) were mixed in 0.06 M phosphate buffer (pH 7.5) with added 0.01 M NaCN in cuvettes and then membrane fractions were added. The cuvettes were incubated at 22°C (34) and absorbance at 550 nm was recorded at 10-s intervals over a 12-min period in a temperature-controlled spectrophotometer (Gilford 250, Gilford Instrument Laboratories, Inc., Oberlin, OH) with an automatic recording device. *d*-LDH standards (Sigma Chemical Co.) and crude cell wall membranes (the latter included as positive membrane controls) were run concurrently.

Fractionation of outer membranes. Outer membranes prepared by EDTA incubation and shearing of whole organisms (EDTA preparations) were suspended in 5–10 ml of an endotoxin-disaggregating buffer containing 0.05 M glycine, 0.001 M EDTA, and 1.5% sodium deoxycholate (NaD) adjusted to pH 9.0 with NaOH (NaD buffer). Outer membrane suspensions were solubilized in this buffer by alkalinization to pH 11.0 briefly; the clear suspensions were then chromatographed at a rate of 10 ml/h (4°C) on a 2.6 × 85-cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated in NaD buffer. Absorption of ultraviolet (UV) light at 280 nm was measured for collected fractions (0–450 ml). The void volume of the column was determined with blue dextran (Pharmacia Fine Chemicals). When required, radioactive counts were measured (Packard Tri-Carb, model 2425, Packard Instrument Company, Inc., Downers Grove, Il.) in 10-μl samples taken from each fraction and suspended in 10 ml of a scintillation fluid consisting of 60% toluene and 40% ethylene glycol monomethyl ether and containing 4 g/liter of Omnifluor (New England Nuclear).

Fractions that showed absorption of UV light at 280 nm were pooled and dialyzed exhaustively (4°C) against 0.025 M glycine adjusted to pH 9.0 with NaOH to free the preparation from NaD. Further purification was accomplished by the addition of ethanol (10%, vol/vol) to facilitate displacement of deoxycholate bound to protein; this was followed by terminal dialysis (4°C) against 0.025 M glycine in 0.15 M NaCl adjusted to pH 9.0 with NaOH (glycine buffer) (16). Protein antigens were concentrated, employing pressure filtration (Amicon Corp.) and used as stocks for subsequent experiments. Protein antigens freed from NaD and suspended in glycine buffer were judged to be relatively dispersed by their ability to remain in solution after centrifugation at 50,000 *g* (25°C) for 60 min.

The remaining smaller molecular size material was collected from later fractions (280–420 ml), exhaustively dialyzed using 8,000 mol wt cutoff tubing (Spectrapor, Spectrum Medical Industries, Los Angeles, CA) against 0.025 M glycine adjusted to pH 9.0 with NaOH and recovered by precipitation in 80% ethanol containing 0.2 M NaCl. For studies of reaggregation, this recovered material, presumed to be LPS, was suspended in Tris–HCl buffer (pH 7.6) and applied to a 40 × 0.9-cm column of Sepharose 4B (Pharmacia Fine Chemicals).

LPSs were also prepared from whole bacteria using a modification of hot phenol water extraction (38). Bacteria were extracted with 45% (vol/vol) aqueous phenol, heated to 65°C, and stirred for 15 min. The mixture was cooled to 10°C and centrifuged at 5,000 *g* for 15 min. The aqueous layer, containing nucleic acids and LPS (presumed), was removed and the extraction was repeated on the remaining material. The combined aqueous layers were exhaustively dialyzed against running water at 4°C and the solution precipitated with 6 vol of cold 0.05 M sodium acetate in 95% ethanol at −20°C for at least 4 h. The precipitate was pelleted by centrifugation (5,000 *g*) for 15 min at 4°C, dried, suspended in distilled H_2O, and ultracentrifuged at 105,000 *g* for 3 h to produce clear pellets. The pellets were washed and ultracentrifuged repeatedly until OD measurements at 280 and 260 nm of the supernatants were <0.01.

Characterization of cell wall-derived antigens

SDS-PAGE. The following preparations were analyzed by SDS-PAGE (39, 40): (*a*) cell wall membrane fractions prepared by spheroplasting and lysozyme treatment followed by density gradient centrifugation; (*b*) outer membranes prepared by EDTA incubation and shearing of whole organisms; and (*c*) outer membrane proteins separated from LPS by NaD chromatography. 14% acrylamide and 0.38% *N,N'*-methylene bisacrylamide (Bio-Rad Laboratories, Richmond, CA) gels were prepared in 0.375 M Tris buffer, 0.1% SDS (pH 8.8), and 25% urea, and poured in a glass mold, 16.0 × 18.0 × 0.15 cm, to a height of 10.5 cm and allowed to harden overnight. A comb with 0.8-cm teeth for slots was inserted into the overlying stacker gel that consisted of 4% acrylamide and 0.1% bisacrylamide and 25% urea in 0.125 M Tris buffer and 0.1% SDS (pH 6.8). Samples for electrophoresis were boiled for 10 min in a solution of 4.4% SDS in 0.18 M Tris HCl, 14.7% glycerol and 1.5% 2-mercaptoethanol (pH 6.8). 20 μg of sample

protein in this solution (15–70 μl) was applied to the gel slots. 5 μl of a similar solution containing 0.25% bromophenol blue, used as a tracker dye, was run in the same lane as low molecular weight standards. After electrophoresis in a running buffer of 0.025 M Tris, 0.2 M glycine, 0.1% SDS at 70 mA constant current for 2 h at 10–15°C, the slab gel was immersed overnight in a staining solution of 0.05% Coomassie Brilliant Blue plus ethanol-acetic acid-water (2.5:1:6, vol/vol/vol). Thereafter, the gel was immersed in a destaining solution of ethanol-acetic acid-water (1:1:8, vol/vol/vol) until the gel background was clear. Silver staining was performed after Coomasie Brilliant Blue staining according to procedures described in the Bio-Rad silver staining kit (41). Retardation factors (R_f) of peptide bands were calculated from known protein standards (Bio-Rad Laboratories) that were run simultaneously.

Chemical analysis. Protein concentrations of preparations were measured with Folin phenol reagent, using bovine serum albumin as a standard (36). The estimation of the content of simple sugars and their derivatives was determined with the phenol–sulfuric acid reaction (42) using glucose (varying concentrations) to generate a standard curve. Amide and ester linked fatty acids were estimated by the method of Snyder and Stephens (43) as modified by Haskins (44), using *N*-acetylglucosamine (amide linked) and tripalmitin (ester linked) respectively, to generate standard curves. Average molecular weight of NT *H. influenzae* LPS-derived fatty acids was obtained from an analysis of two strains reported by Murphy et al. (45), and molar ratios of ester to amide linked fatty acids in NT *H. influenzae* LPS were assumed to be similar to *Salmonella minnesota* LPS with known fatty acid content (46) that was also run in the assay as internal reference. Assays for 3-deoxy-*manno* octulosonic acid (KDO) were attempted using the thiobarbituric acid reaction (47), modified to compensate for the presence of excess polysaccharide (48). KDO standards (Sigma Chemical Co.) were used to generate a standard curve.

