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Immune Enhancement of Pulmonary Clearance of Nontypable *Haemophilus influenzae*

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BALB/c mice systemically immunized by intraperitoneal injection with whole, viable cells of two different strains of nontypable *Haemophilus influenzae* (NTHI) exhibited a markedly enhanced ability to clear the homologous strain of NTHI from the lower respiratory tract. Immunization did not influence the number of phagocytic cells recovered by bronchoalveolar lavage from mice before or after intrapulmonary challenge with NTHI. Immunization also induced the synthesis of relatively large quantities of NTHI-directed antibodies which were detectable in both the bloodstream and the alveolar spaces of the lung. Radioimmunoprecipitation and Western blot (immunoblot) analyses indicated that these antibodies were directed against both the proteins and lipooligosaccharide (LOS) in the NTHI outer membrane. Bactericidal and opsonophagocytic assays determined that the NTHI-directed antibodies in the serum were functional and able to kill or opsonize the homologous NTHI strain. Mice immunized with an NTHI major outer membrane protein-LOS complex also had an increased ability to effect pulmonary clearance of NTHI. Serum and bronchoalveolar lavage fluid collected from these animals immunized with the outer membrane protein-LOS complex contained relatively high levels of antibodies to both of these antigens. The serum from these animals also possessed bactericidal and opsonic activity against the homologous NTHI strain. These results indicate that systemic immunization can enhance the ability of experimental animals to clear NTHI from the lower respiratory tract and suggest that immunoprophylaxis of NTHI pulmonary disease may be feasible.

Nontypable *Haemophilus influenzae* (NTHI) is being increasingly recognized as an important bacterial pathogen. Although it has long been known that this unencapsulated bacterium is an important cause of otitis media in infants and children (27), recent studies have also shown it to be a significant cause of lower respiratory tract disease in adults in the United States (3, 25, 38). Similarly, NTHI-induced pneumonia is apparently an important cause of morbidity and mortality in the pediatric populations of certain developing countries (30).

The fact that NTHI is often found as normal flora in the human upper respiratory tract indicates that this organism most probably gains access to the lower respiratory tract via aspiration of oropharyngeal fluids (12). We have therefore utilized a relevant animal model system in which NTHI is delivered in a bolus challenge to the lungs in order to study the interaction of this pathogen with the lower respiratory tract. An earlier study has already shown that NTHI initially multiplies when inoculated into murine lungs, suggesting that resident defenses are insufficient to effect early pulmonary clearance of NTHI (33). Furthermore, the introduction of NTHI into the lung induces the recruitment of polymorphonuclear leukocytes (PMNs) (34), and the use of a granulocytopenic murine model established the importance of these granulocytes in the early pulmonary clearance of NTHI (33). PMN recruitment was later shown to be dependent on both the C5 component of complement and other chemotaxins (34).

All of the studies described above involved the use of normal animals which were not immunized against NTHI. We have now utilized a systemic immunization regimen to induce the synthesis of NTHI-directed antibodies, which were detectable in both the bloodstream and the alveolar spaces of the lung. This systemic immunization also markedly enhanced the pulmonary clearance of NTHI. More importantly, we have used an acellular outer membrane protein-lipooligosaccharide (LOS) complex to induce pulmonary immunity to NTHI. These results indicate that systemic immunization can enhance the ability of the lower respiratory tract to clear a challenge with NTHI.

MATERIALS AND METHODS

Bacterial strains and culture conditions. NTHI strain BI104, an isolate obtained from the middle ear of a child with otitis media, was kindly provided by Barry Gray, University of Alabama at Birmingham. NTHI strain TN100 was obtained by transtracheal aspiration of a patient with NTHI pneumonia and was generously provided by Steven Berk, Quillen-Dishner College of Medicine, Johnson City, Tenn. The media and culture conditions used for the growth of bacteria were as described previously (33).

Immunization regimen. Broth cultures of strains BI104 and TN100 were harvested during the logarithmic phase of growth by centrifugation at 7,000 × *g* for 10 min. These cells were washed once with phosphate-buffered saline (PBS) and suspended to a final concentration of 5×10^8 CFU/ml in PBS. Ten-week-old BALB/c mice (Cumberland Laboratory, Cumberland, Tenn.) were given 0.1-ml intraperitoneal injections of these cells on day 1 and again on days 28 and 45 of the immunization regimen. Control animals were injected with 0.1 ml of PBS and were housed in cages separate from the immunized animals. Control sera and bronchoalveolar

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lavage fluid (BAL) were collected from PBS-immunized animals on day 60 of the immunization regimen.

Measurement of pulmonary clearance. Immunized and control mice received a bolus inoculum of the homologous NTHI strain on day 60 of the immunization regimen. This inoculum was deposited via an endobronchial catheter into a peripheral segment of the lung which comprised about 25% of the total lung volume (26). Equivalent numbers of mice were killed by cross-clamping the trachea both immediately after inoculation (0 h) to determine bacterial deposition and at 4 and 6 h after the bacterial challenge. The lungs were removed aseptically and homogenized in 4 ml of PBS in a tissue homogenizer (Virtis 45; The Virtis Company, Gardiner, N.Y.), followed by further grinding in a Broeck tissue grinder (Corning Glass Works, Corning, N.Y.). The homogenate was serially diluted in broth, plated on chocolate agar, and incubated at 37°C for 24 h in a candle extinction jar. To compare test groups, the number of CFU per lung from each mouse at each time point was divided by the mean CFU in the lungs of all 0-h mice for that experiment and then multiplied by 100. The resultant number was the percentage of viable bacteria that remained in the lung at each time point.

BAL. The number of PMNs in the alveolar spaces was assessed by bronchoalveolar lavage using a modification of a procedure described previously (26). Mice killed by intraperitoneal injection of sodium pentobarbital (160 mg/kg) had their tracheas exposed and cannulated with PE50 tubing (Clay Adams, Division of Becton Dickinson and Co., Parsippany, N.J.). Heparinized saline (4°C) was carefully injected into the lungs and then aspirated in 0.6-ml portions until a 5-ml volume was recovered. This BAL was collected on ice and subjected to centrifugation at 150 × *g* for 10 min at 4°C. The cell pellet was suspended in Hanks balanced salt solution (HBSS), and the total number of cells was determined with a Coulter counter (model ZBI; Coulter Electronics, Hialeah, Fla.). Differential cell counts of 200 cells each were made on cytocentrifuged, Wright-stained preparations to determine the number of phagocytic cells (alveolar macrophages and PMNs).

Collection of BAL for antibody studies was performed exactly as described above with the following important modifications. Heparin was not used in the lavage, and fetal calf serum was added to the BAL immediately after lavage to a final concentration of 1% (vol/vol). This BAL was then reduced in volume 25-fold in a Minicon macrosolute concentrator (Amicon Corp., Lexington, Mass.). All concentrated BAL samples were stored at −70°C in multiple portions until used for further analysis.

Measurement of bactericidal activity in serum. A modification of a previously published method was used to determine the titers of bactericidal mouse antibody directed against NTHI strain TN100 (18). The serum bactericidal assay was performed exactly as described (18) except that normal adult rat serum adsorbed with cells of NTHI strain TN100 was used as the source of complement. One cycle of freeze-thawing significantly reduced or totally eliminated complement activity in mouse serum (data not shown). Therefore, adult female Sprague-Dawley rats were exsanguinated, and the blood was allowed to stand for 1 h at room temperature. The clotted blood was rimmed and placed in crushed ice for 2 h. The tubes were then centrifuged at 4°C to separate the serum from the cells and the clot. The serum (3.5 ml) was then mixed with 10^{11} broth-grown cells of TN100 (suspended in 2.5 ml of PBS) and allowed to stand in crushed ice for 20 min. The TN100 cells were removed by centrifugation for 15 min at 4°C, and the adsorption step was repeated, followed by centrifugation. The final TN100-adsorbed serum was filter sterilized, dispensed into multiple portions, frozen in a dry ice-ethanol bath, and stored at −70°C. This adsorbed serum had no bactericidal activity against TN100. A 20-μl portion of this TN100-adsorbed rat serum was used as the source of complement in the serum bactericidal assay (18). The bactericidal titer of a mouse serum sample was defined as the greatest dilution which resulted in the killing of 90% of the test bacteria in 30 min.

Opsonophagocytosis assays. Opsonophagocytosis was measured by a modification of the method of Van Furth et al. (37). Venous blood was obtained from a healthy volunteer. Erythrocytes were eliminated by sedimentation at 1 × *g* in a 6% dextran solution, and leukocytes were collected by centrifugation of the resultant supernatant at 110 × *g*. Cells were washed and suspended in HBSS containing 0.1% bovine serum albumin (BSA) to give a final concentration of 2×10^7 leukocytes per ml.

The bacterial suspension was prepared by collecting the cells contained in 5 ml of a late-logarithmic-phase broth culture of NTHI by centrifugation at 3,000 × *g*. Bacteria were washed and resuspended in 50 ml of HBSS. This procedure reliably yielded a stock bacterial suspension of 2×10^8 CFU/ml. This was verified in each experiment by plating serial 10-fold dilutions of the stock suspension onto chocolate agar.

Opsonization was done by incubating 50 μl of the bacterial suspension (10^7 CFU) with 10 μl of serum in duplicate for 30 min at 37°C. Phagocytosis was done by adding the contents of the opsonization tubes (bacteria plus serum) to 0.5 ml of the leukocyte suspension (10^7 leukocytes) in a polycarbonate tube (12 by 75 mm; Becton Dickinson Labware, Cockeysville, Md.). The phagocytosis tubes were incubated for 30 min on a rocker panel at 37°C. At the end of the phagocytosis period, the tubes were centrifuged at 200 × *g* to sediment the leukocytes together with any phagocytosed or adherent bacteria. Supernatants were then plated onto chocolate agar in serial 10-fold dilutions to provide quantitative counts of bacteria remaining in the supernatant. Results are expressed as the percentage of the original bacterial inoculum remaining after 30 min of phagocytosis. In each experiment, a set of tubes was analyzed in which the leukocyte suspension was replaced with HBSS containing 0.1% BSA to ensure that serum opsonic activity was being measured and not the bactericidal activity of the test serum.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was carried out as follows. Serum and BAL were assayed for antibodies to NTHI outer membrane antigens as described by Robertson et al. (29) by using outer membrane vesicles of strains BI104 and TN100 as antigen. These vesicles were prepared by the method of Johnston et al. (14) as modified by Gulig et al. (7). The secondary antibody probe used in this assay was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; heavy and light chain specific; Cooper Biomedical, Malvern, Pa.). The reactivity of this probe with immunoglobulin light chains enables it to detect all isotypes of murine antibody, including IgM (29). Serial dilutions of serum and BAL for use in the ELISA system were made in PBS containing 1% (wt/vol) BSA. ELISA optical density readings between 0.4 and 0.9 were used to calculate the mean ELISA titers. Mean ELISA titers were calculated by multiplying the optical density at 405 nm by the reciprocal of the dilution of serum or BAL from which this reading was derived, with corrections based on readings obtained with control sera and BAL. Sera used in this and

other assays were prepared from blood obtained by retroorbital puncture and were stored at −70°C in multiple portions.