Tests for biologic activity. Skin sites for testing of the dermal Shwartzman reaction, a marker of endotoxin activity (49), were prepared in groups of five 6–8-wk-old New Zealand white rabbits (1–1.5 kg) by intradermal injections of twofold serial dilutions of 0.25-ml samples of (*a*) material recovered from fractions obtained from each of the outer membranes solubilized in NaD and separated on the G-100 column, (*b*) material obtained by hot phenol water extraction of whole organisms, and (*c*) endotoxin derived from *Salmonella typhi* used as a control to establish the sensitivity of the test. Reactions were provoked 24-h later by an intravenous injection of 20 μg of the homologous material (0.5 ml). 6 h thereafter, hemorrhage or necrosis of the sensitized skin sections were recorded as a positive reaction and 50% endpoints calculated (50).

Separation of immunoglobulins

To determine the immunoglobulin specificity of complement (C')-dependent bactericidal activity in normal human serum against NT *H. influenzae,* immunoglobulin M (IgM) was prepared by gel filtration (51) from volunteer serum that had C'-dependent bactericidal activity directed against both strains. 9 ml of the serum was applied to a 1.6 × 85-cm column containing Bio-Gel A-5M (Bio-Rad Laboratories) equilibrated in a 0.1 M Tris and 0.15 M NaCl buffer (pH 7.5) at 4°C. Fractions were assayed for immunoglobulin content by rate nephelometry (Beckman Instruments, Inc.) (52). The effluent containing IgM was pooled, concentrated, and dialyzed against 0.15 M saline. Final adjustment of volume was performed to approximate the concentrations of IgM in whole serum and the preparation sterilized by filtration.

Immunoglobulin G (IgG) was separated by anion exchange chromatography (53) from the same serum. QAE-Sephadex A-50 gel was equilibrated in a 0.048 M ethylene diamine, 0.073 M acetic acid buffer (pH 7.0), then 5 ml of the serum was mixed with an equal amount of buffer and applied to a 13 × 2.6-cm column of the gel mixture (4°). The effluent containing IgG was pooled, concentrated, and dialyzed against 0.15 M saline. The volume was adjusted to approximate the concentration of IgG in whole serum and the preparation sterilized by filtration.

Immunoglobulins were separated from the small volume of convalescent serum available from the patient with pneumonia by affinity chromatography using a protein A Sephrose Cl 4B (Pharmacia Fine Chemicals) column (54). A serum sample was chromatographed onto a 0.9 × 1.5-cm column, and the effluent was collected in 0.1 M phosphate buffer (pH 7.0) and monitored for IgM. IgM containing fractions were pooled, concentrated, and dialyzed against 0.15 M saline. IgG was eluted from the column with 0.1 M glycine HCl buffer, pH 3.0, and the IgG-containing fractions were pooled, concentrated and dialyzed against 0.15 M saline. Immunoglobulin concentrations of the pooled fractions were determined by nephelometry (52) and after the volumes were adjusted to approximate the concentration of IgM (effluent) or IgG (eluant) in the whole immune serum, they were sterilized by filtration.

Immunoglobulin preparations to be stored were kept in 0.15 M phosphate and 1.0 M NaCl (pH 7.0) to minimize the formation of aggregates (55) and dialyzed against 0.15 M saline prior to their use.

Solid-phase immunoadsorption

Serum samples, depleted of specific antibody activity directed against outer membrane proteins (OMPs), and antibodies immunopurified against OMPs were prepared by solid-phase immunoadsorption using purified OMPs covalently linked to Sepharose 4B as the absorbant (54). 2 g of cyanogen bromide-activated Sepharose 4B gel (Pharmacia Fine Chemicals) were swollen and washed with 0.001 M HCl. OMP (10 mg) recovered from the G-100 column was suspended in 10 ml of 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl (pH 8.3) and gently mixed with the swollen gel mixture for 2 h at room temperature. After washing the gel with the $NaHCO_3$ buffer, remaining reactive groups were blocked by incubating the gel with 0.1 M Tris–HCl (pH 8.0) for 30 min at room temperature. The gel was then washed with 0.1 M acetate buffer in 1.0 M NaCl (pH 4.0) followed by 0.1 M borate buffer in 1.0 M NaCl (pH 8.0). The gel was placed into a 1.6 × 2.5-cm column, equilibrated with 0.2 M borate in 0.15 M NaCl, pH 9.0 (borate buffer), and washed free of protein. Mock elution was performed by passing 20 ml of 2.0 M potassium iodide in borate buffer (KI buffer) through protein columns (56). Fractions were collected and monitored for protein antigen leakage (36, 39, 40), but none was detected. Serum samples (0.5–1.5 ml) were equilibrated in borate buffer and applied to the column. Fractions were collected until UV absorbance at 280 nm was no longer detected. The effluent was pooled, concentrated, dialyzed against 0.15 M NaCl, and used in the immunologic assays.

For experiments designed to recover immunopurified antibody by elution of bound antibody from the column, 10 ml of normal human serum with bactericidal activity was absorbed. Potassium iodide buffer was applied to the column to elute the immunoadsorbed antibody. The eluant (10 ml) was pooled dialyzed against 0.15 M saline, concentrated to 3.5 ml, and used in the immunologic assays.

Enzyme-linked immunosorbent assay (ELISA)

Direct binding activity of serum or derivatives of serum for outer membrane antigens was determined by ELISA (57). Polystyrene microtiter plates with round U-bottom wells (Dynatech Laboratories, Inc., Alexandria, VA) were coated at 37°C for 3 h with a solution of OMPs (10 μg/ml) in $NaCO_3$ buffer (pH 9.6) or with LPSs (25 μg/ml) extracted from whole organisms by hot phenol water, the latter electrodialyzed and converted to the triethylamine salt (58), and diluted in barbital acetate buffer (pH 4.6) (16). The 10 μg/ml OMPs and the 25 μg/ml LPS coating concentrations were predetermined to give optimal readings.