SDS-PAGE and SDS-PAGGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane vesicles and other protein preparations was performed as described previously (8, 17). SDS-polyacrylamide gradient gel electrophoresis (PAGGE) of purified NTHI LOS was accomplished as described earlier (18).

RIP analysis. Radioimmunoprecipitation (RIP) analysis with strain BI104 was performed as described previously (7).

Western blot analysis. Outer membrane vesicles were used as the source of outer membrane proteins for Western blot (immunoblot) analysis. Outer membrane vesicles were solubilized (17), and the outer membrane proteins (5 μg of protein per well) were resolved by SDS-PAGE. LOS was purified from strains BI104 and TN100 by the hot phenol-water method of Westphal and Jann (39) and resolved (1 μg/well) by SDS-PAGGE. These antigens were then electrophoretically transferred to nitrocellulose membranes (35). Nitrocellulose strips containing outer membrane proteins were incubated in PBS containing 0.05% (vol/vol) Tween 20 (Tween) for 1 h prior to incubation with 20 μl of serum or 150 μl of concentrated BAL diluted in 10 ml of PBS-Tween. LOS-containing nitrocellulose strips were incubated in PBS containing 2.5% (wt/vol) BSA for 1 h prior to incubation with 20 μl of serum or 200 μl of concentrated BAL diluted in PBS containing 2.5% BSA. Washing of the nitrocellulose strips was performed with PBS-Tween (for outer membrane protein-containing strips) or with PBS alone (for LOS-containing strips). Detection of mouse antibodies bound to the NTHI antigens on the nitrocellulose strips was performed as described previously (18), using antibody probes reactive with all isotypes of mouse antibody.

Purification of an outer membrane protein-LOS complex. A macromolecular complex composed of the major outer membrane protein and LOS of strain TN100 was purified by the method of Munson et al. (21). Animals immunized with this complex received a primary intraperitoneal injection of 20 μg of this protein (containing 2 μg of LOS) suspended in 0.1 ml of complete Freund adjuvant. Subsequent immunizations on days 30 and 45 after the primary immunization involved intraperitoneal injection of 20 μg of this protein in 0.1 ml of incomplete Freund adjuvant. Control animals received injections of complete Freund adjuvant containing PBS.

Statistical analysis. Pulmonary clearance data, the number of total phagocytic cells, and the number of PMNs were compared in immunized and control mice by the Mann-Whitney test for nonparametric analysis (40). Probability values of less than 0.05 were considered significant.

RESULTS

Characterization of bacterial strains. Two different strains of NTHI were used to systemically immunize two different groups of BALB/c mice. NTHI strains BI104 and TN100 possessed different outer membrane protein profiles as determined by SDS-PAGE of outer membrane vesicle preparations (Fig. 1, lanes 1 and 2). These strains also have different cell surface-exposed antigenic determinants in their LOS molecules (27a). Preliminary experiments had determined that strain TN100 grew more extensively than strain BI104 in the lungs of normal mice, so both strains were utilized in the early stages of this study to minimize strain-specific effects on experimental results and to evaluate whether systemic immunization would measurably enhance the clearance of strain BI104 from the lungs.

Effect of immunization on pulmonary clearance. Control and immunized mice were challenged by bolus deposition of 1.7×10^5 to 3.5×10^5 CFU of the homologous NTHI strain in the lungs. Comparison of the numbers of viable organisms of each strain recovered from the lungs of control and immune animals immediately after bolus deposition showed that the immune status of the animals did not affect the challenge organisms differentially at time 0 (Table 1). In addition, these data indicate that homogenization of the lungs of the immune animals did not result in bactericidal killing of NTHI by serum antibodies released from the vasculature of the lung.

Strain TN100 readily multiplied in the lungs of the control animals such that by 6 h postchallenge, the number of viable NTHI had increased by nearly 300% (Table 1). In contrast, substantial pulmonary clearance of TN100 by the TN100-immunized mice was clearly evident as early as 4 h postchallenge, whereas only 5% of the original number of organisms deposited in the lungs of the immune animals were recovered by 6 h postchallenge. Therefore, pulmonary clearance of strain TN100 proceeded rapidly in the immune mice during the same time period when cells of TN100 steadily increased in number in the lungs of the control animals.

Strain BI104 did not grow as extensively in the lungs of the control mice as did strain TN100, such that the control animals reduced the numbers of viable organisms originally deposited in the lungs by 50% in the first 6 h after challenge (Table 1). Nonetheless, animals immunized with strain BI104 still cleared this strain from their lungs much more

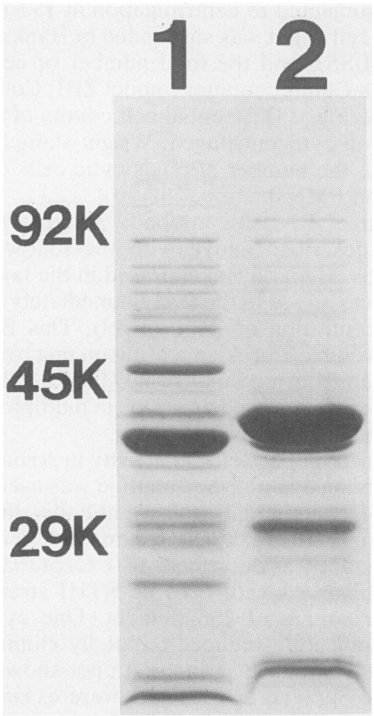

FIG. 1. Comparison of the outer membrane protein profiles of NTHI strains BI104 and TN100. Outer membrane vesicles of these two strains were prepared as described previously (15), and the proteins present in these vesicles (40 μg of protein) were resolved by SDS-PAGE and stained with Coomassie blue. Lanes: 1, strain BI104; 2, strain TN100. Molecular weight position markers are shown on the left side of this figure.

TABLE 1. Effect of systemic immunization on pulmonary clearance of NTHI[a]

NTHI strain	Deposition (10^5 CFU, mean ± SEM)	% Bacteria remaining (mean ± SEM) at:	
		4 h	6 h
BI104			
Control	1.7 ± 0.2	65 ± 6	50 ± 4
Immunized	1.8 ± 0.2	7 ± 1[b]	0.5 ± 0.1[b]
TN100			
Control	2.92 ± 0.3	163 ± 16	296 ± 15.0
Immunized	3.49 ± 0.3	12 ± 2[b]	5 ± 0.3[b]

[a] Each value represents a mean of eight animals at each time.
[b] $P < 0.05$ compared to control.

quickly than did control animals, such that only 0.5% of the original inoculum remained in the lungs of the immunized animals at 6 h postchallenge.

Effect of immunization on cell recruitment. We have previously shown that bolus deposition of NTHI in the murine lung results in the rapid influx of large numbers of PMNs which are crucial for effective early pulmonary clearance of this organism (34). Therefore, we studied recruitment of phagocytes to the alveoli in these experiments to determine whether immunization affects this process of phagocyte recruitment.

The numbers of total phagocytic cells (alveolar macrophages and PMNs) and PMNs were measured in BAL from immune and control mice immediately before challenge and at 4 and 6 h after challenge (Table 2). Immune and control animals had virtually identical numbers of total phagocytic cells immediately prior to challenge, and there were very few PMNs in the BAL from either set of animals. At 4 h after challenge, the numbers of both total phagocytic cells and PMNs increased in the BAL from both immune and control mice, with PMNs increasing drastically. At 6 h after challenge, PMNs represented over half of the cells present in BAL from both control and immune animals. It is important to note, however, that at no time point did the number of total phagocytic cells or PMNs in the BAL from the immune mice differ significantly from the number of these cells in the BAL of the control animals challenged with the same strain of NTHI.

Effect of immunization on antibody response. An ELISA system using outer membrane vesicles of strains BI104 and TN100 as antigen was used to measure what effect immunization had on the levels of antibody to NTHI. Both serum and BAL samples were collected from both sets of immunized mice at the end of the immunization regimen (day 60), and the relative levels of NTHI-directed antibodies in these samples were determined as described in Materials and Methods. The level of antibody to strain BI104 in serum of the BI104-immunized animals rose by 1,000-fold relative to control serum, while there was a >200-fold increase in the antibody titer to BI104 in the immune BAL (Table 3). Similarly, animals immunized with strain TN100 had approximately 1,000-fold more antibody in their bloodstream to outer membrane antigens of this strain than did control animals. The BAL recovered from the lungs of the TN100-immunized animals also contained much more antibody directed against this NTHI strain than did the BAL obtained from control animals (Table 3).

Two different methods were used to determine whether animals immunized with TN100 had higher levels of functional serum antibodies than did control animals. Measurement of bactericidal-antibody titers revealed that the control serum had a titer of only 1:4, whereas the immune serum exhibited a titer of 1:80. Similarly, the use of an opsonophagocytosis assay showed that control serum had little or no antibody opsonic for TN100 (Fig. 2, lane 1). In contrast, the immune serum promoted extensive phagocytosis of TN100, such that the original numbers of NTHI in the opsonophagocytosis assay were reduced by 2 orders of magnitude (Fig. 2, lane 3).

Specificity of antibodies in immune serum and BAL. The results obtained with the ELISA system showed that immunization resulted in a substantial increase in the level of antibody in both serum and BAL to NTHI outer membrane antigens. Two different methods were then used to identify those outer membrane antigens which were targets for these antibodies in the immune serum and BAL.

The first technique involved the use of a highly sensitive RIP system which can detect antibodies directed against proteins exposed on the NTHI cell surface. Serum and BAL were collected on days 7, 14, 28, 45, and 60 in the immunization regimen from animals immunized with strain BI104. RIP analysis of these samples revealed that antibody which immunoprecipitated a major outer membrane protein with an apparent molecular weight of 38,000 (38K) could be detected in serum as early as 7 days into the immunization regimen (Fig. 3A, lane 3). Antibody to two different surface proteins with apparent molecular weights of 100,000 and 130,000 became detectable in serum by 28 days postimmunization (Fig. 3A, lane 5). Antibody which immunoprecipitated the major outer membrane protein became detectable in BAL by 14 days postimmunization (Fig. 3B, lane 4), whereas antibody to the two high-molecular-weight proteins appeared in BAL collected on day 60 in the immunization regimen (Fig.

TABLE 2. Recruitment of phagocytes to the alveoli after intrapulmonary NTHI challenge

Strain and cell type	Phagocyte recruitment (10^6 cells, mean ± SD)[a] at:					
	0 h		4 h		6 h	
	Control	Immune	Control	Immune	Control	Immune
BI104						
Total	1.12 ± 0.04	1.14 ± 0.04	1.18 ± 0.07	0.99 ± 0.03	1.32 ± 0.05	1.28 ± 0.03
PMN	0.002 ± 0.0001	0.002 ± 0.0001	0.29 ± 0.01	0.30 ± 0.01	0.74 ± 0.06	0.71 ± 0.02
TN100						
Total	1.04 ± 0.03	1.10 ± 0.04	1.52 ± 0.05	1.54 ± 0.05	1.98 ± 0.05	1.89 ± 0.04
PMN	0.003 ± 0.0001	0.002 ± 0.0001	0.55 ± 0.02	0.57 ± 0.02	1.15 ± 0.06	1.10 ± 0.05

[a] Each value represents the mean of six to eight animals at each time.