Experiments were performed in duplicate employing 0.25-ml volumes. After coating for 3 h at 37°C, the wells were washed four times

(plates immersed for 10 min each wash) with 0.05% Tween-20 in phosphate-buffered saline (PBS) (pH 7.4) and then reacted with specimens diluted in PBS-0.05% Tween-20 for 1 h at 37°C. Anti-human IgG (γ-chain specific) or IgM (μ-chain specific) conjugated with alkaline phosphatase (Sigma Chemical Co.) was added in a 1:1,000 dilution (optimal dilution predetermined) after another wash and allowed to react for 1 h at 37°C. After the final wash the substrate, disodium *p*-nitrophenyl phosphate in 9.7% diethanolamine buffer (pH 9.8), was added and the reaction allowed to proceed at room temperature; readings were monitored at 405 nm (Micro-ELISA Reader, Dynatech Laboratories, Inc.). A standardized internal control was run concurrently in each experiment and test reactions were read when this control reached a predetermined optical density. Other controls included wells coated with antigen and reacted with conjugate (antigen control), mock-coated wells reacted with serum and conjugate (serum control), and mock-coated wells reacted with conjugate alone (conjugate control). OD of negative controls always were <0.1; readings >0.3 were considered positive and specimen titers were expressed as the limiting dilution that gave 0.3 OD. Conjugates were validated for their specificity in recognizing the designated isotype by ensuring that they did not react with purified heterologous isotypes. This was tested by first coating isotype-specific immunoglobulin onto microtiter plates at concentrations that, when detected by using the homologous conjugate, would give signals similar to those measured in the regular ELISA (above). Then the heterologous conjugates were examined for specificity.

Bactericidal assay

The bactericidal assay employed in this study was a modification of procedures previously described for *Neisseria gonorrhoeae* (18). Reaction mixtures were performed in 12 × 75-mm capped tubes. Test mixtures contained 0.025 ml of complement, 0.10 ml of a dilution of test serum or immunoglobulin fraction, and 0.025 ml of NT *H. influenzae*.

REAGENTS. *Complement.* Freshly drawn serum of an individual with acquired hypogammaglobulinemia containing IgG, 100 mg/dl; IgA, 26 mg/dl; and IgM, 14 mg/dl was allowed to clot for 15 min. The serum was separated by centrifugation at 3,000 *g* for 10 min at 4°C, and then immediately stored at −70°C. Total hemolytic complement activity of the serum averaged 125 $C'H_{50}$U/ml (normal 111±15) (59).

Antibodies. Normal and immune sera, which had been stored at −70°C, and serum depleted of antibodies against OMPs, were employed in bactericidal reactions with the two strains. Purified human serum immunoglobulin fractions and antibodies prepared by immunoabsorption against OMPs were similarly examined.

NT H. influenzae. Organisms were grown overnight (37°C) in BHI broth with added supplements and an aliquot transferred to fresh broth, grown to mid-log phase concentration of ~2×10^8 organisms/ml and diluted 10^{-4} in Geys' balanced salt solution containing 10% bovine serum albumin.

METHOD. Presumed bactericidal antibody (serum, immunoglobulin fraction, or immunopurified antibodies) was added to organisms and the complement was added last. The reaction mixtures were incubated at 37°C with continuous shaking in a water bath (Aquafirm, New Brunswick Scientific Co., Inc., New Brunswick, Edison, NJ). Viable colony counts were performed at 0 and 30 min by plating duplicate 0.025 ml samples of each reaction mixture onto chocolate agar. Killing was accorded to those reactions where greater than a 1 $\log_{10}$ reduction in CFU was observed after the 30-min incubation period, compared to the numbers of organisms present in the same tube after immediate plating (t_0). Titers were assigned to the dilution of serum that achieved 90% killing of the test strain. Active complement controls, heat-inactivated (56°C for 30 min) serum controls, immunoglobulin controls, or immunopurified antibody controls were included in relevant experiments. Organisms maintained 100% or greater viability during the 30-min incubation period in these controls.

Bactericidal inhibition assay

Reagents. Varying concentrations of OMPs and LPSs from the two strains were used to attempt dose-related inhibition of serum bactericidal activity directed against each of the two strains. In experiments that employed patient sera, homologous antigens were tested; in normal sera, antigens derived from both strains were examined.

Method. OMP and LPS preparations were serially diluted in glycine buffer, and incubated (37°C) for 30 min with concentrations of serum that had produced a 1 $\log_{10}$ kill in the bactericidal assay. Thereafter, these absorbed sera were used as the source of bactericidal antibody in the assays. Additional controls included (*a*) the test serum plus complement without antigen to ensure adequate killing by the test serum and (*b*) a companion tube to each absorbed serum that contained only antigen at the specified concentration plus complement to ensure that the antigens were not themselves toxic to the organisms. Percent bactericidal inhibition was expressed according to the formula (18):

$$\%\ \text{inhibition} = 1 - \frac{(\%\ \text{killing, antigen system})^*}{(\%\ \text{killing, control system})^\ddagger}, \text{ where}$$

$$^*1 - \frac{\left(\frac{\overline{CFU}_{t30}}{\overline{CFU}_{t0}}\right)_{\text{antigen+serum+C'}}}{\left(\frac{\overline{CFU}_{t30}}{\overline{CFU}_{t0}}\right)_{\text{antigen+C'}}} \text{ and}$$

$$^\ddagger 1 - \frac{\left(\frac{\overline{CFU}_{t30}}{\overline{CFU}_{t0}}\right)_{\text{serum}\pm\text{C'}}}{\left(\frac{\overline{CFU}_{t30}}{\overline{CFU}_{t0}}\right)_{\text{C'}} \cdot \left(\frac{\overline{CFU}_{t30}}{\overline{CFU}_{t0}}\right)_{\text{serum}}}.$$

The formula includes standardization for the antigen control and also corrects for the survival of organisms in the diluted serum alone. To test independently the antigenicity of OMPs and LPSs used in soluble-phase bactericidal inhibition assays, individual absorbed sera were also tested in ELISA that used microtiter plates coated with the absorbing antigen.

Because these experiments were designed to study the specificity of complement dependent bactericidal antibody for outer membrane antigens of NT *H. influenzae*, it was necessary to ensure that inhibition of bactericidal activity was not the result of complement depletion by the antigens themselves. Accordingly $C'H_{50}$ activity (59) was tested in reaction mixtures, which contained antigen in amounts shown to be inhibitory in the bactericidal assay.

Results

Purification and characterization of NT H. influenzae cell wall antigens. Fractions obtained by final isopycnic centrifugation (preceded by two separate discontinuous gradient centrifugations) of membranes prepared from the middle ear isolate were analyzed for protein content (36). Membranes were prepared by (*a*) EDTA incubation and shearing of whole organisms (EDTA preparations) and (*b*) spheroplasting and lysozyme treatment of organisms (spheroplast/lysozyme preparations). The analysis revealed peaks of 280-nm absorbance at corresponding densities of ρ = 1.22 g/cm^3 for membranes prepared by each of the two methods and two additional peaks at ρ = 1.14 g/cm^3 and ρ = 1.10 g/cm^3 recovered only from the spheroplast/lysozyme preparations (Fig. 1). *d*-LDH activity was present principally in these two low density peaks. Maximal activity in each of these peaks was observed in fractions

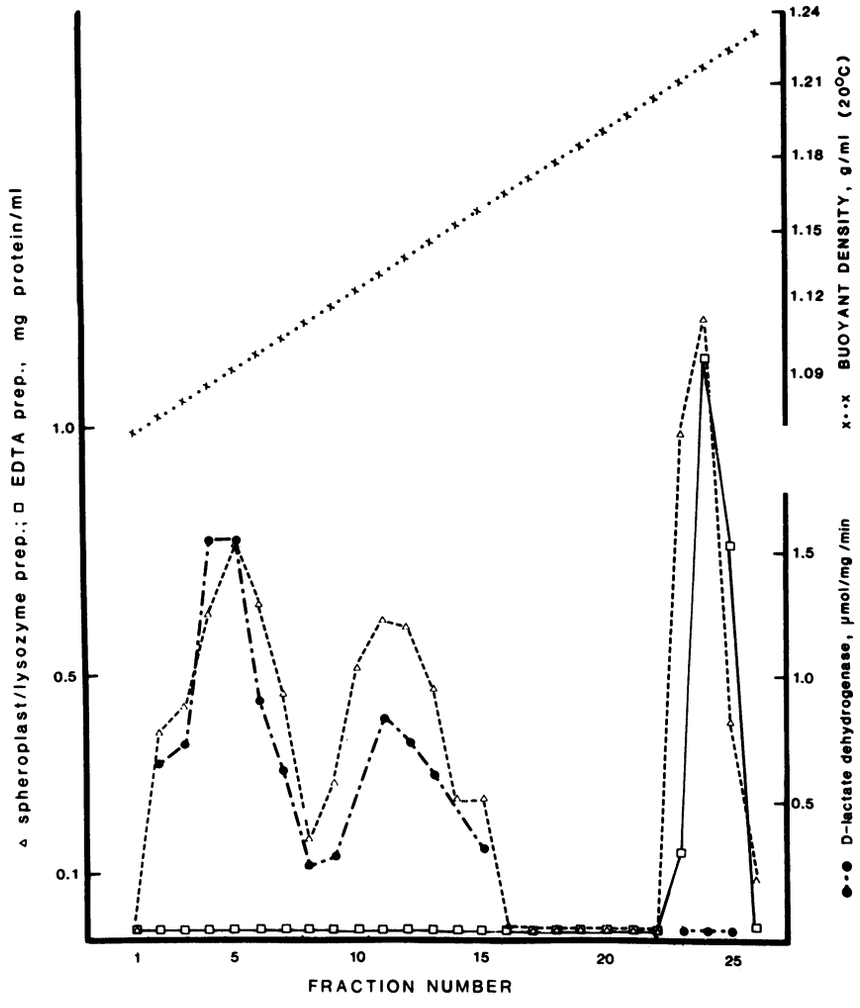

Figure 1. Isopycnic gradient densities of cell wall membranes and outer membranes from NT *H. influenzae* (ear isolate [BCH-2]). (△) Cell wall membranes prepared by spheroplasting and lysozyme treatment of whole cells (spheroplast/lysozyme preparations); (□) outer membranes prepared by EDTA treatment and shearing of whole cells (EDTA preparations); (●) location of D-LDH activity. The first two peaks demonstrate resolution of material from the spheroplast/lysozyme preparations recovered from the lower density interface on the preceeding discontinuous gradient (see text); the third peak (spheroplast/lysozyme and EDTA preparations) represents materials recovered from the higher density interfaces on the preceding discontinuous gradients (see text).

containing maximal protein concentrations (36) (1.56 μmol/mg per min for the ρ = 1.10 g/cm^3 peak and 0.86 μmol/mg per min for the intermediate peak, ρ = 1.14 g/cm^3) (Fig. 1). Crude cell wall membranes exhibited 0.673 μmol/mg per min activity. Each of the two peaks of high density obtained from the EDTA preparation and the spheroplast/lysozyme preparations showed low specific activity at 0.008 μmol/mg per min (34, 60).

Analysis by SDS-PAGE revealed three major protein bands (seen between molecular weights of 31,000 and 45,000) in the high density (ρ = 1.22 g/cm^3) material isolated from the EDTA preparations that had been subjected to gradient density centrifugation (Fig. 2). These were also present in the otherwise similar spheroplast/lysozyme preparations of the same density; an additional band present in this preparation may have been carried over from lower-density fractions in that it was also present in the preparation of intermediate density (ρ = 1.14 g/cm^3) and was one of the two most prominent of many bands seen in the low density (ρ = 1.10 g/cm^3) fractions in the spheroplast/lysozyme preparations (Fig. 2). SDS-PAGE analyses of EDTA preparations had not been changed by density-gradient centrifugation and heating (60°C for 45 min) of organism suspensions prior to EDTA incubation and shearing did not change the analysis of the recovered membranes from those shown in Fig. 2 (lane *5*).

Membranes from the ear isolate prepared by EDTA extraction revealed a single major protein peak after fractionation on the Sephadex G-100 column equilibrated in NaD buffer (Fig. 3). Nucleic acid contribution to the protein peak was estimated at <1.5% ($R_{280/260}$ > 1.25). Radiolabeled material eluted as smaller molecular-sized material. A similar chromatogram was exhibited when membranes from the pulmonary isolate were fractionated by NaD chromatography. SDS-PAGE of EDTA preparations, before NaD chromatography (used to dissociate LPS) and of the protein peak (exemplified as pool *A* in Fig. 3) revealed that the proteins had been freed of lower molecular weight material (Fig. 4). The major OMP band of the ear isolate was calculated at 40,000 mol wt and differed slightly from that of the pulmonary isolate calculated at 39,000 mol wt. Additional bands appeared similar or only slightly different, including bands at 32,000 and 45,000 mol wt and others at higher molecular weights.

Radiolabeled material recovered from the NaD columns (designated as pool *B*, Fig. 3) reaggregated and eluted in the

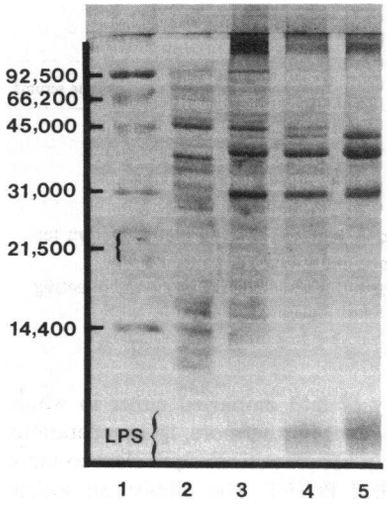

Figure 2. SDS-PAGE of membrane fractions obtained by spheroplasting and lysozyme treatment of NT *H. influenzae* (ear isolate [BCH-2]), followed by density-gradient centrifugation; spheroplast/lysozyme preparation, lanes *2–4;* and EDTA, shear treatment of whole cells (EDTA preparations), lane *5* (see Fig. 1). (*1*) Low molecular weight standards; (*2*) low density (ρ = 1.10 g/cm^3) peak, lysozyme/spheroplast preparation; (*3*) peak of intermediate density (ρ = 1.14 g/cm^3), lysozyme/spheroplast preparation; (*4*) high density (ρ = 1.22 g/cm^3) peak, lysozyme/spheroplast preparation; (*5*) high density (ρ = 1.22 g/cm^3) peak, EDTA preparation.

void of a Sepharose 4B (exclusion size > 4×10^6) column when chromatographed in Tris–HCl. Hot phenol water treatment of whole organisms yielded a high molecular weight antigen, presumed to be LPS, that could be disaggregated with NaD and eluted in the same fractions as pool *B* on the NaD, Sephadex G-100 column (data not shown).