TABLE 3. Effect of systemic immunization on NTHI-directed antibody levels in serum and BAL[a]

Bacterial strain	ELISA titer[b]			
	Serum		BAL	
	Control	Immune	Control	Immune
BI104	45	51,400	2.5	522
TN100	349	456,000	3.8	5,010

[a] Serum and BAL samples were collected from immune and control mice on day 60 of the immunization regimen.

[b] NTHI-directed antibodies were detected by ELISA using outer membrane vesicles of the homologous NTHI strain as antigen. Mean ELISA units were calculated as described in Materials and Methods.

3B, lane 7). Serum and BAL collected from control (unimmunized) animals on day 60 had no detectable antibody to surface proteins of strain BI104 (Fig. 3, lane 2).

Western blot analysis. The RIP system used in the preceding experiments detects only antibodies to surface antigens and does not detect antibodies to outer membrane proteins whose antigenic determinants are buried in the outer membrane (7). Also, a major outer membrane protein of *H. influenzae* type b can be immunoprecipitated either by antibody directed to itself or by LOS-directed antibody reacting with the noncovalent complex formed by this major outer membrane protein and LOS (6). Western blot analysis was therefore used to determine the full spectrum of the immune response to NTHI outer membrane proteins and to detect the presence of antibody to NTHI LOS.

Outer membrane vesicles extracted from strains BI104 and TN100 were used as antigen to detect outer membrane protein-directed antibodies in sera and BAL from the homologous immunized mice. Figure 4 shows that control sera and BAL had no detectable antibody to outer membrane protein antigens of strain BI104 (Fig. 4, lanes 1 and 2) or strain TN100 (Fig. 4, lanes 5 and 6). Sera and BAL from both sets of immunized animals possessed readily detectable quantities of antibodies to numerous different outer membrane proteins of the homologous NTHI strain. It is of interest to note that the complement of outer membrane proteins recognized by the immune serum was qualitatively identical to those recognized by antibodies in the immune BAL for both

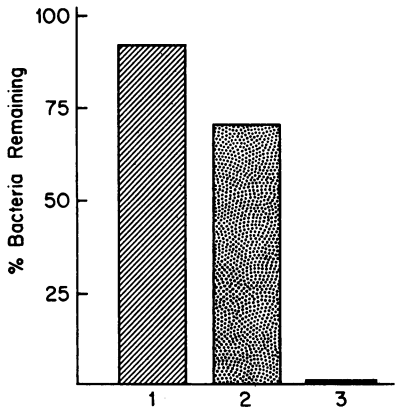

FIG. 2. Opsonophagocytosis of strain TN100 mediated by immune serum raised against whole cells of TN100. Bacteria were incubated with control serum and leukocytes (bar 1), immune serum without leukocytes (bar 2), and immune serum and leukocytes (bar 3). Opsonophagocytosis was measured as described in Materials and Methods.

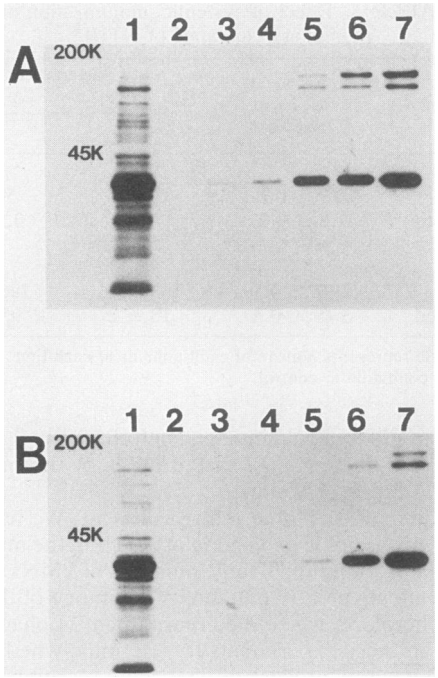

FIG. 3. RIP analysis of serum (A) and BAL (B) from mice immunized with strain BI104. Radioiodinated whole cells of strain BI104 were incubated with sera (100 μl) or concentrated BAL (500 μl) collected at various times during the immunization regimen, and the resultant mixtures were processed as described in Materials and Methods. Lanes: 1, solubilized sample of radioiodinated cells used as antigen; 2, ^{125}I-labeled surface proteins immunoprecipitated by control serum or control BAL. ^{125}I-labeled surface proteins immunoprecipitated by serum or BAL collected during the immunization regimen on day 7 (lane 3), day 14 (lane 4), day 28 (lane 5), day 45 (lane 6), and day 60 (lane 7).

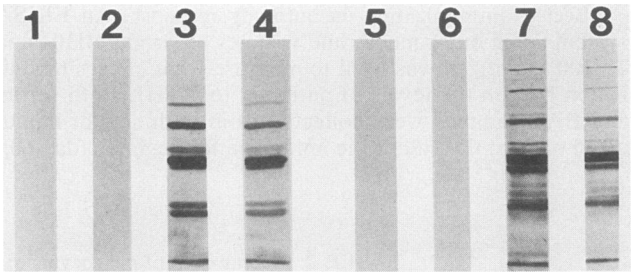

FIG. 4. Western blot analysis of outer membrane protein-directed antibodies in immune serum and BAL. Outer membrane vesicles of strains BI104 and TN100 were used as the source of outer membrane protein antigens. SDS-PAGE and Western blot analysis were performed as described in Materials and Methods. Lanes: 1 and 2, outer membrane proteins of strain BI104 probed with control serum and control BAL, respectively; 3 and 4, outer membrane proteins of strain BI104 probed with sera and BAL, respectively, collected from BI104-immunized animals on day 60 of the immunization regimen; 5 and 6, outer membrane proteins of strain TN100 probed with control serum and control BAL, respectively; 7 and 8, outer membrane proteins of strain TN100 probed with serum and BAL, respectively, collected from TN100-immunized mice on day 60 of the immunization regimen.

strain BI104 (Fig. 4, lanes 3 and 4) and strain TN100 (Fig. 4, lanes 7 and 8).