Disaggregated LPS (mol wt < 14,400) that stained with silver, but not Coomasie Blue, was also most prominent in high density (ρ = 1.22 g/cm^3) material obtained from spheroplast/lysozyme preparations and was absent in the low density (ρ = 1.10 g/cm^3) material (Fig. 2).

Chemical and biologic analysis of separated bacterial fractions. The results of the chemical analyses of fractions prepared from the two strains of NT *H. influenzae* are summarized in Table I. Protein concentrations of outer membrane material freed of LPS (pool *A*, Fig. 3) from each of the strains were >90% (wt/wt). SDS-PAGE revealed that most of the major and minor protein bands found in the intact outer membranes from each of the strains were present and unchanged in outer membrane proteins freed of LPS (Fig. 4). Silver staining of SDS-PAGE of OMPs revealed an absence of stained material in the lower molecular weight regions of the gel where NT *H. influenzae* LPS migrates and is easily detected in quantities as low as 50 ng (experiments not shown) (61). Using this criterion OMPs were <0.25% contaminated with LPS. Protein content (wt/wt) of phenol water-derived LPSs from the two strains ranged from 0.5 to 3.6% in different lots of antigen with an average of 2.1% for the pulmonary isolate and 1.5% for the middle ear isolate; however, none of this showed as peptide bands on SDS-PAGE (experiments not shown). LPS preparations of NT *H. influenzae* contained 0.5% or less KDO as measured by the thiobarbituric acid reaction.

Dermal Shwartzman reactions were performed using purified OMPs and LPSs prepared from each strain. LPSs showed endotoxic activity while purified outer membrane proteins did not elicit a response at the concentrations employed in this assay (Table II).

Bactericidal assays. Bactericidal titers, directed against the pulmonary and middle ear isolates of NT *H. influenzae,* were

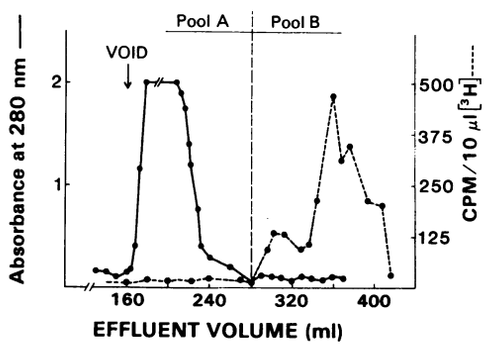

Figure 3. Elution profile of NT *H. influenzae* (ear isolate, [BCH-2]) outer membranes (EDTA preparation) chromatographed on a 2.6 × 85-cm G-100 (Pharmacia Fine Chemicals) column equilibrated in glycine, 0.001 M EDTA, 1.5% NaD buffer (pH 9.0). Outer membranes were suspended in NaD buffer, solubilized by increasing the pH to 11.0, applied to the column, and eluted with the same buffer. *VOID*, void volume measured with blue dextran. (——) Absorbance of 280 nm. (- - -) Radioactivity of outer membranes prepared from NT *H. influenzae* grown in the presence of [^{3}H] sodium acetate.

*Table I. Chemical Analysis of NT H. influenzae Antigens (Percent wt/wt)**

	Pulmonary isolate (BCH-1)			Middle ear isolate (BCH-2)		
	OMPs	LPSs		OMPs	LPSs	
	Pool *A*‡	Pool *B*‡	ϕ Water§	Pool *A*‡	Pool *B*‡	ϕ Water§
	% wt/wt					
Protein‖	94	0.5	2.1	93	<0.5	1.5
Simple sugars¶	4	36	49	5	18	50
Fatty acid content**	3	36	45	3	43	60

* Samples were lyophilized and weighed dry. The amount of designated chemical in the sample was measured by comparing readings obtained for samples with those generated from curves of varying concentrations of indicated standards (see below). Percent wt/wt indicates the amount in the sample (determined from the standard curve) divided by the total amount of sample (dry) used in the assay. Readings represent average values of measurements in at least two lots of each preparation.

‡ Pool *A*, first peak exemplified on the chromatogram in Fig. 3: pool *B*, second peak.

§ LPS extracted from whole cells by hot phenol water (38).

‖ Measured with Folin phenol reagent (36); bovine serum albumin used as standard.

¶ Measured using the phenol sulfuric reaction (42); glucose used as standard.

** Amide and ester-linked fatty acids estimated by the method of Snyder et al. (43), modified by Haskins (44). *N*-acetylglucosamine (amide linked) and tripalmitin (ester linked) used as standards (see text).

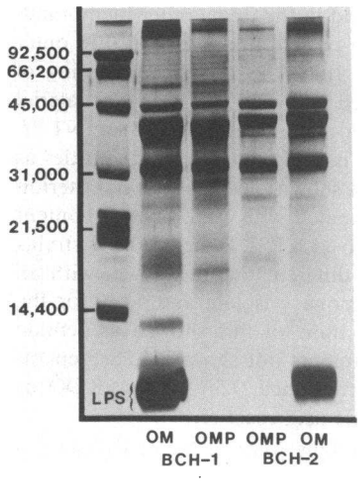

Figure 4. SDS-PAGE of outer membranes (*OM*) prepared by EDTA and shearing of whole cells of NT *H. influenzae* (pulmonary isolate [BCH-1], and ear isolate [BCH-2], see Fig. 2, lane *5*) and of pooled and concentrated outer membrane protein (*OMP*) fractions from the Sephadex G-100 column of the pulmonary isolate (OMP, BCH-1) and the ear isolate (OMP, BCH-2, see pool *A*, Fig. 3). The gel contained 14% acrylamide and was stained with Coomassie Brilliant Blue followed by silver nitrate (see text).

determined in each of two normal human sera tested singly, and a serum pooled from 10 normal adults (containing the two sera tested individually) (Table III). Bactericidal titers in the immune convalescent sera taken from the patients infected with each of the strains showed greater than fourfold higher titer than normals. Percent survival of organisms (CFU) on duplicates of reaction mixtures varied by 10–15%, and titers obtained on successive determinations performed on different days varied by no more than a single twofold dilution.

Bactericidal activities in each of the two purified immunoglobulin preparations (IgM and IgG) and the corresponding normal human serum directed against each strain are shown in Table IV. Normal IgM, at a concentration of 1.1 mg/dl (1% of that in normal serum), killed 95% of colonies of the pulmonary isolate (BCH-1). IgM, prepared by protein A absorption of convalescent serum from the pneumonia patient, and tested at a concentration of 0.3 mg/dl (0.26% of that in whole convalescent serum) also killed >90% of organisms. Killing of this strain by each of the corresponding whole serums was attributed almost completely to IgM, as evidenced

Table II. Sensitizing Doses of Outer Membrane Antigens of NT H. influenzae in Dermal Shwartzman Reactions

Antigen preparation	Sensitizing dose (ID_{50})*	
	Pulmonary isolate (BCH-1)	Ear isolate (BCH-2)
	μg	*μg*
Pool *A*‡ (OMPs)	>640	>640
Pool *B*‡ (LPSs)	24	34
Phenol water-extracted LPSs	80	120
S. typhi endotoxin 0901	<10	<10

* 50% endpoint estimation (50).
‡ Pool *A*, first peak exemplified on the chromatogram in Fig. 3: pool *B*, second peak.

Table III. Serum Bactericidal Antibody Activity Directed against NT H. influenzae

Strain	Normal sera*	Immune serum‡
Pulmonary isolate (BCH-1)	1:96	1:384
Ear isolate (BCH-2)	1:3	1:24

* Titers of two individual adult sera and of a pool of sera from ten adult volunteers (all three specimens gave the same result).
‡ Patient convalescent sera tested against the homologous infecting strain.

by similar killing activity of IgM employed either in whole serum or as a purified fraction. Furthermore, IgG preparations from neither serum used in concentrations equivalent to those in undiluted serums, killed BCH-1. The middle ear isolate (BCH-2) was susceptible to killing by either IgG or IgM purified from normal serum (Table IV).

Inhibition of bactericidal antibody activity. To examine the role of influenzal antigens in the recognition and binding of bactericidal antibodies, we employed both OMPs and LPSs as absorbants of these antibodies. In the initial experiments, direct absorption employing each of the antigens was performed in order to examine dose-response effects of antigen absorption and comparative absorbing capacity of each antigen and the relative differences employing natural versus immune human antibody. Fig. 5 shows the results of direct absorption that used antigens from the pulmonary isolate as absorbants for natural and immune antibody. OMP antigen absorption of both natural and immune antibody proved effective in com-

Table IV. Bactericidal Activity of Normal Human Serum (Titered) against NT H. influenzae and the Corresponding Immunoglobulin Concentrations in the Serum and its Derived Immunoglobulin Fractions

Isolate	IgG	IgM	% Kill
	mg/dl	*mg/dl*	*%*
Pulmonary isolate (BCH-1)			
Normal serum (1:96)*	10	1.1	95
IgM preparation‡	0.1	1.1	91
IgG preparation§	10	0.2	5
Ear isolate (BCH-2)			
Normal serum (1:3)*	334	36	92
IgM preparation‡	3	36	80
IgG preparation§	350	2.3	96

* Normal human serum titered to the killing endpoint (>90%) with complement added separately.
‡ IgM preparation (prepared by gel filtration over Bio-Gel A-5M, [51]); IgM concentration adjusted to coincide with that in titered normal serum that produced killing (1:96 for use against pulmonary isolate, 1:3 for ear isolate).
§ IgG preparation (prepared by anion exchange over QAE-Sephadex A-50 [53]); IgG concentration adjusted to nearly coincide with that in titered normal serum that produced killing.

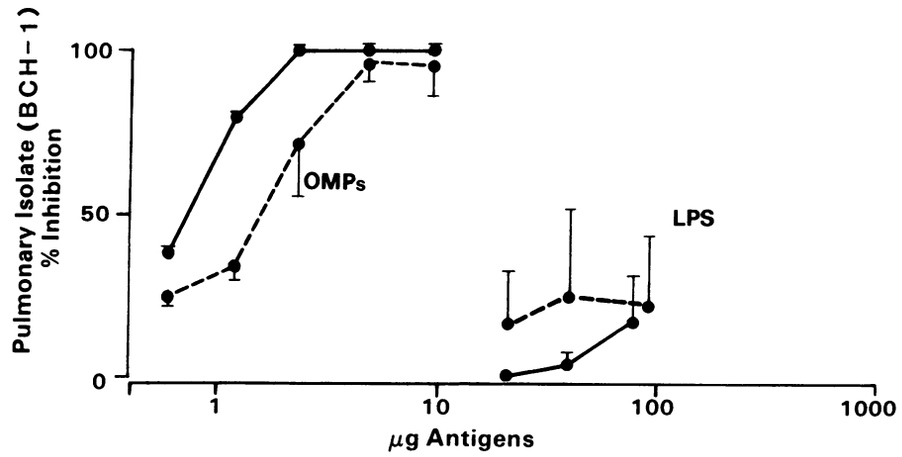

Figure 5. Inhibition of bactericidal activity in human sera by outer membrane antigens prepared from the pulmonary isolate (BCH-1) of NT *H. influenzae:* OMPs and LPS (hot phenol water extraction of whole cells). (——) Inhibition assays were performed with homologous convalescent serum. (- - -) Each of the two normal human sera (mean with range). Amount of OMPs used in the inhibition assays is expressed as added protein measured with Folin phenol reagent (36) in the solutions of OMPs. LPS amounts in the inhibition assays were determined by dry weights.

pletely absorbing bactericidal activity in sera whose bactericidal activity had been diluted to near endpoint. Fig. 6 shows a similar experiment employing antigens from the middle ear isolate that indicates less effective absorption by OMP. For either strain mean inhibition of killing activity in normal or immune sera by phenol water extracted LPS never exceeded 26%. Concentrations of antigens (shown in Figs. 5 and 6) that produced inhibition did not reduce hemolytic complement activity in the reaction mixtures. LPSs extracted with NaD produced no inhibition when tested at concentrations equivalent to those used in testing phenol water-extracted LPSs (data not shown in Figs. 5 and 6). Each outer membrane-derived preparation used in the soluble-phase bactericidal inhibition assays reduced by 81%, or greater, the ELISA titer in serum against the respective antigen when homologous soluble-phase absorptions were performed.