LOS purified from strains BI104 and TN100 by the hot phenol-water method (39) was used as the source of antigen for detecting antibody to NTHI LOS. Control sera and BAL again lacked any detectable antibody to these antigens (Fig. 5, lanes 1 and 2 and lanes 5 and 6), whereas both immune serum and immune BAL contained antibody to the LOS molecules of homologous immunizing strains (Fig. 5, lanes 3 and 4 and lanes 7 and 8).

Effect of immunization with an outer membrane protein-LOS complex. The preceding data indicated that systemic immunization of mice with whole, viable NTHI cells resulted in an immune response which could be correlated with both an enhanced ability to clear NTHI from the lower respiratory tract and with the appearance of antibody in serum and BAL to both NTHI outer membrane proteins and LOS. The presence of numerous antigens in the NTHI outer membrane, however, prevented accurate identification of those antibodies functionally involved in this augmentation of clearance. In addition, it could not be determined whether immunization with the whole organism was required to effect this change in pulmonary clearance ability. It was therefore of interest to determine whether an acellular preparation composed of one or more NTHI surface antigens could induce the synthesis of antibodies which would facilitate early pulmonary clearance of NTHI.

The major outer membrane protein of strain TN100 was purified and analyzed by SDS-PAGE for purity. Coomassie blue staining revealed that this protein, with an apparent molecular weight of 38,000 (38K), was essentially free of contamination with other outer membrane proteins (Fig. 6, lane 2). Analysis of this purified protein preparation by SDS-PAGE followed by silver staining to detect carbohydrate-containing molecules determined that this protein preparation did contain LOS from strain TN100 (Fig. 6, lane 4) as would be expected from the reported noncovalent association between the major outer membrane protein of *H. influenzae* type b and the LOS of this latter organism (6). LOS was present in this outer membrane protein preparation at a concentration of 1 μg/10 μg of outer membrane protein.

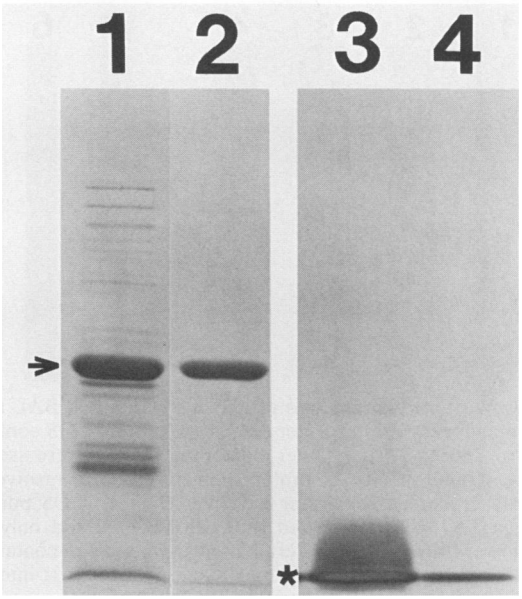

FIG. 6. SDS-PAGE analysis of the major outer membrane protein-LOS complex purified from strain TN100. The proteins present in outer membrane vesicles (30 μg of protein) of strain TN100 (lanes 1 and 3) and the purified outer membrane protein-LOS complex (4 μg of protein) (lanes 2 and 4) were resolved by SDS-PAGE and stained with Coomassie blue to detect proteins (lanes 1 and 2) and with the silver stain of Tsai and Frasch (36) to detect LOS (lanes 3 and 4). In this 10% polyacrylamide separating gel, NTHI LOS migrates just behind the electrophoretic front. The arrow indicates the position of the 38 K major outer membrane protein; the asterisk indicates the position of the LOS. It should be noted that the LOS smear in lane 3 is due to the large amount of LOS in the outer membrane vesicles; the much smaller amount of LOS in the outer membrane protein-LOS complex (lane 4) forms a discrete and very thin band in this gel system.

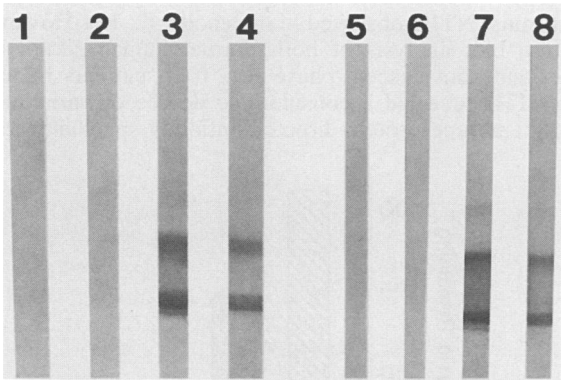

FIG. 5. Western blot analysis of LOS-directed antibodies in immune serum and BAL. SDS-PAGGE and Western blot analysis were performed with purified LOS as antigen, as described in Materials and Methods. NTHI LOS forms a broad band in this polyacrylamide gradient gel; only the relevant lower one-third of each Western blot strip, containing the region where NTHI LOS bands in SDS-PAGGE, is shown in this figure. Lanes: 1 and 2, LOS from strain BI104 probed with control serum and BAL, respectively; 3 and 4, LOS of strain BI104 probed with sera and BAL, respectively, collected from BI104-immunized animals on day 60 of the immunization regimen; 5 and 6, LOS of strain TN100 probed with control serum and BAL, respectively; 7 and 8, LOS of strain TN100 probed with serum and BAL, respectively, collected from TN100-immunized mice on day 60 of the immunization regimen.

Mice immunized with this acellular complex exhibited a greatly enhanced ability to clear a pulmonary challenge with NTHI relative to control mice (Table 4). Western blot analysis revealed that antibodies to the major outer membrane protein were present in both the serum (Fig. 7, lane 1) and BAL (Fig. 7, lane 3) obtained from mice immunized with

TABLE 4. Effect of systemic immunization with an outer membrane protein-LOS complex from strain TN100 on pulmonary clearance of NTHI

NTHI strain and deposition (10^4 CFU)	Immunizing agent	% Bacteria remaining (mean ± SEM) at 6 h postchallenge[a]
TN100 (5.5)	PBS	291 ± 17
	Outer membrane protein-LOS	6 ± 1[b]
TN100 (12)	PBS	358 ± 30
	Outer membrane protein-LOS	16 ± 1[b]

[a] Each value represents a mean of eight animals. These animals were challenged on day 60 in their immunization regimen.

[b] $P < 0.05$ compared to PBS-immunized control animals.

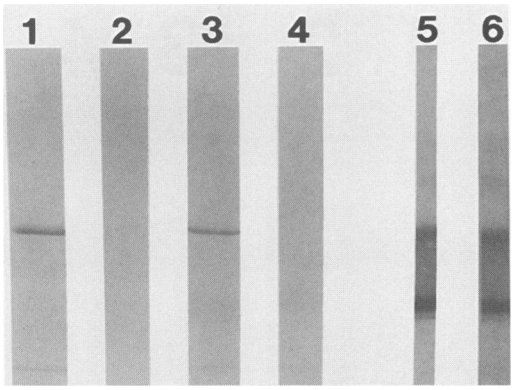

FIG. 7. Western blot analysis of immune serum and BAL from animals immunized with the outer membrane protein-LOS complex purified from strain TN100. Outer membrane vesicles were used as the source of outer membrane protein antigens and were resolved in a 10% polyacrylamide separating gel (lanes 1 to 4). LOS purified from strain TN100 was resolved by SDS-PAGGE, and only the relevant lower third of the Western blot strip (the region containing the NTHI LOS) is shown here (lanes 5 and 6). Lanes: 1, immune serum; 2, control serum; 3, immune BAL; 4, control serum; 5, immune serum; 6, immune BAL. The immune sera and BAL were collected on day 60 of this immunization regimen

the outer membrane protein-LOS complex. In addition, both serum and BAL from these same immunized animals contained detectable levels of antibody to the LOS of strain TN100 (Fig. 7, lanes 5 and 6).

Opsonophagocytosis assays. The enhanced ability of these systemically immunized animals to clear a pulmonary challenge with NTHI, together with the demonstrated presence of outer membrane protein- and LOS-directed antibodies in their serum and BAL, suggested that these antibodies played a functional role in pulmonary clearance. An opsonophagocytosis assay was therefore used to determine whether serum from animals immunized with the outer membrane protein-LOS complex contained antibodies which would enhance the phagocytosis of the homologous NTHI strain. As shown in Fig. 8, there was very little or no detectable phagocytosis of NTHI by a mixture of human leukocytes and control serum from unimmunized animals. Similarly, relatively little killing of NTHI occurred when unheated immune serum was incubated with NTHI in the absence of phagocytic cells. In contrast, a mixture of this same immune serum and leukocytes caused extensive phagocytosis of NTHI. Heating of this immune serum at 56°C for 30 min did not decrease its ability to stimulate phagocytosis of NTHI, indicating that, at least in vitro, complement is not required for this process (data not shown). These data indicate that serum from animals immunized with the outer membrane protein-LOS complex possessed opsonizing activity directed against the homologous NTHI strain. The bactericidal titer of the serum obtained from these same animals was 1:40.

DISCUSSION

Recent studies suggest that NTHI is second only to *Streptococcus pneumoniae* as a cause of community-acquired bacterial pneumonia (3, 25). Despite appropriate antibiotic therapy, patients with chronic systemic illness have a case fatality rate of 10 to 16% from NTHI pneumonia. The failure of antimicrobial agents to prevent a significant residue of mortality from NTHI infections of the lower respiratory tract (25), the importance of NTHI as a cause of otitis media (27), and the continued emergence of antibiotic-resistant NTHI strains (32) all indicate the importance of evaluating the potential for development of a vaccine to prevent NTHI disease.

Most bacterial pathogens which are noted for causing lower respiratory tract disease in humans possess polysaccharide capsules which surround the bacterial cell and function to prevent engulfment of the unopsonized bacterium by phagocytes. Immunization with purified capsular polysaccharide from *S. pneumoniae* has long been known to be effective in preventing pneumonia caused by this organism (1, 20). More recently, purified capsular polysaccharide from *Klebsiella pneumoniae* was shown to be effective in inducing the synthesis of antibodies which could be correlated with immunity to fatal experimental *K. pneumoniae* pneumonia (4). The absence of a polysaccharide capsule on NTHI, however, dictates that noncapsular antigens must be considered in strategies for vaccine development. The two NTHI cell surface components which are obvious targets for a protective humoral antibody response are the LOS and proteins present in the outer membrane of this organism.