The specificity of absorptions involving OMPs from the pulmonary isolate was assessed in experiments that employed solid-phase immunoabsorption of immune and normal sera against the proteins, in that these antigens were most active in the direct (soluble phase) absorption experiments. Immune convalescent serum obtained from the patient with pneumonia underwent >75% reduction in antibody (IgG and IgM) titers (ELISA) against OMPs (Table V) and in bactericidal activity (from 1:384 to 1:48) with solid-phase absorption employing OMPs while 90% of the activity (ELISA) against LPS (IgG and IgM) remained. Two immunoabsorbed normal adult sera likewise each underwent reduction in anti-OMPs and in bactericidal activity (from 1:96 to 1:12) while they maintained 80% or greater of their activity against LPS. No competition of one isotype over the other for the antigens tested was seen in these reactions, as evidenced by endpoint ELISA titers that

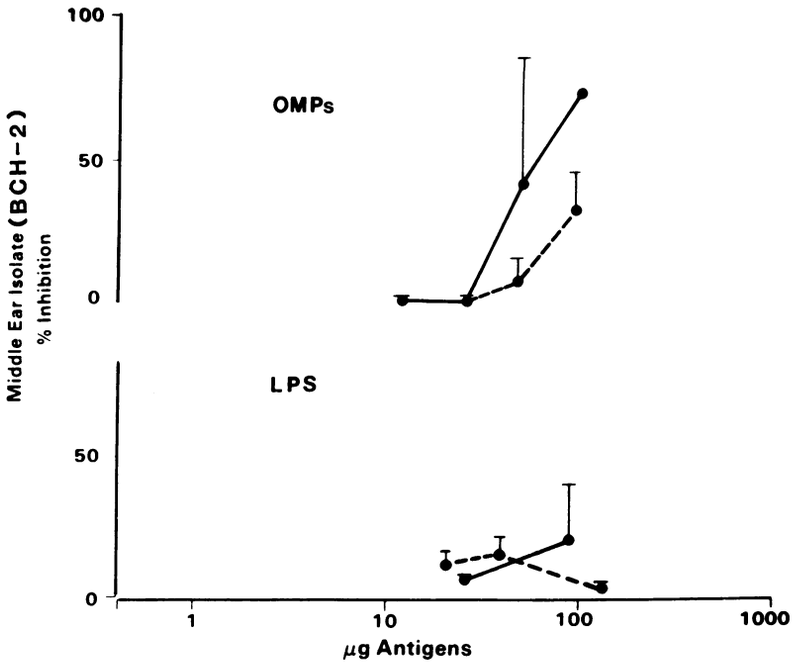

Figure 6. Inhibition of bactericidal activity in human sera by outer membrane antigens prepared from the middle ear isolate (BCH-2) of NT *H. influenzae:* OMPs (Fig. 3, pool *A*) and LPSs (hot phenol water extraction of whole cells). (——) Inhibition assays were performed with homologous convalescent serum. (- - -) Each of two normal human sera (mean with range). Amount of OMPs used in the inhibition assays is expressed as added protein measured with Folin phenol reagent (36) in the solutions of OMPs. LPS amounts in the inhibition assays were determined by dry weights.

were reproduced (range: from −11% to +13%) in the isolated immunoglobulin fractions, IgG and IgM, compared to whole serum.

Concentrated eluants prepared from one of the normal adult sera and recovered from the OMP-affinity column showed antibody (ELISA) activity (IgM and IgG) against OMPs prepared from the pulmonary isolate and bactericidal activity against the isolate. No anti-LPS activity was demonstrated in the concentrated eluants (Table V).

Discussion

Recent investigations have shown renewed interest in the antigenic structure of noncapsular antigens of the species *H. influenzae* (14, 15, 20, 23, 24, 26, 62) and in the presence and functional roles of antibodies directed toward specific antigens present in their outer membranes (62–64). Serum bactericidal antibodies that are directed against surface antigens are associated with protection of the human host against disseminated infection with *Neisseria meningitidis* (17) and encapsulated type b *H. influenzae* (13, 65). Their role in protecting against local disease such as otitis media caused by unencapsulated or NT *H. influenzae* currently is imprecisely understood. However, susceptible children in the age group where this disease is most prevalent may lack bactericidal antibody activity directed against strains of NT *H. influenzae* (27). This activity, however, is present in variable amounts in sera of normal adults (28, 66). Comparisons of both composition and potential for immunogenicity of surface antigens derived from NT *H. influenzae* with the more extensive information that has been reported for *H. influenzae* type b (15, 67) suggest that major molecular differences exist in at least one of the major groups of antigens, the OMPs. Furthermore, although somatic antigens from typable and NT *H. influenzae* may be shared (21, 68), it has been shown that NT *H. influenzae* do not merely represent unencapsulated phenotypes of typable, encapsulated organisms (67, 69, 70). Nontypable strains that share no antigenic cross-reactivity with typable organisms (types a–f) have been subpassaged intraperitoneally in mice to enhance surface antigen production, but no antigens cross reactive with those from encapsulated organisms were detected in these studies (69).

By using gentle techniques to separate outer membranes from whole organisms, we have isolated material that corresponds in its uniform density to purified outer membranes derived from encapsulated *H. influenzae* (22, 32, 71, 72) and other Gram-negative bacteria (18, 34, 60). These outer membranes contain little D-LDH activity indicating relative purity from cytoplasmic membrane and their protein patterns resemble closely that of outer membranes prepared independently by spheroplasting and lysozyme treatment of whole cells (34, 60).

We prepared protein and LPS antigens derived from NT *H. influenzae* outer membranes to investigate the importance of each of these antigens as targets for human bactericidal antibody. OMPs from two strains of NT *H. influenzae* were isolated and purified by using detergent solubilization of whole outer membranes and chromatographic separation of proteins from LPS (18, 60). These proteins were free of endotoxin activity in the dermal Shwartzman reaction; in addition, they were shown to be free of LPS in the more sensitive silver-stained SDS-PAGE. The analysis of OMP patterns by SDS-PAGE showed that they differed in the major band, but that other prominent bands appeared similar in molecular weight. These patterns were generally similar to those previously reported for NT *H. influenzae* (24) with the two major OMPs of each strain falling within the range of 32,000–42,000 mol wt. The patterns seen in the two strains, however, are distinctly different from those that have been reported for *H. influenzae* type b, where a limited number of OMP patterns have been identified (22, 24). These patterns in *H. influenzae* type b have proved useful in a classification of strains used to establish epidemiologic features of *H. influenzae* type b disease and carriage in closed populations (25). Because OMPs derived from *H. influenzae* and other bacterial species, which include

Table V. Antibody Titers (Reciprocal) in Convalescent and Normal Human Sera against NT H. influenzae (BCH-1)*

	OMPs‡		LPSs‡		
Serum	IgG	IgM	IgG	IgM	Bactericidal§
Convalescent serum					
Control	12,229	6,065	4,310	699	384
OMP-immunoabsorbed serum	1,465	1,297	3,863	682	48
Normal human serum (no. 1)					
Control	4,731	555	1,393	2,146	96
OMP-immunoabsorbed serum	1,019	168	1,086	2,210	12
Normal human serum (no. 2)					
Control	2,422	1,204	2,629	1,614	96
OMP-immunoabsorbed serum	611	91	3,085	1,451	12
OMP-eluant	666	47	<62.5‖	<62.5‖	1.5

* Geometric mean titers of three determinations. ‡ Titers measured in serum by ELISA against indicated antigens prepared from the BCH-1 strain. § Bactericidal assay described in the text. ‖ Readings at lowest dilution tested (1:62.5) equaled negative controls.

porins or transmembrane proteins, often are poorly soluble in nonionic detergents (73) or sodium deoxycholate at pH <8.5 (74), we used alkalinization of outer membranes to pH 11.0 in NaD and EDTA followed by chromatographic separation of proteins from LPS and subsequent suspension of proteins at pH 9.0 in glycine buffer to permit relative solubilization (16). Many of the peptide bands seen in the outer membranes of the two strains that we studied were preserved in the protein antigens prepared in this manner.

LPS antigens were purified from NT *H. influenzae* using two methods in this study; deoxycholate chromatography of outer membranes and hot phenol water extraction of whole organisms. We found the physical properties of LPS prepared by either of these techniques to be similar. LPSs derived from NT *H. influenzae* by using either method could be disaggregated with the detergent NaD and reaggregated in aqueous buffer systems, a physical feature characteristic of LPS (75). Although NT *H. influenzae* were composed of lipid and carbohydrate, these antigens contained only small amounts of KDO, a sugar usually present in significant quantities in many LPSs from Gram-negative bacteria but reportedly absent from *Bacteroides* species (76) and low (<1% wt/wt) in encapsulated *H. influenzae* (20). Differences in the limited compositional analysis of LPSs prepared by two different methods as well as differences seen in the Shwartzman test for endotoxicity may have represented selected extractions of LPS species by each of the methods employed. Differences in species of LPS extracted by phenol water and phenol-chloroform-petroleum of the same preparation of organisms have been shown to yield different material (77) and each of these methods respectively have been used to advantage in extracting LPSs from smooth (38) and rough (78) *Salmonella.* In addition, pronounced differences in endotoxic activity has been reported in phenol water-extracted LPS derived from encapsulated *H. influenzae* of different types, as evidenced by requirements for sensitizing doses of 200 μg for type a *H. influenzae* LPS (79, 80) and 10 μg for type b (20) to elicit the dermal Shwartzman reaction.

Protection against the generalized Shwartzman reaction elicited by endotoxin can be accomplished by both IgG and IgM prepared against several heat-killed Gram-negative enteric organisms (81). NT *H. influenzae,* which are uniformly sensitive to the killing action of adult normal human serum (28), may be killed by either of these two major C' fixing human immunoglobulin classes. We have demonstrated that although both IgG and IgM mediated C'-dependent killing of the ear isolate by the normal human serum tested, IgM from the same serum and from immune serum taken from the patient with pneumonia was the principle isotype that killed the more serum-sensitive lower respiratory isolate. Quantitation of isotype specific antibodies against surface-exposed antigenic determinants may be necessary to characterize further these differences, nevertheless, these data are consistent with similar studies involving *Neisseria* showing that natural bactericidal antibodies are principally of the IgM class (16, 82), and that they demonstrate greater efficiency in killing organisms than either IgG or IgA (17). Furthermore, although the immunoglobulin classes of human bactericidal antibodies directed against NT *H. influenzae* have not to our knowledge been reported previously, it has been reported that rabbits immunized with unencapsulated phenotypes of *H. influenzae* types a and b develop both IgM and IgG killing antibodies (63). Heterogeneity in immunoglobulin classes that kill bacteria also has been found in rabbits immunized with living cholera vibrios where vibriocidal antibodies are predominantly 7S whereas administration of killed cholera vaccines results primarily in a 19S antibody response (83). Therefore bactericidal antibodies both natural and elicited by Gram-negative infection or immunization may be heterogenous, they may vary amongst species, and they may be directed against different antigenic determinants (16).

In assessing relative importance of cell wall antigens of NT *H. influenzae* in human immunity, we considered it important to define antigens that were involved in natural as well as in induced immunity. Chemical, physical, and biologic characterization of serologically distinct surface antigens of *H. influenzae* that recognize bactericidal antibodies has been incomplete to date, although much investigative attention has been directed to the polyribose phosphate capsule of *H. influenzae* type b, as an important inducer of functional bactericidal antibody both in experimental animals and humans. Anticapsular antibodies alone, however, may not be responsible for protective immunity to *H. influenzae* type b. Somatic antigens elicit bactericidal antibodies associated with protection in experimental animals (84), and LPS antigens prepared by hot phenol water extraction of *H. influenzae* type b, have been shown to be specific targets for rabbit bactericidal antibodies, although other determinants were not excluded in these studies (14). Passive immunization of infant rats with rabbit antiserum raised against whole *H. influenzae* type b and then depleted of antibodies against polyribose phosphate and LPS by solid-phase immunoabsorption, has been shown to be effective in protecting animals from disseminated infection (64). Furthermore, these protective antisera contained antibodies directed against several outer membrane proteins; removal of these antibodies was associated with loss of the protective activity (64).

Bactericidal antibodies of outer membrane antigens may in some instances be heterogenous and not specific. For example, *Vibrio cholera* endotoxin prepared by aqueous ether extraction of whole organisms inhibits vibriocidal antibody activity (85). This antigen, which has been presumed therefore to be important in cholera immunity is a mixture of LPS and protein; more purified LPS antigen preparations have been less effective in the inhibition reaction. Strains of *N. gonorrhoeae* have been shown to share multiple somatic antigenic determinants with *N. meningitidis* (85). A stepwise reduction of gonococcal bactericidal antibody activity has been demonstrated in rabbit antiserum prepared with viable *N. meningitidis* when the antiserum was first absorbed with surface protein from the immunizing meningococcal strain followed by a second absorption with its LPS (86). While IgM antibodies directed against LPS antigens of *H. influenzae* type b are present in children and adults (87, 88), they may be present in lower concentrations than antibodies against non-LPS somatic antigens (87). Although we have not absolutely excluded the possibility that LPS from *H. influenzae* may be a target for bactericidal antibody in human sera (14), we have demonstrated, in direct absorption experiments using diluted sera, that purified outer membrane proteins more effectively inhibited bactericidal antibody activity than LPS. The use of solid-phase

immunoabsorption to simultaneously deplete sera of both anti-OMP antibodies and bactericidal activity, and the immunopurification of anti-OMP antibodies shown to have bactericidal activity, further support the role of outer membrane proteins as a major target for natural and immune bactericidal antibodies directed against NT *H. influenzae.*

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