Several studies have recently examined serum antibody response to NTHI disease in humans. Patients with NTHI pneumonia possess significant levels of acute-phase serum bactericidal antibody but relatively low levels of opsonizing antibody for their own NTHI isolates (25). During convalescence, however, the level of opsonizing antibody increased significantly; the antigens involved in this immune response were not identified. A more recent study showed that convalescent-phase serum obtained from two patients recovering from NTHI disease contained bactericidal antibody which was primarily directed against NTHI outer membrane proteins; similar results were also obtained with normal human serum (5). Similarly, studies in an animal model system for experimental NTHI otitis media showed that serum antibodies to NTHI outer membrane proteins are primarily responsible for the bactericidal or protective activities against NTHI observed in this model (2, 16). However, Western blot analysis of both normal human serum and acute- and convalescent-phase sera from patients infected with NTHI revealed a considerable degree of variation in outer membrane protein-directed antibody specificities and

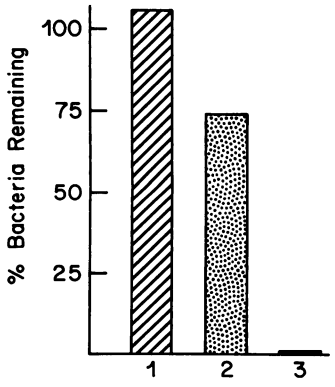

FIG. 8. Opsonophagocytosis of strain TN100 mediated by immune serum raised against the major outer membrane protein-LOS complex of TN100. Bacteria were incubated with control serum and leukocytes (bar 1), immune serum without leukocytes (bar 2), and immune serum and leukocytes (bar 3). Opsonophagocytosis was measured as described in Materials and Methods.

also did not detect a consistent immune response in convalescent-phase serum to NTHI outer membrane proteins (10).

A previous study involving immunization of rats with an aerosol of a temperature-sensitive mutant of *H. influenzae* type b showed that direct immunization of the airway would enhance pulmonary clearance of this encapsulated pathogen (11). In the present study, systemic (parenteral) immunization of mice with whole, viable NTHI resulted in the synthesis of antibodies directed against both outer membrane proteins and LOS of the immunizing strains. Although exact quantities of these NTHI-directed antibodies could not be determined, the immunized animals clearly possessed greatly increased levels of these antibodies in both serum and the alveolar spaces of the lung, relative to the control animals. The appearance of these NTHI-directed antibodies in the immunized animals was associated with a marked enhancement of the ability of these animals to clear NTHI from the lower respiratory tract. More importantly, these NTHI-directed antibodies in the serum of the immunized animals were shown to be functional and to possess both bactericidal and opsonizing activity against NTHI. While these data do not directly identify the particular antibodies responsible for this enhanced pulmonary clearance ability, these findings indicate that systemic immunization with NTHI can have a positive effect on the ability of the lower respiratory tract to clear NTHI and that this enhanced clearance ability can be correlated with the presence of bactericidal and opsonizing antibodies in the bloodstream. That serum IgG antibody can directly enhance pulmonary clearance of NTHI has been demonstrated in passive immunization experiments involving a murine IgG monoclonal antibody to NTHI LOS (E. J. Hansen, T. A. Loftus, and G. B. Toews, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 282, 1985 [manuscript in preparation]). The isotypes of the NTHI-directed antibodies in the immune sera and BAL generated in the present study were not determined, although previous studies indicate that antibody from the alveolar spaces of normal, uninflamed lungs is primarily IgG (15, 28).

Vaccination against NTHI disease of the lower respiratory tract in humans obviously will not involve whole bacterial cells because of the toxicity of the LOS (endotoxin) present in the outer membrane. As a first approximation in determining the feasibility of using an acellular NTHI preparation to immunize against NTHI disease of the pulmonary system, we investigated the vaccinogenic potential of an outer membrane protein-LOS complex from NTHI. Use of this dual antigen system is highly relevant in that both of these outer membrane constituents are targets for antibodies protective against *H. influenzae* type b. Antibodies to outer membrane proteins of *H. influenzae* type b are protective against systemic *Haemophilus* disease in the infant rat model system (17, 21, 31). An LOS-directed monoclonal antibody protects against experimental *H. influenzae* type b disease (6, 9), whereas high titers of LOS-directed antibody can be induced by immunization with covalently coupled conjugates of protein with the nontoxic oligosaccharide from LOS (13, 19). In the present study, the immunization of mice with the NTHI outer membrane protein-LOS complex resulted in the production of antibodies to both of these cell surface immunogens, which in turn correlated with enhanced pulmonary clearance of NTHI. The use of bactericidal and opsonophagocytic assay systems confirmed that the outer membrane protein-LOS complex-directed serum antibodies were indeed functional.

The fact that immunization with an acellular preparation of NTHI surface antigens resulted in enhanced pulmonary clearance of NTHI suggests that immunoprophylaxis against NTHI pulmonary disease may be possible. However, it must be emphasized that the data obtained in this study indicate that immunization can enhance pulmonary clearance of NTHI in an animal model. The ability of immunoprophylaxis to prevent NTHI disease in the human lower respiratory tract will necessarily be influenced by other important variables, including the immunocompetence of the host and, especially in adults in this country, the presence of preexisting pulmonary disease (25). Further evaluation of the microbiological feasibility of such a vaccine requires the identification of specific NTHI surface components which can induce an antibody response protective against most or all strains of this pathogen. Relatively little is currently known about the surface antigens of NTHI, and there appears to be some variation among NTHI strains with regard to the antigenic composition of their outer membrane proteins (22). However, at least one outer membrane protein is both exposed on the NTHI cell surface and antigenically common to all strains of this organism (23, 24). Whether antibody to this protein or other NTHI surface proteins can be protective against NTHI pulmonary disease in an animal model system remains to be determined, although antibody to a major NTHI outer membrane protein was recently reported to be protective against experimental otitis media produced by the homologous NTHI strain (S. J. Barenkamp, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 217, 1986). It has recently been reported that a significant degree of antigenic heterogeneity exists among strains of this pathogen with regard to LOS epitopes (3a, 27a). The identification of NTHI surface antigens with true vaccinogenic potential will require detailed antigenic and biochemical analyses of the outer membrane constituents of this interesting pathogen.

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